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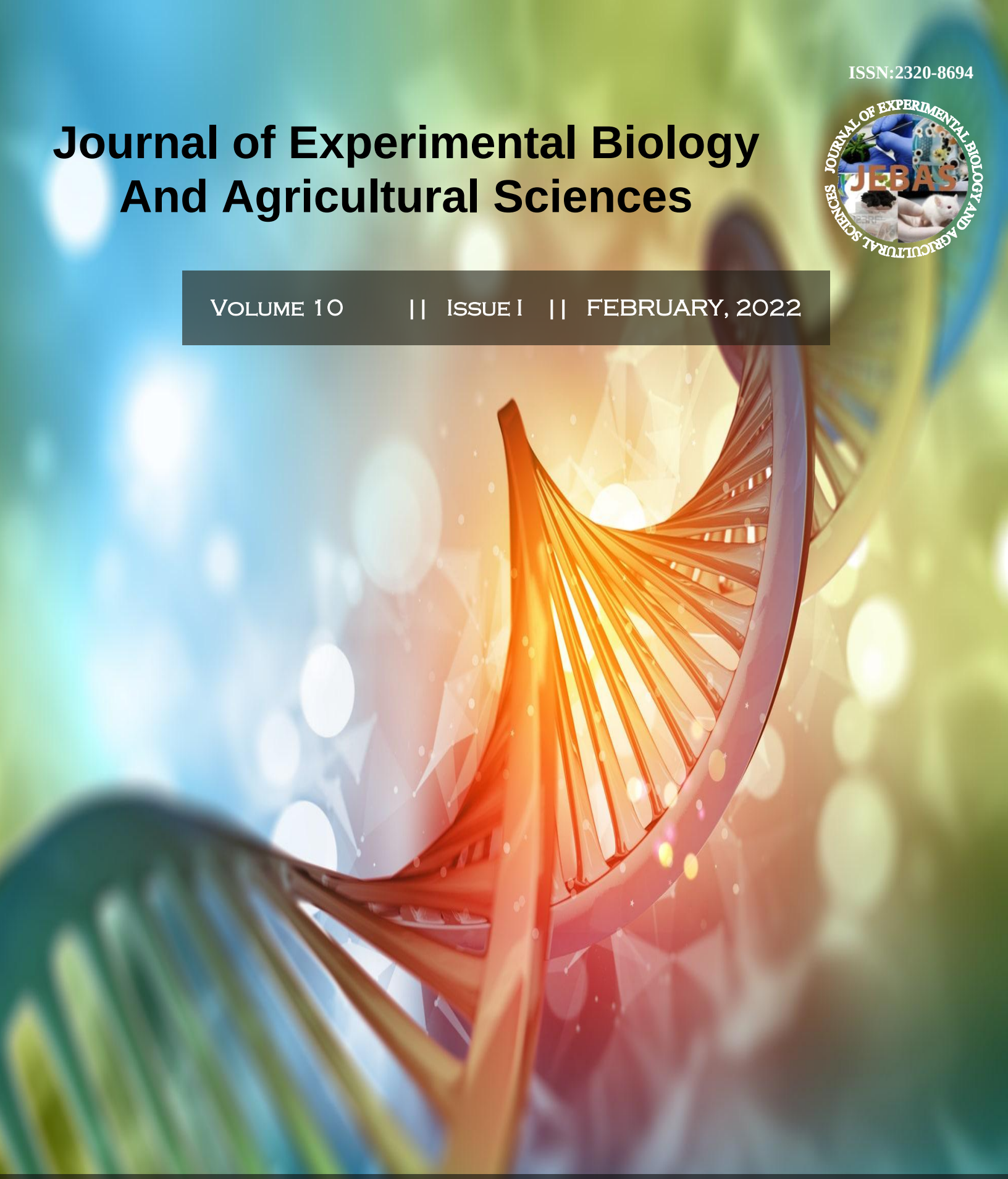

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Table of contents

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Effects of Probiotics, Prebiotics and Synbiotic Supplementation on Cognitive Impairment: A Review

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ABSTRACT

Learning and memory formation are the two essential terms widely used in the field of cognition. Learning can be defined as acquiring new information or skills. Memory is formed due to changes in the neuronal system as a result of continuous stimulus exposure. Both learning and memory are fundamental processes that occur in all living organisms. Memory is broadly categorized into two different categories such as short-term memory (STM) and long-term memory (LTM). Compared to STM, LTM plays an essential role in the day-to-day activities of different living organisms. LTM requires RNA and protein synthesis-dependent mechanisms for memory storage, which lasts up to their lifetime. LTM formation is initiated when the neurotransmitters are released from the presynaptic neuron; further released neurotransmitters bind with their respective receptors present in the postsynaptic neuron and initiate the calcium influx. Calcium influx results in the further activation of molecules involved in the neuronal signaling pathway and results in memory formation. Present review reports the outcome of recent studies which showed that probiotic supplement is responsible for the retrieval of memory in case of memory impairment and its uses in the treatment of neurodegenerative disorders like mild cognitive impairment (MCI), Alzheimer's disease (AD). Recent research studies were shown that probiotic microorganisms may positively regulate neurotransmitter release and increase the calcium influx, brain derived neurotrophic factor (BDNF), and N-methyl-D-aspartate receptor (NMDAR) and plays a pivotal role in the LTM formation in gut-dysbiosed & memory-impaired animal models.

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1 Introduction

Synaptic plasticity refers to the activity-dependent changes that occur in synapses and plays a prominent role in memory formation (Kind and Neumann 2000; Sweatt 2001; Abraham et al. 2019). Several experimental animal models were shown that changes in the synaptic strength are occurred due to repeated stimulus exposure/activity. These changes are referred to as long-term potentiation (LTP) (Malenka et al. 1992; Thiels and Klann 2001; Abraham et al. 2019; Evans et al. 2021). LTP was first identified by terjeløмoin the year 1966. After the discovery of LTP, several studies have been attempted to focus on the change of locus, pre/postsynaptic currents. Recently, it is accepted that postsynaptic signaling mechanisms are most needed for the LTP induction and serve as a primary mechanism to maintain increased synaptic response (Waltereit et al. 2001; Yuste and Bonhoeffer 2001; Evans et al. 2021).

At the initial phase of memory processing in the brain, memory formation may be inhibited by various hindrances like seizures, trauma, inactivation of neuronal pathways, brain lesions, and inactivation of specific transcription factors, translation, or specific blockade of molecular pathways. During the initial memory processing, memory impairment happened due to various reasons such as trauma, seizures, neuronal pathways inactivation/blockade, of inhibition of neuronal transcription factors. Thereby transcription plays an essential role in memory consolidation or its retainment. Long-term plasticity changes result in long-term facilitation (LTF), LTP, and long-term depression (LTD) which requires both transcription and protein synthesis (Baker-Herman and Mitchell 2002; Roberts and Glanzman 2003; Evans et al. 2021; Lin et al. 2021). Several animal studies have been shown that long-term memory (LTM) needed *de novo* protein synthesis during the first few hours of training (Schafe and LeDoux 2000; Scharf et al. 2002; Igaz et al. 2006; Abraham and Williams 2007; Lin et al. 2021; Evans et al. 2021).

The learning process is associated with activity-dependent changes and results in neurotransmitters' release (Lovinger 2010; Bai and Suzuki 2020). Neurotransmitter release results in the activation of neuronal signaling pathway with the help of several proteins like protein kinase A (PKA), extracellular signal-regulated kinase-1/2 (ERK-1/2), mitogen-activated protein kinase (MAPK), and cyclic AMP response element-binding protein-1 (CREB-1) (Yoon and Seger 2006; Ganesh et al. 2010; Ganesh et al. 2012; Mukilan et al. 2015; García-Pardo et al. 2016; Mukilan et al. 2018a, 2018b). Activation of ERK-1/2 results in the phosphorylation of CREB-1 (Peng et al. 2010). Further, phosphorylated CREB-1 results in the activation of the immediate early gene (IEG) cascade and other postsynaptic density protein (PSD), which results in the formation of LTM (Ganesh et al. 2010; Ganesh et al. 2012; Mukilan et al. 2015; Mukilan et al. 2018a, 2018b).

In normal healthy persons, gut microbiota was needed for the proper functioning of the central nervous system (CNS) through endocrine, neural, and immune pathways (Grenham et al. 2011; Moloney et al. 2014; Ma et al. 2019). Pathogenic infection or stress exposure stimulates the secretion of corticotrophin-releasing factors (CRF) via the hypothalamus-pituitary-adrenal axis from the brain. CRF disturbs the gut microbiota and increases the production of endotoxins (Bailey and Coe1999; Mayer et al. 2015; Misak et al. 2020). These endotoxins limit the secretion of serotonin and catecholamines (Linthorst and Reul 1998; Fung et al. 2017; Yang and Chiu 2017). Serotonin and catecholamines increase interaction between the gut microbiota and CNS. By this signaling pathway, CNS maintains homeostasis. Impairment or dysregulation of this signaling pathway resulted in autism, Alzheimer's disease, memory impairment, and neurodegenerative disorders (Linthorst and Reul 1998; Fung et al. 2017; Salami 2021). Recent studies were shown that treatment with probiotic microorganisms was used to overcome the impaired cognitive decline in autism and Alzheimer's disease (Petrof et al. 2013; Choi and Choi 2016; Asl et al. 2019; Morshedi et al. 2020).

Probiotics are beneficial living microorganisms, when it was taken in an appropriate dose it supports the host in many ways like the improvement of cognition, immune system, and also it will supply needed antioxidants (FAO/WHO 2002; Sherman et al. 2009; Kwok et al. 2014). Compared to probiotics, prebiotics was formed by the fermentation of non-digestible ingredients by the beneficial gut microbiota. These prebiotics enhance the growth and activity of beneficial gut microorganisms. The formed probiotic/prebiotic precursor molecules increase the growth and metabolic activity of beneficial microorganisms present in the gut. Thereby it increased the short-chain fatty acid (SCFA) level and also modifies alpha-synuclein protein (Franco-Robles and López 2015; Markowiak-Kopeć and Ślizewska 2020). SCFA were used for the regulation of neurotransmitter release and have a direct effect on the expression level of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF). It will also be shown that SCFA act as an essential molecule for the signaling between the gut microbiota and brain (Montarolo et al. 1986; Dale et al. 1987; Gräff and Tsai 2013; Buffington et al. 2014; Franco-Robles and López 2015; Heyck and Ibarra 2019; Markowiak-Kopeć and Ślizewska 2020).

Recent research reports have shown that the gut microbiota plays an important role in the regulation of gut-brain axis (Wong et al. 2018; Misak et al. 2020). Some gut bacterial strains may also regulate the level of neurotransmitters like γ-aminobutyric acid (GABA)(Wong et al. 2003). Recent studies have shown that various neurotransmitter levels were regulated by some bacterial strains present in the gut. Regulated neurotransmitters may include γ-aminobutyric acid (GABA) (Wong et al. 2003; O'Donnell et al. 2020), serotonin (5-HT), dopamine (DA), and noradrenaline (NA)

(Chen et al. 2017). These neurotransmitters potentially regulate calcium influx and other molecules involved in the formation of LTM (Romo-Araiza et al. 2018; Rezaeiasl et al. 2019; Yang et al. 2020). Present review article trying to explore the role of prebiotics and probiotics in the formation of LTM and its uses in the treatment of memory impairment in neurogenerative disorders like Alzheimer's disease (AD), epilepsy, Parkinson's disease (PD), autism spectrum disorders (ASD).

2 Effect of neuro inflammation on memory impairment

Degeneration of neurons/neuroinflammation within the CNS are associated with the decline in memory formation (Romo-Araiza et al. 2018). Mild cognitive impairment (MCI) is regarded as the intermittent stage of memory decline between healthy apoptosis and dementia (Tajiri et al. 2017; Romo-Araiza et al. 2018; Tobin et al. 2019). MCI results in the loss of neurons in different brain regions like the hippocampus. Loss of neurons may also be related to the defects of mitochondria and oxidative stress. Oxidative stress results in increased levels of proinflammatory cytokines, which results in neuroinflammation. In later stages, there is a probability of conversion of MCI to Alzheimer's disease (Baierle et al. 2015; Zhao et al. 2015; Romo-Araiza et al. 2018; Tobin et al. 2019).

Neuroinflammation is generally associated with cognitive memory decline. This neuroinflammation results in the inflammation of the hippocampal tissue region. Hippocampal tissue inflammation will result in decreased plasticity changes, which results in impaired LTM formation (Di Filippo et al. 2013; Zhao et al. 2019). Decreased plasticity changes result in the low-level synthesis of glutamate and downregulation of N-methyl-D-asparate receptor (NMDAR) in the hippocampus's CA1 and CA3 region (Kumar and Mehta 2011; Bye and McDonald 2019).The downregulation of NMDAR results in impaired LTM formation (Rosi et al. 2005; Di Filippo et al. 2013; Baierle et al. 2015). Other than NMDAR, Brain-Derived Neurotrophic Factor (BDNF) is also down-regulated during neuroinflammation/gut dysbiosis which results in impaired memory formation (Ryan and Nolan 2016; Mora 2013).

3 Role of short-chain fatty acid (SCFA) on memory enhancement

Intestinal probiotic microorganisms are needed for short-chain fatty acid (SCFA) production. This SCFA are made up of carboxylic acids, these carboxylic acid are present with the aliphatic tails of 1-6 carbon. In these 6 carbon molecules, 3 carbon molecules {acetate (C2), propionate (C3), and butyrate (C4)} were

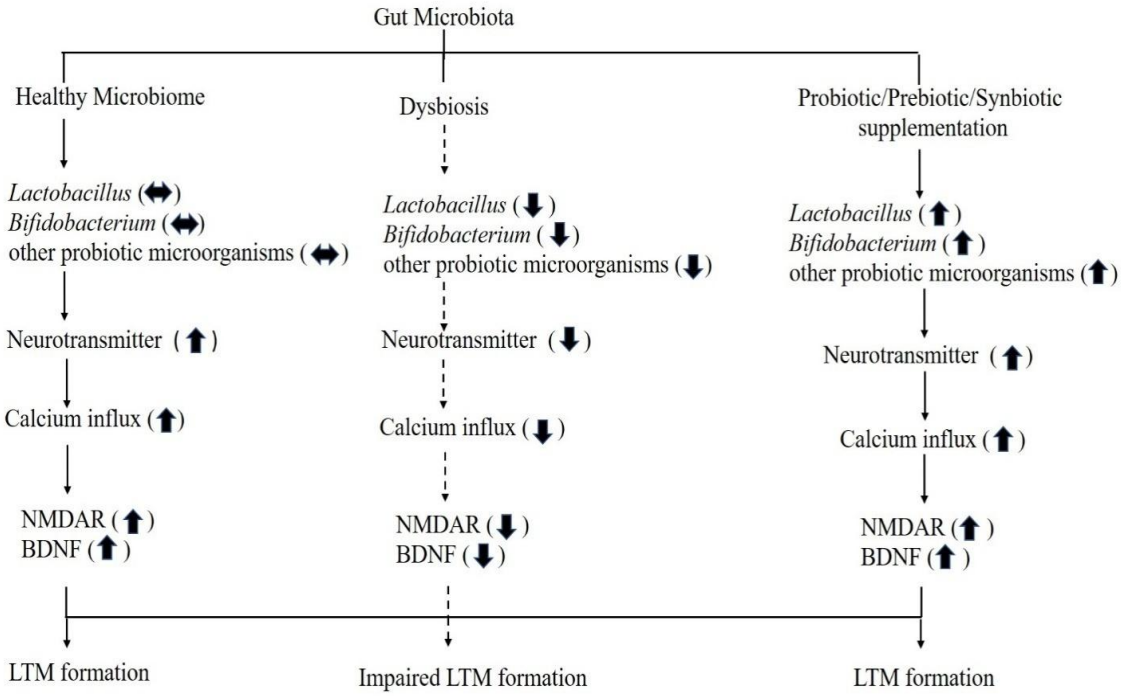

Figure 1 Effect of gut microbiota in the long-term memory (LTM) formation. A) Gut microbiota plays a main role in the development of LTM by the release of neurotransmitters (ex. serotonin) in its normal state and further results in the increase of calcium influx and brain derived neurotrophic factor (BDNF) and N-methyl-D-aspartate receptor (NMDAR). B) dysbiosis affects the normal level of gut microbiota, low level of gut microbiota inhibits the neurotransmitter release, decrease the calcium influx and downregulates BDNF &NMDAR.Low levels of BDNF & NMDAR results in the impaired LTM formation C) Treatment with probiotics restores the normal gut microbiota and restores the LTM memory formation.

produced by anaerobic fermentation of dietary fibers (DF) by the intestinal microorganisms. In these aliphatic tails, three different carbon molecules will be synthesized from the dietary fibers with the help of gut microbiota by anaerobic digestion. Some of the carbon molecules were produced by the Acetate are the most abundant SCFA produced in the gut. Acetate was made from acetyl-CoA from glycolysis (den Besten et al. 2013; Duncan et al. 2002). Propionate and butyrate formation occurs from the carbohydrate metabolism of glycolysis (Louis and Flint 2017). Levels of SCFA decline with age and microbial imbalance. Microbial imbalance results in the increase of pathogenic bacteria Proteobacterium and results in the brain's inflammation (Caracciolo et al. 2014; Romo-Araiza et al. 2018)

Brüssow stated that both probiotics and prebiotics were used for the production of SCFA, increasing neurotrophic factors and neuronal plasticity. Agave inulin a most commonly used prebiotic stimulates the growth of *Enterococcus faecium* (probiotic bacterium), thereby indirectly promoting butyrate synthesis (Huang et al. 2017). Among the three SCFA, butyrate functions as a histone deacetylase inhibitor, enhancing the upregulation of BDNF expression in the different regions of the brain and inhibiting the secretion of proinflammatory cytokines (Park et al. 2016; Kim et al. 2014). Recent research findings were shown that healthy gut microbiota increases butyrate production; the presence of enough butyrate level reduces neuroinflammation and increases BDNF, NMDAR levels in the specific brain region, and upregulation of BDNF, NMDAR results in increased synaptic plasticity (Stilling et al. 2016; Pineda-Rodriguez et al. 2017; Canani et al. 2018).

4 Effect of probiotics on long term memory formation

Several intestinal microbial species influence the physiology, development, and maintenance of an individual's health in the form of gut microbiota. Gut microbiota can be differentiated into three major categories viz., bacteria, viruses, and fungi. These microorganisms regulate intestinal pH and act as a preventive barrier against infectious agents. A healthy level/ equilibrium of intestinal microbiota plays an important role in the maintenance of proper health. A healthy state shows the mutual relationship between gut microbiota and the nervous system (Jiang et al. 2017). Equilibrium of intestinal microbiota was mainly affected by different lifestyles like diet (Gentile and Weir 2018), alcohol consumption (Hillemacher et al. 2018), smoking (Savin et al. 2018), and changes in circadian rhythm (Kaczmarek et al. 2017). However, alteration in the gut microbiota may induce changes in brain activity and also cause neurological disorders like AD (Angelucci et al. 2019). Affected intestinal microbiota equilibrium comes back to normal by consuming probiotics/prebiotics/ synbiotic supplementation (Kaczmarek et al. 2017; Gentile and Weir 2018; Hillemacher et al. 2018; Savin et al. 2018; Hadizadeh et al. 2019).

Probiotics produce beneficial effects on the host's health (Mukherjee et al. 2018). Most bacteria are used as probiotics; probiotic bacteria are needed to synthesize substances required for a host. The presence of a sustainable number of necessary substances prevents inflammation and related diseases (Mukherjee et al. 2018). The most commonly used probiotic strains include *Lactobacillus* and *Bifidobacteria*. Both of these strains are present in yogurt, fermented cheese, and vegetables. Other than probiotics, we also consumed dietary fibers; anaerobic fermentation of dietary fibres results in the formation of SCFA (Daliri et al. 2018; Mukherjee et al. 2018). This type of probiotic microorganisms may synthesize and release different neurotransmitters like serotonin, GABA, histamine, and dopamine (Gareau 2014; Dinan and Cryan 2017). Several gut microorganisms are responsible for the synthesis of neurotransmitters like dopamine, noradrenaline, serotonin, GABA, acetylcholine, and histamine which plays a main role in long-term memory formation (Stanaszek et al. 1977; Tsavkelova et al. 2000; Landete et al. 2007; Shishov et al. 2009; Özoğul et al. 2012; Pokusaeva et al. 2017). However, gut microbiota imbalances play an important role in the deficient synthesis of biogenic amines and neurotransmitters which will have a direct impact on long-term memory formation (Matsumoto et al. 2013). Prebiotic or probiotic treatment increases neurotransmitter level and improves the cognitive functions in memory-impaired animal models (Lyte 2011; Barrett 2012; Dinan 2015; Sarkar et al. 2016; Bermúdez-Humarán et al. 2019).

Neurotransmitters produced by the probiotic microorganisms at first cross the blood-brain barrier and reach the central nervous system (CNS) (Pokusaeva et al. 2017). Among the pool of neurotransmitters, serotonin (5-HT) is synthesized from the amino acid tryptophan. At first, amino acid tryptophan is converted to 5-hydroxytrptophan (5-HTP) with the help of tryptophan hydroxylase. Formed 5-HTP is again reconverted to 5-HT by the aromatic amino acid decarboxylase (AACD) (Adell et al. 2002). This 5-HT is first reported to be involved in learning and memory formation by the 1980s (Altman and Normile 1988). This 5-HT is having multiple neural markers like receptors and transporters (McCorvy and Roth 2015). Further, 5-HT is synthesized from serotonin synthesizing neurons. Serotonin synthesizing neurons are present along the brainstem's midline; the most prominently present in raphe nuclei. Axons of these serotonin synthesizing neurons innervate almost all regions of the brain. Activation of 5-HT receptors stimulates adenyl cyclase (AC), this AC induces the rapid increase of cyclic adenosine monophosphate (cAMP) levels which directly stimulates protein kinase A (PKA) activity. Further, PKA increases MAPK/ERK kinase (Mohamed et al. 2005). ERK 1/2 cascade activation is necessary to consolidate the learning

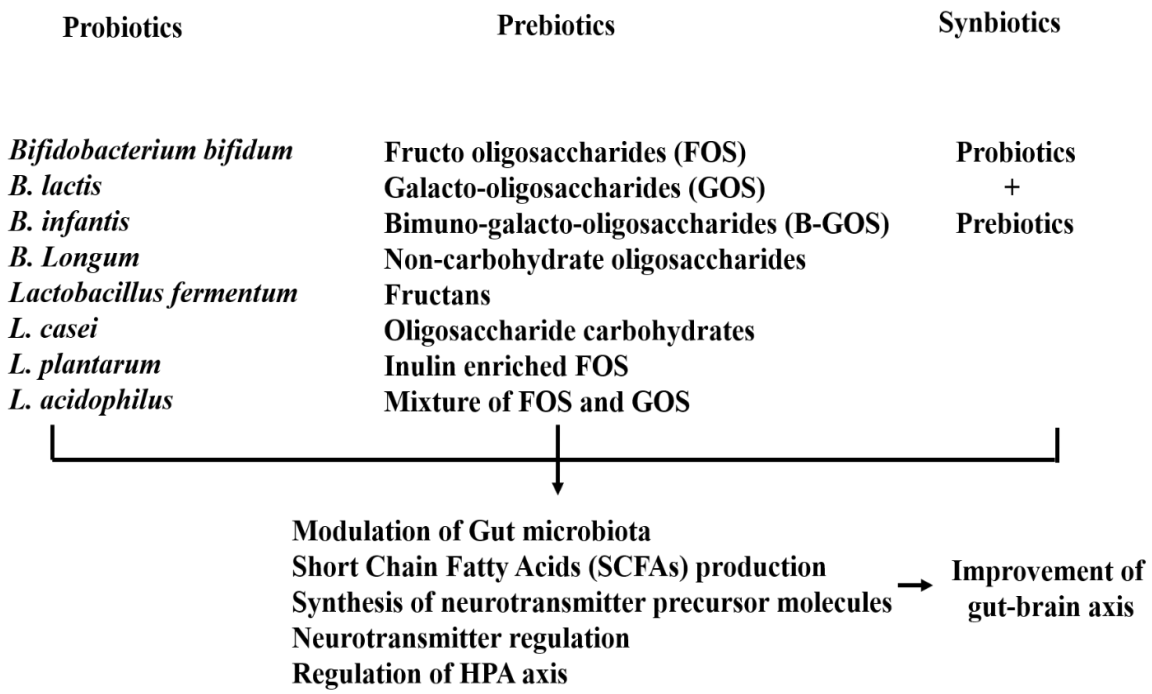

Figure 2 Role of probiotics/prebiotics/synbiotics on the improvement of gut-brain axis

paradigm (Ganesh et al. 2010; Ganesh et al. 2012; Mukilan et al. 2015; Mukilan et al. 2018a; Mukilan et al. 2018b). Compared to the ERK cascade, BDNF is also involved in long-term memory formation. BDNF is needed for stimulating hippocampal-neocortical interaction during the consolidation of memory (Bambah-Mukku et al. 2014). During the consolidation of long-term memory, hippocampal-neocortical interactions were stimulated by the BDNF (Bambah-Mukku et al. 2014; Miranda et al. 2019).

5 Effect of prebiotic and synbiotic supplementation on memory formation

Recent studies were evidenced that early life intake of prebiotic is associated with improvement of cognitive abilities, LTM formation in the early and middle stages of life. During early life, gut microbial colonization is needed for the development of the neuronal structure and its function (Williams et al. 2016). Treatment of neonatal rats with a galacto-oligosaccharide prebiotic (BGOS) increases the expression of N-methyl-D-asparate receptor (NMDAR) subunit-GluN2A, synaptic proteins, and brain-derived-neurotrophic factor (BDNF) in the hippocampal brain region and also alters the neurotransmission during memory impairment. Other than BGOS, oral administration of 2'-fucosyllactose (2'-FL) also showed a higher level of long-term potentiation (LTP) (Williams et al. 2016; Oliveros et al. 2016). Other than prebiotic, synbiotic (probiotic and prebiotic) supplementation is also used for the development of memory in memory-impaired animal models. Romo-Araiza et al. (2018) stated the effect of probiotic and prebiotic supplementation on spatial memory formation.

6 Impact of probiotic supplementation on neural dysfunctions

Recent research findings showed that probiotic supplementation plays an essential role in the prevention of neural dysfunctions by regulating age-related cognitive impairment. Song et al. (2013) tested the effect of prebiotic galactooligosaccharides (GOS) on the neuroprotective effect in amyotrophic lateral sclerosis (ALS). The outcome of the study showed that the administration of GOS reduced motor neuron loss, improved the consequences of atrophy, and also stated the role of probiotic yogurt in the disease onset improvement. Other than GOS, probiotic yogurt administration also delayed disease onset and increased the lifespan of treated mice. Yang et al. (2020) investigated the effect of probiotics on the deficits of intestinal gut microbiota and restores cognitive function using the probiotic preparation of *Bifidobacterium lactis*, *B. bifidum*, *Lactobacillus casei*, and *L. acidophilus* (ProBiotic-4) on SAMP8 (Senescence-accelerated mouse prone 8) mice. Oral administration of ProBiotic-4 for 4, 8, and 12 weeks significantly improves the deficits of the microbiota-gut-brain axis and cognitive function (Yang et al. 2020).

Conclusion

Recent studies were shown that probiotic, prebiotic, and synbiotic supplementation is used as a clinical tool to treat memory decline

in AD, mild cognitive impairment (MCI), and type 2 diabetes mellitus (T2DM) induced memory impairment. The most commonly anti-inflammatory property of the synbiotic supplementation is used for the treatment of neurological disorders and cognition decline in MCI. Compared to MCI, T2DM is a common metabolic disease that leads to memory dysfunction in the brain and is also associated with complications of gut-brain disorders. Some of the important research findings were shown the impact of probiotic, prebiotic, and symbiotic supplementation on the improvement of cognitive complications and plays the main role in the strengthening of neurotransmitter concentration (especially serotonin), BDNF/TrkB/CREB signaling pathways in different brain regions. Strengthening of this neuronal signaling pathway results in the improvement of memory during cognitive decline. Along with this supplementation, SCFAs will also play a positive role on the brain-gut axis. These SCFAs may also be produced by anaerobic fermentation of non-digestible ingredients with the help of certain probiotic microorganisms. The newly formed SCFAs are also involved in the new protein synthesis, enhance histone acetylation and do long-term plasticity changes in the brain. The long-term plasticity changes were enhanced by histone acetylation and could be improved with the help of HDAC inhibitors (HDACi). These SCFAs also act against neuroinflammation and inhibits the expression of proinflammatory cytokines in the presence of butyrate and sodium butyrate. Simply, SCFA increases the levels of BDNF, NMDAR through the supplementation of pre and probiotics along with food. Thereby SCFA plays an essential role in reducing the risk of Alzheimer's disease development due to aging and brain injuries. Other than synbiotic and SCFAs, ProBiotic-4 is also used to treat gut microbiota dysbiosis, which will result in the improvement of cognitive function. However considering the data reviewed here, shown that probiotic, prebiotic, and synbiotic supplementation intake reduces cognitive impairment, memory decline and restores synaptic plasticity and specific gut microbiota changes occur due to lead-led memory impairment, MCI, AD, and neurodegenerative disorders. Other than the restoration of gut microbiota, these supplementations are also used for the restoration of memory impairment and morphological abnormalities of dendritic spines. Thereby present review focused on the effect of probiotic, prebiotic, and synbiotic supplementation on the treatment of memory impairment caused due to aging, MCI, and the role gut-brain axis in the long-term memory formation. This review elaborates the role of gut microbiota on the synthesis of the neurotransmitter, activation of neuronal molecules in long-term memory formation.

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Ethical approval

This article does not contain any studies with human participants or animals performed by the author.

Conflicts of Interest

The author declare no conflict of interest.

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Evaluations of Morpho-Physiological Variances in Soybean Varieties under Low Water Conditions

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ABSTRACT

This study used pot experiments with a completely randomized design (CRD) in triplicates to evaluate physiological and morphological attributes used for the characterization of drought stress tolerance in six soybean varieties (Knap, Mopani, LS677, LS678, Pan1564, and Sonop). Growth and physiological parameters analyzed in this study, included plant height, number of flowers, number of pods, seed number per pod, leaf surface area (LSA), grain yield, and total phenolics, flavonoids, ureides as well as antioxidant activity. Low water conditions caused varied negative effects depending on the level of stress on both morphological and physiological responses of the plants. Enhanced secondary products (ureides, total phenolic, and flavonoid content) were observed in plants subjected to severe water stress, in addition to reduced photosynthetic components and percentage grain yields. However, soybean variety Sonop, LS677, and LS678 consecutively, induced high secondary metabolite accumulations and antioxidant activity possibly preventing the occurrence of excessive oxidative stress damage caused by water shortage. The performance of LS varieties, Knap and Sonop were more prominent than Mopani and Pan1564. Results showed potential tolerance to stress in Sonop, LS678 and LS677, attributed to the strong free radical scavenging activity and maintenance of photosynthetic pigments used to achieve sufficient growth balance in plants.

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1 Introduction

Soybean (*Glycine max* L. Merr.) has been serving as a stable crop for generations and is currently exploited as an industrial crop for applications such as bio-oil extraction and processing, manufacturing of pharmaceuticals, as well as nutraceutical products (Mangena 2021). The above-mentioned attributes and benefits continue to sustain commercial cultivation of soybean as a major alternative source of oils and proteins, further encouraging the acquisition of new and improved genetic traits to enhance plant growth and productivity. To date, the main challenge facing this crop has been the susceptibility of many genotypes to harsh environmental conditions such as water shortage (drought), chilling stress, poor germplasm, and the rapidly diminishing quality of seeds (Hussain et al. 2019). Drought, in particular, is a major problem in soybean agriculture wherein the crop remains highly sensitive to water-deficit stress than any kind of abiotic stress. In 2019/20 projections, the production of soybean in sub-Saharan African countries was averaged at 1.235 compared to 1.366 and 2.865metric tons per hectare for Latin America and East-West Asia, respectively. Reductions recorded in sub-Saharan Africa were due to the frequent acute droughts experienced by the region since 2019 (Engelbrecht et al. 2020). Thus, genotype characterization for selection of stress-resistant properties and breeding of newly improved cultivars, including the conservation of superior genotypes, are available means for up-keeping crops adaptability to conditions disposed to biotic and abiotic stress factors. Drought (also known as water-deficit stress) is one of the most important environmental pressures capable of causing massive reductions in plant growth and development. Drought stress leads to significant decreases of more than 50% losses in crop yield for many species worldwide (Mohamed and Latif 2017). Drought-triggers abscission of leaves, flowers, fruits, and other plant organs, causing morphological and biochemical differentiation and defects in affected tissues. Abscission serves as a mechanism for stress resistance or a marker of severe exposure to abiotic stress (You and Chan 2015). For example, leaf abscission regulated by the interaction of ethylene and auxin is used by biennial and perennial plants to regulate water loss and maintain balanced water levels within tissues (Patharkar and Walker 2016). Death of the whole plant can also occur due to accelerated senescence of tissues in plants exposed to severe water-deficit stress. Furthermore, the physiological influence of abiotic stress needs to be determined, particularly concerning soybean growth and productivity. The anabolic and catabolic processes involving primary metabolites are used by the plants to yield energy required for growth and reproductive activities. However, drought stress triggers major physiological and metabolic changes including reduced photosynthetic pigments, fluctuating amounts of secondary products (ureides, phenolic, and flavonoid contents, etc.), and accumulation of harmful by-products like reactive oxygen species (ROS) (Evert and Eichhorn 2013; You and Chan 2015). This stress subsequently causes adverse effects on plant metabolism by inhibiting the synthesis and utilization of sugars by the source and sink tissues. All metabolic alterations, including morphological growth of soybean plants exposed to drought stress, could provide insights on how a range of adaptations to different stress levels could assist plants in withstanding stressful conditions. This study, therefore, evaluated the physiological and morphological attributes of different soybean varieties for the characterization of these genotypes to identify those showing tolerance against water-deficit stress.

2 Materials & Methods

2.1 Plant materials and study location

Soybean (*G. max*.) varieties (Mopani, Knap, LS677, LS678, Pan1564, and Sonop) were used in this study. The varieties were sourced from the Agricultural Research Council (ARC), Tshwane in South Africa, and evaluated for drought stress tolerance at the University of Limpopo (Sovenga, Turfloop campus), Capricorn District in the Limpopo Province (23.8888° S, 29.7386° E).

2.2 Plant establishment and water stress treatment

Seeds were grown in 35 cm plastic pots containing vermiculite (Greener Tidings Garden Centre, Polokwane, South Africa) in a greenhouse using a randomized block design. For germination and plant establishment, sown seeds were watered twice a week with half-strength Hoagland solution prepared as described by Taiz et al. (2015) and daily with distilled water depending on the moisture content of the medium until they reached Vegetative Growth Stage three (V3). Water-deficit stress was then imposed on the soybean plants by watering once in 5 days to achieve moderate drought stress and once in 7 days for severe drought stress for 3–9 weeks (Mangena 2020a). Soybean plants used as a control were watered daily to saturation depending on the vermiculite moisture. Moisture levels were measured using a Ryobi moisture meter MM-210 (Ryobi, Africa, Midrand, South Africa). Water stress treatments were then terminated after 4–9 weeks when plants reached Reproductive Growth Stage three (R3). Data was recorded in triplicates for both control and water stress-treated plants.

2.3 Morphological data

Plant height was measured from the tip of the plant to the base of the stem just above the level of vermiculite. A metric fluorescent ruler (1 m) (Sigma Aldrich, South Africa) was used to measure the heights of soybean plants. The number of flowers, pods, and seeds per pod produced were countered directly from each plant in all the treatments and control. Leaf surface area (LSA) was estimated from the three leaf samples per plant in triplicates, traced, weighed, and calculated using the formula as indicated below (Campillo et al. 2010).

Calculating formula:

$$\text{LSA} = \frac{25.00\ \text{cm}^2 \times \text{mass of leaf trace}}{\text{mass of 25.00 paper (cm}^2\text{)}}$$

2.4 Relative leaf water content

A total of three middle leaflets of trifoliate leaves per plant were detached and immediately weighed to determine fresh weight. The leaflets were completely immersed in distilled water for 24 hours. After this period, the leaflets were blotted dry using a clean paper towel and then re-weighed to determine the saturated weight. Leaflets were then dried at 60°C in an oven for 24 hours and reweighed until a constant dry weight was achieved. The below formula was then used to calculate the leaf relative water content (RLWC) where *Fm* refers to the fresh weight, *Sw* refers to the saturated mass of leaf samples, and *Dm* to the dry weight of the leaflet samples (Soltys-Kalina et al. 2016).

Calculating formula:

$$\text{RLWC} = \frac{\text{Fw} - \text{Dw}}{\text{Sw} - \text{Dw}}$$

2.5 Physiological data

Leaflet samples per plant were harvested and immediately homogenized in liquid nitrogen and stored at –86°C until used for the determination of total phenolics, total flavonoids, ureides content, and antioxidant activity.

2.5.1 Total phenolics

Total phenolics in harvested leaflets was determined spectrophotometrically using the Folin-Ciocalteau method as described by Torre et al. (1987). A 100 mg frozen powder for each treatment was extracted with 15 mL methanol in 150 mL Erlenmeyer flask on an OrbiShake Platform Shaker (LABOTEC, Midrand, South Africa) for two hours. The extracted plant materials were then filtered into 50 mL volumetric flasks using a coned Whatman No. 1 filter paper (Lasec Group, Johannesburg, RSA). The residues were washed a few times with methanol and the extract volume made up to 50 mL using the methanol. A 500 µL of each extract was thoroughly mixed with 0.5 mL Folin-Ciocalteau (Sigma-Aldrich, Johannesburg, South Africa) in 5 mL distilled water and then allowed to stand for 5 minutes at room temperature. A 1.5 mL of 20% sodium carbonate was added to the extracts, made up to 50 mL with distilled water, mixed, and incubated at 50 °C for another two hours. The mixture was vortexed, and absorbance was read at 765 nm using a Jenway UV-Visible Spectrophotometer (Jenway, Asia Cole-Parmer, China). Total phenolics (*PT*) of the extract was calculated as gallic acid equivalents. The formula below, where *c*- is the concentration of gallic acid in µg/mL, *V*- volume of extract in mL, and *m*- is the weight (mg) of the extract was used.

Calculating formula:

$$\text{PT} = \text{c}\frac{\text{V}}{\text{m}}$$

2.5.2 Total flavonoids

Total flavonoid contents was determined according to the procedure by Zhishen et al. (1999) and Marinova et al. (2005). Aliquots of 500 µL of the extracts were thoroughly mixed with 2 mL distilled water and 1.5 mL of 5 % sodium nitrate (Rochelle Chemicals, Johannesburg, RSA). The mixtures was incubated for 5 minutes at room temperature and 0.15 mL of 10 % aluminum chloride (Rochelle Chemicals, Johannesburg, RSA) was added to each extract and incubated again under room temperature for 6 minutes. After incubation, 1 mL of 1 M sodium hydroxide was added to the extract and made up to 10 mL volume with distilled water and the absorbance read at 510 nm using a Jenway UV-Visible Spectrophotometer (Jenway, Asia Cole-Parmer, China). Total flavonoid (*TF*) contents of the extract was determined as catechin equivalents, calculated as indicated below where; *As*- is the absorbance of extract, *Ac*- is the standard catechin absorbance, *Mc*- is the weight of extract, and *Ms*-is the weight of catechin.

Calculating formula:

$$\text{TF} = \frac{\text{As} \times \text{Mc}}{\text{Ac} \times \text{Ms}}$$

2.5.3 Extraction and determination of ureides

Ureides (allantoin and allantoic) were extracted using 0.2 M NaOH as described by van Heerden et al. (2008). Samples of ground leaf powders were boiled in 1 mL NaOH to convert allantoin to allantoic acids for 20 minutes. The mixture was cooled on ice for 10–15 minutes, centrifuged at 10,000xg for 10 minutes and 350 µL distilled water was added to 50 µL of the extracts. Ureide contents were then determined by reading the absorbance at 525 nm and using allantoin standards as indicated by van Heerden et al. (2008).

2.5.4 Antioxidant assay

The antioxidant activity was determined using a 2.2-Diphenyl-1-picrylhydrazyl (DPPH) method as described by Odhav et al. (2007). About 2.5 mL of plant extracts were thoroughly mixed with 1 mL of 0.3 mM DPPH in 2.5 mL methanol. The mixture was then incubated for 30 minutes at room temperature, followed by an absorbance reading at 518 nm using the Jenway Spectrophotometer. Percentage DPPH scavenging activity was calculated using the formula below, where *AD* is the absorbance

value at 518 nm of methanolic DPPH solution and *AS* refers to the absorbance value at 518 nm for the sample extract.

Calculating formula:

$$\text{DPPH Scavenging activity} = \frac{\text{AD} - \text{AS}}{\text{AD}} \times 100$$

2.5.5 Statistical Analysis

The experiment was conducted in triplicates and repeated thrice. All data was subjected to variation analysis using SPSS statistics program version 26 analysis of variance (ANOVA). The t-test was performed to determine the level of significant differences between means at 5% confidence level.

3 Results & Discussion

3.1 Morphological and reproductive response of plants to water-deficit stress

As indicated in Table 1–3, plant heights were measured based on stem heights determined using a metric ruler. According to the results, water-deficit stress affected soybean growth by decreasing the lengths of stems during moderate (Table 2) and severe (Table 3) water-deficit stress as compared to the control (Table 1). The tallest stems were observed in LS678 in the control, and the shortest was recorded by Pan1564 plants under severe water stress. On average, stem lengths were decreased by at least 5% under moderate stress compared to 15.4% in plants subjected to severe

Table 1 Summary of results on morphological parameters analysed in well-watered soybean plants

Varieties	Plant Height	No. of Flowers/ Plant	No. of Pod/Plant	Seeds/pods	LSA
Knap	39.2 ± 0.42^{a}	10.0 ± 0.00^{a}	22.2 ± 0.32^{a}	3.0^{a}	42.0 ± 0.36^{a}
Mopani	33.2 ± 0.35^{b}	9.0 ± 2.82^{b}	31.6 ± 0.20^{b}	3.0^{a}	114.0 ± 2.14^{b}
LS677	32.5 ± 0.28^{c}	8.0 ± 2.12^{c}	24.4 ± 0.28^{c}	3.0^{a}	45.0 ± 3.14^{c}
LS678	50.1 ± 0.56^{d}	$10.0 \pm 2.12^{d,a}$	$31.3 \pm 0.20^{d,b}$	3.0^{a}	38.0 ± 1.36^{d}
Pan1564	23.4 ± 0.28^{e}	$10.0 \pm 0.0^{e,d,a}$	34.0 ± 0.21^{e}	3.0^{a}	46.0 ± 3.88^{e}
Sonop	40.3 ± 0.28^{f}	$9.0 \pm 0.70^{f,b}$	25.6 ± 0.27^{f}	3.0^{a}	49.0 ± 0.56^{f}

Different superscript letters within columns indicate statistical significance using ANOVA (t-test) where p-value < 0.05 and values with similar letters are not statistically different at the same p-value.

Table 2 Results on morphological evaluation of soybean plants subjected to moderate water-deficit stress

Varieties	Plant Height	No. of Flowers/ Plant	No. of Pod/ Plant	Seeds/pods	LSA
Knap	36.6 ± 0.14^{a}	8.0 ± 0.00^{a}	13.0 ± 0.23^{a}	3.0^{a}	29.0 ± 2.81^{a}
Mopani	32.1 ± 0.21^{b}	8.0 ± 0.70^{a}	18.1 ± 0.01^{b}	2.0^{b}	66.0 ± 0.81^{b}
LS677	31.6 ± 0.28^{c}	8.0 ± 2.12^{a}	17.4 ± 0.02^{c}	3.0^{c}	36.0 ± 0.72^{c}
LS678	39.2 ± 0.28^{d}	8.0 ± 2.12^{a}	22.4 ± 0.14^{d}	3.0^{c}	25.0 ± 0.17^{d}
Pan1564	22.1 ± 0.14^{e}	9.0 ± 4.24^{b}	26.3 ± 0.03^{e}	3.0^{c}	$29.0 \pm 2.81^{e,a}$
Sonop	37.2 ± 0.07^{f}	6.0 ± 0.70^{c}	12.1 ± 0.11^{f}	3.0^{c}	38.0 ± 3.81^{f}

Different superscript letters within columns indicate statistical significance using ANOVA (t-test) where p-value < 0.05 and values with similar letters are not statistically different at the same p-value.

Table 3 Results on morphological evaluation of soybean plants subjected to severe water-deficit stress

Varieties	Plant Height	No. of Flowers/ Plant	No. of Pods/ Plant	Seeds/pod	LSA
Knap	36.6 ± 0.14^{a}	6.0 ± 1.41^{a}	8.10 ± 0.10^{a}	2.0^{a}	21.0 ± 0.00^{a}
Mopani	25.8 ± 0.14^{b}	6.0 ± 0.00^{a}	8.20 ± 0.30^{a}	2.0^{a}	39.0 ± 1.57^{b}
LS677	28.3 ± 0.14^{c}	6.0 ± 0.00^{a}	9.20 ± 0.05^{b}	3.0^{b}	$21.0 \pm 0.19^{c,a}$
LS678	27.3 ± 0.07^{d}	7.0 ± 4.24^{b}	10.2 ± 0.20^{c}	3.0^{b}	16.0 ± 3.98^{d}
Pan1564	21.6 ± 0.77^{e}	7.0 ± 4.24^{b}	11.6 ± 0.02^{d}	3.0^{b}	10.0 ± 1.44^{e}
Sonop	35.3 ± 0.42^{f}	5.0 ± 1.41^{c}	$11.2 \pm 0.02^{e,d}$	3.0^{b}	$19.0 \pm 2..40^{f}$

Different superscript letters within columns indicate statistical significance using ANOVA (t-test) where p-value < 0.05 and values with similar letters are not statistically different at the same p-value.

water-deficit stress. The effects of water stress was more pronounced in LS678 where plant height dropped from 50.1 cm (Table 1) in the control to 27.3 cm of plants subjected to severe water deficit stress (Table 3). A similar drop in stem length was also observed in LS677, Mopani, and then Sonop. Soybean variety Pan1564 and Knap recorded slightly constant stem heights throughout the experiment.

Several studies, such as those of Dong et al. (2019), Hussain et al. (2019), and Mangena (2020b) also showed that drought hampers morphological growth by inhibiting stem/ apical growth of shoots, including grain yield (Figure 1) and metabolism related attributes (Figure 2). Hussain et al. (2019) reported a significant reduction in plant height and grain yield following the exposure of maize plants to water-deficit stress. Under all stress treatments, the growth and yield performance of soybean cultivar LS678 and TGx1835-10E were also found to be lowered by drought stress than their controls (Mangena 2020a). Among these parameters, morphology serves as one of the most important indicators of successful plant growth and development, and a measure of growth rate in relation to plant exposure to water-deficit stress (Dong et al. 2019).

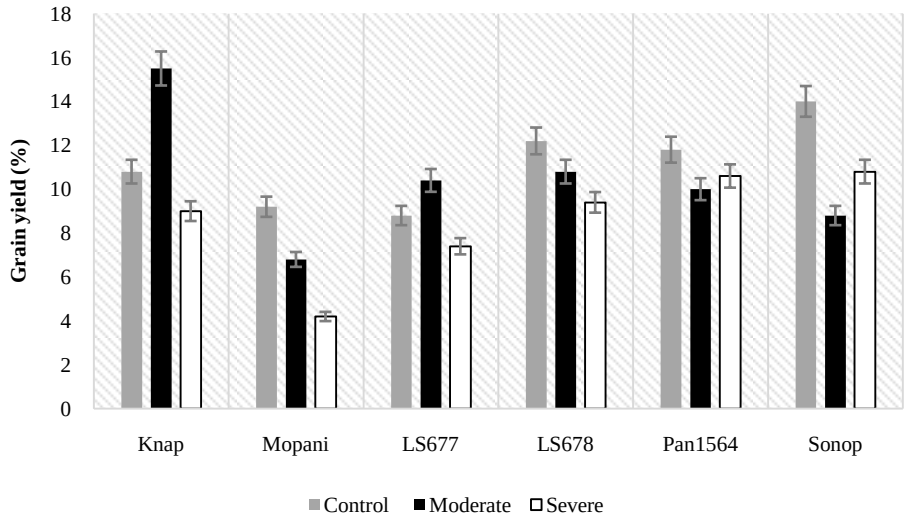

Figure 1 Grain yield of the six soybean varieties analysed at R8 following the exposure of plants to moderate and severe water-deficit stress, as well as well-watered plants used as controls

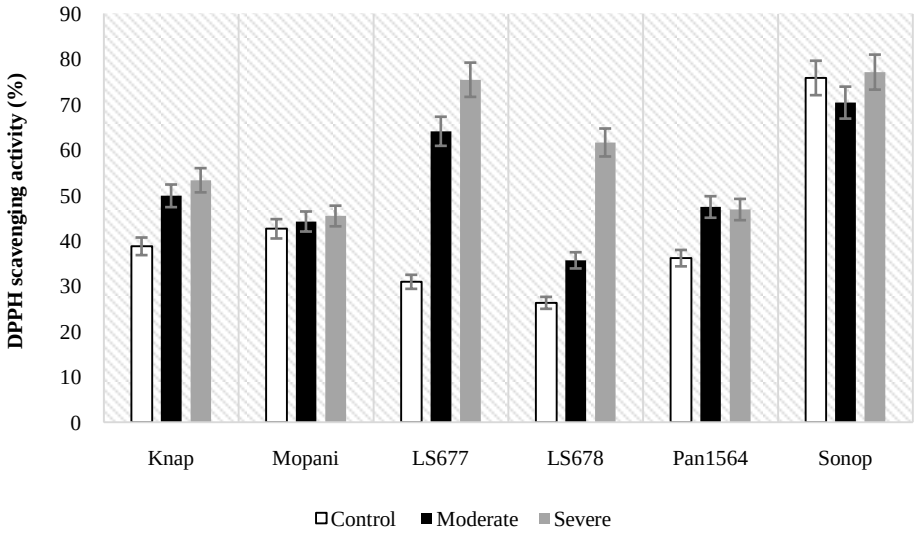

Figure 2 Antioxidant scavenging activity of soybeans analysed after exposure of plants to moderate and severe water deficit stress, and well-watered plants used as controls

The decreasing effects were also observed in leaf surface area (LSA), where the highest average surface area (cm^2) of 114 cm^2 in Mopani was reduced to 66 and 39 cm^2 under moderate and severe water-deficit stress, respectively. All other five varieties (Knap, LS677, LS678, Pan1564, and Sonop) also recorded decreasing LSA values with Pan1564 giving the lowest average of LSA (10 cm^2). Overall, reduced water levels had negative effects on all parameters as indicated by the above observations. Dong et al. (2019) also indicated how leaf area is positively correlated with the amount of sunlight captured by crops, and therefore, directly affecting photosynthetic rates and grain yields. Observations made in this study demonstrated the effects of induced drought stress and changes, especially on total chlorophyll content of plants exposed to moderate (Table 2) and severe (Table 3) water-deficit stress, as well as plants grown under well-watered control conditions (Table 1). As results show, water-deficit stress inhibited the growth of soybean plants in terms of stem height and leaf area (Table 2 and 3) as the level of stress was prolonged. These inhibitory effects became more significant even on reproductive parameters illustrated in Tables 2 and 3, as well as the overall yield in Figure 1.

Both levels of water stress also reduced the number of flowers, pods, seeds/pod, and grain yield in all six soybean varieties (Table 2 and 3). This trend was almost similar to the observation made for the vegetative traits. The highest number of flowers were recorded in Pan1564 (9.0±4.24), followed by a total average of 8.0 flowers in both Knap, Mopani, LS677, and LS678, and the lowest being Sonop with an average total of 6 flowers per plant during moderate drought stress. The prolonged exposure of plants to water-deficit stress elevated the reductions by at least 25% in all varieties and 16% flower reduction in Sonop alone. The results indicate that all reproductive characteristics were altered or dramatically reduced by imposed water stress. Furthermore, grain yields (Figure 1) were also adversely affected by water shortage due to the physical damage of pods and seeds, physiological and biochemical disruptions, as well as molecular changes that are caused by this abiotic stress (Hussain et al. 2018). However, both morphology and reproductive attributes directly reflected the growth and developmental responses of soybean plants to the induced water-deficit stress.

3.2 Physiological response of plants to water-deficit stress

The effect of water-deficit on chlorophyll content (Table 4–6) showed similar trends to those observed for vegetative and reproductive growth parameters (Table 1–3 and Figure 1). The content of photosynthetic pigments was decreased with the decrease in relative leaf water content and also due to the level of water stress (Table 4–6). The reduction in water content of the leaf tissues as indicated in Table 5 and 6 significantly affected the rate of photosynthesis by blocking the transport of energy from

Table 4 Summary of results on physiological parameters analysed in well-watered soybean plants

Varieties	Relative Leaf Water Content (%)	Total Chlorophyll (%)	Total Phenolics (µg/g)	Total Flavonoids (µg/g)	Non-flavonoid phenolics	Leaf Ureides Content (µg/g)
Knap	83.2^{a}	35.1^{a}	24.5 ± 0.14^{a}	19.9 ± 0.43^{a}	4.54 ± 0.07^{a}	0.22 ± 0.39^{a}
Mopani	77.1^{b}	26.5^{b}	26.6 ± 0.08^{b}	19.1 ± 0.67^{b}	7.54 ± 0.70^{b}	0.23 ± 0.09^{a}
LS677	94.0^{c}	36.2^{c}	30.7 ± 0.02^{c}	$19.6 \pm 0.52^{c,a}$	11.1 ± 0.63^{c}	0.28 ± 0.12^{b}
LS678	88.1^{d}	$36.1^{d,c}$	33.1 ± 0.03^{d}	$19.8 \pm 0.57^{d,a,c}$	13.2 ± 0.09^{d}	0.16 ± 0.84^{c}
Pan1564	$94.2^{e,c}$	35.4^{e}	27.3 ± 0.06^{e}	20.8 ± 0.13^{e}	6.48 ± 0.08^{e}	$0.21 \pm 1.02^{d,a,b}$
Sonop	90.0^{f}	$26.3^{f,b,c}$	24.9 ± 0.07^{f}	15.5 ± 0.21^{f}	9.43 ± 0.34^{f}	0.28 ± 0.51^{d}

Different superscript letters within columns indicate statistical significance using ANOVA (t-test) where p-value < 0.05 and values with similar letters are not statistically different at the same p-value.

Table 5 Summary of results on physiological parameters analysed in soybean plants subjected to moderate water deficit stress

Varieties	Relative Leaf Water Content (%)	Total Chlorophyll (%)	Total Phenolics (µg/g)	Total Flavonoids (µg/g)	Non-flavonoid phenolics	Leaf Ureides Content (µg/g)
Knap	69.1^{a}	30.2^{a}	46.3 ± 0.04^{a}	20.6 ± 0.70^{a}	25.6 ± 0.34^{a}	0.26 ± 0.01^{a}
Mopani	74.1^{b}	23.4^{b}	24.7 ± 0.14^{b}	18.7 ± 0.09^{b}	5.99 ± 0.82^{b}	0.34 ± 0.73^{b}
LS677	80.1^{c}	33.3^{c}	48.6 ± 0.04^{c}	19.4 ± 0.33^{c}	29.1 ± 0.02^{c}	$0.37 \pm 0.18^{c,b}$
LS678	88.0^{d}	30.2^{a}	64.0 ± 0.00^{d}	$19.6 \pm 0.03^{d,c}$	44.3 ± 0.18^{d}	$0.29 \pm 0.77^{d,a,b,c}$
Pan1564	83.1^{e}	35.1^{d}	38.6 ± 0.03^{e}	16.4 ± 0.28^{e}	22.1 ± 0.43^{e}	$0.24 \pm 0.21^{e,a,b}$
Sonop	84.1^{f}	$23.2^{e,b}$	24.7 ± 0.02^{f}	14.8 ± 0.91^{f}	9.95 ± 0.41^{f}	$0.30 \pm 1.52^{f,a,c,d}$

Different superscript letters within columns indicate statistical significance using ANOVA (t-test) where p-value < 0.05 and values with similar letters are not statistically different at the same p-value.

Table 6 Summary of results on physiological parameters analysed in soybean plants exposed to severe water-deficit stress

Varieties	Relative Leaf Water Content (%)	Total Chlorophyll (%)	Total Phenolics (µg/g)	Total Flavonoids (µg/g)	Non-flavonoid phenolics	Leaf Ureides Content (µg/g)
Knap	60.0[a]	26.3[a]	30.5 ± 0.06[a]	20.2 ± 0.94[a]	10.3 ± 0.17[a]	0.29 ± 0.95[a]
Mopani	61.0[b]	21.2[b]	32.9 ± 0.03[b]	14.6 ± 0.15[b]	18.3 ± 0.27[b]	0.27 ± 0.38[b,a]
LS677	88.1[c]	31.2[c]	56.2 ± 0.02[c]	18.5 ± 0.03[c]	37.7 ± 0.13[c]	0.22 ± 0.67[c]
LS678	75.2[d]	31.3[d,c]	52.3 ± 0.02[d]	19.0 ± 0.34[d]	33.2 ± 0.97[d]	0.17 ± 1.70[d]
Pan1564	65.0[e]	32.2[e]	35.7 ± 0.05[e]	14.1 ± 0.76[e,b]	21.6 ± 0.28[e]	0.18 ± 0.72[e,d]
Sonop	80.2[f]	21.1[f,b]	28.5 ± 0.11[f]	16.6 ± 0.11[f]	11.9 ± 0.06[f]	0.22 ± 1.6[9f]

Different superscript letters within columns indicate statistical significance using ANOVA (t-test) where p-value < 0.05 and values with similar letters are not statistically different at the same p-value.

photosystem II to photosystem I (Iqbal et al. 2019). This effect led to the decrease in chlorophyll content, especially under extreme water shortage (Table 6), followed by plants exposed to moderate water-deficit stress (Table 5). Mopani and Sonop soybeans recorded the lowest LRWC and total chlorophyll content (%) among all the varieties. Furthermore, shortage of water in leaves and other plant parts possibly caused the production of reactive oxygen species (ROS) which changed the properties of cell membranes (Ahmad et al. 2010) and caused oxidative stress damage to chlorophyll and presumably other molecules such as DNA, lipids, and proteins that were not evaluated in this study.

The oxidative stress that was caused by limited water availability signaled the increase in the production of secondary metabolites (Table 5–6). Phenolics and flavonoids are commonly known as the largest phytochemical molecules with antioxidant properties (Sarkar and Oba 2018). These secondary metabolites were slightly increased under severe water deficit stress (Table 6) than moderate stress (Table 5) in all soybean varieties. As conjectured, the control plants expressed decreased levels of phenolic and flavonoid contents. Observations made were in contrast with findings made by Krol et al. (2014). This study reported decreased levels of phenolic acids in leaves and roots of *Vitis vinifera* under drought stress. Furthermore, the decreased levels of total phenolic compounds in all extracts from grapevine leaves and roots resulted in the lower antiradical activity of the samples obtained from plant parts subjected to drought stress. The study also revealed that long-term drought stress caused a decrease in the level of selected secondary metabolites based on different and specific plant tissues of the grapevine.

However, our results demonstrated high DPPH scavenging activity (Figure 2), suggesting that all extracts obtained from moderate and severe water-deficit stress contained high amounts of antioxidants as indicated in Table 5 and 6, and therefore, possessed high antioxidant activity (Figure 2). LS677, Sonop, and LS678 recorded high antioxidant activity, including high phenol compounds under severe water-deficit stress. These observations are similar to the findings made by many authors who demonstrated that the production of secondary metabolites across species rises under abiotic stress conditions, particularly drought stress (Weidner et al. 2009; Krol et al. 2014; Mohamed and Latif 2017; Habibi 2018; Mangena 2020b).

Conclusion

According to results obtained in this study, reductions in morphological characteristics like plant height and physiological parameters such as chloroplastic pigments dramatically influence growth and productivity in crops. Therefore, these growth attributes could serve as markers in the characterization and selection of soybean varieties that are to be cultivated under low water conditions. Furthermore, improved production of secondary products such as flavonoid and phenolic content, especially under elevated water-deficit stress improved antioxidant activity that indicated reduced sensitivity of selected soybean varieties to this important abiotic stress.

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Conflict of Interest

Authors declare that there is no conflict of interest in publishing this paper.

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Journal of Experimental Biology and Agricultural Sciences, February - 2022; Volume – 10(1) page 21 – 31

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Determination of Trace Elements in Sediments Samples by Using Neutron Activation Analysis

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ABSTRACT

The Juru River is a highly industrialized, urbanized, and agricultural catchment. This study aimed to investigate trace elements in Juru mangrove sediments, including geochemical baselines and enrichment. Sediment was collected from the mangrove in Juru, Penang, Malaysia. A total of eight target elements was examined. Instrumentation activation analysis (INAA) was used to determine the concentration of Fe, V, Cr, Zn and Co. Atomic absorption spectrometry (AAS) was used to determine the concentration of elements that not detectable by INAA (Cd, Pb, and As). In both methods, validated reference material studies were used for validation of the methodology. Metal pollution was estimated using the Enrichment Factor (EF), Geoaccumulation Index (Igeo), Contamination Factor (CF), and Pollutant Load Index (PLI). The EF, Igeo, and CF ranges from 0.45–7.96, -2.18 – 1.95, and 0.33–5.83 respectively. The order of accumulation of the elemental concentration found was Fe > Zn> Cr > V > Pb > As > Co >Cd. The computed mean value of PLI exceeds the unit (PLI > 1).

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1 Introduction

Mangrove ecosystems have high-primary productivity and are coastal forests found in sheltered coasts, estuaries, riverbanks, and deltas in tropical countries. Mangroves are salt-tolerant evergreen trees that grow according to topography, climate, and water salinity. Instead of sand, the soft ground is mostly formed of clay and silt (Sasmito et al. 2020). The inputs of mangrove-derived organic matter into intertidal and river estuaries are important for a variety of fish and shellfish, including many commercial species, because nutrients are often adsorbed onto muddy sediment particles (Hatje et al. 2020). In recent years, the pressures due to increasing population in coastal areas, food production, economic activities, and urban development have caused the resources of the world's mangroves to be degraded (Friess et al. 2019). Growing industrialization and urbanization harmed the environment, particularly in mangrove areas around the world. Naturally occurring and anthropogenic factors trigger its component composition in the mangrove ecosystem (Zhang et al. 2009). Physical activities such as advection and convection, turbulence, diffusion, and others affect trace constituents, which are also influenced by biological processes such as ingestion, excretion, and biodegradation (Brügmann et al. 1999; Naser 2013). Contaminating mangroves and coastal ecosystems with trace and dangerous metals have become a serious threat to coastal ecosystems and humans who rely on marine resources for food and industry. Sediments are an important sink of a variety of pollutants, especially in estuarine ecosystems (Veettil et al. 2019). Metals such as zinc, copper, and chromium are crucial for the functioning of living organisms. However, over-consumption of these metals causes a variety of undesirable effects. Furthermore, due to the bioaccumulation and biomagnification of toxic metals, toxicity increases from low to high trophic levels in aquatic and terrestrial ecosystems will be most severely affected (Talukder et al. 2021; Yunus et al. 2020). Therefore, it is essential to monitor trace elements in sediment to increase knowledge of environmental processes. The elemental composition of nearby pollutant sources and other factors all influence the concentration of elements in sediment (Tiwari et al. 2020).

Many studies have been conducted to determine the level of micronutrients in Malaysian rivers, including Selangor (Kadhum et al. 2015), Sg. Terengganu, Terengganu (Sukri et al. 2018), Sg. Baleh in Sarawak (Chai et al. 2018), Sg. Langat Basin, Sg. Kepayang, Perak (Affandi and Ishak 2018), Sg. Linggi, Negeri Sembilan (Khalaf et al. 2018), Sg. Selangor, Selangor (Othman et al. 2018), Sg. Liwagu and Sg. Mansahaban in Sabah (Tair and Eduin 2018). However, until today there is a lack of information on the content of trace constituents in the mangrove sediments of Malaysia which are suppressed by various pollutants from upstream to downstream rivers.

The trace and toxic elements in sediments are analyzed with established analytical techniques which include alpha spectrometry, X-ray spectroscopy, Auger Electron Spectroscopy (ICP-AES), Atomic Absorption Spectroscopy (AAS), Inductively Coupled Plasma Mass Spectrometry (ICP-MS), and instrument neutron activation analysis (INAA) (Tiwari et al. 2020; Diarra and Prasad 2020). However, each method presents advantages and disadvantages according to a particular task and purpose. The INAA is the most employed method for finding various types of ingredients due to its small sample size, chemical-free procedure, high sensitivity of low detection limit (LOD), concurrent multi-element detection (30-40 elements), negligible matrix interference, samples changed to radioactive materials (Win 2004; Benarfa et al. 2020; Carvalho et al. 2019). In this work, two techniques, instrument Neutron Activation Analytical Instrumentation (INAA) and Atomic Absorption Spectrometry (AAS) were used. The AAS method was used for elements not sensitive to the INAA method. The sensitivity (10 to 30 mg/sample), accuracy, and precision are of the same order for the two techniques and the choice can only be made depending on the urgency or convenience.

Determination of the concentrations of elements in the sediment of mangrove areas are among the first steps in the quantification of the natural and anthropogenic effects on mangrove ecosystems. A high level of contamination can cause hazardous effects on the ecosystem. Thus, the objective of this study is to provide important information on the ecological risk from the mangrove environment by evaluating enrichment factors, contamination factor, pollution load index and the geo-accumulation index. The INAA approach is used to determine the concentration of multi-trace elements in sediments in this work. Alternative method (AAS) was used for those elements not detectable in INAA.

2 Materials and Methods

2.1 Study sites and samples collection

Sediment samples were collected from the mangrove along the Juru River in Penang, Malaysia, as depicted in Figure 1. The sampling was carried out in January 2017 to detect hazardous substances at a trace level of mangrove of Juru River which is roughly a 12 km drive south of Butterworth. The Juru River, 7.95 km in length, flows from Bukit Minyak in the west to the South China Sea. The Juru River also crosses the North-South route, which extends 144.9 km between Ipoh and Alor Setar. The Permatang Rawa River and the Rambai River are two rivers that flow into the upper reaches of the Juru River. The Juru River is a lifeline for Kuala Sungai Juru, where the majority of the residents of Bukit Minyak are fishermen who rely heavily on fishing for their living. Furthermore, the Bukit Minyak area is famous for its

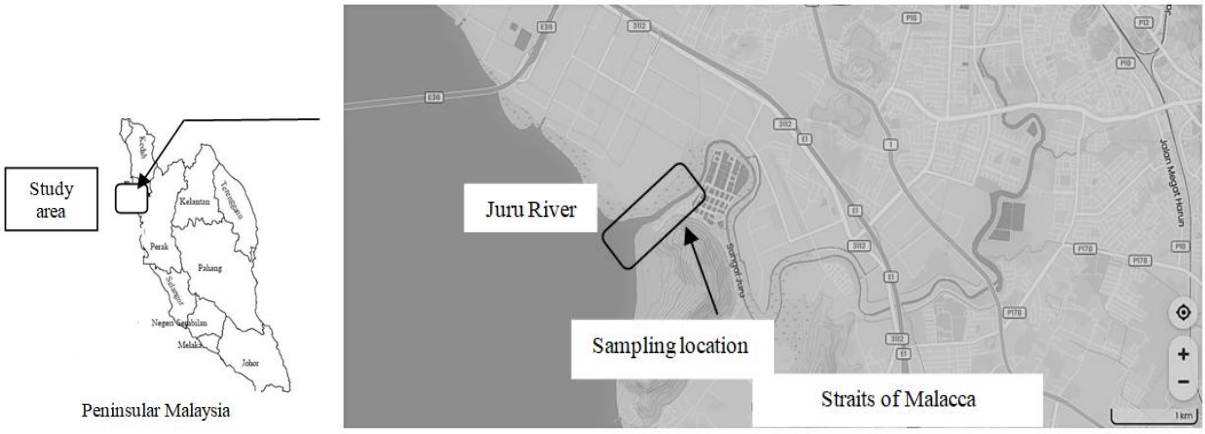

Figure 1 Map showing the sampling site mangrove area along Juru river, Penang, Malaysia

cockle-breeding sector (industrialization). Before the industrial activities, the Juru River was free of pollution and its water was crystal clear. However, since 1970, the environmental landscape has changed as a result of industrial and building projects, as well as human settlements along the Juru River's riverbanks.

For uniformity and to be more typical of the sampling location, fifty sediment samples were collected and mixed. Sediment samples within range of 3.0–5.0 m adjacent to each other and approximately depth 5.0 cm were collected from the intertidal muddy region in the Jura mangrove area by using a pre-cleaned plastic scoop (Kumar et al. 2014; Yap et al. 2002). The collected surface sediment samples were placed in re-sealable polyethylene plastic bags labeled and held in an icebox at 4°C. For moisture removal, surface sediments were dried in an 80°C oven for at least 72 hours on an oven-dry weight. After being crushed in an agate mortar with an agate pestle, the powdered form of sediments was sifted through a 63 µm stainless steel aperture. The samples were stored in plastic pillboxes after being forcefully shaken (Ashraf et al. 2016; Wang et al. 2010; Rezaee et al. 2011).

2.2 Chemical measurement

The powdered samples were divided into four subsamples of approximately 150 mg and 200 mg and stored separately in heat-sealed polyethylene vials for short and long radiation respectively. The half-lives and gamma energies of the radionuclides were used to identify them, and the element concentrations were determined using a comparative method (Kumar et al. 2014; Benarfa et al. 2020). The standard reference materials SRM (IAEA–Soil-7, SL–1 (Lake Sediment) were used as a multi-element comparator. For quality assurance purposes, blank samples, standard reference material, IAEA-Soil-7, and IAEA-SL-1 were irradiated together in a pneumatic transport facility at the research reactor operated at 750 kW (MINT TRIGA) with thermal neutron flux of $4.0x10^{12}$ cm^{-2} s^{-1}. The samples were radiated for 1minute and counted for 5 and 20 minutes after a cooling time of 20 minutes and 24 hours respectively during short radiation. For long radiation, the samples were irradiated for 6 hours and counted for 1hour after a cooling time of 3–4 and 21–28 days respectively. Counting of radiated samples was performed by using calibrated high-resolution HPGe detector. Distance between detector and sample was maintained approximately at 12 cm (short radiation) and 2 cm (long radiation). Specific energy of delayed gamma rays was used to identify the elements present in the sample and their concentration. The efficiency of the gamma spectroscopy system was calibrated with an energy range of 60–2 MeV under the same geochemical configurations by using ^{133}Ba, ^{60}Co, ^{57}Co, ^{137}Cs, ^{54}Mn, and ^{24l}Am standard point sources. All the counting was performed under the condition with a dead time lower than 10% (Kumar et al. 2014).

Approximately 500 mg of powdered sediment sample was digested in ratio 4:1 of nitric acid and perchloric acid (BDH Analar grade). All the digestion tubes were placed into a digestion block at 40 °C for the first hour and then were completely digested approximately at 140 °C for at least 3 hours (Yap et al. 2002). 40 mL of double distilled water was used to dilute the resulting solution. The solution was then filtered out through a Whatman TM No. 1 filter paper Cat No 1001 090 (diameter: 90 mm) into a polyethylene container. A Perkin Elmer Model Analyst 800 and an air-acetylene atomic absorption spectrophotometer (AAS) were used to determine the concentrations of the elements. All the samples were analyzed in three replicates (Yap et al. 2002).

2.3 Heavy metals enrichment and data treatment

Several indexes including the enrichment factor, contamination factor, geo-accumulation index, and pollution load index were considered to assess the metal status for the sampled sediments.

2.3.1 Enrichment Factor (EF)

The enrichment factor is one of the most useful methods for determining if anthropogenic contamination exists based on the computation of the normalization of each element concentration to Fe or Al. The significance of anthropogenic metal inputs was analyzed by enrichment factor (EF) (Zoller et al. 1974) and can be assessed by using the equation below:

$$EF_{metal} = \frac{\left(\frac{M_{exp}}{Fe_{exp}}\right)_{sample}}{\left(\frac{M_{ref}}{Fe_{ref}}\right)_{shale}}$$

Where M_{ref} or Fe_{ref} refers to a common abundant element in the average shale, while M_{exp} or Fe_{exp} refers to element concentration in the experimental sample (Mucha et al. 2003) (Table 1).

2.3.2 Geo-accumulation Index (Igeo)

Muller (1969) developed the Geoaccumulation index (Igeo), which is one of the most dependable indexes for calculating a system's contamination state. Based on the Geoaccumulation index (Igeo), the degree of metal pollution can be divided into seven enrichment classes as given in Table 2. Geo–accumulation index is calculated by the following equation:

$$I_{geo} = log_2\left[\frac{c_n}{1.5 \times B_n}\right]$$

Where C_n and B_n refer to the element's concentration in the sediment samples and its baseline value, respectively. Factor 1.5 is used to remove the effect of background value changes that could be attributed to lithology variations in the sediments (Turekian and Wedepohl 1961; Stoffers et al. 1986).

2.3.3 Contamination Factor (CF)

Contamination Factor (CF) suggested by Hakanson (1980) and requires at least five surficial sediment samples which are averaged to produce a mean pollutant concentration and then compared with baseline pristine reference level (Abrahim et al. 2008). The contamination Factor is calculated by the following equation:

$$CF = \frac{C_{sample}}{C_{background}}$$

Where C_{sample} is the metal concentration and $C_{background}$ represents the mean background value of the metal in sediment (Table 3).

2.3.4 Pollution load index

The pollution load index (PLI) was proposed by Tomlinson et al. (1980). PLI values indicate heavy metal loads near the background level or define as no pollution if the value is lower than one, while the values greater than one indicate soil pollution or contamination (Cabrera et al. 1999). PLI is calculated by the following equation:

$$PLI = \sqrt[n]{CF_1 \times CF_2 \times CF_3 \times .....CF_n}$$

Where CF is the contamination factor; n, number of metals (Table 4).

Table 1 EF classification and sediment contamination status

Index	Classification	Sediment enrichment status	Reference
EF	≤ 2	Low enrichment	Diop et al. 2015
	2–5	Moderate enrichment	
	5–20	High enrichment	
	20–40	Very high enrichment	
	> 40	Extremely enrichment	

Table 2 Igeo classification of the polluted samples

Index	Classification	Sediment pollution status	Reference
Igeo	< 0	Unpolluted	Wang et al. 2015
	0–1	Unpolluted to moderately polluted	
	1–2	moderately polluted	
	2–3	moderately to strongly	
	3–4	Strongly	
	4–5	strongly to extremely strongly	
	> 5	extremely polluted	

Table 3 CF classification and sediment contamination status

Index	Classification	Sediment contanination status	Reference
CF	< 1	low contamination	Nasr et al. 2006
	1-3	moderate contamination	
	3-6	mean considerable contamination	
	> 6	high contamination	

Table 4 PLI classification and sediment contamination status

Index	Classification	Sediment pollution status	Reference
PLI	< 1	no pollution	Zarei et al. 2014
	1-2	moderate pollution	
	2-3	heavy pollution	
	> 3	extremely heavy pollution	

Table 5 The analysis of the standard reference material and comparison with certified values of SL–1 and Soil–7(in mg/kg)

Elements	Standard Value	Measured Value	Recovery (%)	z-score
Fe	67400 ± 1700	67939 ± 1332	101	0.25
Cr	104 ± 9	86 ± 6	83	-0.44
Pb	60 ± 8	49 ± 5	82	-0.33
V	170 ± 15	157.5 ± 21.4	93	-0.03
Zn	223 ± 10	247 ± 17	111	-0.08
As	27.6 ± 2.9	25.5 ± 4.2	92	-0.10
Co	19.8 ± 1.5	20.4 ± 2.9	103	0.06
Cd	1.3 ± 0.8	1.6 ± 0.4	123	0.31

2.4 Statistical analysis

The *Z*-test was used to assess the precision and accuracy of the analytical processes used. The standardized difference *Z* is calculated by taking into account the uncertainty in the SRM measured results as well as the uncertainty in the certified value to determine the analytical method's accuracy. The *Z*-test data is defined by:

$$Z = \frac{c_m - c_{ref}}{\sqrt{\sigma_m{}^2 + \sigma_{ref}{}^2}}$$

Where c_m and c_{ref} referred to measured and reference values of SRM, σ_m, and σ_{ref} are the standard deviation of measured and the reference value of SRM. If the value of Z-test < |3|, it shows that the results of the analysis of the SRM should be in the 99 % confidence interval of the certified value. If the *Z*-test value is < |3|, the results of the SRM analysis should be within the 99 percent confidence interval of the certified value (Alnour et al. 2011, Alfian et al. 2020).

3 Results and Discussion

The quality and validity of the analytical method were checked with standard reference material SRM IAEA–Soil–7 and SL–1. Analytical results of the standard reference material and measured values (concentrations and its standard deviation) of IAEA soil-7 and SL-1 are shown in Table 5. The recoveries between the standard reference material and measured are within the range of acceptable (82–123%) as shown in Table 5.

As shown in Table 5, the Z-test scores calculated by the analysis technique for all elements are within the acceptable range of -3 to +3. It's worth noting that the measured SRM values were randomly distributed (above and below) to the certified levels, indicating that the analytical procedure did not make any systematic errors. The correlation coefficient of the linear regression between the

determined concentrations of IAEA SRM Soiil-7 and SL-1 with those certified values was 0.9997.

The INAA and alternative technique, AAS were used to identify and quantify a total of eight elements in sediments. Iron (Fe) was the most abundant harmful element in seawater at trace levels, while cadmium (Cd) was the least abundant. Overall, target element abundance was found to be in the following sequence in sediments: Fe > Zn> Cr > V > Pb > As > Co > Cd. The composition of natural sediments and their anthropogenic sources around the sampling site may determine the order of abundance of the compounds. The levels of target hazardous elements observed in sediments from the Juru River in Malaysia (the current study) were comparable to data from other sections of the surface sediments collected from Kerteh mangrove forest where the average concentration of Co (8.91µg/g), Cu (29.0 µg/g), Pb (11.7 µg/g), Zn (22.3 µg/g) and Cr (13.2 µg/g) (Yunus and Chuan 2008) and Tanjung Lumpur mangrove forest the average concentrations of Pb, Cu, Co, and Mn were 44.41 µg/g, 32.79 µg/g, 5.79 µg/g and 117.73 µg/g respectively (Yunus et al. 2011). As shown in Table 6, the mean concentration of trace elements of the study area is about the same as other studies except for Co(8.23 mg/kg) which is much lower compared with the concentration of Co (276 mg/kg) from Badovci Lake (Kosovo) (Malsiu et al. 2020). Relative to the USEPA guidelines (PEL Indicators), the concentration of trace element Zn and Pb in the Juru river were much higher than the USEPA limit (USEPA 1994).

Table 6 The concentrations of trace elements (in mg/kg)

Element	Minimum	Maximum	Average	Standard deviation	Other studies	Maximum permissible limit
Fe	33405	37232	34579	1797	35900[a]	-
Cr	78.85	89.04	82.21	4.62	62.8[a]	160*
Pb	27.64	45.25	34.52	9.41	100.94[a] 14.2–79.28[b]	8.1*
V	69.94	87.72	77.13	8.71	88.2[d]	-
Zn	335.24	360.71	348.10	11.44	273.29[a] 168.08–682.31[b]	81.0*
As	9.15	11.47	10.50	0.98	11.2[c]	15.0*
Co	7.70	8.89	8.23	0.49	276[d]	23.0**
Cd	0.572	0.696	0.639	0.063	4.713 0.9–4.8	38.0*

[a]Chen et al. 2016; [b] Ghannem et al. 2016; [c]Li et al. 2008; [d]Avni Malsiu et al. 2020; *USEPA 1994, **PAAS

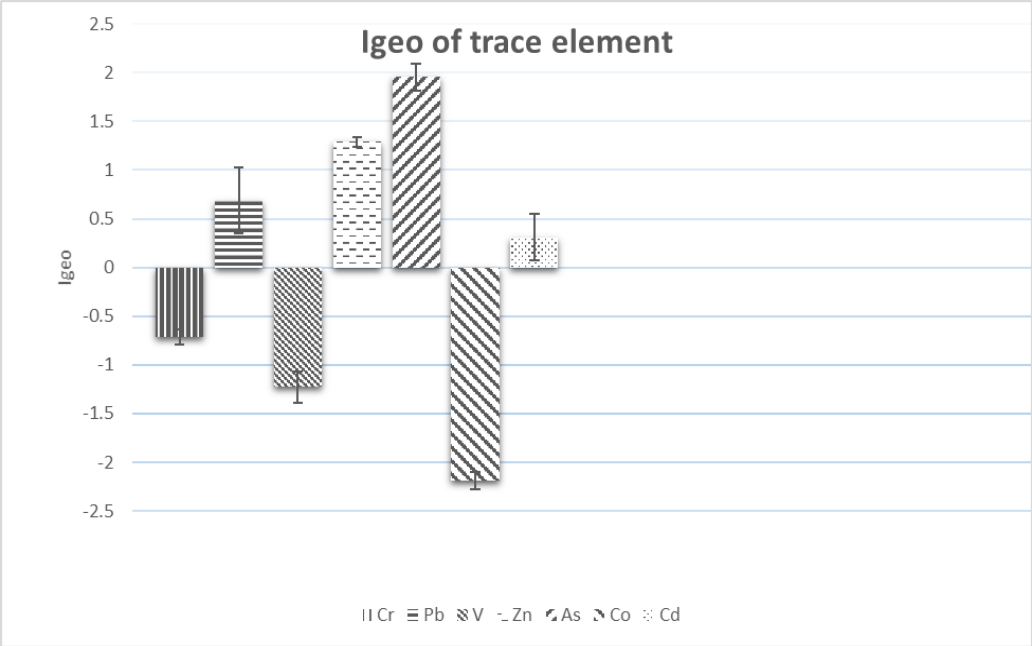

Figure 2 Geo-accumulation index (Igeo) of the elements in the sediment

The Igeo, EF, CF, and PLI of As, Cd, Cr, V, Pb, Zn, and Co in the sediment samples are shown in Figure 2, Figure 3, and Figure 4 respectively. The lowest EF (0.45) was found for the Co while the highest (7.96) for the As. Most of the trace elements showed higher EF values except for Co and V. The EF value of the studied location, Juru indicates minor to moderately severe enrichment of Cd, Pb, Zn, and As. Based on the I_{geo} index as shown in Figure 3, the lowest Igeo found was -2.18 (Co) and the highest 1.95 (As). The EF of As and Zn of the current study is about the same as reported by another study 7.3 and 6.6 respectively (Yii et al. 2020). Most of the trace elements fall in class 0 and 1 are defined as unpolluted and unpolluted to moderately polluted respectively, except for As and Zn which are 1.95 and 1.29 respectively. The CF and PLI were used to assess the status of the heavy metals in sediments (Bhuiyan et al. 2010). Like other parameters, the lowest CF was found 0.33 for Co and the highest 5.83 for As. The Cr, V, and Co showed low contamination. The Pb and Cd reach moderate contamination. The computed mean value of PLI exceeds the unit (PLI > 1).

It was found that all pollution tools (EF, Igeo CF, and PLI) indicate sediments from mangrove areas, Juru river contaminated with As and Zn compared other trace constituents in this work. Special

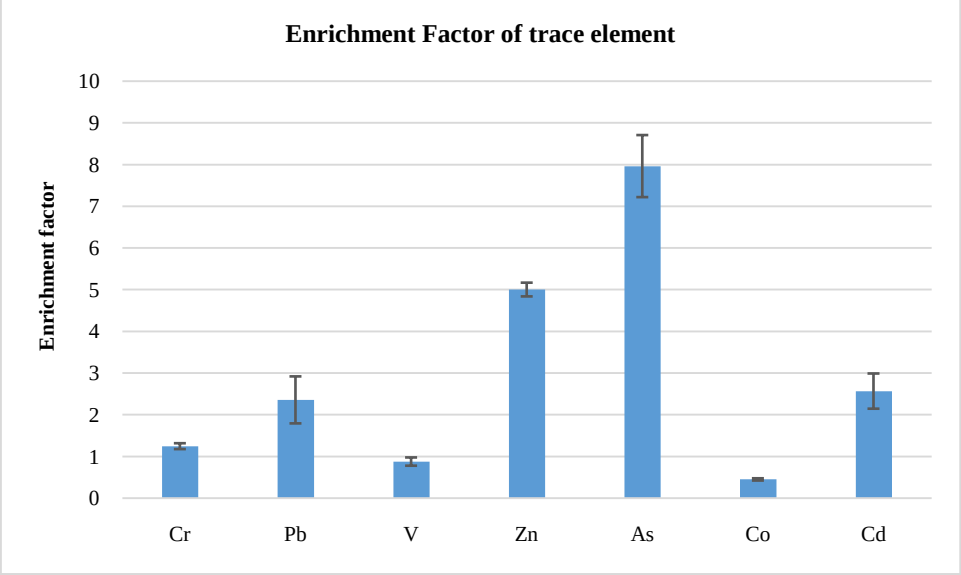

Figure 3 Enrichment Factor (EF) of the trace elements in the sediment

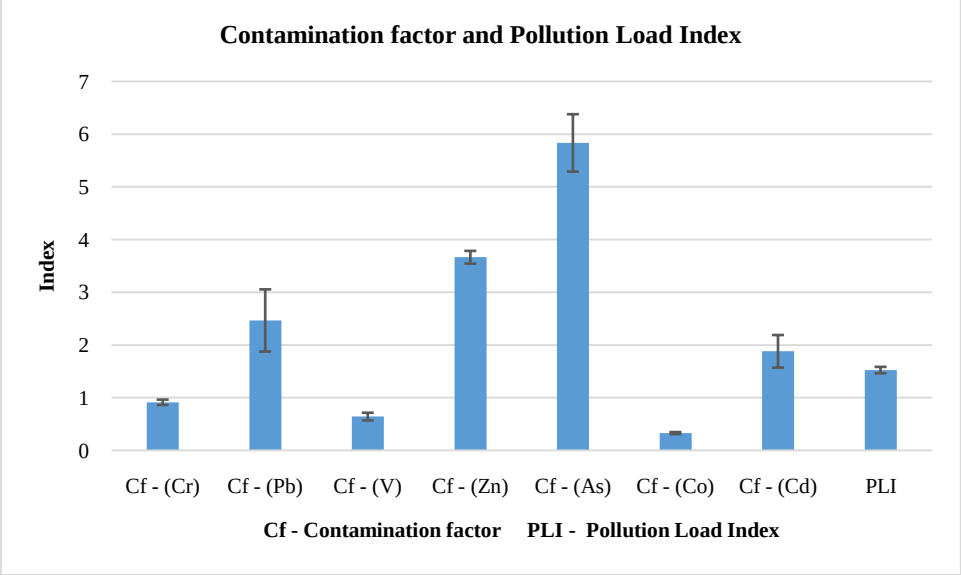

Figure 4 Contamination factor (Cf) and Pollution Load Index (PLI) of the elements in the sediment

consideration needs to be given to As and Zn for their mobility and bioavailability in the aquatic ecosystem. The highest enrichment factor (EF) is mostly due to anthropogenic sources of heavy metals, such as waste disposal, industrialization, urbanization, and others (Gao et al. 2020). This could occur as a result of untreated industrial, residential, and agricultural waste products (Talukder et al. 2021; Jahan and Strezov 2018). As reported by other studies, these could be due to the Juru River receiving a discharge of waste product from several small rivers that flow through urban areas which are active with human activities and originated from non-natural weathering processes or non-crustal materials caused by anthropogenic sources (Yap et al. 2009; Valiela et al. 2002). On the other hand, other activities such as loading and unloading fish from the fisherman and cleaning of boats and maintenance may be also contributing enrichment factors is higher than other sampling locations. Human activities such as tourism, agriculture, aquaculture, and urban sprawl are the main drivers linked to the destruction of mangrove areas. Uneaten fish feed, fish waste, and any organic material that is produced by fish farming activities also destroyed mangrove areas and vary between regions (Al-Shami et al. 2011). There are approximately 300 local fishermen who depend on fish and shellfish catches in rivers and estuaries who are worried about the potential impact on the environment following the implementation of shrimp farming projects in the area near the river Juru (Press Statement, 9 October 2012).

Conclusions

Juru mangrove sediments were found to be significantly impacted by toxic levels of trace metals (Zn and Pb) above the EPA standard. The EF and Igeo values indicate that sediments of mangrove Juru river severely contaminated with As and Zn. The continued discharges of abandoned metals components and effluents from industrial and commercial activities along the Juru River, such as fishing (nets, hooks, etc.), shipping, lumber processing, and outboard engine boats, may have increased the possibility of these metals (As and Zn). As a result, there are serious environmental issues when a bioavailable proportion of hazardous materials directly damages estuarine fish and other biota by incorporating them into the food chain. This study thus provides baseline information on metal concentration and anthropogenic impacts on mangrove forests of Juru and requires the continuous monitoring.

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Conflict of Interest

Nil

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Journal of Experimental Biology and Agricultural Sciences, February - 2022; Volume – 10(1) page 32 – 47

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Effects of Variety, Spacing and Nitrogen Application on Chickpea (*Cicer arietinum)* Growth and Yield in Embu County, Kenya

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ABSTRACT

Chickpea is a pulse crop that is rich in proteins and helps in fulfilling the requirement of protein for vegetarian people thus yield maximization needed. This study aimed to determine the effects of spacing, nitrogen rates, and their interactive effects on the growth and productivity of the selected chickpea varieties. Field experiments were conducted at Mwea, Kenya between 2017 and 2018 involving four varieties (Saina K, Mwanza 2, Chaina I and Chaina III) at a spacing of 50x10cm, 50x20cm, and 50x30cm, and starter nitrogen-fertilizer application rates 0kg, 30kg, 60kg, and 90kg ha^{-1}. A split-split plot design arranged in a 4x3x4 layout was used in the current study. Data related to plant height, biomass, grain yield, and harvest index were collected and subjected to statistical analysis by GLM in SAS 9.4 computer software. Variations occurred in measured traits like the height of crops (34.81-38.00cm), biomass yield (3.31 - 8.08t ha^{-1}), seed yield (0.14 to 1.9t ha^{-1}), and percent harvest index (5 - 45%) was reported. Mwanza 2 expressed the highest plant height, biomass, and grain yield. The highest plant biomass was obtained under 50x10cm spacing, while the highest grain yield weight was reported under 50x30cm spacing x 60kg N ha^{-1}. From the results of the study, it can be concluded that the highest enhanced growth and productivity of chickpea were realized at interactions of Mwanza 2x50x10cmx90kg ha^{-1} nitrogen rate in the study area.

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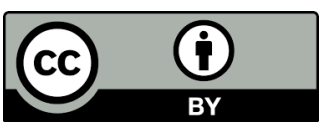

1 Introduction

Chickpea (*Cicer arietinum)* belonging to the Fabaceae family, is an important legume crop that is rich in protein. It has numerous agricultural benefits that made this crop an excellent crop rotation and intercrop mainly with cereal crops. It plays an important role in improving soil health and breaking some diseases and pest cycles (Raimi et al. 2017; Li et al. 2019; Fikre et al. 2020). The inclusion of chickpea in rotation to non-host plants provides sufficient time for decomposition of infested crop residues and reduces the viability of the pathogens (Berrada et al. 2007). Chickpea enhances soil fertility by its biological nitrogen fixation ability. It is estimated to fix up to 140kg N ha^{-1} from the air through symbiotic nitrogen fixation and meets approximately 80% of soil N requirement (Hossain et al. 2016; Schilt-van et al. 2020). In Kenya chickpea crop grows as an intercrop to improve soil fertility and maximize yield (Ogola et al. 2013; Ndukhu et al. 2017). Additionally, the crop act as biocontrol of grassy weeds when used as an intercrop with cereals by covering the ground and smothering the weeds (Banik et al. 2006; Mousavi 2019,). This crop also has the ability of drought tolerance and can survive under scanty rainfall conditions with optimum precipitation of 152-254mm and temperatures range of 18-29^{o}C (McVay and Crutcher 2011; Devasirvatham and Tan 2018; Kaloki et al. 2019a). Its deep root system penetrates deeper soil horizons for moisture (Onyari et al. 2010; Arif et al. 2021). The legumes are a good source of proteins, dietary fiber, vitamins A, C, E, folate, and minerals (Wallace and Zelman 2016; Muehlbauer and Sarker 2017). This crop is used as human food and surplus sold to earn income, while its remnants are used as fodder for animals.

Worldwide, the chickpea crop has the third position after common bean (*Phaseolus vulgaris*) and field pea (*Pisum sativum)*. Internationally, Asia, Europe, America, and Australia are key chickpea producers (Wafula et al. 2021) and accounting 95% of the world's chickpea production (Merga and Haji 2019). The global chickpea production steadily increased from 7.7million tonnes to over 14.2 million tonnes from 2006 to 2019 (FAOSTAT, 2019). According to Merga and Haji (2019), over 1.3 million tons of chickpea from major producers enter the market to supplement the needs of countries unable to meet their production demands. A number of these deficient countries are in Africa where the demand is higher than the supply (Maya and Maphosa 2020). Major chickpea growing areas of Africa are located in the North, West, and Eastern Africa (Maya and Maphosa 2020). These countries contribute about 63% of total African chickpea production (Abebe and Debebe 2020).

In Kenya, chickpea is ranked fourth after common bean, field pea, and cowpeas (Mallu 2015). Its production is dominant in the dryland regions that include parts of the Rift Valley and Eastern regions of Kenya including Mwea, Embu County. Farming systems within Mwea, Karaba location, are rain-fed with limited use of inorganic fertilizers. Pellic vertisol is the dominant soil in the area. Crops grown in the region are drought-tolerant, such as field pea, cowpea, chickpea, and sorghum (Wafula et al. 2021). The annual chickpea productivity in Kenya is low with an average yield of 357kg ha^{-1} (FAOSTAT, 2019). The limited performance of crops might be due to selection from a narrow range of landraces (Rao et al. 2012). The main varieties used in Kenya are (*Desi* 1), ICCV 00108, ICCV 92944 (*Desi* II), ICCV 97126 (*Desi* III), and Saina K, ICCV 00305 (*Kabuli*) (Gaur et al. 2010). Drastic variation in soils and differences in agro-ecological zones contribute to poor crop performance (Jaetzold and Schmidt 1982). In addition, the landrace varieties may not be adaptable to all agro-ecological zones thus, necessitating test performance to determine suitability in growth areas. It, therefore, becomes needful to test the performance of these varieties in specific areas.

Another factor that affects the chickpea performance is sowing spacing due to influence in light interception hence photosynthesis. Spacing affects the number of soil nutrients and water that an individual plant can access (Khan et al. 2010). Initial spacing of 30x10cm tested in Kenya gave yields between 1200 to 1500kg ha^{-1} (Gaur et al. 2010). Due to changing climate and soil fertility, revision of this spacing is necessary to enhance the productivity of the crops (Kamithi et al. 2009; Ngetich et al. 2014). Agajie (2014) reported variation in chickpea yields from 1219kg ha^{-1} (30 x10cm), 1134kg ha^{-1} (30x15cm), 1049kg ha^{-1} (40x10cm), 1019kg ha^{-1} (50x5cm), 733kg ha^{-1} (50x15cm) and 1088kg ha^{-1} (20x10cm) under various spacing. Further, Mallu (2015) also reported that in Kenya biomass of chickpea varieties ranges from 2.9 to 7.4 tonnes per hectare under different spacing growth conditions. Similarly, Kerina et al. (2017) also obtained higher biomass and grain yield for various crops under 45x20cm spacing across various regions.

Over the years, soil conditions have continuously deteriorated due to nutrient mining by continuous cropping without replenishing the nutrients, nutrient export (crop residues transferred to other areas), overcropping, and mono-cropping (Kiboi et al. 2019). In addition, erosion due to erratic and unreliable rainfall and prolonged droughts in arid areas also contributes to nitrogen losses (Kimiti et al. 2009). Cumulatively this contributes to reduced chickpea productivity. Other factors that have contributed to low N in soil include leaching due to floods, increased human activity such as rampant bush clearing and burning (Kerina et al. 2017). Accumulations of these activities have led to the reduction of soil N to about 0.12% of the average plant requirement in semi-arid regions of Kenya (Wafula et al. 2021). Although chickpea can fix nitrogen (N), soils with N deficiency require small doses of starter N-fertilizer at planting time to promote early growth (Lemma et al. 2013; Dar et al. 2021). Thus, legumes also need N-fertilizer

application to boost their productivity (Li et al. 2016). Variability in N-levels, spacing, and narrow chickpea germplasm in growing areas of Kenya, may be responsible for depressed yields of chickpea in Embu county. This study, therefore, investigates the effect of variety, plant spacing, level of nitrogenous fertilizer at sowing time, and their interactions on plant growth and grain yield of chickpea in Embu County.

2 Materials and methods

2.1 Site description and environment

Field experiments were conducted in the Karaba area of Mwea Ward, Mbeere South Sub-County, Embu County, Kenya (Figure 1). The study site lies at coordinates 0°46'14.4822'S and 37° 22'23.79324'E and an altitude of 980 meters above sea level. Karaba receives bimodal rainfall patterns with annual rainfall amounts ranging from 600 to700 mm according to MIAD weather data, resulting in growing seasons between March to May and October to December (Wafula et al. 2021). In addition, the average minimum and maximum temperatures fluctuate between 18-20°C and 19-24°C (KMD 2017) respectively. However, over the cropping period, precipitation recorded was 264.3mm (LR2017), 408mm (SR2017), and 787.1mm (LR2018), while minimum and maximum temperatures recorded ranged from 13-16°C and 22-29°C respectively (Figure 2). Site selection for field trials was due to the uniformity of the soils and environmental conditions among many farmers in the local area (Figure 1) (Wafula et al. 2021).

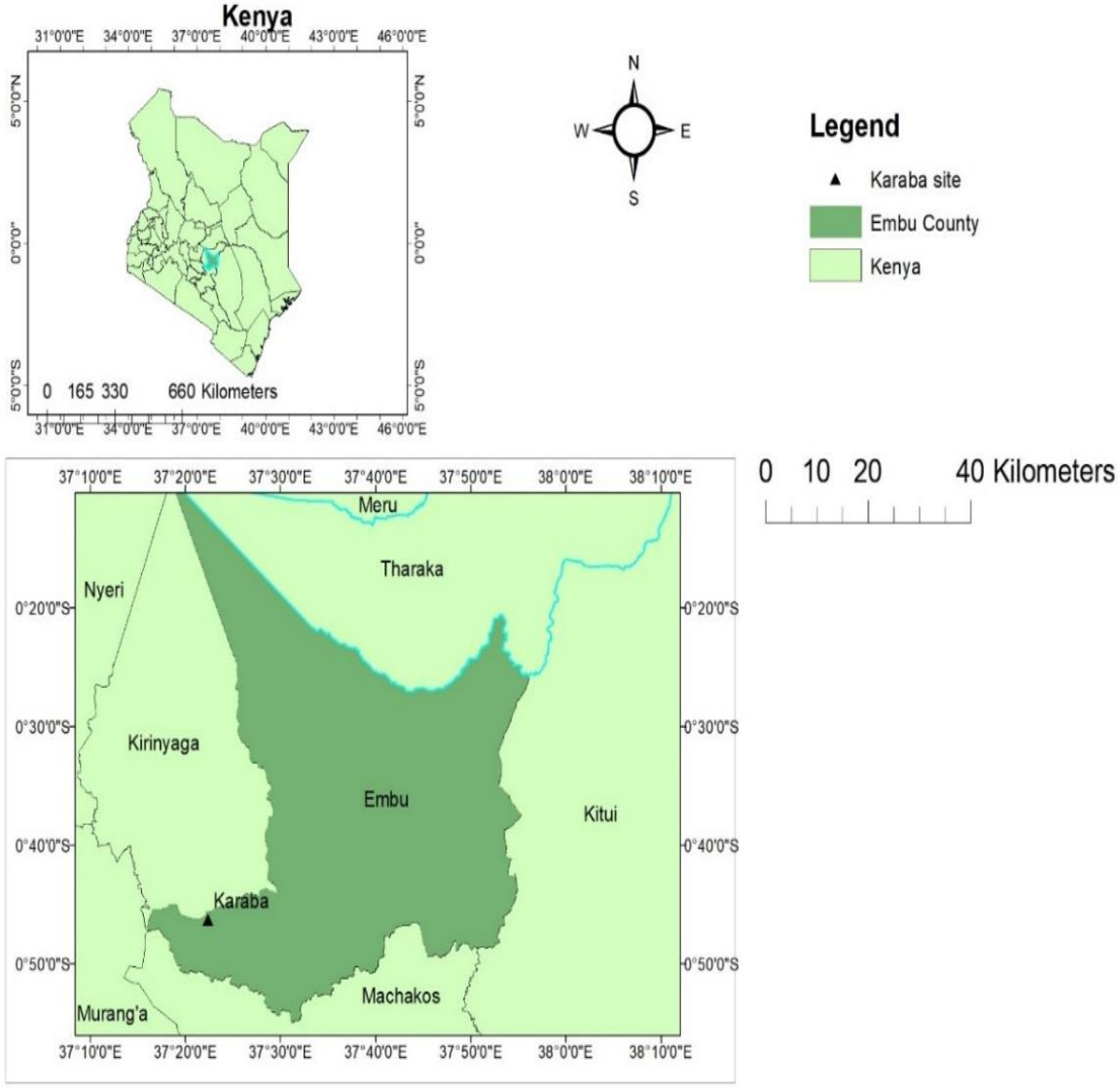

Figure 1 Map of the study area. Karaba. Explain the scale

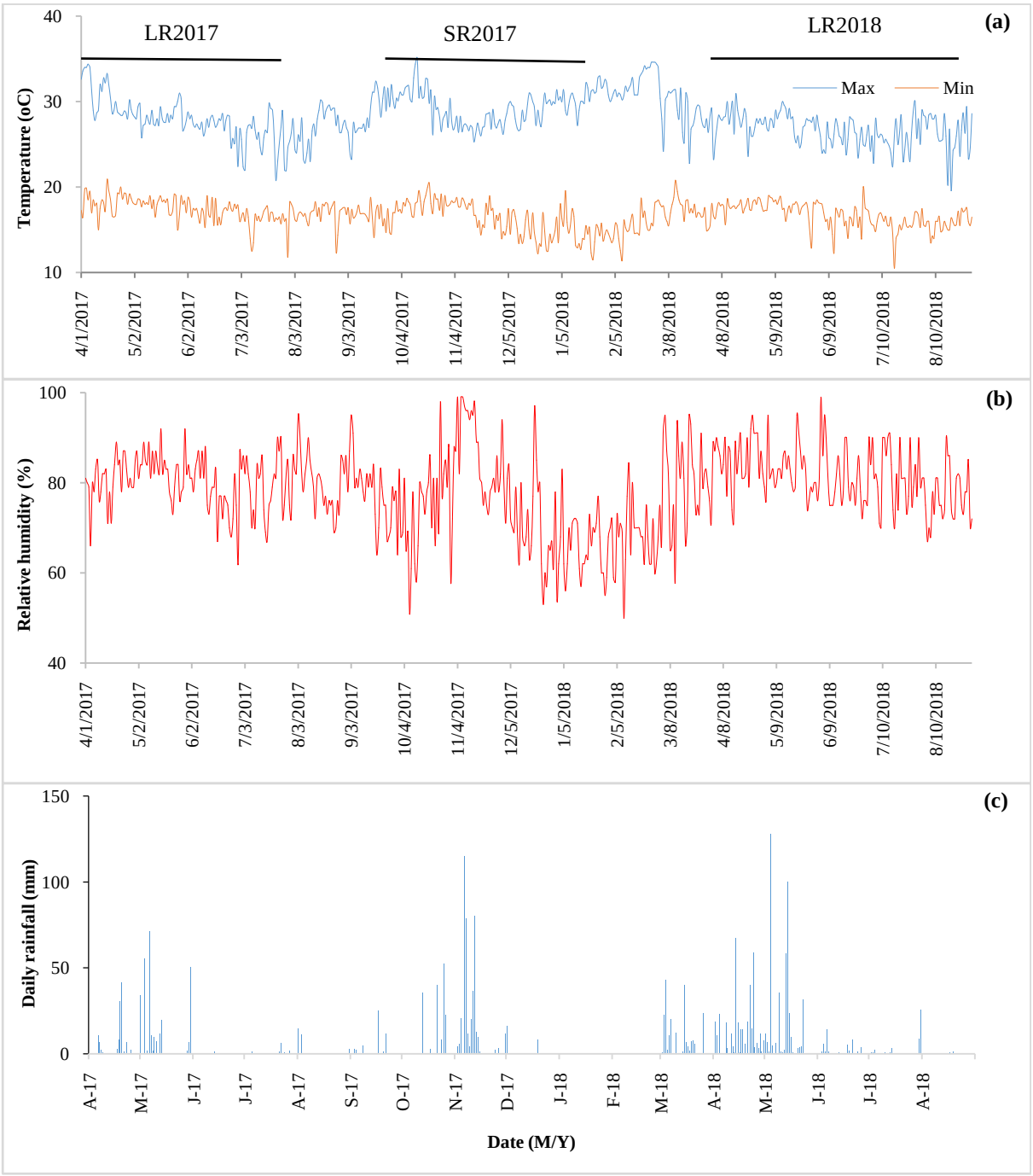

Figure 2 Daily weather data a) maximum and minimum temperature (^{0}C), b) relative humidity (%) and c) rainfall (mm). Max indicates maximum and min indicates minimum. LR is the long rains and SR is the short rains. Source: (KMD 2017; KMD 2018). In X-axis first leter stand for month and 17= 2017 while 18=2018.

2.2 Setting the field trials

The experiment was a split-split plot design (SSPD) of a 4x3x4 factorial arrangement. Each plot have a 3x3m size and had six rows of the variety having 30, 15, and 10 planting holes per row concerning spacing used. Accordingly, the arrangement of factors within the plot was chickpea variety (main plot), spacing (subplot), and inorganic fertilizer (sub-sub plot). Land preparations were done in February 2017, before the onset of long rain using a tractor-mounted with a disc plow to remove hardpans, followed by

harrowing using a disc harrow for the first season (LR2017), then leveling using a hoe (jembe). A hoe was used in subsequent seasons in September for season 2 (SR2017) and in February for season 3 (LR2018) for all field operations.

Sourcing of planting materials was from Kenya Seed Company, Nakuru, while di-ammonium phosphate (DAP) was collected locally from Mwea. DAP fertilizer is recommended for the area by the Ministry of Agriculture, Kenya (MoA, 2013). Two seeds were sown per hole at a spacing of 50x10cm, 50x20cm, and 50x30cm that were thinned two weeks after germination to leave one seedling per hole and enable obtaining the required plant population of 200000, 100000, and 70000 per hectare. Application of fertilizer was carried out at planting time, using four rates of N_0 (0kg N ha^{-1}), N_{30} (30kg N ha^{-1}), N_{60} (60kg N ha^{-1}), and N_{90} (90kg N ha^{-1}). Application of pre-emergence herbicide glyphosate @ 480g/L (Weedall) and organo-phosphate @ 26.25EC (Sumithion super) pesticide was carried out after planting to control weeds and crickets correspondingly. The crop was harvested when 90% of leaves had dried and dropped on the ground.

2.3 Data collection

Within each plot, a 1.5x2m section was purposively marked, and from this, data were collected on plant height and total biomass every after two weeks at the stages of 56, 70, and 84 DAS, while grain yield data were only recorded at 84DAS. For the estimation of biomass, three plants were uprooted, bagged, and transported to the University of Embu and oven-dried at 68^{o}C for 72 hours. The weights were taken in grams using an electronic balance (Model BA2204B, Machine number 040683), and an average per plant was determined. For grain yield, three plants were manually harvested and sun-dried to a constant weight, and the weight was recorded at 13% moisture content using equation 1 given by Ngetich et al. (2014). Harvest index was determined by calculating the ratio of average grain yield to biomass per plant, and then multiplied by 100 (Patil et al. 2021). Standard management practices were carried out when necessary (Kalungu and Harris 2013).

$$Grain\ yield\ (t\ ha-1) = Grain\ weight * \frac{SMC\%}{AMC\%} \quad \text{Equation 1}$$

Where AMC means actual moisture content of sample obtained using a moisture meter, SMC means Standard moisture content (13%).

Data were analyzed using the Generalized Linear Model (GLM), (Rutherford 2011) in SAS 9.4 software (SAS 2015). Mean separation was done using Turkey's honestly significant difference (HSD) test and Duncan's multiple range test (DMRT).for interactions means at a 95% confidence level.

3 Results

3.1 Chickpea plant height

Plant height significantly varied ($P<0.05$) across the seasons (Table 1). At 56 days after sowing (DAS) Chaina I and Chaina III had the highest plant height, while between 70 and 84 DAS Mwanza 2 had the highest plant height in both the long and the short rainfall (Table 1). In addition, the effect of spacing was significantly high at 56DAS (50x10cm) in LR2017 compared to other spacing. During SR2017, both 50x10cm and 50x30cm had a similar height of 34.9cm and 34.8cm respectively, while 50x20cm scored the least (33.8cm). Nitrogen fertilizer rates N_0 and N_{30} had significantly high differences compared to N_{60} and N_{90} in plant height at 56 and 70DAS apart from the LR2017 growth condition (Table 1). At 84DAS, N_{90} was significantly superior in height than other N-rates over the LR2017 growth conditions. However, in SR2017 and LR2018 plant growth was significantly reduced (Table 1).

3.2 Effects of imposed treatment on dry biomass, grain yield, and harvest index

Results of the study suggested that Saina K and Mwanza 2 have the highest biomass under LR2017 and SR2017 growth conditions compared to Chaina I and Chaina III (Table 2). The biomass decreased as spacing increased from 50x10cm (8.16t ha^{-1}) in LR2017 to 3.07t ha^{-1} in 50x30cm. Additionally, an application rate of N_{60} and N_{90} had a significant effect ($P<0.05$) on biomass during LR2017 and SR2017 compared to the other N-rates (Table 2).

Genotype Mwanza 2 had a significantly higher grain yield weight than other varities in LR2017, but Saina K registered significantly higher grains yield than the others in the cropping season of SR2017. However, under LR2018, Saina K and Mwanza 2 had significantly higher grain yield than the other two varieties. Spacing of 50x30cm gave the lowest grain yields in SR2017 but yielded better than other spacing in LR2017 although, no significant difference was reported in the yield in LR2018. Under the LR2017 and LR2018 growth season, N_{60} had the highest grain yield of 1.59t ha^{-1} and 2.00t ha^{-1} respectively (Table 2). In LR2017, Mwanza 2 (45%) gave the highest harvest index but it was not significantly different from Chaina I (43%) variety, whereas, in SR2017, Saina K performed better than all the other varieties. Spacing of 50x30cm posted a better harvest index in SR2017 and LR2018. However, in LR2017, spacing of 50x20cm posted the highest harvest index but it was not significantly different from 50x30cm. Harvest index was also significantly

Table 1 Plant height as influenced by variety, spacing and nitrogen application rate during the three cropping seasons in Mwea

[1]Treatment	Height 56 DAS (cm)			Height 70 DAS (50% maturity for Chaina varieties)			Height 84 DAS (50% maturity for Mwanza 2 & Saina K))		
Variety	LR 17[2]	SR 17	LR 18	LR 17	SR 17	LR 18	LR 17	SR 17	LR 18
Saina K	33.8^{b}±0.30	32.4^{b}±0.48	32.4^{c}±0.45	35.1^{a}±0.30	34.6^{c}±0.48	34.1^{c}±0.46	35.8^{b}±0.24	36.7^{b}±0.35	36.39^{b}±0.35
Mwanza 2	34.6^{a}±0.36	34.0^{b}±0.68	34.0^{b}±0.68	35.7^{a}±0.38	37.5^{a}± 0.49	36.3^{a}± 0.27	37.7^{a}± 0.47	38.0^{a}± 0.47	37.80^{a}± 0.41
Chaina I	33.8^{b}± 0.22	36.8^{a}± 0.40	35.6^{a}±0.29	35.4^{a}± 0.31	36.1^{c}± 0.44	35.5^{a}± 0.32	36.4^{b}± 0.28	35.1^{c}± 0.38	36.04^{b}± 0.27
Chaina III	34.6^{a}±0.29	34.8^{b}±0.35	34.7^{ab}±0.39	34.8^{a}±0.45	35.6b^{c}±0.45	35.1^{c}± 0.31	35.8^{b}± 0.33	34.8^{c}± 0.55	35.50^{b}± 0.31
Spacing									
50x10 cm	34.8^{a}±0.20	34.9^{a}± 0.20	34.5 ^{a}±0.20	35.87^{a}±0.21	35.7 ^{a}± 0.22	35.2^{a}± 0.21	36.4^{b}± 0.19	35.0^{b}± 0.19	35.92^{b}± 0.20
50x20 cm	33.4^{b}±0.18	33.8^{b}±0.19	33.8 ^{a}±0.24	34.9^{b}±0.19	35.7 ^{a}± 0.20	35.2 ^{a}±0.21	35.7^{c}±0.19	36.4^{a}±0.20	36.24^{b}±0.20
50x30 cm	34.2^{b}±0.25	34.8^{a}±0.49	34.2 ^{a}±1.01	35.0^{b}±0.21	36.4 ^{a}± 0.45	35.3 ^{a}±1.01	37.2^{a}±0.21	37.1^{a}± 0.46	37.14^{a}± 1.08
Nitrogen									
N_0	34.3 ^{a}±0.40	33.5^{b}± 0.62	33.8^{b}±0.43	35.2 ^{a}±0.35	35.3^{bc}±0.42	34.6^{b}±0.42	35.6^{c}±0.20	36.2^{ab}± 0.43	35.48^{b}± 0.30
N_{30}	34.0 ^{a}±0.42	33.5^{b}± 0.37	33.7^{b}±0.25	34.9 ^{a}±0.52	35.1^{c}± 0.26	34.9^{b}±0.41	36.3^{cb}±0.54	35.5^{b}± 0.25	36.29^{ab}± 0.50
N_{60}	34.0 ^{a}±0.29	35.9^{a}± 0.27	34.4^{a}±0.65	35.0 ^{a}±0.32	36.4^{ab}± 0.24	35.3^{ab}±0.45	36.4^{b}± 0.35	36.2^{ab}± 0.31	36.70^{a}± 0.54
N_{90}	34.4^{a}±0.27	35.1^{a}± 0.41	34.9^{a}± 0.36	35.8 ^{a}±0.42	36.9^{a}± 0.53	36.2^{a}± 0.35	37.4^{a}± 0.31	36.6^{a}± 0.44	37.26^{a}± 0.42

Treatments (Variety, Spacing and Nitrogen). Cropping seasons: [LR17 (long rains 2017), SR (short rains 2017) and LR18 (long rains 2018)]. Nitrogen rates: [N_0 (0kg N ha^{-1}); N_{30} (30kg N ha^{-1}); N_{60} (60kg N ha^{-1}) and N_{90} (90kg N ha^{-1})]. Sources of interactions: Variety (V), Spacing (S), N (Nitrogen), VS (Variety x Spacing), VN (Variety x Nitrogen), SN (Spacing x Nitrogen) and VSN (Variety x Spacing x Nitrogen). Means with same letter in a column are not significantly different at P≤0.05; Ns means not significant at P≤0.05.

Table 2 Total biomass, grain yield and harvest Index as influenced by variety, spacing and nitrogen application rate

Treatment[1]	Total biomass (t ha^{-1})			Grain yield (t ha^{-1})			Harvest index (HI%)		
Variety	LR2017[2]	SR2017	LR2018	LR2017	SR2017	LR2018	LR2017	SR2017	LR2018
Saina K	6.58^{a}± 0.64	8.08^{a}± 0.54	5.12^{b}±0.56	1.49^{b}± 0.09	0.87^{a}±0.06	1.91^{a}±0.11	33^{c}±3.50	12^{a}± 0.65	45a±1.56
Mwanza 2	6.29^{a}± 0.56	7.74^{a}± 0.48	7.13^{a}± 0.30	1.92^{a}± 0.15	0.47^{b}± 0.06	1.95^{a}±0.16	45^{a}±5.34	6^{b}± 0.55	40a± 3.06
Chaina I	4.24^{b}± 0.25	3.31^{c}± 0.25	4.29^{bc}±0.19	1.55^{b}± 0.12	0.14^{c}±0.01	1.23^{b}±0.13	43^{a}± 4.72	5^{b}± 0.70	40a± 3.08
Chaina III	3.68^{c}± 0.31	4.38^{b}± 0.25	4.24^{c}± 0.28	1.21^{c}± 0.08	0.19^{c}± 0.01	1.07^{b}±0.25	38^{b}±3.04	5^{b}±0.40	35a±3.46
Spacing									
50x10 cm	8.19^{a}± 0.26	8.00^{a}± 0.26	7.51^{a}± 0.24	1.15^{c}± 0.06	0.50^{a}± 0.07	1.49^{a}±0.06	17^{b}± 0.91	5^{c}± 1.47	25^{b}± 1.43
50x20 cm	4.33^{b}± 0.19	5.98^{b}± 0.20	4.56^{b}± 0.14	1.52^{b}± 0.08	0.41^{b}± 0.09	1.65^{a}±0.09	38^{a}±1.70	7^{b}± 2.41	44^{a}±2.33
50x30 cm	3.07^{c}± 0.12	3.65^{c}± 0.17	3.51^{c}± 0.24	1.96^{a}± 0.08	0.35^{b}± 0.09	1.49^{a}±0.14	37^{a}±2.35	9^{a}±2.05	52^{a}±2.90
Nitrogen									
N_0	4.33^{c}± 0.41	5.23^{c}± 0.45	4.91^{a}±0.44	1.45^{a}± 0.11	0.31^{c}± 0.05	1.20^{b}±0.21	44^{a}± 4.62	7^{a}± 0.65	33^{b}±3.84
N_{30}	5.10^{b}± 0.45	5.85^{b}± 0.38	4.92^{a}±0.58	1.55^{a}± 0.04	0.39^{bc}±0.12	1.34^{b}±0.12	43^{a}± 0.78	7^{a}±1.99	35^{ab}±3.76
N_{60}	5.84^{a}± 0.47	5.96^{ab}±0.53	5.62^{a}±0.60	1.59^{a}± 0.11	0.44^{b}± 0.11	2.00^{a}±0.08	36^{b}± 2.51	7^{a}±3.63	50^{a}±0.69
N_{90}	5.52^{ab}± 0.48	6.46^{a}±0.59	5.33^{a}±0.58	1.58^{a}± 0.15	0.52^{a}± 0.08	1.63^{ab}±0.21	37^{b}± 4.96	7^{a}±0.82	41^{ab}±3.40

Treatments (Variety, Spacing and Nitrogen). Cropping seasons: [LR17 (long rains 2017), SR (short rains 2017) and LR18 (long rains 2018)]. Nitrogen rates: [N_0 (0kg N ha^{-1}); N_{30} (30kg N ha^{-1}); N_{60} (60kg N ha^{-1}) and N_{90} (90kg N ha^{-1})]. Sources of interactions: Variety (V), Spacing (S), N (Nitrogen), VS (Variety x Spacing), VN (Variety x Nitrogen), SN (Spacing x Nitrogen) and VSN (Variety x Spacing x Nitrogen). Means with same letter in a column are not significantly different at P≤0.05; Ns means not significant at P≤0.05.

(P<0.05) affected under LR2017 and LR2018 growth conditions by nitrogen application. Application of N_0 and N_{30} generated the highest harvest index of 44% and 43% in LR2017 respectively while for the LR2018, N_{60} gave the highest harvest index of 50% but this was statistically similar to N_{30} and N_{90} (Table 2).

3.3 Interactive effects between treatments

3.3.1 Synergetic effects of varieties and spacing on plant height and yield indicators

The effects of interactions between chickpea varieties and spacing on plant height and yield indicators are as shown in Table 3. The highest plant height was recorded for Mwanza 2 in all spacing compared to other varieties; however, Chaina Ix50x10cm spacing posted the highest plant height of 34.8cm at H56DAS. Further, Chaina I and Chaina IIIx50x20cm gave higher heights at 56 DAS as compared to other spacing (Table 3). All varieties expressed the highest biomass under spacing 50x10cm (Table 3). Further, Mwanza 2x50x10cm spacing gave the highest biomass of 10.6 t ha^{-1} compared to the lowest Chaina I that had 2.78t ha^{-1}. Further, this treatment gave the highest grain yield (1.67t ha^{-1}) at 50x30cm over the cropping period. Saina K and Chaina III performed better under 50x20cm spacing while Mwanza 2 and Chaina I were found better than others at the 50x30cm spacing (Table 3). Among the all tested varieties, the highest harvest index was reported at 50x30cm growth conditions.

3.3.2 Interactive effects between varieties and nitrogen rates on height and yield attributes

Mwanza 2xN_{90} gave the overall highest plant height at 56, 70 and 84 DAS compared to the other varieties and N-rates. However, each variety improved height at different levels of nitrogen, among the tested varieties, Saina K had high heights at N_{30}, Chaina I at N_{90}, while Chaina III gave the highest value at N_0 (Table 4). The N_0 treatment resulted in the lowest biomass in all varieties while N_{90} gave the highest biomass in Mwanza 2 (7.74t ha^{-1}). Additionally, Saina K and Chaina I gave their maximum biomass at N_{60}, while Chaina III was maximum at N_{30}.

Saina K recorded the highest overall grain yield under N_{60} (1.89t ha^{-1}) while the worst performer was Chaina III under N_{90}. However, Mwanza 2 gave maximum yields at N_{90} (1.73t ha^{-1}), Chaina I, and Chaina III at N_{60}. Kabuli varieties (Mwanza 2 and Saina K) performed poorly under low N, while Desi varieties (Chaina I and Chaina III) performed lowest under high nitrogen rates. The highest harvest index was reported for the Chaina I (37%) under N_0 while the lowest index was reported for the Chaina III under N_{90}. Saina K and Mwanza 2 expressed their maximum index under N_{60}, and N_{90} respectively (Table 4).

3.3.3 Interactive effects of varieties, spacing and nitrogen rates on plant height and yield parameters

Mwanza 2 and Chaina I accumulatively performed better at 50x10cmxN_{90} compared to other varieties (Table 5). At a spacing of 50x20cmx$N_{60,}$ Chaina III gave better performance and gave 36.2cm height which is superior to the rest combinations. At 50x30cmxN_0, plant height was better among all varieties except in Chaina I which performed better at 50x30cmxN_{30} (Table 5). Further, Mwanza 2 gave a better plant height of 36.7cm at 50x30cmxN_{90} compared to other varieties.

In all interactions involving N_0 for biomass production, Mwanza 2 was better than other varieties apart from under 50x30cmxN_0 where Saina K was better (Table 5). Interactions of varieties under N_{30} fertilization rate resulted in Mwanza giving the highest biomass at 50x10cm, 50x30cm except under 50x20cm where Saina K was superior. Additionally, Mwanza 2 gave better biomass under interactions of N_{60} and N_{90} except under 50x30cm where Saina K was a better yielder.

In all interactions where the N_0 rate was applied, Mwanza 2 performed better in grain yield than all varieties except under 50x30cmxN_0 where Chaina I marginally gave the highest results (Table 5). Mwanza 2 performed better where N_{90} interactions were used except under 50x20cmxN_{90} where Saina K recorded the highest grain yield. In addition, Saina K performed better than other varieties at interactions of 50x10cmxN_{60}, and 50x20cmxN_{60} (Table 5). Additionally, Chaina III gave the highest grain yield with interactions of 50x10cmxN_{30} of 1.38t ha^{-1} compared to other varieties, while Mwanza 2 gave the highest grain yield under 50x20cm x N_{30} and 50x30cm x N_{30} of 1.37 and 1.76t ha^{-1} respectively.

Significant harvest index among interactions occurred under Saina K and Chaina I with 50x10cmxN_0, and 50x10cmxN_{90} giving higher results of 20% compared to Mwanza 2 and Chaina III (Table 5). Interactions of 50x10cmxN_{30} gave the lowest harvest index for Chaina I but higher results in the other varieties, while in the case of 50x10cm x N_{60} lowest harvest index was reported for Mwanza 2. Additionally, interactions of Chaina I with 50x30cmxN_0 gave a better harvest index of 60% compared to other varieties. Saina K, Mwanza 2, and Chaina III reported a high harvest index under N_{30}x50x10cm and N_{30}x 50x20cm while Chaina I was highest under N_{30}x50x30cm. Nonetheless, Saina K, Chaina I, and Chaina III gave a higher harvest index with interactions 50x10cmxN_{60} compared to Mwanza 2, while Saina K gave the highest index of 30% under 50x20cmxN_{60}. However, all varieties expressed a better harvest index under 50x30cmxN_{60}, with Mwanza 2 giving a superior index of 60%. Saina K and Chaina I gave a higher harvest index of 20% under 50x10cmxN_{90}, while Saina K, Mwanza 2, and Chaina I were better under 50x20cmxN_{90}. Mwanza 2 had a superior harvest index of 50% under 50x30cmxN_{90} interactions.

Table 3 Synergetic interactions between variety and spacing on plant height, total biomass, grain yield and harvest index

Treatments	SainaK	Mwanza2	ChainaI	ChainaIII	SainaK	Mwanza2	ChainaI	ChainaIII	SainaK	Mwanza2	ChainaI	ChainaIII
	H56				H70				H84			
50x10cm	34.0^{b}±0.47	34.7^{a} ± 0.38	34.8^{a} ±0.38	34.5^{b} ±0.38	35.1^{b}±0.40	36.3^{a}± 0.41	35.4^{b}± 0.39	34.6^{c}± 0.41	36.0^{b}±0.33	36.8^{a}±0.39	35.5^{c}±0.40	34.3^{d}± 0.42
50x20cm	33.1^{b} ± 0.52	33.2^{b} ± 0.58	34.4^{a} ±0.31	34.2^{a} ± 0.53	34.4^{d}±0.44	36.4^{a}± 0.46	35.6^{b}± 0.36	34.9^{c}± 0.39	36.5^{b} ± 0.39	37.5^{a} ± 0.39	35.7^{c} ±0.38	35.4^{c} ±0.43
50x30cm	33.0^{c}±0.52	35.3^{a}±0.58	34.0^{b} ± 0.37	35.1^{a}±0.35	34.3^{c}±0.42	36.8^{a}±0.40	35.9^{b}± 0.45	36.0^{b}±0.46	36.3^{b}±0.35	39.2^{a}±0.52	36.3^{b}±0.38	36.3^{b} ± 0.41
Yield attributes												
	Tb (tha^{-1})				GY (tha^{-1})				HI%			
50x10cm	9.38^{b}± 0.56	10.6^{a}± 0.70	5.63^{c}±0.41	5.98^{c}± 0.37	1.21^{a}± 0.09	1.16^{a}± 0.14	0.89^{a}± 0.14	0.92^{a}± 0.16	17.2^{c}± 0.01	12.5^{c}± 0.02	16.1^{c}± 0.02	16.3^{c}± 0.02
50x20cm	6.32^{a}± 0.56	6.56^{a}± 0.41	3.42^{b} ±0.25	3.52^{b}± 0.33	1.64^{a}±0.27	1.51^{a}± 0.16	0.86^{b}±0.09	0.76^{bc}± 0.07	32.1^{b}± 0.05	29.7^{b}± 0.03	28.9^{b}± 0.04	27.2^{b}± 0.04
50x30cm	4.07^{a}± 0.22	3.99^{a}± 0.28	2.78^{b} ±0.32	2.79^{b}± 0.33	1.43^{a}± 0.17	1.67^{a}± 0.15	1.17^{b}± 0.16	0.79^{bc}± 0.21	40.9^{a}± 0.05	50.0^{a}± 0.03	43.4^{a}± 0.05	34.4^{a}± 0.09

Variety (Saina K, Mwanza 2, Chaina I and Chaina III). H56 means 56 days after sowing; H70 means 70 days after sowing; H84 means 84 days after sowing. N-rates: N_0 (0kg N ha^{-1}), N_{30} (30 kg N ha^{-1}), N_{60} (60kg N ha^{-1}) and N_{90} (90kg N ha^{-1}). Tb means Total biomass; GY means economic grain yield; tha^{-1} means tonnes per hectare; HI means harvest index.

Table 4 Synergetic interactions between variety and nitrogen on plant height and yield attributes

Varietyx N	SainaK	Mwanza2	ChainaI	ChainaIII	SainaK	Mwanza2	ChainaI	ChainaIII	SainaK	Mwanza 2	ChainaI	ChainaIII
	H56				H70				H84			
N_0	33.0^{b}±1.21	33.3^{b}± 1.24	34.6^{a}±0.85	34.5^{a}±1.07	35.0^{b}±0.66	35.7^{a}±0.66	35.3a±0.74	34.6b±0.91	36.3^{a}±0.73	36.3^{a}±0.99	35.2^{b}±0.87	35.9^{a}±1.17
N_{30}	33.7^{b}±0.76	33.0^{c}± 1.64	35.8^{a}±0.81	33.9^{b}±0.66	34.5^{c}±0.52	36.2^{a}±0.71	35.7^{b}±0.51	34.6^{c}±1.03	36.6^{a}±0.57	37.0^{a}±1.06	36.2^{b}±1.00	34.6^{c}±1.45
N_{60}	33.9^{c}±1.58	34.4^{b}± 0.98	35.6^{a}±0.88	35.7^{a}± 0.81	34.1^{c}±1.03	36.4^{a}± 0.74	35.5^{b}±0.59	35.8^{b}± 0.42	35.8^{b}± 1.12	38.5^{a}± 1.68	35.7^{b}±1.09	35.7^{b}± 0.98
N_{90}	34.2^{b}±0.43	36.1^{a}±1.39	35.6^{a}±0.57	34.7^{b}±0.69	34.9^{d}±0.61	37.7^{a}±0.85	36.1^{b}±0.71	35.6^{c}±0.57	36.4^{b}±0.94	39.5^{a}±1.49	36.3^{b}±0.78	35.2^{c}±1.13
Yield attributes												
	Tb (tha^{-1})				GY (tha^{-1})				HI%			
N_0	5.5^{b}± 0.77	6.9^{a}± 1.11	3.1d±0.37	3.8^{c}± 0.42	1.1^{a}±0.09	1.2^{a}±0.23	0.9^{a}±0.14	0.7^{a}± 0.08	26.5^{b}±0.04	26.3^{b}±0.06	36.6^{a}±0.06	23.3^{c}±0.04
N_{30}	6.3^{a}± 0.92	6.2^{a}± 0.79	4.2^{b}±0.56	4.5^{b}± 0.68	1.1^{a}±0.13	1.4^{a}± 0.20	1.0^{a}±0.19	1.0^{a}± 0.18	25.0^{c}±0.04	30.9^{a}±0.05	28.3^{b}±0.06	27.8^{b}±0.05
N_{60}	7.39^{a}±0.90	7.27^{a}±1.126	4.49^{b}±0.68	4.08^{b}±0.73	1.89^{a}±0.29	1.4^{a}± 0.21	1.0^{b}±0.15	1.0^{b}± 0.31	36.0a±0.07	29.9c±0.07	26.7d±0.05	32.0b±0.11
N_{90}	7.2^{b}±1.11	7.8^{a}± 1.37	4.0^{b}±0.61	4.0^{b}±0.55	1.7^{a}±0.3	1.7^{a}± 0.23	1.0^{b}± 0.16	0.6^{b}± 0.07	32.7^{b}± 0.06	34.1^{a}±0.06	26.3^{c}±0.04	20.8^{d}±0.04

Variety (Saina K, Mwanza 2, Chaina I and Chaina III). H56 means 56 days after sowing; H70 means 70 days after sowing; H84 means 84 days after sowing. N-rates: N_0 (0kg N ha^{-1}), N_{30} (30 kg N ha^{-1}), N_{60} (60kg N ha^{-1}) and N_{90} (90kg N ha^{-1}). Tb means Total biomass; GY means economic grain yield; tha^{-1} means tonnes per hectare; HI means harvest index.

Table 5 Effect of synergetic interactions between varieties, spacing and nitrogen rates on plant height and yield attributes

	K1	K2	D1	D2	K1	K2	D1	D2	K1	K2	D1	D2	K1	K2	D1	D2
Treatments	H84cm				Tb (t ha^{-1})				GY (t ha^{-1})				HI%			
50x10cmxN_0	33.5^{b}±0.31	34.1^{a}±0.53	34.3^{a}± 0.67	34.0^{a}± 0.92	5.8^{b}±0.14	10.7 ^{a}±0.3	4.7^{c}±0.57	5.1^{c}±0.34	0.9^{a}±0.07	1.1^{a}±0.18	0.6^{b}±0.06	0.6^{b}±0.04	20^{a} ±0.02	10^{a} ±0.02	20^{a} ± 0.01	10^{a} ± 0.02
50x10cmxN_{30}	33.1^{c}±0.49	33.5 ^{c} ±0.74	35.8 ^{a} ±0.87	34.7^{b}± 0.74	7.6^{a} ±0.35	8.0^{a}±1.75	6.7^{b}±0.20	6.8^{b} ±0.41	1.1^{a} ±0.16	1.1^{a} ±0.13	0.8^{a} ±0.09	1.4^{a} ±0.39	20^{a} ± 0.02	20^{a} ±0.03	10^{b} ± 0.02	20^{a} ± 0.05
50x10cmxN_{60}	33.2^{c}±0.73	34.9^{b}±0.89	35.7^{a}± 1.26	35.7^{a}± 1.07	8.4^{b}± 1.01	10.4^{a}±0.13	7.1^{c}±0.64	6.8^{c}± 0.75	1.5^{a} ±0.13	0.9 ^{c} ±0.05	1.3^{a} ±0.15	1.2^{b}± 0.09	20^{a} ±0.02	10^{b}±0.01	20^{a} ± 0.03	20^{a} ± 0.01
50x10cmxN_{90}	33.8^{c}±0.75	36.6^{a}±0.66	36.0^{b}± 0.52	33.8^{c}± 0.46	8.4^{b}±0.99	14^{a}± 0.98	5.5^{d}±1.18	6.8^{c}±0.38	1.4^{a} ±0.13	1.6^{a} ±0.22	0.9^{b} ±0.44	0.6^{bc}± 0.05	20^{a} ± 0.04	10^{b} ± 0.02	2 ^{a} ± 0.07	10^{b} ± 0.01
50x20cmxN_0	31.8^{c}±0.38	31.5^{c}±1.08	34.2^{a}± 0.56	33.3^{b}± 0.95	6.3^{b}±1.01	8.8^{a}±0.38	2.6^{d}±0.22	5.1^{c}± 0.34	1.1^{a} ±0.09	1.2^{a} ±0.34	0.9^{a} ±0.08	0.9^{a} ± 0.06	30^{b} ±0.03	20^{c}±0.05	40^{a} ± 0.02	20^{a}± 0.04
50x20cmxN_{30}	33.5^{b}±0.72	33.6^{b}±0.46	35.1^{a}± 1.05	33.0^{c}± 0.89	6.7^{a}±0.17	4.9^{b}±0.33	3.7^{d}±0.20	4.1^{c}± 0.31	1.1^{a} ±0.15	1.4^{a} ±0.20	0.8^{b} ±0.16	0.8^{b} ±0.08	30^{a} ± 0.03	30^{a} ± 0.03	20^{b} ± 0.05	30^{a} ± 0.04
50x20cmxN_{60}	31.6^{d}±0.81	34.1^{c}±1.05	35.4^{b}± 0.78	36.2^{a}± 0.59	7.2^{a}± 1.00	7.3^{a}±0.80	2.4^{b}±0.34	2.1^{b}±0.13	2.1^{a} ±0.50	1.4^{b} ±0.17	0.7^{c}±0.16	0.5^{c} ± 0.07	30^{a} ± 0.06	20^{b} ± 0.04	20^{b} ± 0.04	20^{b} ± 0.04
50x20cmxN_{90}	31.3^{b}±0.34	35.0^{a}±0.48	35.5^{a}± 0.90	35.2^{a}± 0.64	5.9^{b}±1.25	7.2^{a}±0.46	2.7^{d}±0.53	3.6^{c}±0.44	2.3^{a} ±0.65	2.2^{a} ±0.13	1.1^{b}±0.15	0.8^{b} ± 0.15	40^{a} ± 0.05	40^{a} ± 0.03	40^{a} ± 0.04	30^{b} ± 0.07
50x30cmxN_0	33.9^{c}±0.71	34.3^{c}±0.76	35.2^{b}± 0.79	36.2^{a}± 0.94	3.5^{a}± 0.38	3.1^{a}±0.23	2.0^{c}±0.26	2.5^{b}±0.22	1.2^{a} ±0.11	1.4^{a} ±0.11	1.4^{a} ±0.11	0.7^{b}± 0.09	40^{c}± 0.06	50^{b} ± 0.04	60^{a} ± 0.04	30 ^{d}± 0.04
50x30cmxN_{30}	32.8^{c}±0.53	32.0^{d}±0.89	36.6^{a}± 0.90	34.0^{b}± 0.82	3.8^{b}± 0.13	5.0^{a}± 0.52	2.0^{c}±0.23	3.6^{b}±1.16	1.0^{bc}±0.06	1.8^{a} ±0.11	1.3 ^{b} ±0.29	0.7^{c} ± 0.09	30^{c} ±0.02	40^{b} ± 0.03	50^{a} ± 0.05	30^{c}± 0.06
50x30cmxN_{60}	33.7^{c}±0.80	34.1^{c}±1.32	35.8^{a}± 0.60	35.2^{b}± 0.63	4.9^{a}± 0.20	4.4^{b}± 0.46	5.2^{a}±0.85	2.7^{c}±0.26	2.1^{a}± 0.32	2.1^{a}± 0.07	1.2^{b}±0.25	1.4^{b}± 0.77	60^{a} ±0.09	60^{a} ± 0.04	40^{c} ± 0.07	50^{b} ± 0.33
50x30cmxN_{90}	32.3^{c}±0.63	36.7^{a}±1.03	35.2^{b}± 0.82	35.0^{b}± 1.19	4.8^{a}± 0.18	4.3^{b}±0.41	3.0^{c}±0.69	2.2^{d}± 0.21	1.4^{a}±0.14	1.5^{a}± 0.19	0.9^{b}± 0.22	0.5^{b}± 0.04	40^{b}± 0.03	50^{a}± 0.06	30^{c}± 0.07	30^{c}± 0.01

Variety (K1 means Saina K,K2 means Mwanza 2, D1 means Chaina I and D3 means Chaina III); Spacing (50x10, 20 and 30cm); N_0 (0kg N ha^{-1}), N_{30} (30kg N ha^{-1}), N_{60} (60kg N ha^{-1}) and N_{90} (90kg N ha^{-1}); Tb means Total biomass; GY means economic grain yield; tha^{-1} means tonnes per hectare; HI means harvest index

Table 6 Synergetic effects of season, variety, spacing and nitrogen rates on traits under study

Source of Variation	H56	H70	H84	Total biomass	Grain yield	Harvest index
Season	0.0851	0.0006***	0.1582	<0.0001***	<0.0001***	<0.0001***
Season *V	<.0001***	0.0065	0.0001***	<0.0001***	<0.0001***	0.0052*
Season *S	0.4149	0.0264**	<0.0001***	<0.0001***	<0.0001***	<0.0001***
Season *V*S	<.0001***	0.0181**	0.0089*	<0.0001***	<0.0001***	0.0004***
Season *N	<.0001***	0.1872	0.0154**	0.02**	0.003*	<0.0001***
Season *V*N	<.0001***	0.0537**	0.0044*	<0.0001***	0.03**	0.0061*
Season *S*N	<.0001***	<.0001***	<0.0001***	0.0001***	<0.0001***	<0.0001***
Season *V*S*N	<.0001***	<.0001***	<0.0001***	<0.0001***	0.04**	0.0045*

Sources of variation, season, V indicates variety, S indicates spacing and N indicates nitrogen application rates, P-values in bold signify non-significance. *, ** and *** means P is significant at <.01, <.05 and <.001.

3.3.4 Interactions of season, variety, spacing, and nitrogen application rates on all traits

Interactions between season, variety, spacing, and nitrogen application rates induced a significant effect ($P<0.05$) on the various chickpea performance traits (Table 6). Interactions of season x variety, season x variety x nitrogen, Season x Variety x Spacing x Nitrogen influenced a significant effect on all studied traits.

4 Discussions

In the current study, Mwanza 2 had significantly higher vegetative growth at 70 and 84DAS this was followed by the Chaina I and Chaina III over 7% at full maturity. The differences in height were realized at the later stages of vegetative growth. *Kabuli* varieties, under which Mwanza 2 are classified, are taller than *Desi* varieties (Chaina I and Chaina III) (Onyari et al. 2010; Mallu 2015).

In this study, spacing did not show a clear pattern in various growth stages although variations were observed under different spacing. The tallest plants were observed under 50x30cm spacing in all the seasons. These findings corroborate with findings of Agajie (2014) and Under Newly (2011), in chickpea plants and faba beans respectively under wider spacing. Similarly, other works established differences in plant height across different legume crops under varied spacing (Tuarira and Moses 2014; Alemayehu et al. 2015). The utilization of soil nutrients such as added nitrogen is reported to significantly influence chickpea growth, expressed in terms of plant height (Fazle et al. 2014). Though nitrogen is known to promote plant growth, the level varies and depending on the time of nutrient application (Dhima et al. 2015; Caliskan et al. 2008). These results are in agreement with the findings of the current study. At 56 and 70 DAS plant height of the chickpea was conspicuously lower in the N_0, $N_{30,}$ and N_{60} compared to the N_{90} application rates. In later stages of growth (84DAS), significance in plant height between N_{90} and the other rates (N_0, $N_{30,}$ and N_{60}) reduced, mainly due to senescence (Kherif et al. 2021). The final plant height after N_{30} to N_{90} fertilization gave taller plants indicating that increased N-application rates can positively increase plant growth of chickpea. The increase in plant height under N-fertilized plots is due to readily available Nitrogen from inorganic fertilizer that stimulated crop growth (Namvar et al. 2011; Fazle et al. 2014; Goa and Ashamo 2016).

In the current study, plant biomass reflects the amount of sunlight, water, and mineral resources that a plant can capture and turn into plant mass (Sims et al., 2012; Macák et al. 2020). The spacing of 50x10cm had significantly higher biomass compared to the other wider spacing. This was more prominent in Saina K, which produced more than 59% biomass compared to the other varieties. On the other hand, Mwanza 2, which is a *Kabuli*, had higher biomass across the seasons with over 78% compared to the *Desi* varieties (Chaina I and Chaina III). Differences in biomass production were due to variations in adapting to environmental conditions among varieties (Alemu et al. 2014). Mwanza 2 had a taller stature than Chaina III, reflected in all parts of the plant. This enabled the plants to capture more photosynthetically active radiation (PAR) for increased biomass (Goa 2014; Devi et al. 2019). Results, however, contradict the findings of Mekuanint et al. (2018), who found a non-significant effect of variety in biomass production between two chickpea varieties under three-level spacing with blended fertilizers.

Under 50x10cm growth conditions, the biomass of 5.1t ha^{-1} was recorded for chickpea. This was 62% higher than that of 50x30cm spacing. Thus, increased plant density enables chickpeas to produce more biomass (Vaghar et al. 2013). However, the findings contradicted with the findings of Gezahegn et al. (2016), who reported that plant spacing increased biomass in *Vicia faba* and *P. vulgaris* respectively. N-fertilizer application also increased plant biomass, especially in early growth stages. On average, the N_{60} and N_{90} rates had over 20% more biomass than the control rate (N_0).

This corroborates with the findings of Onyari et al. (2010), those who reported 4.6 to 13.6 t ha^{-1} chickpea biomass in Kenya. Hence, *Kabuli* varieties with closer spacing and adequate N-fertilizer have the potential of producing higher biomass yield under the Mwea conditions in Kenya. For other legumes, biomass yields of 3.0 and 2.6 t ha^{-1} have been reported for Bambara nuts when 45 and 0 kg-N ha^{-1} were applied respectively (Uchara et al. 2013).

Crop yield is important in agriculture as it shows the amount of produce harvested per unit area for a given time (Keerthi et al. 2015). Seasons provide different weather conditions for the growth and development of different varieties; hence, they can convert resources to outputs in form of yields (Sellami et al. 2021). In this study, *Kabuli* (Mwanza 2 and Saina K) varieties gave higher grain yield than *Desi* type (Chaina I & Chaina III) grown in three seasons in Mwea, Kenya, indicating that they are better adaptable to the growth conditions. In contradiction Księżak and Bojarszczuk (2020), reported a higher grain yield in *Desi* than *Kabuli* under various cropping methods. In addition, Shumi et al. (2020), reported significant differences in grain yields of Chickpea varieties in different seasons.

In the LR2017 season, the amount of rainfall was less than normal and the grain production in the 50x30cm spacing performed better than other spacing, which underscored the effect of competition for resources on chickpea, crop production. However, in LR2018 season (rainfall more than normal) had no significant differences in the grain yield among the three plant spacing. This implied that reduced rainfall and wider spacing favored higher grain yield, while higher rainfall promoted vegetative growth at the expense of grain yield (Onyari et al. 2010). Similar observations were reported by Tamiru et al. (2020), who found higher grain yield under wider intra row spacing of 15cm in chickpea in drier environments.

Application of N_{60} gave the highest chickpea grain yields in two seasons compared to lower N-rates, though not significant from N_{90}. However, in all seasons, N_{60} was significantly higher than N_0 and N_{30} growth conditions in SR2017 and LR2018 seasons. Observation shows that starter nitrogen of about N_{60} needs to be applied for chickpea production as, low starter nitrogen led to low yields under the conditions of Mwea, Kenya. These results are in agreement with the observation of previous researchers who suggested that the application of nitrogen fertilizers enhances chickpea yield (Uddin et al. 2014*).* Similarly, Dar et al. (2021), established the highest yield of 2,023kg ha^{-1}under high N-application (30kgha^{-1}) compared to lower rates (0, 15kg N ha^{-1}), and the highest rate of 45 kg ha^{-1} N (1940kg ha^{-1}). In addition, Khaitov and Abdiev (2018), reported high grain yield (1.68 t ha^{-1}) under 75kgha^{-1} N fertilization compared to lower rates and the highest rate of 100kgha^{-1} nitrogen. Starter doses of 20 to 40kg N ha^{-1} are widely recommended (Dar et al., 2021), but the findings of the current study show that increasing N-rates up to 60kg ha^{-1} can increase the grain yield of chickpea.

The harvest index determines crop potential as it describes the capacity of a crop to allocate biomass into reproductive parts, and is thus used as a measure of reproductive efficiency (Wnuk et al. 2013). The *Kabuli* varieties had a better harvest index than the *Desi* varieties. However, various previous researchers reported that the harvest index of *Kabuli's* is reportedly inferior to that of *Desi* (Richards et al. 2019; Maya and Maphosa 2020). Mekuanint et al. (2018**)** reported contradictory results of a high harvest index in *Desi* (54%) compared to *Kabuli* (48%). Bhardwaj and Hamama (2015) reported similar results in mung bean (*Vigna radiate*) that show the influence of variety on harvest index. Spacing of 50x30cm and 50x20cm were ideal since they had a better harvest index than 50x10cm in all seasons. An increase of HI over 9% under wider spacing could be due to limited competition for growth factors that led to more assimilates partitioned into seed grain. Similarly, Mekuanint et al. (2018) reported an index of 53.25% under wider spacing. The 50x10cm spacing posted the lowest index in all seasons indicating the area cannot support denser populations that promote vegetative growth at the expense of grain yield. Similarly for other legumes, Kerina et al. (2017), and Agajie (2014) reported significantly lower harvest indices under small spacing than higher spacing in cowpea, Lablab, common bean, and chickpea.

Application of N_0 and N_{30} rates generated the highest harvest index of 44% and 43% in LR2017 that could be attributed to the interaction with overall environmental conditions such as heat stress during the reproductive stage enhancing dry matter allocation into the seed (Muruiki et al. 2018). The results are in agreement with Doaiy et al. (2019), whose reported significant effects on yield and yield components under high nitrogen fertilization. Tamagno et al. (2018) established similar findings in *Glycine max* production. However, the highest harvest index value of 50% under N_{60} treatment in all seasons, indicates it was the best-suited N-rate for chickpea production under the study. Related findings were noted by Onyari et al. (2010), Wnuk et al. (2013), and Ndukhu et al. (2017) those who reported significant improvement in harvest index under nitrogen application. However, Seval et al. (2020) reported a shrinking harvest index in chickpea varieties under N-application.

Interactive effects indicate that a combination of two or more factors simultaneously affects the outcome of a chickpea crop or cropping system yield (Onyari et al. 2010). Enhanced plant growth under wider spacing was due to less competition for nutrients and water (Wafula et al. 2021; Onyari et al. 2010). Muruiki et al. (2021) also reported differences in plant height among chickpea varieties ranging from 35cm to 53cm. However, Mekuanint et al. (2018) noted non-significant results in height among chickpea

varieties. Some varieties of chickpea are indeterminate, thus, growing after the vegetative stage. Indeterminate growth prolongs the reproductive period, which could be associated with higher plant height (citation). Interactions of Mwanza 2 x 50x20cm gave the highest biomass (10.6t ha^{-1}) over the study period, suggesting that increase in plant population likely increased biomass. However, Mwanza 2 and Chaina I x 50 x 30cm spacing, gave better grain yield than other varieties showing that high biomass did not translate to high grains in all varieties. Saina K and Chaina III x 50x20cm interaction also gave a higher yield than others did. High yield is due to the efficient utilization of growth factors under various interactions (Tehulie and Yimam 2021). The highest harvest index was noted under interactions of all varieties x 50x30cm. Similarly, significant interactions between variety x spacing on chickpea heights, biomass, grain yield, and HI have been reported (Abdullah et al. 2019). This might be due to crop population, and adaptability to environmental conditions. Consistently across the observed traits, biomass decreased with increased spacing while grain yield increased with increased spacing. This implied that an increase in plants population led to an improvement in chickpea biomass that did not translate to high grain yields.

Interactions between varieties and nitrogen on measured parameters revealed significant results. Each variety improved height at different levels of nitrogen, with Mwanza 2 giving the highest heights under the N_{90} rate at all days of observation. Further, the observed interactive effects of variety and nitrogen (SN) on measured traits could be due to varied competitive capacity under spacing rates and nitrogen release. This conforms with the findings of Amiri et al. (2021) in soybeans that established heights of 64.26cm to 97cm under different N-rates. Other findings are by Purushothaman et al. (2014), Naderi et al. (2021) and Basal and Szabó (2020) in chickpea are also in agreement with the findings of the present study. In contradiction, Pasqualone et al. (2021) established a higher mean plant in *Desi* (79.39cm) than *Kabuli* (68.83cm) under various nitrogen treatments (30, 40, 100Kg N ha^{-1}). Although *Desi* varieties expressed lower biomass than *Kabuli*, both gave low biomass under N_0, but their maximum varied under various nitrogen treatments. The reverse was noted under harvest index with *Desi* (Chaina IxN_0) expressing the highest harvest index (37%) compared to other varieties, while *Kabuli* varieties (Mwanza 2 and Saina K)xN_0 performing lowest. Further, the findings of Devi et al. (2019) and Lemma et al. (2013), also established increased grain yield under variety x nitrogen fertilizer treatments than control. Finally, the joint interactive effects of variety, spacing, and nitrogen (VSN) application factor underscore the importance of joint consideration of the above factors on promoting chickpea production. The interaction between variety, spacing, and fertilizer application was significant on all the measured variables except for grain yield and harvest index in the LR2018. The significant seasonal and treatment (variety, spacing, and nitrogen application rates) interactions on chickpea performance are due to environmental differences including rainfall amounts. The study findings were consistent with previous studies of Kaloki et al. (2019b) that reported significant effects between variety, environmental, and management interactions.

Conclusion

From the results, *Kabuli* varieties performed better in the study area than *Desi* varieties. Under variety x spacing interactions, Saina Kx50x10cm, Mwanza 2, Chaina I, and Chaina IIIx50x30cm were ideal for plant height. All varieties x 50x10cm gives high biomass, while 50x30cm was appropriate for high grain yield and harvest index. VarietyxN_{60} encouraged high plant height, biomass, and grain yield across most of the varieties, though Mwanza 2 was best under N_{90}. All varieties improved harvest index with increasing N level except Chaina I, which attained its maximum at N_0. It is therefore important to note the following interactions enhanced grain yield, Saina K x50x20cmxN_{90}, Mwanza 2x50x30cmxN90, Chaina Ix50x30cmxN_0, and Chaina IIIx50x10cmxN_{30}. To increase harvest index, all varieties to grow under 50x30cmxN_{60} interactions, except Chaina I. However, narrow spacing is recommended for biomass production. Further research can be conducted for similar and other environmental factors using these varieties to validate findings.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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Effect of Drainage Channels on Vegetation Diversity of Tropical Peatswamp Forest of Sebangau National Park, Indonesia

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ABSTRACT

Peat swamp forests are playing important role in climate change by carbon storage, biodiversity conservation, and crucial local livelihoods. The construction of drainage channels in Sebangau National Park, Indonesia negatively affects the Peatland ecosystem and degrades the vegetation diversity. This research aims to study the composition and vegetation diversity of secondary peat swamp forests in Sebangau National Park (SNP), especially around large and small drainage channels. For the observation of vegetation composition and diversity, each observation block consisted of 3 transects that were 300 m apart from each other, and perpendicular to the channel. For observations on small drainage channel blocks, transects are made to continue the previous transect at a distance of 500 m from the end of the large drainage channel. On each transect, 5 plots of vegetation were made using the plot line method with a distance of 50 m between each plot. A total of 15 plots of 30mx30m size were prepared for each drainage channel category. Observations were made on the growth rate of seedlings in a 2m x 2m plot, poles in a 5m x 5m plot, saplings in a 10m x 10m plot, and trees in a 20m x 20m plot. The results of the study showed that *Shorea* spp., *Combretocarpus rotundatus*, *Cratoxylum arborencens*, and *Calophyllum* sp. are the dominant plant species of the study area. Overall 92 species were reported from the Large Drainage Channel block and 86 species from the Small Drainage Channel block. Further, the Species Diversity ranged between 1.43 - 1.57 while Species Richness ranged from 16.80 – 23.03, and the

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Evenness Index ranged from 0.83 – 0.92 at all levels of vegetation growth. Results of the study can be concluded that the channel dimensions do not have any effect on species number, diversity index, species richness, and species evenness at all levels of vegetation growth. The Similarity Index of species at seedlings, saplings, and poles is more than 50%, while at the tree level it was reported less than 50%.

1 Introduction

Peatland ecosystems are always associated with water-saturated conditions, high organic matter, and flooded anaerobic conditions. The peatland ecosystem is of global importance because of its high carbon storage, role in global climate change, higher biodiversity, and importance in community livelihoods (Joosten 2015; Wildayana 2017). Parish et al. (2008) suggested that tropical peatland covers one-third of global wetlands, and occupied the area of 440,000 to 600,000 km^2. From the total tropical peatland area, the maximum areas are distributed in Southeast Asia, South America, and Africa's Congo Basin (Page et al. 2011; Gumbricht et al. 2017). Further, it has the most diverse and most threatened peatland environment and is associated with significant carbon emissions by natural decomposition, fires, and biodiversity loss (Page et al. 2006; Yule 2010; Turetsky et al. 2015). In Southeast Asia, particularly in Malaysia, Sumatra, and Kalimantan, peat forest cover has declined from 119,000 km^2 to 46,000 km^2 from 1990 to 2015, while agricultural areas on peatland increased from 17,000 km^2 to 78,000 km^2 during the same study period (Miettinen et al. 2016). Small-scale farmers, industrial oil palm, and expansion of paper pulp are some responsible factors for this agricultural conversion which put this peatland agricultural system is in danger of extinction (Miettinen et al. 2016; Wijedasa et al. 2018; Tan et al. 2021).

Peat swamp forest has very high biodiversity and is a habitat for various flora and fauna including 1,524 plant species, 123 mammals, 268 birds, 75 reptiles, 27 amphibians, and 219 fish species (Page et al. 1997; Yule 2010; Posa et al. 2011; Posa et al. 2011). Along with this, PSF is also an important ecosystem for the primates *Pongo* spp., clouded leopard (*Neofelis diardi*) and cat species, Storm stork (*Ciconia stormi*), white-winged duck (*Asarcornis scutulata*) (Silvius & Verheugt 1986; Morrogh-Bernard et al. 2003; Cheyne et al. 2011; Cheyne et al. 2014).

Thus, national and international efforts have been needed to be enhanced for conserving the remaining peat swamp forest (PSF). Some of the important challenges which affect efforts to restore degraded peatlands are increasing the groundwater level on drained peatlands and the development of economically competitive crop species suitable for paludiculture (Wosten et al. 2008; Wichtmann et al. 2010; Uda et al. 2017; Uda et al. 2017; Evans et al. 2019; Tan et al. 2021). Damage to the peat ecosystem causes disturbance to this diversity (Mishra et al. 2021). Tropical peatland degradation generally begins after converting PSF into a nonforest area for agriculture, smallholder plantations, and industrial forest plantations, which is usually accompanied by drainage channel construction. Changes in land use from PSF to open peat cause serious damage to the ecological function of the peat as a carbon sink and store, in addition to biodiversity. The decrease in forest cover, mainly due to drainage, is also associated with a decrease in the groundwater level, which impact the characteristics of peat soils including the processes of decomposition and compaction that result in an increase in bulk density (Wösten et al. 2006a; Sherwood et al. 2013; Sumargana et al. 2016; Uda et al. 2017; Cooper et al. 2019a; Evans et al. 2019; Sinclair et al. 2020). Bulk density of peat soil is important in regulating the hydrology of peatlands by influencing groundwater storage capacity (Rydin and Jeglum 2015), and reducing the hydraulic conductivity of peat (Päivänen 1973), water retention, and increasing flooding (Hooijer et al. 2012; Könönen et al. 2015; Evers et al. 2017; Evans et al. 2019). The change from PSF to other uses that reduce land cover also changes hydrological functions, especially surface runoff. Anshari et al. (2010) reported that conversion of PSF to open areas by removing vegetation and drainage reduced the C/N ratio, organic acid, and peat soil compaction and increased bulk density and pH of peat soil as a direct result of drainage. Thus, changes in the physical and chemical characteristics of peatlands tend to cause changes in the vegetation structure and composition of peatlands, but empirical studies to investigate the effect of changes in the properties of these peat soils are still limited, especially in tropical peat.

Sebangau forest of Kalimantan, Indonesia, have diverse biodiversity and have more than 215 tree species, 92 non-tree plant species, 73 ant species, 66 butterfly species, 297 spider species, 41 Komodo dragon/damselfly species, 55 fish species, 11 species of amphibians, 46 species of reptiles, 172 species of birds, and 65 species of mammals (Husson et al. 2018). Sebangau National Park (SNP) is one of the Indonesian PSF ecosystems which has relatively good conditions in carbon storage and water regulation as compared to the surrounding area. Therefore, it is necessary to manage it wisely and sustainably because SNP's peat swamps are believed to have high economic and ecological value (Taman Nasional Sebangau, 2011; Khalwani et al. 2017). Resort Mangkok or commonly called SSI (former HPH PT, Sinatra Sebangau Indah), is included in the working area of the National Park

Management Section, Sebangau II, Pulang Pisau Regency. From the 1970s until the mid-1990s, various illegal logging activities were rampantly carried out by people in the Sebangau area. Along with this, the research area was faced forest fires during the long dry season of 1992, 1994, 1997, and 2002. In the early 1970s, the Sebangau River is one of the important transportation routes which mainly used for timber transportation. These activities cause the Sebangau peat-swamp forest area to lose water and damage the hydrological function of the area, causing drought and flammability in the dry season (Taman Nasional Sebangau, 2016).

In general, the PSF ecosystem is easily disturbed, and once it is disturbed it will be difficult to return to its original state. Excessive drainage and fires in the Mangkok Resort area of Sebangau National Park peat swamp ecosystems are likely to cause changes in the structure of the vegetation that grows in these localities. This may also influence the properties of peat soil due to excess drainage which has implications for vegetation growth. Thus, changes in the physical and chemical characteristics of the peatlands will most likely cause changes in the structure and composition of the vegetation of this area. Further, changes in land use from PSF to open peat which is usually associated with drainage seriously damage the various ecological functions including biodiversity (Wösten et al. 2006b; Sumargana et al. 2016; Uda et al. 2017; Cooper et al. 2019b). Furthermore, it has also an impact on the characteristics of peat soil, including the process of decomposition and compaction (Sherwood et al. 2013; Evans et al. 2019; Sinclair et al. 2020).

The expected recovery is a restoration that leads to the original ecosystem, although this is very difficult and takes a very long time, especially with continued disturbances in the ecosystem (Kimmins 1997). The progress of the recovery process can be measured by several factors, one of which is by looking at the composition of the type and structure of the vegetation in the area. This research was conducted to evaluate the effect of large drainage channels and small drainage channels on the plant composition and diversity due to changes in hydrological function in the peat swamp forests.

2 Materials and Methods

The current study was carried out in the area of Mangkok Resort, Sebangau NP, Central Kalimantan, Indonesia (Figure 1). Field observations and data collections were carried out in 2018. Geographically SNP is located at 1°54' – 3°08' South Latitude and 113°20' – 114°03' East Longitude. Further, the study area is located between the 3 regencies/cities namely Palangka Raya City, Katingan Regency, and Pulang Pisau Regency. The topography consists of coastal lowlands with altitudes ranging from 2 to 8 MSL and it is generally a waterlogged wetland area (swamp). The park is covered by deep peat with a thickness of more than 3 m, the study area is also suffered by repeated fire in different areas since 1997, and includes former concession and illegal logging areas with small drainage channels constructed. In the Mangkok Resort research area, water channels were previously used for logging and transporting. This area of about 88 ha and is allocated by SNP for research-based tourism development, including biodiversity research, peatland restoration, and social communities. Various efforts including the construction of 45 canal blocks/dams have been carried out for rehabilitation. The constructed dams have been divided into 3 categories including 10 permanent, 9 semi-permanent, and 26 simple dams (Taman Nasional Sebangau 2016). Hydrological rehabilitation was carried out by constructing large channel blocks in 2006 and small channel block in 2016 (Balai Taman Nasional 2015).

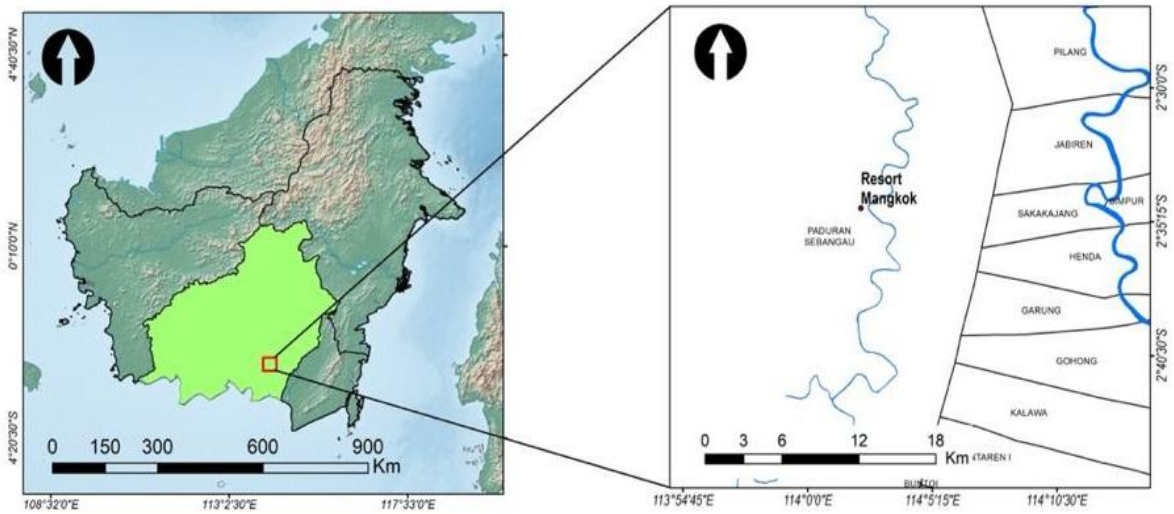


Figure 1 Research location at Sebangau National Park, Central Kalimantan, Indonesia

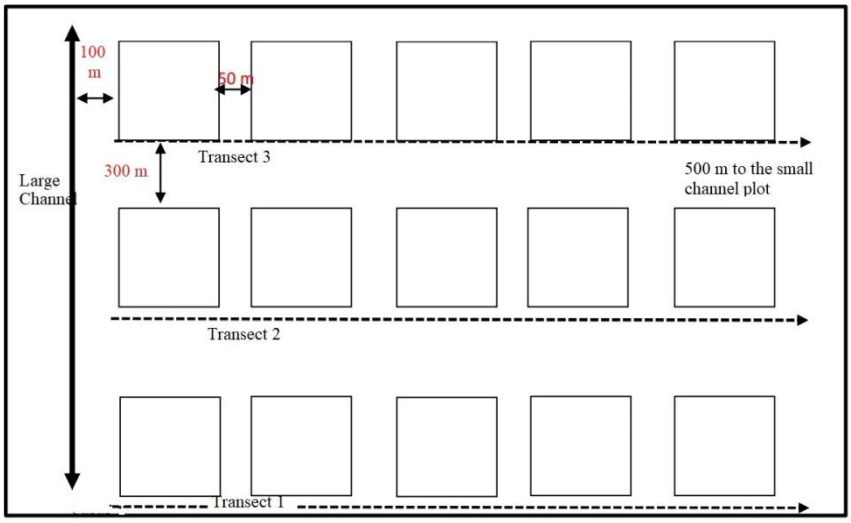


Figure 2 Research Plot Placement Design

: Channel row; : Transect of placement of observation plots; : Distance between lines and observation plots; 2m x 2m : Plot for seedlings, woody vegetation with height <1.5m; 5m x 5m : Plot for saplings, woody vegetation with height >1.5m and a diameter <10cm; 10m x 10m : Plot for poles level, woody vegetation with diameter 10 cm-<20 cm (all subplots); 20m x 20m : Plot for trees, woody vegetation with diameter >20cm (subplots a, b, d, e)

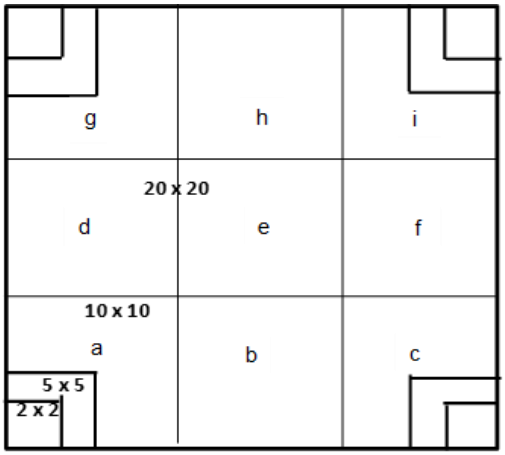


Figure 3 Plot design for Vegetation Measurement

Various observations related to the vegetation diversity including seedlings, saplings, poles, and trees were undertaken from the large and small drainage channels area of Resort Mangkok, National Park of Sebangau. Path design, vegetation category size definitions, and plot placement are shown in Figures 2 and 3.

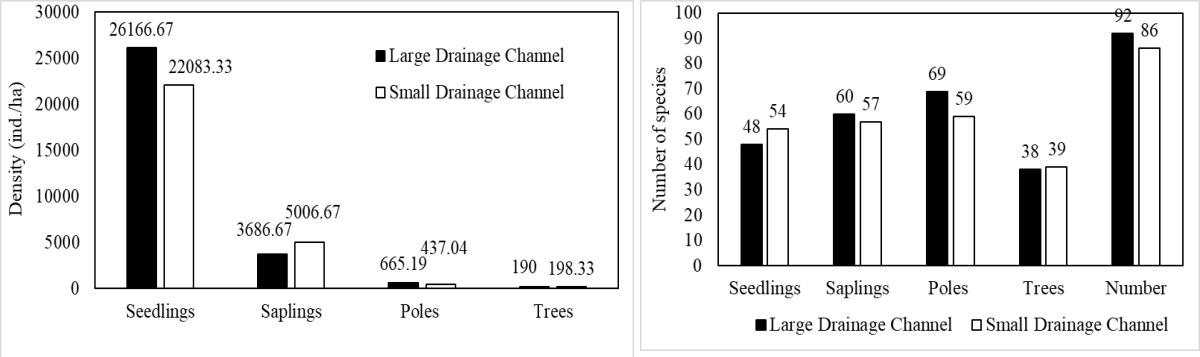
Observation blocks in the study area are located on 2 drainage channels located at Mangkok Resort: the large drainage channel was >10 m wide while the small drainage channel was 2.5 m wide. Vegetation observation blocks were placed systematically near to each channel. Each block was made up of 3 transects separated from each other by 300 m and located at a distance of 50 m from the channel. In each transect, 5 vegetation plots were made using the plot line method (Kusmana 1997). The observation transect on the large channel begins at a distance of 100 meters, perpendicular to the channel. While the small channel transect starts at a distance of about 1000 m from the large channel. Thus, the distance between the end of the large channel observation transect and the beginning of the small channel transect is about 500 meters. The dimensions of the plot was 30 m × 30 m and the total number of plots was 30 (15 for each drainage channel size category). Observations were made on the growth rate of seedlings in a 2m x 2m plot, poles in a 5m x 5m plot, saplings in a 10m x 10m plot, and trees in a 20m x 20m plot Overall, the sample plot area in each research block was 1.35 hectares.

2.1 Data Collection and Analysis

The biodiversity index of species for each plot was calculated using standard parameters including importance value index (IVI), species diversity index, species richness index, species evenness index, and species similarity index (Table 1). Data from vegetation inventory are analyzed to determine the composition and dominance of the species. The dominance of a species will be indicated by the importance value index. Plant density indexed (INP) for pole and tree-level vegetation is the sum of Relative Density (RD), Relative Frequency (RF), and Relative Dominance (RD), while for seedling and sapling level vegetation, the INP value is calculated by the sum of RD + RF (Indriyanto 2006). For the species diversity index, we used the calculation of the Shannon - Wiener Index (Magurran 2004), while for the calculation of the species richness index Margalef Index was used (Margalef 1958). Pielou's evenness index (E_{Pielou}) (Magurran 2004) was used for species evenness index, while for the species similarity index Kent's (2011) method was used.

Table 1 Equations for calculating index diversity of species

Index	Equation	Description	References
Important value index	Relative Density + Relative Frequency + Relative dominance		Indriyanto 2006
Relative Density	$\frac{\text{The density of a species}}{\text{Total Density of all species}}\ x\ 100$		
Species Density	$\frac{\text{Number of a species}}{\text{Total Area Sampled}}$		
Relative Frequency	$\frac{\text{Area of plot in which species occur}}{\text{Total Area Sampled}}$		
Frequency of Species	$\frac{\text{Area of plot in which species occur}}{\text{Total Area Sampled}}$		
Relative Dominance	$\frac{\text{Dominance of a species}}{\text{Total Dominance of all species}}\ x\ 100$		
Dominance of species	$\frac{\text{Total basal area of a species}}{\text{Total Area Sampled.}}$		
Index of Species Diversity (H')	$H' = -\sum_{i=1}^{s}\left(\frac{ni}{N}\right)\ln\left(\frac{ni}{N}\right)$ H' = Shannon - Wiener Index ni = Number of individuals species N = Total number of individuals	The magnitude of the Shannon-Wiener species diversity index is as follows: a. H' > 3, species diversity is abundant with a high number of individual wealth. b. H' 1 ≤ H' ≤ 3, species diversity is moderate. c. H' < 1 indicates that the diversity of species is low or low	Magurran 2004
Index of Species Richness (R)	$Dmg = \frac{S-1}{\ln N}$ Dmg = Margalef's Index S = Number of species observed N = Total number of individuals of all species in the sample Ln = Natural logarithm	Margalef's index (R) criteria are: a. R<3.5; low density b. 3.5<R<5.0; medium density c. R>5.0; high density	Margalef 1958
Index of Species Evenness (E)	$E = \frac{H'}{\ln S}$ E = Index of Species Evenness (0-1) H'= Shannon_Wiener Index S = Number of Species	The evenness index ranges between 0 and 1; 0 indicates that the evenness level is very uneven; whereas if the value is close to 1, almost all species that exist, have the same abundance (Magurran, 2004)	Magurran 2004
Similarity Index (SI)	$SI = \frac{2C}{(A+B)}$ SI= Sorenson Similarity Index A= Number of species from sample A B= Number of species from sample B C= Number of similar species in both samples A and B	SI>50 means the area has the same species in the community; SI<50%, then there are differences in the types of community constituents in the area or do not even have the same types.	Kent, 2011



(a) (b)

Figure 4 (a) Density and (b) Number of Species at all Growth Level

3 Results and Discussion

3.1 Species Composition and Dominance

Total 112 tree species have been reported from the vegetation analysis near the large and small channel blocks observation of Sebangau. The number of species reported from the large drainage channel blocks was not different from the reported from the small drainage channel (Figure 4). Around the large channel block, there were a total of 92 species, consisting of 48 seedlings, 60 saplings, 69 poles, and 38 adult tree species (Annexure 1). Meanwhile, around the small drainage block, there were a total of 86 species, consisting of 54 seedlings, 57 saplings, 59 poles, and 39 adult tree species. The conditions in these 2 blocks with a decrease in vegetation density with a pattern like a letter "J" upside down (Figure 4a), indicated that the stand structure was still normal and the regeneration process was running well. Based on the density values of the 2 studied blocks the pattern is as seedlings > saplings > poles > trees. These results are in line with several previous researchers Indriyanto (2006), Sidiyasa (2009), and Hidayat (2014) who reported similar regeneration processes. Competition for space utilization, soil nutrients, and sunlight causes the normal pattern of the regeneration process. In addition to the influence of environmental factors, it turns out that changes in vegetation density are influenced by growth and deaths (Indriyanto 2006), which leads to a reduction in the number of surviving individuals at each growth stage (Kusmana and Susanti 2015). One of the indicators of forest restoration is the creation of natural regeneration, which is marked by the growth of natural regeneration and the resilience of species diversity. However, not every species can regenerate because it is possible to change the dominant species at each growth stage.

In the case of types of species, a wide variation was reported between the studied drainage channel blocks, and the species found in large drainage channel block are not reported from the small drainage channel block. The major species reported from the large drainage channel block are *Aglaia* sp. (Bangkuang Napu), *Alstonia spatulata* (Pulai), and *Anisoptera* sp. (Katimpun), while *Ardisia* sp. (Kayu Bulu), *Artabotrys suaveolens*, *Blumeodendron kurzii* (kenari) are the major species which found in small drainage channel blocks. Furthermore, tree species that are always reported from the large channel are *Calophyllum inophyllum* (Kapur naga), *Calophyllum nodusum* (Kakal), *Calophyllum soullatri* (Takal), *Calophyllum* sp. (Panaga jangkar).

In general, the species composition obtained from this study was not so much different than the other studies in different locations of SNP. Similarly, the number of species reported from the PSF block ranged from 100 to 113 species (Page et al. 1999; Mirmanto 2010; Nugroho 2011; Siahaan et al. 2014). Furthermore, Nugroho (2011) suggested that among the studied species the dominant species was *Palaquium leiocarpum* Boerl.v. The findings of Kalima and Denny (2019) and Qirom et al. (2019) are slightly different from the present study and they reported fewer species ranging from 47 – 96 species from the different locations of SNP. Furthermore, the composition and types of species outside SNP are also very diverse; Astiani (2016) stated that the results of research on degraded PSF in Riam Berasap, West Kalimantan and found 108 species from the three types of land cover namely > 10 years after logging (low), 5-10 years after felling (medium), and degraded forest (open area or former fire). This variation in the number of species shows that PSF species composition is very dynamic, and these species variations are related to the location and time of observations. Similar types of diversity in species compositions were reported by Page et al. (1999), Mirmanto (2010), and Mofu (2011). Page et al. (1999) found that in PSF habitat sub-types peat depth increases towards the center of the Sebangau peat dome.

The absence of a significant difference in the number of species between the Large Channel Block and the Small Channel Block indicates that the channel size does not affect the number and types of the vegetation. So far there has been no publication that directly relates the dimensions of the canal to the groundwater level on peatlands, but the water level in the canal and the distance from the

Table 2 Dominant Species along with the Large and Small Drainage Channels

Observation Block	Growth Level	Local Name	Latin Name	IVI (%)
Large Drainage Channel	Seedlings	Tatumbu	*Syzygium havilandii*	17.74
		Gerunggang	*Cratoxylum arborescens*	16.86
		Tatkal	*Callophyllum soullatri*	13.45
	Saplings	Kemuning	*Xanthophyll eurhynchum*	15.09
		Malam-Malam	*Dyospyros bantamensis*	13.68
		Gentalang	*Palaquium sp.*	11.85
	Poles	Gerunggang	*Cratoxylum arborescens*	44.17
		Belangeran	*Shorea balangeran*	14.81
		Trending	*Campnosperma coriaceum*	13.21
	Trees	Belangeran	*Shorea balangeran*	33.25
		Rahanjang	*Xylopia fusca*	24.47
		Gerunggang	*Cratoxylum arborescens*	18.66
Small Drainage Channel	Seedlings	Kemuning	*Xanthophyll eurhynchum*	26.72
		Jambu Hutan	*Syzygium sp.*	23.98
		Jambu-Jambu	*Syzygium incarnatum*	15.37
	Saplings	Kemuning	*Xanthophyll eurhynchum*	22.53
		Jambu Hutan	*Syzygium sp.*	20.38
		Belangeran	*Shorea balangeran*	10.16
	Poles	Belangeran	*Shorea balangeran*	29.88
		Malam-Malam	*Dyospyros bantamensis*	18.31
		Manggis Hutan	*Garcinia banana*	18.22
	Trees	Malam-Malam	*Dyospyros bantamensis*	28.57
		Belangeran	*Shorea balangeran*	28.30
		Tumih	*Combretocarpus rotundatus*	25.55

canal significantly change the characteristics of peat, especially bulk density (Astiani et al. 2017; Sinclair et al. 2020). The results of the study showed that drainage ditches in peatland landscapes lowered the water level more than 3 times, and it reached from 11.7 cm to 37.3 cm. The impact of this drainage channel on the water level is worse in dry months (July-August). The lowering of the peat water level makes changes the microclimate of the soil, especially the temperature and water content of the peat. Astiani et al. (2017) also reported that changes in land use on peat along with drainage development also affected the peat water level.

As shown in Table 2 dominance of the species is described by the IVI value of standing vegetation. The upright vegetation in the study area was dominated by *Shorea balangeran* (Balangeran) which were present at almost all growth levels of both studied blocks. Further, *S. balangeran* is a commercial species that is almost extinct in its natural habitats (Wardani and Susilo 2016) and can be categorized as a species that is resistant to growth and regeneration in burned forests (Atmoko 2011). In addition, this tree species cannot tolerate shaded conditions and it required light for its normal growth and that's why it is well acceptable under the studied environmental conditions (Wibisono et al. 2005). Species dominance also came from pioneer species, such as *Combretocarpus rotundatus*, *Cratoxylum arborescence*, and *Calophyllum* sp. and this indicates that this location is highly disturbed by drainage, fire, or waterlogging. In peatlands affected by fires, species from the genus Calophyllum are generally found, and the species such as *C. rotundatus*, *Palaquium sp*., and *C. arborencens* were reported from the studied areas also (Astiani 2016; Simbolon 2004; Yulianti et al. 2009). The channel of the study area causes excessive drainage, which results in repeated droughts and fires in the Mangkok Resort area. The results of the current study suggest that repeated fires made changes in vegetation composition and disrupt natural regeneration processes.

The findings of Hoscilo et al. (2011) also suggested that repeated fires causes land cover changes and mostly it is dominated by non-timber vegetation such as ferns or tree mosaics.

3.2 Species Diversity Index (H')

The species diversity index is a parameter that can describe the state of succession or community stability (Goodman 1975; Subiandono and Heriyanto 2016; Pamoengkas et al. 2019). Further, a community can keep itself stable, despite disturbances to its components. Kalima and Denny (2019) stated that forest communities consist of various types of plants, the older the forest stand, the species diversity becomes higher. The diversity index values for each growth stage at the large drainage channel block and small drainage channel block are presented in Figure 5. All values of the species diversity index (H') at various growth stages fall between 1.43 and 1.57, which means the species are abundant and well distributed. The results of the calculation of the diversity index also show that the size of the drainage channel does not affect the value of the species diversity index, although the highest H' value was observed for the large drainage channel block (1.46-1.57) but it was lower than the peat swamp forest area of Sumatra, which ranges from 2.89-2.96 (Istomo and Aziz 2021).

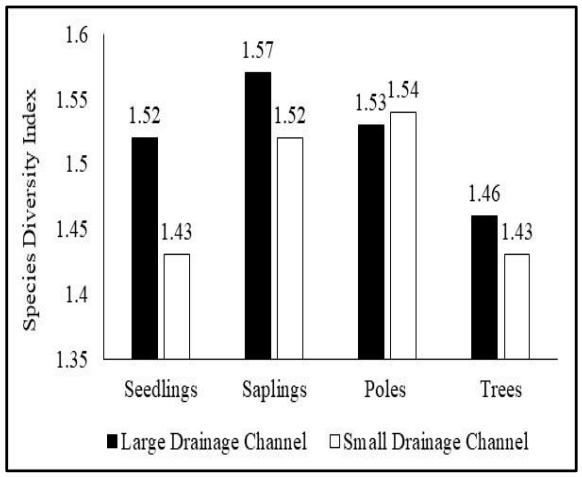


Figure 5 Index of Species Diversity at all Growth Level for both types of channel

3.3 Species Richness Index (R)

Ismaini et al. (2015) stated that species richness is the number of species in a community, where a larger number of species found have the highest species richness index. The Margalef richness index divides the number of species by natural functions, showing that an increase in the number of species is inversely proportional to an increase in the number of individuals. According to Magurran (2004), an R-value <3.5 indicates low species richness, while a value of 3.5 to 5 indicates moderate species richness, and an R-value > 5 indicates high species richness. The species richness index in the large drainage channel block is in the range of 16.80 – 23.03 while it was reported 18.31- 20.93 for the small drainage channel (Figure 6). The highest species richness value 23.03 was at the poles growth stage of the large drainage channel. The species richness index of the Sebangau research area is higher than other research sites of the peat swamp forest area of Sumatra where it was reported between 5.18-6.51 (Istomo and Aziz 2021).

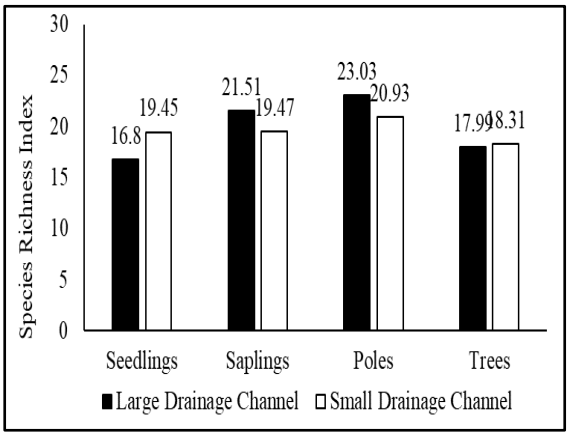


Figure 6 Index of Species Richness at all Growth Level

3.4 Species Evenness Index (E)

The evenness index value is used to measure the degree of evenness of the abundance of individual species in the community. It describes the balance between one community to another. It is stated that if the value of E is close to 1, the evenness is high (Soegianto 1994; Sidauruk 2016). According to Kusnadi (2015), the highest evenness index is directly proportional to the species diversity index. According to Magurran (2004), if an evenness value is close to 1 indicates that a community is more evenly distributed, whereas it is increasingly unevenly distributed if the value is close to zero. The evenness index value is strongly influenced by the diversity index and the number of species. The evenness index is directly proportional to the species diversity

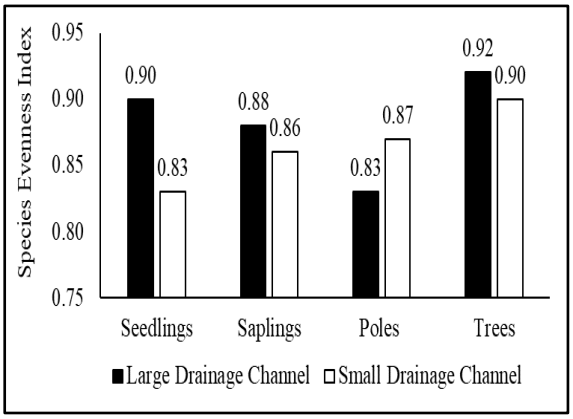


Figure 7 Index of Species Evenness at all Growth Level

index, the higher diversity index values also have the higher evenness index values (Odum 1993). Further, if the species diversity index is low and the number of species is also low the evenness index value will be also small.

As shown in Figure 7, the Evenness Index (E) of the two studied blocks is high with a value range of 0.83 – 0.92 at various growths stages; and this was slightly higher compared to Gunawan et al. (2012), where the E value was reported between 0.77 and 0.84. The highest values were found for adult trees near both large and small drainage channel which suggest that these are the species with a relatively equal and even number of individuals. Further, the species evenness index for both the studied block was very high which suggested that species are evenly distributed in both the studied blocks. This is also suggested that the channel size has no significant effect on the resulting evenness index value.

3.5 Species Similarity Index (SI)

Results related to the similarity values for all growth levels have been given in Table 3, which suggests that the similarity values between large and small channel blocks are in the range of 46.75 - 73.44%. The similarity of species was shown at the various growth stages of seedlings, saplings, and poles with SI values > 50%, while in the case of adult trees SI value was reported 46.75% which showed higher dissimilarity. Djufri (2003) suggests that if the SI value is greate0072 than 75% the similarity criterion is very high, while when it is in the range of 50 - 75%, 25 – 50%, and < 25% it comes under the categories of high similarity, low similarity, and very low similarity respectively. Many tree species are that are present in the large channel are not found in the small channel and vice versa. This phenomenon occurs because the forest conditions are relatively nonhomogeneous. Barbour et al. (1987) suggested that individuals of the same type will occupy relatively homogeneous microsite conditions because these species naturally have developed adaptation and tolerance mechanisms to their habitat. Loveless (1983) also suggested that physical and chemical factors along with animals and humans' activities also determined the types of plant or plant community.

According to the Taman Nasional Sebangau (2016) reports, the forest fire is more common around the large channel and this might be responsible for the smaller value of the similarity index. Further, variations in environmental, physical, and chemical conditions and the interactions between the study area species also affect the similarity index, and as a result study area also had low category vegetation similarity (Loveless 1983). Barbour et al. (1987) suggested that individuals of the same type occupy relatively homogeneous microsite conditions and this helps species to naturally developed adaptation and tolerance mechanisms for their habitat.

Conclusion

The channels were constructed as part of a previous logging concession in the PSF area of Mangkok Resort, SNP. Results of the study suggested that although the construction of channels resulted in the over-drainage which developed the dry conditions and increased the chances of forest fires but after this also vegetation composition did not show any significant changes in the study area. Further, in terms of variations in distance from the river and peat depth, the number of species recorded in this study is higher than the previous studies in the same location, but it is not so much different from the studies conducted in other locations of SNP. Study area vegetation is dominated by *Shorea spp, C. rotundatus, C. arborencens,* and *Calophyllum* sp. Results of the study also suggested that the dimensions of the drainage channel seem did not affect the number of species, species diversity index, species richness index, and evenness index. Further to better establish the influence of the existence of channels on the composition and diversity of vegetation types in SNP and other degraded PSF areas the consistency can be tested by making observation blocks on the channel that have been recently rehabilitated.

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Table 3 Species Similarity Index (SI %) at Observation Block

Growth stage	Number of Species		Number of Species found in both blocks	SI (%)
	Large Drainage Channel	Small Drainage Channel		
Seedlings	48	54	31	60.78
Saplings	60	57	41	70.09
Poles	69	59	47	73.44
Trees	38	39	18	46.75

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Annexure 1 List of Species Found at the Research Site

Species	Family	Large Channel	Small Channel
Acronychia pedunculata	Pteridaceae	√	√
Adinandra sp.	Pentaphylacaceae	√	√
Aglaia rubiginosa	Meliaceae	√	√
Aglaia sp.	Meliaceae	√	X
Alseodaphne coriacea	Lauraceae	X	√
Alstonia spatulata	Apocynaceae	√	X
Anispotera sp.	Dipterocarpaceae	√	X
Antidesma coriaceum	Phyllanthaceae	√	√
Antidesma diandrum	Phyllanthaceae	√	√
Archidendron borneense	Fabaceae	√	X
Ardisia sp.	Primulaceae	X	√
Artabotrys suaveolens	Annonaceae	X	√
Artocarpus sp.	Moraceae	√	X
Baccaurea bracteata	Phyllanthaceae	√	√
Barringtonia longicephala	Meliaceae	√	√
Beilschmiedia glabra	Lauraceae	√	√
Blumeodendron kurzii	Euphorbiaceae	X	√
Calophyllum inophyllum	Calophyllaceae	√	√

Species	Family	Large Channel	Small Channel
Calophyllum nodusum	Calophyllaceae	√	√
Calophyllum soullatri	Calophyllaceae	√	√
Calophyllum sp. 2	Calophyllaceae	√	√
Calophyllum sp. 3	Calophyllaceae	√	√
Calophyllum sp. 4	Calophyllaceae	√	√
Calophyllum venulosum	Calophyllaceae	√	√
Campnosperma coriaceum	Anacardiaceae	√	√
Carallia brachiata	Rhizophoraceae	√	X
Castanopsis tungurrut	Fagaceae	X	√
Combretocarpus rotundatus	Anisophylleaceae	√	√
Cratoxylum arborescens	Hypericaceae	√	X
Crudia tenuipes	Fabaceae	√	X
Cryptocarya crassinervia	Lauraceae	√	√
Dacrydium pectinatum	Podocarpaceae	√	X
Dactylocladus stenostachys	Crypteroniaceae	X	√
Dialium sp.	Fabaceae	√	√
Diospyros bantamensis	Ebenaceae	√	√
Diospyros perfida	Ebenaceae	√	X
Diospyros pseudomalabarica	Ebenaceae	√	√
Diospyros siamang	Ebenaceae	X	√
Diospyros sp. 2	Ebenaceae	X	√
Dipterocarpus borneensis	Dipterocarpaceae	√	X
Dryobalanops beccarii	Dipterocarpaceae	√	X
Dyera lowii	Apocynaceae	√	√
Elaeocarpus glaber	Elaeocarpaceae	√	√
Elaeocarpus mastersii	Elaeocarpaceae	√	√
Elaeocarpus palembanicus	Elaeocarpaceae	X	√
Elaeocarpus parvifolius	Elaeocarpaceae	√	√
Eugenia elmerii	Myrtaceae	√	√
Evodia speciosa	Rutaceae	√	X
Fagraea racemosa	Gentianaceae	√	√
Ficus sp. 1	Moraceae	√	√
Ficus sumatrana	Moraceae	√	X
Garcinia bancana	Guttiferae	√	√
Gardenia tubifera	Guttiferae	√	√
Gluta sp.	Anacardiaceae	√	X
Gnetum cuspidatum	Gnetaceae	√	X
Gonystylus bancanus	Thymelaeaceae	√	√
Gymnacranthera farquhariana	Myristicaceae	X	√
Horsfieldia sp.	Myristicaceae	√	√

Species	Family	Large Channel	Small Channel
Ilex cymosa	Aquifoliaceae	√	√
Ilex hypoglauca	Aquifoliaceae	√	X
Koompassia malaccensis	Fabaceae	√	√
Lithocarpus conocarpus	Fagaceae	√	√
Lithocarpus sp.	Fagaceae	√	X
Litsea lanceolata	Lauraceae	X	√
Litsea noronhae	Lauraceae	√	√
Litsea sp.	Lauraceae	X	√
Litsea sp. 1	Lauraceae	√	√
Lophopetalum javanicum	Celastraceae	√	X
Madhuca motleyana	Sapotaceae	√	√
Mallotus sp.	Euphorbiaceae	√	√
Mezzettia sp.	Annonaceae	√	√
Microcos sp. 3	Malvaceae	√	√
Myristica maxima	Myristicaceae	X	√
Myristica sp.	Myristicaceae	X	√
Neoscortechinia kingii	Euphorbiaceae	√	√
Nephelium maingayi	Sapindaceae	√	√
Palaquium ridleyi	Sapotaceae	√	√
Palaquium sp. 1	Sapotaceae	√	√
Palaquium sp. 2	Sapotaceae	√	√
Parartocarpus venenosa	Moraceae	√	√
Platea sp.	Icacinaceae	X	√
Pouteria malaccensis	Sapotaceae	√	√
Pternandra coerulescens	Melastomataceae	X	√
Sandoricum beccarianum	Meliaceae	√	√
Santiria apiculata	Burseraceae	√	√
Shorea balangeran	Dipterocarpaceae	√	√
Shorea sp. 2	Dipterocarpaceae	√	√
Shorea sp. 3	Dipterocarpaceae	√	X
Shorea teysmaniana	Dipterocarpaceae	X	√
Shorea uliginosa	Dipterocarpaceae	√	√
Stemonurus secundiflorus	Stemonuraceae	X	√
Sterculia sp.	Malvaceae	√	√
Syzigium lineatum	Myrtaceae	√	√
Syzigium sp. 1	Myrtaceae	X	√
Syzigium sp. 3	Myrtaceae	√	√
Syzygium chloranthum	Myrtaceae	√	√
Syzygium havilandii	Myrtaceae	√	√

Species	Family	Large Channel	Small Channel
Syzygium incarnatum	Myrtaceae	X	√
Syzygium zeylanicum	Myrtaceae	√	X
Tarenna fragrans	Rubiaceae	√	X
Tetractomia tetrandra	Rutaceae	√	√
Tetramerista glabra	Tetrameristicaceae	√	√
Tristaniopsis merquensis	Myrtaceae	√	√
Tristaniopsis sp. 2	Myrtaceae	√	X
Urophyllum sp.	Rubiaceae	√	X
Vatica sp.	Dipterocarpaceae	√	√
Xanthophyllum eurhynchum	Polygalaceae	√	√
Xanthophyllum stipitatum	Polygalaceae	√	X
Xerospermum sp.	Sapindaceae	√	√
Xylopia fusca	Annonaceae	√	√

Note: √ = Available; X = Not available

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Differentiation Effect of Two Alkaloid Fractions from Vietnamese Lycopodiaceae on Mouse Neural Stem Cells

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Huperzia serrata

Lycopodium Alkaloid

Neuronal Disease

Mouse Neural Stem Cell

ABSTRACT

Various Lycopodium alkaloids have been studied for their various biological activities including anti-inflammatory, antioxidant, immunomodulatory, and neuroprotective activities. Moreover, these alkaloid compounds have high potential in the treatment of neuron degenerative disease. This study has been carried out to test the effect of *Huperzia serrata* (Thunb.) Trevis, and *Lycopodium clavatum* L alkaloid fractions on the mouse neural stem cells (NSCs). Firstly, the alkaloid fractions were used to verify its toxicity on NSCs. The multiple concentrations of alkaloid fractions from *H. serrata* (0.044; 0.088; 0.175; 0.35; 0.7; 1.4 mg/ml) and *L. clavatum* (0.031; 0.063; 0.125; 0.25; 0.50; 1.0; 2.0 mg/ml) have been used for the treatment of NSCs at period of 48h incubation. Results of the study suggested that the IC_{50} value of *H. serrata* and *L. clavatum* was 0.56 mg/ml and 0.50 mg/ml, respectively. Then, the NSCs were differentiated in the presence of 5 and 10 µg/ml of alkaloid fraction from *H. serrata*; 0.625 and 1.25 µg/ml of alkaloid fraction from *L. clavatum* for 6 days. Here, we observed the primary NSCs treated with alkaloid fraction extract from *H. serrata* showed the increased gene expression level of early neuron *TUBB3* and neuron-specific cytoskeleton *MAP2*. On the other hand, the *L. clavatum* alkaloid fraction increased the expression of neural stem cell marker genes (*Nestin* and *PAX6*) and decreased neuron marker genes. In conclusion, these results established that alkaloid fraction from *H. serrata* promoted differentiation of the mouse NSCs to neuron cells, and *L. clavatum* extract had a capacity for stemness maintenance.

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1 Introduction

Neural stem cells (NSCs), located in the central nervous system, are multipotent cells that can self-renew, proliferate and differentiate into neurons and glial cells (Fujita 2003; Noctor et al. 2001; Takouda et al. 2017). NSCs are present not only in the developing brain but also in specific areas such as the subventricular zone (SVZ) and hippocampus of the adult brain (Temple 2001; Gage 2000). Recent studies indicate that NSCs can be activated after neuronal injury and migrate to the injured sites to replace the lost neurons (Nakatomi et al. 2002; Russo et al. 2011). NSCs can be cultured as neurospheres and later on develop into an *in vitro* neural network. Primary NSCs culture provides an *in vitro* model to identify drugs that can protect NSCs or increase neurogenesis from NSCs. This raised the potential for the treatment of neural damage and neurodegenerative diseases (Gage 2000).

The Lycopodiaceae plants belong to club moss, mainly native to tropical mountains characterized by low evergreen herbs with needle-like or scale-like leaves. The Lycopodiaceae has been widely known as the traditional herbal medicine for a long time to treat medical ailments such as swelling, rheumatic fever, myasthenia gravis, schizophrenia (Ma and Gang 2004). These plants are rich in alkaloids that have high potential bioactivity properties with a range of applications in therapy and research. The lycopodium alkaloids have a unique structure of heterocyclic skeletons such as C16N1, C16N2, and C27N3. Until now, more than 400 lycopodium alkaloids have been described (Kitajima and Takayama 2011; Siengalewicz et al. 2013). The Lycopodium alkaloids have been shown various biological activities such as anticancer, antiviral, anti-inflammatory, hepatoprotective, and immunomodulatory activities. Recently, these alkaloids have been used to treat an aneurysm, chronic lung, and bronchial diseases also. Further, most of the alkaloids derived from Lycopodium were demonstrated their potential effects on neuron diseases (Ayer 1991; Zangara 2003; Kitajima and Takayama 2011; Siengalewicz et al. 2013). Huperzine A was extracted from *H. serrata* (Thunb.) Trevis and used as a folk medicine in the treatment of inflammation, swelling, and fever. The scientific evidence showed that these compounds also have other pharmacological activities such as anti-inflammation, antioxidation, protection of cellular organelles from some neurotoxic, and regulation of nerve growth factor (NGF) (Wang and Tang 2007; Zhang et al. 2008; Wang et al. 2011). Huperzine A is a potent, specific, selective, and reversible inhibitor of acetylcholinesterase (AChE) (Friedli and Inestrosa 2021). This compound also has some effects on the learning and memory of animals. Li et al. (2021) reported the clinical effect of Huperzine A on elderly patients with vascular dementia. The result indicated that the co-treatment of Huperzine A and hyperbaric oxygen had a significant effect on cognitive function and serum hypoxia-inducible factor-1α level (Li et al. 2021). Using the meta-analysis of randomized clinical trials on Alzheimer's disease patients indicated that Huperzine A seemed to have significant effects on cognitive as well as activities of daily living, and improved global clinical assessment (Yang et al. 2013). *L. clavatum* is a rich source of alkaloids and triterpenoids. Recently, Dymek et al. (2021) developed a technique to extract *L. clavatum*, *L. annotinum*, and *H. selago* active ingredients by using the pressurized liquid extraction method. These methanolic extracts showed bioactivity as anti-AChE (Dymek et al. 2021). Three new triterpenoids of *L. clavatum* were isolated from the methanol extract via combined chromatographic separation techniques. The primary bioactivities screening showed that these new triterpenoids had a cytotoxic and anti-inflammation effect (Giang et al. 2022). *In vivo* experiment in the rat model of Parkinson's disease, lycopodium extracts also reduced the loss of dopaminergic neurons by suppressing oxidative stress and neuron inflammation (Jayaraj 2019).

In this study, two lycopodium alkaloid extracts from Vietnam were used to check the effect on the differentiation stage of primary NSCs. The data demonstrated the maintain NSCs and activating the differentiation of NSC. These results highlight the scientific evidence of these lycopodium alkaloids in application to the treatment of neuron degenerative disease.

2 Materials and Methods

2.1 Plant Materials

The whole dried plants of *H. serrata* and *L. clavatum* were collected from mountain regions of Vietnam. The plants were identified by the trained taxonomist. After their identification, the voucher specimens were deposited at the Museum of Biology, Faculty of Biology, University of Science, Vietnam National University, Hanoi, (accession number HNU 024659 for *L. clavatum* and HNU 024660 for *H. Serrata*.

Further, 80 g of whole plant powder was extracted with MeOH and 0.5% NaOH, sonicated for 10 minutes, and refluxed three times. The crude extracts of *H. serrata* (18.39g) and *L. clavatum* (18.69g) were dried under reduced pressure and resuspended in 200 ml of 1N HCl and then partitioned with EtOAc (ratio 1:1) (Chuong et al. 2014). The final concentration of alkaloid fractions of *H. serrata* and *L. clavatum* was 6.61g and 5.19g, respectively. The alkaloid fractions were dissolved in DMSO for further study.

2.2 Primary NSCs culture and differentiation condition

The primary neuronal stem cells were kindly received from Animal Cell Laboratory, Center for Life Science Research (CELIFE), University of Science, Vietnam National University, Hanoi. The cells were maintained on neuropan basal medium

Table 1 List of primers sequences

Primers	Sequences
Nestin	F: 5′- GGTGGGCAGCAACTGGC -3′
	R: 5′- CAGCTTGGGGTCAGGAAAGCC -3′
Pax 6	F: 5′- CTGGAGAAAGAGTTTGAGAGG -3’
	R: 5′- CTG CTGCTG ATA GGAATGTG -3’
MAP2	F: 5′- AAGTCACTGATG GAATAAGC -3’
	R: 5′- CTCTGCGAATTG GTTCTG -3’
TUBB3	F: 5′- GCCTCCTCTCACAAGTATG -3’
	R: 5′- CCTCCGTATAGTGCCCTT -3’
GAPDH	F: 5′- GTGGCAAAGTGGAGATTGTTGCC -3’
	R: 5′- GATGATGACCCTTTTGGCTCC -3’

(PAN-biotech) supplemented with 2% B27 (without retinoic acid), 10 ng/ml bFGFvà, 10 ng/ml EGF, and 1% antibiotic mixture (50 U/ml penicillin, 50 µg/ml streptomycin, >1 ng/ml amphotericin B). For differentiation reduction, neurospheres were harvested on day 6 and then transferred to plates with poly-D-lysine (PDL)-coated surface containing 1% fetal bovine serum (FBS), neuropan basal medium, supplemented with mixture antibiotic and without the growth factors. All cells were culture at 37°C in a humidified incubator with 5% CO_2 (Zhu et al. 2019).

2.3 Lycopodium alkaloid fraction treatment conditions

Lycopodium alkaloid fractions were dissolved in DMSO and diluted in a growth medium to make the final 1% concentration of DMSO. For control experiments, the same volume of DMSO was added. The cytotoxic activities of isolated alkaloids were evaluated at different concentrations and determined by the Promega Cell Titer-Glo® Luminescent Cell Viability Assay kit. Primary NSCs were cultured in 96 wells of opaque black culture disk (SPL 31496). The plant extracts were added 0.044; 0.088; 0.175; 0.35; 0.7; 1.4 (mg/ml) for *H. serrata*; 0.031; 0.063; 0.125; 0.25; 0.5; 1.0; 2.0 (mg/ml) for *L. clavatum* for 48h (Ma et al. 2013). The measure of the cell viability was followed by the instruction. Briefly, after 48 hours of treatment, the medium was replaced by CellTitter-Glo Luminescent solution, then the cells were lysis by shaker at 500 rpm in 10 minutes. The disks were rested at room temperature for 5 - 10 minutes and read the luminescent signal at 535 nm by a multi-plate reader (Berthold Tristar LB 942). Cell viability curves were drawn, and IC_{50} was calculated using GraphPad Prism software version 8. To test the effect of alkaloid fractions in the differentiation of the primary NSCs, 5 and 10 µg/ml of *H. serrata* extract; 0,625 and 1,25 µg/ml of *L. clavatum* extract were added in the differentiation medium. The morphology was observed on day 2 and day 6. The images were captured by phase-contrast microscopy.

2.4 RNA extraction and quantitative Reverse-Transcription polymerase chain reaction (qRT-PCR)

Total RNAs were extracted using the RNeasy Mini Kit (Qiagen, 74104), and cDNAs were generated by reverse transcription using the qScript cDNA (Quantabio, 95047). qRT-PCR was performed using 2 µl of cDNA in 25 µlGoTaq® qPCR Master Mix (Promega). The mouse gene-specific primers were synthesized and used in the quantification of transcripts are shown in table 1.

Mouse *GAPDH* was used as an internal control. Real-time PCR reactions were carried out using the Applied Biosystems 7500 Real-Time PCR System. Results are expressed as fold induction in mRNA levels as calculated by the ΔΔCT method (Livak and Schimittgen 2001).

2.5 Statistical analysis

Statistical analysis was performed using the paired t-tests for in vitro data and the nonparametric Mann-Whitney test for in vivo data with GraphPad Prism version 8.0 software (GraphPad Software). Results are presented as means ± SEM (standard error of the mean). Data with a P value less than 0.05 were considered significant.

3 Results and Discussion

3.1 Effect of alkaloid fractions on the morphology of neural stem cells

In the current study, the primary mouse neural stem cells (NSCs) were received from Animal Cell Laboratory, CELIFE, VNU University of Science, Hanoi. To confirm the stemness of cultured cells, semi-quantify RT-PCR was performed to check the expression of neural progenitor/stem cells marker genes. As shown in Figure 1A, the neurospheres were formed, and the high

expression of *Nestin* and *PAX6* was detected but not for other marker genes for differentiation. This result indicated that these cells had the neural stem cell status.

Cytotoxicity of two alkaloid fractions of *H. serrata* and *L. clavatum* was calculated as the percentage of cell viability by using CellTiter-Glo® assay. The multiple concentrations of alkaloid fractions from *H. serrata* (0.044; 0.088; 0.175; 0.35; 0.7; 1.4 mg/ml) and *L. clavatum* (0.031; 0.063; 0.125; 0.25; 0.50; 1.0; 2.0 mg/ml) were used to treat NSCs at period of 48h incubation (Figure 2). Cell viability analyses designated that alkaloid fractions caused development inhibition of NSCs in dose-dependent manners. At low concentrations, both *H. serrata* and *L. clavatum* extracts had little effect on cell viability. Further, the concentration level of 0.35 mg/ml of *H. serrata* and 0.25 mg/ml of *L. clavatum* started cell survival inhibition. Both plant extracts showed a less cytotoxic effect on the primary NSCs with the IC_{50} value of 0.56 mg/ml (*H. serrata*) and 0.50 mg/ml (*L. clavatum*).

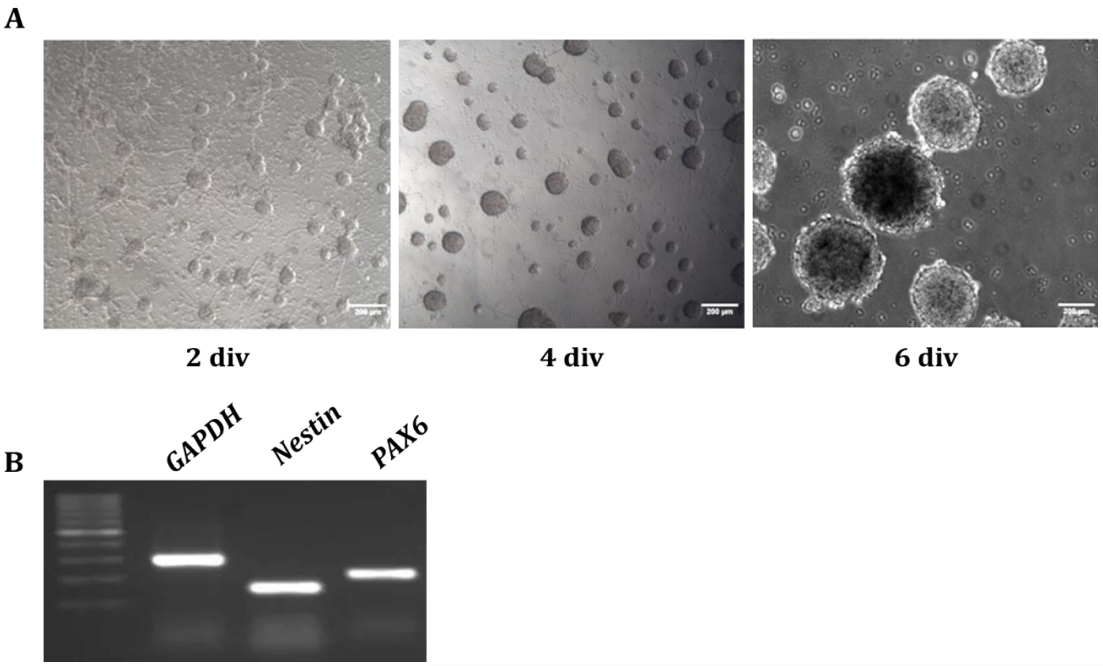

Figure 1 Identification of the mouse primary NSCs. A - Representative images of neuro-sphere in continuous culture at 2, 4, 6 days *in vitro*. Scale bar = 200 µm. B - The expression of neural stem cell marker genes, *Nestin*, *PAX6*, was determined by RT-PCR.

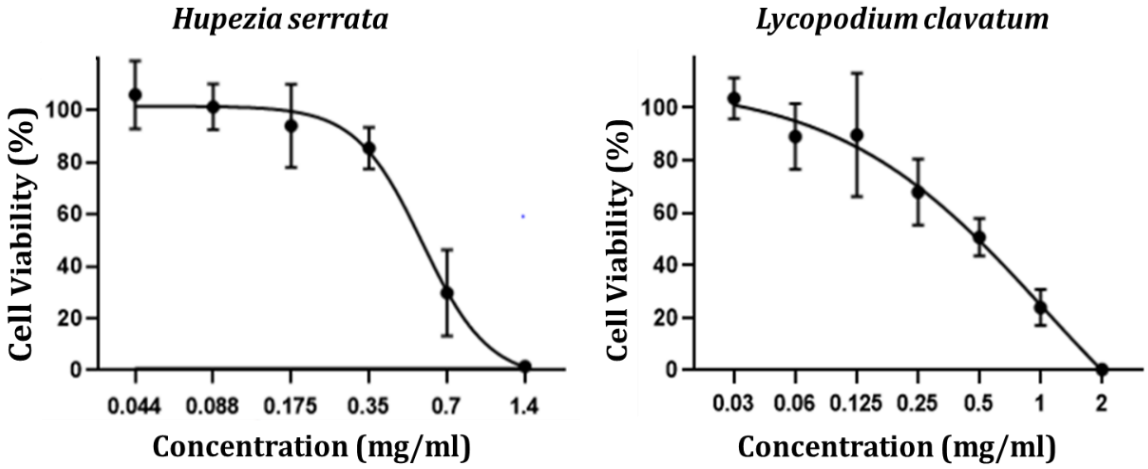

Figure 2 Toxicity effect of the Lycopodium alkaloid fractions on primary mouse neural stem cells after 48 h post-treatment. A - *Hupeziaserrata* IC_{50} = 0.56 mg/ml. B -*Lycopodium clavatum* IC_{50} = 0.50 mg/ml.

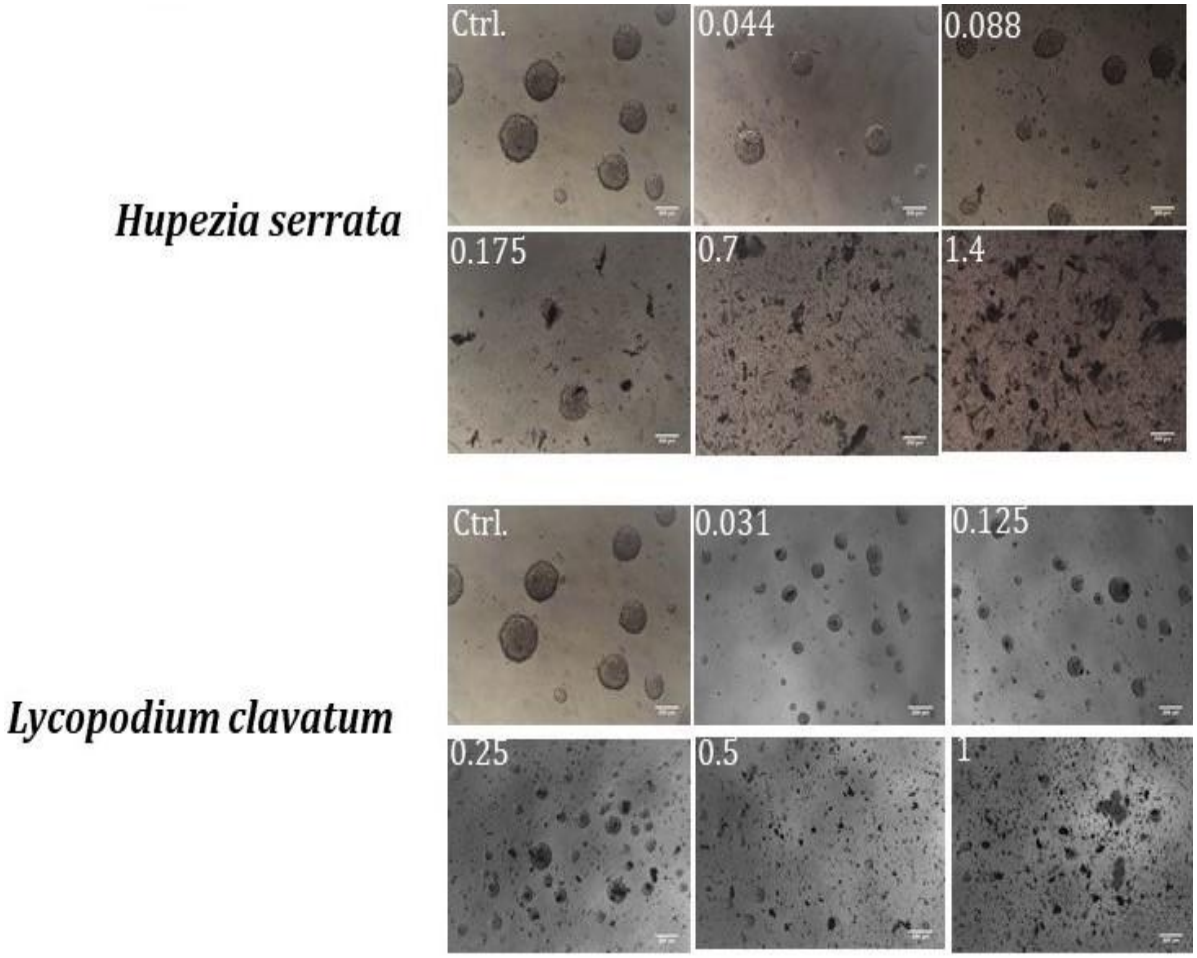

Figure 3 Imagines of the morphological changes induced by the alkaloid fraction of various concentrations of *H. serrata*and *L. clavatum* on the primary NSCs after 48 h post-treatment. The cells were observed by using a phase-contrast microscopy. Scale bar = 200 µm.

To identify the effect of two alkaloid fractions, changes in cell morphology were also observed. The primary NSCs were exposed to multiple concentrations of alkaloid fractions of *H. serrata* and *L. clavatum* and observed under a phase-contrast microscope. 48h of post-treatment, the primary NSCs indicated the most prominent effects on both alkaloid fractions. Results presented in figure 3 showed that the number of cell death increased correspondingly with an increase of alkaloid fraction concentrations. At the lower concentration, 0.044 mg/ml of *H. serrata* extract and 0.031 mg/ml of *L. clavatum* extract, the neurospheres remained forming. Nevertheless, at the higher concentrations, the shape of the primary NSCs reduced in size, became shrunken, and broke into small pieces. Furthermore, at the highest concentration (1.4 mg/ml for *H.serrata* and 1.0 mg/ml for *L. clavatum*), the primary NSCs turned to black, completely fractured, and showed signs of detachment from the surface of the wells denoting cell death. Untreated primary NSCs (control) had no change in morphology.

3.2 Effect of Alkaloid fractions extracted from *H. serrata* and *L.clavatum* on the differentiation of NSCs

To identify bioactivities related to the differentiation of primary NSCs, we determined the effect of the alkaloid fractions under the differentiation condition. The primary NSCs were differentiated in the presence of DMSO or Lycopodium alkaloids extract dissolved in DMSO, and the morphology was observed on day 2 and day 6. Here, 5 and 10 µg/ml of alkaloid fraction from *H.serrata*; 0.625and 1.25µg/ml of alkaloid fraction from *L.clavatum* were added to the differentiation medium. These concentrations did not show any effect on the viability of primary NSCs in the differentiation medium. Similarly, no neuron cell morphological difference was reported between the control and treated cells on day 2 (Figure 4). However, after 6 days of post-treatment, the cells treated by *H.serrata* extract were in more density than control at both concentrations (5 and 10 µg/ml). On the contrary, the cells treated with *L. clavatum* have less density as compared to the control at

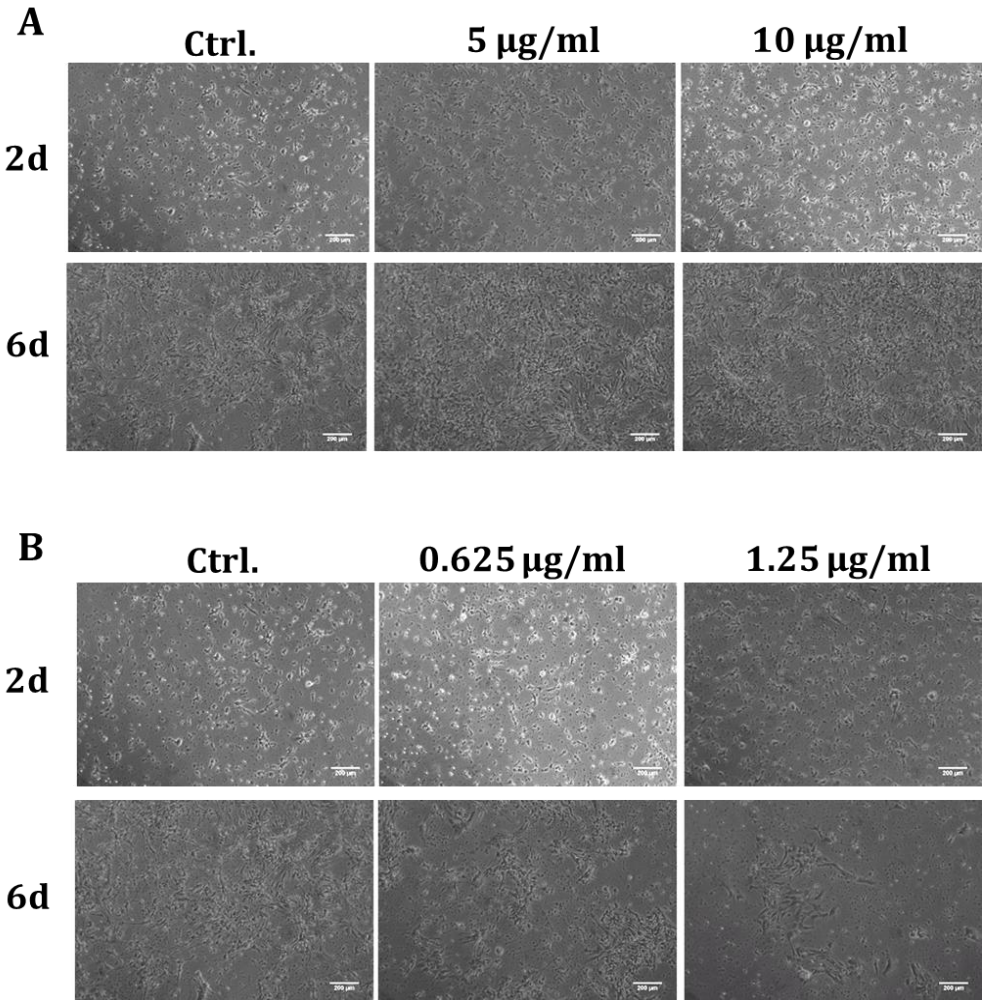

Figure 4 Lycopodium alkaloid fractions affect to differentiation of primary NSCs. A, B - Primary NSCs were exposed to alkaloid fractions from *H. serrata*and *L. clavatum* under the differentiation condition. The images were takenon day 2 and day 6. Scale bar = 200 µm

0.625 and 1.25µg/ml. These results indicated that the alkaloid fraction of *H.serrata* promoted the differentiation of primary NSCs, while the alkaloid fraction of *L. clavatum* seemed to suppress the differentiation of primary NSCs.

3.3 Effect of Lycopodium alkaloid fractions on the expression of genes related to the differentiation of NSCs

Findings of the current study suggested that both alkaloid fractions from *H.serrata* and *L.clavatum* affect to differentiation of primary NSCs, the expression of marker genes of neural stem cells and neuronal cells were also determined in the current study. *Nestin* and *PAX6* genes were selected as markers for neural stem cells and *TUBB3* and *MAP2* genes were selected as markers for neuron cells. As shown in Figure 5A, the expression of *Nestin* and *PAX6* was reduced in treated cells compared to the untreated sample after 2 days of post-treatment and remained till day 6. NSCs have the potential to differentiate into neurons and form a functional neural network. The differentiated neuronal marker *TUBB3*gene (early neuron) significantly increased after 6 days of post-treatment with alkaloid fraction from *H.serrata* extract. Moreover, the *MAP2* gene, a neuron-specific cytoskeletal protein also showed high upregulated on day 6 in comparison to the control sample. These results demonstrated that the alkaloid fraction from *H.serrata* treatment promotes NSCs differentiation to neurons.

In the case of primary NSCs exposed to alkaloid fraction from *L. clavatum* under differentiation condition, the expression of *Nestin* gene had no significant difference to control, and PAX6 was increased on day 6. On the contrary, the expression of neuronal marker genes (*TUBB3* and *MAP2*) was decreased in comparison to control (Figure 5B). These data suggested that the alkaloid fraction from *L. clavatum* could maintain stemness.

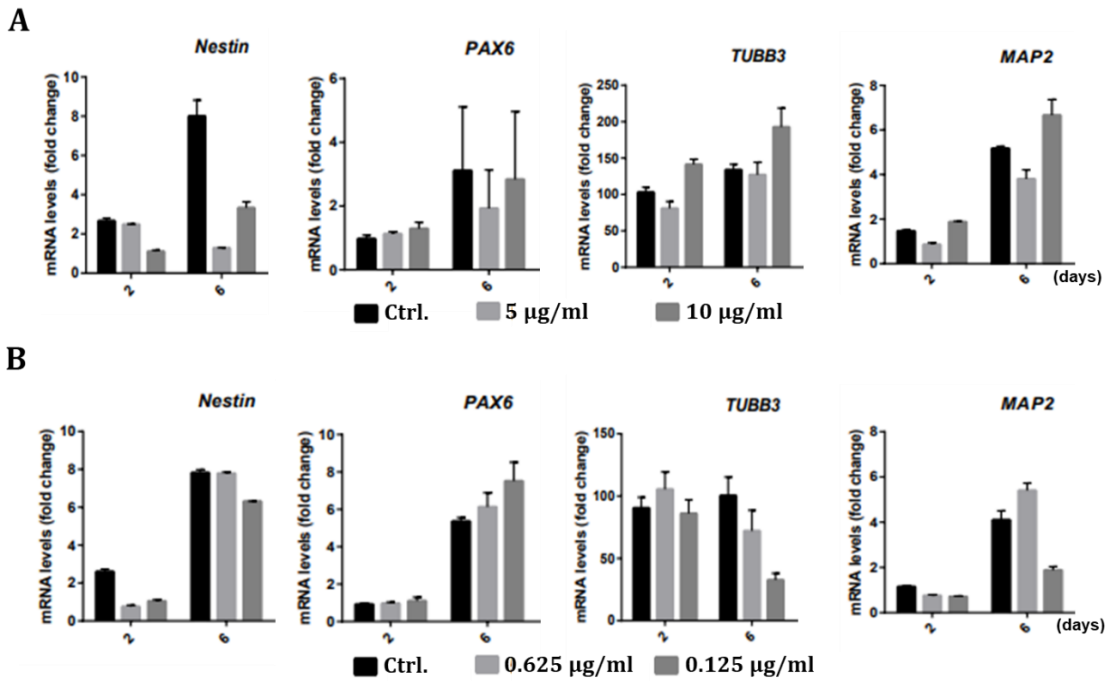

Figure 5 qRT-PCR analysis for mRNA expression levels of the Lycopodium alkaloid fractions affect the expression of genes related to differentiation of NSCs. A, B - mRNA levels of NSCs treated with alkaloid fraction of *H. serrata*and alkaloid fraction of *L. clavatum* were quantified by qRT-PCR. Data are presented as mean ± SEM.

4 Discussion

In this study, we examined the effect of alkaloid fraction from two Lycopodiaceae collected in Vietnam on the primary NSCs. First, we evaluated the effect of these extracts on the morphology of primary NSCs and then determined the cytotoxicity when the cells were exposed to the plant extracts. We found that these extracts affect the primary NSCs on dose-manner. The value of IC_{50} of *H.serrata* and *L.clavatum* was 0.56 mg/ml and 0.50 mg/ml, respectively. This data showed a less cytotoxic effect of two lycopodium alkaloid fractions on the primary NSCs.

It has been reported that lycopodium alkaloids were used in the treatment of neurogenerative diseases such as Ma et al. (2013) demonstrated that Huperzine A promoted the proliferation of cultured mouse embryonic hippocampal NSCs. Huperzine A protects the neural stem cells against Aβ-induced apoptosis in neural stem cells and microglia co-culture system by inhibiting cell apoptosis through restraining microglia's inflammatory response induced by Aβ1-42 (Zhu et al. 2015). In Panama, the Lycopodiaceae family has been screened for their anticholinesterase inhibitory and antioxidant activities, and the results showed that only *L. clavatum* subsp. clavatum showed strong AChE inhibition (Calderón et al. 2013). α-onocerin was isolated from the chloroform extract of *L.clavatum* was shown as an acetylcholinesterase inhibitor (Orhan et al. 2003). Similarly, Hanif et al. (2015) reported that *L. clavatum* on memory functions and cerebral blood flow in memory-impaired rats. There are several papers published about the bioactivities of Lycopodiaceae extract from Vietnam. Chuong et al. (2014) isolated six Lycopodium alkaloids from Vietnamese *H. squarrosa*, among them, lycosquarosine A and acetylaposerratinine have shown strong AchE inhibitory activity (Chuong et al. 2014). Two other Lycopodium alkaloids were identified and named fawcettidine, and 12-epilycodoline N-oxide from *H. phlegmaria* was collected from Vietnam. The compounds showed moderately AChE inhibitory effects (Thu et al. 2019). However, there is a lack of study on the bioactivities of these Lycopodiaceae extracts on the NSCs and their differentiation. Here, under the differentiation condition, the primary NSCs were treated with *H. serrata* derived alkaloid fraction showed the capability into mature neurons as indicated by the increased gene expression level of early neuron *TUBB3* and neuron-specific cytoskeleton *MAP2* but not to neural stem cell marker *Nestin* and *PAX6* gene. On the other hand, the alkaloid fraction from *L. clavatum* showed opposite bioactivities in the differentiation condition. With increased neural stem cell marker genes and decreased differentiation neuron marker genes compared to control, *L. clavatum* alkaloid fraction potential played a role in stemness-maintaining function. Taken together, these results established that alkaloid fraction from *H.serrata* promoted differentiation of the primary NSCs to neurons, and *L.clavatum* extract had a capacity of stemness maintaining.

Conclusions

In summary, our study calculated that the value of IC_{50} of *H. serrata* and *L. clavatum* was 0.56 mg/ml and 0.50 mg/ml, respectively. The lycopodium alkaloid from *H. serrata* showed the effect on the differentiation of NSCs to neuron cells, and the lycopodium alkaloid from *L. clavatum* has the potential to maintain the stemness of primary NSCs.

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Author's contributions

H.N.T. Tran, H.T.T. Nguyen, D.H. Nguyen, T.D. Nguyen performed the experiments. T.T. La and K.T.T. Nguyen collected the plants and identified them, Q.H. Nguyen and L.T. Nguyen critically revised the manuscript for important intellectual content. M.H.T. Hoang developed experimental concepts and designs, analyzed and interpreted data, and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Journal of Experimental Biology and Agricultural Sciences, February - 2022; Volume – 10(1) page 73 – 82

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Protective effect of *Crocus sativus* stamens extract on gentamicin-induced nephrotoxicity and oxidative damage in rat kidney

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KEYWORDS

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Oxidative stress

Nephroprotective

ABSTRACT

Crocus sativus is a medicinal plant supposedly possessing various biological activities. Currently, it is evaluated only by the medicinal properties of its stigma and many parts of this plant are unused. This work contributes to the valorization of *C.sativus* stamens by exploring the property of methanolic extract to prevent gentamicin-induced nephrotoxicity in rats. Twenty Wistar rats (weight 250 ± 30g) were assigned into four equal groups (n = 5), and among the assigned groups, group 1 was given only distilled water (Control), group 2 received intraperitoneal (i.p.) injection of gentamicin (GEN) 80 mg/kg/d, group 3 received the combination of gentamicin (80 mg/kg/d, i.p.) and oral administration of a lower dose of *C. sativus* methanolic extract (250 mg/kg/d), while the group 4 received the combination of gentamicin (80 mg/kg/d, i.p.) and oral administration of a higher dose of *C. sativus* methanolic extract (500 mg/kg/d). The injection of gentamicin for the nephrotoxicity induction and post-treatment with methanolic extract was carried out once a day for 15 days. For nephrotoxicity evaluation, biochemical and histopathological analyses were performed. The estimation of serum and urinary creatinine, blood urea nitrogen, sodium levels was carried out with the help of Architect Ci 4100 Analyzer. Oxidative stress was assessed by the determination of renal malondialdehyde (MDA) and catalase (CAT) levels. The results of the study suggested that gentamicin injection induced a significant ($p < 0.01$) elevation in serum renal

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biochemical parameters and oxidative stress indices. The methanolic extract of *C. sativus* significantly ($p < 0.05$) reduced serum creatinine, urea, and sodium levels, with an improvement in the histopathological results of gentamicin-induced alterations. Furthermore, pretreatment with plant extracts improved hepatic antioxidant status, by the elevation of the CAT and reducing the lipid peroxidation level (MDA) in tissues. The present study suggests that the methanolic extract of *C. sativus* stamens has an interesting nephroprotective effect on the renal lesions induced by GEN in modulating renal parameters and oxidative stress on *Wistar* rats.

1 Introduction

The kidney is an organ at risk for drug toxicity because maximum drugs are eliminated by the kidney either by glomerular filtration (GF) or proximal tubular secretion (Ghuysen 2005). Drug-induced nephropathies are usually lead to the appearance of acute kidney diseases that only persist for the treatment period. However, some chronic kidney disease progress may develop chronic end-stage renal failure and given the high prevalence of acute nephrotoxicity (Karie et al. 2010). Further, aminoglycosides, oxytetracycline, and nonsteroidal types of anti-inflammatory drugs might be nephrotoxic and can induce acute kidney injury (Conly et al. 1994).

Gentamicin (GEN) is an aminoglycoside and is frequently used to inhibit Gram-negative bacteria. Although it is widely used and has a positive effect, but nephrotoxicity is the most important obstacle that limited its use (Reiter et al. 2002; Morales-Alvarez 2020). It was reported that 10-30% of patients treated with GEN face acute renal failure. Unfortunately, treatment with GEN for more than 7 days shows some symptoms of nephrotoxicity (Ali 2003). GEN causes several histopathological and biochemical changes in the kidney of humans and experimental animals. Some drugs including antioxidant compounds, hormones, minerals, and vitamins play a significant role in the treatment of GEN-induced kidney damage (Ghaznavi et al. 2018; Huang et al. 2019).

On the other hand, some medicinal plants also confirmed a protective effect against GEN nephrotoxicity. *C. sativus* belongs to the genus Crocus, family Iridaceae and grows in the Eastern Mediterranean region. Iran, Morocco, Italy, and Spain are the largest producers of saffron (Khan et al. 2020). The stigma of the saffron is well known for its antioxidant, antimicrobial (Lahmass et al. 2018), anti-inflammatory (Hosseinzadeh and Younesi 2002), and anti-depression (Moshiri et al. 2006) activities. Currently, *C. sativus* is only valued by its stigmas and much of the saffron by-products such as tepals and stamens are not used and are usually discarded during stigma development (Sánchez-Vioque et al. 2012a). Recently, some pharmacological studies have been suggested the medicinal values of saffron tepals extract and found hepatoprotective (Ouahhoud et al. 2021; Omidi et al. 2014), antidepressant (Akhondzadeh Basti et al. 2007), anti-oxidant (Sánchez-Vioque et al. 2012b; Wali et al. 2020), and antifungal (Khoulati et al. 2019) properties. Till now the medicinal properties of stamens is poorly studied. Therefore, the present study has been carried out to valorize the renoprotective effect of *C. sativus* stamens against the GM-induced nephrotoxicity in *Wistar* rats.

2 Materials and Methods

2.1 Chemical Compounds.

Gentamicin sulfate (GEN) was purchased from Sigma-Aldrich Chemicals (St.Louis, MO, USA). Standard kits for urea, creatinine, and sodium assay were purchased from Biosystems, Spain. Thiobarbituric acid (TBA), Trichloroacetic acid (TCA), Glutathione, and hydrogen peroxide (H_2O_2) were purchased from Sigma Chemicals, Germany. All other reagents used in this work were of high quality and analytical grade.

2.2 Plants material and extract preparation

C. sativus plants were collected from Oujda-East of Morocco in 2019. The collected plants' specimen was identified by the trained taxonomist and deposited in the herbarium of the Department of Biology, Mohamed First University, Oujda, Morocco under the reference number HUMPOM210. The stamens were collected manually during the period between late October and early November. For obtaining the methanolic extracts, the stamens were dried at room temperature and after drying the stamens were powdered, from this 50 g of powder was macerated in 500 ml of 80% methanol (Merck Company, Germany) for 24 hours at room temperature (Montoro et al. 2012). This was followed by the filtration of the extract by a paper filter and evaporated in a rotary evaporator at 40 °C and finally, the obtained extracts were stored at -20°C until their use. This operation was repeated several times to prepare a sufficient amount of extract. The selected concentrations were prepared by the dissolving extract in distilled water.

2.3 Animals

Twenty male and female *Wistar* rats (weight 250 ± 30g) were collected and acclimatized for seven days in wire cages at constant temperature (21 ± 2°C) and in a 12h light/dark cycle with free access to food and water at the animal house of the Department of Biology, Faculty of Sciences, Oujda. Rats were cared for in compliance with the internationally accepted guideline for the care

and use of laboratory animals, published by the US National Institutes of Health.

2.4 Experimental Protocol

Rats were randomly assigned to four equal groups and each group has five animals. Among the assigned groups, animals of group 1 received only vehicle (distilled water 1 ml/kg) and served as a control, animals of group 2 were intraperitoneally (i.p.) injected by the gentamicin (80 mg/kg) for 15 days, animals of the group 3 orally received a methanolic extract of *C. sativus* stamens at a dose of 250mg/kg body weight and after two hours, 80 mg/kg; GEN was injected i.p. to rats for 15days, similarly, animals of the group 4 orally received the higher dose of *C. sativus* stamens methanolic extract (500 mg/kg body weight), and after two hours, 80 mg/kg, GEN was injected i.p. to rats for 15 days.

2.5 Collection and analysis of urine

To evaluate the diuretic activity, total urine volume and dietary intake were measured at the start (T_0) and end of the experiment for all rats groups. All animals were kept separately in metabolic cages, and after 24 hours urine samples were collected on days 0 and 15 of the establishment of the treatment (Ouahhoud et al. 2019). After determining the urine volume, the samples were stored at -20 ℃, and were analyzed for creatinine, urea, total protein, and electrolytes, including sodium with the help of Architect Ci 4100 Analyzer.

2.6 Sample collection and Biochemical assays

Twenty-four hours after the last dose of intraperitoneal injection of gentamicin, all animals were anesthetized under a light ethyl ether and sacrificed. The blood samples were collected from the abdominal aorta and were put into dry blood collection tubes (Bernardi et al. 1996). Then, the blood was centrifuged at 4 ℃ for 10 min at 3000 rpm to separate the plasma. Thereafter, the collected plasma sample was stored at -20 ℃ until biochemical analysis.

2.7 Determination of lipid peroxidation

Malondialdehyde (MDA) is a biomarker commonly used to estimate lipid. peroxidation. In this study, MDA level was determined from the kidney tissue as per the method of Draper & Hadley (1990). The excised kidneys were rinsed in 0.9% NaCl and homogenized in iced phosphate buffer (0.1 M, pH 7.4). Each supernatant from the organ homogenate extract (0.5 ml) is mixed with 1 ml of trichloroacetic acid (TCA 30%) and centrifuged at 2500 rpm for 10 minutes. After this, 1ml of 0.67% solution of thiobarbituric acid (TBA), and 0.5 ml of supernatant are incubated for 15 minutes at a temperature of 90°C. The absorbance of the TBA-MDA complex was determined at 532 nm by using a double beam UV Visible spectro-photometer (UV-Visible spectrophotometer T80+). Lipid peroxidation was expressed in nmol of MDA produced per gram (g tissue/mg of protein) using the 1.56×10^5M molar extinction coefficient:

2.8 Determination of catalase activity (CAT)

Catalases are present in a large number of tissues. They are tetrameric enzymes involved in the cell's defenses against oxidative stress (Aebi 1984). The enzymatic reaction is initiated by adding to 20 µl the tissue homogenate prepared in a 100 mM phosphate buffer solution (pH = 7.5), the substrate H_2O_2 at a concentration of 0.5 M. The amount of the supernatant (S) must be determined according to the amount of protein, which should be between 1 and 1.5 mg an amount of 10 to 20µl of diluted supernatant. Then, monitoring the degradation of H_2O_2 to O_2 at 240 nm by using a double beam UV Visible spectro-photometer (UV-Visible spectrophotometer T80+) after 15s and 60s of this addition. The catalase activity was determined according to the below formula, and the results are expressed in µmol of H_2O_2 per minute and mg of protein. The catalase activity is determined according to the following relationship: IU/g = ((2.3033) /ΔT) x (logA1/A2). Here, A1: Absorbance in after 15 seconds, A2: Absorbance after 1 minute, T: Interval of time per minute.

2.9 Assessment of histopathology

The histopathological analysis of the kidneys of all animals was performed to examine microscopically the lesions. Pieces of a kidney from each group were immediately fixed in 10% neutral formalin and dehydrated in alcohol (50~100%), embedded in paraffin, and cut into 4~5 µm thick sections using the Leica RM2235 hand-rotated microtome. These sections were stained with hematoxylin-eosin. Tissue damages in kidneys, cortex, external and internal medulla were studied using optical microscopy (Optika microscopy, Italy). The ratio of glomerulus dilation/ Bowman space in all groups was estimated using "MESURIM_PRO" software, the scale was taken from "Optika microscopy digital USB camera" microscopic imaging software, and the Bowman's space/glomerulus ratio was calculated using the following formula:

$$\text{Ratio} = (\text{BSA}/\text{GS}) * 100$$

Here, BSA = Bowman's space area in mm²; GS = Glomerulus surface mm²

2.10 Statistical analyses

The results are expressed as mean ± standard error of the mean (SEM) and were analyzed by Graph Pad Prism 5 Software using one-way ANOVA to compare the multiple-group. $P < 0.05$ was considered statistically significant.

Table 1 Effect of methanolic extract on the kidney weight and the body weight change on gentamicin induced nephrotoxicity in *Wistar* rats

Groups	Average relative kidneys weight (g/Kg)		% body weight change	
	Right kidney	Left kidney	Day 7	Day 14
Control	3.33 ± 0.16	3.12 ± 0.19	+ 7.09 ± 1.24	+11.62 ± 0.52
GEN (80 mg/kg)	3.67 ± 0.11	4.00 ± 0.15 *	+ 3.66 ± 2.47	+9.76 ± 2.87
250 mg/kg extract + GEN (80 mg/kg)	3.88 ± 0.22	3.91 ± 0.18	+ 8.04 ± 1.53	+ 12.12 ± 3.62
500 mg/kg extract + GEN (80 mg/kg)	3.64 ± 0.24	3.65 ± 0.27	+ 8.98 ± 1.56	+ 12.68 ± 3.87

The data are expressed as mean ± SEM (n = 5); $^{*}P < .05$, $^{**}P < .01$ compared to the control group; 250 mg/kg methanolic extract + GEN 80 mg/kg, while 500 mg/kg methanolic extract + GEN 80 mg/kg

Table 2 Effect of methanolic extract treatment on the urinary volume, hydric intake, and dietary intake.

Groups	urinary volume (ml/24 h)	Hydric intake (ml/24 h)	Dietary intake (g/ 24 h)
Control	13.1 ± 0.6 $^{\#\#}$	31 ± 1.6$^{\#}$	30.5± 3.4
GEN (80 mg/kg)	24± 2.6 **	48 ± 4.6	37.88 ± 2.1
250 mg/kg + GEN (80 mg/kg)	9.2 ± 0.8 $^{\#\#}$	42 ± 3.2	30.28 ± 3.2
500 mg/kg + GEN (80 mg/kg)	13.2 ± 1.6 $^{\#}$	44 ± 2.1	40.88 ± 1.1

The data are expressed as mean ± SEM (n = 5); $^{*}P < .05$, $^{**}P < .01$ compared to the control group; $^{\#}P < .05$, $^{\#\#}P < .01$ Compared to the GEN group; 250 mg/kg methanolic extract + GEN 80 mg/kg, while 500 mg/kg methanolic extract + GEN 80 mg/kg

3 Results

3.1 Effect of extract treatment on body and kidney weight

The results related to the body and kidney weight revealed that daily injection of gentamicin (80 mg / Kg) and the treatment with 250 and 500 mg/kg/day of the methanolic extract did not induce any significant change in the bodyweight of rats compared to the control (Table 1). Furthermore, no significant change was reported in the weight of the right kidney in the mice treated with *C. sativus* methanolic extract as compared to the control group while the weight of the left kidneys in rats treated with GEN showed some increases as compared to the control group. However, the mice treated with the *C. sativus* methanolic extract did not induce a significant increase in the left kidney weight as compared to the GEN group.

3.2 Effect of extract treatment on urinary volume, hydric intake, and dietary intake

Table 2 showed that water intake was significantly decreased in the untreated control. While in the extract-treated rats, the water ratio was less than in the rats treated with GEN groups. The volume of 24 hrs urine samples was also higher in the untreated rats than in the control ($p<0.01$). In contrast, the treatment with methanolic extract at 250 and 500 mg/kg significantly reduced the urinary volume as compared to the untreated GEN group ($p<0.01$ and $p < 0.05$ respectively). Further, food intake remained consistent in the control rats, as well as in the treated and untreated groups.

3.3 Effect of extract treatment on biochemical parameters of serum

Table 3 depicted the effect of *C. sativus* stamens methanolic extracts (250 and 500 mg/kg) on the serum levels of urea, creatinine, and sodium concentration in rats injected with gentamicin. When compared to the standard control, serum urea and creatinine levels in the GEN group increased significantly ($p<0.05$). In addition, rats injected with gentamicin and then treated with methanolic extract at a dose of 250 mg/kg showed a significant ($p<0.01$) decrease in serum urea and creatinine levels as compared to the GEN treated groups. Further, rats treated with the higher dose of *C. sativus* stamens methanolic extracts (500 mg/kg) remarkably reduced ($p<0.01$) the serum creatinine and urea level ($p<0.05$) as compared to the GEN group. Besides, the treatment with the methanolic extract reduces the levels of creatinine and urea for both doses ($p<0.01$) as compared to the controls. Table 3 also suggested that the GEN reduced serum sodium levels compared to control while the treatment with both extracts (250, 500 mg/kg) significantly improves ($p<0.01$, $p<0.05$) the serum sodium levels compared to the GEN group, respectively. Besides, the extract at 250 mg/kg significantly ($p<0.05$) increases serum sodium compared to controls.

Table 3 Effect of methanolic extract on the serum level of urea, creatinine and sodium in GEN-treated rats.

Groups	Serum creatinine (mg/dl)	Blood urea nitrogen (mg/dl)	Blood Sodium Level (nmol/l)
Control	0.60 ± 0.01	35.4± 1.3	150.8± 7.7
GEN (80 mg/kg)	0.66 ± 0.01*	36.6± 0.7 *	138.8± 2.9
250 mg/kg + GEN (80 mg/kg)	0.51 ± 0.02 $^{\#\#*}$	29.2±1.5$^{\#\#**}$	181.2 ± 6.7$^{\#\#*}$
500 mg/kg + GEN (80 mg/kg)	0.53 ± 0.005 $^{\#\#**}$	30.2± 2.4$^{\#}$	174.4± 13.7$^{\#}$

The data are expressed as mean ± SEM (n = 5); *P < .05, **P < .01 compared to the control group; $^{\#}$P < .05, $^{\#\#}$P < .01 Compared to the GEN group; 250 mg/kg methanolic extract + GEN 80 mg/kg, while 500 mg/kg methanolic extract + GEN 80 mg/kg

Table 4 Effect of methanolic extract on the urinary level of urea, creatinine and sodium in GEN-treated rats.

Groups	Urine creatinine (mg/24 h)	Urine urea nitrogen (mg/24 h)	Urine sodium (nmol/24 h)
Control	703.3 ± 63.8	3816± 157.06	234.4±16.4
GEN (80 mg/kg)	318.6 ± 24.5 *	1822.2 ± 107.9	140.8± 9.5
Extract 1 (250 mg/kg) + GEN (80 mg/kg)	589.05 ± 50.6 $^{\#}$	3078.4±318.9 $^{\#}$	225.2 ± 30.8 $^{\#}$
Extract 2 (500 mg/kg) + GEN (80 mg/kg)	482.9 ± 48.8 $^{\#}$	2565.4± 346.5	204.6 ± 23.8 $^{\#}$

The data are expressed as mean ± SEM (n = 5); *P < .05, compared to the control group; $^{\#}$P < .05, Compared to the GEN group; 250 mg/kg methanolic extract + GEN 80 mg/kg, while 500 mg/kg methanolic extract + GEN 80 mg/kg.

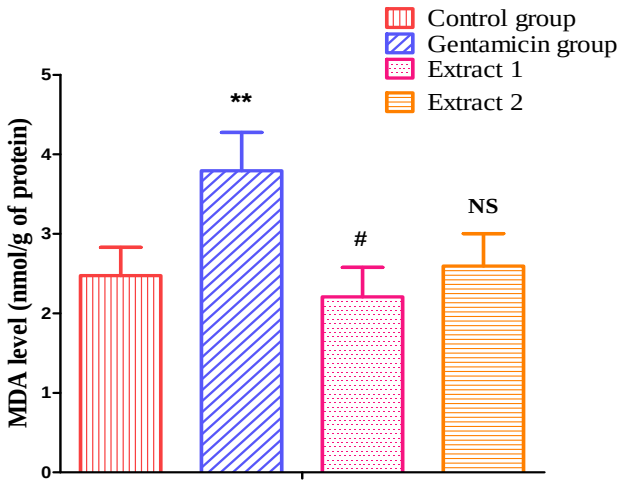

Figure 1 Effect of methanolic extract of the *Crocus sativus* stamens on MDA production in the kidneys of GEN-treated rats. Extract 1: methanolic extract (250 mg/kg + GEN 80 mg/kg), extract 2: methanolic extract (500 mg/kg + GEN 80 mg/kg). *P < .05, **P < .01 compared to the control group. #P < .05, Compared to the GEN group. NS. Not significantly different Compared to the GEN group.

3.4 Effect of extract treatment on urinary biochemical parameter

Table 4 indicates that the daily injection of gentamicin (80 mg/Kg) caused a significant ($p<0.05$) decrease in the concentration of urinary creatinine and no significant reduction was reported in the urinary urea and sodium as compared to the control. While, a significant elevation was observed in creatinine, urea, and sodium levels in the urine of rats intoxicated with GEN and treated with the extract at 250 mg/ml compared to the GEN group ($P < 0.05$). The extract at a dose of 500 mg/ml significantly ($P < 0.05$) increased the creatinine and sodium levels while no significant changes were reported in the level of urea as compared to the control.

3.5 Effect of extract treatment on oxidative stress marker and antioxidant enzyme activities

Figure 1summarized the protective effects of *C. sativus* stamens methanolic extract on MDA production in the kidneys of GEN-treated rats. Lipoperoxidation is manifested in the GEN group by a significant increase in MDA ($p < 0.01$), compared to the control

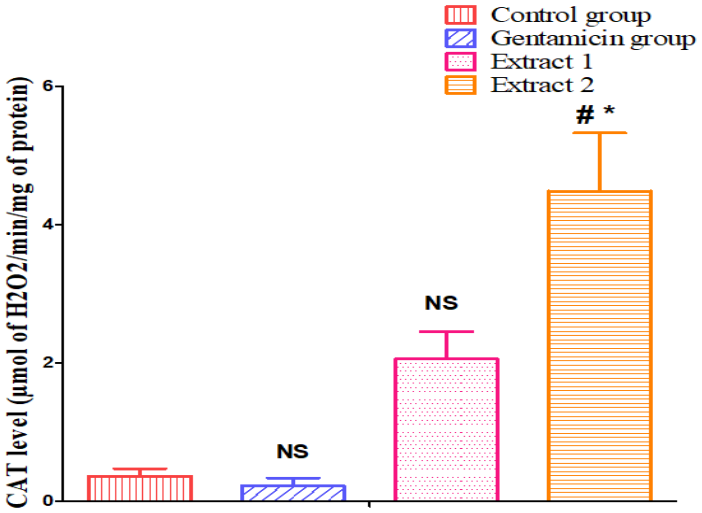

Figure 2 Effect of methanolic extract of the *Crocus sativus*stamens on catalase activity of GEN-treated rats. Extract 1: methanolic extract (250 mg/kg + GEN 80 mg/kg), extract 2: methanolic extract (500 mg/kg + GEN 80 mg/kg). Values are expressed as mean ± SD (n = 5). *P < .05 compared to the control group. #P < .05, Compared to the GEN group. NS. Not significantly different Compared to the GEN group.

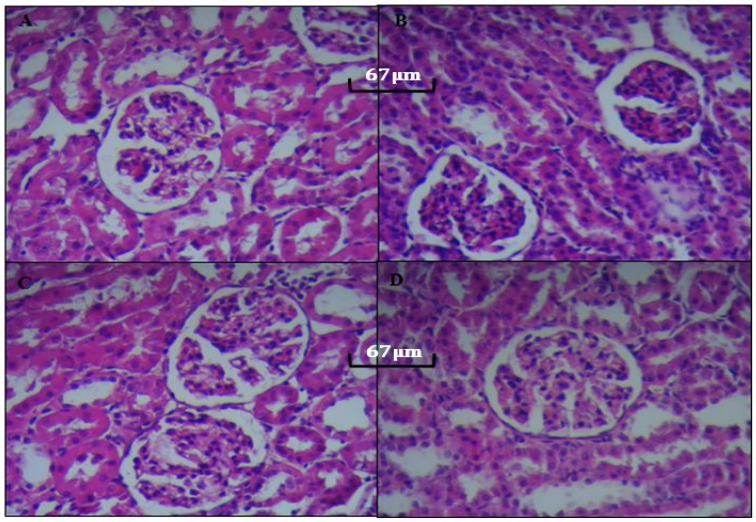

Figure 3 Photomicrographs of rat's kidneys from different experimental groups stained with H&E. Normal histopathological view of a renal section in control rats (A), the treatment with Gentamicin sulfate (80 mg/kg/day) shows degeneration, tubular necrosis, and dilation, glomeruli hypertrophy (B). Section from rat treated with GEN (80 mg/kg/day) plus methanolic extract (250 mg/kg/day) reveal near to normal structure (C). Renal sections of GEN 80 mg/kg + methanolic extract 500 mg/ kg renal sections showed a remarkable recovery of the glomerulus with tubules with moderate Bowman's capsule dilation (D).

group. Treatment with methanolic extract at a concentration of 250 mg/ml significantly ($p < 0.05$) decreases the lipid oxidation in rats, whereas 500 mg/ml concentration has a non-significant effect on the reduction of MDA value in the control group. Furthermore, figure 2 suggested that GEN administration caused a decrease in the activity of the defensive enzymes CAT compared to the control group. Although methanolic extract at 250 mg/kg treatment caused an insignificant increase in the CAT activity in the GEN-treated group compared with the non-treated GEN group, while methanolic extract 500 mg/kg induced a significant increase in renal CAT of GEN-treated rats ($P < 0.05$).

3.6 Effect of extract treatment on histopathological alterations in the kidney cell

Figure 3 depicted the sections of renal tissue light microscopic examination from each animal group. Histopathological assessment of gentamicin treated group kidneys showed massive

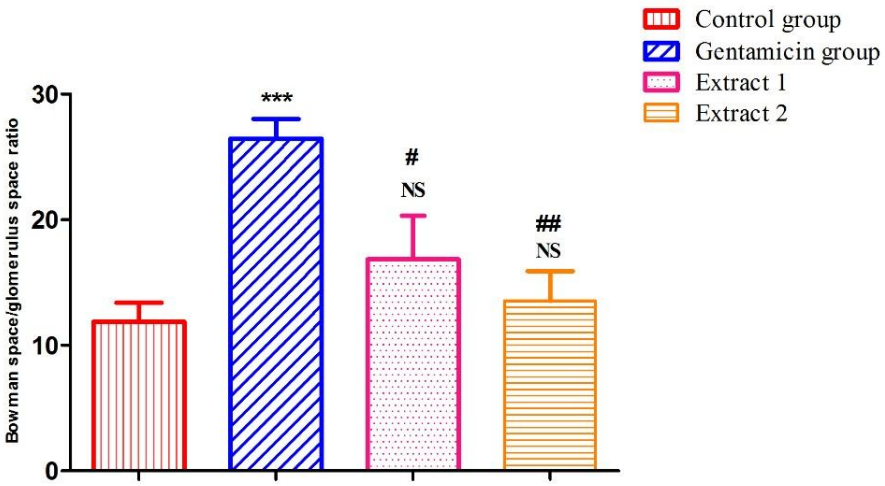

Figure 4 Effect of Methanolic extract *Crocus sativus* stamens on o glomerulus dilation/ Bowmanspace ratio in the kidney of gentamicin sulfate-treated rats. Extract 1: methanolic extract (250 mg/kg + GEN 80 mg/kg), extract 2: methanolic extract (500 mg/kg + GEN 80 mg/kg). Values are expressed as mean ± SD (n = 5). $^{*}P < .05$ $^{***}P < .001$ compared to the control group. $^{\#}P < .05$, $^{\#\#}P < .01$, Compared to the GEN group. NS. Not significantly different compared to control group.

and severe damage. Gentamicin induced renal injury that caused tubular necrosis and dilation. Further, glomeruli hypertrophy showed multifocal renal tubular degeneration and coagulative necrosis in the cortex. Figure 4 suggested that the Bowman space/glomerulus ratio increases significantly ($p < 0.001$) in the GEN treated group as compared to the control group. This means that GEN induces glomerulus dilatation and also causes some alterations in the basement membrane and expansion of the Bowman capsule. However, treatment with *C. sativus* stamens methanolic extract at variable doses has a significant protective effect ($p < 0.01$) against GEN-induced glomerulus damage. In mice treated with GEN + higher dose of methanolic extract (500 mg/kg) renal sections showed a remarkable recovery of the glomerulus with tubules with moderate Bowman's capsule dilation.

4 Discussion

Aminoglycosides have long been used against bacterial infections and are widely used in clinical practice. However, drug-related toxicity is the main constraint to clinical treatment with aminoglycoside antibiotics, despite their positive effects (Vysakh et al. 2018). The aminoglycosides accumulate in the proximal tubule epithelial cells, causing nephrotoxicity. Due to high efficacy, gentamicin is the first choice of aminoglycosides in treating gram-negative infections (Choi et al. 2011). It induces direct nephrotoxicity through the accumulation in the renal cortex. In the current study, GEN was injected at a dose of 80 mg/kg (ip) successively for 15 days. As expected, the administration of GEN induced renal damage, which was associated with increased biochemical parameters, urea, and creatinine. This study showed that impaired renal function was correlated with increased kidney weight and these results are confirmed by the findings of Ghaznavi et al. (2016)**.** The results of this study indicated that daily treatment of rats with a methanolic extract (250 and 500 mg/kg/day) for 15 days prevented GEN-induced nephrotoxicity in kidney weight.

Plasma levels of creatinine and urea are crucial parameters of kidney function. An increase in creatinine means impaired kidney function or kidney disease (Gilbert et al. 1989; Hasanvand et al. 2019; Edeogu et al. 2020). The present study showed that intraperitoneal injection of GEN (80 mg/kg) induced significant nephrotoxicity ($p<0.05$) by increasing serum creatinine, urea, sodium, and urine volume. These results are consistent with several previous studies (Raju et al. 2011; Janjua et al. 2014; Salama et al. 2020) which found that daily administration of GEN resulted in a significant elevation in serum, urine urea, and creatinine concentrations as compared to the control group.

The pathophysiology of gentamicin renal toxicity is complex and still unknown. Recent literature has shown that GEN produces reactive oxygen species (ROS) in the kidney (Choi et al. 2011; Ozbek 2012), and has induced an elevation in lipid peroxidation and a decrease in the levels of antioxidant molecules (Kang et al. 2013). Elevated levels of MDA, as an indicator of lipid peroxidation, were shown in the GEN-treated group. In contrast to this, increased MDA levels were significantly reduced ($p < 0.05$) by methanolic extract of *C. sativus* stamens at 250 mg/kg. Whereas at a higher concentration (500 mg/kg), the MDA value was not significantly reduced, indicating that the generation of reactive oxygen species in GEN-induced nephrotoxicity has been eliminated and mitigated the effect of lipid peroxidation.

Interestingly, methanolic extract administered restored the renal activities and CAT level in GEN treated groups. Furthermore, the elevation in MDA and increase in CAT activities in the GEN + methanolic extract could be due to the antioxidant properties of *C. sativus* stamens extract.

After filtration, GEN as a non-metabolized element is reabsorbed by the renal tubules and enters in the tubular cells after their binding to phospholipid membrane receptors that induced structural or functional changes (Balakumar et al. 2008). Accumulation of GEN in kidney tissue leads to severe kidney damage, involving total tubular necrosis and dilatation, hypertrophy of the glomeruli is evidenced in multifocal renal tubular degeneration. Coagulant necrosis has been also observed in the cortex. In addition, some changes such as inflation and congestion have been also reported in the glomeruli, while damage to the basement membrane and expansion of Bowman's capsule was also reported during the study. These findings are in agreement with the results of Josiah et al. (2020) and Okokon et al. (2011). The histopathological results confirmed the changes in the biochemical parameters. Simultaneous administration of methanolic extracts improved the GEN-induced alterations in the renal tubules and glomeruli. Antioxidant compounds show a preventive effect against gentamicin-induced toxicity in rat kidneys (Khan et al. 2009). *C. sativus* stamens is a by-product generated during stigma collection has been evaluated as a potent antioxidant compound (Montoro et al. 2012).

Conclusion

Results of the study can be concluded that treatment with both the concentrations of methanolic extract would decrease and prevent GEN-induced nephrotoxicity in rats. Administration of methanolic extract resulted in a significant decrease in kidney weight, biochemical parameters, as well as CAT and MDA concentrations in the kidneys. These results suggest the antioxidant properties of *C. sativus* stamens methanolic extract may cause the renoprotective effect.

Author contributions

All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Conflicts of Interest

The authors declared no potential conflicts of interest to the research, authorship, and/or publication of this article.

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Journal of Experimental Biology and Agricultural Sciences, February - 2022; Volume – 10(1) page 83 – 89

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Effects of *Rauwolfia serpentina* L. Benth. ex Kurz (Serpentina) and *Costus igneus* Nak. (Insulin plant) leaves crude extracts on the blood glucose levels of alloxan induced albino rats

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Diabetes

Hypoglycemia

Phytochemicals

Insulin

Dietary supplement

Oxidative stress

ABSTRACT

The prevalence of treatment failures from dietary patterns and oral medications associated with diabetes have generated adverse effects and are oftentimes expensive. Recently, food-based therapies such as *Rauwolfia serpentina* (serpentina) and *Costus igneus* (insulin plant) have been received much attention due to the urge for an alternative and safe solution against diabetes. Thus, the hypoglycemic effects of serpentina and insulin plant leaf crude extracts were determined on the blood glucose level of test rats. Twenty-four alloxan-induced male albino rats were subjected to this experimental study distributed into six groups in a completely randomized design. The negative control (NEG) comprised of diabetic rats receiving no treatment; while the positive control (MET) comprised of diabetic rats treated with metformin; experimental groups include IN1X and IN2X for the diabetic rats treated with extracts of insulin plant leaves administered once and twice daily and SER1X and SER2X for the diabetic rats treated with extracts of serpentina leaves administered once and twice daily. Results of the study revealed that both serpentina and insulin plant leaves crude extract demonstrated hypoglycemic effects due to the presence of zinc that potentiated insulin action. Further, the insulin plant improved glucose and insulin levels due to quercetin which reduced oxidative stress and protects DNA damage, β-amyrin and β-L-arabinose methyl glucoside which builds-up insulin for glucose metabolism. The presence of significant phytochemical contents in the insulin plant has been attributed to the stimulation of β cells. In conclusion, insulin plant leaf crude extract elucidated better hypoglycemic activity than the serpentina plant leaf crude extract in the blood glucose levels of alloxan-induced diabetic rats.

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1 Introduction

The body's response to blood sugar is coordinated by a variety of mechanisms. To avoid the build-up of glucose in the blood, the insulin regulation, secretion, uptake, or breakdown of insulin in the body must not fail (Mahmoud et al. 2017). Drugs like alloxan monohydrate are used for introducing diabetes in animals (Qinna and Badwan 2015). According to Rohilla and Ali (2012), alloxan selectively destroys the insulin-producing β cells found in the pancreas and causes diabetes. Alloxan monohydrate has been widely used to induce experimental diabetes due to its pancreatic-selective β-cell toxin that induces random, rapid, and irreversible severe necrosis of β-cells and damages the pancreas (Ramadan et al. 2017). The insulin-producing cells in the pancreas are selectively destroyed and appeared to build a redox cycle with the development of superoxide radicals (Ojo et al. 2016). With a consequent lack of insulin secretion caused by alloxan induction, it has been widely used to induce experimental diabetes mellitus and many studies have been performed using this model to performed pancreatic damage (Helal et al. 2013).

There are several approaches currently available to treat diabetes including insulin therapy and treatment with biguanides such as metformin (Cheng and Fantus 2005). In recent years, several major insulin-sensitizing agents have been developed, including metformin as a modern oral hypoglycemic agent from the derivatives of a natural plant product (Lee et al. 2006). The frontline anti-diabetic drug metformin also known as dimethyl biguanide was developed from a plant-based molecule of *Galega officinalis* (Annadurai et al. 2012). Metformin causes weight reduction, improved insulin sensitivity, and lipid-lowering in both human and animal models of insulin resistance cell (Saenz et al. 2005) which is thought to have beneficial effects by activating the stress-activated kinase protein that signifies cellular stress in the treatment of type 2 diabetes and obesity (Leverge et al. 2003). Unfortunately, biguanide therapies have restricted viability with several side effects including hypoglycemia and weight gain. Therefore, the quest for alternative drugs from medicinal plants, such as *R. serpentina* (serpentina) and *C. igneus* (insulin plant), is fundamental to overcoming these problems (Kazeem et al. 2015).

Serpentina is globally recognized as antihypertensive phytomedicine and is a medicinally significant plant utilized to obtain drugs. It is said to appear in Sanskrit as an Ayurvedic medicine named Sarpgandha and Chandra which are referring to an antidote for snake-bite (Malviya and Sason 2016). Serpentina is a small annual shrub belonging to the family Apocynaceae that grows erect to a height of 30-110 cm in moist and shady places with a dark green slender stem, lance-shaped leaves with hairless blades measuring up to 2 cm long by 2.5 cm wide (Kumar et al. 2012). Known for its diversified ethnomedicinal usefulness, serpentina can cure snake bites, gastrointestinal tract disorders, breast cancer, and skin problems (Azmi et al. 2015). Many present studies emphasize the potential of serpentina as an anti-fungal, anti-inflammatory, anti-oxidant, anti-proliferative, anti-cancerous, anti-diuretic, anti-dysentery, and tranquilizing agent (Malviya and Sason 2016). The dynamic constituent of the serpentina is utilized adequately as commercial drugs in modern science (Azmi and Quereshi 2016).

On the other hand, the insulin plant has gained increased popularity in recent years due to its incredible cure for diabetes and contains a very potent effect of anti-diabetes (Aruna et al. 2014). Insulin plant, previously known as *Costus pictus*, is commonly referred to as fiery costus, spiral flag, spiral ginger, step ladder, or insulin plant. It is a perennial flowering plant belonging to the family of Costaceae and was recently separated from the family of Zingiberaceae due to the presence of spirally arranged leaves and rhizomes and no content of essential oils (George et al. 2007; Joshi et al. 2013). Due to its anti-hyperglycemic activity, the leaves of the insulin plant were utilized generally as a dietary food supplement in the management of diabetes (Gireesh et al. 2009). The leaves have been accounted to build insulin pools in the blood and assist with working up insulin in the body (Annadurai et al. 2012). Many diabetics claimed a lowering blood glucose levels after consuming the leaves of the insulin plant which lead to it being named insulin (Shetty et al. 2010). Although the insulin plant is being generally utilized for diabetes, there are still no available details about its formulation (Aruna et al. 2014) and possible business opportunities (Annadurai et al. 2012). Thus, this study was conducted to identify the effects of serpentina and insulin plant leaves crude extracts on the blood glucose levels of alloxan-induced albino rats.

2 Materials and Methods

2.1 Research Design

This is an experimental study carried out on 24 test rats that were subjected to the induction of alloxan monohydrate to increase blood glucose levels. The animals were distributed equally in a complete randomized design (CRD) employed with 6 groups namely, NEG (negative control) composed of diabetic rats receiving no treatment, MET (metformin drug) as a positive control composed of diabetic rats treated with commercial antidiabetic drug metformin, the animals of group IN1X and IN2X are diabetic rats treated with the extracts of insulin leaves administered once daily and twice daily, respectively, while the members of group SER1X and SER2X are diabetic rats treated with the extracts of serpentina leaves administered once and twice daily. Each group has four rats including replicates. Ethics review certification was issued by the DLSU-D Ethics Review Committee (DERC) before the conduct of the study.

2.2 Acclimatization and Maintenance of Laboratory Rats

Control and experimental rats were raised as per the protocols of the Philippine Association of Laboratory Animal Science (PALAS) Code of Practice for the Care and "Use of Laboratory Animals" in the Philippines and John Hopkins University "Use of Experimental Animals (2002)". Rats were individually caged and kept in good condition in a well-ventilated and well-lighted room at room temperature in a veterinary clinic. Fifty grams of food pellets were fed to rats 3 times daily at 6 am, 12 pm, 6 pm, and water was provided *ad libitum.*

2.3 Preparation and Administration of Drug Inducer

After the acclimatization period, alloxan monohydrate was used to induce diabetes in rats through a single intra-peritoneal injection of a freshly prepared solution in normal saline at a dose of 150 mg/kg body weight once a day for three successive days (Azmi et al. 2015).

2.4 Preparation of Different Treatments

Standardized Metformin Gludin® oral hypoglycemic tablets were prepared based on the weight of rats using the standard dose of 500 mg/kg and further diluted in distilled water. Fresh leaves of the test plants about 2 kg were collected from a local horticultural farm. The leaves were collected, cleaned, and washed with distilled water, shaded dried for 21 days, and coarsely powdered; this powder was well soaked in a plastic container at about 8L 95% laboratory-grade ethyl alcohol. It was kept overnight at room temperature. The supernatant was collected and evaporated to dryness using a rotary evaporator (Heidolph®) and the final residue was lyophilized using a lyophilizer (Azmi et al. 2015; Ashwini et al. 2015; Nicolas et al. 2016).

2.5 Administration of Treatment

Prepared oral hypoglycemic tablets and leaf crude extract treatments were administered to diabetic rats via the oral route using the gavage method. A standard dosage of 500 mg/kg body weight for the Metformin and 250 mg/kg body weight for the leaf crude extracts were given at every 24-hr interval during the entire period of the experiment. The treatments were inserted into the mouth of the albino rats using the flexible ball tip of a needle. The rats were restrained by holding onto the scuff or the loose folds of the skin on the neck and back portion of the rat. Once the rat was properly restrained, the gavage needle was inserted carefully into the mouth of the rat to ensure proper administration of treatment. The administration started 72-hours post-induction of alloxan monohydrate at an interval of every 24 hours thereafter for 21 days (Gireesh et al. 2009; Shetty et al. 2010; Nicolas et al. 2016).

2.6 Data Collection and Statistical Analysis

Blood samples were collected 3 hours after the administration of the treatments. A drop of blood was placed on the blood glucose test strips and the strip was inserted on a glucose meter. The reading displayed on the screen was recorded. The results were expressed in terms of a milligram per decilitre of blood. Rats with fasting blood glucose of more than 200 mg/ dl were considered diabetic and included in the experimentation after 72-hour post-induction of alloxan monohydrate. Results of blood glucose levels were compared with the standard hematologic data for rats to investigate the efficacy of serpentina and insulin plant leaves crude extract against diabetes mellitus (Gireesh et al. 2009).

Two-way analysis of variance (ANOVA) was used to compare for significant differences between the treatments with serpentina and insulin plant leaves crude extract. Likewise, a posthoc test was used to find out if a significant interaction among the groups in the hematologic data. All statistical analyses were done at a 5% probability level.

3 Results

3.1 Blood Glucose Test Analysis

Blood glucose tests performed in positive control and treated rats showed remarkable results (Figure 1). The blood glucose level of all treatment groups showed a significant increase at post-induction of alloxan monohydrate. Administration of alloxan monohydrate led to a highly significant increase in the blood glucose level of the rats when compared to the pre-induction period.

One-week post-treatment, all rats that received different treatments showed a notable decrease (>23 mg/dl) of blood glucose level. The highest value of blood glucose decrease was observed from the IN2X group while the NEG group has the least decrease. After two weeks, the IN1X group continued to show a decreasing blood glucose level. On the other hand, IN2X and MET group started to increase their blood glucose level while SER2X is almost the same as the status of the NEG (within 222-263 mg/dl). However, all treatment groups had not reverted to normal blood glucose levels (>200 mg/dl). After three weeks, the MET group showed the highest decrease of blood glucose level while the lowest decrease was observed from the IN1X group. On the other hand, the constant blood glucose level was observed from the IN2X group. Comparing the decrease, SER1X and NEG group have a comparable increase of blood glucose level, while SER2X group already started to show the highest increase of blood glucose value.

It is worth noting that the decrease (or increase) during the 1st week, 2nd week, and 3rd week were highly significantly varied ($p<0.05$) within treatment groups (Table 1). During the first week, IN2X, IN1X, and MET groups, statistically, have a

comparable decrease while NEG has the least decrease among the treatments. Hence, while constant blood glucose level was observed in IN2X during the 2nd and 3rd week, it has statistically the highest decrease among the treatments for three weeks. Although NEG has the least decrease during the 1st week, it has statistically the lowest increase during the 2nd week. On the other hand, a significant decrease in IN1X was observed during the 2nd week only, however, the MET group started to decrease significantly with the highest decrease observed among the treatments during the 3rd week. Comparing the decrease in the blood glucose level of the different treatment groups during the 1st week, 2nd week, and 3rd week, the IN2X group has the highest decrease at 221 mg/dl while SER2X has the lowest decrease at 225 mg/dl.

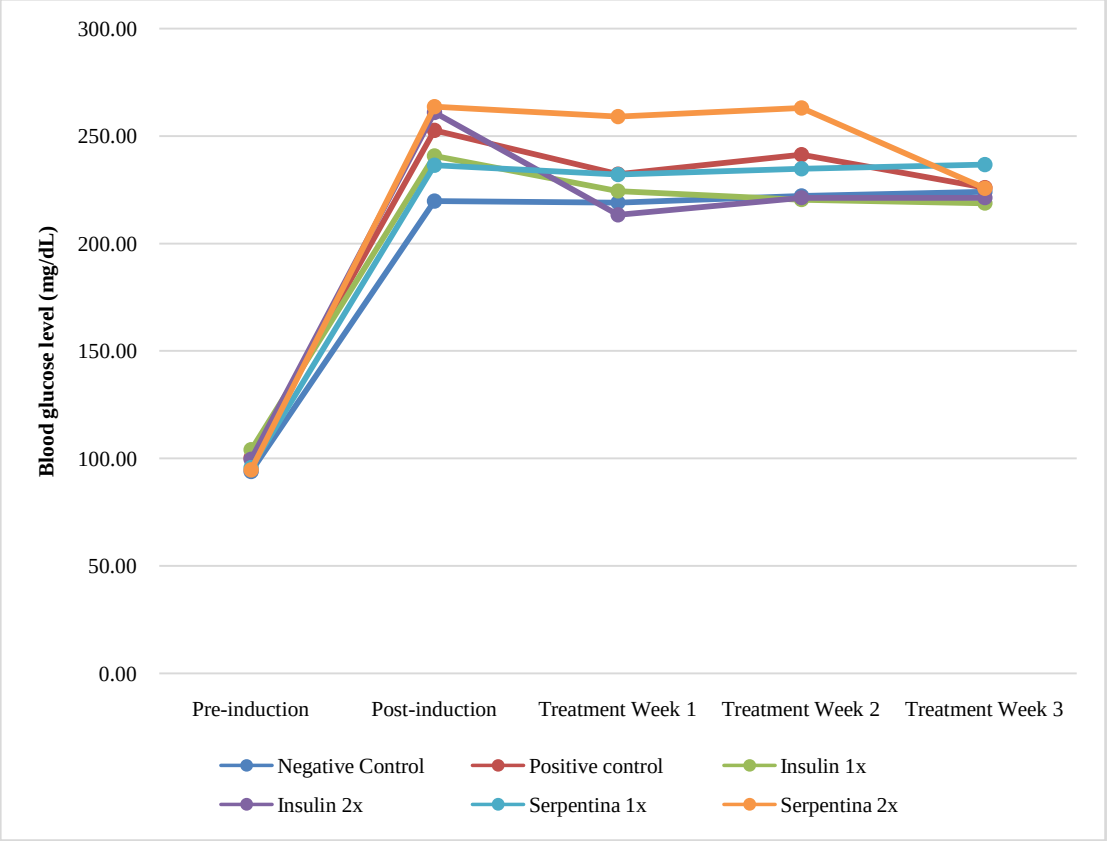

Figure 1 Average blood glucose level on rats prior to and after alloxan monohydrate induction and post treatment

Table 1 Average decrease (increase) of blood glucose value (mg/dl) on rats prior to and after alloxan monohydrate induction and 3 weeks after treatment

Treatment Group	Pre-induction	Post-induction	Post-treatment after 3 weeks	Difference
NEG	94.00±1.41^{A}	219.67±11.90^{B}	224.00±13.95^{B}	4.33±3.09^{X}
MET	103.33±9.39^{A}	252.67±8.65^{B}	226.00±12.33^{C}	-26.67±4.50^{Y}
IN1X	104.00±6.16^{A}	240.67±12.23^{B}	218.67±14.73^{C}	-22.00±3.56^{Y}
IN2X	99.67±4.92^{A}	261.00±11.86^{B}	221.33±7.72^{C}	-39.67±8.50^{Z}
SER1X	95.67±1.25^{A}	236.33±12.28^{B}	236.67±11.79^{B}	0.33±0.94^{X}
SER2X	94.67±6.02^{A}	263.67±28.24^{B}	225.66±28.61^{C}	-18.92±0.69^{Y}

**Normal values (Emordi et al.2016; Qinna and Badwan 2015; Helal et al. 2013);* Different letters denote statistically significant ($p<0.05$). Letters ABC compare the blood glucose levels during pre- and post-induction of alloxan and after 3-week treatment. Letters XYZ compare the difference (3-week treatment - post-induction) of blood glucose level between treatment groups; Legend: NEG = negative control, MET = metformin, IN1X = Insulin leaf crude extract administered once daily, IN2X = Insulin leaf crude extract administered twice daily, SER1X = Serpentina leaf crude extract administered once daily, SER2X = Serpentina leaf crude extract administered twice daily.

4 Discussion

Insulin plant leaf crude extract has shown a potent anti-diabetic effect and ameliorates hyperglycemia which enhanced insulin secretion (Gireesh et al. 2009; Joshi et al. 2013; Ashwini et al. 2015). The administration of insulin plant leaf extract on rats showed the highest marked decrease in the blood glucose level measured in mg/dl in the serum one week post-treatment than the administration of serpentina plant leaf extract in double dosage. These findings can be attributed to the strong α-amylase inhibitory activity of the insulin plant, which was not observed in the properties of serpentina. The enzyme α-amylase hydrolyzes the α-bonds of large α-linked polysaccharides such as glycogen and starch to yield glucose and maltose. In addition, α-amylase inhibitors bind to α-bond of polysaccharide and prevent the breakdown of polysaccharide into monosaccharide and disaccharide, thus preventing the absorption of glucose in the bloodstream (Aruna et al. 2014). Therefore, even in a single dose, the insulin leaf crude extract showed improvement in the blood glucose level of rats than the administration of serpentina leaf crude extract twice a day. Moreover, based on the results of phytochemical analysis, insulin plant crude leaf extract contains highly significant quantities of polyphenolic compounds, such as flavonoids, than serpentina crude leaf extract. Consumption of foods high in polyphenolic compounds was associated with a lower risk of diabetes (Hanhineva et al. 2010). Further, recent studies have demonstrated that insulin plant leaf crude extract was effective in preventing insulin resistance by improving insulin sensitivity at the peripheral level through its anti-oxidant and inflammatory effects that involve stress-sensitive signaling cascade at the molecular level (Ashwini et al. 2015). The lowest decrease of blood glucose level was observed in IN1X three weeks post-treatment but proved to be effective in maintaining the constant value of blood glucose level and body weight as shown in the IN2X group. Hence, it is interesting to note that the prolonged use of the insulin leaf crude extract may potentiate the hypoglycemic action due to the release of insulin-sensitizing action against oxidative stress and regeneration of the β cells in a slow action after three weeks. This agreed with the findings of Emordi et al. (2016) that show a dramatic decrease in plasma glucose concentration as a result of the slow recovery of insulin releases from the regenerated β cells of the damaged pancreas. The blood glucose lowering effect of insulin plant crude leaf extract was due to its role as an antioxidant enzyme that protects against free radicals that contribute in the oxidative stress by scavenging oxygen free radicals, caused by the pancreatic β cell necrosis which released abundant free radicals (Hoque et al. 2011). One of the noteworthy aspects of the insulin plant leaf crude extract was the anti-inflammatory actions of phenolic compounds that are hence the possible explanation for the improvement in insulin sensitivity and glycemic control seen in the study of Dragan et al. (2015). This conformed to the study of Pitchai et al. (2010) that the productive effects were majorly due to the presence of various secondary metabolites which enhance the versatile ethnopharmacological properties of medicinal plants with almost negligible side effects. Therefore, this study led to the idea that insulin plant leaf crude extract showed an anti-hyperglycemic effect and improvement in insulin sensitivity in the alloxan-induced diabetic rats with no side effects exhibited.

It was worth noting that the effect of serpentina leaf crude extract in lowering the blood glucose level was slower as compared to insulin plant leaf crude extract, primarily due to its mode of action in stimulating the release of insulin from the pancreatic β cells through ATP-sensitive potassium channels (Akbar 2011). Both short and long-term antidiabetic activities of serpentina have been reported by Azmi and Quereshi (2016). Advanced computational studies on alkaloids of serpentina highlighted the role of insulin receptor activators and aldose reductase inhibitors, which strengthens the anti-diabetic activity of this plant (Pathania et al. 2013). In this study, the administration of serpentina on diabetic rats caused by an alloxan-induced drug proved effective as expressed by the decreased level of blood glucose one week post-treatment. The hypotriglyceridemic effect of serpentina was due to its ability to enhance insulin sensitivity for the receptor due to a significant amount of alkaloids and polyphenolic compounds which are involved in improving glucose and lipid homeostasis (Azmi et al. 2015). Therefore, serpentina can be effective in improving insulin resistance which can improve glucose uptake in target tissues and stimulate anabolic processes of insulin-like glycogenesis and lipogenesis. Further, the hypoglycemic effect of serpentina was also due to its extra-pancreatic action via inhibiting fructose absorption in the intestine and reducing insulin resistance. However, it was not observed in this study, since based on the result, constant supplementation of serpentina for two weeks post-treatment recorded the lowest decrease of blood glucose. In the same manner, a similar result was reported by Nicolas et al. (2016) that the effect of serpentina was slower in decreasing the blood glucose level.

Conclusion

Based on the result of this study, the antioxidant activities of the insulin plant might have elucidated better compared to that of serpentina since the insulin plant caused a more consistent decreasing pattern of blood glucose level which was also comparable to metformin drug. The insulin plant exerts an antidiabetic effect by stimulating insulin secretion from the pancreas under insulin-resistant conditions. Insulin plant leaf extract decreases hyperinsulinemia by improving insulin sensitivity through its anti-oxidant and anti-inflammatory effects caused by β-amyrin. At a molecular level, the insulin plant decreased activation

of molecules involved in stress-sensitive signaling cascade and resulted in the downregulation of pro-inflammatory cytokines. In addition, the presence of quercetin, a class of flavonoids, in the insulin plant, plays a vital role in the reduction of oxidative stress, low-grade inflammation, and down-regulates the gene expression and production of the pro-inflammatory cytokines in the blood cells. The high content of phenolic compounds and flavonoids in the insulin plant was postulated to contribute to its antidiabetic activity through scavenging oxygen free radicals. Hence, the blood glucose lowering effect of insulin plant leaf crude extract was due to its role as an antioxidant enzyme that protects against free radicals that contribute to oxidative stress which leads to the induction of systemic insulin resistance cells.

Furthermore, serpentina obtained the lowest reduction in the blood glucose level in the entire period of three-week post-treatment. The gradual reduction of blood glucose level by serpentina was due to its mode of action that stimulates the release of insulin from pancreatic β-cells through ATP-sensitive potassium channels. Moreover, the anti-diabetic activity of serpentina is due to the increase in glucose utilization by delaying or preventing glucose absorption that is caused by the potential inhibitors of alpha-glucosidase and alpha-amylase that have been yielded in the plant. But since, in this case, alloxan-induction caused permanent diabetes due to the destruction of the β cells and therefore, the available pancreatic β cells to be stimulated were not present or if present, in insignificant number.

Conflict of Interest

The authors would hereby like to declare that there is no conflict of interests that could arise.

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Effect of probiotic *Bacillus* spp.-supplemented feed on the growth, length-weight relationship, and condition factor of Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

This study was carried out to evaluate the effect of two probiotic *Bacillus* spp. (RM10 and BFAR9) on the growth, length-weight relationship (LWR), and condition factor (k) of Nile tilapia (*Oreochromis niloticus*). For this, one hundred thirty-five fingerlings (1.12±0.08 g weight and 1.26±0.15 cm length) were divided into three groups (Control, RM10, and BFAR9) and distributed into nine circular concrete tanks. The fish were fed with commercial (control) and *Bacillus* spp. supplemented diets at 5% of body weight for 56 days. The results of the study revealed better (P<0.05) growth concerning average body weight (ABW - 17.12±0.71g), specific growth rate (SGR - 4.89±0.22 g·day^{-1}), absolute growth (AG - 16.02±0.78 g), and feed conversion ratio (FCR - 1.31±0.09) in the group fed with *Bacillus* sp. RM10 as compared to the control (ABW- 13.25±2.34g; SGR - 4.41±0.17g·day^{-1}; AG - 12.13±2.25g; FCR - 1.62±0.11). The LWR in all experimental treatments showed a significant correlation (P<0.05) with an R^2 value of 0.988, 0.966, and 0.979 for Control, RM10, and BFAR9, respectively. The k value revealed that all treatments are in good condition as k value is greater than 1 (1.913, 2.038, and 1.896 for control, RM10, and BFAR9 respectively). The result of the current study revealed that application of *Bacillus* sp. RM10 improves the growth and feed utilization in Nile tilapia.

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1 Introduction

Aquaculture is playing an important role in increasing fish production and helping to meet the growing demand of the global population for fishery products. One of the most popular aquaculture species are the tilapias (*Oreochromis* spp.), which is the worldwide second most farmed fish (Prabu et al. 2019). These species are native to Africa and the Middle East and were introduced into over 90 countries for aquaculture and fisheries (Watanabe et al. 2002; Prabu et al. 2019). The global production of tilapia in 2015 was estimated at 5,576,800 MT (Prabu et al., 2019). In the Philippines, tilapia is the second most farmed fish with an estimated production of 277,006 MT in 2018, which contributes to 12.02% of the total aquaculture production of the country (Bureau of Fisheries and Aquatic Resources 2018). Tilapia production has quadrupled over the past decade and continued to increase global production rapidly due to its suitability for aquaculture, stable market prices, and the increasing demand for food fish as a result of the growing population (Prabu et al. 2019). However, this intensification led to the proliferation of antibiotic-resistant microorganisms as a result of the improper use of chemotherapeutic agents to improve the growth and disease resistance of the fish. As an alternative, recent studies explored the use of beneficial bacteria or probiotics to encourage more eco-friendly aquaculture (Balcazar et al. 2006). *Bacillus* spp. are widely used as a probiotic microorganism for aquatic animals to promote growth performances, disease resistance, and better immune response (Balcazar et al., 2006). These bacteria are suitable probiotic candidates since they are generally non-pathogenic and non-toxic when fed to fish, can survive under harsh environmental conditions, and produce antimicrobial substances as compared to other probiotics (Kuebutornye et al. 2019).

The need for sustainable aquaculture has encouraged exploration into the use of probiotics on aquatic organisms. The original interest was focused on their use as a growth promoter and to improve the health of animals; however, new extents have been found, such as their effect on reproduction or stress tolerance, although this requires a more scientific development (Cruz et al. 2012). The information on probiotics has increased in the past years and currently, it has been established that they have an antimicrobial effect on their host organism (Cruz et al. 2012). Probiotic bacteria enact their antimicrobial effect by amending the intestinal microbiota of their host through secretion of antibacterial substances (bacteriocins and organic acids), prevention of pathogen adhesion to the intestine, competing for nutrients essential for pathogen survival, and production of antitoxin effect (Cruz et al. 2012). Furthermore, probiotics are also capable of modifying the immune system, regulating the allergic response of the body, and reducing the proliferation of cancer in mammals. As a result, when probiotics are provided in a certain concentration these are positively affecting the host's health (Myers 2007).

Previous studies also reported a significant effect of *Bacillus* spp. on growth, disease resistance, and hematological parameters of Nile tilapia (Soltan et al. 2016; Sutthi et al. 2018; Elsabagh et al. 2018; Kuebutornye et al. 2020; Won et al. 2020; Ghalwash et al. 2021) and other aquatic species (Hauville et al. 2016; Munir et al. 2016; da Paixão et al. 2017; Kong et al. 2017; Wang et al. 2017; Amoah et al. 2021; Saravanan et al. 2021). In this present study, the effect of two previously isolated *Bacillus* spp. on the growth performance, length-weight relationship, and condition factor of Nile tilapia was evaluated.

2 Materials and Methods

2.1 Preparation of Bacteria and Experimental Diets

Two probiotic strains viz., BFAR9 and RM10 of *Bacillus* spp. with GenBank accession numbers: MH919302 and MH919308, isolated from African nightcrawler earthworm (*Eudrilus eugeniae*) were used in this study (Samson et al., 2020). Overnight cultures of the probiotic strains were suspended in a 250 mL nutrient broth and incubated at 37 °C for 24 hours. The suspensions were aseptically transferred into sterile test tubes, pelleted, washed, and suspended in Phosphate-buffered Solution (PBS). The bacterial density was adjusted using the McFarland standard. Dilutions were carried out using PBS to have a similar bacterial density (McFarland, 1907).

Experimental diets were prepared using a commercial feed (31% crude protein, 5% lipid, 8% crude fiber, and 12% crude ash) as the basal diet (control). The probiotic-treated diets were prepared to contain a single strain of *Bacillus* spp. at 10^8 CFU·g^{-1} of feed. The selected isolates were sprayed on the basal diet and air-dried for 2 hours before storing at 4°C.

2.2 Experimental Fish and Set-up

One hundred thirty-five *O. niloticus* fingerlings (FaST strain) were obtained from the Freshwater Aquaculture Center, Central Luzon State University, Science City of Muñoz, Nueva Ecija, Philippines. Experimental fish were allowed to acclimatize for 7 days before the experiment. The fingerlings were randomly distributed into 9 circular concrete tanks (200-L capacity) and divided into 3 experimental groups (three replicates per group). Each group consists of 45 fish, which had an initial weight and length of 1.12 ± 0.08 g and 1.26 ± 0.15 cm, respectively. Prepared diets were given thrice daily (8:00, 12:00, and 16:00), initially at 5% of fish body weight per day for 56 days. Sampling was done biweekly to measure the length and weight of the fish and adjust the feeding ration. The water quality parameters such as temperature, dissolved oxygen, and pH were measured daily using the YSI multiparameter (YSI 556 - YSI Incorporation, Yellow Spring, USA)

2.3 Data Collection

At the end of the feeding trial, all fish were measured and weighed. The specific growth rate (SGR), absolute growth (AG), feed conversion ratio (FCR), survival rate (SR) and condition factor (k) were measured as follows (Bagenal 1978; Fulton1904):

$$SGR = \frac{(\ln [\text{final weight}] - \ln [\text{initial weight}])}{(\text{time interval in days})} \times 100$$

$$AG = \text{Final weight (g)} - \text{Initial weight (g)}$$

$$FCR = \frac{\text{Amount of feed given (g)}}{\text{Weight gain (g)}}$$

$$SR\ (\%) = \frac{\text{No. of individuals at the end of the experiment}}{\text{No of individuals at the beginning of the study}} \times 100$$

$$k = 100 \times \frac{\text{Weight (g)}}{\text{Length (cm)}^3}$$

2.4 Length-weight relationship

The length and weight of fish were tabulated and analyzed after 56 days. The relationship between the length (L, cm) and weight (W, g) was calculated by regression equation (Keys 1928)

$$W = \alpha \times L^b$$

Here α represents the antilog of the intercept of the regression curve, and b is the regression coefficient. The degree of association between L and W was calculated by the correlation coefficient (r). In the length-weigh relationship, the value of exponent b provides information on fish growth; when b = 3, the increase in weight is isometric, while b > 3 means increase of weight is positive allometric, and if b < 3, the increase of weight is negative allometric (Tesch 1968).

2.5 Statistical Analyses

Results were analyzed by one-way analysis of variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT) at a significant level of $P<0.05$ using R Statistics software. Results are presented as means ± SD. The LWR was calculated using linear regression analysis in Microsoft Excel using the log-transformed data of weight and length.

3 Results

3.1 Growth Performance and Survival

The data on growth performance, survival, and condition factor of experimental fish are presented in Table 1. Results of the study revealed that the initial length and weight measurements of the fish had no significant difference among the groups. Further, no significant differences between the two probiotic-fed groups in terms of length and weight; however, significantly higher ($P<0.05$) ABW (17.12 g), SGR (4.89 g·day^{-1}), and AG (16.02 g) was observed from *Bacillus* sp. RM10-fed group compared to the control group (ABW = 13.25 g, SGR = 4.41 g·day^{-1} and AG = 12.13 g). On the other hand, no significant difference was observed in the length of the fish among all treatments. Furthermore, in terms of feed efficiency, the results of the current study showed significantly lower FCR in RM10 (1.31) compared to the control (1.62). The survival rate of the experimental fish also revealed that there are no significant differences in all treatments. After 56 days of culture, all treatments have ≥80 % survival. The water quality parameters recorded during the experiment are temperature 26.7 ± 0.8 ºC, pH 7.8 ± 0.2, and dissolved oxygen level 4.65 ± 1.16 ppm.

Table 1 Growth performance, feed utilization, survival, and condition factor of Nile tilapia fed with probiotic *Bacillus* spp.

PARAMETERS	TREATMENT		
	Control	RM10	BFAR9
Initial Body Length (IBL) (cm)	3.89 ± 0.44^{a}	3.91 ± 0.19^{a}	3.82 ± 0.19^{a}
Initial Body Weight (IBW) (g)	1.11 ± 0.10^{a}	1.11 ± 0.11^{a}	1.14 ± 0.09^{a}
Final Body Length (FBL) (cm)	8.83 ± 0.53^{a}	9.44 ± 0.24^{a}	9.18 ± 0.16^{a}
Final Body Weight (FBW) (g)	13.25 ± 2.34^{b}	17.12 ± 0.71^{a}	16.02 ± 0.28^{ab}
Feed Conversion Ratio (FCR)	1.62 ± 0.11^{a}	1.31 ± 0.09^{b}	1.54 ± 0.15^{ab}
Specific Growth Rate (SGR) (g day^{-1})	4.41 ± 0.17^{b}	4.89 ± 0.22^{a}	4.71 ± 0.11^{ab}
Absolute Growth (AG) (g)	12.13 ± 2.25^{b}	16.02 ± 0.78^{a}	14.85 ± 0.20^{ab}
Survival Rate (SR) (%)	83.33 ± 15.28^{a}	80.00 ± 10.00^{a}	90.00 ± 17.32^{a}
Condition Factor (k)	1.91 ± 0.02^{a}	2.04 ± 0.11^{a}	1.90 ± 0.08^{a}

Data are given as mean ± SD (n=3). The mean values in the same row with different superscript letters are significantly different at $P<0.05$.

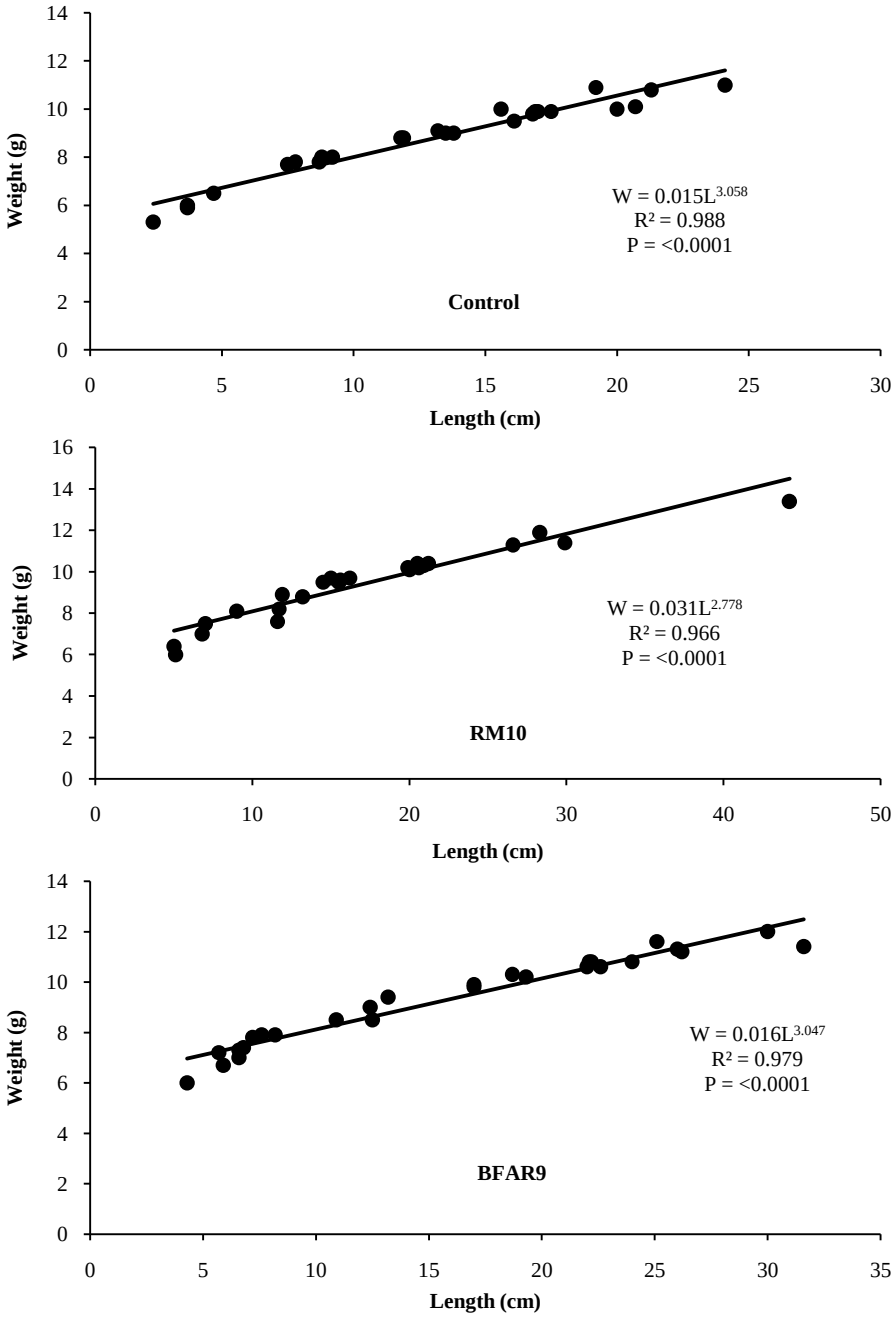

Figure 1 Logarithmic regression of weight and length data of Nile tilapia fed with probiotic *Bacillus* spp.

3.2 Length-weight Relationship and Condition Factor

The length-weight relationship of the non-probiotic-fed and probiotic-fed groups was evaluated after the feeding trial. The logarithmic regression of LWR and coefficient of determination values (R^2) are presented in Figure 1. Results showed a significant correlation (P<0.05) between the length and the weight among all experimental groups with an R^2 value of 0.988, 0.966, and 0.979 for Control, RM10, and BFAR9, respectively. Moreover, the value of slope (b) in BFAR9 (3.047) and Control (3.058) is close to 3, except for RM10 (2.778). No significant differences were reported in the condition factor on all treatment groups. However, the condition factor revealed that the experimental fish in all groups are in good condition as demonstrated by the k-value. All groups that have k-value is greater than 1 considered as good one (Control = 1.913, RM10 = 2.038, and BFAR9 = 1.896).

4 Discussion and Conclusions

The applications of probiotics in aquaculture have already been reported for several years. The increasing demand for an eco-friendlier approach in combating the emerging diseases in aquaculture and intensifying of production has turned the industry to explore the use of these microorganisms. Probiotic application has already been proven to increase aquaculture production by improving the growth and survival of the cultured species. In the present study, the application of the probiotic *Bacillus* spp. improved the growth performance of the fish. Higher ABW and growth parameters (AG and SGR) were observed in the experimental group fed with *Bacillus* spp. RM10. Previous research studies have already reported the beneficial effect of *Bacillus* probiotics application on fish growth. Similar findings were reported in Nile tilapia (Apún-Molina et al. 2009; Zhou et al. 2009; Ridha and Azad 2012; Soltan et al. 2016; Sutthi et al. 2018; Opiyo et al. 2019; Rahman 2019;), white shrimp, (*Litopenaeus vannamei*) (Nimrat et al. 2012; Zokaeifar et al. 2012), rainbow trout (*Oncorhynchus mykiss*), and African catfish (*Clarias gariepinus*). Previous studies showed the colonization ability of *Bacillus* spp. in the gut of the fish. This ability aids in imposing the beneficial effects of probiotic bacteria through stimulation and production of digestive enzymes, enhanced organic acid production, reducing the antinutritional factors of feed ingredients, and collectively maintaining a healthy gut; thus, improving the digestion and nutrient absorption of the host. This is demonstrated by the lower FCR value of the group fed with *Bacillus* sp. RM10, which showed that the probiotic application improved the feed utilization of the fish. On the other hand, the application of *Bacillus* sp. BFAR9 did not result in any significant improvement on Nile tilapia growth. Similar findings were observed in, tambaqui (*Colossoma macropomum*) (da Paixão et al. 2017), gilthead sea bream (*Sparus aurata*) (Arığ et al. 2013), and Nile tilapia (Shelby et al. 2006; Silva et al. 2015). Although growth improvement is not evident in these reports, a significant increase in the villi height of the intestine (Silva et al. 2015) and digestive enzyme activities (Arığ et al. 2013) revealed that the application of *Bacillus* probiotics have a positive effect on the digestion of the fish. Therefore, the effect of probiotic bacteria can be possibly observed in other aspects of the host, such as enhanced immunity, digestion, pathogen prevention, stress tolerance, and reproduction. Furthermore, it is also possible that the effect of a certain strain of probiotics varies in every species of fish. Similarly, the probiotic effect of *Bacillus* spp. might be due to differences in each strain or species of bacteria.

In the present study, the effect of *Bacillus* probiotics application in the LWR and condition factor of Nile tilapia was evaluated. The results showed a significant correlation between the length and weight of the fish. In the LWR, the value of slope (b) provides information on fish growth, b <3 is said to be negative allometric, b = 3 is isometric and b >3 is positive allometric. Isometric growth pattern (*i.e*., the proportional increase in length and weight that gives fish ideal shapes) was observed in BFAR9 (3.047) and control (3.058) as indicated by their slope value, which is close to the value of ideal growth (b = 3), as suggested by Froese (2006). Although experimental groups fed with *Bacillus* spp. RM10 demonstrated a lower slope value (2.778) than the ideal growth value, it is still within the acceptable range of 2.5–3.5 which was estimated by Froese (2006). Furthermore, the condition factor in all treatments showed that the experimental fish are in good condition ($k \geq 1$). This condition factor is widely used in fisheries and general fish biology studies. It is calculated from the relationship between the weight of a fish and its length, to describe the condition of that individual. Previous studies have utilized this parameter to evaluate the overall condition of aquatic organisms as affected by several factors and for fish stock assessment purposes. The present study demonstrated the beneficial effect of the probiotic *Bacillus* spp. application in the growth of Nile tilapia. Further intensive investigations are required to evaluate the role of these microorganisms in disease resistance, stress tolerance, and fish reproduction.

In conclusion, this study revealed that supplementation of *Bacillus* sp. RM10 in the diet of Nile tilapia improves the growth performance and feed utilization of the fish. Therefore, based on the result of this study, the application of *Bacillus* sp. RM10 in the diet can increase the production and lessen the feed requirements of Nile tilapia.

Abbreviations

Average Body Weight (ABW), Specific Growth Rate (SGR), Absolute Growth (AG), Survival Rate (SR) and Feed Conversion Ratio (FCR)

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Conflict of Interest

The author declares no conflict of interest.

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Evaluation of haematological and behavioural changes in *Channa punctatus* (Bloch) on short-term exposure to a commercial-grade synthetic pyrethroid pesticide

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LC_{50}

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Behaviour

ABSTRACT

This study aims to assess the acute toxicity of commercial-grade Cypermethrin (10% EC) and evaluate the hematological and behavioral alterations in a freshwater fish *Channa punctatus* upon short-term exposure to Cypermethrin. A four-day static acute toxicity test was performed to estimate the median lethal concentration (LC_{50}) value of Cypermethrin. During the acute toxicity test, the behavior of the control and cypermethrin exposed fish was critically observed and recorded. After completing the acute toxicity test, the hematological effects of Cypermethrin in *C. punctatus* were evaluated using two sublethal dosages (0.08 mg/L and 0.12 mg/L). Results of the study revealed that this pesticide induced significant mortality in *C. punctatus* with a 96-h L_{50} value of 0.263 mg/L. Cypermethrin exposed fish showed hyperactivity, irritability, erratic swimming, frequent surface visit, etc. Exposure to sublethal concentrations of Cypermethrin for a short period resulted in a significant decline ($P<0.05$) in total erythrocytes count (TEC), packed cell volume (PCV), mean corpuscular volume (MCV), and hemoglobin (Hb) concentration as compared to control groups. In contrast, pesticide-exposed groups had a significant increase ($P<0.05$) in mean corpuscular hemoglobin concentration (MCHC) and total leucocyte count (TLC). It is apparent from the results of the study that this commercial formulation is toxic to the studied fish. This study also revealed hematological and behavioral alterations in *C. Punctatus* which could be used as biomarkers for incipient Cypermethrin intoxication.

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1 Introduction

Pyrethroids are synthesized from pyrethrins derived from *Chrysanthemum cinerariifolium* (Ullah et al. 2019). These are used as possible alternatives to some organochlorine, carbamates, and organophosphorus pesticides (Saxena and Seth 2002). Cypermethrin is a potent wide-spectrum synthetic Pyrethroid pesticide sold under the different trademarks (de Moraes et al. 2018; Sharma et al. 2021) and is extensively utilized against agricultural pests, household insects. Along with this, it is also used in fish farming as a chemotherapeutic to ectoparasites and biological vectors of fishes (de Moraes et al. 2018; Overton et al. 2019). In Indian natural waters, the level of Cypermethrin is found to be significantly higher than the acceptable threshold (Arisekar et al. 2019; Maurya et al. 2019; Sharma et al. 2021). The widespread use of pesticides in modern agriculture contributes to agricultural nonpoint source pollution. Pesticides are introduced into Indian natural water bodies through various natural processes such as surface run-off and soil erosion, posing a threat to drinking water resources as well as affecting a broad range of non-target phytoplankton, zooplankton, and higher trophic organisms such as fish and fish predators (Mondal et al. 2018; Rajmohan et al. 2020; Kalyabina et al. 2021). Cypermethrin is known to be toxic to aquatic organisms, especially to fish (de Moraes et al. 2018). Pyrethroids such as cypermethrin can readily permeate inside the gills, even at meager quantities in the water, rendering fish the most susceptible to the pesticide (Shaluei et al., 2012). As fish cannot adequately metabolize pyrethroids, their susceptibility to aqueous pyrethroid exposure is exacerbated (Borges et al. 2007).

By modulating the activities of many enzymes and metabolites, pesticide accumulation in tissue causes many physiological and biochemical changes in fish (Sarma et al. 2013). Pesticides alter the rate of enzymatic reactions and may also inhibit certain enzymes. Pesticide exposure in fish results in a considerable reduction in total protein levels, an increase in blood glucose levels, and significant alterations in total bilirubin, albumin, urea, inorganic phosphate, and cholesterol levels in serum. Pesticides also induce the production of reactive oxygen species (Ullah et al. 2019; Gonçalves et al. 2021). Blood serves as a pathophysiological indicator of the body since it is susceptible to both external and internal perturbations (Ismail et al. 2018). Assessments of hematological parameters might be effective for monitoring pesticide stress and can provide valuable information on the physiological responses of fish to changing environmental conditions (Agrahari et al. 2006).

The synthetic pyrethroid insecticide affects the central nervous system of fish through inhibition of acetylcholinesterase, which leads to hyperstimulation of nicotinic and muscarinic cholinergic receptors present the central nervous system. It ultimately causes a variety of behavioral inconsistencies (Borges et al. 2007; Ullah et al. 2019; Tsai and Lein 2021).

Though several authors reported the toxic impact of Cypermethrin on different fish species, still there is a paucity of scientific documentation on tropical freshwater fishes including *Channa punctatus* (Bloch) with combined biomarker approaches. *C. punctatus* also known as the spotted snakehead, is a tropical freshwater teleost found throughout the Indian subcontinent. These are the representatives of the Order Anabantiformes and are extremely valuable commercially. This species meets most of the requirements of a model species, including easy year-round availability and easy acclimatization under laboratory conditions. This study was aimed to investigate the acute toxicity of commercial-grade Cypermethrin and evaluate hematological and behavioral changes in *C. punctatus* upon short-term intoxication with Cypermethrin.

2 Materials and Methods

Healthy *C. punctatus* specimens (average length of 14.02 ± 0.13 cm and average weight of 42.34 ± 1.71g) were obtained from local unpolluted water bodies. The fish specimens were acclimatized under laboratory conditions for seven days after collection. During this period, the fish were given a pelleted commercial fish meal once a day. The accumulated wastes were siphoned off daily. A commercial formulation of Cypermethrin (10% EC) with the trade name Ustaad™, manufactured by United Phosphorus Limited (UPL), India, was used in this investigation, which was procured from the local market.

2.1 Physico-chemical parameters of the diluent medium

The temperature, pH, and Total dissolved solids (TDS) were measured with the help of a portable instrument. Standard methods recommended by APHA (2012) were used for the estimation of dissolved oxygen (DO) and alkalinity. The mean and standard deviation (SD) of the water quality parameters are represented in Table 1.

Table1 Physico-chemical characteristics of the aqueous medium

Parameters	Value
Alkalinity	115.29 ± 13.37 mg/L
DO	6.58 ± 0.16 mg/L
TDS	690.35 ± 38.71 mg/L
pH	7.3 ±.01

2.2 Acute toxicity bioassay

Four days static acute toxicity test was performed to estimate the median lethal concentration (LC_{50}) value of Cypermethrin. Before determining the actual concentration of the test solution for the definitive test, a rough range-finding test was performed (Sarma et al. 2013; Ghosh et al. 2021). Under normal day/light conditions, a set of 10 randomly chosen fish specimens were subjected to

different concentrations of the test chemical (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, & 0.7 mg/L) in the definitive test. Frequent monitoring was made to observe the mortality of the fishes. The number of dead fishes was also recorded at the end of 24, 48, 72, 96 hours of exposure. As soon as feasible, the dead specimens were removed.

2.3 Behavioural Observations

The behavior of the control and cypermethrin exposed fishes was critically observed and recorded (qualitative analysis) during the acute toxicity test. Behavior of fish such as swimming pattern (normal/erratic), activity pattern (hypoactive/normal/hyperactive), the extent of mucous secretion (no mucous secretion/low mucous secretion/heavy mucous secretion), frequency of surface visit, etc. was taken into consideration for this study.

2.4 Observations on Hematological Parameters

Following the completion of the acute toxicity tests, the hematological effects of Cypermethrin were assessed using two sublethal dosages of 0.08 mg/L and 0.12 mg/L (30% and 45 % of the 96-hour LC_{50} value, respectively). A pesticide-free control group was also prepared. Water was exchanged every 24 hours in the treatment sets to keep the concentration of Cypermethrin unchanged during the exposure period. Two sets were prepared, one of which was kept for up to 7 days and the other for 14 days. A pelleted fish meal was fed to the pesticide-exposed groups and the control group once daily. No mortality was reported during the experimentation periods. Fishes from each group were anesthetized on the 7th and 14th days before the blood was taken from them. Blood was collected by caudal puncture using 3ml sterile plastic syringes rinsed with an anticoagulant (1% EDTA). Neubauer's Haemcytometer was used to determine the TEC (Total Erythrocyte Count) and TLC (Total Leukocyte Count) using RBC and WBC diluting fluid, respectively (Agrahari et al. 2006). The acid-hematin method was used to calculate the concentration of hemoglobin (Hb) (Sood 2010). The microhematocrit method (Schalm et al. 1975) was used to determine Packed Cell Volume (PCV). Using standard formulas, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated (Dacie and Lewis 1991).

2.5 Statistical Analysis

LC_{50} values for 24, 48, 72, and 96 hours were determined using Finney's Probit Analysis method with 95% confidence limits, slope, and intercept values (Finney 1971). For hematological studies, the obtained values are presented as mean ± SD. One-way ANOVA was performed to discern the variance among groups. Dunnett's test was applied to establish comparisons between the control group and each of the treated groups. All the statistical analyses were performed using the Statistical package SPSS 21.0.

3 Results

Results of an acute toxicity test revealed that mortality in *C.punctatus* is proportional to the toxicant concentration. No mortality was observed in the control group during the experiment. The percentage of *C. Punctatus* mortality exposed to 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 & 0.7 mg/L cypermethrin after 96 hours is presented in table 2.

Table 2 Percentage mortality of *C. punctatus* after 96 h of exposure to Cypermethrin

Concentration	No. of alive fishes at 96 hours	% of mortality at 96 hours
0	10	NIL
0.1	10	NIL
0.2	7	30%
0.3	4	60%
0.4	2	80%
0.5	1	90%
0.6	0	100%
0.7	0	100%

Table 3 LC_{50} Values of the Cypermethrin to *C.punctatus* at Different Exposure Period (with 95% confidence limit, slope and intercept)

Exposure Period (hr)	LC_{50} mg/L	95% confidence limit		Slope ± SE	Intercept ± SE
		Upper	Lower		
24	0.40	0.34	0.46	7.30± 4.53	2.83 ± 0.62
48	0.37	0.30	0.42	6.36 ± 1.5	2.95 ± 0.62
72	0.32	0.27	0.38	6.52 ± 1.39	3.15 ± 0.64
96	0.26	0.18	0.31	5.29 ± 4.0	3.07 ± 0.67

Based on Probit analysis, the LC_{50} values of Cypermethrin for *C.punctatus* after 24, 48, 72, and 96 hours were 0.409, 0.371, 0.328, and 0.263 mg/L, respectively. As the span of exposure increased, the slope function decreased. Table 3 shows all the median lethal concentration (LC_{50}) values (with 95 % confidence limits) for different exposure periods.

Throughout the study period, the fish in the control group appeared to be active and healthy. Fishes kept in lower concentrations of cypermethrin (0.1 mg/L and 0.2 mg/L) showed behavior similar to the control group. Hyperactivity and irritability continued for the first 24 hours of exposure at higher concentrations (>0.2 mg/L); thereafter, gradually slowed down. Erratic swimming behavior increased with the increasing concentrations and exposure time. The frequency of surface visits of fish exposed to Cypermethrin increased as the toxicant's concentration increased (Surface visits were observed to be more frequent at 0.6 mg/L and 0.7 mg/L). However, at each concentration, as exposure time progressed, the frequency of surface visits reduced progressively. In the treated groups, there was a substantial rise in opercular activity. When comparing the treatment and control groups, it was found that opercular activity reduced steadily over time in the treated groups. Heavy mucus secretion, gulping of air, convulsions, and loss of equilibrium was evident before death. During the experiment, it was typical to see animals lying motionless at the bottom of the aquarium for extended periods.

In the present study, exposure of fish to two sub-lethal concentrations (0.08 mg/L and 0.12 mg/L) of Cypermethrin caused significant alterations in the haematological parameters of *C. punctatus*. The changes in control and cypermethrin-treated groups are presented in Table 4. One way ANOVA revealed that there was a statistically significant difference in TEC [F (2,27) =134.34, P<0.05], Hb [F (2,27) =75.83, P<0.05], & TLC [F (2,27) =212.43, P<0.05] between at least two sets. Further, statically significant difference was also evident in case of other hematological parameters like PCV [F (2,27) =1181.87, P<0.05], MCV [F (2,27) =385.04, P<0.05], MCH [F (2,27) =9.44, P<0.05], & MCHC [F (2,27) =107.94, P<0.05].

Dunnett's test found that the mean value of TEC, PCV, MCV & Hb decreased significantly (P<0.05) as compared to control. After clearing Levene's test for homogeneity in variance, Student's t-test revealed that hematological changes are not statistically significant between exposure tenure.

4 Discussion and Conclusions

Synthetic pyrethroids are used indiscriminately, which has various unforeseeable implications for non-target organisms. This study has evaluated the acute toxicity and hematological and behavioral changes in *C. punctatus* exposed to sublethal concentrations of commercial-grade cypermethrin for a short period.

The results of the present study showed that Cypermethrin induced significant mortality in *C. punctatus* with a 96 h L_{50} value of 0.263 mg/L. Borges et al. (2007) and Das and Mukherjee (2003) reported that the 96 hours LC_{50} value of Cypermethrin in *Rhamdia quelen* and *Labeo rohita* was 0.193 mg/L and 0.139 mg/L,

Table 4 Hematological parameters of *C.punctatus* exposed to two (0.08 mg/L &0.12 mg/L) sublethal concentrations of Cypermethrin

Parameter	Days of Exposure	Control	0.08 mg/L Concentration	0.12mg/L Concentration
TEC ($\times 10^6$ mm^{-3})	7	3.16 ± 0.05	2.91 ± 0.06	2.71 ± 0.04
	14	3.09 ± 0.02	2.8 ± 0.02	2.6 ± 0.03
Hb (gm/dL)	7	11.47 ± 0.4	10.68 ± 0.32	9.53 ± 0.09
	14	10.85 ± 0.25	10.7 ± 0.35	9.29 ± 0.06
PCV (%)	7	34.53 ± 0.4	29.84 ± 0.07	24.22 ± 0.12
	14	34.24 ± 0.32	28.91 ± 0.09	23.26 ± 0.41
MCV (10^{-4}fL)	7	109.45 ± 2.85	102.64 ± 2.03	89.27 ± 1.32
	14	110.96 ± 1.37	103.12 ± 1.06	89.34 ± 1.06
MCH (10^5pg)	7	36.82 ± 0.83	36.77 ± 1.7	35.11 ± 0.51
	14	35.17 ± 0.77	38.17 ± 1.39	35.69 ± 0.39
MCHC (g%)	7	33.21 ± 1.33	35.81 ± 1.09	39.33 ± 0.48
	14	31.7 ± 0.7	37.01 ± 1.14	39.45 ± 0.78
TLC (10^3 mm^{-3})	7	17.84 ± 0.6	19.88 ± 0.53	20.89 ± 0.05
	14	17.62 ± 0.1	20.29 ± 0.52	21.07 ± 0.11

Table 5 LC_{50} of Cypermethrin in various fish species

Fish Species	LC_{50}value (Hour)	Reference
Rhamdia quelen	0.193 mg/L (96 h)	Borges et al. (2007)
Labeo rohita	0.139 mg/L (96 h)	Das &Mukherjee (2003)
Catla catla	4.43 µg /L (96 h)	Vani et al. (2012)
Tor putitora	63 µg/L (96 h)	Ullah et al. (2015)
Cirrhinus mrigala	150µg /L(96h)	Vasantaraja et al. (2012)
Channa punctatus	0.4 mg/L(96h)	Kumar et al. (2007)
Danio rerio	0.27 mg/L (72 h)	Xu et al. (2010)
Channa punctatus	0.263 mg/L(96h)	This study

respectively. On the other hand, Xu et al. (2010) and Kumar et al. (2007) obtained a relatively higher LC_{50} value in *Danio rerio* and *C.punctatus* as compared to this study (Table 5). The toxicity of Cypermethrin varies with fish species (Borges et al. 2007). Pyrethroid toxicity is incredibly reliant on the stoichiometry of the compound. Toxicity variations between formulations with the same ingredients may be influenced by the carriers, inert substances, physiology, sex of the test specimens, and the physio-chemical properties of the aqueous media (Kumar et al. 2007; Sarma et al. 2013; Ghosh et al. 2021).

Stress-induced behavioral alterations are the most sensitive indicators of pesticide toxicity (Nwani et al. 2013). Exposure to Cypermethrin has resulted in behavioral changes in *C.punctatus*, among the reported behavior changes, irregular or erratic swimming, frequent surface visit, sinking to the bottom, and dyspnea are the most common ones. These findings were consistent with the findings of Polat et al. (2002), in *Poecilia reticulata*, Kumar et al. (2007), in *C.punctatus*, and Borges et al. (2007) in *Rhamdia quelen*. Synthetic pyrethroids put on hazardous effects on the nervous system of fish by altering the permeability of sodium channels and other voltage-gated channels like calcium and chlorine gated channels (de Moraes et al. 2018; Soderlund 2011; Ullah et al. 2019). Disturbances to these channels lead to different neurobehavioral changes, including hyperirritability (de Moraes et al. 2018). The acetylcholinesterase enzyme is active at both neuronal and neuromuscular junctions. Different behavioral inconsistencies can be attributed to acetylcholinesterase inhibition by the pesticide at the neuromuscular junction, culminating in impeded neural transmission and aggravated acetylcholine at nerve endings (Singh et al. 2018; Ullah et al. 2019).

In this study, *C.punctatus* exposed to sublethal concentrations of Cypermethrin showed a significant decrease ($P<0.05$) in TEC, Hb, MCV. Similar observations were reported in *Catla catla* by Vani et al. (2012), in *Alburnus tarichi* by Özok et al. (2018), and in *Anabas testudineus* by Velmurugan et al. (2016). The findings of this study are also in agreement with the findings of Saxena and Seth (2002) in the same species. Further, Jayaprakash and Shettu (2013) reported a significant decrease in the Hb content, TEC, and PCV in *C. punctatus* exposed to another pyrethroid pesticide, deltamethrin. Borges et al. (2007) and Montanha et al. (2014) observed an increase in Hb content, whereas Das and Mukherjee (2003) reported a reduction in Hb content with no associated change in TEC due to Cypermethrin exposure. Anemia is generally thought to be caused by a decrease in hemoglobin and TEC levels (Saleh and Marie 2016). Inhibition of erythropoiesis is most likely the cause of anemia. Furthermore, due to a drop in RBC and Hb, the oxygen-carrying capacity of blood in pesticide-exposed fish decreases (Adhikari et al. 2004). In this study, significant elevation in leukocyte count was observed, consistent with the findings of Velmurugan et al. (2016) in *Anabas testudineus* and with Adhikari et al. (2004) in *Labeo rohita.* This substantial increment could be ascribed to general immune response and defense against Cypermethrin (Khan et al. 2018). It is difficult to perform a meaningful comparative evaluation of the effect of Cypermethrin on the hematological profile of fish because of the variability of concentrations used, the period of exposure, and the fish species.

This study unraveled that despite low, sub-lethal and short-term exposure, the xenobiotic (commercial formulation of Cypermethrin) was able to elicit hematological adjustments and behavioral changes in *C.punctatus*. Hematological and behavioral alterations in *C.punctatus* could be used as biomarkers for incipient Cypermethrin intoxication. It was also evident that this species is more resistant to Cypermethrin's effects compared to other fish species. The changes reported here can be reflected in other parameters related to growth, reproduction, etc. which need to be investigated in future studies.

Conflict of Interest

The authors declare no potential conflict of interest.

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An intensive study on pesticides contamination and its removal in fruits and vegetables collected from Ghaziabad, India

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QUECHERS extraction

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DMRT

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ABSTRACT

These days pesticides are extensively applied in agriculture to increase productivity; although these pesticides increase productivity but also have a negative impact on the consumer. Thus, pesticide exposure in agricultural products must be decreased. The present study attempted to assess pesticide residues in samples of apple, tomato, and brinjal and determine the efficacy of washing solutions in pesticide removal. For sample preparation, the QuEChERS method was employed, and prepared samples were analyzed using gas chromatography-mass spectrometry. Results of the study revealed that among the collected samples, 58.33 percent samples were showing lower pesticide residues as compared to the maximum residue limit (MRL) while 12.5 percent of the samples were showing higher pesticides residues as compared to the suggested MRL. Further, from the collected fruits and vegetable samples, the presence of the chlorantraniliprole, carbendazim, beta endosulfan, chlorpyrifos, malathion, carbaryl, thiomethoxam, DDT, and flubendiamide were detected in the range of 0.0–1.41 mg/kg. Among the detected pesticides, chlorpyrifos and flubendiamide were the most commonly detected pesticides. Effectiveness of different washing solutions was studied, which indicated a significant reduction in residues of all the washing solutions compared with the control ($p < 0.05$) and concluded that ascorbic acid and sodium bicarbonate solution was very effective in pesticides removal compared with water and chemical alone.

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1 Introduction

Pesticides are extensively used in worldwide agriculture to control pests. The use of pesticides helps in increasing crop yield, protecting crops from insects and pests, and extending their storage life. Pesticides are often used in developing countries to boost food production (Omwenga et al. 2021; Akoto et al. 2015). Although excessive use of pesticides increases agricultural production, on the other hand, excessive use of pesticides has negative health consequences. The acute neurotoxic insecticides, namely organophosphates (OPs), carbamates (CBs), and pyrethroids (PYs), have different modes of action in humans and other mammals, are among the several pesticide categories to which humans are exposed through their diet (Jardim et al. 2018). Health risks of pesticides range from headaches and nausea to long-term effects such as cancer, congenital disorder, dysgenesis, and endocrine disruption (Jallow et al. 2017). The levels of organochlorine pesticides (OCP) are elevated in patients with colorectal cancer (Abolhassani et al. 2019). Most of the applied pesticides may exist in the environment as residues. Pesticide residues are the remains of an active component, metabolite, or byproduct of a pesticide. Further, some pesticides are persistent, and they accumulate in the environment. Accumulation of pesticides in the soil, water, and from here to different plant parts is the most common route through which these pesticides enter into the food chain and are showing biomagnification (Kaushik et al. 2009). Biomedical studies from US and Canada have demonstrated pesticide residues in biological fluids (Omwenga et al. 2021; Haines et al. 2017; Centers for disease control and prevention 2019).

Insecticides, fungicides, and herbicides are commonly used for controlling pests, and these products exhibit high pesticide levels (Nguyen et al. 2020). According to the ICAR-National Institute of Agriculture Economics and Policy Research report, the annual pesticide consumption in India was approximately 0.29 kg/hectare per year in 2016-17 (Subash et al. 2018). Pesticide consumption is highest in Maharashtra, followed by Uttar Pradesh, Punjab, and Haryana (Subash et al. 2018). Due to a lack of knowledge, farmers tend to apply pesticides near the harvest period just before reaching the end-user in the market. Thus, to ensure the safety of consumers, countries around the world have set up the maximum residual limit (MRL). In India, “The Food Safety and Standard Authority of India” under the Ministry of Health and Family welfare utilizes the Good Agricultural Practice data for MRL fixation (FSSAI Guidance Note. 13/2020). The MRL is calculated based on the estimated food consumption for each crop is less than 80% of the permissible daily intake and a risk assessment. Estimated food intake is calculated by multiplying the MRL by food. Thus, food commodities contaminated with pesticides must be monitored, whereas actions to remove pesticides from the food crops are vital for food safety.

After China, India is the world's second-largest producer of fruits and vegetables. Fruits and vegetables consist of nutrients that are necessary for the human body to grow and heal itself. Fan et al. (2015) reported that the amount of pesticides used in vegetable crops is three times higher than those used for cereal crops. The most common fruits and vegetables that can be consumed directly and are affected by high pesticide residues are apples and tomatoes respectively. Washing and peeling are the most commonly used procedures for removing pesticide residues. Rinsing with water alone is not effective. Several studies have been investigated the efficacy of washing solutions in removing pesticide residues. The use of a 10% acetic acid solution to remove organochlorine and organophosphorus pesticides has been reported to be effective by Abou Arab (1999). Krol et al. (2000) reported the efficacy of brief rinsing with water. Polat & Tiryaki (2020) confirmed that acetic acid and citric acid washing treatments are more efficient than tap water. Additionally, Rasolonjatovo et al. (2017) concluded that a blend of washing solutions is more effective than a single solution in removing pesticide residues. Although solvents used for pesticide removal can remove the residues, they cause toxic effects. Thus, the use of non-toxic washing solutions, which are easily available at home or can be easily prepared at home, has been attempted. The present study monitored the pesticide residues in fruits and vegetables to determine and mitigate the risks to human health, understand their translocation, and identify an effective washing solution for the removal of pesticides from the surface of fruits and vegetables.

2 Materials and methods

2.1 Raw materials

Raw apple, tomato, and brinjal samples (5–6 kg) were collected twice from the four different locations of Ghaziabad, Uttar Pradesh, India local market within 30 days during January 2019. Samples were refrigerated and analyzed within 1 week (Satpathy et al. 2012).

2.2 Extraction of pesticide residues

5.0 g of homogenized sample was extracted with acetonitrile followed by the addition of magnesium sulfate, sodium chloride, trisodium citrate dihydrate, and potassium hydrogen citrate sesquihydrate. The extract clean-up was done using primary secondary amines and magnesium sulfate followed by evaporation of extract under a nitrogen evaporator at 35°C. Final reconstitution was done with ethyl acetate and injected into the gas chromatography-mass spectrometry (GC–MS)/MS as suggested BS EN 15662:2018.

2.3 Instrument conditions

The experimental setup was as per the Jallow et al. (2017) descriptions. The sample was injected in splitless mode on GC–MS/MS with Autosampler (7000B, triple quadruple with Mass hunter software) having capillary column (HP-5MS; 30 m × 0.25 mm × 0.25 µm). The column flow rate was 1.0 mL/min, and the initial column temperature was 60°C. The injector temperature was set at 300°C. The column oven ramping was set at an initial temperature of 60°C, with a 1.0 minute hold time. The temperature was raised at the rate of 40.0°C per min to 170°C with no hold time and at the rate of 10.0°C to 310°C within 3.0 min (Tables 1 and 2).

Table 1 Chromatographic conditions for GC-MS/MS

Column	HP-5MS, 30m x 0.25mm x 0.25µm
Column oven	60°C
Injector temperature	300°C
Injection Mode	Split less
Carrier gas	Helium
Flow rate	1.0mL/min
Injection volume	1.0µL
Run time	22.75min
Mode	MRM
Source temperature	220°C
Interface Temperature	310°C
Solvent delay	3.00 min

Table 2 Oven ramping

Rate (°C/min)	Final temp (°C)	Hold time (min)	Run time (min)
---	60	1.0	1
40.0	170	0.0	3.75
10.0	310	3.0	20.75

2.4 Translocation and pesticide removal in different washing solutions

Pesticide mix solution of detected pesticides was prepared with an effective concentration of 100 ppm in ethyl acetate. Pesticides mix was applied on the surface of the apple, tomato, and brinjal samples. A total of 50 µL of pesticide mix stock (100 ppm) was applied and kept for 24 h at room temperature for pesticide deposition.

To study translocation, six spiked apple peel and pulp samples were analyzed along with surface pesticides by treating the surface with ethyl acetate after 24 h of spiking. Surface pesticides and peel were analyzed for tomatoes and brinjals, too.

To compare the translocation characteristics of pesticides in fruits and vegetables, the translocation factor (TF) was calculated using the following equations (Klinhom et al. 2008; Felizeter et al. 2012; Wang et al. 2019).

$$\text{TF for surface to peel} = C_{peel}/C_{surface}$$

$$\text{TF for peel to pulp} = C_{pulp}/C_{peel}$$

$$\text{TF for surface to pulp} = C_{pulp}/C_{surface}$$

Here, C_{peel} (mg/kg), $C_{surface}$ (mg/kg), and C_{pulp} (mg/kg) are the measured concentrations of a pesticide in the peels, surface, and pulp of fruits and vegetables, respectively.

To analyze washing solution effectiveness, one sample was analyzed to estimate the pesticide concentration in the control sample. Then, treatments with four different washing solutions, namely 0.1% ascorbic acid, 0.1 N sodium carbonate, ascorbic acid, and sodium bicarbonate mixture, and warm water, were administered. The treated samples were immersed in 200 mL of washing solution for 15 min with gentle agitation after every 5 min (Satpathy et al. 2012) and then rinsed with 200 mL tap water for 2s. After washing, fruit and vegetable samples were air-dried for 30 min. The samples after washing were collected and extracted as per the extraction method and analyzed for pesticide residues.

2.5 Statistical analysis

All treatments were replicated thrice. The effects of washing solutions on percentage reduction of pesticides were determined using two-way analysis of variance, with the pesticides serving as main effects and the four washing solutions serving as minor effects. Significant differences between treatment effects and interactions were determined following a significant F-test ($P < 0.05$) by using all pair-wise comparisons of treatment means with DUNCAN's option (IBM SPSS) (Felizeter et al. 2012). A p-value of <0.05 was considered statistically significant.

3 Results

3.1 Recovery study

The method was validated through sensitivity/linearity, specificity, the limit of quantification (LOQ), matrix effect, recovery/ trueness, precision, within lab reproducibility, and robustness. Six blank samples were spiked at LOQ levels (10 µg/kg) quantified against the calibration curve. Mean recoveries of the standard thiabendazole, chlorantraniliprole, carbendazim, pp-DDT, lambda cyahalothrin, betaendosulfan, chlorpyrifos, permethrin, malathion, carbaryl, cyfluthrin, thiomethoxam, and flubendiamide added to

the pesticide-free vegetables and fruits at 0.01 mg/kg were 97% , 104.66% , 99.74% , 87% , 101% , 81% , 86% , 97.8% , 99.57% , 106.91% , 84.78% , 99.98% , 96.11% , and 105.42% , respectively. LOQ recoveries were within the acceptance criteria of 70–120%, with an associated relative standard deviation (RSD) of ≤ 20%. The recovery percentage in the present study was higher than 80%, indicating a validated analytical procedure. Further, the average recovery criteria as per SANTE/12682/2019 was 70 –120% at each level. RSD should be <20% at each level. Based on the validation results, the present method was considered suitable for the estimation of pesticide residues in fruits and vegetables. The precision and accuracy were within the prescribed limits in the concentration range.

3.2 Multiple residue detection in fruits and vegetables

A total of 24 fruit and vegetable samples were used for pesticide residue analysis, and the ranges of detection levels are summarized in Table 3. Among the studied samples, 09 samples were free from residues. The pesticide residues in 14 samples were less than MRL and while in one sample it was reported higher than the recommended MRL value (Table 4). Further, among the apple samples, 9 (37%) samples exhibited no residues, and 15 (63%) samples had residues less than MRL. Among the studied tomato samples, 6 (25%) samples exhibited no pesticides residues, 15 (62%) samples exhibited residues less than MRL, and 3 (13%) samples exhibited residues greater than the MRL recommendation. Among the brinjal samples, 12 (50%) samples exhibited no residues, and 12 (50%) samples exhibited residues less than MRL (Figure 1). A high percentage of contaminated samples were observed in tomatoes (75%).

The most common pesticides observed in fruits and vegetables were carbendazim (15 samples), chlorpyrifos (12 samples), flubendiamide (15 samples), and chlorantaniliprolein (6 samples). Beta endosulfan, malathion, and carbaryl were observed in 3 apple samples; while thiomethoxam and DDT were observed in 6 and 3 tomato samples, respectively. In 3 tomato samples, chlorpyrifos exceeded its MRL. The residue content of fruits and vegetables is illustrated in Table 5 and Figure 2. In 37.5 percent of the samples, there was only one pesticide residue, whereas, in 29.1% of the samples, there were multiple pesticide residues.

Table 3 Pesticides level detected in selected fruits & vegetables collected from Ghaziabad location

Pesticides Name	Pesticides detected in Apple (n=8)	Pesticides detected in Tomatoes (n=8)	Pesticides detected in Brinjal (n=8)
Thiabendazole, mg/kg	ND	ND	ND
Chlorantraniliprole, mg/kg	ND	ND	0-0.033
Carbendazim (MBC) , mg/kg	0.0 - 0.023	0.0 - 1.41	ND
Lambda Cyahalothrin, mg/kg	ND	ND	ND
BetaEndosulfan, mg/kg	0.0 - 0.035	ND	ND
Chlorpyrifos, mg/kg	0.0 - 0.021	0-0.21	ND
Permethrin, mg/kg	ND	ND	0.0- 0.022
Malathion, mg/kg	0.0 - 0.018	ND	ND
Carbaryl, mg/kg	0.0 - 0.035	ND	ND
Cyfluthrin, mg/kg	ND	ND	0.0-0.025
Thiomethoxam, mg/kg	ND	0-0.026	ND
DDT, mg/kg	ND	0-0.032	ND
Flubendiamide, mg/kg	ND	0.0-7.96	0.0-6.76

Table 4 Pesticides Residues in analysed Samples

Produce	Without Residue	With Residue < MRL	With Residue > MRL
Apple	3	5	0
Tomatoes	2	5	1
Brinjal	4	4	0

Table 5 Co-occurrences of pesticides Residues in Samples

Number of Residues	% of Samples	No of Samples
0 residue	33.3	8
1 Residue	37.5	9
2 Residue	20.8	5
>3 Residue	8.3	2

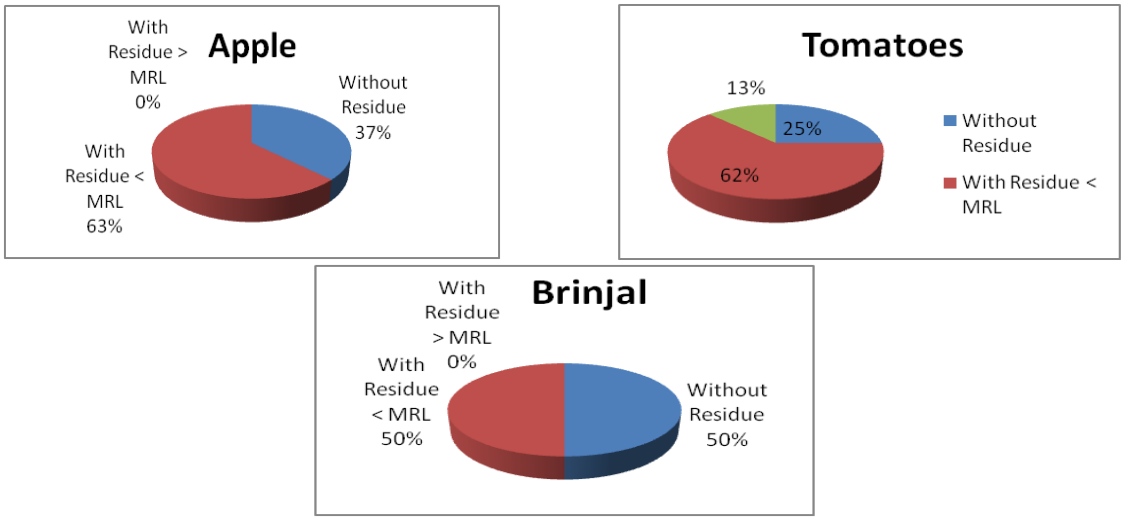

Figure 1 The detected pesticides frequency in whole , tomato and brinjal samples compared to maximum residual limits (MRL) along with frequency of MRL exceedance.

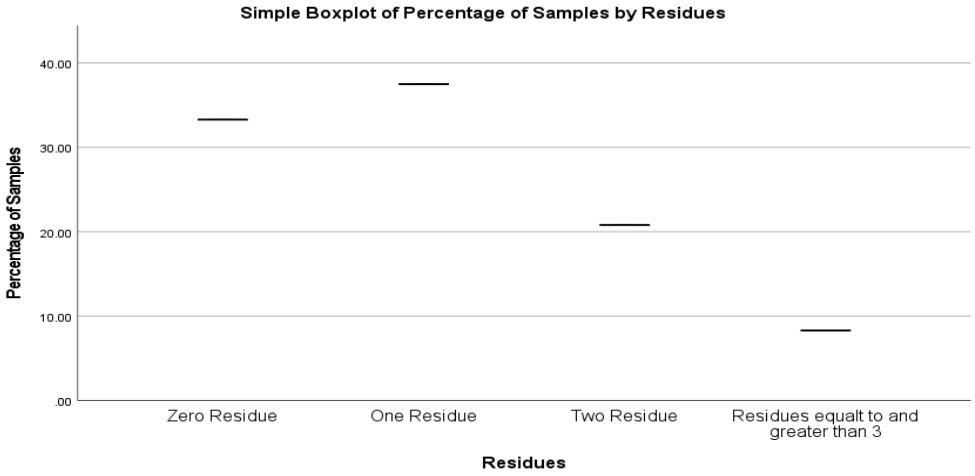

Figure 2 Co-occurrences of pesticides residues in samples

3.3 Translocation study

The translocation ability of a plant is the ability to transfer its pesticides or chemicals to different parts such as the surface, peel, and pulp (Wang et al. 2011). Penetration factor (PF) is calculated as the ratio of pesticide concentration in the peel to the pulp. This factor can be used to study peel to pulp translocation and is calculated as the ratio of surface concentration to peel concentration to determine the translocation of pesticides from surface to peel (Lozowicka et al. 2020); the higher the PF values, the better is the translocation between the skin and pulp.

$$\text{PF for surface to peel} = C_{surface}/C_{peel}$$

$$\text{PF for peel to pulp} = C_{peel}/C_{pulp}$$

For the apple samples, the PF of surface to peel ($C_{surface}/C_{peel}$) and that of peel to pulp (C_{peel}/C_{pulp}) ranged from 0.02 to 5.28 and 0.0 to 189.16, respectively. The highest PF of surface to peel ($C_{surface}/C_{peel}$) was observed for beta endosulfan (average value, 4.91). In the case of C_{peel}/C_{pulp}, higher PF (118.63) was observed for malathion, followed by carbaryl (48.07) and chlorpyrifos. Higher PF value of malathion and carbaryl indicated that the maximum pesticide concentration penetrated and moved to the pulp after 24 h. High PF values indicate better translocation between the skin and pulp.

For the tomato samples, PF ranged from 4.29 to 16.95. Higher PF was observed for flubendiamide (average value, 14.22), followed by thiomethoxam (average value, 7.49). For the brinjal samples, the average PF of 37.34 was observed for flubendiamide (Figure 3, 4, and 5).

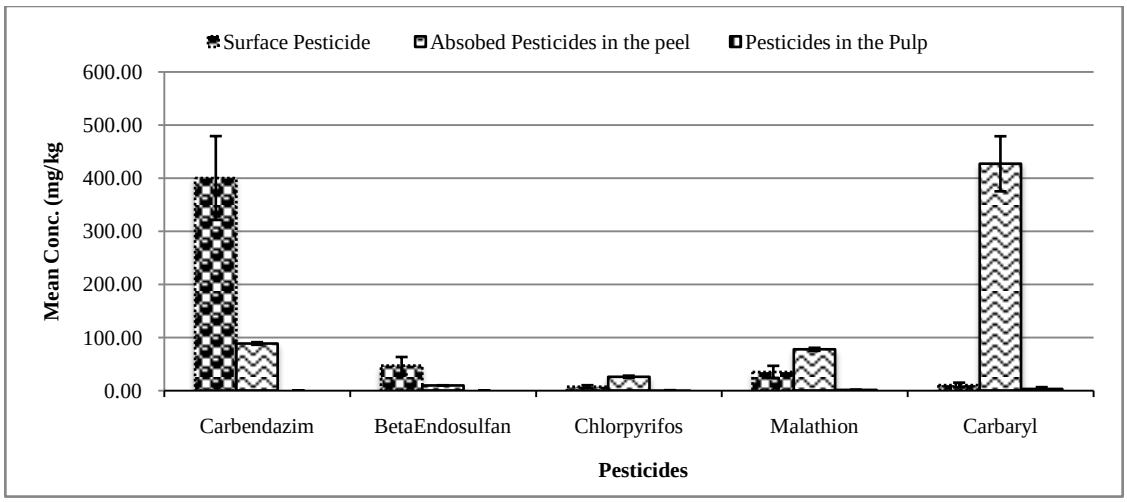

Figure 3 Translocation of pesticides residues in apples

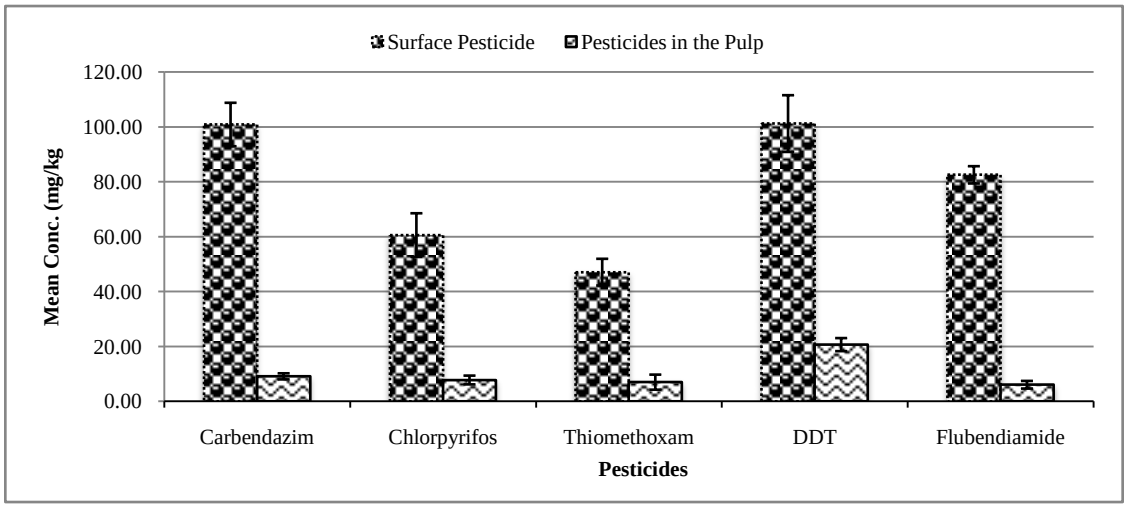

Figure 4 Translocation of pesticides residues in tomatoes

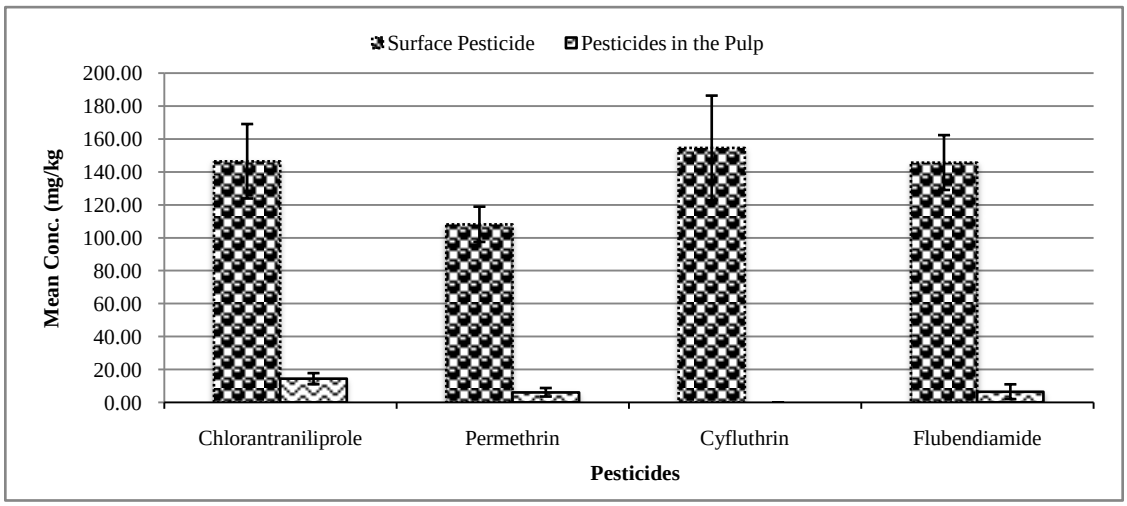

Figure 5 Translocation of pesticides residues in brinjal

Table 6 Reduction in pesticides residues after washing treatment

Pesticides name	% Reduction water	% Reduction in 0.1 % Sodium Carbonate	% Reduction in 0.1 % mixtue of absorbic acid and sodium carbonate (70:30 v/v)	% Reduction in 0.1 % Ascorbic Acid
Apples				
Carbendazim (MBC)	88.16	91.80	96.05	66.10
BetaEndosulfan	83.96	82.08	83.75	65.52
Chlorpyrifos	87.52	88.97	86.91	61.74
malathion	97.12	97.83	97.93	21.67
carbaryl	96.43	97.14	97.42	39.77
Tomatoes				
Carbendazim (MBC)	93.94	93.17	95.12	69.31
Chlorpyrifos	90.58	90.88	94.85	70.85
Thiomethoxam	98.06	71.54	98.06	87.80
DDT	55.03	64.35	66.53	66.53
Flubendiamide	64.51	63.76	87.76	48.47
Brinjal				
Chlorantraniliprole	77.99	90.86	93.74	31.21
Permethrin	59.76	91.73	88.64	64.82
Cyfluthrin	97.09	96.61	97.70	82.73
Flubendiamide	58.63	83.38	71.74	58.45

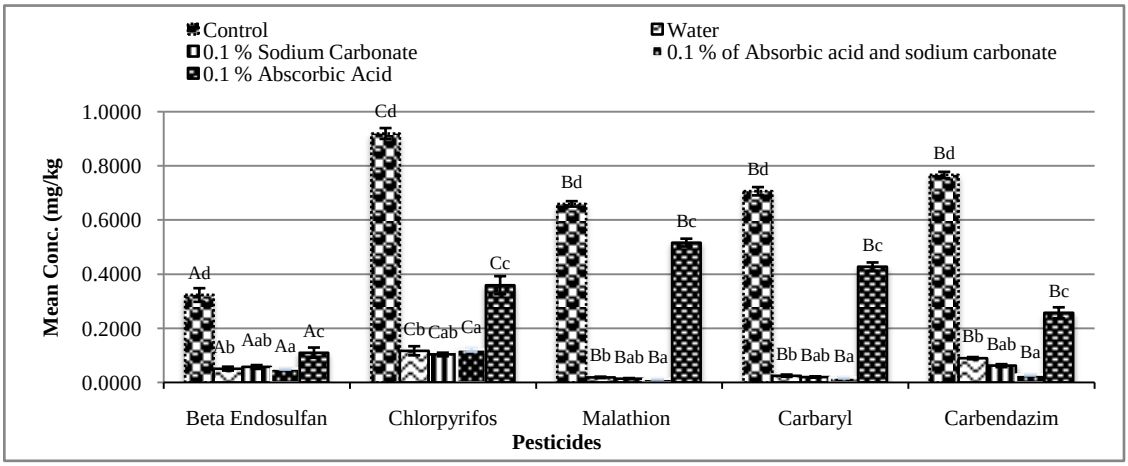

Figure 6 Different washing solutions effectiveness on removal of different pesticides in apples. All the values are mean of triplicates ± S.D. ANOVA significant at $p < 0.05$. Different capital letters indicate significant difference when compared between different pesticides and small letters indicate significantly different values when compared between different treatments (DMRT, $p < 0.05$).

3.4 Pesticide reduction study

Table 6 presents the data for the percentage reduction of pesticides in the apple, tomato, and brinjal samples. To determine the percentages of pesticide reduction, the samples were spiked with a pesticide mix solution and washed after 24 h with 0.1% ascorbic acid; 0.1% sodium carbonate; and a mix solution of ascorbic acid and sodium carbonate in a ratio of 50:50 v/v and water. The concentration after washing treatment was compared with the concentration obtained from untreated samples. In the untreated apple samples, the total concentrations of carbendazim, beta endosulfan, chlorprifos, malathion, and carbaryl in apple were 0.76, 0.32, 0.94, 0.66, and 0.71 ppm, respectively. In tomato samples, the concentrations of carbendazim, chlorpyrifos, thiomethoxam, DDT, and flubendiamide were 0.82, 1.1, 0.91, 0.49, and 0.85, respectively, whereas, in brinjal samples, the concentrations for chlorantraniliprole, permethrin, cyfluthrin, and flubendiamide were 0.58, 1.23, 0.55, and 0.73 ppm, respectively (Figure 6, 7, and 8).

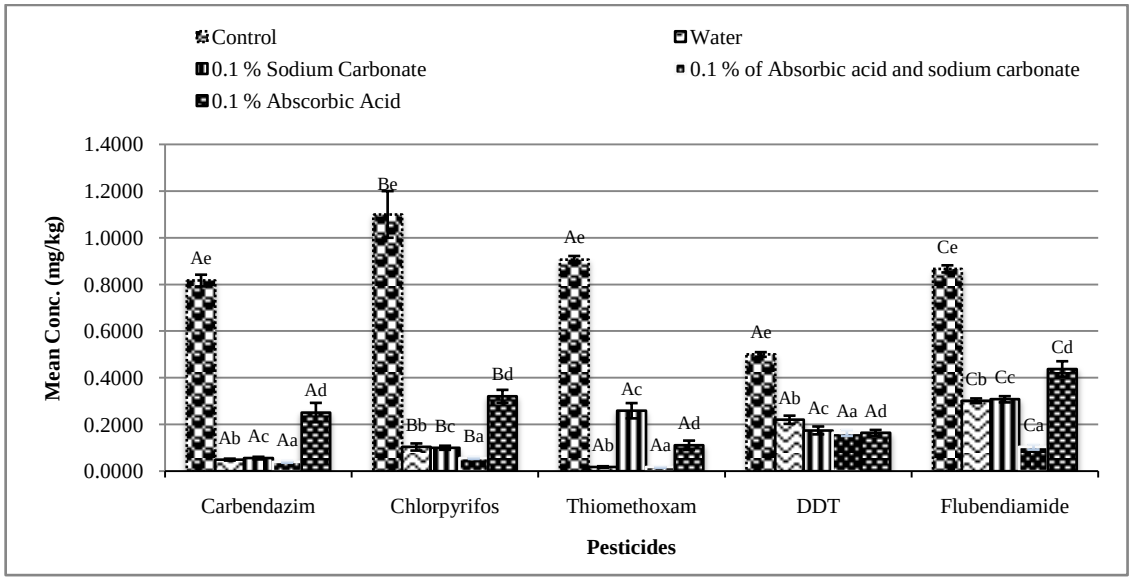

Figure 7 Different washing solutions effectiveness on removal of different pesticides in tomatoes. All the values are mean of triplicates ± S.D. ANOVA significant at $p < 0.05$. Different capital letters indicate significant difference when compared between different pesticides and small letters indicate significantly different values when compared between different treatments (DMRT, $p < 0.05$).

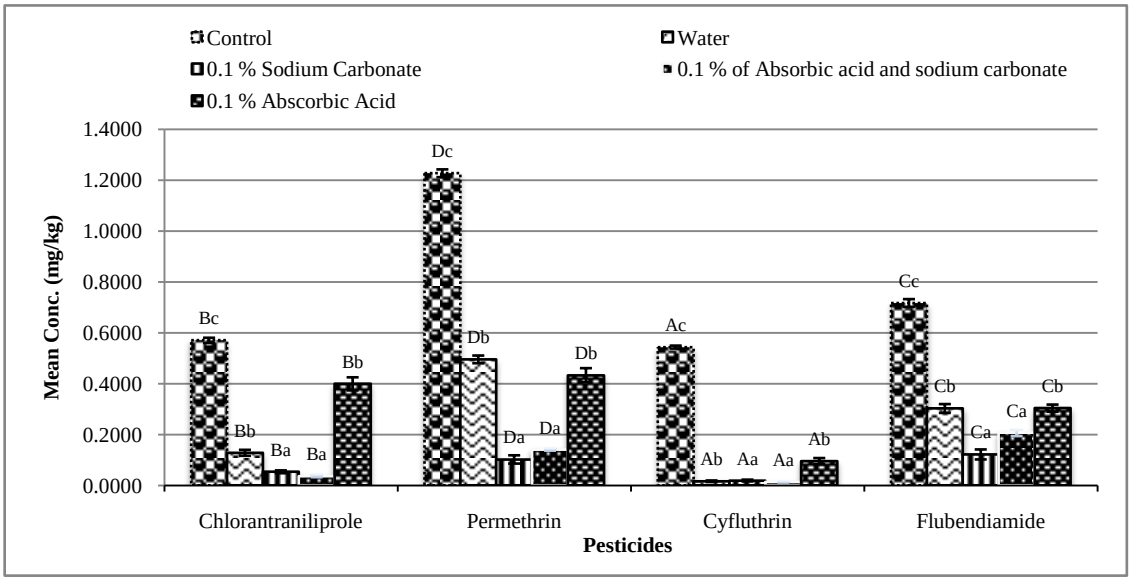

Figure 8 Different washing solutions effectiveness on removal of different pesticides in brinjal. All the values are mean of triplicates ± S.D. ANOVA significant at $p < 0.05$. Different capital letters indicate significant difference when compared between different pesticides and small letters indicate significantly different values when compared between different treatments (DMRT, $p < 0.05$).

In the apple samples, malathion percentage reduction ranged from 21.67 to 97.93% and was more in the mixed solution, this was followed by the combination of sodium carbonate, water, and ascorbic acid which exhibited 21% reduction in malathion. Similarly, the carbaryl percentage reduction ranged from 39.77 to 97.42%, and the highest reduction was reported in mixed solution while the lowest reduction was observed in ascorbic acid solution. Mixed solution and washing treatment with water results were comparable in apples. In tomatoes, a higher reduction was observed in thiomethoxam (71.54–98.06%), carbendazim (69.31 – 95.12 %), and chlorpyrifos (70.84 –94.85%), whereas less reduction was observed in DDT (55.03%–66.53%). The mixed solution reduction results were superior in tomatoes. In brinjal, a high reduction was observed in cyfluthrin, followed by chlorantraniliprole. However, a low reduction was observed in flubendiamide.

Pesticide reduction in water ranged from 55.03 to 98.06%. In the present study, the residue reduction was not directly correlated to pesticide solubility in water. The water solubility of thiomethoxam, malathion, and chlorpyrifos was highest among all the pesticides. The percentage reduction of thiomethoxam was 87.80% in tomatoes, malathion residue reduction was 21.67% in apples, chlorpyrifos residues reduction was 61.74% in apples, and 70.85% in tomatoes (Figure 6, 7, and 8).

4 Discussions

Chlorpyrifos levels in tomatoes were higher than the MRL in the present study. Similarly, higher chlorpyrifos concentrations in tomato was reported by Elgueta et al. (2017). The presence of chlorpyrifos in vegetables is consistent with the study by Kunyanga et al. (2018). Chlorpyrifos is a widely used insecticide that is highly efficient against various pests (Amoah et al. 2006; Angioni et al. 2011; Omwenga et al. 2021). It is also registered for use in fruits and vegetables in India as per FSSAI (Food Safety Regulation of India). Chlorpyrifos is persistent in nature, can be magnified, and bioaccumulates in plants. The World Health Organization, Food and Agricultural Organization, and US Environmental Protection Agency have classified chlorpyrifos to be moderately or highly hazardous to human health (WHO Programme on chemical safety 2009; Roberts & Reigart 2013; Omwenga et al. 2021). In the present study, the presence of DDT (banned agrochemicals) was also detected from the tomatoes. This finding is concurrent with that of Dari et al. (2016). Pesticides organophosphates and carbamates are safer than organochlorines such as DDT, aldrin, and dieldrin (Fernandez et al. 2000). Although DDT is banned, it is still available in the market in the unlabeled form and packaging material. Uneducated farmers are unaware that DDT has been prohibited. Further agricultural chemicals that are not intended for apple and tomato production are utilized in their production, which is an alarming situation that necessitates the investigation on how these pesticides reached the market (Dari et al. 2016). Lipid content, rate of rainfall, soil properties, soil organisms, and temperature affects the occurrences of pesticides in different food matrices (Bento et al. 2016). Multiple residues might be the consequence of persistent insecticides being absorbed by plants (Zhang et al. 2017) and poor agricultural practices of using more than one pesticide during application (Omwenga et al. 2021). Jallow et al. (2017) exhibited multiple residues resulting from different pesticide applications. Because of the low perception of pesticide toxicity and the strong demand for the crop, growers sometimes do not wait for residues to wash off before harvesting (Agyekum et al. 2015).

Several herbicides, as well as the majority of organophosphate and organochlorine insecticides and some carbamate insecticides, translocate into plants at non-lethal concentrations (Edwards 1975). Pesticides have a variety of chemical characteristics and modes of action including absorption, adsorption, and elimination (Agyekum et al. 2015; Li et al. 2019; Lozowicka et al. 2020). Pesticide absorption and movement in plant tissues are ascertained by physical and chemical properties. Further, some translaminar pesticides only move a short distance from the surface into the tissues, although some non-systemic pesticides do not penetrate into the plant tissues or only penetrate in a small amount (Hou et al. 2016; Lozowicka et al. 2020). Some organic compounds may volatilize from the soil and penetrate into the plant leaves through the cuticle or stomata (Collins et al. 2006). Different penetration abilities may be due to differences in the cuticle and epicuticular wax and dust on the fruit surface. These waxes and cuticles make a non-uniform surface which acts as a barrier for pesticides (Yang et al. 2016; Kvesitadze et al. 2015). Yang et al. (2016) reported that systemic pesticides can penetrate faster and deeper into fresh produce. However, the present study exhibited that although the systemic pesticide moved faster from the surface to peel and accumulated there, but these could not penetrate much into the pulp as compared to the non-systemic pesticides. Systemic pesticides such as carbendazim translocated from surface to peel. This finding is concurrent with that of Yang et al. (2016). Łozowickaa et al. (2020) reported that chlorpyrifos is one of the main compounds found in the apple peel. In the present study, chlorpyrifos translocated from the surface to peel and from peel to pulp much faster than other pesticides. Similarly, Łozowickaa et al. (2020) reported the highest concentrations of non-systemic acaricide, Carbaryl, insecticides, and chlorpyrifos from the apple peel. Differences may be observed in the adjuvant used in commercial pesticide formulations, and this is based on the lipophilic nature of the pesticide, the concentration of pesticides employed, application duration, and sampling (Finizio et al., 1997). In the whole apple, high levels of non-systemic acaricide such as beta endosulfan and non-systemic carbamates such as carbaryl may be attributed to the presence of micro-fissures in the skin due to abiotic or pest damage in an apple during the maturation stage, allowing entry into the peel (Lozowicka et al. 2020). Agyekum et al. (2015) exhibited dieldrin, fenitrothion, chlorpyrifos, lindane, and DDT in the peel of tomatoes. The peel retained higher organophosphorus and organochlorine pesticides than the other fractions. In the present study, chlorpyrifos and DDT concentrations in the tomato peels were high and these findings are supported by the findings of Agyekum et al. (2015). The concentration of organochlorine pesticides in the current study was similar to that reported by Mbakaya & Ngowi (1994). The present study results are concurrent with the findings of Kumari et al. (2002; 2003) and Deka et al. (2005).

As per the solubility of pesticides, the thiomethoxam concentration reduction was high while malathion reduction was less as per its solubility, which may be due to the presence of wax and dust on

the apple surface. Pesticide removal efficacy will depend on the fruit surface. Thus, no correlation was observed between pesticide solubility in water and pesticide reduction after washing. These findings are consistent with those of other studies (Satpathy et al. 2012; Youssef et al. 1995; Izumi 1999). Percentage reduction in ascorbic acid was less in the case of all applied pesticides except DDT. Thus, the mixture of ascorbic acid and sodium carbonate was selected. For removal of the pesticides residues, 0.1% mixture of ascorbic acid and sodium carbonate (70:30 v/v) was found to be effective. Additionally, 94.85% of chlorpyrifos, which is found in vegetables, can be removed using this washing solution. The movement of chemicals from one part of a plant to another also depends on the physiochemical properties of the chemical (Pullagurala et al. 2018; Zhang et al. 2017).

In the present study, the solution of sodium carbonate and ascorbic acid was observed to be more effective than water in removing pesticide residues. Alessandra exhibited that pesticides residue removal by sodium bicarbonate was more efficient than water (Rodrigues et al. 2017). These findings are concurrent with that of the present study. Rasolonjatovo et al. (2017) also concluded that a blend of washing solution is more effective than a single solution in removing pesticide residues as it exhibits a synergetic effect, which is in agreement with the findings of the present study. Yu-Shan et al. (2013) confirmed that mixing sodium bicarbonate with water forms carbonic acid, can remove pesticides through oxidation. Polat & Tiryaki (2020) confirmed that acetic acid and citric acid washing treatments were more efficient than tap water treatment. Additionally, Holland et al. (1994) exhibited that water solubility was the most significant parameter for pesticide residue. Although highly soluble pesticides can be easily removed from agricultural commodities (Randhawa et al. 2014; Holland et al. 1994; Lozowicka et al. 2020). Water alone was not found effective in the removal of pesticide residues. As per FSSAI Guidance Note 13/2020, pesticide residues in fruits and vegetables were reduced using methods such as scrubbing with a soft brush. According to the FSSAI guidance, washing with water removes 75%–80% of pesticide residues from the surface of fruits and vegetables, which supports the current study findings. However, a chemically prepared solution was better and more effective than water alone and a single washing solution because the chemicals present in mixed solutions work in synergy by interacting with each other.

5 Conclusions

The presence of pesticides on fruits and vegetable samples collected from the Ghaziabad district underlines the requirement to educate farmers on the usage and dosage of pesticide residues. A checklist of approved and banned chemicals to the food commodity should be supplied to the farmers. Agrochemical assistance must be provided to farmers for the selection of agrochemicals and their application at the right time and quantity. Furthermore, proper and frequent monitoring of pesticides available in local fruit and vegetable markets is required. Penetration behavior of pesticides was observed on the surface, pulp, and peel of fruits and vegetables. Physiochemical properties affect pesticide adsorption, absorption, and penetration. Additionally, some systemic pesticides penetrate deeply. This study will facilitate the design of guidelines for the safe application of pesticides to apple, tomato, and brinjal plants. Furthermore, a study of the effect of washing solutions exhibited that the solution of ascorbic acid: sodium carbonate (30:70 v/v) is more effective in reducing the pesticide residues from fruits and vegetables than water and single chemical alone. Pesticide reduction along with the physicochemical properties of pesticides also depend on the active components of the chemical that interact with each other and effectively reduce the pesticide level. Thus pesticide residues can be reduced from fruits and vegetables using a mixture of chemical washing solution and peel removal.

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Conflict of interests

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Ethical issues

There are no ethical issues to declare.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Effect of Feed Additive on the Mineral Composition of Quail Blood

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ABSTRACT

Blood serum microelement composition of egg-laying quails raised in the urban environment is of great scientific and practical interest. This study was carried out to evaluate the effect of a feed additive on the mineral composition of quail blood. To stimulate metabolism and egg productivity, quails of the experimental group were fed with a supplement containing magnesium, vitamins B, L-carnitine at the dose of 0.25 ml/liter of water for 120 days. At the age of 120 days, the blood serum micronutrient composition of experimental (10) and control (10) birds were measured by mass spectrometry followed by mathematical processing of the data obtained under laboratory conditions. Figuratively, all studied elements are divided into 4 groups i.e. macronutrients (calcium, phosphorus, potassium, sodium, and magnesium); essential elements (iron, copper, zinc, selenium, molybdenum, chromium, manganese, cobalt); roughly essential (silicon, boron, arsenic, lithium, and nickel) and roughly toxic (aluminum, titanium, lead, mercury, antimony, and cadmium). Results of the study revealed that the blood serum of a control group have a wide range of studied mineral content components while in the experimental group, egg-laying quails showed a decrease in phosphorus (18.30%), iron (12.29%), copper (6.76%), zinc (6.92%), molybdenum (18.80%), arsenic (14.00%) and cadmium (12.50%), as well as increases the concentration of magnesium (5.85%), manganese (28.31%), nickel (39.40%), lithium (8.32%), titanium (11.96%), lead (16.13%), mercury (13.34%) and antimony (14.29%) relative to the control group. The data obtained indicate that the feed additive had an ambiguous effect on the metabolism and led to changes in the concentration of certain trace elements in the blood serum, which in turn influenced the levels of other elements. The higher content of Ni, Li, Ti, Pb, Hg, and Sb in the blood serum of experimental laying quail stimulated the activity of enzymes, metabolic processes, which contributed to an earlier start of egg-laying.

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1 Introduction

The current stage of civilization is characterized by a global escalation of industrial activity with the involvement of a vast amount of chemical elements (Sergeev et al. 2004). Changes in the upper layers of the earth's crust undoubtedly affect the chemical composition of living organisms (Ershov et al. 2020), and also affect the concentration levels of biogenic elements of the living organisms, this serves as an ecosystems variability indicator. At the end of the last century, it was believed that living organisms' composition includes about 70 elements, but today there are 81 such elements (Pletneva 2008). All these biogenic elements have their definite functional role and concentration in the body (Polyanskaya 2014) and accordingly, these elements can be divided into three main categories i.e. macroelements (C, O, H, N, P, S, Ca, Na, Mg, etc.), trace elements (Mn, Cu, Zn, Co, Ni, I, F), and ultra-trace elements (As, B, Br, Li, Ni, V, Cd, Pb, etc.). These ultra-trace elements entered the organism's metabolism from an evolutionary point of view (Bgatov 1999), but their importance for animal organisms is extremely high since they take the most active part in metabolic processes occurring in the body.

In a living organism, chemical elements act in a complex way, and the elemental composition of the organism is very labile for several substances (Zholnin 2006). Nevertheless, the ecological peculiarities of certain territories affect the physiological indicators and the formation of adaptation mechanisms in farm animals and poultry (Krivonogova et al. 2013).

To meet human needs for essential proteins, vitamins, and trace elements, quail eggs and meat are valuable dietary products (Afanasyev et al. 2015; Chaunina 2016). The formation of a healthy, highly productive stock of laying quail and the creation of functional foods, complying with GOST R 52349-2005, is impossible without the use of biologically active feed additives (GOST 2005; Lysenko and Shirina 2013; Koshaev et al. 2011; Kuchina and Khurshkainena 2019). Various feed additives, such as ToxiNon, Api-Spira, birch tinctures, and Profort, are used to increase the meat and egg productivity of birds (Kletikova et al. 2019; Tereshchenko 2020; Chekhunova 2021; Kotarev et al. 2021). Effects of feed additives on the mineral composition of blood serum have been reported by various researchers. Gerasimenko (2006) noted that the copper content in the serum of geese changes with age and it was reported 0.55±0.007 mg/L, 0.50±0.011 mg/L, and 0.61±0.006 mg/L in young, 30-day, and 180 days old geese (Gerasimenko 2006). Es'kov and Kir'yakulov (2009) reported the presence of various micronutrients such as mercury (2.50±0.13 µg/L), lead (1.01±0.038 mg/L), cadmium (14.10±1.67 µg/L), zinc (4.49±0.873 mg/L), copper (2.34±0.234 mg/L), manganese (198.00±11.4 µg/L), magnesium (22.60±1.25 mg/L), selenium (1.94±0.37 µg/L), cobalt (137.00±14.10 µg/L) from the blood of white-fronted goose. In geese of the Italian white breed, the concentration of iron in the blood was established and it was reported minimum (4.14±0.02 mg/L) in 20 days old goslings and maximum (5.01±0.07 mg/L) in 60 days old goslings (Nikulin et al. 2004). Similarly, Mukhamedyarova (2021) also reported the presence of various micronutrients and trace elements in the blood serum of chickens and suggested that preparations Synbilite and Nabikat reduced the content of lead, nickel, and cobalt. Despite the great contribution of scientists to the study of the trace element composition of blood in birds, the determination of the mineral spectrum in quail, which includes 24 indicators, has not yet been carried out before. The purpose of this research was to evaluate the effect of a feed additive on the concentration of minerals in the blood serum of laying quail.

2 Material and Methods

This study was carried out during 2020-2021 at the Department of Obstetrics, Surgery, and Non-communicable Animal Diseases. To achieve the goal of the study, 2 groups of 1-day-old Japanese quails breed were formed. All the recommended standards conditions and microclimate parameters were used (Pigareva 1971). The used experimental diet was based on Purina® balanced feed. The control group of quails was provided pure boiled water, while for the experimental group feed additive was added to the water at the rate of 0.25 ml/l of water and was fed until the age of 120 days. The used feed additive contains magnesium, vitamins B, and carnitine belongs to type F and is used to increase the safety and productivity of farm animals and poultry.

At the end of the study, blood serum was collected from the 10 quails of control and 10 quails of the experimental group at the age of 120 days. Blood samples were taken from the axillary vein in the morning before feeding. Determination of the trace element composition was performed in a licensed laboratory using the mass spectrometric method. During the complete study, the standard procedure and guideline of the Ministry of Health, Russia dated 2004 "Guidelines on methods of quality and safety control of biologically active food additives" have been used (Guidance R 4.1.1672-03). Sample preparation was carried out as per the guideline of GOST 26929-94 (GOST 26927-86 guideline of Raw materials and food products). The content of toxic elements was carried out as per the GOST 26930-86 raw materials and food products guideline with the help of the atomic absorption method. While the concentration of arsenic was determined by the GOST 30178-96 raw materials and food products guideline. Mathematical processing of the data was carried out with the help of the Excel table processor.

3 Results and Discussion

The range of calcium, magnesium, and phosphorus in the blood serum of the control quail group was over 5% while the birds of

the experimental group showed a tendency of potassium and calcium decreases, and a significant decrease ($p \leq 0.05$) in phosphorus (18.30%), and an increase in magnesium (5.85%) concentration was reported from the experimental animals (Table 1). Probably, the feed additive containing magnesium contributed to the integration of macronutrients in metabolic processes, which contributed to the maintenance of catalytic and immunobiological processes in the body (Medvedsky et al. 2016).

Further, the concentration of Fe, Cu, and Zn in the blood serum of control of laying quail was 12.29, 6.76, and 6.92% higher than in the experimental group, respectively ($p \leq 0.05$) (Table 2). It is known that heavy metals contained in the feed are accumulated in the parenchymatous organs, tissues, and eggs of quail (Tarasenko 2015), at the same time, many of them are essential components of the body of birds and perform vital functions, which is especially important during egg-laying. Oviposition is associated with an increase in the intensity of anabolic processes in laying hens, metalloenzymes have obligatory participation in this, and various minerals such as iron, copper, and zinc which play a special role during this period (Lopes-Alonso et al. 2005). Further, the presence of various trace elements are also important for the functioning of respiratory enzymes and regulate the processes of biological oxidation, ATP generation, the inclusion of Fe in the heme, exchange of nucleic acids, and various other metabolic processes (Patrick 2003; Karomatov and Turaev 2017).

The concentration of Fe, Cu, and Zn in laying quail of the experimental In case of molybdenum, it was reported 18.80% higher in the quail control group as compared to the experimental group, this might be a prerequisite for the development of urolithiasis (gout) because excess molybdenum stimulates the synthesis of xanthine oxidase and intensifies purine metabolism (Sherkhov et al. 2015), stimulating the formation of uric acid which is harmful to the organisms and deposited in the joints, tendons, visceral organs. Along with this, the birds of the control group had 28.31% less manganese than the experimental group, which may be a prerequisite for lower concentrations of B vitamins (Miroshnichenko et al. 2007).

No significant deviations were reported in the content of selenium, cobalt, and chromium in both control and experimental group of laying quails (Table 2), which suggested the normal functioning of the antioxidant system, coenzymes, thyroid function, and metabolism of carbohydrates, lipids, cholesterol, and their integration into the egg's protein (Taylor 1985; Kavtarashvili et al. 2017).

Table 1 The concentration of macronutrients content in the blood serum of the quails

Parameter	Control group (mg/l)	Experimental group (mg/l)
Calcium	87.21±4.46	83.31±0.18**
Potassium	169.59±7.13	165.73±2.24
Magnesium	23.76±1.34	25.15±0.11*
Sodium	3200.03±114.32	3292.18±17.68*
Phosphorus	78.98±4.15	64.49±1.83**

Values given in the table are average of ten replicates and represent as mean ± SD, * significantly not different from the control group ($p \leq 0.05$); ** Significantly different from the control group ($p \leq 0.05$)

Table 2 The concentration of essential trace elements in the blood serum of the quails

Parameter	Control group (mg/l)	Experimental group (mg/l)
Iron	1536.00±237.43	1345.60±21.32**
Copper	762.40±38.56	710.80±13.28**
Zinc	707.18±46.84	658.24±9.68**
Selenium	128.80±13.37	125.20±1.16
Molybdenum	29.67±3.23	24.09±1.27**
Chromium	0.69±0.13	0.70±0.05
Manganese	0.53±0.08	0.68±0.02*
Cobalt	0.24±0.06	0.24±0.01

Values given in the table are average of ten replicates and represent as mean ± SD, * significantly not different from the control group ($p \leq 0.05$); ** Significantly different from the control group ($p \leq 0.05$)

Table 3 The concentration of roughly essential trace elements in the blood serum of the quails

Parameter	Control group (mg/l)	Experimental group (mg/l)
Silicon	315.70±6.24	301.70±4.38
Boron	34.02±1.23	34.59±1.46
Arsenic	4.07±0.08	3.50±0.02**
Lithium	3.30±0.33	4.60±0.28*
Nickel	3.85±0.62	4.17±0.22*

Values given in the table are average of ten replicates and represent as mean ± SD, * significantly not different from the control group (p≤0.05); ** Significantly different from the control group (p≤0.05)

Table 4 The concentration of roughly toxic trace elements in the blood serum of the quails

Parameter	Control group (mg/l)	Experimental group(mg/l)
Antimony	0.28±0.07	0.32±0.02*
Aluminum	4.53±0.54	4.70±0.11
Titanium	1.864±0.134	2.236±0.026*
Lead	0.31±0.08	0.36±0.02*
Mercury	0.30±0.06	0.34±0.04*
Cadmium	0.16±0.02	0.14±0.01

Values given in the table are average of ten replicates and represent as mean ± SD, * significantly not different from the control group (p≤0.05); ** Significantly different from the control group (p≤0.05)

Further, an imbalance in the trace elements of an individual can cause a malfunction of the physiological and biochemical characteristics of the poultry organism and lead to changes in the direction and intensity of metabolism at the cellular level (Sadovnikov and Shusharin 2016). In the case of silicon and arsenic, quail laying hens of the control group did not show much significant increase (P≤0.05) in silicon (4.4%) but the concentration of arsenic increased by 14% as compared to the experimental group (Table 3). According to Drogalev (2017), silicon is an important trace element in the "architect" of the body and forms the "foundation" of the functioning of all systems. It interacts with various organic compounds which are important for the body of birds (Nikolaenko and Andrenko 2020). The concentration of arsenic was reported less in the experimental group and this might be due to the influence of tested feed. Kaletina (2008) suggested that arsenic has a negative impact on the bird's body and inhibits the oxidative processes in mitochondria. Furthermore, the interaction of arsenic with thiol groups of proteins, cysteine, glutathione, lipoic acid, causes disorders of sulfur, selenium, and phosphorus exchange, and slows the egg formation and egg-laying process.

The blood levels of lithium and nickel in the control group of laying quail were lower than in the experimental group by 39.40 and 8.32% (p≤0.05). The biological role of lithium and nickel is not fully understood but various evidences suggested that lithium deficiency leads to stunting, and may associate with the birth of weak or nonviable offspring (Anke et al. 1983), while its addition to the diet stimulates the growth of birds with less feed intake. (Khomchenko and Naumova 2005). Further, Nickel is actively involved in enzymatic reactions and can accumulate in the keratinized tissues of birds, mainly in the feathers, without causing harm to the bird (Akhmetgalieva 2014). The higher content of lithium and nickel in the experimental group of quail stimulated ovogenesis and the onset of the productive period.

Further, the experimental diet-fed animals have a higher concentration of boron (1.68%), which stimulate the formation of boron esters which is associated with the various biomolecules as S-adenosyl-methionine, NADPH, AMP, ADP, ATP, etc. and accelerate the metabolic rate and beginning of oviposition (Becker and Bykov 2018).

Results presented in Table 4 revealed that the blood serum of laying quail in the control group have less aluminum (3.76), titanium (11.96), lead (16.13), mercury (13.34), and antimony (14.29%), while the level of cadmium was reported 12.50% higher than the experimental group (p≤0.05).

Kanzhigalina et al. (2013) suggested that minerals that take part in the building of the body, including heavy metals, cannot be considered pollutants because they are playing important role in various metabolic activities. The importance of studying these elements is since the effects of this antimony in the body are

similar to those of arsenic (Alyakhnovich and Novikov 2016). Aluminum is antagonistic to calcium, magnesium, phosphorus, zinc, and copper, but it is involved in the formation of the skeleton, cartilage, connective tissue, and regeneration (Shekeeva 2017). Although mercury belongs to the group of thiol poisons, blocking sulfhydryl (thiol) groups but it influences the activities of more than 50% of proteins-enzymes, activates the synthesis of immunoglobulins in microbes, enhances cooperative interaction of T and B-lymphocytes, interleukin-2 and γ-interferon formation (Hemdan et al. 2007; Martynova et al. 2014; Shinetova and Bekeeva 2017). Titanium metabolism disorder in the body is often a diagnostic sign of several diseases because it activates metabolic processes, improves blood composition, reduces urea and cholesterol content, participates in embryogenesis processes, increases general motor activity, while it reducing stress sensitivity and aggressiveness in the animals (Arefieva et al. 2010; Maslovskaya 2015). Cadmium is also a natural element and is considered a cancer-causing agent, it makes several pathological changes in the body and negatively affects the enzyme systems and many trace elements metabolism. Cadmium also can accumulate in vital organs like kidneys, liver, bones, blood, spleen, lungs, heart, and muscles (Argunov and Sviridov 2003). Akhmetgalieva (2014) suggested that in small concentrations Cd can stimulate the growth of some animals by affecting the carbohydrate metabolism, synthesis in the liver hippuric acid, and the activity of some enzymes. Vakhutkevich and Snitinskiy (2014) suggested that cadmium is a part of some special protein that binds and transports heavy metals in the body, and is involved in their detoxification. Lead is considered a broad-spectrum protoplasmic poison that mainly causes changes in the blood, cardiovascular and nervous systems and is synergistically involved in increasing the toxicity of other metals (Ershov et al. 2020), disrupting enzymatic activity, provoking disruption of hemoglobin synthesis, DNA replication, reducing nonspecific body resistance (Shaposhnikova and Bolgova 2012). Along with these negative effects, lead can initiate the processes of free radical oxidation, leading to an increase in lipid peroxidation, a decrease in the activity of catalase and superoxide dismutase (Nabil et al. 2011). At the same time, low lead concentrations stimulate protein biosynthesis and modulate the activity of immunocompetent cells (Patil et al. 2006). Analysis of the data and literature suggested the vital necessity of antimony, aluminum, titanium, lead, mercury, and cadmium for laying quail.

Conclusions

In the current study, first time the content of 24 minerals in the blood serum of Japanese quail was studied from the Ivanovo region of Russia. Further, to the best of our knowledge, this is the first study carried out to study the effect of a feed additive containing magnesium, B vitamins, and L-carnitine on the dynamics of mineral substances in laying quail. The blood serum of the control group quails showed a higher content of iron, copper, zinc, molybdenum, phosphorus, cadmium, and arsenic. Against the background of the use of a complex feed additive, a significant increase in magnesium, manganese, nickel, lithium was noted. Further, the experimental diet increased the rate of various minerals which stimulate the various enzymatic processes and led to the beginning of egg-laying 7-12 days earlier in quail laying hens of the experimental group compared with the control group. The established concentrations of mineral substances in the blood serum of the laying hens of the control and experimental groups did not adversely affect the health status, hematological parameters, and basic metabolic parameters. Consequently, the results of the current study can be considered the preliminary data for Japanese quail laying hens bred in the Ivanovo region of Russia for egg production and subsequent incubation.

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Conflict of interest

There is no possible conflict of interest declared as concerns the publication of this paper.

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Effect of Thyme aqueous and alcoholic extract on the Beef Mincemeat shelf life extension

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ABSTRACT

Antioxidant and antimicrobial activity of thyme has been well established against various microorganisms. This study was carried out to investigate the effect of aqueous and alcoholic extract of thyme on beef mincemeat quality. Three differential concentrations (0.4, 0.8, and 1.2 mg/ml) of both thyme extracts were used for the beef mincemeat preservation. Untreated meat samples were considered as the control group while the extracts treated beef mincemeat are stored at 4°C for 7 to 14 days. To validate the extract's ability to prolong the storage period at 4 °C, various bacteriological indicators like total plate count, presence of total *coliform*, *Salmonella*, *Shigella*, and *Staphylococcus aureus* count were assessed. The results of the antimicrobial assay of aqueous and alcoholic extracts of thyme at different concentrations showed that the aqueous extract had significant inhibitory action on the growth of a wide range of bacteria compared to the alcoholic extract. Thus, the thyme aqueous extracts can be efficient and promising as preservatives for meat and its products, especially at high concentrations to inhibit bacterial growth.

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1 Introduction

Biochemical changes and enhanced microbial activity are the most common events that occur during food spoilage, especially in the case of inadequate preparation, handling, packaging, and storage (Tshabalala et al. 2021). Many factors such as lipid peroxidation may encourage worsening the food quality and safety as a result of the formation of hydroperoxides (Fratianni et al. 2010). Peroxidation may further cause the deterioration and degeneration of other secondary products (Djordjevi et al. 2019). Undeniably, refrigerated delivery has made the international supply of consumable foods feasible. However, proper packaging conditions and refrigeration are not always sufficient to secure the food from spoilage, contamination, and undesirable changes (Fratianni et al. 2010). Perishable types of food, such as meat, spoil easily due to the favorable growth conditions for various microorganisms. Besides this, many undesirable chemical and enzymatic activities lead to unpleasant odors and an unacceptable taste (Djordjevi et al. 2019) which make food unsuitable for human consumption (Vencato et al. 2020). Since ancient times, people used various traditional preservation methods such as salting, drying, smoking, fermentation, and canning whose primary function was to limit decay and rottenness (Zengin and Baysal 2014). These methods are also used to prevent meat spoilage and prolong its shelf life (Hernández et al. 2018). Recently, various advanced methods such as refrigeration, freezing, and chemical preservation have been directed towards preserving food without affecting its flavor, nutritional quality, and texture (Zengin and Baysal 2014). Chemical preservation is an artificial method that is based on applying some chemicals such as nitrates, benzoates, sulfites, and sorbates for preservation (Ukrainets 2016). It is well established that these chemical preservatives have a negative effect on human health owing to their allergic and carcinogenic causes if they are used or consumed excessively (Mischek and Krapfenbauer-Cermak 2011). Therefore, there is an urgent need of replacing these chemical preservatives with natural products (based on herbs and medicinal plants as food additives). Various researches establish that natural preservatives can be used as an alternative to enhance food quality without altering food taste (Ukrainets 2016). Antimicrobial and antioxidant activities of natural derivatives against food spoiling microbes have been reported by various researchers (Ukrainets 2016; Emeka and Chiamaka 2020; Efenberger-Szmechtyk et al. 2020; Pateiro et al. 2021; Salman et al. 2021a), these antimicrobial and antioxidant activities are associated with the content of bioactive natural compounds that are present in these plants (Salman et al. 2021b). Further, these natural additives have proven their ability and effectiveness to reduce the effects of oxidative damage, delay in the emergence of objectionable flavors, and improve the food status (color of meat dyes) (Efenberger-Szmechtyk et al. 2020). Therefore, professional interest in studying the properties of these natural food additives has increased considerably.

Thyme is one of the bioactive herbs used as culinary (flavoring agent and spice) and therapeutically agent (Garca-Dez et al. 2017). Plant extract of thyme contains many active ingredients such as monoterpene phenols, carvacrol, thymol, p-cymene, and other monoterpenes, such as -pinene, 1,8-cineole, camphor, linalool, and borneol which might attribute the medical effect of the plant. (Emeka and Chiamaka 2020). Further, thyme's antibacterial properties were notably established against pathogenic bacteria (Wesolowska and Jadczak 2019).

The addition of thyme essential oil to dried meat products showed bacterial inhibition activity against foodborne pathogens and it was similar to the other selected essential oils (Garca-Dez et al. 2017). Agrimonti et al. (2019) investigated the antimicrobial synergetic effect of various essential oils including cinnamon, thyme, and oregano against a wider range of microorganisms and reported that the thyme components have significant microbial inhibition, especially against the relatively resistant gram-negative. Robust action of thymol and carvacrol against *P. aeruginosa* or *S. aureus* has been also reported by Di Pasqua et al. (2006). These plant extracts inhibit the growth of many microorganisms by desolating the plasma membrane, disturbing the pH, and misbalancing the inorganic materials (Agrimonti et al. 2019, Nieto 2020, Yang et al. 2021). Thus, there is a possibility to increase the quality of meat and decrease microbial deterioration by using plant extracts. Therefore, the current study was conducted to investigate the potential role of thyme aqueous and alcoholic extracts as a preservative agent to prolong the shelf life of mincemeat by the inhibition of bacterial growth.

2 Materials and Methods

2.1 Preparation of thyme extracts

Thyme leaves were procured from the local markets of Al-Najaf province, Iraq. Collected leaves were washed using tap water, followed by using sterile water. The dried plant's leaves were crushed immediately before the assay using an electric grinder, followed by grinding. Alcoholic extracts of collected thyme leaf were prepared by adding 100 gm of the leaves powder to 1000 ml of 70% (v/v) aqueous ethanol in a closed conical flask for 24 hrs at room temperature in the dark. The extract was filtered through cheesecloth, and the residue was re-extracted three times using the same solvent. The filtrate obtained was evaporated in a vacuum evaporator at 40 °C and stored at lower temperature till the completion of the study. The test extracts solution was prepared from the stock solution by dissolving the required amount of ethanol and the concentration was set 0.4, 0.8.and 1.2 mg/ml. Similarly, the aqueous extracts were prepared by adding 100g of ground sample to 1000 ml of sterilized water and leaving for 30 minutes on a magnetic stirrer, followed by filtering using cheesecloth. The obtained filtrate was evaporated in a vacuum

evaporator at 40 °C and frozen until used. The concentrations were prepared as mentioned previously as the alcoholic extracts.

2.2 Preparation of mincemeat samples

Samples of beef meat were procured from local markets in Al-Najaf, Iraq, and transferred to the laboratory for processing. The samples were cut into pieces and then minced using a meat grinder. From the prepared samples, the first 250gm of mincemeat sample was stored as a control sample without any extract additives. The remaining samples were divided into six different parts (250 grams for each concentration) and treated with the 0.4, 0.8, and 1.2 percent aqueous and ethanol extracts of thyme.

2.3 Microbial Count

Microbiological analysis was performed using the plate counting technique. The microbial count was carried out as per the recommendation of the American Public Health Association for food examination (APHA 1992). The analysis was based on counting the TPC, *coliform* bacteria, *Salmonella*, *Shigella*, and *Staphylococcus aureus*. Nutrient agar was employed for TPC. As per the bacterial strains, the culture medium was changed and McConkey agar medium was used for the determination of *coliform* bacteria, S.S. agar was used for the investigation of *Salmonella- Shigella*, and MSA for counting *S. aureus*. Homogenized samples (extracts with the mincemeat) were serially diluted up to 10-fold and dilutions D4, D5, and D6 were selected for the bacterial isolation and placed in a sterilized petri dish. The plates were performed in triplicates and incubated at 37°C for 24–48 hours on the days specified in the research schedule (7 days–14 days). Based on the selective media and morphological characterization the strains of bacteria were identified and calculated.

2.4 Statistical Analysis

All the obtained results were expressed in mean ± SD (standard deviation) in this study. Differences between means of data were compared by the least significant difference (LSD) calculated by using the Statistical Analysis System (SAS Institute, Inc., Cary, NC).

3 Results

The results of an antibacterial assay of the aqueous and alcoholic extracts of thyme on the bacterial reduction count were evaluated and represented by figures 1-4. The mincemeat samples were stored at 4 °C and observations were taken after 7 and 14 days of storage for different concentrations (D 4, D5, and D6). Results presented in figure 1 revealed the effect of various aqueous (TW) and alcoholic extracts (TA) concentrations on the total bacterial count after 7 days of culture. A significant effect of both thyme extracts was reported on the total bacterial count and meat samples treated by thyme extract have significantly lower bacterial colony count as compared to the untreated control samples. Further, a gradual reduction was reported with increasing concentrations and the least bacterial count was reported when samples were treated by high concentration (1.2 mg/ml), particularly in the case of aqueous extracts (TW). The growth reduction was variable between the dilutions of meat samples (D4-D6) and it was reported highest in D6 for both types of extracts. After 7 days of the treatments, the least bacterial growth was reported for *coliform* bacteria, *Salmonella*, *Shigella*, and *S.aureus* as compared to the

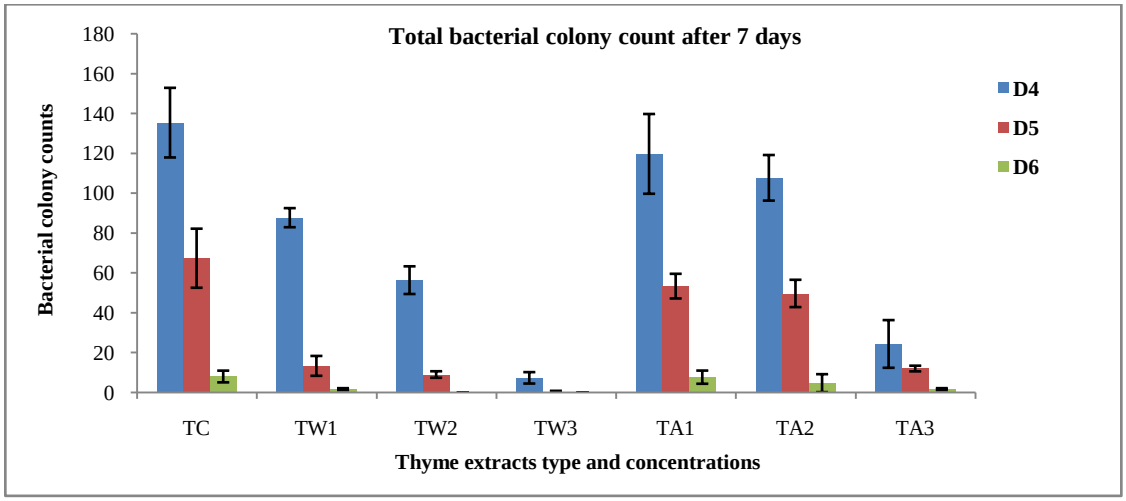

Figure 1 Effect of aqueous and alcoholic thyme extracts on the total bacterial colony count at different concentrations and dilutions on day 7 of storage at 4ºC. Values given in bars are mean of count, the error bars represent the SD (standard deviation); TC (control); TW1-TW3 (0.4 mg/ml, 0.8 mg/ml, and 1.2 mg/ml aqueous extracts respectively); TA1-TA3 (0.4 mg/ml, 0.8 mg/ml, and 1.2 mg/ml ethanol extracts respectively); D4-D6 (Dilutions of the cultured samples)

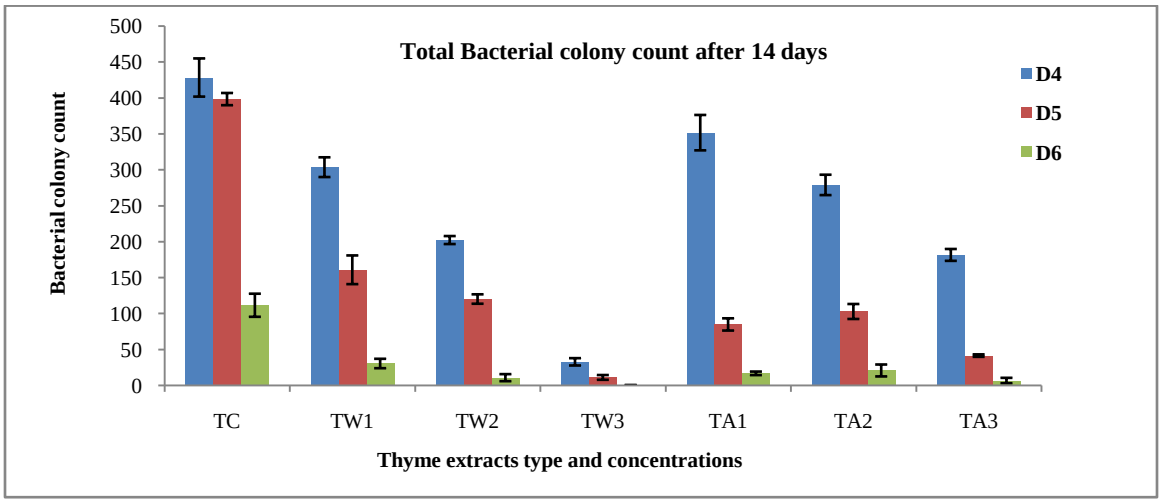

Figure 2 Effect of aqueous and alcoholic thyme extracts on the total bacterial colony count at different concentrations and dilutions on day 14 of storage at 4°C (Values given in bars are mean of count, reset all figure legends and abbreviations are similar to the figure 1)

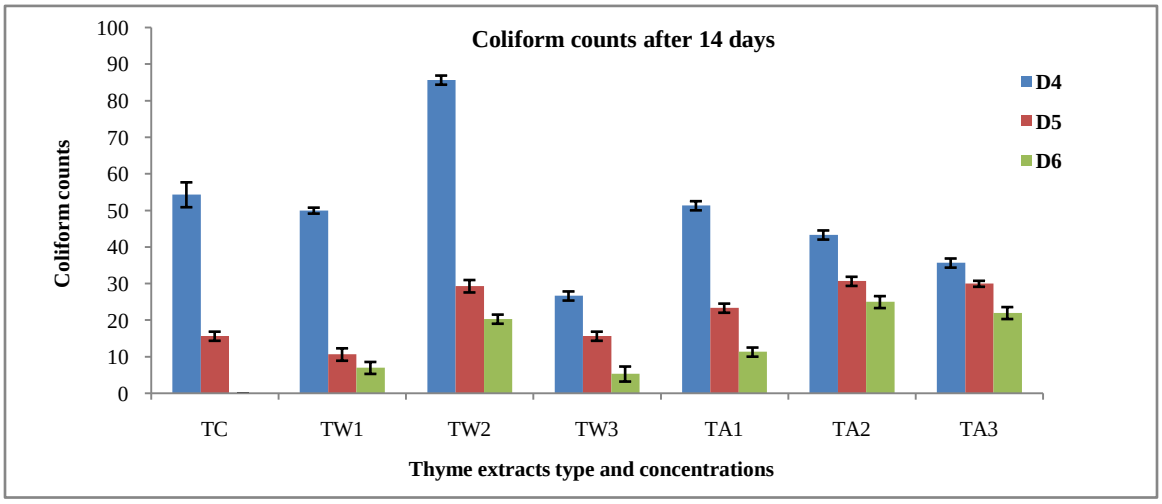

Figure 3 Effect of aqueous and alcoholic thyme extracts on the coliform bacterial counts at different concentrations and dilutions on day 14 of storage at 4°C (Values given in bars are mean of count, reset all figure legends and abbreviations are similar to the figure 1)

control. On14th day of storage the total bacterial count was dramatically increased as compared to the 7 days of storage (Figure 2). However, a significant decline ($P< 0.05$) was reported at the concentration 1.2 mg/ml and D6 in aqueous extracts compared to the control.

After 14 days of storage, in the case of coliform bacterial count variable results were reported which are not following any predetermined pattern. Among the tested aqueous extract concentration, the least coliform count was reported from the lowest concentration (TW1), this was followed by the highest concentration (TW1) while concentration TW2 appeared to be less efficient in reducing the number of coliform bacteria colonies count. In the case of alcoholic extracts, there were statistically significant differences were identified ($P > 0.05$) between the concentrations and examined sample dilutions. However, there are significant differences ($P > 0.05$) recorded between the control and both extracts but interestingly no coliform was observed at D6 in the control (Figure 3).

Further, in the case of *Salmonella* and *Shigella* count, aqueous and alcoholic extracts were not showing any significant difference ($P > 0.05$) between the various concentrations, dilutions, and control treatments. Among the tested concentrations and dilutions, treatment TW3 was found effective in reducing the count of *Salmonella* and *Shigella* (Figure 4). Whereas in the case of *S.aureus, no bacterial growth was reported* between variations concentrations and dilutions, and the highest *S.aureus* growth was reported from the control treatments at lowest sample dilutions (Figure 5).

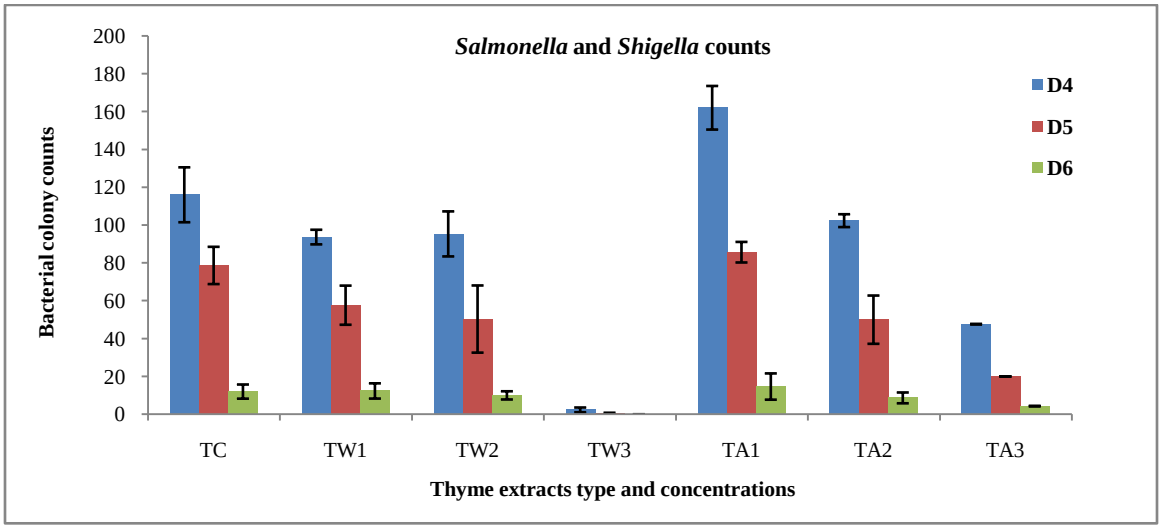

Figure 4 Effect of aqueous and alcoholic thyme extracts on the *Salmonella* and *Shigella* counts at different concentrations and dilutions on day 14 of storage at 4°C (Values given in bars are mean of count, reset all figure legends and abbreviations are similar to the figure 1)

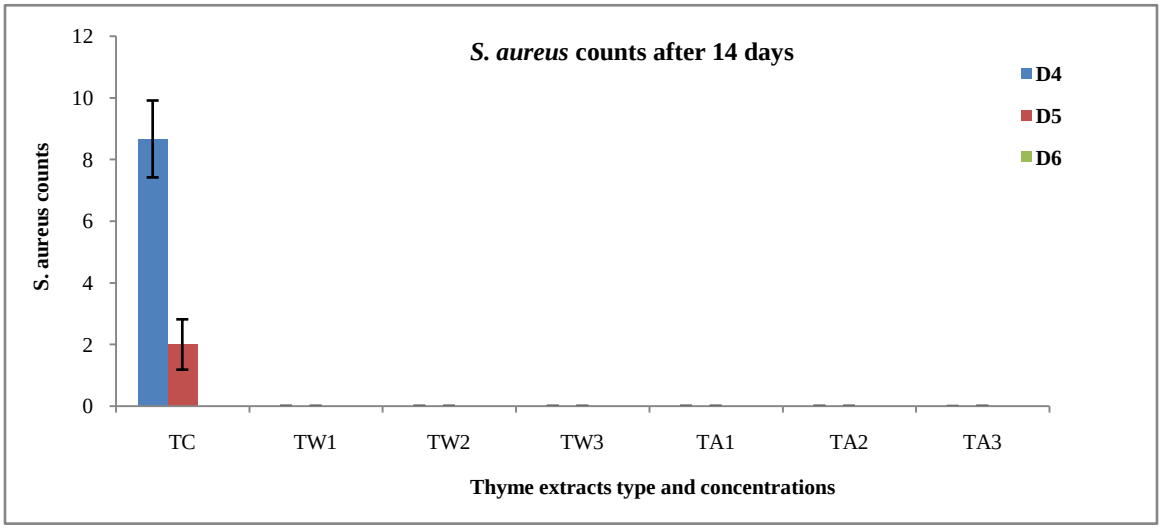

Figure 5 Effect of aqueous and alcoholic thyme extracts on the *S.aureus* counts at different concentrations and dilutions on day 14 of storage at 4°C (Values given in bars are mean of count, reset all figure legends and abbreviations are similar to the figure 1)

4 Discussion

One of the most important obstacles to food manufacturers in the presence of various harmful microbes that may deteriorate the food during storage and transportation. Increasing the human concern regarding the harmful effect of chemical preservatives, generate the need to explore plant-based natural preservatives (Nieto 2020). These natural food preservatives that have been traditionally applied for preservative purposes are safe, cost-effective, and also appetite boosters (Ali et al. 2018). The antimicrobial activity of various plant extracts against foodborne pathogens has been well explored (Garca-Dez et al. 2017; Ali et al. 2018; Agrimonti et al. 2019; Salman et al. 2021b).

Thyme is one of the most popular herbs in the food industry due to its flavor, fragrance qualities, and medicinal properties. In the current study, the efficiency of two solvent extracts (aqueous and alcoholic) was evaluated for their antibacterial action on cooled mincemeat samples during different periods (7 and 14 days). Furthermore, different gradual concentrations were applied, and the results on the seventh day of storage showed that with increasing the extract concentration, the total viable count of bacteria was reduced, in particular of D6 dilution and aqueous extracts (TW) in comparison with the non-treated samples. However, on day 14 of storage, the total plate count was slightly increased, but the number was reduced again at D6 serial dilution and aqueous extract (TW), especially for the highest concentration

(1.2 mg/ml). Among the tested solvents, the aqueous extract was found superior over the alcoholic extract and this might be due to the polar nature of most active ingredients, these components are competently dissolved in water and they retain their actions against the bacterial selected parameters (Abubakar and Haque 2020). Despite the safety and no toxicity effects involved with water extracts, water is not sufficient as a solvent. Therefore, different solvents (semi-polar and non-polar) are applied instead to ensure the bioavailability of the active constituents. Correspondingly, Hernández et al. (2018) recorded that viable counts significantly decreased when they applied thyme essential oils to prolong the shelf-life of dried meat. Undoubtedly, thyme is well-known to possess pharmacological relevance, and the presence of rich bioactive content may interfere with and disturbed bacterial growth (Nieto 2020).

The results of the test for coliform bacteria revealed that no growth was observed on the seventh day of storage. However, when the period was extended to 14 days, the situation was slightly different. The bacterial growth was observed at all concentrations and dilutions but in different manners. The results of the current study are in agreement with the findings of Boskovic et al. (2015), those who investigate the effect of some thyme constituents against a range of coliform bacteria and obtained similar results. Similarly, Duckova et al. (2012) reported the bactericidal effects of thyme oil at a concentration of 0.05% against *E. faecalis* and *E. mundtii* strains at 6 and 25 ℃ storage. Therefore, these outcomes can be explained by the impact of the natural extracts on the collapse of negative bacteria cell walls, these extracts also deplete the lipopolysaccharides and increase the permeability of the cytoplasmic membrane for ATP and some ions, which facilitated cell death (Boskovic et al. 2015). Trajchev et al. (2020) also evaluated the bactericidal efficacy of some medicinal plant extracts including thyme against the *Salmonella* spp. that are resistant to the majority of commercial antibiotics and are reported that thyme extract exhibited the most killing efficacy against the *Salmonella* spp. Additionally, the results of the current study suggested that aqueous extracts increase the bioavailability of active components and completely inhibited the growth of *S.aureus* on day 14 at all concentrations and dilutions. It is also well established that some pathogens, including *S. aureus*, *S. enterica*, and *S. typhimurium* are sensitive to thymol and carvacrol, and their biocidal action results in fundamental and functional membrane disintegration(Chouhan et al. 2017). Moreover, thyme has also proved its potential in vitro antibacterial activity against food pathogens such as *Salmonella*, *S.aureus*, *E. coli*, *Klebsiella*, *Pseudomonas*, and *Enterococcus* at different concentrations (Boruga et al. 2014).

Conclusion

From the current research, it could be concluded that the aqueous extracts of thyme can preserve mincemeat from different bacterial contaminants. Therefore, these natural additives could be safely used by meat processors to improve the quality and extend the shelf life of meat products. Obviously, plant extracts prepared from different plant parts are rich in bioactive materials. Hence, they offer excellent substitute agents of synthetic antibacterial. According to the results, there are potential and promising applications of these materials as preservatives for preventing the spoilage and deterioration of different types of meat and meat products. Although these extracts are safe and accessible, further research is needed to investigate their effects on the sensory and nutritional quality of meat products.

Conflict of Interests

The authors declare that there is no conflict of interest

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Journal of Experimental Biology and Agricultural Sciences, February - 2022; Volume – 10(1) page 131 – 134

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Kisspeptin is Testosterone independent regulator of Sexual Motivation in Male Rats

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ABSTRACT

Kisspeptin is the peptide product of the KISS-1 gene and endogenous agonist for the Kiss1 receptor. It is well known that kisspeptin acts centrally, and stimulates the secretion of gonadoliberin (GnRH). Further, Kisspeptin also interacts with other neuropeptides such as neurokinin B and dynorphin to regulate GnRH pulse generation and plays a key role in sexual behavior. This study aimed to evaluate the effect of kisspeptin on male rats' sexual motivation and its dependence on testosterone levels. In this study total of 50 copulation naive male Wistar rats were collected and divided into 5 groups (10 rats in each group), among these first group received only saline (control), the second group has been given 20µg buserelin acetate (GnRH analogue), the third group has been given intranasally kisspeptin-10 (3ng), the fourth has been received intraperitoneally kisspeptin-10 (30ng) and the fifth group has been given Yoquimbine 200 µg. Behavioral effects were registered in the open-field reward-proximity chamber with a female in the estrous phase of the cycle over the transparent perforated wall for 10 minutes in red light. Blood samples were collected from the tail vein after 30 minutes of the substance administration and in the collected blood samples, testosterone concentration was measured by the ELISA method. All animal groups were compared with each other by the ANOVA test and correspondent "post hoc" paired tests of Newman–Kruskall– Wallis test and Dunn's test. Intranasal administration of buserelin acetate increased the concentration of testosterone but did not affect sexual motivation in rats. Further, intraperitoneal administration of Kisspeptin-10 enhances testosterone concentration and sexual motivation. While intranasal administration of kisspeptin-10 didn't enhance testosterone level but increased sexual motivation. Results of this study showed some effects of kisspeptin along with the independent regulation of steroids.

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1 Introduction

Originally Kisspeptins are proteins encoded by the endogenous metastasis suppressor neuropeptides produced by the KISS1/kiss1 gene and are key regulators of reproductive function. Several isoforms including kisspeptin (KP)-54, KP-14, KP-13, and KP-10 exist with a common RF-amide C terminus (Clements et al. 2001; Kotani et al. 2001). Further, Kisspeptin is an endogenous ligand for the kisspeptin receptor (KISS1R) (Lee et al. 1999). All of the isoforms can activate the kisspeptin receptor due to a common C-terminal decapeptide. It is well known that kisspeptin centrally acts via the kisspeptin receptor and stimulates the secretion of gonadotrophin-releasing hormone (GnRH). Loss of kisspeptin signaling causes hypogonadotrophic hypogonadism in humans and other mammals (Silveira et al. 2010). Kisspeptin interacts with other neuropeptides such as neurokinin B and dynorphin and regulates the GnRH pulse generation (Bai et al. 2014; Wolfe et al. 2018). Adekunbi et al. (2018) and Sieme et al. (2004) suggested that Kisspeptin also plays a significant role in sexual behavior. Gonadally intact testosterone replaced male kiss1r knockout mice fail to display an olfactory partner preference despite normosmia, as evidenced by spending a comparable amount of investigatory time with male and female mice (Stephens et al. 2017). This study was carried out to evaluate the effect of kisspeptin on male rats' sexual motivation and its dependence on testosterone levels

2 Materials and Methods

2.1 Experimental Animals and treatments

For this study 50 copulation naive male Wistar rats (age 100 days, weight 250±30g) were collected and were divided into 5 groups (each group is of 10 rats). Among the formulated groups, the first group animals were intact as considered as control, while the remaining four groups were administrated with hormonal and non-hormonal regulators of sexual behavior. In the experimental group, the animals of the first group received intranasally GnRH analogue Buserelin acetate (Pharm sintez, Russia) @ 2 µg/µl and intraperitoneally saline. The second group received 0.15µg/µl Kisspeptin-10 (Institute of Ultra Pure Biochemical Preparations, Russia) intranasally and saline intraperitoneally. The third group received intranasally saline and 0.15µg/µl Kisspeptin-10 intraperitoneally. The fourth group received intranasally saline and 1mg/ml Yoquimbine HCl (Zdorovie pharmaceuticals, Ukraine) intraperitoneally.

2.2 Behavioural tests

The Open-field reward-proximity chamber made of plexiglas was used for assessment of the appetitive behavior for sexual reward. Open-field arena (85 × 35 × 50 cm high) had a chamber over the transparent perforated wall made of Plexiglas (15 × 35 × 50 cm high) which was mounted at one end. The front perforated wall allowed the subjects to approach and investigate (i.e., sniff) the animals (estrous female rat) in the chamber but prevented tactile contact or copulation. On the day before the appetitive behavior testing, all the subjects were habituated for 30 min in the open-field arena. The Male's behavior was recorded on video in the darkroom with red light for 10 minutes. The open-field and stimulus-cage were wiped clean with 3% hydrogen peroxide between subjects to eliminate olfactory cues. As the measures of sexual incentive motivation, for each animal, the time spent on sniffing the stimulus-cage (sniffing time, i.e., nose-point within the perforated wall) and latent time before were recorded.

2.3 Testosterone assay

Blood samples were collected from the tail after 30 minutes of the substance administration. Serum was separated with a centrifuge (8000 rpm). The samples were frozen and stored at -80^{o}C until the ELISA was performed. Testosterone concentrations in serum were measured by solid-phase ELISA using the Testosterone test system - EIA Kit (Alkor-Bio, Russia).

2.4 Statistical Analysis

GraphPad Prism v.5 and SPSS SigmaStat 3.0 software were used for statistical processing of the obtained quantitative data. Kolmogorov-Smirnov normality criterion was used to evaluate the correspondence of random value distributions to Gaussian ones. To compare control and experimental groups, the Wilcoxon nonparametric criterion for paired comparisons and the method of single-factor dispersion analysis with subsequent multiple intergroup comparisons by the Newman-Kales criterion was used. The data are presented as "mean ± SD".

3 Results

The first purpose of this study was to measure behavioral aspects of sexual motivation like the number of trying to reach the female and latent time before it. Results of the study revealed that intranasal administration of buserelin acetate didn't act on latent time before trying to reach the female (7.9±3.5 sec. vs 8.0±3.5 sec. in control) and reduced the number of trying (9.8±1.3 sec. vs 13.2±2.0 sec. in control). Further, intranasal and intraperitoneal administration of kisspeptin-10 reduced latent time before trying to reach the female (3.9±1.7 sec. and 5±1.6 sec.) but only intranasal administration induced the number of trying (19.5±3.0 vs 14,3±2,0) (Figure 1, 2). In the second step of this study, quantitative determination of testosterone in blood serum was also carried out. Buserelin acetate increase testosterone level about three times (47.5±19.5 nmol/ml vs 14.5±6.2 nmol/ml in control). No significant differences were found between testosterone level in rats after intranasal or intraperitoneal kisspeptin-10 administration

and control animals (11.2±3.0 nmol/ml vs 13.1±4.0 nmol/ml). Yoquimbine HCl also didn't enhance the testosterone level (13.2±4.0 nmol/ml) (Figure 3).

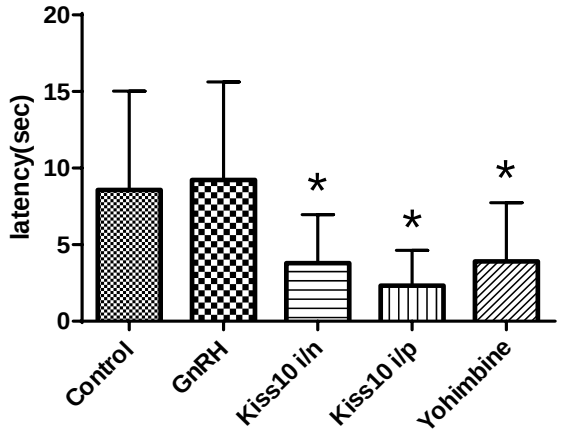

Fig 1 Latent time in sexual motivation test

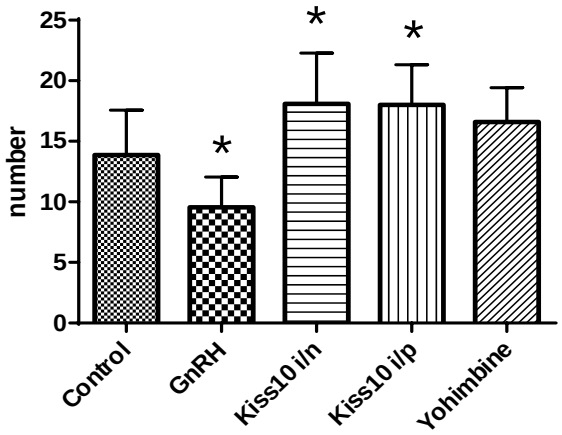

Fig 2 Number of trying to reach female in sexual motivation test

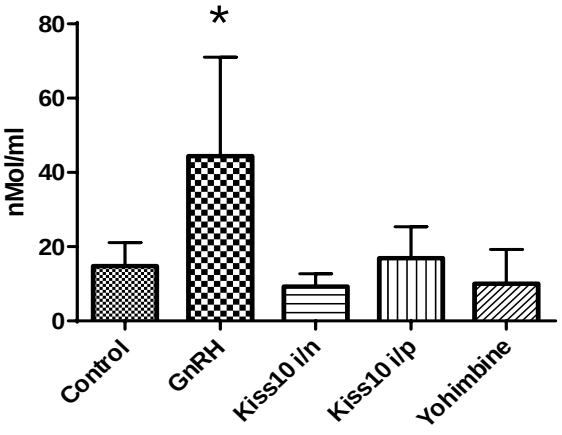

Fig 3 Testosterone in blood serum

4 Discussions

A strong relationship between kisspeptin, GnRH, and sexual steroids has been reported by previous researchers. The initial observations of the current study also describing the essential role of kisspeptin signaling in puberty emerged that kisspeptin neurons are also relying on steroid feedback regulation to GnRH neurons. However, till now the role of kisspeptin signaling outside of the hypothalamus was unknown. The posterodorsal medial area of the amygdala (MePD), where the kisspeptin-responsive neurons were found, is particularly associated with pheromone-related reactions, which suggests that kisspeptin may affect sexual behaviors (Lee et al. 1999). Immunohistochemistry data regarding amygdala kisspeptin neurons, identifying that they receive vasopressinergic and dopaminergic inputs (Pineda et al. 2017). It suggests interplay with key behavioral neuropeptides implicated in social behavior and motivational behavior (Meyer-Lindenberg et al. 2011; Tissen et al. 2019). Results of the study support the idea of the steroid-independent mechanism of kisspeptin effects. Low doses of buserelin acetate can induce a transitory increase of testosterone and may be helpful to stimulate libido in some mammals (Sieme et al. 2004; El-Khawaga et al. 2011).

Conclusion

Intranasal administration of buserelin acetate increased the concentration of testosterone but did not affect sexual motivation in rats. Intranasal administration of kisspeptin-10 didn't act testosterone but increased sexual motivation. Intraperitoneal administration of kisspeptin-10 acts on both testosterone concentration and sexual motivation behavior. This study has shown some effects of kisspeptin realized with the steroid-independent mechanism.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Relationship between temperature, temperature-humidity index and amount of food intake of Sheep

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Food intake

Hot season

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Temperature-humidity index

ABSTRACT

This study aimed to identify the relationship between temperature, temperature-humidity index (THI), and the amount of dry matter food intake (DMI) by sheep. Twelve Phan Rang (Ninh Thuan province) sheep belonging to three age groups of 6, 9, and 12 months (4 heads of each age group) raised in Thua Thien Hue province were fed with natural grass for two seasons: hot season (April-August) and cold season (November-February). Daily temperature, humidity, and food intake were recorded. The results of the study revealed that temperature and THI were closely correlated ($P<0.05$) with the amount of food intake by sheep. When the temperature was in the range of 29.5°C to 32.5°C and increased by 1^{o} C, the DMI of sheep decreased by 14.7 g/BW/day. When the value of THI was more than 28.5 and rose by 1°C, the DMI of sheep decreased by 16.2 g/BW/day.

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1 Introduction

Sheep are isothermal animals and adapted to ambient temperature conditions. In Vietnam, since the 18th century, sheep were raised in the Southern Central Region and the practice continues until now. Marai et al. (2007) stated that when the ambient temperature is going down, sheep needs more energy to maintain their body temperature and increase their food intake. Previous studies showed that sheep dry matter intake (DMI) was significantly reduced when they suffered heat stress (Savage et al. 2008; Alhidary et al. 2012; Tadesse et al. 2019). According to Marai et al. (2007) and Tadesse et al. (2019), the free grazing time regime also has a significant effect on the DMI of sheep. When it experienced cool temperature (from 7:00 AM to 11:00 AM) the DMI reached a higher level and when the temperature rose from 11:00 to 19:00 it is going down. The influence of seasons on DMI and digestibility of sheep was also reported by various researchers (Kamalzadeh et al. 1997; Li et al. 2012; Goetsch and Johnson1999). The lower DMI of sheep in summer or dry season and the warm temperature was found to be lower than that in autumn (Goetsch and Johnson 1999) as compared to cold season or cold environment (Li et al. 2012). Food intake was not only affected by ambient temperature but it is also affected by humidity. Therefore, many researchers have been proposed to study the effect of the temperature-humidity index (THI) to assess the heat stress of sheep (Paim et al. 2012; Marai et al. 2009; McManus et al. 2008).

In recent years Phan Rang sheep have been raised on large scale in the Thua Thien Hue province. Climate difference between the two provinces does influence the sheep husbandry practices due to the corresponding impact on their food intake. Statistics showed that the weather indexes in Thua Thien Hue and Ninh Thuan provinces differ markedly, in which the annual rainfall in Thua Thien Hue is much higher than that of Ninh Thuan (3,877 vs. 1,687 mm/year), the air temperature is lower (24.7^{o}C compared to 27.4^{o}C), and the air humidity is higher (87.3% vs. 79%). However, no study has been carried out on the effect of climate on sheep husbandry performance in this area. Therefore, this experiment aimed to identify the influence of temperature and humidity on the food intake of Phan Rang sheep raised in Thua Thien Hue, and recommend suitable solutions for sheep farmers to better the efficiency in livestock production.

2 Materials and methodology

2.1 Study sites and animal samples

The experiment was conducted on 12 Phan Rang sheep (males and females) with the three age groups ranging from 6, 9 to12 months (4 sheep in each age group) bought from Ninh Thuan province and nurtured at Thuy, Livestock Research Center, University of Agriculture and Forestry, Hue University, Thua Thien Hue province. Before the experiment, Helmin and worms were removed from sheep by orally administering with 12 mg/1 kg BW albendazole (Han-Dertil-B, Hanvet).

2.2 Feeds, feeding, and composition

Sheep were fed with daily collected natural grasses, dried at 60^{o}C for 72 hours, and daily left over's were also collected and weighted to calculate daily dry matter intake

As per the National Research Council US (1981) the dietary composition of nature grass is as follows: DM = 20.5%; OM = 87.9%; CP% = 10.5; NDF = 60.1% and Total of Energy (3742 Kcal/kg, 12.1 % of Ash).

2.3 Housing and management

Sheep were numbered for tracking and captured individually in each cage whose dimension was 0.8 x1.5 m. All cages were placed on floors leveled approximately 0.2 m higher than the ground, with holes in the floor for manure collection. The barn has enough windows to ensure ventilation systems on top. In each cage, there is a trough for roughage, a trough for fine food, and drinking water. All sheep were kept in cages throughout the examination time and only left for free grazing once at weekends when no data is collected.

2.4 Data and variables

For collecting the data regarding the barn's temperature and humidity of the study area an automatic Hygro - Thermometer (France) was used at the following time points: 1:00; 4:00; 7:00; 10:00; 13:00; 16:00; 19:00 and 22:00 on consecutive days in both seasons. The hygro-thermometer was placed at a height similar to that of an adult sheep, which is 0.8 m from the ground and 0.6 m from the barn floor. The daily recording of THI at different times of the day during the experiment was calculated using the formula of Marai et al. (2000): THI = T^{o}C – [(0,31 – 0,31*RH/100)(T^{o}C - 14,4)], in which T ^{o}C; air temperature ^{o}C; RH: air humidity (%).

Daily food intake was collected at 5 meals (7:00, 9:00, 13:00, 16:00, and 21:00 h) and the amount of food to be fed was 3% (DM) of the sheep weight; while daily left over's were also collected and weighted to calculate daily dry matter intake. Sheep were weighed at the beginning of each stage and the average weight in each stage, which served to calculate the intake per mass weight.

2.5 Data analysis

Data were recorded by Microsoft Excel and processed using descriptive statistics and analysis of variance (ANOVA) through

the model (GLM) on Minitab version 15.10 (2010). Nonlinear regression analysis was carried out by using the following quadratic equation: Y = ax2 + bx + c; where Y is the amount of food intake (g DM/kg BW); x: is temperature or THI, according to the method by Tadesse et al. (2019.

3 Results

3.1 Variables (temperature, humidity, and THI)

The results of the study showed the variation in temperature, humidity, and THI at 8 timelines of the day (1:00, 4:00, 7:00, 10:00, 13:00, 16:00, 19:00, and 22:00, consecutively) during two seasons i.e. hot season (HS) and cold season (CS) and are illustrated in Figure 1. Results of the study revealed that the temperature and THI of the cages in the cold and hot seasons tended to fluctuate according to the general rule, which is "the lowest value was reported from 1 to 4 o'clock and increased gradually later on". These parameters reached the maximum at 13 o'clock, and then gradually decreased until 22 o'clock. Further, trends of humidity changes are antipodes with temperature and THI, and it reached the peak from 1 to 4 o'clock and decreased gradually, later on, these results correspond with Tadesse et al. (2019). The temperature was lowest between 22:00 and 4:00 of the following day, with an average of 21.1^{o}C in CS and 27.9^{o}C in HS. The highest temperature was at 13 o'clock and at this time, the average temperature was reported to be 26.70℃ in the cold season and 35℃ in the hot season. The difference in temperature during the cold season was greater than in the hot season, which was realized in the early morning at 7 o'clock with a temperature of 9^{o}C of the hot season, and 13:00 o'clock of the cold season. Results of three hotter months (June to August) of the summer season showed that the amount of food intake has been changed by the time (Table 1). Further, food intake frequency results of cold seasons 3 months (from December to February of the following year) also showed that there is an effect of temperature and humidity food intake (Table 2). An average of THI also indicated different values between hot and cold season and a food intake (Table 3), and suggested the impact of temperature stress on the sheep food intake.

Table 1 Fluctuation of the temperature and humidity in the hot season (n= Fn)

Temperature (^{0}C) \ Humidity (%)	< 50	50 - 60	61 - 70	71 - 75	76 - 80	81 - 90	91 - 100	Total
24						2		2
25					1	4		5
26					3	14	5	22
27				6	13	31		50
28			6	8	29	60		103
29			9	27	49	20		105
30			13	21	19	8		61
31		1	29	9	4	3		46
32		6	24	5	2			37
33	1	14	39	1				55
34	3	18	12	3	3			39
35	4	41	14	2	1			62
36	7	18						25
37	9	7	1					17
38								
39			1					1
40								
Total	24	105	148	82	124	142	5	630

Fn = Number of appeared frequency in amount changes

Table 2 Fluctuation of the temperature and humidity in the hot season (n= Fn)

Humidity (%)		< 50	50 - 60	61 - 70	71 - 75	76 - 80	81 - 90	91 - 100	Total
Temperature (^{0}C)	16							1	1
	17						3	13	16
	18						5	19	24
	19						2	34	36
	20						5	33	38
	21						19	25	44
	22			1	2	2	19	31	55
	23				2	1	22	27	52
	24			2	1	3	22	10	38
	25				1	5	22	12	40
	26			3		5	19	1	28
	27			2	2	9	7		20
	28			2	4	1	3		10
	29			4	7	1			12
	30		1	6	1		1		9
	31			5	1				6
	32		2	3					5
	33		4	4					8
	34	1	1						2
	Total	1	8	32	21	27	149	206	444

Fn = Number of appeared frequency in amount changes

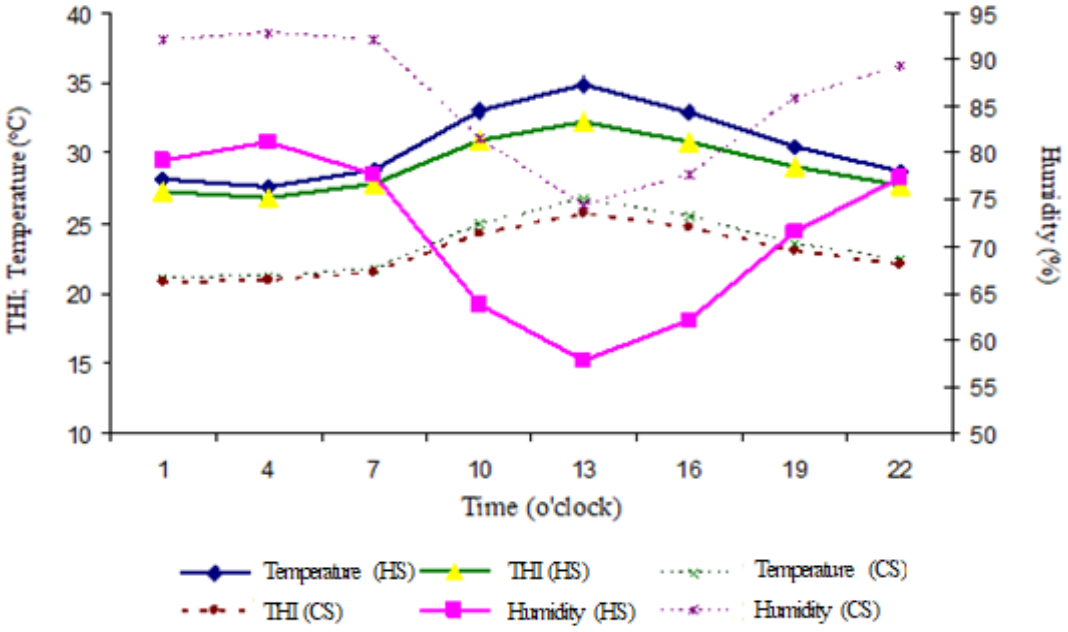

Figure 1 Variation in temperature, humidity, and THI in seasons

Table 3 Frequency of THI occurrence at predetermined hours daily in the hot and cold seasons (n = Fn)

Hour in a day	Hot season				Cold season			
	≤ 22.2	22.3 – 25.6	25.7 – 28.5	> 28.5	≤ 22.2	22.3 – 25.6	25.7 – 28.5	> 28.5
1		5	75	4	43	16	1	-
4		8	73	-	40	19	1	-
7		2	69	16	34	20	6	-
10		-	9	78	14	20	19	7
13		-	-	83	11	20	17	12
16		1	6	73	18	17	18	7
19		1	25	58	28	20	11	1
22		1	63	18	31	23	6	-
Total		18	320	330	219	155	79	27

Fn = Number of appeared frequency in amount changes

3.2 Relationship between temperature and feed intake

The relationship between the temperature and food intake of sheep have been represented in Figure 2, this suggested a correlation between the amount of food intake and the barn temperature, as follows: Y1 = -0,0874x12 + 3,0284x1 + 23,861 (R2 = 0.81; P = 0.001), where Y1: amount of food intake (gDM/kg BW/day); x1: temperature (0C). According to Tadesse et al. (2019), the R2 value is more important than the P-value when using regression analysis. When calculating the amount of food intake of sheep at every 0.5°C temperature difference, it was reported that when the temperature was equal to or higher than 22.5°C, the average amount of food intake of sheep was 49.3 gDM/kg BW/day (100%). With the increase of temperature, the amount of food intake of sheep tended to decrease, but not much. Further, when the temperature was in the range of 22.5°C to 29.5°C, the amount of food intake of sheep decreased approximately 9.2 gDM/BW/day, and overall 18.7% reductions were reported as compared to equal to or lower than 22.5°C. Remarkably, when the temperature increased >29.5°C, the food intake dropped by approximately 11.6 gDM/BW/day and showed a decrease of 23.5% compared to the temperature ≤ 22.5°C (P<0.05) (Table 4).

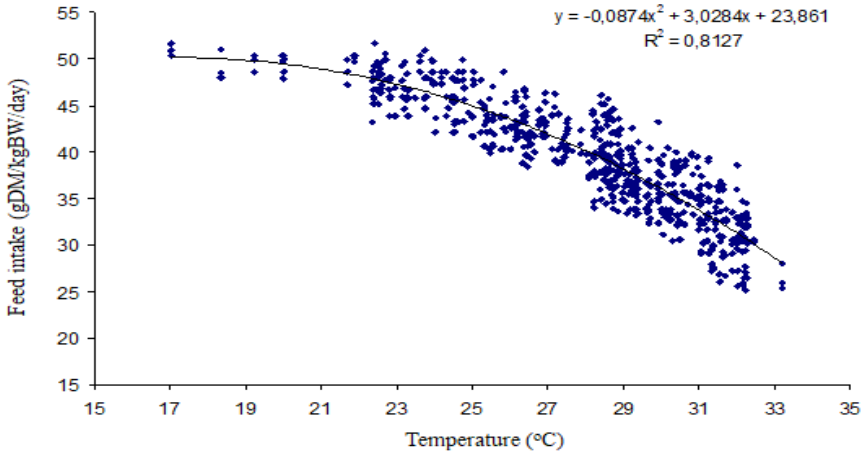

Figure 2 The relationship between barn temperature and food intake of sheep

Table 4 Temperature milestones affecting feed intake

Temperature (°C)	Food intake (gDM/BW/day)	
	Fluctuation range	M ± SEM
≤ 22.5	47.6 – 51.0	49.3^{a*} ± 0.98
22.6 – 26.3	42.7 – 47.4	45.2^{b} ± 0.85
26.4 – 29.5	36.8 – 42.9	40.1^{c} ± 0.98
> 29.5	26.4 – 36.1	37.7^{d} ± 0.91

*Data with different exponents in the same column are significantly different (P<0.05)

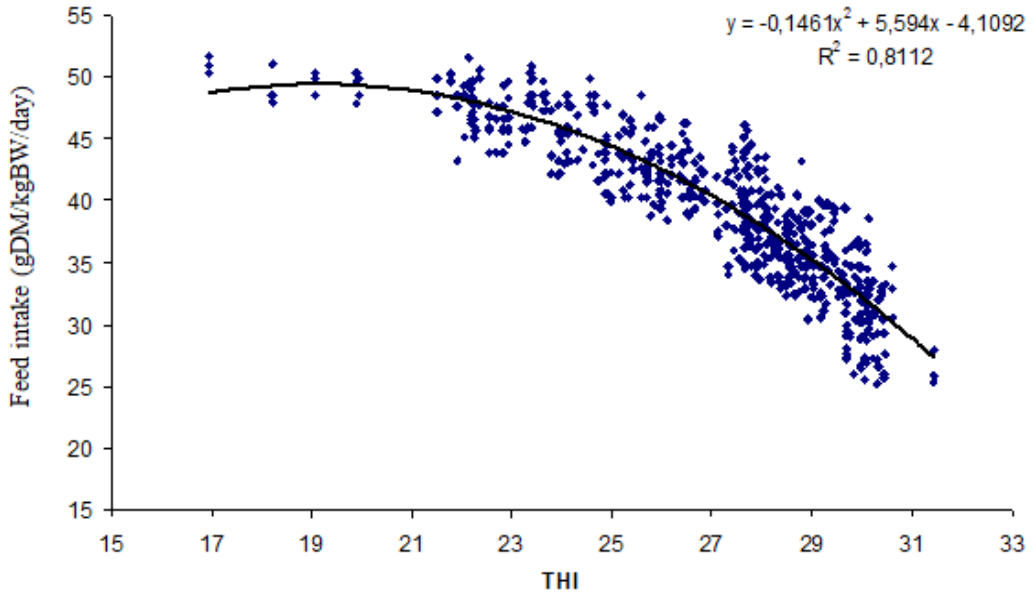

Figure 3 The relationship between THI cage and food intake of sheep

Table 5 THI milestones affecting feed intake

THI	Food intake (g DM/BW/day)	
	Fluctuation range	M ± SE
≤ 22.5	48.0 – 51.0	49.11^{a*} ± 0.75
22.6 – 26.3	46.1 – 47.7	46.78^{ab} ± 1.15
26.4 – 29.5	43.6 – 47.6	45.15^{b} ± 0.81
> 29.5	39.3 – 42.6	41.09^{c} ± 0.81
≤ 22.5	26.4 – 36.0	32.27^{d} ± 0.81

*Data with different exponents in the same column are significantly different (P < 0.05)

3.3 Relationship between THI and feed intake

The relationship between the THI and food intake of sheep is illustrated in Figure 3 and it showed a correlation between THI and food intake of sheep as the following equation: Y2 = -0,1461x22 + 5,594x2 - 4,1092; R2 = 0,81; P = 0,001; where Y2: the amount of food intake (g DM/kg BW/day); x2: THI.

The calculation of the THI value showed that when the THI was ≤ 22.2°C, the average food intake of sheep was 49.11 gDM/kg BW/day (100 %). Further, when the THI was > 22.2 - 25.6°C, the amount of food intake of sheep was not significantly different (P > 0.05) but with the increase to temperature from 25.6 - 28.5°C increased the rate of THI which slightly decreased the rate of sheep food intake (approximately 8.02 gDM/BW/day), it was showing overall 16.3 %, reduction as compared to the THI at ≤22.2°C. Similarly, when the temperature range between >28.5 - 31.5°C, THI was also increased and the food intake of sheep decreased remarkably, with an average reduction of 16.84 gDM/BW/day and it was approximately 34.3% less as compared to THI ≤22.2°C (P< 0.05) (Table 5). Further, with the increase of THI value from>28.5 - 31.5°C and for every increase of 1°C, the food intake of sheep decreased by 6.1 - 15.2 g/DM/BW/day (compared to THI ≤ 22.2°C). Specifically, when THI increased from 28.5 - 29.5°C, 29.5 - 30.5°C, and 30.5 - 31.5°C, food intake decreased by 11.4, 17.6, and 19.6 gDM/BW/day respectively.

4 Discussions

According to Srikandakumar et al. (2003), at a temperature of 32°C and 65% of humidity, sheep started to suffer from heat stress. Under conditions of high ambient humidity and low

temperature, the mechanism of heat loss through the skin does not work, so the sheep must increase the respiratory rate to expel heat (Srikandakumar et al. 2003; McManus et al. 2008; Alhidary et al. 2012). Thus, it is noteworthy that in hot seasons, although the temperature is high, the frequency of occurrence of high humidity levels accounts for a large proportion. Thua Thien Hue Province, where the air contains a large amount of water vapor, is one of the regions with the highest air humidity in Vietnam. In the cold seasons, high air humidity (> 80 %) accounted for a large proportion (79.95 % of the total measuring hours) and was predominantly associated with low air temperatures (< 27 0C). High air humidity combined with low air temperature can cause cold stress in sheep. Thus, in the cold seasons, high humidity (> 80 %) accounted for a large proportion. In winter, Thua Thien Hue province experiences prolonged drizzles and northeast monsoons causing quite high humidity.

When THI rises, body temperature (Srikandakumar et al. 2003; Marai et al. 2007; 2009; Alhidary et al. 2012), respiratory rate (Srikandakumar et al. 2003; Marai et al. 2007; Savage et al. 2008; Alhidary et al. 2012), and skin temperature of the sheep found increases (Bhatta et al. 2005; McManus et al. 2008; Marai et al. 2009), and this temperature rise reduced the food intake (Marai et al. 2007; Savage et al. 2008; Alhidary et al. 2012). This point is worth noting when raising sheep in hot seasons in Thua Thien Hue, where high air humidity accounted for a large proportion of time in both hot and cold seasons. These results suggested that during the hot seasons when the temperature and the humidity were both reported high, it could cause stress in sheep. These weather features are very different from those of Ninh Thuan province and other localities across the country where sheep are raised (Table 3). At temperatures of >29.5 - 32.5°C, for every 1°C increase, the amount of food consumed by sheep decreased by 12.2 - 17.2 g/DM/BW/day. Specifically, when the temperature increased from 29.5 - 30.5, 30.5 - 31.5, and 31.5 - 32.5°C, the amount of food intake decreased by 12.2, 14.7, and 17.2 gDM/BW/day, respectively. The results of this study are consistent with previous studies by Ames and Brink (1977) and Keyserlingk and Mathison (1993).

According to Ames and Brink (1977), when the temperature was in the range of 5 – 30°C, the food intake of sheep did not change but when the temperature was < 5°C, the amount of food intake increased; and when the temperature was > 30°C, the amount of food intake decreased. Similarly, Keyserlingk and Mathison (1993) reported that when the temperature is in the range of 5 – 21°C, there was no influence on the food intake of sheep. According to Alhidary et al. (2012), when the environment temperature exceeded 30°C, heat stress occurred, resulting in a decreased DMI of sheep. The dry matter intake of Merino sheep (Australia) at room temperature of 16 – 24°C was 1008 g/day (3.33% BW) and it reduced 234 g/day (23.2 %) and reported 774 g/day (2.65 % BW) at 28 – 38°C (Alhidary et al. 2012). Similarly, Savage et al. (2008) reported that the DMI of sheep at room temperature of 20°C was 1,578 g/head/day (3% BW) and at a temperature of 30 – 40°C was decreased to 1,136 g/day (2.4% BW).

Other authors stated that when sheep expose a high ambient load heat, they will lose energy to maintain a stable body temperature, leading to an increased respiratory rate, water intake, and reduced food intake (Alhidary et al. 2012; Savage et al. 2008; Marai et al. 2007). However, sheep are less sensitive to heat stress than other cattle under the same environmental conditions. In addition, water shortages, unbalanced diets, and undernutrition conditions increase the heat stress in sheep (Marai et al. 2007). Studies have shown that the food intake of sheep is significantly reduced in high-temperature environments (Marai et al. 2007; Savage et al. 2008; Alhidary et al. 2012). Other studies have proven similar results, which showed higher food intake in autumn than in summer (Goetsch and Johnson 1999), higher food intake was reported in cold environments as compared to the warm environments (Li et al. 2012), and lower food intake was reported in the hot-humid and hot-dry environments as compared to the cold-dry and cold-humid environments (Guerrini 1981). According to Pluske et al. (2010), the environmental temperature during the year affects the amount of food intake of sheep. Thus, the results of this experiment are completely consistent with the above studies.

Conclusions and Recommendations

Temperature and THI were strongly correlated ($P < 0.05$) with the food intake. In the condition of temperature which was in the range of 29.5 - 32.5°C, for every 1°C increased, the sheep food intake decreased by 14.7 gDM/BW/day and the THI value was > 28.5. For every increase of 1°C value, the food intake of sheep decreased by 16.2 gDM/BW/day.

The natural conditions of Thua Thien Hue province (700 km north to Binh Thuan province) can be suitable for the application of sheep housing and farmers can raise these animals with adaptation to increase the efficiency of householders' income.

Ethics approval and consent to participate

All animals and samples were applied as per the international, national, regional, and institutional guidelines for animal care and rules in Vietnam. The approved animal slaughter license number is HU VN 0004.

Conflict of Interest

The authors report no conflict of interest

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Authors' contributions

All authors discussed and designed the experiments. Bui Van Loi, Nguyen Xuan Ba, Le Duc Ngoan, and Nguyen Quang Linh conducted the main experiments and data analysis. All authors wrote, read, and agreed on the final manuscript.

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Journal of Experimental Biology and Agricultural Sciences, February - 2022; Volume – 10(1) page 144 – 149

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Molecular Docking studies of Apigenin, Kaempferol, and Quercetin as potential target against spike receptor protein of SARS COV

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ABSTRACT

COVID-19 has been categorized as a pandemic in early 2020 and is known to cause by Severe Acute Respiratory Syndrome Coronavirus (SARS–CoV2). Numerous investigators and people in the scientific community are trying to find a superlative way to avert and cure the ailment by using phytochemicals. Abundant studies have revealed that flavonoids can be very operative in averting virus-mediated infection. The purpose of this study was to accomplish molecular docking studies among plant-derived flavonoids (Apigenin, Kaempferol, and Quercetin) and spike receptor (PDB ID: 2AJF) protein of coronavirus. Pyrx virtual screening tool and biovia discovery studio visualizer were utilized in the current molecular docking investigations. Outcomes of docking studies exposed that selected phytochemicals have interacted with targeted spike receptor protein with binding energies in the range of -6.3 to -7.3 kcal. In conclusion among the various selected ligands, quercetin may be a better inhibitor for the deactivation of SARS-Coronavirus.

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1 Introduction

In December 2019, the breakout of Corona-virus or COVID-19 in Wuhan was an awful disease that caused millions of deaths worldwide (Sood et al. 2020). Throughout history, this coronavirus has killed more human beings than any other infectious disease. In the early month of January 2020, COVID-19 had been characterized as a pandemic by the World Health Organization (WHO). According to the Report of WHO to date, there are 398,785,192 confirmed cases and 5,771,443 deaths in 206 Nations (WHO 2020). Corona-viruses are a diverse group of viruses that belongs to the family *coronaviridae*. They are composed of a long RNA strand. Their genome is the largest among all RNA viruses. They are named after the crown-like spikes present on their surface. The main infection is caused by Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) (Sood et al. 2021; Vashishth and Tehri 2020). SARS-CoV-2 Ibeta coronavirus belongs to the *coronaviridae* family, similar to Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV). It is one among the 36 coronaviruses in the family of *coronaviridae* within the order Nidovirals. Members of this family are mainly known to cause respiratory or intestinal diseases in various creatures including humans (Tuli et al. 2021a). Recent research suggests that natural plant-based compounds such as phytochemicals including flavonoids, alkaloids, and others may be useful in the development of safe SARS-CoV treatments (Talwar et al. 2020; Vardhan and Sahoo 2020; Silveira et al., 2020; Tuli et al. 2021a; Tuli et al. 2021b;). In the year 2020, Vardhan and Sahoo (2020) pursued in silico computational analysis of phytochemicals including glycyrrhizic acid, limonin, 7-deacetyl-7-benzoylgedunin, maslinic acid, corosolic acid, obacunone, and ursolic acid and suggested their utility as promising drugs to target proteins of SARS-CoV-2. Similarly, Hall and Ji (2020) explored the in-silico potential of Zanamivir, Indinavir, Saquinavir, and Remdesiviras proteinase inhibitors. In the present study, three molecules from the flavonoid class were chosen to investigate the binding affinity with spike protein of coronavirus.

2 Materials and methods

In this present study of in silico docking, we have used different online bioinformatics servers, databases, and tools that help us to study the docking interaction of flavonoids such as Apigenin, Kaempferol, and Quercetin against spike protein of SARS-CoV.

2.1 Software's Used in Docking interaction

We have used Pyrx virtual screening tool and Biovia discovery studio visualizer. PyRx is a free and open-source virtual screening tool. It is a mixture of numerous software like AutoDockVina, AutoDock 4.2, Mayavi, Open Babel, and others. PyRx includes a docking wizard with an easy-to-use user interface which makes it a valuable tool for Computer-Aided Drug Design. Biovia discovery studio is a software suite that enables life science researchers to analyze and model molecular structures, sequences, and other data. Vina and AutoDock 4.2 are the docking software used by PyRx. The software provides tools for displaying and editing data as well as performing simple data analysis. Discovery studio offers many tools for working with and visualizing data.

2.2 Retrieval of Three-Dimensional Structure

The Three-dimensional Structure of the SARS coronavirus spike receptor-binding domain with PDB ID:2AJF (Figure 1) was retrieved from the online database RCSB protein data bank and which was later on viewed in PyMol software. The energy minimization and optimization of the target molecule were studied in the Swiss Protein Databank Viewer. In the 3-D structure of PDB ID:2AJF, all the water molecules were removed which were not involved in ligand interaction, and all the missing atoms and valences were corrected (Rivas 2019).

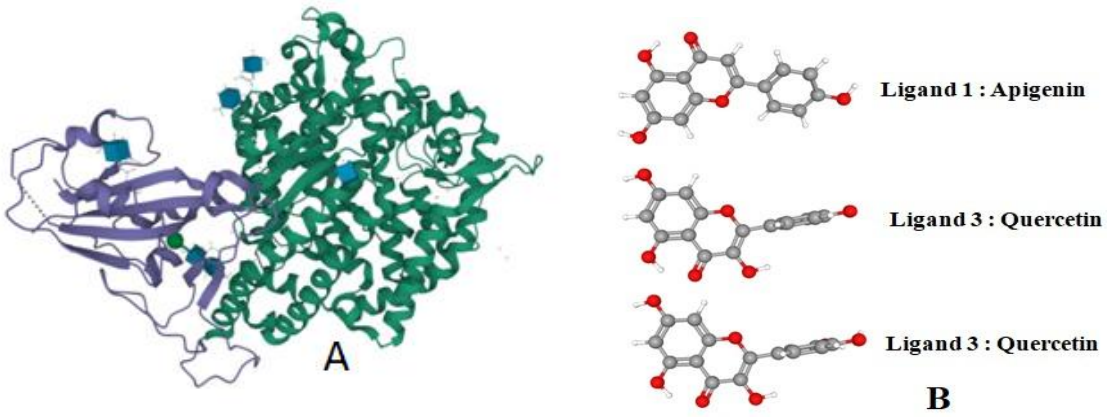


Figure 1 (A) Three-dimensional structure of SARS coronavirus spike receptor-binding domain with PDB ID:2AJF; (B) Ligands used in the study derived from PubChem Apigenin, Kaempferol and Quercetin

Table 1 Prediction of Molecular properties of Apigenin, Kaempferol and Quercetin by Molinspiration and 'Lipinski's rule of five

Properties	Ligands		
	Apigenin	Kaempferol	Quercetin
Molecular Weight	270.24	286.24 g/mol	302.23
XLogP3	1.7	2.46	1.5
Hydrogen Bond Donor Count	3	4	5
Hydrogen Bond Acceptor Count	5	6	7
Rotatable Bond Count	1	1	1
GPCR ligand	0.07	-0.10	-0.06
Ion channel modulator	-0.09	-0.21	-0.19
Kinase inhibitor	0.18	0.21	0.28
Enzyme Inhibitor	0.26	0.26	0.36
Protease inhibitor	-0.25	-0.27	-0.25
Nuclear receptor ligand	0.34	0.32	0.36
Topological Molecular polar surface area	90.89	111.13	131.35
Molar Refractivity	73.99	76.01	78.03

2.3 Selection and Preparation of Ligands and Prediction of Molecular Properties

The Ligands used in the study Apigenin, Kaempferol, and Quercetin are chosen from plants and herbs sources. All the three ligands were retrieved by PubChem (Figure 1) and saved in MOL SDF format and energy minimization, hydrogen bonds, and geometrical confirmations were done and modified in Marvin-Bean Package. The online tool Lipinski rule of 5 (Lipinski et al. 2001) and Molinspiration [www.molinspiration.com] were used for screening of identified ligands and it was found that all the three ligands were found to obey the Lipinski rule of 5. All the three ligands were used for the study that is shown in Table 1 with their physiochemical characteristics and Lipinski rule of 5 criteria.

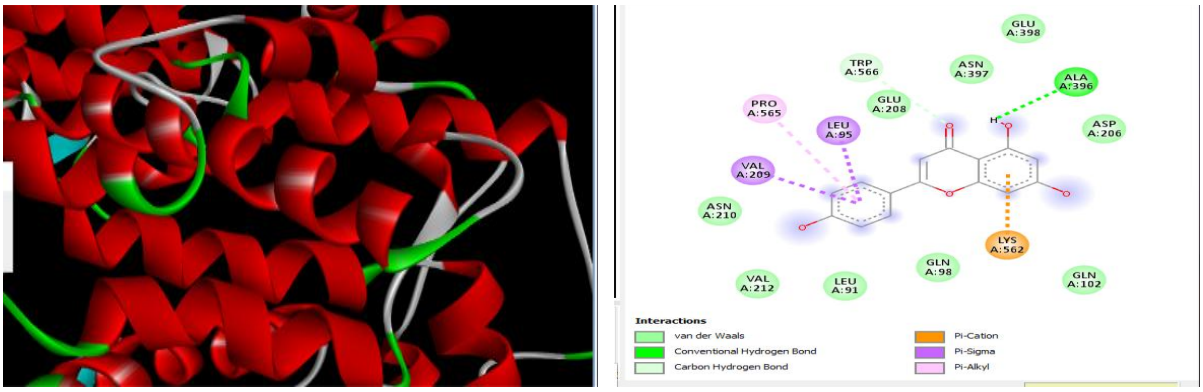
2.4 Molecular docking

The virtual screening is used to study the Ligand and Protein binding affinity along with the binding of the drug targets, protein receptors /or enzymes for interactions. We have used a free version of PyRx software to study molecular docking. All the default docking algorithms were used, and all the coordinates X, Y, and Z were set in the grids which were placed in the active site pocket center, and the lowest binding energies were the best suitable for interactions (Trott and Olson 2010)

3 Results and discussion

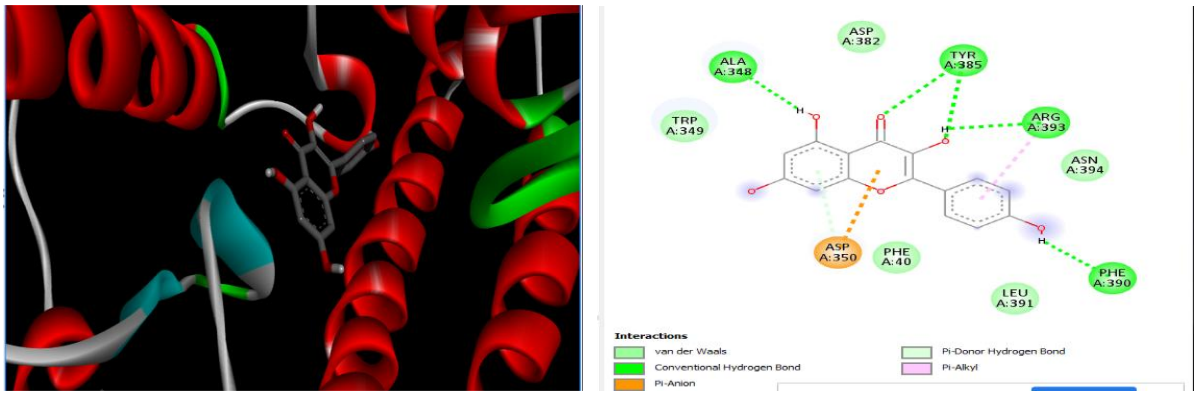
Previously, constructing and designing a drug was a lengthy, costly, and time-consuming process that took more than ten years and millions of dollars to complete. Computer aided drug design, also known as molecular docking, has become popular in recent years as a way to study protein-ligand interactions and properties such as hydrophobicity, binding energy, hydrogen bond donor-acceptor, geometry complementarily, and electron distribution. These interactions between ligands and receptors aid in the development of new medicines or therapies to treat deadly diseases (Tuli et al. 2020; Tuli et al. 2021c). The scientific community and pharmaceutical companies are concentrating on novel compounds with the aid of molecular docking techniques to speed up the drug development process. The root mean square deviation values (RMSD) were used to evaluate the docking results for the tested ligands with the receptor protein in this analysis. These RMSD values were based on the coordinates between the atoms and their conformational changes. The binding energy (kcal/mol) data enables us to investigate and compare the binding affinity of various ligands/compounds with their respective target receptor molecules. Binding energy represents the sum of total internal energy minus the energy which is linked to the unbound system. The lower binding energy indicates a higher affinity of the ligand for the receptor. In other words, Lower the binding energy the most favorable is docking results. The ligand with the highest affinity can be selected as a possible drug candidate for further research. The result of docking studies revealed that the ligands had good binding energy with the target molecule (Figure 2, 3, and 4) 2AJF, spike receptor protein of SARS-CoV, ranging from -6.3 to -7.3 kcal (Table 2, 3 and 4). Recently, Hagar et al. (2020) investigated the anti-covid activities of N-Heterocycles by using computational tools (Hagar et al. 2020). Similarly, several phytochemicals including nelfinavir, lopinavir,

kaempferol, quercetin, luteolin-7-glucoside, demethoxycurcumin, naringenin, apigenin-7-glucoside, oleuropein, curcumin, catechin, epicatechin-gallate, zingerol, gingerol, and allicin were investigated as anti-covid therapeutics by using in silico tools (Khaerunnisa et al. 2020). Terpenoids based molecules have also been utilized to study in silico molecular interactions against covid associated proteins. The results are acknowledged to have inhibitory actions of Ginkgolide A against novel COVID-19 protease.



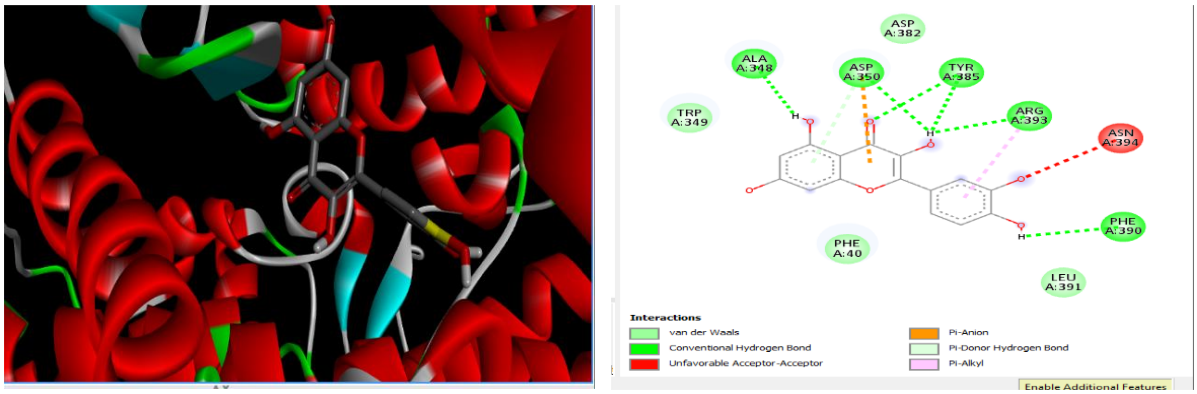
(A) (B)

Figure 2 Conformational changes observed due to the binding of Apigenin with PDB ID:2AJF (A) shows surface area interactions of Apigenin with receptor binding domain of SARS-CoV (B) is 2D interactions of Apigenin and receptor protein



(A) (B)

Figure 3 Confirmational changes observed due to the binding of Kaepmferol with PDB ID:2AJF (A) shows surface area interactions of Kaempferol with receptor binding domain of SARS-CoV (B) is 2D interactions of Kaempferol and receptor protein.



(A) (B)

Figure 4 Confirmational changes observed due to the binding of Quercetin with PDB ID:2AJF (A) shows surface area interactions of Quercetin with receptor binding domain of SARS-CoV (B) is 2D interactions of Quercetin and receptor protein.

Table 2 Interactions of Apigenin with receptor protein and values of binding energy and RMSD

Ligand	Binding Affinity	rmsd/ub	rmsd/lb
2ajf_5280443_uff_E=233.26	-7.3	0	0
2ajf_5280443_uff_E=233.26	-7.2	6.891	3.559
2ajf_5280443_uff_E=233.26	-6.9	13.973	12.242
2ajf_5280443_uff_E=233.26	-6.8	20.256	17.001
2ajf_5280443_uff_E=233.26	-6.8	16.906	14.701
2ajf_5280443_uff_E=233.26	-6.8	15.177	14.141
2ajf_5280443_uff_E=233.26	-6.7	16.785	14.446
2ajf_5280443_uff_E=233.26	-6.7	14.581	12.753
2ajf_5280443_uff_E=233.26	-6.6	32.537	29.924

Table 3 Interactions of kaempferol with receptor protein and values of binding energy and RMSD

Ligand	Binding Affinity	rmsd/ub	rmsd/lb
2ajf_5280863_uff_E=362.50	-7	0	0
2ajf_5280863_uff_E=362.50	-6.9	6.928	4.355
2ajf_5280863_uff_E=362.50	-6.8	6.472	5.494
2ajf_5280863_uff_E=362.50	-6.7	6.376	3.474
2ajf_5280863_uff_E=362.50	-6.7	7.519	6.346
2ajf_5280863_uff_E=362.50	-6.6	15.16	14.177
2ajf_5280863_uff_E=362.50	-6.6	6.56	2.158
2ajf_5280863_uff_E=362.50	-6.5	39.152	37.253
2ajf_5280863_uff_E=362.50	-6.5	7.298	5.063

Table 4 Interactions of quercetin with receptor protein and values of binding energy and RMSD

Ligand	Binding Affinity	rmsd/ub	rmsd/lb
2ajf_5280343_uff_E=380.43	-7.1	0	0
2ajf_5280343_uff_E=380.43	-7	7.378	3.781
2ajf_5280343_uff_E=380.43	-6.9	26.609	22.918
2ajf_5280343_uff_E=380.43	-6.9	14.91	14.03
2ajf_5280343_uff_E=380.43	-6.8	44.95	44.112
2ajf_5280343_uff_E=380.43	-6.7	6.041	3.612
2ajf_5280343_uff_E=380.43	-6.7	7.4	3.615
2ajf_5280343_uff_E=380.43	-6.7	35.398	32.864
2ajf_5280343_uff_E=380.43	-6.6	6.445	3.707

Conclusions

Natural flavonoids were verified *in silico* for their possible interactions with spike receptor protein of COVID by using molecular docking tools to validate their drug-like candidature. The study revealed that quercetin was a better inhibitor for the inactivation of SARS-Coronavirus and should be pursued as a promising drug candidate for this virus. Further in vitro and clinical studies are needed to design effective COVID-19 drugs if the high efficacy function of quercetin is taken into account.

Conflict of interest

The authors declare no potential conflict of interest.

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Pterocarpus angolensis: Botanical, Chemical and Pharmacological Review of an Endangered Medicinal Plant of India

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ABSTRACT

Herbal products for primary health care are gaining huge interests of the people and the various healthcare professionals. This is mainly because of the local availability and cost-effectiveness of plant remedies over expensive modern treatments. *Pterocarpus angolensis*, a deciduous plant belonging to the family of Fabaceae is mainly found in the tropical regions of Africa. This tree is rich in medicinal properties which are immensely used by the locals in Africa for the treatment of ringworm infections, ulcers, urinary schistosomiasis, skin injury, etc. The extracts of *P. angolensis* are treasured in Africa for their effectiveness against many diseases like gonorrhea, mouth diseases, diarrhea, etc. It is reported to have inhibitory activity against various pathogens like E*scherichia coli, Staphylococcus aureus,* and *Salmonella typhimurium* because of the high concentration of bioactive compounds like flavonoids, tannins, and other phenolic compounds in the bark and leaves of the tree. Various research papers demonstrated the polar and nonpolar constituents of this plant showing antimicrobial, anti-plasmodial activities against *Streptococcus agalactiae, Candida krusei,* etc. In India, very few of these plants have been reported to be alive in the Darjeeling district, West Bengal. But, lack of proper documentation or research paper led to negligence related to the importance of this species and it has already been listed in the IUCN Red List of threatened species. The main objective of this review is to spread awareness about the conservation of the plant possessing such remarkable properties. Secondly, to provide an overview of the phytochemical screening of various important medicinal constituents that this plant possesses and this might lead to change in the field of modern medicine.

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1 Introduction

Human beings have been known to use plants as medicine for many health related problems since the pre-historical period. This practice forms the origin of much of modern medicine. Many drugs which are used even today are of plant origin. Quinine (from cinchona bark), morphine (from the opium or poppy seeds), aspirin (from willow bark) are well known examples (Vickers et al. 2001). There has been a huge increase in the consumption of herbal products in the last three to four decades. According to WHO's report published in 2010 about three fourth of the world, the population depends on herbal products for their day-to-day health care (WHO 2010). Firstly, the cost of the present-day hospital treatments is too costly as compared to traditional medicines (Maroyi 2013). Secondly, resistance to drugs has led people to resort to more affordable traditional medicine. Lack of efficacy of modern-day treatment is reported by The World Health Organization (Brand Features 2018). Moreover, scarcity of medicines in developing countries has also compelled people to depend on herbal medicines (WHO 2013). Herbal supplements have been used to treat various ailments such as asthma, rheumatoid arthritis, migraine, gastroenteritis, and cancer (Maroyi 2013).

P. angolensis commonly known as the Blood wood tree is a deciduous tree belonging to the family Fabaceae, which is mostly found in southern and eastern Africa. The sap of the tree resembles blood and hence the name Bloodwood. The tree is famous for its quality timber (Mojeremane and Lumbile 2016). Moreover the sticky deep red or maroon-colored sap is used as a dye for fabric. Also, medicinal values of treating several ailments like ringworm, ulcers, and urinary schistosomiasis (Chipinga et al. 2018; Ndamba et al. 1994), antibacterial and antifungal properties of this plant are well documented. Hence exploitation of the tree for above mentioned medicinal as well as a commercial utility has already made it marked as a "decreasing" species in the IUCN Red-List of threatened species (Barstow and Timberlake 2018).

On Indian soil, *P.angolensis* is known as Ram Supari. It has been reported to be alive in the Darjeeling district of West Bengal but is surprisingly few. Therefore, the tree needs to be conserved immediately. Because of the lack of proper documentation of ethnobotanical and pharmacological aspects of this plant in India, the importance of this plant has been completely neglected to lead to the impending extinction of this species from India.

Considering the above facts, this review provides an overview of the botanical, chemical, and pharmacological aspects of *P.angolensis*. It presents the various beneficial properties of the plant in the field of medicine as well as its commercial value. The main aim of this review is to provide an overview of the phytoconstituents that the plant possesses and to spread awareness about the conservation of the plant with such significant medicinal properties.

2 Botanical Aspect

P.angolensis is a deciduous tree having a slightly flat crown with a high canopy. It reaches up to 16 m high and reaches 28 m under favorable conditions. They have straight stems that are occasionally swollen at the base (Mojeremane 2016). The leaves exhibit alternate arrangements and are drooping in nature (Takawira-Nyenya et al. 2005). The plant has sweet-scented, orange-yellow flowers that have distinctive round pods surrounded by brown and papery wings (Mojeremane 2016) which are pear-shaped and are bisexual (Takawira-Nyenya et al. 2005). Very few stems in the younger age classes are present currently. The reason behind this is the exacerbation by loggers to supply timber for commercial purposes (Stahle et al. 1999). This indicates that it is under threat of extinction and therefore calls for immediate measures to conserve it.

3 Chemical or Phytochemical Aspect

The family of Leguminosae has been substantially investigated and various secondary metabolites with a wide range of diversity in their chemical structures have been reported to date (Ramawat and Mérillon 2008). Phytochemical investigations of different parts of *P.angolensis* have revealed the presence of many phytochemicals such as flavonoids, isoflavonoids, pterocarpans, triterpenes, epicatechins, deoxybenzoin, and chalcones (Abouelela et al. 2019) (Table 1). The non-polar extracts of such plants are mainly composed of essential oils and other bioactive compounds. These are obtained from different plant parts such as flowers, bark, wood, twigs, leaves, and roots(Abubakar and Majinda 2016). Preliminary screening of the extracts of the plant from various parts revealed the presence of a high concentration of tannins in the bark, saponins are found in the root as well as bark. Researchers have confirmed phytochemicals such as tannins, flavonoids, terpenes are mainly found in the leaves (Chipinga et al. 2018).

3.1 Non-polar Profile

Nonpolar (*n*-hexane and chloroform) extraction of the stem bark followed by GC-MS analysis is performed to analyze the different metabolites. Identification is done by comparing the acquired spectra with the existing NIST library (National Institute of Standards and Technology). The structures of the phytochemical are elucidated using NMR (Nuclear Magnetic Resonance) spectroscopy. GC-MS (Gas Chromatography-Mass Spectroscopy) analysis of the chemical constituents from the n-hexane and chloroform extracts reveals the presence of glycosides, ketone, saturated and unsaturated fatty acids, alcohols, and sterols. Ten volatile phytoconstituents, viz. tetratriacontane 11, n-hexadecanoic

Table 1 List of compounds isolated from *P. angolensis*

NOS.	COMPOUND NAME	SOURCE (PLANT PART)	REFERENCES
1.	Liquiritigenin	Wood	Maurya et al. 1984
2.	Prunetin	Wood	Seshadri 1972
3.	Muningin	Wood	Bezuidenhoudt 1987
4.	Epicatechin-3-O-galate	Stem Bark	Noufou et al. 2012
5.	Epicatechin(4b-8)-epicatechin(B2)	Stem Bark	Samie et al. 2009
6.	(-)-Epicatechin	Bark	Bezuidenhoudt 1987
7.	Hexamer of Epicatechin	Stem Bark	Samie et al 2009
8.	Isoliquiritigenin	Wood	Seshadri 1972
9.	α,2'-Dihydroxy-4,4'-di-methoxy chalcone	Wood	Bezuidenhoudt 1981
10.	(-)-Pterocarpin	Wood	Shah 1975
11.	Angolensin	Wood	Seshadri 1972
12.	α (S)-4-O-methylangolensin	Wood	Bezuidenhoudt 1980
13.	(αR,1"R,4"S,4"αR,8"αR)-4-O-α-cardinylangolensin	Wood	Bezuidenhoudt 1980
14.	(αR,1"R,4"S,4"αR,8"αR)-4-O-T-cardinylangolensin	Wood	Bezuidenhoudt 1980

acid 1, 7-dehydrodiosgenin 12, stigmasta-3,5-dien-7-one 14 , lupeol 13, octadecanoic acid 10, friedelan-3-one 15, hexadecanoic acid, methyl ester 9 and tetradecanoic acid 8 are reported to be the most abundant in the n-hexane extract of *P. angolensis*. While from the chloroform extract 1-octacosanol 16 was reported to be present (Abubakar and Majinda 2016).

3.2 Polar Profile

Phenolic compounds are found to be universal in almost all plants as secondary metabolites. Various researchers have estimated the different phenolic compounds in *P. angolensis* using Folin-Ciocalteu's reagent. Mainly phenolic compounds are present in the water extract of the plant. However, some amounts of these compounds are also present in methanolic extracts. Leaves of *P.angolensis* are reported to have more polyphenols as compared to barks (Santos et al. 2020). Further analysis of the phenolic profile by HPLC-DAD (High-Performance Liquid Chromatography with Diode Array Detection) verified the presence of gallic acid, caffeic acid, and taxifolin in the crude extracts and fractions of *P.angolensis* but not in abundance. The presence of hexadecanoic acid and lup-20(29)-en-3β-ol has been revealed in the GC-MS analysis of a methanolic extract of the leaves (Santos et al. 2020). The thick bark of the plant that secretes blood-red sap is found to be rich in organic compounds such as tannin (76.7%) and mining when damaged (Mojeremane 2016).

4 Pharmacological Aspect

Many researchers have revealed that the various parts of the plants such as the bark, roots, flowers, sap, and seeds have different medicinal values and are used to treat various ailments. The red sap rich in various organic compounds is to stop nose bleeding. It is also efficient in killing ringworms and curing ulcers. Several studies have reported that the sap is used in treating eye cataracts, malaria, skin inflammations, and urinary schistosomiasis. Cold infusion from the bark is used as a remedy for rashes as well as to relieve headaches, mouth ulcers, corneal ulcers, and various stomach-related disorders (Mojeremane 2016). The seeds are crushed to form powders that are used as a wound dressing (Takawira-Nyenya et al. 2005) to speed up healing. Apart from the above-mentioned benefits of the plant, various researches have revealed the surprising pharmacological properties of the plant.

4.1 Antiplasmodial Activity

In a study, some researchers have explored the efficacy of the *P. angolensis* extract to inhibit the survival of the malarial parasite *Plasmodium falciparum*. Although malaria is a treatable disease, the growth of parasites resistant to antimalarial drugs is growing day by day (WHO 2017a & b).

Heat shock proteins (Hsps) are universal chaperone molecules that facilitate protein folding. Hsp70 family of chaperones has been reported to be expressed by the malarial parasite. The Hsp70s show a low basal ATPase activity (Kampinga and Craig 2010). Hsp 70s in their ATP-bound state, exhibit low affinity for substrates, which results in the release of the substrate. It expresses six members of the Hsp 70 family out of which two of them are located in the cytosol-PfHsp70-1 and PfHsp70-z (Shonhai et al. 2007). These two Hsps are thought to function towards facilitating protein folding for the survival of the parasite. It has been studied

that the maintenance of protein quality is important for the survival of the parasite as about 24 percent of the parasite proteome is made up of arginine repeats that tend to aggregate under stressful conditions that the parasite encounters during its life cycle (Singh et al. 2004). So the two proteins have been considered as potential targets to inhibit the survival of the parasite.

Growth inhibition assays were conducted using *P .falciparum* 3D7 that were maintained at the blood stage. Chloroquine was used for the validation of the antimicrobial sensitivity of *P. falciparum.* It was used as a positive control. *P. falciparum* 3D7 grown in absence of extracts was used as a negative control. The treatment of parasite cultures with *P.angolensis* extracts resulted in the inhibition of the growth of the parasite. They concluded that *P.angolensis* plants contain certain compounds which interfere with ATP binding by Hsp70 or do not allow the chaperones to bind the substrates. The result from this study has interestingly revealed that 50% ethyl acetate and 50% hexane fraction extract of *P.angolensis* exhibits potent antiplasmodial activity. However, the exact phytochemicals in that inhibited the parasite survival is unknown and further can be investigated (Zininga et al. 2017).

4.2 Anti-inflammatory Activity

In various diseases, including venereal diseases, inflammation becomes a prime risk factor and sometimes leads to various complications in the treatment (Mulaudzi et al. 2013). Among the different phenolic compounds, flavonoid has been reported to have the maximum anti-inflammatory property (Cushnie et al. 2005). The extracts of this deciduous tree are treasured for their anti-inflammatory property due to their high concentration of tannins and flavonoids (Chipinga et al. 2018; Cai et al. 2019). In a study, the scientist aimed to evaluate the anti-inflammatory property of the extracts of *P. angolensis*. They evaluated the anti-inflammatory activity against cyclooxygenase (COX-1 and COX-2) enzymes. They reported that the dichloromethane and petroleum ether extracts of the *P.angolensis* bark exhibited good anti-inflammatory activity in both COX-1 and COX-2 assays (Mulaudzi et al. 2013).

4.3 Antimicrobial Activity

Various scientists have reported that the extracts of *P.angolensis* shows anti-inflammatory activity and also high inhibitory activity against various bacteria such as *Staphylococcus aureus, Escherechia coli,* and *Salmonnella typhimurium* (Luseba et al. 2007). On further research, scientists have confirmed the anti-fungal property of the plant. They analyzed the phytochemicals present in the plant parts and evaluated the effectiveness against pathogenic bacteria such as *Staphylococcus aureus, Streptococcus agalactiae,* and fungus *Candida krusei.* Minimum inhibitory concentration (MIC), minimum bactericidal concentration/minimum fungicidal concentration (MBC/MFC) of crude extracts against pathogens was determined. They confirmed that the extracts exhibited antimicrobial activity. The antimicrobial activity varied in accordance to part of the plant used, the polarity of the bioactive compound, and concentration. They confirmed that more antimicrobial activity was exhibited by methanolic and dichloromethane extracts as compared to aqueous extracts (Chipinga et al. 2018). Various studies have revealed that the chloroform extracts *P. angolensis* stem bark showed activity against *Bacillus subtilis* with MIC of 50 µg (Abubakar and Majinda 2016).

Scientists have extracted and demonstrated the structures of seven different compounds from the ethanol extract of *P.angolensis* mainly phthalate and epicatechin and its derivatives. These compounds were tested against *S .aureus* and *Entamoeba histolytica*. The presence of epicatechin and derivatives in the stem bark extract revealed strong antibacterial activities against *S.aureus* but weak activities against *E.histolytica* (Samie et al. 2009).

Researchers have revealed that results phloem sap has bioactive compounds that possess antimicrobial activity of the plant against *S. aureus, Escherichia coli,* and *Pseudomonas aeruginosa*. The antimicrobial efficacy of the phloem sap was evaluated using the agar diffusion technique. They used tryptone – soya agar medium and impregnated it with the test organisms. However, the results obtained were proved to be negative as there was no formation of the clear zone for any of the test organisms. Further, they conducted the GC-MS analysis of the phloem saps and identified various compounds from the GC profile. The compounds identified are lactic acid, glycerol, phosphoric acid, succinate, D-ribofuranose, 3, 4-dihydroxybenzoic acid, silane, and D-fructose (van der Riet et al. 1998). However, with regards to lactic acid, it was found that, when used in combination with some other compounds such as sodium or potassium sorbate, it showed antimicrobial activity against the gram-negative test organism (Kim and Hearnsberger 1994)

Campylobacter sp. has been listed on the World Health Organization (WHO) list of global priority pathogens for research and development of new antibiotics (WHO 2017a; WHO 2017b). They belong to the groups of zoonotic bacteria which are the leading causes of human bacterial gastroenteritis (Cheng and Fischer 2018). Researchers have revealed the efficacy of *P.angolensis* extract and expressed the antibacterial activity as minimal inhibitory concentration (MIC) by the broth microdilution method. They confirmed that the ethyl acetate extract of the bark of the plant showed MICs of 90μg/ml (Hlashwayo et al. 2020).

4.4 Antioxidant Activity

The various reactive oxygen species induce oxidative stress which may result in DNA as well as protein damage that can lead to

aging and diseases such as cancer. Various studies have revealed the antioxidant property of *P.angolensis* that helps in scavenging the free radicals, thus inhibiting oxidative stress. The crude extract and the different fractions of *P.angolensis* were evaluated by the researchers to investigate the different phenolic compounds. Techniques such as UV-Visible and FT-IR (Fourier Transform Infrared Spectroscopy) were used to analyze the spectroscopic characteristics of the extract fractions. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and reducing power assays were used to assess the antioxidant efficacy. The results obtained showed that the fraction containing a low phenolic concentration displayed the highest DPPH radical scavenging activity while the fraction with high phenolic content exhibited the highest reducing power activity. Spectroscopic and IR spectroscopy details obtained by them revealed both the fractions from the stem bark are antioxidant rich fractions (Traoré et al. 2017).

4.5 Wound Healing activity

Healing of wounds occurs by the reconstruction of injured skin by the involvement of various cells (epithelial and mesenchymal cells) and molecules (cytokines and growth factors). The natural bioactive compounds extracted from the plants have always revealed wound healing activity from ancient times by causing the proliferation of fibroblasts and keratinocytes. Plant extracts contain growth factors and various cell adhesion molecules (Talekar et al. 2017)

Various studies have revealed the wound healing potential of *P.angolensis* and the researchers have confirmed that the methanolic extract of bark and leaf of the plant showed wound healing activity and clearly showed the decrease in size of the injuries (Santos et al. 2020).

Conclusion

This review presents an overview of the different phytochemical compositions and bioactivities of *P.angolensis*. As described; the plant exhibits versatile benefits such as anti-plasmodial activity; wound healing, antioxidant activity, anti-bacterial and anti-fungal activity, and many more. Firstly, as no research or proper documentation has been done with this plant in India to date, the detailed phytochemical screening of plant extract and various benefits presented in the review will enable us to systematically explore the possible medicinal impact of this plant in Indian perspective. Secondly, the objective of this review is to spread awareness about the conservation of the plant possessing such remarkable properties which can usher in a paradigm shift in the practice of medicine and encourage the people to cultivate and propagate such a threatened species and in protecting this valuable resource from extinction.

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Conflict of interest

All the authors declare that there is no conflict of interest.

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Utilization of Agro Waste for Beneficial Product Formulation

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ABSTRACT

In the past few years, we have been very familiar with the waste hierarchy concept of the 3 R's, Reduce, Reuse and Recycle. This review article aims to suggest a possible way to reuse the agro-waste sector. It will focus on the zero waste food industry. While consuming our day-to-day food unknowingly we throw away some of the important portions of fruits and vegetables which can help us fight diseases and stay healthy. Therefore, we need proper management to utilize these beneficial components present in those fruit scrapes. An abundant amount of food waste is been produced during the processing of food from the different food industries. In addition to this, agro wastes like peels, seeds, etc. are also generated from fruit and vegetable agriculture. This paper mainly focuses on the agro-waste of the food industry, which can be consumed when the bioactive compound is extracted and is available as a functional food. The bioactive compounds have the potential to control blood pressure, diabetes, inflammation, etc. Thus, by incorporating these bioactive compounds we can enhance the quality of food. Recently functional food is consumed by a large population for its beneficial effect on our body.

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1 Introduction

Agricultural wastes or agro wastes refer to the waste products produced directly from agricultural activities throughout the processing as well as the growing of raw agricultural harvests such as vegetables, flowers, fruits, crops, etc. These agricultural wastes eventually contain various materials that are beneficial to humans. Numerous studies have already revealed that various types of agro-waste like peels, husks, etc. of fruits possess natural antimicrobial properties (Sadh et al. 2018). The increasing practice of agricultural production generated a large number of agricultural crop residues, by-products, etc. In accordance with the increasing farming system in developing countries, the production of agricultural waste is also increasing (Obi et al. 2016). These huge amounts of agricultural wastes and biomass produced from agriculture-based industries were burnt or naturally transformed into organic compost in the previous days (Upadhyay and Harshwardhan 2017).

1.1 Global Scenario of waste generated from Food and By-products

Annually throughout the world, a huge number of by-products and food wastes were generated from food industries and various other sources during food processing, storage, transportation, etc (Helkar et al. 2016). These agro wastes like trimmings, bagasse, peels, stems, shells, bran, seeds, damaged fruits, etc. possess a greater nutritional value in comparison to the actual final product (Torres et al. 2018). Nowadays a significant amount of by-products and wastes are produced as a result of the increasing demand for minimally processed fruits (Torres et al. 2018). These by-products and food wastes contain several valuable components like polysaccharides, fats, proteins, flavor compounds, fibers, phytochemicals, and bioactive composite which are valuable to health (Helkar et al. 2016). Therefore, the initiative has been taken to minimize the costs associated with their disposal and ensure their sustenance in the environment as these residues and by-products have added nutritional value in them (Lima et al. 2018). By proper utilization of these by-products and making functional food ingredients and functional food products, sustainable and stable economic growth of food industries is possible with increment in income (Helkar et al. 2016).

1.2 India's hunger and malnutrition

One of the major causes of malnutrition in India is economic inequality. The diet of people below the poverty line is poor in both quality and quantity. The problem of hunger and malnutrition arises not from the non-availability of food or agro product but unawareness of various agro by-product utilization as well as the inaccessibility of the available food. Therefore, we require alternative nutritional sources to accomplish these nutritional

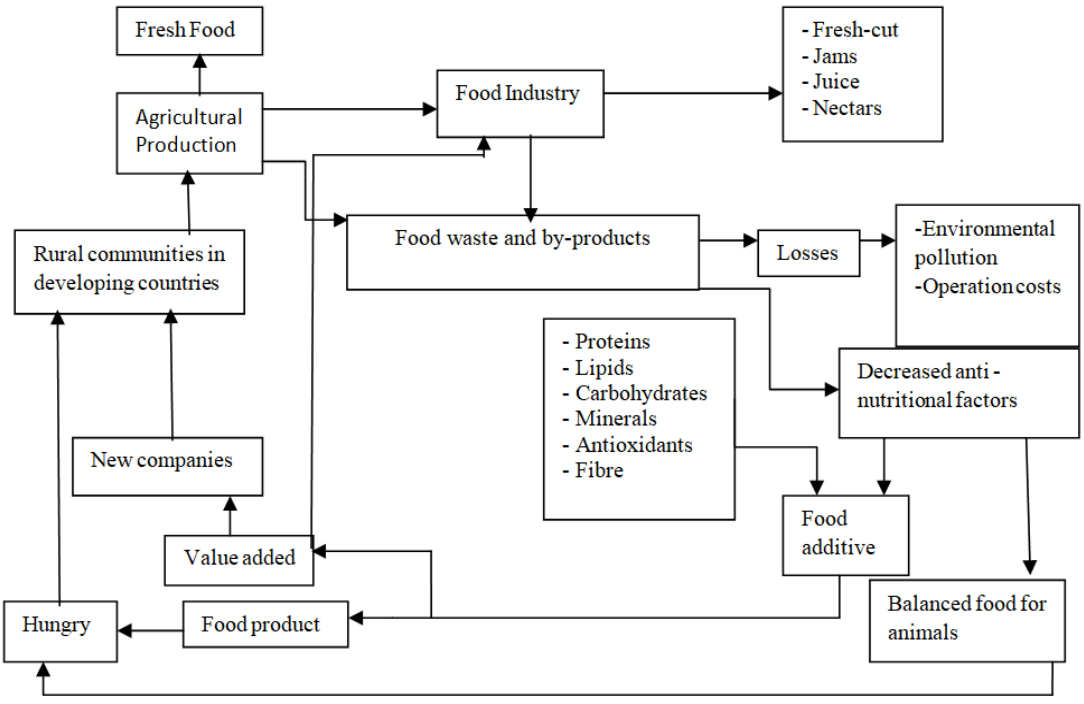

Figure 1 Hypothetical illustration of the utilization of food waste generated by-products which can lead to significant minimization of hunger (Torres et al. 2018).

problems of society. Food wastes and by-products generated from food industries are enriching in proteins, bioactive compounds, lipids, starch, micronutrients, and dietary fibers, etc. In developing countries, a large amount of agro wastes and by-products remain produced (Torres et al. 2018). To mitigate the problem of protein deficiency and associated malnutrition in any developing country (Helkar et al. 2016) like India, it is high time to focus on these agro wastes and turn these huge potential by-products or agro wastes into wealth. During industrial processing of several fruits (eg-pineapple), it has been observed that most of the fruit is discarded as residue. The high quantity of waste produced from fruits and vegetables during their processing as residues are then processed to extract its beneficial components (Sagar et al. 2018). Proper utilization of industrial by-products, which can serve as raw material for the functional food industry, can improve severe health conditions, reduce nutritional problems, can provide an economic advantage to the industry, and can minimize the factors contributing to the mismanagement of waste (Helkar et al. 2016). The conceptual diagram of the utilization of food waste and by-products is represented in Figure 1.

2 Agro waste produced from Industries

Among the numerous sources of agro-waste producers, the food industry plays a pivotal role. Some important fruits and vegetables used in processing in developing countries are tropical and sub-tropical fruits like banana, mango, grape, etc., and vegetable crops tomato, pumpkin, etc.

2.1 Fruit Industry

2.1.1 Banana (*Musa acuminata*)

According to the total production (56.4 million metric tons), banana ranks fourth behind rice, corn, milk. It is used broadly because of its nutritional importance. Banana is cultivated in over 122 countries worldwide (Ehiowemwenguan et al. 2014). In past, there are many studies show that parts of bananas can be utilized to treat various diseases. The agro-waste part of the banana is its peel that has antibacterial activity against microorganisms (Kapadia et al. 2017). The peel and pulp of fully ripe bananas possess antifungal (prevent fungus disease in tomato plants) as well as antibiotic principles (against *Mycobacterium*) (Ehiowemwenguan et al. 2014). Many enzymes like polyphenol oxidase and gelling agent pectin are present in a banana skin. The extract of banana peel is also used individually or in combination in cream or ointment, helping to mitigate pain, itching, and swelling (Chabuck et al. 2013). Dopamine, norepinephrine, serotonin are found in the ripe peel and pulp. Dopamine and norepinephrine help to increase blood pressure whereas serotonin stimulates intestinal smooth muscle (Ehiowemwenguan et al. 2014).

2.1.2 Mango (*Mangifera indica*)

Mango is the most abundant tropical seasonal fruit consumed both in fresh or processed form throughout the world. About 25% of its production is used extensively to make puree, pickles, jam, jelly, juice, etc (Liu et al. 2017). Skins of mango are the most important by-products of mango processing. The by-products of mango have been efficiently utilized. A significant amount of phytochemicals, hemicellulose, cellulose, pectin, protein, lipids, polyphenols, carotenoids, etc. are present in mango waste, therefore they can be used in the production of nutraceuticals as well as a functional food (El-Faham et al. 2016).

2.1.3 Grape (*Vitis vinifera*)

The grape is widely cultivated because of its high nutritional content, good taste, and economically significance in making juice, jam, wine, and raisins (Nile et al. 2013) and for other multipurpose use (Shah et al. 2019). Almost 75% of the production is being used for manufacturing wine. The wine industry generates solid waste like peels, seeds, and stalks which contribute up to 20% of the total biomass (Amorim et al. 2019). The phytochemicals extracted from the juice, seed, skin of grapes are carotenoids, melatonin, phenolics, flavonoids, stilbenes. These phytochemicals have potential antioxidant, anti-apoptotic, anti-inflammatory, anticancer as well as antimicrobial activity. These compounds also possess cardioprotective, hepato-protective effects (Yang and Xiao 2013). Phytochemicals such as tannins, epicatechin, anthocyanins, catechin, flavonols, flavan-3-ols, epigallocatechin, gallocatechins, and epicatechin gallate, proanthocyanidins (typically hexamers), or procyanidins obtained from grapefruit have been reported for their bioactive properties. Acylated procyanidins, an ester of gallic acid was obtained from grape seeds extract (GSE) (Imran et al. 2017)

2.1.4 Pineapple (*Ananas comosus*)

Pineapples are an economically significant fruit worldwide. Large amounts of solid as well as liquid wastes are produced from the pineapple canning industry that causing disposal problems (Li et al. 2014). Researchers have made efforts to process these wastes to minimize the problems associated with disposal and to ensure their sustenance (Lima et al. 2018). The peel waste generated from pineapple cannery can serve as a well off source of various bioactive compounds that bears antioxidant activities (Table 1). Bromelain extracted from fruit, stem, peel of pineapples (Li et al. 2014) is a mixture of various enzymes like endopeptidases, glycoprotein, glycosidase, carbohydrates, cellulases, protease inhibitors (Pavan et al. 2012). The glycoprotein (proteolytic enzyme) is the primary ingredient in crude bromelain, which is present along with other insoluble materials, such as organic acids, minerals, colored pigments, an inhibitor of protease, and organic solvents (Rathnavelu et al. 2016). Due to its various biochemical

Table 1 Antioxidant activity of pineapple

	Water content(% w/w)	Ethanol (% v/v)	Yield (% w/w)	Antioxidant activity(mg.ml-1)	Total Sugar (%w/w)
Fresh PPW	85.5	0	5.16	0.2 ± 0.009	3.69
		15	6.48	0.4 ± 0.004	7.51
		35	6.57	0.4 ± 0.003	7.16
		55	6.94	0.5 ± 0.020	7.40
		75	5.70	0.6 ± 0.040	6.23
		95	4.18	0.6 ± 0.001	5.65
Dried PPW	5.43	0	24.67	0.8 ± 0.050	ND
		15	30.95	1.3 ± 0.090	
		35	28.93	1.2 ± 0.001	
		55	45.38	0.9 ± 0.070	
		75	30.09	0.9 ± 0.070	
		95	30.37	1.2 ± 0.110	

ND= Not determined PPW= Pineapple peel waste (Saraswaty et al. 2013)

Table 2 Nutrient content of pomegranate peel (per 100g)

Composition	Content
Total solid	94.50
Water content	5.40
Entire sugar	17.70
Reducing sugars	4.34
Protein	4.90
Crude fibre	16.30
Fat content	1.26
Ash	3.40

(According to Khan et al. 2017)

and different pharmacological beneficial properties, this is used as a potential therapeutic agent.

2.1.5 Pomegranate (*Punica granatum*)

Pomegranates (*Punica granatum*) have a prominent medical history and possess remarkable medicinal properties (Bassiri-Jahromi 2018). The nutrient content of Pomegranate fruit peel, which is referred as the solid waste produced in a large quantity at the time of processing, is depicted in Table 2 (Fourati et al. 2019). The extracted and isolated bioactive compounds and by-products are rich in antioxidants of the polyphenolic class, which include tannins and flavonoids (Khan et al. 2017). Ellagitannins and anthocyanins are the bioactive compounds, which makes pomegranate an important fruit by protecting against inflammatory diseases and also have radical (free) scavenging activity (Sorrenti et al. 2019), anti-metastatic, anti-proliferative and anti-invasive, anticancer, antiviral effects as reported previously. Approximately every part of the pomegranate including the fruit juice, peel, flowers, arils, bark, phytochemicals has been tested for antimicrobial activities (Bassiri-Jahromi 2018). Pomegranate by-products, punicalagin can inhibit the growth of pathogenic *Escherichia coli*, *Staphylococcus aureus*, *Clostridium perfringens*, *Pseudomonas aeruginosa*, etc. Moreover, a significant increase was found in the population of useful bacteria including *Bifidobacterium* sp. and *Lactobacillus* sp.

2.2 Vegetable agriculture

Biological wastes are abundantly generated in a prominent amount from the agro-food industries as well as from vegetable agriculture.

2.2.1 Corn silk

Corn silk is the agro-waste produced from corn and is used as a medicine. The stigmas of the maize flowers (female) are referred as corn silk. Fresh CS looks like soft silk threads of 10-20 cm in

Table 3 Tocopherol and carotenoid analysis in various parts of pumpkin in *C. pepo* and *C. moschata*

Tocopherol and carotenoid	Part	*C. pepo*	*C. moschata*
α Tocopherol	Peel	4.45 ± 0.75	6.15 ± 2.19
	Flesh	1.40 ± 0.01	1.54 ± 0.99
	Seed	21.33 ± 3.36	25.74 ± 0.73
γ Tocopherol	Peel	0.66 ± 0.09	ND
	Flesh	ND	0.52 ± 0.01
	Seed	61.65 ± 17.66	66.84 ± 4.90
β carotenoid	Peel	39.48 ± 0.24	68.30 ± 2.02
	Flesh	1.48 ± 0.05	5.70 ± 1.50
	Seed	17.46 ± 18.29	7.15 ± 1.50
β cryptoxanthin	Peel	0.15 ± 0.02	0.13 ± 0.03
	Flesh	ND	ND
	Seed	0.16 ± 0.16	ND

ND- Not Determined (According to Rahman et al. 2019)

length and it can be light green or yellow-brown. The main constituents of CS are vitamins, carbohydrates, proteins, minerals (eg.-potassium, calcium), fiber, etc. (Guo et al. 2009). Corn silk tea has the potential to improve health conditions such as reducing edema, decreasing inflammation, improving obesity, lowering blood pressure, and therefore used for the treatment of hypertension (Shi et al. 2019).

2.2.2 Papaya (*Carica papaya*)

Carica papaya is also known as 'papaya' (Roshan et al. 2014) is referred to as the powerhouse of nutrients. Papaya generally requires one year to grow, which has made it an outstanding choice from a socioeconomic viewpoint in countries that produce it (Oliveira and Vitória et al. 2010). Papaya is rich in powerful antioxidants, vitamin B, vitamin E, vitamin C, vitamin A, fibre, magnesium (Aravind et al. 2013). Papaya or papaya seeds is used to treat male fertility. Papaya fruits contain lycopene (Alotaibi et al. 2017). The digestive enzyme-papaintha present in papaya is effective against trauma, allergies, sports injuries. The nutrient content of papaya helps to improve the cardiovascular system and prevent colon cancer (Aravind et al. 2013).

2.2.3 Tomato (*Solanum lycopersicum)*

Tomato (*Solanum lycopersicum*) is a widely eaten vegetable either in fresh or processed form and is included in the family Solanaceae (Isah et al. 2014). Phytonutrients, fibre, vitamin C, A, B complex, and 14 types of carotenoids are mainly present in tomato fruit. The redness of tomatoes is because of the occurrence of lycopene. The carotene present in tomatoes can impart orange coloration. The strong red color in tomatoes is an indication of the presence of a high quantity of lycopene. Several important components like phytoene, lycopene, gamma-carotene, neurosporene and phytofluene are derived from tomatoes. The antioxidant property of lycopene helps to mitigate prostate cancer as well as other cardiovascular diseases (Sidhu et al. 2017; Giovannucci et al. 2002).

2.2.4 Pumpkin (*Cucurbita spp*.)

Pumpkin (*Cucurbita spp*.) is coming from the family *Cucurbitaceae* and is cultivated and consumed throughout the world for its agricultural, commercial, and decorative uses (Amin et al. 2019). The agro-waste i.e., pumpkin seeds discarded at the time of processing has significant nutritional values. They provide an excellent source of protein and the best quality oil having a unique aromatic flavor (Devi et al. 2018). In addition, they are also good sources of minerals, mono-unsaturated fatty acids, dietary fibre, and health-benefiting vitamins. Decreasing sodium intake and consuming adequate potassium is important for treating high blood pressure (Rahman et al. 2019). Pumpkin seeds are generally consumed as a raw, cooked, or roasted form which is enriched with minerals mainly zinc, phosphorous, magnesium, potassium, and selenium. These compounds help the prevention of diseases such as arthritis, cancer, prostate, etc. Therefore, seeds are referred as regarded valuable for the food industry because of their valuable constituents like fibres, protein, minerals, phytosterols, fatty acids (Patel 2013). The antioxidants vitamin E, vitamin C, carotenoids, and tocopherol have therapeutic effects to improve eye health and prevent degenerative damage. The tocopherol and carotenoid content of pumpkin are shown in Table 3. A decrease in risk and progression of age-related macular degeneration has been shown with a higher intake (3 or more servings per day) (Rahman et al. 2019). Different pumpkin extracts containing antioxidant activity helps in the treatment of diabetics, vascular injury, etc. (Yadav et al. 2010).

2.2.5 Oyster Mushroom

During packaging, the ends of the mushroom are left out for cleaner packaging purposes. However, these thrown portions of mushroom contain an equal amount of vitamin D and active compounds as the body of the mushroom. They can be collected for more utilization of the compounds. In recent times, mushrooms are included efficiently in the normal human diet. Therefore, consumption of certain types of species increases. The genus

Pleurotus generally refers to as Oyster mushroom, comprises about 40 different species (Deepalakshmi and Mirunalini 2013). Some bioactive compounds like polysaccharides; polysaccharide-proteins; polysaccharopeptides; functional proteins such as pleuron glycoprotein, nebrodeolysin, ubiquinone-9 ubiquitin-like peptide; proteoglycans and lectin, glucans, etc are extracted from genus Pleurotus (Oloke and Adebayo 2015). Unsaturated fatty acids are present in mushroom fruiting bodies. One of the pivotal components responsible for the dry matter of mushrooms is protein however protein content of the sample can vary (Wani et al. 2010). It has low-fat content in comparison to protein and carbohydrates. It is reported that a small amount of vitamin C is present in mushrooms, the wild variety of mushrooms possess different types of vitamins (Ghosh et al. 2019).

3 Bioactive Compounds present in Food waste and its Disease Management

3.1 Norepinephrine

Norepinephrine, found in the banana peel is a bioactive compound. Noradrenaline (norepinephrine) is a neurotransmitter found in both the central nervous system and peripheral nervous systems i.e., in cell bodies of the pons and medulla (Sagar et al. 2018). The main function of noradrenaline is associated with the fight-or-flight response where the body is ready to react to an emergency. Depletion of norepinephrine resulted in depression, which is contributing to significant potential morbidity, mortality, suicide, medical illness, etc. (Moret and Briley 2011). Noradrenaline belongs to the catecholamine class is derived from phenylalanine and tyrosine. It serves as a drug for blood pressure, regulates flexor muscles, and is distributed in the medulla oblongata as well as in the dorsal vagal nucleus (Terbeck et al. 2016). Recent reviews have explored the role of the noradrenergic system in emotional memory (Tully and Bolshakov 2013).

3.2 Angiotensin

Angiotensin is extracted from corn silk. It is mentioned in folk medicine that corn silk tea is used for anti-hypertensive healthcare. Angiotensin-converting enzyme (AcE) helps to maintain homeostasis of blood pressure (Li et al. 2019). The first angiotensin-converting enzyme (AcE) inhibitor is Captopril; which drug is used widely in different types of cardiovascular (CV) diseases. Losartan was the first angiotensin receptor blocker (ArB). Both ArBs and AcE inhibitors are commonly used in patients with heart disease, hypertension, diabetes, chronic kidney disease, and coronary artery disease (Messerli et al. 2018).

3.3 Proanthocyanidins

From grape skin and seed, a broad class of potential bioactive compound or polymerized polyphenol component named Proanthocyanidin (PC) is isolated. PC is well known for its free radical scavenging activity (Jing et al. 2015) and thereby provides protection against different neurodegenerative diseases as well as cardiovascular diseases like atherosclerosis, restenosis, and heart failure following angioplasty. Oxidative stress is the primary step, due course of time, it develops various vasculopathies, neurodegenerative diseases (epilepsy, Parkinson's disease, Alzheimer's disease), cardiovascular diseases, etc. (Yang et al. 2018). Experimental evidence both in *in-vivo* and *in-vitro* models explored the beneficial antioxidant property of grape seed proanthocyanidins extract (Dixon et al. 2004).

3.4 Carotenoids

Carotenoids is a major bioactive compound of mango enriched with vitamin A. It is a fat-soluble pigment that consists of greater than 700 compounds that are required for the different coloration (red, orange, and yellow). Most carotenoids are chemically hydrocarbons that consist of 40 carbon atoms and two terminal rings (Mezzomo and Ferreira 2016). They are mostly derived from dark green leafy vegetables, colored fruits, etc (Gouado et al. 2007). Carotenoids help in quenching singlet oxygen, removing peroxy radicals, inhibiting cell proliferation, modulating carcinogen metabolism, immune response (Mezzomo and Ferreira 2016). Studies showed that Carotenoids are highly lipophilic molecules and perform as an antioxidant agent by inhibiting the incidence of diverse ROS-mediated disorders by scavenging ROS. By preventing the oxidation of membrane structures Lipoproteins, their major transporter mitigates the mortality (Fiedor and Burda 2014). The chemical structure of Carotenoids reveals functional conjugated double bonds, which are prone to be attacked by electrophilic compounds (Venkateswarlu and Reddy 2014). It is reported that with the increasing uptake of carotenoid-rich food, the risk of occurrence of different types of cancer, atherosclerosis, and other diseases is reduced (Fiedor and Burda 2014).

3.5 Papain

The papain is a natural enzyme (proteolytic) extracted from the latex of papaya's unripe fruits that digest protein and is used as a meat tenderizer, as digestive medicine, in pharmaceutical, tanning, and brewing, and in the manufacturing of chewing gum (Foda et al. 2016). Extensive proteolytic activity is shown towards proteins, polypeptide chains, amino acid esters, etc. Mostly the peptide bonds formed between the basic amino acids (eg. - lysine, arginine, histidine), leucine, glycine are cleaved by Papain (Olmoss 2012). The conditions of acidity for the optimum action of papain are found to be pH 10 (Frankel 1917). The cross-linkages of collagen which give stability to the collagen fibrils are hydrolyzed by Papain and thus they become weaker upon exposure to papain gel. In dental care, dentin papain-based gel is used as a useful bioactive

compound for the removal of chemomechanical dental caries. Sports injuries, allergies, etc can also be cured by using Papain (Olmoss 2012).

3.6 Tocopherol

The methyl-substituted derivatives of tocol are termed as 'Tocopherol'. Tocotrienols with an unsaturated isoprenoid sidechain and tocopherols with a saturated side chain are collectively called as tocols and are found in edible oils (Rizvi et al. 2014). Common salad oil is pumpkin seed oil. It is a rich dietary source of Tocopherol. Tocopherol is a class of organic compounds many of which have vitamin E. Pumpkin seed oil helps in the reduction of prostate size, lowering hypertension and hypercholesterolemia as suggested in some research (Stevenson et al. 2007). A diet rich in pumpkin seeds can lower the levels of different types of cancer. There are also potential health benefits to be gained from the different carotenoids pigments found in pumpkin seed oil (Dar et al. 2017). Cardiovascular functions are found to be improved by gamma tocopherol. The vessel-relaxing nitric oxide generated by the action of the enzyme nitric oxide synthase helps to improve endothelial function. Here tocopherol helps in this process by enhancing the function of nitric oxide synthase (Rizvi et al. 2014). The antioxidant potential of tocopherols plays a significantly important role in the therapeutic effects of pumpkin seed oil. The pumpkin seed oil in roasted form contains higher levels of alpha and gamma-tocopherol as compared to the roasted sunflower oil (Stevenson et al. 2007). Due to its free radical scavenging activity, it helps to reduce mutations, inhibit cancer cell progression by blocking the cell cycle process in different ways (Rizvi et al. 2014). Tocopherols and especially their oxidation product tocopheryl quinone helps in the inhibition of platelet aggregation so can be used as anticoagulants (Brigelius-Flohé et al. 2002).

3.7 Bromelain

Bromelain commercially obtained from the fruit or stem of the pineapple, it belongs to a protein-digesting enzyme that contains different thiol endopeptidases and other different components like peroxidase, escharase, phosphatase, etc. Bromelain possesses antithrombotic, anti-inflammatory activities (Pavan et al. 2012). Pineapple juice uptake helps in the reduction of blood cholesterol (Debnath et al. 2012). Bromelain has comprised of sulfhydryl proteolytic enzymes which have protein-digesting and milk-clotting and coagulating properties (Orsini 2007). Blood coagulation, inflammation, and certain types of the tumor may all be reduced by therapeutic doses of bromelain when taken as a dietary supplement (Joy 2010). It may also help to ease arthritis pain, heal the early stages of an injury, prevent and treat sports injuries, joint aches, tendonitis (Debnath et al. 2012).

3.8 Ellagitannins

Ellagitannins are comprised of a complex class of polyphenols characterized by one or more hexahydroxydiphenoyl (HHDP) moieties esterified with sugar, usually glucose (Klewicka et al. 2016). With over 500 compounds reported, they represent the largest group of tannins. They are easily labilized in solution. Upon hydrolysis with acid and/or base, their hexahydroxydiphenoyl (HHDP) group converted into the gallic acid dimeric derivative, ellagic acid after spontaneous rearrangement (Erukainure et al. 2018). The antimicrobial potential is exhibited by ellagitannins. In an instance, they inhibit the activity of methicillin-resistant *Staphylococcus aureus*. Ellagitannins and ellagic acid inhibit cancers of the mouth, breast, esophagus, etc., and reduce inflammation by reducing the level of pro-inflammatory cytokines IL-6 and increasing IL-10, anti-inflammatory cytokines which can hamper the initiation of carcinogenesis (Lipińska et al. 2014).

3.9 Lycopene

Lycopene is a red-colored predominant carotenoid pigment, phytochemical found in tomatoes and other red fruits that possess potential antioxidant activity (Alda et al. 2009). 2.5 times higher lycopene levels are present in the tomato skin than the pulp and its core. At the time of processing of tomatoes, most of the portion is lost as skin and seeds (Knoblich et al. 2005). Increased lycopene intake promotes the reduction of the incidence of prostate cancer (Elbadrawy and Sello 2011). Lycopene has several different cardiovascular beneficial effects, such as an anti-inflammatory (Mozos et al. 2018), antioxidative, cardioprotective, etc.

3.10 Lovastatin

Lovastatin is clinically useful as a potent anti-hypercholesterolemic agent. It can reduce serum cholesterol levels by inhibiting its synthesis or increasing the number of LDL (low-density lipoprotein) receptors and their catabolism and also slows the progression of atherosclerosis (Hunninghake et al. 1987; Schimmel et al. 1997). Lovastatin treatment had shown to the prevalence in patients with Alzheimer's disease. Upon injection with a high dose of lovastatin, the results revealed that the high dosage stimulated high bone formation both *in-vivo* and *in-vitro*, and also the injection of lovastatin at particular sites heal the femoral fractures and decreased the cortical fracture gap (Radha and Lakshmanan 2013).

4 Production of Functional Food by Utilizing Isolated Bioactive Compounds

All foods are considered as somewhat functional food as they are responsible for the taste, smell, and quality of food. Nowadays lots of experiments are performed to boost their inherent qualities as

Table 4 Functional food currently in the Indian market

Company name	Product description	Base product	Main components	Health claims
Yakult	Probiotic drink	Fermented milk	Skimmed milk, glucose-fructose syrup, live *Lactobacillus casei*	Helps in digestion and build immunity
Amul	Probiotic yogurt and drink	Yogurt	Probiotic yogurt or dahi	Improves intestinal microflora and aid better digestive health
Dabur	Chaywanprash	Jam	Amla, giloy, and more than 40 other natural ingredients	Strengthens body internal defense mechanism
Himalaya	Green tea Tulasi	Tea	Tulasi, black pepper, mint, cardamom, fennel, and ginger	Aids digestion and relieves flatulence
Nestle	Magi vegetable multigrain noodle	Noodle	Ragi, corn, jowar, and wheat	High protein content
Kellogg's	Kellogg's K	Cereal	Wheat, rice, oats, honey	Naturally cholesterol free, 8 essential vitamins, only 2% fat, high vitamin B complex
Britannia	High fibre biscuits	Biscuits	Wholesome wheat	Source of protein and fibre, low-fat food
ITC sun feast	Marie light	Biscuits	Enriched with natural wheat fibre with 0% trans-fat and 0% cholesterol	Low fat and high in fibre and protein
Fortune	Rice bran fortified oil	Oil	Tocopherols	Cholesterol-lowering potential, prevent or delays heart disease, cataracts, macular degeneration, prostate, and other cancer.

Source: Self-created based on a market survey by authors.

they may become helpful in improving some disease conditions (Hasler 2002).

Functional food can be (i) a natural food, (ii) a food with the added component, (iii) a food with the removed component, (iv) a food with modified components, (v) a food in which the bioavailability has been modified or (vi) any combination of these (Henry 2010). The demand for functional food is ever-growing, especially in the Indian market (Table 4) which is determined by concern about healthy diet, lifestyle, and increase in life expectancy and to enhance the quality of life. Hence, health plays an important role in the research according to the behavior of the consumer towards the functional food (Ali and Rahut 2019).

For a long period, we have overlooked the power of peel from fruits and vegetables. Researchers have found that peels, seeds, and other agro waste parts of fruits contain more percentage of bioactive compounds than the fruit. For proper utilization of these compounds, these can be extracted and added in conventional food like juice, puree, jam, tea, etc. to make them functional food and to boost their quality.

4.1 Norepinephrine

The neurotransmitter norepinephrine extracted from banana peel contains minerals, amino acids, proteins, glycoproteins, and antioxidants. During the processing, it is to be kept in mind to retain both natural norepinephrine and epinephrine in the extract as in Green tea. This helps to increase body thermogenesis. Intake of banana in the form of banana pure or the form of extract has health benefits in humans (Medasani 2013).

4.2 Carotenoid

Mango is the most demanded fruit in India and is also known as the king of fruits. Many industries produce jam, jellies, juice, pickles and generate a lot of mango peel waste too. As this fruit is high in carotenoid and antioxidants but the availability is only for a few months, therefore we can extract the bioactive compounds and put them in the products for making it an improved conventional food.

4.3 Angiotensin

Corn skill is a great source of angiotensin. Angiotensin is a bioactive compound, which has proved to treat cardiovascular diseases. Corn skill tea is very popular in hilly areas as it lowers blood pressure. We can transfer this medicinal property of corn skill into regular tea. If corn skill is added in tea bags then we can naturally cure patients with high blood pressure.

4.4 Proanthocyanidins

The wine industry produces a large amount of grape skin and seed waste. The peel of the grape is rich in proanthocyanidins, which is an antioxidant and reduces oxidative stress-associated diseases. If

this bioactive compound can be extracted and yet again be added to the wine, they can produce wines, which are beneficial for health and reduce cardiovascular disorder, neuron disorder. The proanthocyanidins can also be added to tetra-packed grape juice.

4.5 Papain

Papaya jam and papaya sauce are famous around the world for their extensive proteolytic activity. Papain is a bioactive compound, which is found in a large amount in the papaya skin. The enzyme is widely used to cure dental caries, sports injuries, and allergies. If papain is extracted and added in candies, such candies will be healthy for your teeth. Similarly, for quick recovery from sports injuries, sports person can eat candies for relief from pain.

4.6 Tocopherol

Tocopherol is found in pumpkin oil. Pumpkin oil is beneficial for hypertension, hypercholesterolemia, and arthritis. A good source of vitamin E is pumpkin oil. Pumpkin oil can be used as a dressing on salad as it will enhance the taste and is beneficial for health.

4.7 Bromelain

Bromelain extracted from pineapple is used as a medicine for pain, muscle soreness, and many other conditions as it has benefits on health. The pineapple juice and jam industry only used the pulp and the skin and stem is thrown away. The stem of a pineapple is rich in bromelain. If bromelain can be extracted and added to the pineapple jam and juice it will produce a functional refreshment.

4.8 Ellagitannins

Ellagitannins are found in the skin and seeds of pomegranate and possess antimicrobial activity against bacteria, fungus, viruses. The juice industry produces a lot of waste from pomegranate seeds. The solid waste can be used as refreshment pills as they help to fight against bacteria present inside the mouth.

4.9 Lycopene

Lycopene is a form of carotenoid. Tomato peel is rich in lycopene. Industries, which produce tomato sauce, create a lot of tomato peel waste. If lycopene is extracted and added to sauces like tomato, chili, soya, etc. then this sauce will be beneficial for health.

4.10 Lovastatin

Lovastatin is a drug, which is extracted from the oyster mushroom. In mushroom industries, the end of the stem (stipe) of the oyster mushroom is thrown away. The stipe contains enough amount of lovastatin as the cap (pileus) of the mushroom. If the waste can be collected and lovastatin is extracted, we can produce the low-cost drug for high blood pressure and cardiovascular disease.

5 Scenario of India's Population and Hunger

In the year 2015, the United Nations (UN) take the commitment to accomplishing zero hunger by 2030 which is regarded as an important step towards the Sustainable Development Goals (SDGs). This new commitment needs significant implications, demands a wide array of research besides the conventional analysis of energy intake, and takes into consideration all the necessary nutrients for proper nourishment (Ritchie 2018).

The increment in population was negligible before the year 1921 and therefore is considered as the 'Great Divide' in Indian demographic literature. In between 1911-1921, a marked reduction was observed in the population with an average growth rate of 1.2 percent between the years 1921-1951 and 2.2 percent per annum during the 30 years from 1951 to 1981. There was a threefold increase occurred in India's population in the last 90 years. India's estimated total population from 2014 to 2020 is 1,369 million. Due to overpopulation, comes the crises of food and employment. Foodgrain production increased to 174.4 million tonnes in 1991 from the early 50 million tonnes production in 1950-51. Table 5 represents an increase in population growth and grain production since 1961 (Bhagat 2000).

Table 5 Relation between the increase in population growth and grain production (India, 1961-91)

Year	Population (million)	Food production(million tonnes)	Per capita availability per annum (Kg)
1961	439.2	81.3	185
1971	547.9	103.5	188
1981	683.3	130.8	191
1991	844.3	174.7	207
2001	1027	204.5	199

Source: Census of India, 1971, 1981 and 1991.
Centre for Monitoring India's Economy. India's Agricultural Sector: A Compendium of statistics Mumbai. Economic Survey 2001-02 (Bhagat 2000)

Table 6 Benefits of functional foods to serve the malnutrition children

AGE (Year)	FUNCTIONAL FOOD	BIOACTIVE COMPOUNDS	HEALTH BENEFITS	RECOMMENDED AMOUNT OF INTAKE
0.5 – 1	Milk powder (Rich in vitamin A)	Carotenoid	Increases immune response Healthy eyesight	Once daily
0.5 - 1	Milk powder (Banana flavour)	Norepinephrine	Increase motor skills Increases communication skills Healthy neurons	Once daily
1 - 3	Cerelac (Rich in vitamin E)	Tocopherol	Strong bones Good muscle reflex	Twice daily
1 - 3	Custard (Pineapple flavour)	Bromelain	Increase immunity Quick healing effect	Once daily
1 - 3	Custard	Proanthocyanidins	Neural protection	Once daily
3 - 5	Cerelac	Carotenoid Norepinephrine	Healthy eye Increase motor skill	Twice daily
3 - 5	Biscuits	Tocopherol Lycopene	Increase motor skill Increase immune system	Twice daily

Source: Self-created based on a market survey by authors.

6 Hypothesis to Serve Malnutritional Children

An adequate, nutritious, and balanced diet is vital (Mishra 2012) to mitigate the problem of malnutrition. India right now has self-sufficient food grains. Therefore, there is no problem with limiting the food supply. The problem is due to the food sequestration as well as lack of proper provisioning. Sustainable food security demands (i) sufficient food availability, (ii) adequate resources, (iii) proper utilization of the available foods, adequate water, and good sanitation (Kumar and Kumar 2013). Yet a large number of children suffer from malnutrition. The relative price of food stuffs has remained unchanged over the past 20 years (Saxena 2018).

India has a population of 1.11 million contributing around 17 percent of the world's population. Due to this large population size hunger, malnutrition and food insecurity are evident in India (Acharya 2009). The higher child malnutrition rate in India is contributed by many factors. First, Indian women's nutrition, inappropriate feeding, and rearing practices for young children due to their low social status, the practice of marriage at an early age, low weight of females at the time of pregnancy, and poor education. Underweight women give birth to babies having low birth weight. They are more prone to malnutrition due to inappropriate caring, low social status, poor quality of consumed food, unhygienic lifestyle, bad medical facilities, and difficulty in obtaining food (Saxena 2018). Our hypothesis is to serve the malnutrition children between 6 months to 5 years shown in Table 6, by providing the bioactive compounds in baby food at a cost-effective price. As we can see, the mother is not well nourished so she cannot serve the baby. If our functional baby food can reach every door of nursing mothers then we can lower the amount of hunger in India.

7 Discussions and Conclusion

From various industrial activities, a lot of agro-waste and by-products are generated. For example, during the processing of raw agricultural products, residues or agro wastes are produced. The accumulation of these discarded agro wastes can lead to environmental pollution, which ultimately affects human health. However, by utilizing proper food industrial waste management techniques like "3 R", these wastes can be converted into useful ones. The by-products produced from agro-industries or food industries are considered as a good source of proteins, minerals, amino acids, and bioactive compounds, etc. In an instance, Norepinephrine extracted from the banana peel, carotenoid isolated from mango peel waste, angiotensin comes from corn silk, the drug lovastatin extracted from the waste part oyster mushroom, etc. are some important bioactive compounds that have antimicrobial, antioxidant, anti-carcinogenic, cardio-protective, etc. potential. Therefore, these agro wastes can serve as a raw material for the development of functional foods. By utilizing these by-products efficiently from the food industry, it helps to decrease the negative cost along with the reduction of the amount of environmental wastes and can directly affect the economy of the country. Proper utilization of industrial by-products is needed in developing counties like India to reduce the problem of hunger and malnutrition by increasing the availability of functional food. This can create a zero-waste food industry in the future.

Abbreviation

GSE - Grape Seeds Extract, CS - Corn Silk, PC – Proanthocyanidin, ND - Not Determined, PPW - Pineapple Peel Waste, AcE - Angiotensin-converting Enzyme, ArB - Angiotensin receptor Blocker, TNF - Tumour Necrosis Factor, HHDP – Hexahydroxydiphenoyl, ROS – Reactive Oxygen Species

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Impact of the non-biodegradable plastics and role of microbes in biotic degradation

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Management

ABSTRACT

Plastic is a group of elastic organic compounds whose definition has radically changed from being a large family of useful polymers to an indispensable part of life. We might say we are residing in the “era of plasticene”. If we simply pause and look around, we would realize that a majority of things in our daily life comprise plastic polymers. Currently, the international production of these polymers has spiked to around 300 million metric tons annually. Surprisingly about 50 percent of the products are discarded within a year of fabrication. Once discarded ‘outside’ they end up ‘somewhere’ and start exerting their disruptive consequences. Despite its enormous utility, it is now being increasingly known that these polymers are surely not without their downsides. Several steps are taken and even more, are being investigated so the mayhem of plastic doesn't prove for a "no pilot in cockpit" situation. Here we have conducted a review work of the available literature on various biological entities that can utilize plastic while at the same time focusing our attempts to assemble information regarding the probable enzymes that do it. We have also provided a report on the effect of different plastics on the ecosystem and the various management alternatives out there.

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1 Introduction

The plastic sector, among the quickest expanding industry, has begun its mass-scale production since the 1950s nevertheless its program in day to day life dates back a hundred years ago (Geyer et al. 2017). The property of elasticity of plastic has led to a marked increase in its usage in various daily life activities. As a result, its manufacturing volume right now has far exceeded 300 million tons per year. A major portion of the global plastic create comprises polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polyethylene terephthalate (PET), polystyrene (PS), polyurethane (PU), and nylon.

The marine ecosystem is one of the worst-hit environments by these polymers where they are gathered most. Especially in the five suburban gyres typically at their zone of convergence (Cózar et al. 2014). One of the most visible effects of marine plastic pollution is the formation of plastic islands in oceans and rivers, such as the Great Pacific Garbage Patch, frequently called a "gyre within a gyre"(Howell et al. 2012).

Due to oxidation, the surface of plastics develops micro-cracks and pits, which are broadly prone to fracturing due to humidity and temperature stress or scraping against sand particles generating a lot of microparticles. This phenomenon is often observed in UV introduced plastics particles such as high-density polyethylene, low-density polyethylene, or polypropylene (Andrady 2011).

A major drawback in petrochemical-derived plastic waste control is that it requires a long time to be degraded by abiotic variables such as UV or temperature and is very immune to absolute degradation by microbes due to its properties such as large molecular mass, absence of specific functional moieties for the enzymes to act upon, degree of crystalline nature and high hydrophobic nature (Urbanek et al. 2018).

Even though plastic polymers are difficult to degrade but certainly, their degradation is not impossible. Certain enzymes can make this possible. In general, molecules of large molecular mass cannot be hauled as such across the cell wall. They are divided up into smaller subunits that are more hydrophilic and then be degraded by intracellular metabolism (Shah et al. 2008). Therefore, many microbes secrete certain extracellular enzymes that can carry out this chopping activity which breaks down large hydrophobic polymers into smaller monomeric units. Consecutively, easy biotic degradation and mineralization followed by microbial assimilation (Gewert et al. 2015).

If somehow, we could enhance the potential of these enzymes (many of which are already isolated) and cost-effectively harness their activity, then we can come up with an instrument to degrade plastic efficiently. It is now increasingly realized that microorganisms can colonize almost all plastics that enter the marine earth. These colonizations are guided by the material properties and ecological variables like oxygen accessibility, levels of temperature, salinity level, and light exposure (De Tender et al. 2015).

As per a study conducted across the coastal Baltic stations, households of *Hyphomonadaceae* (largely the *Hyphomonas*) and *Erythrobacteraceae* (largely the *Erythrobacter*) which were known to metabolize Polycyclic Aromatic Hydrocarbons (PAH) were found in abundance colonizing the microplastics (Oberbeckmann et al. 2017).

2 Impact of microplastic

The microplastic travels far and wide along the ocean surface layer circulation driven by wind thereby, and its impacting places many kilometers away from the source of contamination (Thevenon et al. 2014). Various studies have shown the microplastics are amassing in a huge variety of marine organisms which range from zooplanktons and mussels (Caruso 2015), decapod lobsters such as *Nephrops norvegicus* (Murray and Cowie 2011), sea turtles such as *Chelonia Mydas* (Caron et al. 2018) to the large blossom Fin whale (Fossi et al. 2012) and various types of fishes where it may cause esophageal toxicity and pathology as detected with the experiment on adult medaka (Rochman et al. 2013). In 2013, in a study on short-tailed shearwaters, it was found that frequently Persistent Organic Pollutants (POP) are leached from ingested plastics to the cells of the bird ingesting it (Tanaka et al. 2013). This is of major concern because those organisms that have their daily diet exposed to a good deal of plastics are possibly under threat. Wilcox et al. (2018) attempted to establish a connection between the mortality of turtles and their ingestion of these kinds of plastics. They found that as low as 14 pieces of plastic in the gut of an animal could activate a 50 percent likelihood of its mortality.

In a nutshell, the disposed of compound fragments are usually hazardous as these xenobiotic compounds, once ingested by birds mistakenly may result in their death by interfering with the normal physiological processes like digestion. The reason can be due to blockage of the gastrointestinal tract (Ryan 1987). The disposed of plastic products can not only degrade to form smaller microplastics but also can break down further to even smaller nanoplastics of size ranging from 1nm to 1000nm based on a recent opinion. These nanoplastics because of their small size have a large surface area thereby having high absorption and adsorption properties (Gallo et al. 2018). Due to this, the nanoplastics could be expected to harbor more heavy metals contaminants and toxic organic chemicals compared to microplastics. In humans, both micro and nano variants of plastic debris can cause DNA damage, osteolysis, inflammation, apoptosis, necrosis of tissues, oxidative stress, and variations in gene expression and consequently in protein

expression (Lusher et al. 2017). A recent publication even reported the possible ingestion of microplastic clutter by jellyfish (Macali et al. 2018). Exploration of the effects of microplastic from the terrestrial background has resulted in the observation that it reduces the sorption capability of certain organic pollutants from the soil, thereby speeding up groundwater contamination (Metzelder et al. 2019).

3 Non-Biodegradable plastics, their effects and microbial arsenals for degradation

3.1 Nylon

Nylon, the multiple varieties of which are already available in the current market, are synthetic polyamides made from different reactions. There is the inherent polymerization of ω-amino acids, copolymerization of diacid and diamine, or the polymerization involving the ring-opening of the lactam that forms the nylon polymer. Nylon-6 and nylon-66 are the major members of the family sharing about 90% of its production worldwide. These are predominantly used in making fishing nets, ship hulls, disposable plastics, textile fibers, bristles for toothbrushes, and several other items. So clearly they would likewise be the major polluting variants of nylon. However, in the open environment, many rogue microbes have been discovered to exhibit some quantity of degradation of the polyamides or their replicating units say, for example, there are microbes like *Pseudomonas aeruginosa* that was discovered to degrade ε-caprolactam that is practically absolutely needed for the synthesis of nylon-6and was found to grow on the surface of the 6-Amino hexanoate cyclic dimer, a common by-product from nylon factories or *Flavobacterium* sp. K172 (IFO14590) was found to use up at least one percent of the same (Negoro 2000) or another microbe *Geobacillus thermocatenulatus* that has been discovered to degrade nylon 66 and nylon 12 (Tomita et al. 2003).

The breakdown of a cyclic dimer of 6-amino-hexanoate requires the assistance of three enzymes. All of these three might be very instrumental in degrading the polymer. The first is the 6-aminohexanoate-cyclic-dimer hydrolase (nylA), an amidase signature hydrolase having a catalytic triad of Ser^{174}- *cis*-Ser^{150}-Lys^{72} that was found to degrade the dimer into its monomeric units. It achieves this function by carrying out a nucleophilic attack of the serine in its catalytic site on the carbonyl moiety of the amide bond followed by the formation of an acyl-enzyme complex and finally, a two-step deacetylation process with the help of a water molecule, ultimately regenerating the functional enzyme for another round of catalysis (Yasuhira et al. 2010). The second enzyme is 6-aminohexanoate-dimer hydrolase, two-domain penicillin recognizing serine reactive hydrolase of 392 amino acid residues and has been reported to act via a nucleophilic attack of a serine residue at 112^{th} place of the enzyme and ultimately deacetylating the substrate through the formation of an acyl-enzyme complex (Negoro et al. 2007). The next enzyme is Endo-Type 6-Aminohexanoate-Oligomer Hydrolase of 355 amino acid residues transcribed from the nylC gene and utilizes the linearized trimer, tetramer, or pentamer of 6-aminohexanoate through an endo-type response (Kakudo et al. 1993). According to an article printed in 2018, two enzymes, nylD1 and nylE1 have been discovered to play a substantial role in the metabolism of their 6-aminohexanoate oligomer after it has been converted to the individual monomers. The nylD1 transcribes a molecule known as aminohexanoate aminotransferase that converts the 6-amino hexanoate to adipate semialdehyde through a pyridoxal phosphate (PLP) dependant pathway while utilizing α-ketoglutarate, pyruvate, and glyoxylate as acceptors. Further, the adipate semialdehyde dehydrogenase, another enzyme transcribed by the nylE1 receptor uses an NADP+ cofactor and converts the adipate semialdehyde to adipate (Takehara et al. 2018).

An interesting study claims that alkalophilic bacteria *Agromyces* sp. KY5R strain and *Kocuria* sp. strain KY2 produces 6-Aminohexanoate oligomer hydrolases that have much better thermal stability, affinity, and catalytic function under alkaline pH (Yasuhira et al. 2007). In 2009, researchers discovered another type of enzyme, aryl acrlamidase that could cleave off amide bonds in adipic acid bishexyl amide and aggravate the surface degradation of nylon-6 (Nagai et al. 2014). According to a Sudhakar et al. (2007), several marine bacterial species identified as *Bacillus cereus*, *Bacillus sphericus*, *Vibrio furnisii*, and *Brevundimonas vesicularis* have been reported to act upon both Nylon 6 and nylon 66 (Sudhakar et al. 2007). In the same study, the authors have hypothesized that as a substrate for bacterial degradation nylon 66 is much more favored than nylon 6. This behavior may be explained in terms of the basic structure of the 2 polyamides. Nylon 66 has about twice the amount of carbon-nitrogen bonds compared to nylon 6 which is a favorable target for the bacterial enzyme because less energy is required to break this bond in comparison to the carbon-carbon bonds of nylon 6. Because of this reason, nylon 66 falls a simple prey to bacterial degradation. Lignin-degrading fungus like the white-rot fungi IZU-154 is also reported to attack nylon-66 by oxidative metabolism (Deguchi et al. 1997).

3.2 PVC

Polyvinyl chloride (PVC) is a thermoplastic polymer of vinyl chloride monomeric units which was serendipitously discovered by Baumann in 1872. Originally hard and brittle, this polymer is made soft by the inclusion of a plasticizer such as di-n-butyl phthalate. It is utilized for making garden hoses, raincoats, pipes, insulation cables, shower curtains, simulated leather, water tank, or electrical insulator. Besides its elastic usage, it's also potentially toxic. The inhalation of PVC dust was reported to induce lung cancer

(Wagoner 1983). Also, it's vinyl chloride repeating units are extremely dangerous and are known to cause hepatic angiosarcoma, brain tumor, and other severe problems such as pneumoconioses, lymphatic and hematopoietic cancer, and emphysema (Kielhorn et al. 2000). Vinyl chloride has an anesthetic effect and its inhalation leads to irregular heartbeats in dogs (Oster and Carr 1947). Other indicators of chronic exposure include dizziness, headache, lack of breath and respiratory disease (Fralish and Downs 2021), and acroosteolysis that's a medical condition characterized by the presence of osteolysis and Raynaud phenomenon in the palms precisely in the terminal phalanges (Lopez et al. 2013). Also, there are pieces of evidence that indicate the cause of asthma and allergies as a result of the use of phthalate plasticized PVCs (Jaakkola and Knight 2008). A way out must be investigated to handle the PVC-related wastes and by-products that accumulate in the environment.

At the research level, several microbes have been detected to biodegrade these polymers and entities related to them. Sumathi et al. (2016) have discovered a low molecular weight PVC that could be biodegraded by *Cochliobolus* sp. by using an enzyme typically of 60-80kD known as laccase (Sumathi et al. 2016). This enzyme was obtained from the Japanese lacquer tree *Rhus vernicifera* in 1883 by Yoshida and later on isolated from fungus by Bertrand and Laborde (Viswanath et al. 2014). Hence, it has the possibility to be present in a variety of host organisms and is truly a green enzyme as would be discussed in the upcoming text.

The enzyme comes under the class of oxidoreductase and is a blue oxidase (due to the T1 copper) that is dependent upon oxygen as an electron acceptor for its purpose (Vishwanath et al. 2014). This cupredoxin could be monomeric, homodimeric, heterodimeric, or multimeric (Jaiswal et al. 2015). It could act upon a broad spectrum of substrates due to its low specificity especially a wide range of aromatic and non-aromatic compounds such as inorganic ions, phenolic and non-phenolics compounds (Nguyen et al. 2016).

This enzyme, laccases have been isolated from *Pycnoporus sanguineus* and *Trametes versicolor* which could break down bisphenol A (Barrios-Estrada et al. 2018). The laccase obtained from *Anthracophyllum discolor* could biodegrade polycyclic aromatic hydrocarbons (PAH)(Acevedo et al. 2011). Additionally, in a study, it was discovered that a laccase from an ascomycete *Stachybotrys chartarum* could act upon 2,2′-azino-di-(3-ethylbenzthiazolinsulfonate) (ABTS). It is now known that *Aspergillus nidulans* can produce as many as six different laccases with different substrate specificities (Mander et al. 2006) Therefore, it's highly evident that such a resourceful enzyme could degrade a variety of xenobiotic entities and not just PVC.

The catalytic site of laccase consists of four copper atoms in different oxidation states of which one is in type 1 and paramagnetic ‘blue copper’, another type-2, and the remaining two in type-3 oxidation states and exist as a diamagnetic copper-copper set. There is a characteristic cupredoxin fold which is two β-sheets organized like a Greek-key barrel with approximately seven antiparallel β-strands with varying sequences interconnected by hairpin loops. This enzyme contains three homologous domains and the third domain harbors type 1 copper. The trinuclear cluster (TNC) made of one type 2 and the rest two are type 3 dimer, exists betwixt the first and third domains with the second domain exclusively functioning to orient the other two domains so that the TNC is properly formed. An electron transfer chain has also been reported between the type1 copper ion and the trinuclear cluster (Arregui et al. 2019).

This enzyme works by either directly oxidizing the substrate to its respective radical or by an in-direct oxidation process for those substrates that have their ionization potential higher than the redox potential of the type 1 copper center (Agrawal et al. 2018). The whole cascade is initiated once the substrate associates with the binding site where it's stabilized by numerous hydrophobic residues. For example, in the instance of the laccase of *T.versicolor*, the substrates are stabilized by Phe162, Leu164, Phe265, Phe332, and Pro391 and also by an Asp206 which influences its orientation by interacting with His 458. For each enzymatic cycle, four substrate molecules are required. Following the proper binding, the substrates are modulated by the removal of one electron from each of its four molecules by the type1 copper ion. This copper ion transfers the electrons to the TNC with assistance from a highly conserved cysteine-histidine electron transfer bridge. Next, the oxygen enters the TNC site and gets reduced to water. At first two of the four migrating electrons are contributed to it resulting in the formation of a laccase-peroxide intermediate. At length, two more electrons are provided to crack the peroxide linkage. Parallel to this hydrogen ions are provided from the 424th glutamic acid residue to produce water molecules with the help of a semi-conserved aspartate at the 77th position present at the trinuclear cluster interface (Arregui et al. 2019).

Laccase (isolated from *Cochliobolus* sp.) can hamper PVC due to its structural relatedness to chlorophenolic compounds which are known to be degraded by the enzyme (Sumathi et al. 2016). Similarly, xylotrophic basidiomycetes such as *Phanerochaete chrysosporium* and *Trametes (Coriolus) versicolor* also have been reported to degrade polychlorinated phenols though it might not play any pivotal part in its mineralization (Ricotta et al. 1996). *Trametes pubescens* were found to act against chlorophenol mix at a pH of 6 and a temperature of 40 degrees (Gaitan et al. 2011). *Panus tigrinus*, basidiomycete was found to hamper chlorophenol (Rabinovich et al. 2004). A phytopathogenic fungus, *Rhizoctonia praticola* was detected to degrade chloroanilines. However, the question of whether they could degrade PVC similar to

Cochliobolus sp. has to be investigated. The answer could be yes or it could be no.

Aside from that, plasticized PVC has also been reported to be colonized by *Aureobasidium pullulans*. In the same study *Alternaria alternate*, *Alternaria infectoria*, and *Purpureocillium lilacinum* are reported to nicely degrade plasticized PVC (Webb et al. 2000). Members of the genera *Pseudomonas*, *Bacillus*, and *Chelatococcus* have been found to hamper PVC, *P. citronellolis* partially degrading the PVC materials without sterilization (Giacomucci et al. 2019). Further, PVC was also found to be degraded by white-rot fungi like *Pleurotus* sp. and *Poliporus versicolor* (Kirbas et al. 1999). There might be many other enzyme systems to hamper the PVC; therefore, further extensive investigation is necessary. But what turns out is that the main difficulty is the time required for complete degradation of PVCs deposited in the open environment due to different anthropogenic activities is very long. Hence, the biggest challenge of the hour is to bioengineer the enzymes to improve their efficiency.

3.3 Polyurethane (PU)

Polyurethane (PU) is a class of condensation plastic polymer that was innovated as a rubber substitute during the second world war. This elastic polymer manufactured chiefly by the reaction of a diisocyanate with a diolcould also incorporates a wide range of modifications due to the presence of different aromatic compounds, ester, urea, or ether. Due to this, it finds its usage in a wide array of fields such as building, textile, automobile parts production and recently in a high number of biomedical implementations like artificial skin, adhesive for soft tissues, tissue engineering scaffolds, vascular prostheses, devices for drug delivery, pericardial patches, artificial nerve graft and dressing of wounds (Krasowska et al. 2015).

Despite being so malleable in usefulness this polymer also has significant disadvantages once it's disposed of in the environment. Due to this, it has been enlisted among the top polluting plastic polymers on the planet. For example, spraying of PU foam for thermal insulation in homes and offices emits tiny particles of urethane indoors which have been reported to irritate lungs and cause dyspnea and asthma. Studies also have shown that objects made up of PU could emit volatile organic compounds (VOCs), which can cause acute respiratory harm (Huang and Tsuang 2014). In 2016 multiple cases of allergies, irritation of the throat, bleeding of the nose, burning sensation in the eyes, and tightening of the chest have been reported in children who practiced routinely in running tracks made from PU (Wu et al. 2019). The observation had incited lots of societal perturbation especially, among the parents and their kids. In light of the circumstance, research was conducted by a group of specialists. The results showed that there is the emission of xylene, toluene diisocyanate, and unpredictably very low concentrations of sulfur dioxide which can have a profound effect on long-term exposure, especially in the shape of impairments like chronic obstructive pulmonary disease (COPD)(Wu et al. 2019).

The polyurethanes comprise so-called "sections", which are actually alternating blocks. Every one of these blocks could be either the hard sections or the soft sections depending on their chemical nature. Polymers of different natures could be produced by varying the proportion of soft and hard sections (Krasowska et al. 2015). This family of polymers encompasses both non-biodegradable and biodegradable polymers. But it's fascinating to say that the recalcitrant non-biodegradable ones have been reported to be biodegraded by certain microbes.

The biodegradation of polyurethane by the fungus was first confirmed by Derby and Kaplan (1968). They also suggested that compared to polyether polyurethane, the polyester version is a better choice of substrate for fungal biodegradation. They further reasoned that for significant enzymatic degradation to occur, the presence of long unbranched carbon string separating the urethane linkages along with the presence of three neighboring methylene groups is very essential (Darby et al. 1968). Similarly, Hedrick and Crum (1968) reported the growth of *Pseudomonas aeruginosa* and *Cladosporium resinae* on the polyester polyurethane baffles of aircraft gas tanks (baffles are utilized to decrease the sloshing and projectile impact). Later on, Crabbe et al. (1994) reported four species of fungi i.e. *Curvularia senegalensis*, *Fusarium solani*, *Aureobasidium pullulans*, and *Cladosporium* sp. from garden soil could use up the polymer. Additionally, they found out that *C. senegalensis* used an esterase-based enzyme for its actions (Crabbe et al. 1994).

Subsequently, Nakajima-Kambe et al. (1995) reported the active degradation of polyester PU by *Comamonas acidorvorans*. The microbe behaves so with the assistance of a 522 amino acids long esterase transcribed by a gene named Pud A. In its catalytic site, it exhibits a high degree of homology with the serine hydrolase and was discovered to possess the Ser-His-Glu catalytic triad. Three-dimensionally this catalytic site is close to the surface binding domain (Nomura et al. 1998). The surface binding domain was pointed out to be hydrophobic and is considered to entail the hydrophobic PU substrate followed by catalytic degradation (Akutsu et al. 1998).

Fungal isolates from sand infected with PU had deteriogenic activity on the polymer and was found primarily to be due to urease activity (Loredo-Treviño et al. 2011). Numerous researches in the literature show a vast array of microbes that could degrade polyester polyurethane such as the five strains *of Nectria gliocladioides*, seven strains of *Geomyces pannorum* and one strain of *Penicillium ochrochloron* (Barratt et al. 2003) endophytic

fungus *Pestalotiopsis microspora* isolated from *Taxus wallachiana* from Ecuadorian Amazonian region is found to act on PUR via serine hydrolase activity under both aerobic and anaerobic conditions (Russell et al. 2011) and various other organisms such as *Candida rugosa* (Gautam et al. 2007), *Pseudomonas chlorophis*, *Phoma* sp., *Geomyces pannorum*, *Alicycliphilus* sp., *Pseudomonas flourescens*, *Acinetobacter gerneri*, *Bacillus subtilis*, *Enterobacter agglomerans* and *Serratia rubidaea* (Mahajan and Gupta 2015), and *Acinetobacter gerneri* (Howard et al. 2012). From *P.chlorophis* three PUase enzymes, one of 65KDa and another of 31KDa, and a third of 60KDa have been identified (Howard 2002).

Lately, studies exhibited the chance of biodegrading the highly recalcitrant polyether polyurethane that was previously regarded as highly resistant to microbial degradation. A study demonstrated that *Cladosporium cladosporioides*, *Aspergillus fumigatus*, and *Penicillium chrysogenum* could attack the polyether polyurethane by considerable esterase activity and low urease activity (Álvarez-Barragán et al. 2016). Again, another study demonstrated that *Alternaria*sp. can do the same by a coalition of physical and chemical procedures. Physically it disrupts the structural arrangement of its substrate by its innumerable hyphae while using enzymes like urethanase and urease to carry out the degradative activity (Matsumiya et al. 2010). Reports suggest that cholesterol esterase can hamper Polyether based PUs liberating free amines (Christenson et al. 2006). However, the activity might be too low.

3.4 Polyethylene Terephthalate (PET)

Polyethylene terephthalate (PET) is a condensation polymer in which the monomeric units of ethylene glycol and terephthalic acid are combined by ester linkages (Sinha et al. 2010). This polyester is a fine illustration of a thermoplastic polymer and can be glass-like amorphous in its purest form. This polymer is commonly utilized in fabrics and food packaging industries and the production of microwavable packaging and drinking bottles. Besides its tremendous advantages, it has certain disadvantages which can no more be ignored. In its microplastic type, it's reported to damage *Parvocalanus crassirostris*, a copepod. The outcomes are a decline in its population density along with a radical effect on its survivability and reproductivity (Heindler et al. 2017). A recent study reports that terrestrial organisms such as *Achatina fulica* (snail) are adversely affected by the microplastic fibres of the polymer. The various effects include substantial damage to the intestinal villi and enhancement of oxidative stress within the body by upregulating the malondialdehyde level and lowering the level of glutathione peroxidise (Song et al. 2019). Recent data reports some pieces of evidence about the leaching of certain amounts of phthalate which could act as endocrine disruptors and other genotoxic and carcinogenic compounds in bottles used to store various items (Rastkari et al. 2017). This might occur under conditions of long term storage. The PET polymers are indeed very lasting. This makes the substances to be utilized in various programs in day to day life but once such substances are eventually worn out, they are finally disposed of. This then turns the boon of durability into the bane of ecological burden.

Many enzymes have been reported to degrade PET polymers such as lipase (*Thermomyces lanuginosus*, *Rhizopus chinensis*, *Candida antarctica*, *Burkholderia* sp.), cutinase (*Fusarium solani*, *Humicola insolens*, *Penicillium citrinum* & some members of *Thermobifida* genera) (Kawai et al. 2019).

A breakthrough in the quest for a bio-based enzyme to degrade synthetic PET polymers came in 2016 with the discovery of *Ideonella sakaiensis* 201-F6, a gram-negative aerobic bacterium in Japan (Tanasupawat et al. 2016). Since then, much work was done to understand the degradation of the polymer by the bacterium. The deterioration of the PET polymers is carried out by two different enzymes- one is the PETase that could breakdown PET into mono-(2-hydroxyethyl) terephthalate (MHET) and another is MHETase that degraded the end product of PETase to ethylene glycol and terephthalic acid (Palm et al. 2019).

PETase is a α/β-hydrolase fold enzyme protein comprising of three polypeptide chains and a serine based catalytic triad of Ser-Glu-Asp (precisely, S133-H210-D179). The nucleophilic serine is polarized by the histidine residue which in turn is stabilized by the aspartic acid residue in the catalytic site whose flexibility is maintained by the disulphide bonds (Fecker et al. 2018). The catalytic cleavage occurs after the proper binding of the substrate. For this purpose, the wobbling kind of conformation of the tryptophan at the 156th position is very important. Once the substrate binds to the enzyme, there is a conformational change in the enzyme which displaces the carbonyl moiety of the leading hydrophobic ring into the middle of the substrate-binding section where the catalytic nucleophilic attack is carried out by the triad. During this process, the oxyanion hole acts as a substrate polarizer, thereby polarizing the ester linkages and stabilizing the intermediate. Subsequently, there is the nucleophilic attack of water just after the formation of the acyl-enzyme complex (Chen et al. 2018).

The MHETase comprises a typical lid domain (that confers substrate specificity) and a catalytic α/β-hydrolase fold domain. It is a type of feruloyl esterase with a molecular weight of 65KDa. Its lid domain is lodged in between the β strand and the fifteenth α helix. The enzyme has a calcium-binding website and five disulfide bonds. It possesses a typical catalytic triad of serine, histidine, and aspartic acid (S225, H528, D492) along with the oxyanion hole like the PETase (Palm et al. 2019).

Experts are already giving a lot of effort to make the PETase industrially available. In reality, according to recent work, the researchers have improved the expression of the enzyme by using the secretion system of *Escherichia coli*. They had used a Sec-dependant and SRP-dependant signal peptide from *E. coli* to generate an extracellular enzyme capable of degrading PET. However, the activity of this extracellular PETase was discovered to be lower than the cytosolic version expressed in *E. coli* RossettaGami-B which could generate a degraded product of 4.8 mg/L post 36 hours of incubation (Seo et al. 2019). The MHETase has been successfully propagated in *E. coli* BL21 by using the pUCIDT plasmid vector (Janatunaim and Fibriani 2020).

In 2019, there was a successful demonstration of PETase production by a diatom called *Phaedactylum tricornutum*a though its main problem was poor growth (Moog et al. 2019). After green algae, *Chlamydomonas reinhardtii* (industrially GRAS organism) was shown to produce PETase (Kim et al. 2020). Using the metagenomic approach a different group of researchers had identified a novel cutinase homolog out of leaf-branch compost effective at degrading PET (Sulaiman et al. 2012). Lately by using a similar strategy about 504 possible PETase generating genes have been identified mainly in organisms of the genera *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* (Danso et al. 2018).

3.5 Polypropylene (PP)

Polypropylene is a thermoplastic polymer made up of repeating units of propylene monomers. Stereochemically, it appears in three distinct conformations: isotactic where the methyl substituents are oriented on the same side, syndiotactic where methyl groups frequently alternate on the other hand, and atactic where methyl groups are randomly oriented. This polymeric compound is widely used in the production of strappings, movies, sheets, injection mouldings, auto parts, fibres, and fabrics (Maddah 2016). This versatile polymer was found by Rehn and Natta in 1954 and commercialized in 1957 (Andrady and Neal 2009).

It is quite similar to polyethylene but is conducive with tougher mechanical characteristics along with higher heat resistance but consecutively low resistance to chemicals such as non-polar solvents or oxidizing agents (Scalenghe 2018). As a result of its high susceptibility to oxidation by dint of its chemical structure, it is capable of creating a lot of free radicals (Sternschuss et al. 2012). If such a reaction occurs in the human body due to the consumption of a microplastic contaminated diet then it is likely to induce oxidative stress. On the other hand, the intensity of damage caused due to the consumption of microplastics from the human body requires additional investigation. Already there's evidence that exposure to polypropylene flock can cause the onset of interstitial lung disease along with peribronchial thickening in some cases (Atis et al. 2005).

Polypropylene is one of the most frequently discovered plastics in oceans worldwide and is known to accumulate polychlorinated biphenyls (Teuten et al. 2007). In an experiment conducted by using *Daphnia magna*, it was discovered that leachates from this polymer were relatively not as toxic as plasticized PVC (Lithner et al. 2011). Further, explorations on this subject would be very helpful because its influence on the environment is certainly not unmanifested if not overtly extensive.

Degradation of the polymer may be carried out by chemical means but the use of microbes in this pursuit could be a green strategy. Fortunately, some data support the possibility of the development of this strategy. For example, some researchers claim the possibility of PP degradation by reactive oxygen species (ROS) created by sulfate-reducing bacteria (Cacciari et al. 1993). Inside the body polypropylene implants are reported to be assaulted by the myeloperoxidase enzyme of macrophages (Iakovlev et al. 2015). The myeloperoxidase is a porphyrin ring containing an enzyme that takes up hydrogen peroxide to produce hypochlorous acid which is a powerful oxidant. All these studies suggest that reactive oxygen species may play a main part in the degradation of the polymer.

3.6 Polystyrene (PS)

Polystyrene is a versatile thermoplastic xenobiotic polymer that is a byproduct of free radical addition polymerization of styrene and is highly recalcitrant. The benzene derivative styrene was found in 1831 from the exudates of *Liquidambar orientalis* and *L. styraciflua* (Miller et al. 1994). It is widely used in the production of toys, radio, TV cabinets, and thermal insulators such as Styrofoam. It might be strong or foamed and is mostly made in four distinct varieties, i.e., general-purpose polystyrene (GPPS), high impact polystyrene (HIPS), polystyrene foams, and expanded polystyrene (EPS) foam.

Certain environmental problems arise when various products of those polymers are unprecedently discharged as wastes. One of the clearest problems is the contamination brought on by microplastic formation. A noteworthy effect of such microplastic has been observed from the *Crassostrea gigas*, an economically important oyster (served as seafood), where there is a lowering of their sperm motility and the oocyte diameter and number thereby decreasing their reproductivity and inhabitants (Sussarellu et al. 2016). The effects were investigated from the medaka (*Oryzias melastigma*) and were found to induce oxidative stress, negative regulation of the hypothalamus-pituitary gonadal axis, retardation of offspring development, and hampering of gonadal maturation (Wang et al. 2019). A team of scientists has discovered that

polystyrene nanoparticles that have positively charged amino alterations and are of sizes of about 52nm are toxic to *Daphnia magna*. The group has reported the possibility of such things crossing the blood-brain barrier of fish and accumulating inside thereby bringing about alterations in the brain and behavioral changes (Mattsson et al. 2017). According to a current analysis, the PS nanoparticles might negatively impact the microbial population from the soil by exerting some sort of antimicrobial properties where dehydrogenase activity and other enzymatic activities related to N-(leucine-aminopeptidase), P-(alkaline-phosphatase), and C-(β-glucosidase and Cellobiohydrolase) cycles in the soil are detrimentally lowered (Awet et al. 2018). Styrofoam has been proven to accumulate heavy metal mercury after being disposed of. This may indicate the possibility of mercury bioaccumulation and biomagnifications (Graca et al. 2014). Again, styrene is known to cause lymphohematopoietic cancers and hence is a possible carcinogen (Huff and Infante 2011). Therefore, the toxicological evaluation of polystyrene has to be further examined. A group of researchers while expressing their concerns regarding the effects of polyester microparticles within the human body discovered that such entities might cause mitochondrial depolarization, thereby interrupting energy productions from the individual Caco-2 epithelial cell lines (Liu et al. 2019). However, based on a current experiment (based on exposure concentrations) with environmentally realistic conditions to examine the exposure patterns, another group reported that micro-sized particles on accidental ingestion impose a negligible impact on mammals (Stock et al. 2019). Such type of contradictory observations indicates that there is a need for extensive investigations because the issue of absorption of toxic contaminants of hydrophobic nature is a well-proven truth and cannot be ignored. Hence all these polymeric wastes influence the environment but to what extent, is a matter that has to be further explored.

Several works focused on this field are already done. There are reports of *Pseudomonas aeruginosa* degrading modified polystyrene (PS) polymeric materials. Also, PS scents and powder are degraded by *Rhodococcus ruber*. A fungal species, *Curvularia* sp. has been found to show some degradative action towards pre-oxidized atactic polystyrene (Ho et al. 2017). Also, it has been found that *Bacillus* sp. and *Pseudomonas* sp. could degrade brominated HIPS by an enzymatic depolymerase action (Mohan et al. 2016). Such action has also been reported in *Enterobacter* sp., *Citrobacter sedlakii*, *Alcaligenes* sp., and *Brevundimonas diminuta*, all of which can hamper HIPS to some extent (Sekhar et al. 2016). Esterase activity of *Lantinus tigrinus* was found to biodegrade polystyrene (Tahir et al. 2013). A few years ago an interesting discovery was made that the larvae of *Tenebrio molitor* (commonly referred to as mealworms) could use Styrofoam as a supply of food. The gut of the organism was found to possess a firmicutes *Exiguobacterium* sp. that can biodegrade the polymer (Ho et al. 2017). Further investigations have resulted in the discovery of a super worm, the larvae of *Zophobas atratus* which may digest and mineralize styrofoam with the potential credit moving to its gut microbiota (Yang et al. 2020). Two species of an extremophilic gram-positive bacterium, *Exiguobacterium sibiricum* strain DR11 and *Exiguobacterium undae* strain DR14 were reported to exhibit reassuring degradation of polystyrene (Chauhan et al. 2018). Other microorganisms like *Microbacterium* sp., *Paenibacillus urinalis* were also found to degrade the polymer. As per a study conducted in Mumbai, *Bacillus subtilis* was found to have maximum degradative action within this context concerning different microbes which were isolated out there. A smart strategy to degrade this highly recalcitrant polymer once its utility expires is by integrating biodegradable molecules in its backbone during the manufacturing of the polymer as exemplified by the degradation of the starch-polystyrene copolymer by *Bacillus coagulans* (Ho et al. 2017).

To the best of our knowledge, there is absolutely no thorough mechanism of microbial degradation of polystyrene deciphered till now. However, the degradation of styrene has been well analyzed. It is well known that the microbial degradation of styrene is dependent upon two different trajectories. One manner is by targeting the aromatic ring to get direct cleavage. As detected in *Rhodococcus rhodochrous* NCIMB 13259, there's lead ring hydroxylation bringing about the formation of styrene cis-glycol via enzymatic catalysis of a dioxygenase. The product of the reaction is then dehydrogenated to 3-vinyl catechol. The reaction then divides into a meta cleavage pathway, wherein, one sub-chain precidely the 3-vinyl catechol degrades to acetaldehyde and pyruvate with the help of many enzymes such as catechol 2,3-dioxygenase, semialdehyde hydrolase, hydratase, and aldolase involving many intermediates. At another part, 2-vinyl muconate is formed as the end product. The second pathway is the oxidation of the vinyl side chain that finally contributes to the formation of phenylacetic acid through certain intermediates. The phenylacetic acid is then converted into phenyl-acetyl-CoA which enters the tricarboxylic acid cycle via the formation of different intermediates (Mooney et al. 2006). The existence of a well-organized styrene biodegradation mechanism in microbes is a clear sign of the prospect of not only discovering more efficient polystyrene degrading microbes but also engineering the present ones to boost the rate of doing so.

3.7 Polyethylene (PE)

Polyethylene is a polyolefinic chain growth thermoplastic polymer that is made from unsaturated ethylene units. This homopolymer is stable at high temperatures (precisely as much as 290° C in the absence of oxygen). Even after such elevated temperatures, it does not completely degrade to its most straightforward monomeric units; instead breaks down into forms that are smaller units with

comparatively fewer degrees of polymerization (Aggarwal and Sweeting 1957). It is economical, adaptable, and extremely resistant to chemicals and may act as a marvellous electrical insulator (Bhakta 2017).

Depending on the method of manufacture it may assume more or less branched construction. There are various sorts of polyethylene depending upon the molecular arrangement and texture with slightly varying properties. The most frequently used ones are low-density polyethylene (LDPE), high-density polyethylene (HDPE), and linear low-density polyethylene (LLDPE) (Gulmine et al. 2002).

3.7.1 LDPE

The low-density polyethylene is a branched-chain polyolefin with a high degree of branching and with a low density and melting point of 110 degrees (Bhakta 2017). However, it is highly vulnerable to stress cracking, has UV resistance, and is highly flammable. Largely used for fabricating plastic bags, dispensable bottles, and tubing.

3.7.2 HDPE

A cheap, thermoplastic polymer that is made by coordination polymerization while utilizing the Ziegler-Natta catalyst. This linear polymer has a high density and a high melting point of about 130 degrees Celsius (Bhakta, 2017). It has higher resistance and strength in comparison to the LDPE. Consecutively, it is employed in the production of pipes, containers, and moulded hardware. However, it has low UV and heat resistance.

3.7.3 LLDPE

The linear low-density polyethylene is a polyolefinic copolymer with a linear backbone with short-chain branches. It is manufactured by utilizing α-olefin and ethylene monomeric units in the existence of a catalyst, precisely the metallocene or the Ziegler-Natta catalyst (Krupa and Luyt, 2001).

Apart from these three, there are different variations of polyethylene that are available in the form of Ultra-high-molecular-weight polyethylene (UHMWPE) (Kurtz et al., 1999), cross-linked polyethylene (PEX) (Xiong et al. 2017), medium-density polyethylene (MDPE) (Bashford 1997) and very-low-density polyethylene (VLDPE) (Mathot et al. 1989).

It is now that we've started to realize that these versatile commodities with such a wide assortment of skills are not without their disadvantages. Usually, the fact that it might form microplastic has resulted in plenty of concerns. As such kinds of entities not only can act as vectors for toxic chemicals but also are damaging themselves. They contribute to bioaccumulation and biomagnification with plenty of side effects. Starting from lower-order organisms such as *Mytilus galloprovincialis* also known as the Mediterranean mussel, these microparticles may cause tumultuous altered developments of the digestive tract by lowering the intestinal content and thinning of epithelial cells from the tubules. Also, it boosts the necrotic development of various body cells like those of their mantle and gonads due to haemocytic aggregation (Thomas et al. 2018). Disruptive effects were studied in sediment-dwellers such as *Chironomus tepperi* (the non-biting midge) and was discovered that particles of 10-27 µm caused retarded development, congestion of the digestive tract, and increased mortality among the inhabitants (Ziajahromi et al. 2018). Evaluation of microplastic ingestion in zebrafish revealed considerable accumulation from the larval digestive tract and also disproportionate transcriptional agitation typically in the genes involved in processes such as glycolysis, purine metabolism, and lysine metabolism to name a few which were strangely sorted out in 14 days post-exposure (LeMoine et al. 2018). Earthworms (*Eisenia fetida*) which play an important role in soil fertility on exposure to 100-200 µm low-density polyethylene incurred substantial damages to their skin while experiencing an increased activity of the catalase enzyme and enhanced concentration of malondialdehyde, signalling the occurrence of oxidative stress (Chen et al. 2020). These particles are cytotoxic to microalgae and have been found to interfere with the anaerobic sludge management by reacting with traces of oxygen via its electron donor sites (generated by the action of UV) resulting in the formation of $O_2^{\bullet-}$ and H_2O_2 which could cause oxidative stress to the microbes (Wei et al. 2019). Recently some conflicting results have also come up that state that microplastics probably have no significant impact on zooplanktons. It might however in the fishes cause a lowered rate of egg hatching with often the conditions being premature (Beiras et al. 2018). But such problems could not be neglected concerning the increasing signs of toxic effects in organisms of varying sophistication levels. In mice, it might activate inflammation and intestinal dysbacteriosis (Li et al. 2019). It is now increasingly suspected that nano and microplastic exposure may be neurotoxic to human beings just like in different organisms where it might change the neurotransmitter activity (Prüst et al.2020). Already, there is a lot of evidence that we are frequently subjected to such particles. Not only that we are also exposed to many different natural products as leachates of plastic products such as PEX (cross-linked polyethylene) pipes (Lund et al. 2011). Very recent research focused on the feeding behavior of goldfish has found out that the ingestion of microplastics occurs while food intake with the green and black particles eaten more than the red, blue, or white ones. It also discovered that particles of more than 2mm were automatically discarded even if ingested (Xiong et al. 2019). This type of monitoring indicates that there's a varying proclivity level for different microplastics. Some are more damaging than others. Genetic insights into the chemoreception of

Daphnia pulex have been conducted. The analysis has identified 58 gustatory receptors (Grs) which were included in their unique tasting and smelling process, in a nutshell, the chemosensing of the environment (Peñalva-Arana et al. 2009). In light of these works, it is now thought that the surface eco-corona of nano plastic particles plays a significant part in promoting ingestion by organisms such as *Daphnia magna* (Balakrishnan et al. 2019).

In the last few years, a great deal of focus is given to the exploration of different microbes which could act as possible degraders of plastic polyethylene. Efforts were given to solve the enzymatic mechanisms that enter interplay. It is now being increasingly recognized that such endeavours would be very difficult due to the lack of functional groups capable of hydrolysis. However, pre-treating such waste issues with heat, UV, or oxidizing agents could enhance the degradation process. This can be attributed to the fact that such treatments produce functional groups (such as carbonyl groups) which are easily acted upon by microbial enzymes such as laccase, manganese peroxidase, and lignin peroxidase aiding in the process of degradation.

The manganese peroxidase, a heme-based enzyme, requires a hydrogen peroxide as an acceptor of two electrons producing a specific intermediate (compound I) which oxidizes the substrate to make free radicals along with the other intermediate (compound II) that oxidizes Mn^{2+} into Mn^{3+}. The generated cation chelated by the fungal malonate and oxalate results in the development of reactive radicals which may act upon non-phenolic chemicals (Chowdhary et al. 2019). In this context, an innovative strategy was adopted by Ehara et al. (2000) where they successfully degraded the polyethylene with this enzyme by using tween 80 in place of hydrogen peroxide.

The lignin peroxidase, a ferric ion-based enzyme, works similarly by utilizing hydrogen peroxide and a distinctive oxo-ferryl intermediate (Falade et al. 2017). However, these enzymes work mostly upon lignin whose redox potential is way lower than polyethylene chains, hence appreciable degradation is not achieved (Zimmermann and Wei 2017). Therefore, the quest for an efficient PE degrading enzyme continues.

Many microbes discovered so far are predicted to degrade the polymer by the above-mentioned enzymatic processes. Typical examples within this context are *Trichoderma harzianum* (laccase & manganese peroxidase) (Sowmya et al. 2014), *Phanerochaete chrysosporium* (manganese peroxidase) (Iiyoshi et al. 1998), *Trametes versicolor* (laccase) (Fujisawa et al. 2001), *Penicillium simplicissimum* (laccase, manganese peroxidase) (Ramalingappa et al. 2014), *Rhodococcus ruber* (laccase)(Santo et al. 2013), *Bacillus cereus* (laccase, manganese peroxidase) (Rajeswari et al. 2015) and *Klebsiella pneumonia* (laccase, peroxidase, lipase, tyrosinase) (Srivastava et al. 2017).

According to Sangale et al. (2019) *Aspergillus terreus* and *Aspergillus sydowii* have been discovered to be the most efficient one of the consortium of 109 fungal members. However, not many enzymatic accounts were referred to in the same. Microbes of genera *Fusarium*, *Aspergillus*, *Pseudomonas*, *Brevibacillus*, *Lysinibacillus* (Muhonja et al. 2018), *Streptomyces* (Anthony et al. 1992) are expected to have some degradation activity. Furthermore, microbes such as *Mucor rouxii* NRRL 1835, *Rhizopus oryzae* NS5 (Awasthi et al. 2017), *Micrococcus luteus*, *Proteus vulgaris*, *Staphylococcus aureus* (Priyanka and Tiwari 2011), *Rhodococcus ruber*, and combined cultures of *Aspergillus niger* and *Lysinibacillus xylanilyticus* (Esmaeili et al. 2013) are discovered to have some degradative action. Recently, it's been indicated that the marine fungus *Zalerion maritimum* could use PE microplastics hence may have a tremendous future program in bioremediation (Paco et al. 2017). Apart from all this, a new study claims that the gut of *Galleria monella*, commonly called the greater wax moth harbours a microbe, *Enterobacter* sp. D1, that could degrade polyethylene by an extracellular oxido-reductase enzyme (Ren et al. 2019). Similar results are reported by *Plodia interpunctella* (Indian mealworm) and *Achroia grisella* (lesser waxworm). While in the former two PE degrading strains of *Enterobacter asburiae* YT1 and *Bacillus* sp. YP1 was identified from the gut, from the latter, it is not clear whether the effect is due to the gut microbes or due to an exceptional enzyme produced inside its own body (Kundungal et al. 2019). The action of *Pseudomonas* sp. E4 requires appreciation as researchers found out that it might act upon entirely untreated polymer with the assistance of its alkaline hydroxylase (alkB) gene product (Yoon et al. 2012). Presently a thorough investigation has resulted in the discovery of two cyanobacterial species *Phormidium lucidum* and *Oscillatoria subbrevis* that can degrade low-density polyethylene via laccase and relatively less manganese peroxidase activity(Sarmah and Rout 2018). Different species of algae such as Bacillariophyceae, Chlorophyceae, and Cyanophyceae were found to colonize the surface of polyethylene but how far they could degrade the polymer in the near future is a matter that has to be explored (Sharma et al. 2014). Some important microorganisms, their synthesized enzymes and impact of these on plastic degradation have been represented in table 1.

4 Present Management

Despite the exclusive attempts to find the one perfect solution to fight against plastic pandemonium, the war continues while every day the environmental conditions due to plastic pollution get more and more desperate. Presently, in this COVID era, some serious pollution is occurring due to the worldwide disposal of these one-time use PPE kits much of which are plastic based products. While there are groups of researchers or bioengineers that are struggling

to locate the best degrading enzyme or to find others who are analyzing the threat factors and designing a proper management strategy from the existing amenities. Plastic management is a way to lower the burden of xenobiotic plastics in the environment, sustainably. It widely involves the recovery of plastic waste, recycling, and reusing them.

The first step in this process is the grouping of plastic wastes deposited in the environment followed by shredding to reduce the sizes, then washing with detergent to remove contaminants and sorting based on their substance build-up (Goodship 2007). The sorting could be done manually based on certain identification codes or it might be automated. But the manual process is labour-intensive with significant chances of human error. The newer automated methods are very powerful in sorting out the polymers using high precision technology. These methods are classified under three heads:

(i) The dry sorting techniques (such as detection utilizing near-infrared radiation and X rays, atmosphere sorting, electrostatic sorting, mechanical sorting, and sorting by melting)

(ii) The wet sorting techniques (such as sink float sorting procedure, hydrocyclones, and selective dissolution), and

(iii) The chemical sorting techniques (such as hydrolysis, glycolysis, and hydroglycolysis) (Ruj et al. 2015)

Often the combination of automated and manual techniques is included. After this, the particles are melted followed by compression to produce the pellets which may be reused to make several materials like water packing bottles. Contrary to thermoplastics, the thermosetting polymers cannot be remoulded or staged rather they're used as reinforcing fillers in thermoplastic polymers that lower their production price. Often the plastic materials are exposed to thermal degradative processes such as pyrolysis, hydrogenation, or gasification for energy recovery and also to reduce the quantities (Goodship 2007). These processes are much better in comparison to simple combustion in the open air. Energy recovery processes are greatly used in countries such as Japan. Plastics may be a replacement for coal due to their high calorific values. The proper combustion of plastic particles is potentially carried out in blast furnace and cement kilns during co-incineration with zero risks of toxic emissions. The residual slag by-product might be utilized in construction (Singh and Sharma 2016).

Rather than discarding carelessly in the open environment, disposing of plastic wastes in landfills is an old option but it may cause problems particularly the leaching of toxicants. However, in addition to all scientific strategies, we feel there is a good need to create more public awareness through several programs. No great feat in this subject could be achieved as long as the general public languishes in innominate darkness.

In the past few years, many campaigns have been launched to propagate the ill effects of plastic. Alternatives to polyethylene carry bags such as bags made of paper, fabric, or jute should be promoted among the general public. Amidst all these developments, a few controversies have arisen. One of them is the incineration of plastics. As the growing amounts of waste incinerated, several intellectuals have started questioning whether such huge public investment in this industry as it is turning out to be may hinder the advancement of waste recycling processes. Plastic waste management has a behavioural part that often sparks consumer uncertainties regarding the sorting out of wastes. For example, individuals might easily distinguish between plastic and paper but, often they fail to classify whether it is packing or non-packaging plastic (Henriksson et al. 2010). Perhaps more awareness and providing means for effortless access to simplified information might appreciate better recycling rates. Another way of decreasing plastic contamination might be to decrease the production of petroleum-based plastics and increase the use of bio-based plastics as they're easily degradable in the environment. But within this context care should be taken that the sale price of such biodegradable plastic should be just like that of the petrol-based counterparts or else the customers may be discouraged from buying them.

Conclusion and Future prospects

In the present situation, about 99 percent of the plastic generated is based on fossil fuels. While the bio-based ones claim to be a suitable replacement we have got a bigger issue to deal with. The issue is how to deal with those that are already accumulated after their specific utility expired. We just cannot wait for a few thousand years to let them all go away naturally. We do not even know to what extent they are impairing our ecosystem. The microbes, typically the ones that go rogue and develop in a variety of challenging environments might help in the process. However, much more conclusive works regarding their proteomics have to be carried out. We should attempt to reduce our everyday life usage of Petro-based plastics and every time we do we say a big no to the query raised by the first Brandt Commission- “Are we to leave our successors a scorched planet of advancing deserts, impoverished landscapes, and ailing environment?”. Driven by our ambitious vision to have the most durable commodities in our daily life we have unknowingly opened pandora’s box of silent pollution. Therefore, we must find an absolute solution for this plastic mayhem before it could prove to be the hideous “Mr Hyde” for our beloved ecosystem.

Table 1 Various microbes and their enzymatic effect on the plastics degradation

Type of Plastic	Usage examples	Potential degraders	Enzymes	References
Nylon	fishing nets, plumbing pipes, sewage pipes	Bacteria of family *Bacillaceae, Nocardiaceae, Vibrioceae, Caulobacteriaceae, Microbacteriaceae, Micrococcaceae* and fungus of *Phanerochaetaceae*	6-aminohexanoate-cyclic-dimer hydrolase, 6-Aminohexanoate-dimer hydrolase and endotype 6-Aminohexanoate oligomer hydrolase , Aminohexanoate aminotransferase & Adipate semialdehyde dehydrogenase, Aryl acrlamidase	Negoro 2000; Takehara et al. 2018; Nagai et al. 2014
Polyvinyl chloride	garden hose, raincoat, pipes, insulation wires	Bacteria of family *Chelatococcaceae, Bacillaceae, Pseudomonadaceae* and fungus of family *Pleosporaceae, Polysporaceae, Aspergillaceae, Ceratobasidiaceae*	Laccase	Arregui et al. 2019
Polyurethane	construction, textile, automobile production, artificial skin	Fungi of the family *Pleosporaceae, Bionectriaceae, Aspergillaceae, Sporocadaceae* and bacteria of the family *Moraxellaceae, Bionectriaceae, Comamonadaceae, Pseudomonadaceae*	Polyurethanase, Serine hydrolase, cholesterol esterase, urease	Russell et al. 2011; Christenson et al. 2006
Polyethylene Terephthalate	textiles, and food packaging	Fungi of the family *Trichocomaceae, Rhizopodaceae, Ustilaginaceae* and bacteria of the family *Nocardiosporaceae*, a proteobacteria *Ideonella sakiensis*	cutinase & lipase, MHETase, PETase	Kawai et al. 2019; Palm et al. 2019; Fecker et al. 2018
Polypropylene	automobile parts, fibres and fabrics	Certain Sulphate reducing bacteria, macrophages(inside body)	Myeloperoxidase	Iakovlev et al. 2015
Polystyrene	Toys, thermal insulator, television cabinet	Bacteria of the family *Pseudomonadaceae, Nocardiaceae, Enterobacteriaceae,Caulobacteriaceae* fungus of the family *Polyporaceae*, Pleoporaceae, larvae of *Zophobas atratus, Zophobas morio*	Esterase	Tahir et al. 2013
Polyethylene	Packaging, disposable bottles	Fungi of the family *Hypocreaceae, Polyporaceae, Aspergillaceae, Phanerochaetaceae* Bacteria of *Bacillaceae, Morganellaceae* and Cyanobacteria of the family *Oscillatoriaceae*, larvae of *Galleria monella, Plodia interpunctella*	Manganese peroxidise lignin peroxidise & laccase , alkaline hydroxylase	Wei et al. 2019*;* Yoon et al. 2012

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Conflict of interest

We declare that there is no conflict of interest.

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Journal of Experimental Biology and Agricultural Sciences, February - 2022; Volume – 10(1-BIONEXT-2021) page 190 – 200

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An overview of heat-stress response regulation in Gram-negative bacteria considering *Escherichia coli* as a model organism

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ABSTRACT

Response to heat stress (HSR) is a key stress response for endurance in *Escherichia coli* mediated by transcriptional factor σ-32. Even though there has been extensive investigation on the contribution of proteins and chaperones in retaining protein stability in cells under stress conditions, limited information is available regarding the dynamic nature of mechanisms regulating the activity of the highly conserved heat shock proteins (Hsps). Several gene expression-based studies suggest the pivotal role of Hsp70 (DnaK) in the regulation of the expression of heat shock genes (Hsg). Direct interaction of Hsp70 with σ-32 may regulate this function in *E. coli*. Recent studies revealed that localization of σ-32 to the membrane interior by SRP-dependent pathway enables them to function appropriately in their role as regulators. The contributions of different cellular components including cell membrane remain unknown. Other cellular components or σ-32 interfere with polypeptides which could play a crucial role in cell survival. Sigma factor monitors and preserves outer membrane integrity of *E. coli* by stimulating the genes regulating outer membrane proteins (OMPs) and lipopolysaccharide (LPS) assemblage as well as through expression of small RNAs to down-regulate surplus unassembled OMPs. σ-E activity is regulated by the rate at which its membrane-encompassing anti-sigma factor, RseA is degraded. Mutations in *rseA* are reported to constitutively increase the sigma (E) activity that is validated at both genetic and biochemical levels. In this review, the basic mechanism of heat stress regulation in gram-negative bacteria has been elaborated using *E. coli* as a model organism.

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1 Introduction

1.1 Bacterial stress response

Bacteria and other microorganisms have evolved to endure in inconsistent environmental conditions that may sometimes be adverse to their existence. A multitude of stress response programs empowers the bacteria to engender appropriate responses to diverse environmental challenges like heat shock, oxidative stress, anti-microbial agents, and nutritional restrains. A well-coordinated and effective response is generated by a complex network of regulatory systems at a global scale that helps them to preserve a stable cellular equilibrium under multiple stresses simultaneously (Scott et al. 2010). These pathways recruit suites of transcriptional regulators to remodel the cellular proteome that facilitates adaptive changes in microorganisms in variable and extreme environmental conditions (Giuliodori et al. 2007; Fiebig et al. 2015). Transcriptional regulation in response to environmental change can be deduced using three models of signal transduction (Figure 1): (i) One component system: One-component regulators are simple and abundant; consisting of a sensory input domain controlling its adjoining output domain that functions as the DNA binding domain (DBD) (Ulrich et al. 2005), (ii) Two-component system: The two-component systems comprises of a sensory protein with histidine kinase activity along with a response regulatory receptor. The response regulator stereotypically consists of a phosphoryl group-transfer domain and an output domain that interacts with the DNA. Translocation of a phosphoryl group from a His residue on the kinase to an Asp residue on the receiver domain is mediated by the sensory region of histidine kinase that in turn controls the DBD output domain (Ulrich et al. 2005), and (iii) Use of alternative sigma factors: Binding of σ-factors to core RNA polymerase (RNAP) impart promoter selectivity to regulon of gene-expression. Adaptation to non-optimal conditions by the implementation of compensatory physiological changes can be attributed to the induction of new σ-factors or by regulating its activity (Helmann 2010).

2 Types of the bacterial stress response

Stress response systems not only help in the survival of the microorganisms but can also play a vital part in the disease-causing ability of virulent microbes. Some of the most significant stress response systems of bacterial origin include: (i) Heat shock response which is primarily controlled by σ-32 that protect at elevated temperature, (ii) Envelope stress response regulates cellular integrity with the help of sigma factor σ -E together with Cpx dual-component system, (iii) Cold shock response is generated in stress condition induced by cold temperature aided by expression of RNA chaperones and ribosomal factors, (iv) General stress response modulates gene expression globally depending on the σ factor S and enables cell growth under a variety of adverse situations, (v) (p)ppGpp-dependent stringent response controls the overall physiological response of the cell when there is a limitation of nutrients through the reduction in protein synthesis capacity of the cells (Yura and Nakahigashi 1999).

3 Heat shock response (HSR)

Ritossa (1962) first recorded the event of heat shock response in a strongly amplified form of interphase chromosomes isolated from salivary glands of *Drosophila melanogaster* flies when they were

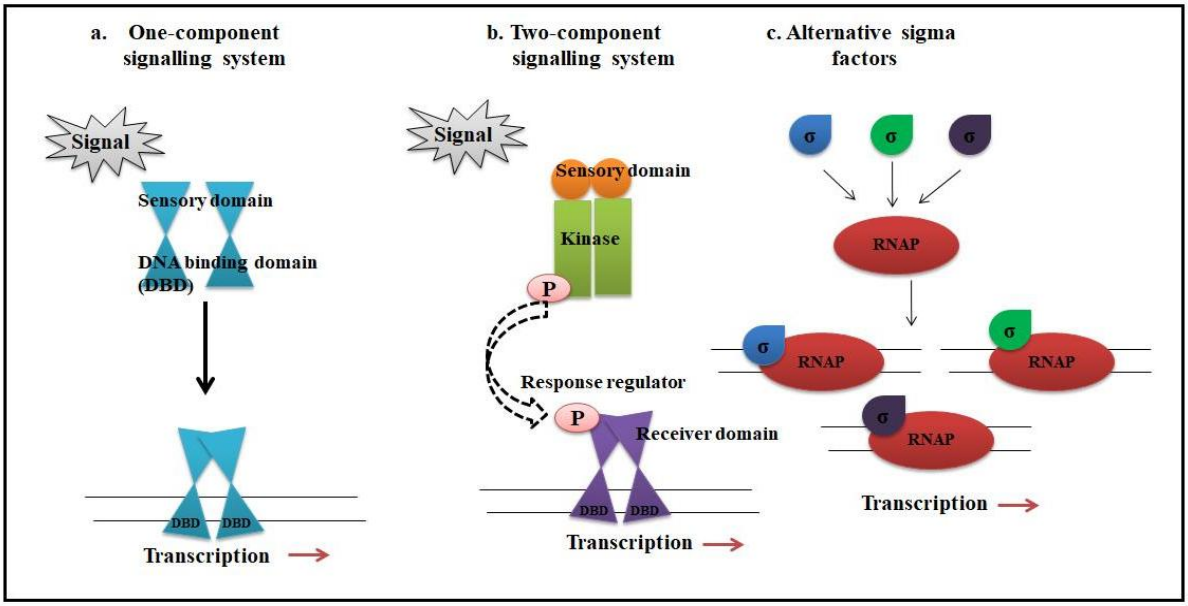

Figure 1 Modes of transcriptional regulation in response to environmental stress a. one-component signalling system, b. two—component signalling system and c. alternative sigma factor (Adapted from Fiebif et al. 2015)

shifted from their usual growth temperature to 37°C. The next step of HSP activity was detected by Tissières et al. (1974) followed by the invention of the σ-32 (σ-H) as a substitution sigma factor that is in charge of HSG expression in *E. coli* (Feng et al. 2019).

All microorganisms are equipped with well-coordinated genetic programs that allow them to survive under stressful situations. A harmonization of expression of genes that orchestrate several vital cellular processes as well as structures like those involved in DNA metabolism, determination of cell membrane composition, and house-keeping genes play a pivotal role in the improvement of bacterial stress tolerances. In the majority of the cases, cells get acclimatized to stress conditions due to elevated expression of a particular class of proteins that is dedicated to deal with the stress factor. A panel of stress proteins known as heat shock proteins (HSPs) exhibit significantly enhanced expression following a drastic elevation in temperature in course of HSR (Schumann 2016). HSR is a cytoprotective response induced by heat shock and other stressful conditions, represented by a specific program of gene expression that empowers the cells to survive and pull through from otherwise fatal circumstances. It constitutes increased production of a panel of molecular chaperones (HSPs) from the family of heat shock genes (HSGs) (Wierstra 2013). The HSPs function in regulating precise folding and localization of proteins, decreasing protein denaturation and inhibiting the clumping of oxidized proteins (Vabulas et al. 2010).

A dedicated panel of sensory biomolecules (DNA, RNA, proteins) of bacterial origin called Thermo sensors can perceive temperature variations and transduce signals from one cell to another that direct gene expression outputs. As soon as stresses signal is recognized, it acts as a stimulus to trigger specific regulatory mechanisms controlling the expression of HSPs: Positive as well as negative regulations. While positive regulation readdresses the RNA polymerase to a subgroup of designated promoters with help of specific substitute sigma factors, the negative regulation is facilitated by repressors of transcription (Roncarati and Scarlato 2017). It is mention-worthy that while several species of bacteria implement either positive or negative mechanisms solely, there are some species exhibiting coexistence of these two differing strategies. Therefore, HSG expression can be regulated by two major mechanisms: recruitment of (a) alternative sigma factors and (b) transcriptional repressors (Schumann 2012, 2016).

3.1 Principal mediators of HSR

It is crucial to maintain proteostasis in a normal cell as conservation of protein folding is essential for preserving their functionality which in turn controls the balance of cellular homeostasis. Perturbation in the programming of protein homeostasis can induce protein dysfunction that may lead to lethal consequences as severe as cell death. Critical physiological processes of the bacteria could be adversely affected by the alteration of protein structures that ultimately lead to cell damage or death (Díaz-Villanueva et al. 2015). Therefore, heat shock response confers protection against hyperthermia by immediate induction of several families of HSPs acting as molecular chaperones that impart protection from cellular stress and damage induced by protein misfolding (Vabulas et al. 2010).

Interestingly, some HSPs have copious prevalence in all metabolic conditions as they are also essential when the bacteria are growing normally. GroEL (also called Cpn60) along with DnaKem bodies two chief chaperone families Hsp60 and Hsp70, respectively in bacteria. Both of them are reported to contribute significantly to protein assembly even during the growth phase of microorganisms devoid of any stress. However, their action becomes more domineering during HSR. Accompanied by their co-chaperones GroES (also called Cpn 10) and DnaJ-GrpE, together with ATP hydrolysis, they can interact with hydrophobic moieties of unstructured proteins and promote proper folding. Another group of HSPs of bacterial origin constitutes a multi-component system of proteases that are dedicated for the clearance of non-functional polypeptides from stressed cells. The proteases comprise of subunits like ClpA/ClpX and HslU with substrate recognition attribute that when associated with respective catalytic subunits ClpP and HslV respectively remodel substrate polypeptides. The altered polypeptides are then subjected to deterioration by proteolysis (Missiakas et al.1996; Wawrzynow et al.1996). HSP family also comprises of members such as Lon, FtsH, and DegPthat conglomerate chaperone activity with protease activity on a single polypeptide. Small HSPs are a diverse panel of proteins that are committed to provide protection to proteins in unfolded conformation by binding with them until they will be re-assembled to functional form by ATP-dependent chaperones (Matuszewska et al. 2005). Also, the HSPs are directly or indirectly associated with microbial pathogenesis. In this context, there are many instances where molecular chaperones serve miscellaneous purposes other than mere protein folding. For instance, a GroEL paralog of *Mycobacterium smegmatis*, named GroEL1, exhibit no activities related to heat shock but is associated with the establishment of biofilm and synthesis of mycolic acid (Ojha et al. 2005). Also, human gastric pathogen *Helicobacter pylori* possess a GroES homolog that along with its co-chaperonin function, contributes to its pathogenicity by participating in the storage and trafficking of one of its virulence factors Ni^{2+} ions (de Reuse et al. 2013). There are also instances where molecular chaperones might act as virulence factors directly. It is anticipated that during host-microbe interaction, numerous species of bacteria utilize cell surface GroEL and DnaK chaperones for adhesional though this concept demands experimental validation. The chaperones also contribute to cell-to-cell communication, induction of pro-inflammatory cytokine production as well as in apoptosis (Henderson and Martin 2011).

3.2 Regulatory mechanism of HSR

In physiological temperatures, the conformation of a macromolecule permits either only the basal level of HSG expression or is maintained in an inactive form until it receives the stimulus of heat shock. HSR is regulated by RNA, DNA, and proteins with thermo-sensory properties (Schumann 2012). Nucleic acid thermo-sensors can acquire two different temperature-dependent secondary structures that allow either a ground-level or elevated level of gene expression at mRNA as well as protein levels. The chaperones sequester functional σ factors rendering them unavailable or maintaining transcriptional repressors in an active form at physiological temperatures. Following the shock due to elevated temperature, as the inactivated proteins get titrated by chaperons the transcription factors will prevail in its non-functional state allowing the σ factors to interact with the core enzyme of RNA polymerase. With the propensity of denatured proteins being subjected to refolding or protease-mediated degradation, the chaperones will have greater scope to regulate protein turnover with help of transcriptional regulators.

4 Heat-shock transcription factors (HSF)

A group of proteins functioning as HSFs is responsible for regulating HSP expression at transcriptional levels. Hsf1 is the most well-studied transcription factor of all the reported ones that is crucial for HSR (Pirkkala et al. 2001). Hsf1 mostly has cytoplasmic localization as a non-functional monomer, in association with Hsp70 and Hsp90 under normal physiological conditions (Vabulas et al. 2010). Hsf1 dissociates from this complex when exposed to the selective pressure of thermal stress. Hsf1 then forms a trimeric complex followed by phosphorylation and the phosphorylated protein then enters into the nucleus promptly to activate the expression of HSP genes. Hsf1 activation is associated with its detachment from the chaperone complexes which is proposed to be triggered by the interaction of the accumulated unfolded proteins with Hsp70 and Hsp90 (Figure 2) (Jacobs and Marnett 2010). Likewise, electrophiles like HNE, nitroalkenes, sulforaphane, and 15d-PGJ2 can react with target cysteine residues on Hsp90 and Hsp70 covalently, prompting them to dissociate from Hsf1. This is followed by elevated accumulation

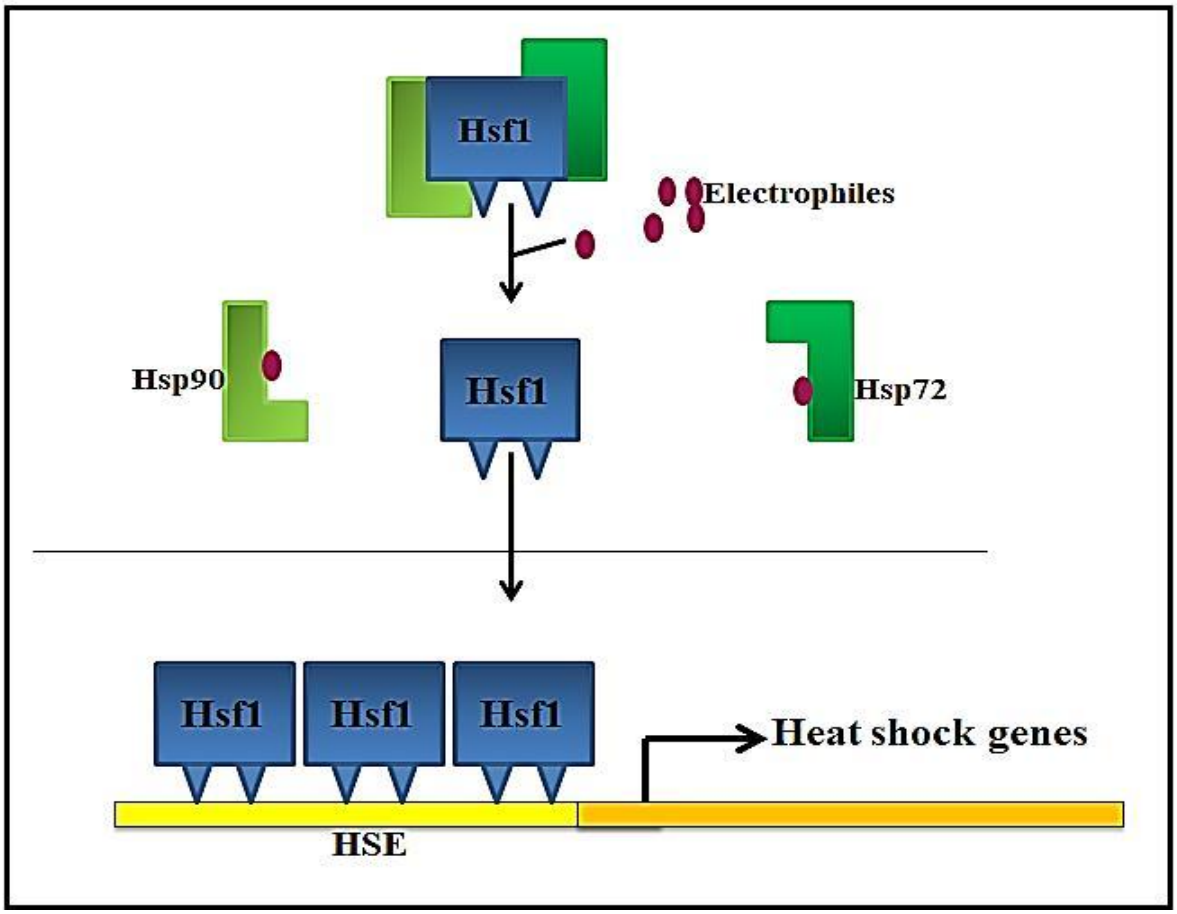

Figure 2 Pictorial representation of mode of action of HSF: Under the selective pressure of thermal stress and accumulation of unfolded proteins, in presence of electrophiles inactive monomer of Hsf1 is detached from Hsp70 and Hsp90 which then forms a trimeric complex followed by phosphorylation. This is followed by entry of the phosphorylated protein into nucleus to activate HSP gene expression.

of Hsf1in the nucleus and subsequent HSR activation (Vabulas et al. 2010; Kansanen et al. 2011).

5 Heat-shock regulations in *E. coli*

Escherichia coli K-12 strain was the first candidate microorganism subjected to heat-shock response related studies in bacteria where the operons dedicated for HSR are equipped with defined promoters regions identified by σ-32 that perform the role of a transcriptional activator. The half-life of σ-32 is short as the proteolytic activity of the gene product of *hflB*(*ftsH*) leads to its degradation (Yura 2019)**.** Every bacterium possesses one primary sigma factor (*E. coli*: σ-70) with multiple alternative σ factors. While the housekeeping σ factors control the expression of genes essential for bacterial growth and propagation, the alternative counterparts are activated only when cells are subjected to certain stressful events affecting their cellular physiology. There are reports of the existence of six, sixteen, and even sixty-two varieties of substitute sigma factors in *E. coli* K12 strains, *B. subtilis*, and *Streptomyces coelicolor* respectively. In *E.coli* two diverse forms of regulators of heat shock σ-32 and σ-E have been reported till date. Following a heat shock (for instance, an abrupt elevation in temperature from 30°C to 42°C) buildup of unfolded polypeptides in the cytoplasm prompts σ-32 activation. In ambient temperature σ-32 is produced in nominal concentration with a half-life <1 min. On the other hand, amassing of denatured proteins in the periplasm triggers activation of σ-E.

5.1 σ-32 heat shock regulon

Three different mechanisms function in consortia to endure stress due to elevated temperature and to preserve protein stability and functionality in viable cells by regulating σ-32 activity (i) at 30°C a partial secondary structure formation causes sequestration of the Shine-Dalgarno sequence of rpoH mRNA coding for σ-32. So at this temperature translation is witnessed only at a minimal level. However, following a temperature change to 42°C, RNA strand separation leads to an elevated level of protein expression (Yura 2019), (ii) when the temperature is low then either DnaK/DnaJ/GrpE or GroEL/ES chaperone system promotes majority of σ-32 molecule sequestering. Once a heat shock is triggered, the chaperones detach from σ-32 allowing them to bind to the denatured proteins through the process of chaperone titration (Gamer et al.1992). Overexpression of any one of these chaperone systems rapidly inhibits σ-32 activity while overexpression or depletion of chaperon substrate increase or decrease σ-32 activity, and (iii) at 30°C, majority of σ-32 molecules in free conformation are directed to FtsH protease or ClpXP protease either through association with signal recognition particle or modification by ubiquitin-like protein respectively for denaturation (Kanemori et al. 1997; Xu et al. 2015; Miyazaki et al. 2016).

Once subjected to thermal stress, there is a fast rise in the concentration and functionality of σ-32 due to increased expression of rpoH. Concurrent to this, the buildup of unfolded proteins in the cytoplasm causes momentary sequestration of the chaperones (5–10 min) that in turn leads to stabilizes σ-32. Both mechanisms lead to a prompt 12- to 15-fold surge in σ-32 content with a10 minute half-life ensuing the induction phase (Schumann 2016).

5.2 RpoH or σ-32: Controller of HSR

The sigma factor RpoH (also known as σ-32) is the prime regulator of expression of a majority of the heat-shock genes such as proteases and chaperones in *E. coli* as a component of the heat-shock regulon (Yura and Nakahigashi 1999). Transcriptional regulation of *rpoH* during translation is attributed to secondary structure formation in mRNA and post-translationally by FtsHand other proteases. The secondary structure of the RNA thermometer (RNAT) encompassing the 5'-UTR to a portion of the coding region of the *rpoH* transcript controls translation efficiency. At normal temperature, the closed conformation of RNAT prevents ribosome binding to the transcript while elevated temperature prompts melting of the secondary structure allowing ribosome to initiate the process of protein synthesis (Nagai et al. 1991; Yuzawa et al. 1993). At physiological temperatures, DnaK/DnaJ/GrpE and GroEL/GroES chaperone systems associate with RpoH and guide it for FtsH-mediated degradation. Stress induced by heat shock enhances intracellular concentration of misfolded proteins and dissociates the chaperone systems from RpoH and permits it to accompany the core RNA polymerase (RNAP) inducing transcription of HSGs many of which translate for chaperones and proteases (Gamer et al. 1996; Horikoshi et al. 2004). These proteases as well as chaperone took part in recovery from the heat-inflicted impairment of activity. As a result HSR is turned down rendering sufficient concentration of existing DnaK/DnaJ to sequester RpoH again (Blaszczak et al. 1995; Gamer et al. 1996) (Figure 3). Studies reveal that amino acid residues L^{47}, A^{50}, and I^{54} located on a superficial α-helix in the 2.1 region of amino-terminal domain of RpoH protein is associated with protein stability and this is the segment that binds with core-RNA polymerase. However, this area 2.1 of RpoH is vital although not adequate enough for denaturation by FtsH. In C region of RpoH that is located in the center of the sigma factor another region has been identified that binds with the RNA polymerase involving amino acid residues A^{131} and K^{134} which has also been reported to be linked with RpoH decay (Obrist et al. 2009). This location in the C region is identified as the turnover component for proteolytic degradation by FtsH protease. An RpoH fragment encompassing 37–147 amino acids consisting of regions 2.1 and C is found to be degraded by FtsH. This observation signifies that these two sites are adequate for precise identification of RpoH by FtsH and subsequent degradation (Obrist and Narberhaus 2005; Obrist et al.

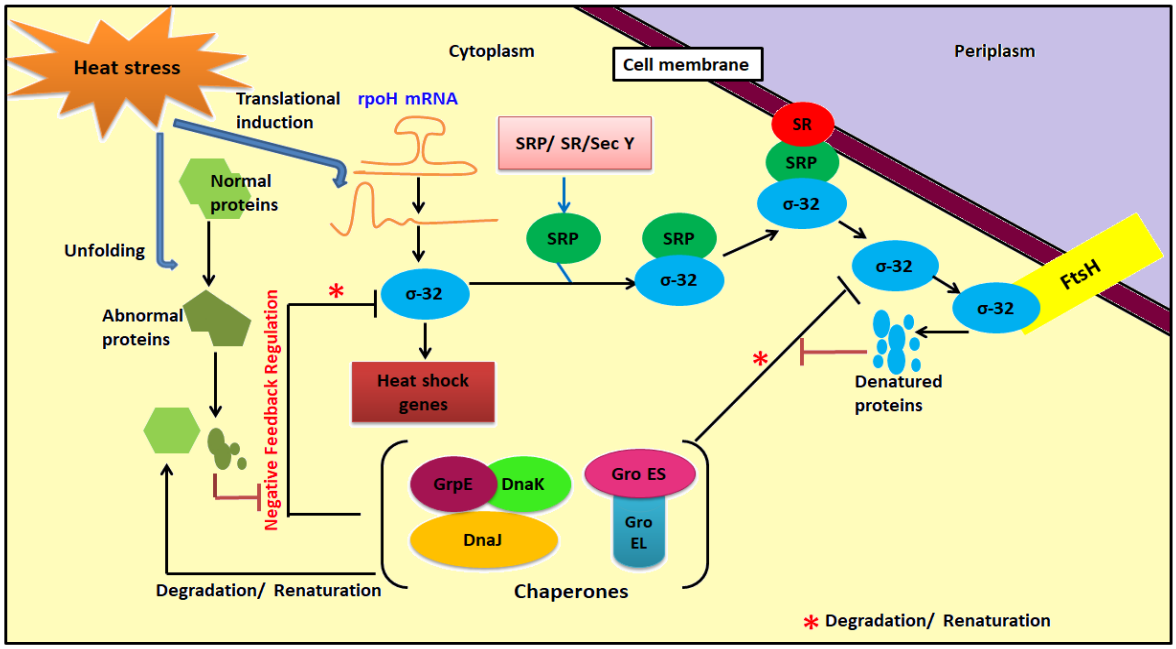

Figure 3 Pictorial representation of regulation of σ-32 mediated heat-shock response in *Escherichia coli:* σ-32 is the master regulator of transcription of most of the heat-shock genes in *E. coli*. σ-32 expression is regulated at translational level by secondary structure formation in mRNA and post-translationally by FtsH and other proteases. Rise in temperature allows melting of the secondary structure followed by protein synthesis.At normal temperatures, chaperone systems DnaK/DnaJ/GrpE and GroEL/GroES associate with σ-32 and guide it to FtsH-mediated degradation. Thermal stress elevates intracellular concentration of misfolded proteins, detaching the chaperone systems from σ-32 and allowing it to induce expression of heat-shock genes. Also, SRP/FtsY-dependent pathway recruits σ-32 first to the inner membrane followed by transfer to the chaperone-dependent system to induce FtsH-induced degradation.

2007, 2009). There is direct interaction of DnaJ with region 2.1triggering conformational changes that are further presumed to induce DnaK-attachment to the site 3.2 of RpoH (Rodriguez et al. 2008; Suzuki et al. 2012; Noguchi et al. 2014; Miyazaki et al. 2016).

Apart from *dnaK/dnaJ/grpE*, *groEL/groES*, and *ftsH*, the *ffh* gene is a component of the σ-32 regulon (Nonaka et al. 2006). Ffh protein coupled with the 4.5S RNA constitutes the signal recognition particle (SRP) which along with its membrane-bound receptor FtsY, directs RpoH degradation. Independent of DnaK/DnaJ binding, the signal peptide binding site of Ffh binds associates with the hydrophobic residues in the amphipathic helix of site 2.1 of RpoH assigned with the function of homeostasis control. Thus, the SRP/FtsY-dependent pathway recruits RpoH first to the inner membrane followed by handover to the chaperone-based system to prompt FtsH-induced lysis (Lim et al. 2013; Miyazaki et al. 2016) (Figure 3).

5.3 FtsH and regulation of σ-32-mediated HSR

FtsH protease is a membrane-anchored AAAmetallo protease that is crucial for maintaining intracellular stability of σ-32 along with other proteases like HslVU (Herman et al. 1995; Tomoyasu et al. 1995). However proteolytic enzymes like HslVU may also cause σ-32 denaturation significantly (Kanemori et al. 1997). Three of the well-characterized substrates of FtsH include LpxC, the main biocatalyst of LPS biosynthesis; RpoH/σ-32, the alternative heat-shock σ-factor, and YfgM, a membrane-bound protein with a dual role, concerned with cytosolic stress adaptation and periplasmic chaperone activities. Through degradation of LpxC this universal protease regulates the ratio of phospholipid and LPS in the outer membrane. Thus, the concentration of lipid and sugar residues of lipopolysaccharides together with free forms of SecY protein is regulated not only by σ-32 but also by FtsH. Therefore both of them contribute to the regulation of membrane protein transport (Ito and Akiyama 2005; Bittner et al. 2016). During HSR, the growth rate of the cells is slow, so a lesser amount of LPS is required and hence LpxC level is adjusted by FtsH-dependent degradation.

In ambient growth conditions under normal temperature σ-32is subjected to FtsH-mediated degradation through DnaK/DnaJ chaperones. Under heat stress, the chaperones are released from σ-32by non-functional proteins leading toσ-32 stabilization. σ-32 in free form the associated with RNA polymerase to trigger heat-shock regulon expression.

6 Heat shock regulon of σ-E

σ-E or σ-24 belongs to the family of extra-cytoplasmic function σ-factors that have the potential to respond to a diverse form of stress

stimuli like cell envelope stress and oxidative stress (Helmann 2002). In *E. coli*, the *rpoE* gene of operon rpoE-rseA-rseB-rseC encodes for σ-E. An antagonist sigma factor of σ-E, named RseA, is a transmembrane protein that prevents the collaboration of σ-E with core RNAP by entrapping it to the membrane (Missiakas et al. 1997). A periplasmic protein named RseB interacts with RseA functioning as a co-anti-sigma factor (Cezairliyan and Sauer 2007). RseC protein, located in the inner-membrane, positively regulates σ-E activity through a mechanism yet to be deciphered. In course of a cell envelope stress, the outer membrane proteins (OMPs) together with lipopolysaccharides mediate the detachment of σ-E from its anti-σ factor through proteolytic cleavage. The C-terminal domain of unfolded/ denatured OMPs is identified by DegS protease that causes detachment of RseB from RseA along with concomitant inactivation of the latter anti-σ factor in its periplasmic milieu (Walsh et al. 2003; Grigorova et al. 2004; Mecsas et al. 1993; Lima et al. 2013). Then, within or in close vicinity to the transmembrane region RseP proteolytically activates RseA releasing a shortened version of the latter in the cytoplasm (Kanehara et al. 2002, 2003). In the end, the remaining fraction of RseA will undergo complete degradation by one of the several cytoplasmic proteases (Chaba et al. 2011). Finally, upon cytoplasmic release σ-E will bind to RNAP directing it to σ-E-dependent promoters to activate σ-Eregulons comprised of a total of 89 transcriptional units.

Research in the last few years has demonstrated that the thermal stress-related response of *E. coli* forms the basis of the heat-shock response characteristic of other bacteria. *E. coli*, *P. aeruginosa*, *V. cholera*, and other members of γ2 and γ3 purple bacteria are reported to be unique as the sole regulator of their HSGs appear to be σ-32 with no additional explicit control element being identified. In other eubacterial groups manifold regulatory mechanisms dictating HSG transcription have been recorded. For instance, in *Bacillus subtilis*, a minimum of three clusters of heat-shock genes have been identified, among which only one is triggered byσ-B (Yura et al. 1993).

7 Insight from ribosomal profiling

Zhang et al (2017) performed ribosome profiling to excavate translational regulation in *E. coli* under heat stress. Alteration in ribosomal footprints was found to coincide with changes in transcript level upon thermal stress. Concerning transcript level and translational efficiency, expression profiling revealed upregulation of 58 genes with simultaneous down-regulation of 57 genes under thermal stress compared to normal conditions. Gene ontology and KEGG pathway-based analysis of the functional implications of this altered gene expression profile revealed a significant correlation of the two-component system pathway with heat stress in terms of 5 genes, namely, *rstA, frd, dcuB, phoB* and *pstS*. RstA is a translational controller of effector proteins of the two-component system that responds to environmental stimulus together with RstB. On the contrary, genes associated with cellular growth, amino acid biosynthetic pathways (*metE, asd, serA, mtn*), and ribosomal assembly (*rps K* and *rps Q*) along with translational efficiency (*infA* and *inf C*)are down-regulated (Zhang et al. 2017).

8 Possible application of the *E.coli* HSR

The HSR is instrumental in conferring the cell with protection against adverse conditions that elevate the levels of denatured proteins, namely, viral infection, heat shock, high alcohol concentrations, UV irradiation, oxidative stress, heavy metals, and recombinant protein production (Zhao et al. 2005; Li et al 2007). These stress factors can be detrimental to the cells altering their biological activities (Carroni et al. 2014). In the confrontation with such stressful situations, cellular responses are manifested in form of synthesis of HSPs like proteases and chaperones. If there is misfolding or unfolding of proteins, chaperones assist in protein renaturation (Müller et al. 2013). The HSR mechanism, though very complex, has a great possibility of being utilized in different approaches to synthetic biology. The creation of a library of biological components that are large in number, with predictable activities as well as can be designed, are suitable for easy integration into complex genetic systems (Seo et al. 2013; HoynesO'Connor and Moon 2016). For instance, enhanced complexities of pathways necessitate the expansion of the array of inducible promoters and other regulatory components that are available. Many of the members comprising the HSR mechanism of *E.coli* are suitable for becoming parts of a toolkit of well-studied biological components that in turn can be implemented for the construction of devices/systems, like biosensors and microbial cell factories. Such devices have a wide range of applications in environmental remediation, health care, or industrial sectors (Rodriguez et al. 2008). The sensing unit can be composed of a heat-shock promoter region stimulated by triggering the *E.coli* HSR by the diverse form of stress conditions like heat shock or elevated levels of chemicals. Heat shock promoter activation mediates the expression of a reporter gene. Such biosensors are highly sensitive to diverse applications. Moreover, this strategy can also be employed to translate a protein of interest producing a compound of therapeutic significance in the industry such as antibiotics (Rodriguez et al. 2008; Carroni et al. 2014).

9 Discussions

In this review, we have emphasized on the significance of an abrupt reaction of the bacterial population to temperature alterations and appraised the main mechanisms are adopted to counteract impending cellular damage. There are a plethora of diverse strategies availed by bacterial cells to regulate the highly conserved defense strategy of HSR. Regulation of HSG gene expression is proficiently realized by proteins with specialized

regulatory potentials which exert their beneficial or adverse effect on the gene expression by the RNAP. For reprogramming of gene expression in response to stress perception, both positive as well as negative regulatory modes, alone or in combination, orchestrate with posttranscriptional machinery to be utilized by bacteria. The model organism *E. coli* has been used for a long as the central archetype for elucidating the basis of stress-instigated HSG transcription through the usage of alternative σ-factors. After extensive research over several years, σ-32 homeostasis is achieved when chaperone-mediated modulation of σ-32 stability is complemented by regulation at the RNA and protein level. Also, the recent identification of SRP-SR co-translational targeting system-mediated localization of the heat-shock sigma factor in the inner membrane augments the understanding of the intricate mechanism of heat shock response regulation. Incorporation of Tn5 transposon upstream of *ftsY* gene encoding SR, the receptor for SRP significantly increased σ-32 concentration in presence of excess chaperones. This observation confirmed that for the proper functioning of heat shock regulation it is essential for σ-32 to be transmitted to membrane interior by SRP-SR-SecY pathway (Lim et al. 2013; Miyazaki et al. 2016). Nevertheless, further experimentations are required to understand other mechanistic facets of the HSR driven by σ-32 and other molecules in *E. coli*.

Conclusion

HSR is a highly preserved defense strategy in bacteria against environmental stress that is regulated by a plethora of diverse strategies. There is a panel of dedicated regulatory proteins to reprogram the expression of heat-shock proteins through positive and/or negative modes of controlling transcription as well as posttranscriptional regulatory mechanisms.

The model organism *E. coli* is an archetype to elucidate the mode of stress-induced activation of HSG expression based on the usage of alternative σ factors. Several studies have revealed the existence of a multifaceted regulatory cascade of gene expression control in combination with the regulation of σ32 stability and homeostasis driven by chaperons. Recent studies on the localization of heat-shock σ-factor internal to the plasma membrane through SRP-SR co-translational targeting system has further enhanced the understanding of the mechanism of HSR in bacterial cells. In *E. coli* HSG expression is controlled solely by the positive mechanism of regulation. σ-32 or σ-E/ σ-24 take part in heat-shock regulation with σ-32 playing an important part in recognizing signal from both cytoplasm and inner membrane, while σ-E/ σ-24 is devoted to stress response beyond the cytoplasm.

Several studies have shown that HSR in *E.coli* involves complex interactions where chaperones and proteases regulate functionality and stability of σ-32 respectively involving feedback loop. With the help of such feedback loops, the system becomes capable of functioning if there is any variation in its physical parameters, particularly in their permissible range. Further elaborate studies will be vital for exploring diverse aspects of the multifaceted HSR schemes.

Conflict of Interest

Nil

Authors' Contribution

Conception: Deborupa Paul and Sanmitra Ghosh; Literature review: Deborupa Paul and Sanmitra Ghosh; Data interpretation: Deborupa Paul and Sanmitra Ghosh; Drafting the manuscript: Deborupa Paul and Sanmitra Ghosh; Supervision: Sanmitra Ghosh

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Journal of Experimental Biology and Agricultural Sciences, February - 2022; Volume – 10(1-BIONEXT-2021) page 201 – 210

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Artificial Womb Technology: A Roadmap to a changing Medico-Legal Landscape

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ABSTRACT

Scientists worldwide have tried to replicate birth processes for years, which have resulted in many new infertility solutions like in vitro fertilization (IVF) or surrogacy, but Artificial Womb Technology (AWT) is the most advanced and unique. AWT proposes an alternative to conventional pregnancy and childbirth. Presently, there is no prototype of an artificial womb for people. The innovation is particularly in its early stages. However, we do have to think about the scientific moral, and legal issues before racing into this innovation. We also need to deal with social, religious economic, and health issues. Here in this paper, we have specifically done a critical analysis of the bioethical issues concerning this upcoming technology. A transdisciplinary approach encompassing both the legal and scientific viewpoints, concerns, and suggestions related to this new technology has been discussed. We strongly suggest a worldwide discussion and be ready with a strong framework before we practice AWT, a venture whose outcomes are yet awaited.

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1 Introduction

Innovation that can support the artificial growth of human fetus will change the most significant and changeless notion of human multiplication that has been there since forever: that a baby should gestate in a woman's body' (Jennifer 2006). Two groups of researchers from Australia, Japan, and the United States got some optimistic outcomes from examining organism development in artificial womb models, pulling in huge media consideration, in 2017 and 2019 (Partridge et al. 2017; Usuda et al. 2019), also, more as of late a third exploration group situated in the Netherlands declared that they had gotten a considerable fund to build up an AW prototype (Davis 2019). The progressing efforts of these researchers (Table 1) and clinical specialists are gaining significant headway towards improving an AW tool that can mimic the cycle of normal pregnancy in ectogenesis.

1.1 History

Chronologically, the recorded data finds the mention of Ectogenesis in the early twentieth century. At that time, it found alchemists like Paracelsus writing about Ectogenesis as future technological development (Hedesan 2014). Despite so, we can find references to Ectogenesis through various mythologies around the world. It is beyond doubt that Hindu mythological scriptures and even Epics are storehouses of unexplained references in biology and technology.

One such that seems quite relevant is a direct reference from the Epic – 'Mahabharata'. The storyline depicted one royal queen named Gandhari, unable to deliver a baby, falling victim to miscarriage. Then Saint Veda Vyasa who was her father-in-law put the parts of the disintegrated embryo into jars, which later became the "Kaurava Princes" (Kalra et al. 2016).

Christopher Kaczor categorized Ectogenesis into "partial ectogenesis" and "complete ectogenesis". The former deals with some part of the gestation outside the natural womb, whereas the latter involves complete incubation in an artificial womb. These divisions have been monumental in categorizing further research into the subject (Kaczor 2010).

In 1932, Aldous Huxley presented a dystopia having a concept of the artificial womb (AW) in his science fiction novel "Brave New World" (Huxlay 1932). Emanuel M. Greenberg, a New York doctor and inventor developed a design for an artificial womb and filed a patent for the same in 1955 (Greenberg 1955). Dr. Yoshinori Kuwabara, a researcher from Juntendo University in Japan, published his work on partial Ectogenesis. His research involved gestating a goat embryo outside the maternal womb in an artificial environment made out of a plastic container containing solution mimicking amniotic fluid (Kuwabara et al. 1989).

In 2001, Hung C L from Cornell University crafted an artificial uterus by using biodegradable scaffolding, and in vitro cultured human endometrium cells. She grew a surplus human embryo obtained from fertility banks for about six days before the experiment was terminated (Liu et al. 2001). While there has been little development in the field, it is also fixed with skepticism from researchers. An eminent fertility specialist in California, David Adamson accepts that AWs are still many years away from being clinically possible because of immunological and cardiovascular issues (Rosen 2003).

Table 1 Timeline of the developments in Artificial Womb Technology

Year	Development in AWT	Reference
Earlier 20th century	Paracelsus Wrote about Ectogenesis as future technological development.	Hedesan 2014
1932	Aldous Huxley Presented a story version of the artificial womb in his novel "Brave New World".	Huxlay 1932
1955	Emanuel M. Greenberg, a New York doctor and inventor, created a design for artificial womb and filed patent for this.	Greenberg 1955
1989	Yoshinori kuwabara and his group from Japan were able to gestate a goat embryo in an artificial environment made up of plastic container having solutions mimicking the amniotic fluid.	Kuwabara et al. 1989
2001	H C Liu of Cornell University crafted an artificial uterus using biodegradable scaffolding, and in vitro cultured human endometrium cells.	Liu et al. 2001
2015	Miura and his group developed an “ex-vivo uterine environment (EVE)” therapy utilizing an artificial placenta for providing nutrients and gas exchange to developing lamb.	Miura et al. 2015
2017	Partridge and his group from USA were able to provide physiological support to extremely premature lamb for up to 4 weeks	Partridge et al. 2017
2017	Dr. Flake, presently a foetal surgeon at a Children's Hospital in Philadelphia received the "Scientist of the Year Philly Geek Awards" for delivering premature lambs in "Bio bags".	Tom 2017
2020	Dr. Guid from MMC, Netherlands, proposed to develop a protocol for an artificial womb within 5 to 10 years after receiving a new €2.9 million grant from the EU program “Horizon 2020” for researchers in Eindhoven.	*TU/e 2020*

In 2017, Dr. Flake, presently a fetal surgeon at a Children's Hospital of Philadelphia received the "Scientist of the Year Philly Geek Awards" for delivering premature lambs in "Bio bags". These were fluid-filled AWs that mimicked the mother's womb to quite a realistic level (Tom 2017). A subsequent research group in Australia devised an elective plan named the “EVE (ex-vivo uterine environment) platform' for extended survival of a fetus in an artificial environment (Usuda et al. 2019).

2 Project Artificial Womb on Human Beings

The development of human beings through artificial means outside the mother’s womb is controversial, particularly when we are not sure of justified advantages. The U.S. Office for Health and Human Services, British Medical Research Council, and The World Medical Association, in any case, all acknowledge that non-therapeutic exploration in such conditions may be legitimate as it can save the life of a premature baby or in conditions where a mother is medically unable to continue the gestation.

Brazier and Alghrani reason that the capability of the artificial womb to “improve the care of premature babies provides a strong case for permitting ethically approved research”. Subsequently, the AWT trial can be defended, albeit the truth, as indicated by Brazier and Alghrani, is that investigation into Ectogenesis being in any underlying concern's general benefits is debatable. The utilitarian significance of advocating the use of artificial womb technology is that in such cases the research includes the prementioned convention, strong examination plan, defined protocol, and systems intended to guarantee the assurance of subjects and to guarantee the creation of generalizable information. The research should be allowed in case of clinical premature birth (Alghrani and Brazier 2011). Administrative offices, like FDA (Food and Drug Administration) in the US or the TGA (Therapeutic Goods Administration) in Australia would necessitate broad clinical proof before these devices could be used for ectogenesis. FDA has a risk-based device classification based on the safety and efficiency of a medical device. Class I, II, and III devices represent low to moderate risk, moderate to high risk, and high risk, respectively. AWT is probably going to fall in class II/ III. The FDA has the power to require that gadgets of high-risk category settle pre-market endorsement. So they can't be by, and large utilized before clinical examinations have exhibited their wellbeing and viability. Studies with the prospective for significant danger should be supported by an Institutional Review Board (I.R.B.) and the F.D.A. Comparable progressive characterization frameworks, with measures requiring broad proof for medical devices to access high risk to patients, have been set up in Australia and Europe (Romanis 2020a). There are two classifications for the use of Artificial Womb for ectogenesis i.e. (a) ex vivo pregnancies or Complete ectogenesis: the embryo is managed with IVF strategies and it is straightforwardly embedded into the artificial womb for the whole incubation; and (b) ex utero pregnancies or partial ectogenesis: the embryo begins the natural pregnancy in the womb of a mother and later it is implanted into an artificial womb for the remainder of the development.

In the Eindhoven University of Technology (Netherlands), Scientists have got a Future and Emerging Technologies fund from the European Union Program, “Horizon 2020” of just about 2.9 million Euro to develop a protocol for an artificial womb within 5-10 years. The replica, which is being created, would incubate babies with artificial conditions for development and breathing (Davis 2019). Outstandingly, if there should be an occurrence of untimely parturition, the artificial womb will be a sufficient replacement for the unfavorable conditions of the mother's uterus. It will give an indigenous habitat to the infant so that it faces the least disturbances due to the transition from a natural womb to an artificial environment. In contrast to existing incubators, the Aws would be like a natural ambiance. Fluids, oxygen, and supplements would surround the child within the artificial placenta and will interface with the umbilical cord. Premature birth has been a significant issue, influencing more than 1 out of 10 children worldwide. Prematurely born children might have more medical conditions or require remaining in NICUs (Neonatal Intensive Care Units), which help in supporting their cardiorespiratory capacity and advancement to the entire period of gestation. Despite development in medication for premature babies, NICUs are not a very good standby for the safe and protective environment of the mother's womb (Brado et al. 2021).

An artificial womb may be a better alternative to an incubator and artificial respiration owing to its close resemblance with the natural womb. Guid Oei, a renowned gynecologist at Maxima Medical Center(Netherlands), said "Using this artificial womb, we want to help extremely premature babies through the critical period of 24 to 28 weeks,"

"Over the next eight years, we're going to develop these technologies, and come up with the first prototypes of the artificial womb. Once these have been carefully tested, we want to help the first extremely premature baby in our artificial mother in eight years' time in the first clinical tests. That's quite the challenge," says Oei. (TU/e 2020)

3 Ethical Issues

One cannot pick a side in the battle of ethics when it comes to a matter such as Ectogenesis. It could be beneficial in saving the lives of premature babies, helping in fecund couples, giving gay and transgender people new procreation alternatives, and aged parents to experience parenthood. AWT could provide a substitute for conventional pregnancy and childbirth. The fetus could avail a healthier environment free from drugs and alcohol contamination

supplemented with an absolute equilibrium of nutrients, sound, temperature, and movement. AWT will also help to balance gender inequalities.

Smajdor, a supporter of ectogenesis, argues that “the fact that women have to gestate and give birth to have children, whereas men do not, is a prima facie injustice that should be addressed by the development of ectogenesis” (Smajdor 2007, 2012)

On the other hand, we find experts such as Dr. Randy Morris of the University of Illinois, School of Medicine, and Dr. Foreman, (Bioethics Group, David Geffen School of Medicine at UCLA) inquiring about the capability of AWT to recreate the complicated technique at service throughout the regular pregnancy. Their main doubt is regarding the AWT's capacity to perpetuate the amount of blood flow needed to sustain a child during pregnancy (LaFee 2004). Dr. Nobuya of the Kuwabara research group also acknowledges the concern raised due to challenges faced by the technology. The amount of extracorporeal blood needed to endure one extrauterine gestation would be excessively costly (David 2010).

3.1 The Morality of Artificial Womb Technology

Though Ectogenesis showers some magnificent opportunities to the couple who cannot conceive, it ethically violates the natural law of birth. Ethically and according to the natural law of birth, a baby is meant to be born in a mother's womb and whenever this baby delivering method is manipulated by human intervention, and then it becomes unethical. So, if such a technique is not considered to be ethically correct, then what will be the future of the "Artificial Womb" technique in society or the world at large? This question remains unanswered till the ethical boards of society agree.

3.2 Religious Issues

Technology has not always been accepted with open arms. There is always friction from the orthodox part of society. From Galileo's derivation of the sun being in the center to Ectogenesis, all questions religious teachings and beliefs, leading to social aggression upholding the moral permissibility of such scientific developments. Even though the official and authoritative teaching of the Catholic Church has not yet given a conclusive pronouncement on the official authority of AWT. "Donum Vitae" (Roman Catholic published “Instruction on Respect for Human Life in its Origin and on the Dignity of Procreation”) addresses it straightly. It says, “*These procedures are contrary to the human dignity proper to the embryo, and at the same time they are contrary to the right of every person to be conceived and to be born within marriage and from marriage*” (Donum vitae 1987). The maternal instincts are put into question when a woman opts for such a procedure to preserve her physical appearance and limitations on food and alcohol consumption and to elude the physical problems in the ordeal of pregnancy and childbirth.

Secular bioethicist, Deane Wells and his collaborator Peter Singer, famously write "*freedom to choose what is to happen to one's body is one thing; freedom to insist on death of a being that is capable of living outside one's body is another*" (Shook and Gelfand 2006).

The greatest challenge to the Church's and other religious beliefs would be when homosexual men could use the technology to 'procreate' without the need of the opposite sex to serve as a surrogate where male-derived embryonic stem cells could be used to develop female oocytes (Rosen 2003).

This could be the gateway to someday creating a fetus out of the genetic elements of both homosexual couples. With the growing acceptance of the LGBTQ community worldwide, AWT will be helpful to cater to their procreation requirements.

3.3 Social Issues

AWT infringes women's "right to choose" in many ways. It should be a mother’s choice whether to remove an embryo developing in her womb in case of any unavoidable medical emergency. If a developing embryo or a fetus is not operable and the mother wants it to be removed out of her womb, it's her choice. The worst-case scenario would find women being forced to have their fetuses withdrawn and gestated out of their bodies. This could be a serious issue in countries where a conservative mindset dominates society. There is universal concurrence that artificial womb technology is appreciated both to support preterm and to diminish others of the loads leaned merely on them for giving birth to offspring. AWs could reduce the loads set only on ladies in multiplication, because the innovation may decrease the requirement for pregnancy.

We live in a society where an invention comes with many criticisms before it gets approved on a large scale. So, an "artificial womb" technology is not an exception to that. It will also have to be approved by society for complete success.

3.4 Economic Perspectives

If such a procedure gets to be funded by private business organizations, then the people at the helm of these organizations would be quite the authority to conclude how, when and for whose advantage it should be put to use. In the bounds of state, private insurance companies, this could be an escape from covering the unpredictable cost of traditional childbirth. This could create a divide in a society where AWT would be the flag bearer of the privileged class and conventional pregnancies related to those who cannot afford it. Some crucial points to be noted in this regard are (i) Considering the cost incurred in AWT, it is presumed that such

a technology will be only helpful for rich people urging for parenthood, (ii) Another crucial point is that if the machine goes wrong and the procedure does not execute positively, who will be responsible for such a loss? Will it be the doctors, institution, or the prospective parents who will bear the cost?, (iii) Considering that even if the doctors and/or organizations are held responsible, will they compensate and give it another try for their mistake? There is the least probability for such compensation, and (iv) Moreover, if we blame the machine (the artificial womb) for the loss, it does not matter as a machine is nothing but a non-living object operated by humans. So, in this technique, we are simply leaving the fate of a living being to a non-living object.

3.5 Global Perspectives

Artificial womb technology is not quite the global talk even though it has a multitude of implications. When the technology is commercialized, it is bound to create a rift between those nations that can afford it and allow it to be practiced within their jurisdiction and those that cannot. It will tend to give an air of authority to those with the affordability of such technology.

Moreover, AWT also tends to boost the economy for those countries that have lenient regulations regarding such advancement. Countries like India and Israel have favored destinations for fertility tourism where assisted reproductive technology is becoming an increasing source of income. Medical tourism is an untapped resource and outsourcing that can be a massive backbone in developing stunted economies (Salama et al. 2018)

AWT can be the holy grail to countries that regulate reproduction to control the population. A higher population density means lesser resources and a backward economy. Countries having an unbalanced gender ratio may use such technology to manipulate the male-female ratio. Other than that, AWT can be the gateway to designer babies. Genetic manipulations can help remove defective genes that can cause genetic disorders in the future. AWT will not only shift the present perceptions of society regarding women but also elevate them to a certain extent (Bulletti et al. 2011).

3.6 Mother Is More Than a Uterus

Recent studies have shown that a mother's mental state directly or indirectly influences the fetus—a mother's posture, rest cycle, diet, and voice, all influence the baby. According to Janet Dipietre, a researcher at the John Hopkins Fetal Development Project, "*the maternal context provides an environment that goes far beyond the direct circulatory system connection*" (Don 2015).

Janet Di Pietro, in his study, reports that It can be good for the fetus to be under moderate levels of stress. Janet and Baltimore reports that light anxiety or stress in an otherwise healthy and stable pregnancy could activate the nervous system to mature more speedily, and could stimulate cognitive and motor development in a developing embryo (Sandman and Davis 2012; Viltart and Vanbesien-Maillot 2007; Buss et al. 2012)

Sensory and neuronal mechanisms of the brain for hearing develop by the 30th week of pregnancy, and a study shows that babies while being in the mothers' womb can hear their mothers speak during the last 10 weeks of pregnancy and convey, maybe, in their own way, what they heard after their birth (Moon et al. 2013). As an embryo develops, it is continually attaining information from its mother. It isn't simply hearing her pulse or any other music she may play, but the emotional and social behavior of the mother may also feed signals through her placenta. "*We believe that the human fetus is an active participant in its own development and is collecting information for life after birth*," (Sandman et al. 2012)

During the Dutch Famine in the year 1944, starving mothers giving birth during that time found their babies huddled with problems like obesity or diabetes. Researchers of the University of California- Irvine established the fact that a growing fetus demanded consistency in the mother's health pre and post-birth. Mothers remaining healthy/unhealthy both before and after birth showed no change in the development of the fetus. Whereas mothers who became healthy from being depressed after birth seemed to be down the pace of the child's development (Guller et al. 1984).

All of this comes down to one very simple question, "Does the limits of human science permit the replication of something as complex as the Uterus?"

Since 1982 when the first experiments of implantations (Bulletti et al. 1986) outside the human body began, the maintenance of an extracorporeal profusion led to sustain ex-vivo human uteri have been followed (Bulletti et al. 1988a). Yet the complications remain the same. When in 1989 it faced a judgmental panel of political groups that upheld the banner of Ethical issues, the experiments being conducted in Italy came to a sudden halt. One among its significant challengers were the opinions voiced against it in the journal "Fertility and Sterility" **(**Bulletti et al. 1988b). Before AWT becomes a reality, this article needs to address some major issues like:

i. Human placenta and the possibilities of its replication. Connecting a human placenta to an artificial womb in cases where the fetus has been removed from the womb after conception (Callaghan et al.1963).

ii. Uterine transplantation and immune rejection.

iii. Artificial suppliers and disposers being apt replacers of natural ones when the fetus is developed in a separate environment.

iv. The various kinds of psychological and physiological build up the fetus undergoes in the complete absence of a maternal host when in an artificial environment (no influence of maternal mental state and alcohol or tobacco intake).

v. Next in line is the capacity of the process of dialysis in waste disposal. What effect can it have on the mainstream purification capacity of the infant's body post-birth when it might be possible that the fetus learns to purify its own blood completely out of imitation of the mother's purification process (Completely Hypothetical) (Bulletti et al. 1997). ECMO (Extracorporeal Membrane regeneration) and its capacity to oxygenate blood is validated but only in the case of a goat fetus. So, what are the complete guarantees this process can provide for a human fetus without future effects (Alexander et al.1968).

4 The legalities of choosing ex utero development: Indian Perspective

The innovative advancement should be pointed towards giving advancement to individuals' life. According to Dr. Flake, the objective of AWT research is to provide "physiologic help" by mimicking the environment in the uterus as intently as could be expected, keeping more untimely infants to survive and creating better security for them. Dr. Flake further added that one cannot compare a child developed in an artificial womb with the sound fetus in the mother's womb, but rather one can compare it with the extremely premature infant born and continued with the help of existing incubator technology, which often leads to life-threatening complications. However, he further pointed out that there may be some other complications also in such infants. He is concerned that the benefits of the technology may be overshadowed by the harm it poses (Yuko 2017).

Legally it is important to contemplate the protections accessible for authorities furnishing the end of pregnancy to proceed with development ex utero. Finishing the pregnancy is viewed if all else fails because N.I.C. is no assurance that an immature baby will survive (Romanis 2020b).

"The Medical Termination of Pregnancy Act, 1971" ("MTP Act" hereinafter) in India prescribes grounds under which termination of pregnancy is possible. According to the "Medical Termination of Pregnancy Act, 1971" the decision to terminate a pregnancy and convey embryo preterm should only be explored when (MTP Act 1971);

(i) the pregnant woman would face grave mental or physical harm in case of a continued pregnancy; or

(ii) in case the child is allowed to be born, it is highly expected to have severe mental and/or physical abnormalities.

4.1 "The Medical Termination of Pregnancy (Amendment) Act, 2021"

In India, if a pregnancy is terminated medically within 12 weeks after conception, one doctor's opinion is required, while two doctors' opinions are required if the pregnancy is terminated between 12 and 20 weeks. "The Medical Termination of Pregnancy (Amendment) Act", which may pave the way for AWT, enables abortion on the advice of one doctor up to 20 weeks, and two doctors in the case of certain groups of women between 20 and 24 weeks. This Act also establishes state-level Medical Boards to decide whether a pregnancy can be aborted after 24 weeks if there are significant foetal abnormalities (Medical Termination of Pregnancy (Amendment) ACT 2021)

4.2 Right to life *vs* Right to choice

Across the world, there is an ongoing debate on right to life and the right to choice. Depending on the foetal health and risk to the pregnant woman, different countries have different rules and time limits for allowing abortions. On one hand, choosing to end a pregnancy is a choice that is part of a pregnant woman's reproductive rights and on the other hand, the state also should protect life and should provide for the protection of the fetus. To strike a balance between these two rights is very important and the decision should be taken very carefully. In these situations, the AWT can be of great help to protect the rights of both the mother and the child. Both may survive by following this technology.

The Indian Penal Code also has provisions regarding causing of miscarriage, of injuries to unborn children, of the exposure of infants, and of the concealment of births, starting from Section 312-318 (Indian Penal Code 1860). Even a pregnant lady can be held liable in certain cases if she causes herself to miscarry without following the law. The Constitution of India under Article 21 provides and protects the cherished Right to life (The Constitution of India as on 9th December 2020)

4.3 "The pre-natal diagnostic techniques (regulation and prevention of misuse) amendment act, 2002"

In India, female foeticide is rampant. The "Pre-Natal Diagnostic Techniques (Regulation and Prevention of Misuse) Amendment Act, 2002" provides for (a) Sex determination after or before conception, (b) Regulations of prenatal diagnostics to determine sex-linked disorders, congenital abnormalities, metabolic disorders, chromosomal abnormalities, and inborn errors of metabolism, and (c) preventing female foeticide due to misuse of sex determination

The prime feature of this Act is to safeguard the girl child from female foeticide (Patnaik and Kejriwal 2012). In the case of ectogenesis, we are uncertain as to how far it will be possible to

keep the sex of the child a secret to their parents. This warrants the need of a regulated system to be developed to restrict any type of manipulation or identification of the gender of the baby developing ex-utero ("The pre-natal diagnostic techniques (regulation and prevention of misuse) amendment act 2002")

4.4 "The Assisted Reproductive Technology Act, 2021": Future Roadmap

Section 2 (1)(a) of this Act defines "Assisted reproductive technology" with its grammatical variations and cognate expressions, means, "all techniques that attempt to obtain a pregnancy by handling the sperm or the oocyte outside the human body and transferring the gamete or the embryo into the reproductive system of a woman."

The "Assisted Reproductive Technology (Regulation) Act 2021" also has provisions to establish State Boards, National Board, and National Registry of Assisted Reproductive Technology (ART) in India to accredit and supervise ART Banks and ART clinics. The objective of this is to ensure the ethical nature of these services provided and to protect all the rights of all persons (including surrogates) while providing the maximum benefit to all stakeholders within a recognized framework of ethics and good medical practices. Offences under the Act ("The Assisted Reproductive Technology (Regulation) Act 2021") states: "Any medical geneticist, gynecologist, registered medical practitioner or any person shall not (a) abandon, disown or exploit or cause to be abandoned, disowned or exploited in any form the child or children born through assisted reproductive technology; (b) sell human embryos or gametes, run an agency, a racket or an organisation for selling, purchasing or trading in human embryos or gametes; (c) import or help in getting imported in whatsoever manner, the human embryos or human gametes; (d) exploit the commissioning couple, woman or the gamete donor in any form; (e) transfer the human embryo into a male person or an animal; (f) sell any human embryo or gamete for research; or (g) use any intermediates to obtain gamete donors or purchase gamete donors".

The proposed offenses will be penalized with fines ranging from five to ten lakh rupees for the first contravention. In cases of subsequent contraventions, punishment will range between imprisonment for three to eight years, and fines between 10 to 20 lakh rupees. Any clinic or bank that advertises or offers sex-selective Assisted Reproductive Technology will face a sentence of five to ten years in prison, a fine of Rs 10 lakh to Rs 25 lakh, or both.

It's high time to start thinking about some of the ethico-legal issues related to trial AWT before they are viewed as a clinical alternative. Vocalist and Wells contended in 1984 that the capacity of current medication to guarantee the endurance of preterms implied that Ectogenesis was at that point an incomplete reality. Both Cannold and Alghrani allude to partial Ectogenesis as a reality shown by the 'development of premature children in incubators' (Horn and Romanis 2020). Subsidiary legal issues like experimenting with human embryos have stunted development in the sector for the past few years. In a highly populous country like India where there is a strong democratic framework with multiple views, the acceptance of something new is challenging. It has been almost over a few decades since in-vitro, surrogacy, and many such technologies have emerged and accepted by society. Laws should be put up where Ectogenesis will become a procedure only open to legally proven necessities.

5 Probable Misuse of Awt

In the wrong hands, this technological development may be lethal. The probable misuses of AWT may be:

5.1 Human Trafficking

Globally, the commodification of human beings has transcended below the lowest of standards. Human Traffickers have set up a black market where human beings are bought and sold like commodities for various purposes. By using AWT these criminals may misuse it for satisfying illegal purposes.

5.2 Genetic modifications to make a designer baby

Opening a myriad of options, starting from choosing what genes to keep and what not. Babies born in an artificial womb would surely be free of certain genetic discrepancies and might be completely different from their biological parents.

5.3 Cheap labor

Humans could be grown as a crop through AWT and put to labor. There may be ownership over an individual born artificially, and slavery may get a new dimension.

5.4 Organ harvesting

Ectogenesis would be a boon to such miscreants. Developing a fetus just to harvest its organs would cause an increase in crime and destroy and devalue the life of a human being.

5.5 Human lab rats for scientific experiments

In a world where it is even inhuman to use animals for scientific experiments, how can we even consider humans. Whereas considering that Ectogenesis may provide the opportunity to artificially conceive babies, a single individual can produce babies like a crop with all legal rights upon them as guardian subjecting them to lab experiments. There is also a hypothetical scenario where artificially grown humans could be subjected to colonization

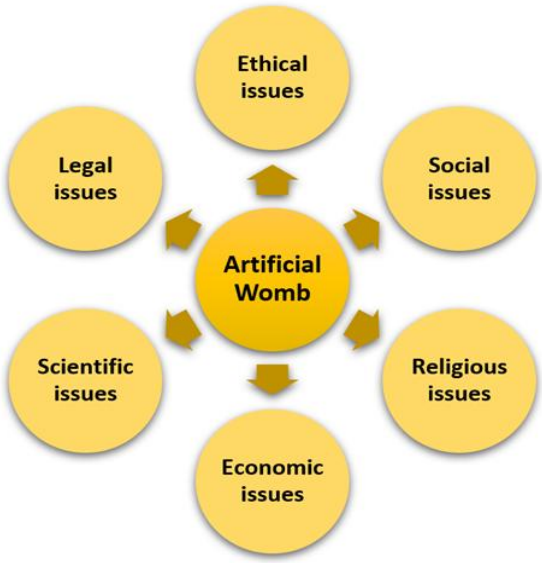

Figure 1 Bioethical and other issues related to artificial womb technology. The diagram highlights six significant issues which has been raised in the future implementation of artificial womb.

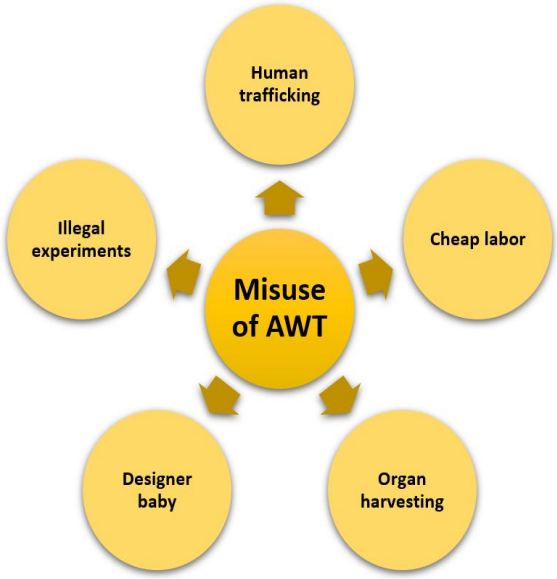

Figure 2 The diagram shows the probable misuse of Artificial Womb Technology (AWT). It highlights the five significant areas where AWT might be misused.

experiments on other planets giving them a very less percentage of survival and more chances of a horrific existence and finally death.

Conclusion

Artificial womb is a genuine and significant innovation and something deserving a moral discussion. In such a scenario, the need for rules emerges. Embracing ethical pluralism, the law will be held responsible for encouraging or restraining scientific technology. Significantly, the innovative advancement is pointed towards giving an improvement to individuals' life.

However, even before it becomes a reality it has initiated debates on many related issues (figure 1) and has raised concerns over its probable misuses (figure 2). It is additionally critical to consider every part of the issues including philosophical, clinical, bioethical ones, likewise the advantages and disadvantages of it. Presently, there is no prototype of an artificial womb for humans. The innovation is very much in its early stage. But we require to be prepared for our upcoming developments in the following decades.

When something so complex like Ectogenesis demands a green light, ethical and legal issues are bound to arise. We need to work out the critical scientific studies that have brought up loopholes in its mechanism. By the depiction, it is a high-level life supportive network, but the uncertainty of outcomes for any individual subject implies that there is the capability for irrational dangers of antagonistic impacts.

Therefore, to conclude, it is significant that we begin thinking about a portion of the ethico-legal issues inalienable to trial Artificial Womb innovation before they are considered a clinical choice.

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Applications of Artificial Intelligence in Healthcare

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Medical diagnosis

ABSTRACT

Now in these days, artificial intelligence (AI) is playing a major role in healthcare. It has many applications in diagnosis, robotic surgeries, and research, powered by the growing availability of healthcare facts and brisk improvement of analytical techniques. AI is launched in such a way that it has similar knowledge as a human but is more efficient. A robot has the same expertise as a surgeon; even if it takes a longer time for surgery, its sutures, precision, and uniformity are far better than the surgeon, leading to fewer chances of failure. To make all these things possible, AI needs some sets of algorithms. In Artificial Intelligence, there are two key categories: machine learning (ML) and natural language processing (NPL), both of which are necessary to achieve practically any aim in healthcare. The goal of this study is to keep track of current advancements in science, understand technological availability, recognize the enormous power of AI in healthcare, and encourage scientists to use AI in their related fields of research. Discoveries and advancements will continue to push the AI frontier and expand the scope of its applications, with rapid developments expected in the future.

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1 Introduction

Artificial Intelligence (AI) is a technology that supports machines with similar intelligence as human beings, to perform some tasks given by a human, like facial recognition for identification of individuals and voice recognition with virtual assistants like Alexa and Siri. Driverless vehicles or self-driving cars are also assisting elderly or blind passengers. Google DeepMind has instructed machines to read retinal signals with the same precision as a skilled specialist. Babylon, the wellbeing application, claims its chatbot can breeze through General Practitioners tests (He et al. 2019).

AI in healthcare promises noble repayment to patients. To determine the best approach for the customization of medicine, researchers need to evaluate comprehensive patient data alongside broader aspects to track and recognize sick and relatively healthy people, contributing to a better understanding of biological indicators that can indicate a change in health (Ahmed et al. 2020). Various aspects of patient care and administrative procedures among suppliers, payers, and pharmaceutical corporations could be managed by AI. AI devices are already surpassing radiologists when it comes to diagnosing critical tumors and advising researchers on how to establish consortia for expensive clinical trials (Davenport and Kalakota 2019).

Machine learning can enhance clinical decision support (CDS) for clinicians and healthcare workers. This gives the means to increase revenue potential. Machine learning, a division of AI, categorizes patterns, using algorithms and information to commit automated perceptivity to healthcare providers. AI uses advanced algorithms to 'learn' characters from a large section of healthcare data, followed by taking advantage of the obtained perspective to serve clinical practice (Jiang et al. 2017). Additionally, it can be equipped with the knowledge and self-correcting abilities to perk up its correctness based on feedback. An AI program supports doctors by supplying them with up-to-date health care knowledge from newspapers, journals, and professional procedures to alert them of appropriate treatment. AI uses tools to discover complex relationships that cannot be simplified to an equation. Neural networks, a part of AI, similarly interpret data to the human Central Nervous System (CNS) via an immense number of interconnected neurons. Such interpretation allows Machine learning (ML) structures to address difficult problem solving exactly as a clinician could, by deliberately evaluating facts to draw reasonable conclusions (Buch et al. 2018).

AI and robotics are already a part of our healthcare system. Rather than entirely replacing the work of physicians and other healthcare professionals, AI devices will support and enhance their jobs. Artificial intelligence can assist healthcare practitioners with a variety of jobs, such as administrative tasks, clinical documentation, patient outreach, and also in the areas like image analysis, medical device administration, and patient monitoring. Hence, we need to keep tabs on recent AI advancements (Ahmed et al. 2020). Figure 1 represents twelve major applications of AI in healthcare and these are (i) Medical Diagnosis, (ii) Robot-Assisted Surgery, (iii) Clinical Trial, (iv) Training, (v) Fraud Detection, (vi)

Figure 1 Twelve major applications of Artificial Intelligence in Healthcare.

Drug Discovery, (vii) Stroke management (viii) Cardiac Tissue Chips, (ix) Artificial Neurons, (x) Plastic surgery, (xi) Organ Transplantation and (xii) Spinal Cord Surgery. In this review article, we have discussed how Artificial Intelligence is being used in various healthcare and allied products or applications.

1.1 Artificial Neural Network and AI-Devices

The non-linear relationships between input and output variables are involved in artificial neural networks (ANN), which is comparable to how the human brain works (Park and Lek 2016). In 1958, the artificial neural network was introduced and became very popular due to the increase in capacity and complexity of data (Ravi et al. 2017). ANNs consist of an input layer, a hidden layer, and an output layer. Input data is obtained directly by the input layer by inserting a function into each node. The nodes in the hidden layer then receive a balanced linear combination as input from all the components in the input layer and use a non-linear transformation activation feature. The output layer does a similar function to the hidden layer. It receives signals from the hidden layer and generates an outcome with an activation function (Han et al. 2018).

AI devices involve two major categories – (a) Machine learning (ML), for analyzing organized facts such as genetics, electrophysiological, and imaging data. In therapeutic applications, ML methods evaluate a patient's attributes and infer the likelihood of disease outcomes. Precision medicine is the most prevalent application of conventional machine learning in healthcare, evaluating the therapy actions that are likely to be helpful in a patient based on the outcomes of prior patients (Lee et al. 2018). (b) Natural language processing (NLP) enhances and expands standardized medical data by extracting knowledge from unstructured data sources such as clinical notes or medical publications (Spasic and Nenadic 2020). The goal of NLP methods is to turn texts into structured computer-readable data that can be studied using ML approaches. Natural Language Processing primarily functions as human-computer interaction. The NLP systems can evaluate vast amounts of scientific data efficiently and help maintain inappropriate spam off the files. In healthcare, NLP helps to isolate complex data. The usage of Artificial Intelligence in NLP allows healthcare to collect critical data in real-time from trustworthy sources. The virtual healthcare assistants use models activated by medical terminology through interactions using NLP (Murff et al. 2011).

1.2 AI-Assisted Medical Diagnosis

AI is commonly used in medicine and can facilitate the advancement of therapy, taking care of chronically ill patients, recommend specific interventions for complicated diseases, and improve the quality of medical care. Furthermore, the improvement of various AI approaches has resulted in early disease identification, diagnosis, and referral management (Shen et al. 2019). Tencent, a Chinese mobile services firm has launched two new AI-based medical imaging devices 'AI Medical Innovation System (AIMIS) Medical Image Cloud' and 'AIMIS Open Lab', which will help with medical data management and stimulate the development of medical AI applications. Patients can access their images created by CT scans, MRIs, and X-rays on the Tencent AIMIS Medical Image Cloud, which will allow comfortable and reliable sharing of patients' medical information. Tencent AIMIS Open Lab may create medical AI applications by sharing Tencent's medical AI abilities with external parties such as scientific research institutions, universities, and research and engineering innovation companies. Tencent's 'AIMIS Image Cloud' connects medical institutions at all levels in the Medical Treatment Combination via cloud-based Picture Archiving and Communication Systems (PACS), helping physicians to undergo examinations in primary medical centers and acquire professional diagnoses virtually. Physicians can use Tencent's real-time interactive multimedia facilities to conduct online consultations and concurrent collaborative image operations to communicate more effectively while dealing with complex cases (Hithaishi 2020). The team plays digital health platform's AI-powered augmented workflow solutions to help minimize the strain of fundamental repetitive chores and improve diagnostic precision while evaluating medical pictures. It allows users to quickly access solutions for operational, therapeutic, and strategic decision support while also assuring future-readiness through flexibility and scalability (Siemens Healthcare 2021). The AI-RAD Companion Chest CT is a computed tomography device assistant based on AI. Through AI-powered algorithms, the AI-RAD companion automates the post-processing of image datasets. Regular processes with repeated actions and high patient numbers can be automated to assist radiologists to focus on more relevant issues. This method can compute severity scores in about 10 seconds per case, compared to 30 minutes for hand comments. These findings can be utilized to quickly determine the severity of the pulmonary infection and track the course of irregularities in COVID-19 patients (Gouda and Yasin 2020). These advancements represent how AI has caught up with human intelligence, starting from the day it was founded and extending into the future. It is anticipated that AI may surpass human performance in specific activities in the future, which can become beneficial for humans (Hosny et al. 2018). AIRad companion helps in increasing the accuracy of diagnosis through AI-powered algorithms while rendering medical images thereby helping radiologists reduce workload and error rates.

1.3 Robot-Assisted Surgery

Robot-assisted surgical devices allow surgeons to perform different surgical procedures in a patient's body via incisions.

Compared to standard surgical approaches, this type of surgery can help to reduce pain, blood loss, scars, infection, and post-operative recovery time. While examining the surgical site in three dimensions, a computer and software program helps a surgeon efficiently operate surgical equipment connected to the robotic arms through small incisions (Lanfranco et al. 2004). A laparoscopic approach is a surgical process that includes making small incisions into the abdomen (less than one centimeter) to introduce short, thin tubes (trochars) through which long, slender equipment is inserted. These instruments are used by the surgeon to manipulate, cut, and suture tissue. Laparoscopic treatment is generally less painful for the patient, and recuperation is much faster. The growth of robotic technologies has revolutionized controlled access surgery by resolving some of the shortcomings of the laparoscopic technique (Garry 2006). Robotic systems do not substitute the surgeon or conduct duties individually, instead, these systems have the capabilities that boost flexibility and ergonomic performance. They are operated by the surgeon, which is why they are referred to as 'master-slave systems'. They include: (a) the master console involving the user port that allows the operator to see a 3D representation of the operating area, manipulators for monitoring instruments, and a monitor panel for adjusting camera position and focus; and (b) the slave unit is mounted on the side of the patient where the instruments and the camera are connected and operated with the robotic arms (Singh et al. 2018).

1.4 Virtual Nursing Assistant

Virtual nursing assistants support patients in hospitals for an illness or surgery. Sally and Walt, the virtual personal healthcare assistants from iCare Navigator, connect with empathy to enable patients to take an active role in their health and rehabilitation. They can be accessed through a tablet or hospital TV set (Sadhika 2019). Studies have shown that if the patient's healthcare assistant is not a human, they do not feel criticized for asking questions. Sally and Walt have as much patience as it takes to ensure that patients understand their recommended instructions and discharge details to take proper care of them upon discharge. As a result, patients participate honestly and fearlessly, and they are praised for their dedication (Barrett et al. 2019). TeleHealth services, which created iCare Navigator, claims that it leverages electronic health records from a patient and utilizes machine learning to create a customized connection. iCare Navigator is developed on cloud-based technologies and a sophisticated artificial intelligence system that constantly tracks and examines the patient's response, attitude, receptivity, sensitivity, and overall commitment to providing fully customized patient experiences (Raleigh 2017).

1.5 Clinical Trial Assistance

Because of regulatory uncertainty, risk avoidance, and apprehension about rapidly emerging technologies (machine learning and wireless health monitoring tools and sensors) clinical drugs have largely stagnated during the past 30 years. Another significant obstacle in the drug research phase is the recording of the outcomes of most modern clinical trials with normal patient effects that do not readily turn into individualized medical decisions at the standard point of care (Shah et al. 2019).

The goal of artificial intelligence is to change clinical decision-making procedures. Since it can leverage the large quantities of genomic, biomarker, and phenotype data collected throughout the healthcare system, including patient records and guidance systems, to enhance the protection and quality of healthcare outcomes (Magrabi et al. 2018).

Research centers, biotechnology firms, and development companies examined the use of AI and ML, generally in three major areas are (i) machine-based learning to determine the therapeutic effects of molecular products and drug discovery targets; (ii) utilizing neural networks and optimization strategies on diagnostic images (e.g., retinal scans, autopsy samples and body surfaces, bones, and vital organs) to allow for quicker detection and monitoring of progression of diseases and dynamic algorithms for a numerical increase of current therapeutic and diagnostic data sets; (iii) working with deep-learning methods on integrative information sources such as the combination of genetic and therapeutic data to identify new prediction method (Shah et al. 2019).

1.6 Training

Improving ML models requires well-structured training data regarding a fairly stable process over time. Divergence from this results in over-fitting where AI puts undue emphasis on false associations in records (Buch et al. 2018). Along with reforming the traditional way doctors operate, two of the most awaited problems are the black box problem and liability issues. Mount Sinai Hospital's Black Box engineers have developed a deep learning algorithm that was tested on 700,000 patient results. This algorithm could predict with high precision, the onset of a condition such as schizophrenia. This is much more remarkable given that even for professionals, this disease is hard to diagnose. The main issue with this approach is that there is no way to tell how the machine generates this prediction or what variables are considered. This concept is called the Black Box phenomenon (Paranjape et al. 2019).

Recent advances in computing capacity and data collection have culminated in a new area requiring digital stored information processing to gain new knowledge. Whereas clinical trials and costly prospective research have mostly found the standard treatment and data science's inclusion into the medical sector, which has the potential to significantly increase the rate at which

information is generated and the range of issues that can be addressed (Celi et al. 2016). Through the accelerated digitalization of health care, electronic health records (EHRs) promote innovative ways of collecting and accessing useful knowledge, which can be used for better decision-making. Doctors have to use their knowledge and skills to handle evidence, monitor AI software and use AI apps to make educated decisions (Paranjape et al. 2019).

1.7 Fraud Detection

Healthcare fraud is classified into three categories: (a) People (e.g., doctors, dentists) or provider institutions (e.g., hospitals) that commit healthcare fraud; (b) often companies engage in unethical practices targeting other service providers (e.g., laboratory services) or suppliers of medication and medical products by collecting fees on payout; (c) fraudulent activities related to caregivers can often include certain classes, e.g., patients or insurers. Initiatives to counter fraud and abuse in the healthcare sector can be categorized into the 3 types of initiatives that aim to stop, track, and respond to fraud and abuse (Joudaki et al. 2015).

A few auditors manage thousands of healthcare reports through conventional techniques to prevent fraud. The problem is that they do not have much time for each claim; they focus on specific aspects of the claim instead of the overall picture of the provider's activity. Hence, this approach takes time and is inefficient. Electronic medical records and increased use of computer-based applications have provided new ways to help diagnose fraud and misconduct. Technologies in ML and AI technologies are bringing fraud detection solutions that are automatically generated to the forefront (Bauder and Khoshgoftaar 2018). The core aspect of 'knowledge discovery from databases (KDD) is data mining, which includes the application of techniques that analyze the data, establish specific models and find previously unknown trends within the data. Data mining can help third-party payers, like health insurance organizations, derive valuable information from numerous cases and classify a limited subset of cases or applicants for further review and inspection of fraud and abuse (Joudaki et al. 2015).

The data mining tools used in the analysis of healthcare fraud were classified into two basic strategies – Supervised and Unsupervised methods. Unsupervised techniques are deployed, where no previous collections of valid and fraudulent findings exist. Unsupervised techniques usually measure the characteristics of one claim in comparison to other claims to decide whether they relate to one another or vary from each other. This also explicit the order and correlation rules between records identify anomaly records or related records in classes. Supervised techniques involve a dataset of identified fraud / genuine cases to create a model that will allocate the observation to either fraudulent or non-fraudulent based on rating. They require trust in identifying the documents accurately. They help identify previously identified trends of fraud and misconduct. Therefore, the models will be revised periodically to represent innovations in dishonest activities and regulatory and settings adjustments (Bolton and Hand 2002).

1.8 Drug Discovery and Other Research

Deep neural networks (DNN) and recurrent neural networks (RNN) are two types of artificial neural networks that help AI develop faster. Artificial Intelligence technologies generated broad attention in pharmaceutical science, as deep learning algorithms showed superior results in the prediction of properties. The application of AI for early drug development has been greatly expanded, e.g., the *de novo* development of chemical compounds and peptides and formulation preparation (Hessler and Baringhaus 2018).

Deep Neural Network (DNN) is composed of several stages of non-linear functions, such as several hidden layers of neural networks. Deep learning approaches focus on understanding feature hierarchies in which features are built at higher levels of hierarchy using features at lower levels. In deeper structures, even better outcomes may be obtained when each layer is retrained with an unsupervised learning program (Gudivada et al. 2016). The Tox21 challenge, which took place in 2014, was the scientific community's "largest" endeavor to test computational algorithms for predicting toxicity. Using specially devised assays, 12,000 pharmaceuticals and chemicals from the environment were tested for 12 different hazardous effects. As part of the "Tox21 Data Challenge" (Tox21 challenge), the efficiency of computational algorithms for preliminary assessment of toxicity had been analyzed to determine their potential to reduce in vitro research and animal testing (Mayr et al. 2016). DNNs demonstrate equal or better output than other machine learning strategies, for example, for various properties ranging from predicting biological behavior, ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) characteristics to physicochemical parameters. The lack or presence of identified toxicophores had been implemented as a descriptor in addition to physicochemical descriptors and extended connectivity fingerprinting (ECFP). The DNN can isolate molecular characteristics that are allegedly associated with recognized toxicophoric components. These networks tend to know more complex concepts in the different hidden layers (Hessler and Baringhaus 2018).

Researchers at TCS Innovation Labs in Hyderabad, India, are harnessing artificial intelligence (AI) to acquire novel compounds which could attack specific portions of the novel coronavirus (SARS-CoV-2). The researchers began by training the generative deep neural network model on a sample of approximately 1.6 million drug-like small compounds from the ChEMBL database and then they retrained the network with protease inhibitor

molecules and finally, they examined how strongly they bonded with chymotrypsin-like protease, which is the target protein (Lee et al. 2014). They found 31 candidate molecules out of which two molecules were extremely similar to aurantiamide, an antiviral compound that exists naturally (Desikan 2020).

Since AI is increasingly changing the medical world, specialization on the subject has also significantly increased in recent years; emphasizing the need for a systematic analysis of study findings and developments of Artificial Intelligence in Medicine (AIM). IBM Watson-Oncology has occupied medicine for treating people with cancer with comparable or greater effectiveness than human specialists. Microsoft's Hanover Project at Oregon has examined scientific evidence to customize the care choice for a person's cancer. The National Health Service of the United Kingdom (NHS) used Google's DeepMind tool to identify health threats by examining smartphone application data and diagnostic photos obtained from NHS patients (Bali et al. 2019).

Introducing such high-throughput methods to biology and disease provides the pharmaceutical industry with both obstacles and prospects to discover possible therapeutic strategies. Recent improvements have contributed to an increase in participation in utilizing machine learning (ML) technologies in the pharmaceutical industry. The eminent drug development strategy aims to produce medications (small molecules, antibodies or peptides or newer techniques such as short RNAs or cell therapies) to improve the disease condition by amplifying the function of a molecular target (Liu et al. 2019). Despite a recent revival in phenotypic tests, launching a product development plan demands - the target modulation which will lead to modulation of the disease status. Based on the available data, target recognition and prioritizing refers to the process of selecting the target. The subsequent phase requires validating the function of an identified target for disease utilizing physiologically relevant ex vivo and in vivo approaches (target validation). As an example machine learning can be used to evaluate broad datasets with knowledge of a putative target's function to form assumptions about possible cause and effect based on known genuine targets. To identify morbidity-related genes that are also druggable, a tree-based meta-classifier that specializes in the topological network of transcriptional, protein-protein, metabolic interactions, along with tissue expression and subcellular localization, has been developed (Costa et al. 2010). Jeon et al. (2014) developed a support vector machine (SVM) classifier for breast, pancreatic and ovarian cancers using different sets of genomic data to identify proteins as drug targets or non-drug targets.

1.9 AI Application for Stroke Management

Stroke is a widespread and consistently-occurring disease due to which over 140,000 people dies in the US. Hence, studies on stroke prevention and care are of considerable significance. AI methods have been used in an increasing number of stroke-related trials in recent years, especially in the three key fields of stroke care – early disease prediction, diagnosis, and treatment (Astrakas et al. 2012).

Scientists from the UK and USA claim the AI system will forecast heart attacks and strokes accurately. Kristopher Knott, a British Heart Foundation research associate, and his colleagues performed the largest yet cardiovascular magnetic resonance imaging (CMR) and AI study. CMR is a procedure that tests blood circulation to the heart by measuring how much of a single cardiac muscle contrast product takes up; the greater the blood flow, the less probable obstructions may exist in the cardiac arteries. Interpreting the reports is laborious and time-consuming; it is often more qualitative than quantitative. To build a more insightful strategy, they created an AI system that reviewed images and learned to identify symptoms of impeded blood flow. Researchers discovered that the AI model performed well when the device was tested on scans of over 1,000 patients who wanted CMR because they were either at risk of developing cardiac failure or had already been diagnosed, in determining which individuals were more likely to suffer from a heart attack or stroke.

Machine learning programs may be able to distinguish an ischemic stroke from a hemorrhagic or any other type of stroke, reducing the risk of ignoring cases such as meningitis, coma, encephalitis, acute demyelination, abscess, and subdural hematoma. In 2018 the FDA allowed an AI algorithm to be used in triage support for clinical decision-making, called Viz. AI Contact can interpret CT scans to identify symptoms of stroke in visual videos, allowing a tentative diagnosis. The device can alert a neurovascular specialist via smartphone or laptop upon detecting a stroke case, allowing the specialist to focus on the most critical cases while the radiologist can study less urgent photos. This AI-enabled system management will provide timely treatment for patients who might not take the regular examination protocol without endangering their health or even their life (Liebeskind 2018).

With scientifically validated, data-driven technologies, Rapid AI allows physicians to make quicker, more precise diagnosis and recovery decisions for stroke patients. Clinicians globally are enhancing patient safety and outcomes every day with a proven, respected network built by stroke experts and are used in more than 1300 hospitals worldwide. NeuroView, a medical technology startup, also aims to automate the prediction of stroke defects in the area. A common goal shared by many of today's more well-known professional organizations, healthcare infrastructure firms, and even hospitals that are increasingly partnering together to solve common challenges, developing their own departmental and consumer-friendly AI practices. Nonetheless, researchers'

achievements during the last decade should not be underestimated. It casts a largely positive forecast for what healthcare practitioners, patients, and businesses assume to see in the future of AI in healthcare (Tran et al. 2019).

1.10 Cardiac Tissue Chips (CTCs)

The heart has usually been one of the most challenging organs to imitate in artificial organ and organ-on-a-chip technology studies. Research teams at Birmingham's University of Alabama have created biomimetic cardiac tissue chips (CTC)–cell culture systems, which can reliably simulate complicated blood flow pressures involved with shifts in the pressure volume of the heart. Researchers hope that by detecting harmful medications before they reach clinical trials, these tissue chips will speed up research into medicines and medical devices and enhance health. The cardiac tissue chip (CTC) prototype consists of entrapped cardiac cells which could be grown in three-dimensional (3-D) fibres and exposed to hemodynamic loading for simulating shifts in the pressure-volume relationship of the left ventricle. Several parameters related to cardiac function, heart rate, peak-systolic pressure, end-diastolic pressure-volume, as well as systolic and diastolic length proportion, can be monitored accurately, causing cardiac cell cultures under developmental, normal, and pathological conditions (Rogers et al. 2019).

The CTC can replicate the pressures involved with an overload of both pressure and volume (Kong et al. 2019). The experiments with cardiomyogenic cell line-derived H9c2 cells, showed that under pathological hemodynamic pressure, the culture inside the CTC generates changes in morphology and gene expression that are comparable to those reported with hypertrophic and dilated cardiomyopathy (Rogers et al. 2019). The cells inside the CTC undergo accelerated cardiac hypertrophy remodeling and fibrosis under pressure overload, whereas the cells susceptible to prolonged volume overload, encounter major adjustments in the cellular size due to thinning and elongation of the designed tissue. According to these findings, CTC is capable of being utilized to build significant designs in which hemodynamic loading and unloading could be replicated correctly to simulate heart illnesses. However, platforms like Organ-on-chip and CTC produces a huge amount of data (related to shape, size, structure, interaction, and composition) that needs to be analyzed. This can be very complex to analyze using traditional computational methods. In such cases, AI and ML can do the job more efficiently. Since CTCs can offer a clearer picture of the course of illness and medication toxicity, scientists expect to find more about these problems sooner (Fermini et al. 2016). Now the researchers are focusing on combining the CTCs with chips describing other organs and tissue coupled with AI. Since these broader tissue chips may replicate human physiology more precisely for science and drug testing (Mencattini et al. 2019; Rogers et al. 2019).

1.11 Artificial Neurons

Alzheimer's disease is a neurodegenerative condition that entails gradual destruction of neurons with emotional, behavioral, and motor implications, losing the afflicted person's spirit, and is traumatic not just for patients but also for their families (Oboudiyat et al. 2013). However, experts are pursuing potential approaches in nanotechnology that may greatly improve the wellbeing of sufferers. The World Health Organization (WHO) designated dementia as a priority condition in 2008 in a part of its campaign called the mental health gap action program (mhGAP). In 2010, the global dementia population was estimated to be around 35.6 million individuals, and it is anticipated to nearly double every 20 years, surpassing 65.7 million in 2030 and 115.4 million in 2050. Every year, over 7.7 million new incidences of dementia are discovered around the world, or once every four seconds (Tanna 2013). A recent study showed an unusual immunoglobulin (Ig) accumulation in the brain parenchyma of AD tissues, along with the distinct neurons that demonstrated such vascular-derived antibodies to be degenerative and apoptotic. Later studies found that these Ig-positive neurons possessed classical complementary elements, C1q and C5b-9, that were more often spatially aligned to Ig-negative neurons with reactive microglia. As a result, the mere existence of anti-neuronal autoantibodies in previously rejected serum can have no pathogenic implications, given no Blood-Brain Barrier (BBB) defect is prevalent that would allow the harmful effects of such autoantibodies to reach their targets (D'Andrea 2005).

A multinational research team has created artificial neurons that could be inserted into the brain to repair damage sustained by neurodegenerative diseases including Alzheimer's disease. The chips developed by the team are silicon-based miniature machines, built on biological ion channels that simulate the function of actual neurons (Abu-Hassan et al. 2019). The aim is to make such chips to reverse the harm done by autoimmune reactions, repairing the nervous circuit's key functions. In reality, they reflect linking bridges that disrupt a neural canal. The silicon chips, which functions like biological neurons, need just 140 nanoWatts of electricity, which is one-billionth of the power supplied by the microprocessors used to create artificial neurons, rendering the silicon chips ideal for use as medical implants or in other bioelectronic devices. The next target for scientists is to investigate the least intrusive and non-surgical approaches for administering deep brain stimulation to promote access to this care for people with Alzheimer's disease, making it possible to endorse the application of artificial intelligence (University of Bath, 2019).

1.12 Plastic Surgery

Cosmetic surgeons are creative and are always on the frontlines of modern scientific developments. Starting from the development of

skin grafts through transplantation, the profession of plastic and reconstructive surgery has ever advanced immensely, due to our ability to integrate innovations quickly and effectively. Snapchat's AI technology has indeed begun to affect the world of plastic surgery. Using AI processing technology to discern facial expressions, Snapchat puts filters on people's faces to dramatically change its portrayal (Ameer et al. 2013). Snapchat is a smartphone app available for both Android and iOS devices, built for communication purposes. Evan Spiegel, one of the company's co-founders, is in charge of designing this mobile application. The app's basic feature is that any picture, video, or message you share is viewable to the receiver for a short time before being unavailable (Velten et al. 2007). People seeking plastic surgery to look like their filtered selfies have become known as "Snapchat dysmorphia" as a result of AI advancements (Ameer et al. 2013).

Data-driven surgical modelling applications that can define empirical asymmetries in pre-operative photographs can offer feedback on the finest tactic towards attaining a favored cosmetic result. Picture modifying software that promotes such abilities raise unrealistic expectations and are incapable of compensating for the constraints of cosmetic operation reality. In terms of surgical screening, AI systems have now been created to categorize patients upon whom certain therapies are too dangerous and to exclude them from the pre-operative environment. Both depend on unintuitive risk factors concealed in previous surgery mishaps data, and both have significant health and economic contributions (Kim et al. 2019). Through the creation of plastic surgery monitoring operations and results (TOPS), the General Register of Autologous Fat Transfer (GRAFT), the Regional Surgical Quality Improvement Plan (NSQIP), Cosmet Assure, and ASAPS CLOUD databases, the cosmetic surgery group has recently shown their ability to integrate massive data. This reflects the willingness that requires processing data from plastic surgery systematically, as well as a forthrightness to artificial intelligence upheaval. Finally, the American Society for Cosmetic Plastic Surgery (ASAPS) initiated the Aesthetic Neural Network (ANN), which is a preliminary method for refining treatments and modelling economics (Chandawarkar et al. 2020).

Health decisions about wound treatment are based on an assessment of wound features, such as dimensions and location, as well as patient-related parameters, such as skin texture, genetic details, and living environment. The seriousness of certain casualties meets the human eye clearly which signifies the emergence of artificial intelligence to render assessment easy and more effective (Yeong et al. 2005). By matching wound pictures against explicit measures of the patient's body, a thinking machine may estimate the magnitude of infected/damaged tissues (Kim et al. 2019).

In addition to other smart imaging techniques, angiograms from computed tomography (CT) based on AI-assisted analysis may help surgeons develop surgical flaps. The new equipment allows doctors to view three-dimensional (3D) photographs as strips, thereby saving lapse of time and improving reliability in their ability to see the human body's inner framework. While radiologists must divide CT angiography images into 64 slices for precise diagnosis, a computer can evaluate all slices of a 3D imaging specimen at the same time. While conducting a separate flap procedure, cosmetic surgeons very often choose the same configuration of flaps. Nevertheless, AI's fast, integrative thought capabilities may assist surgeons in developing a strategy that can be customized to each particular patient.

Cutaneous wound infections may extend into osteocytes, causing gradual swelling and osteomyelitis. During the diagnosis phase, there are many problems raised for osteomyelitis because of the time it takes to display some sort of osseous lesion for simple radiographic images. AI-assisted radiographic image assessment may reduce the amount of time required for osteomyelitis to become detected on a radiographic photograph. Radiologists, in a similar manner, have used temporal subtraction, a function of computer-assisted diagnosis, for strengthening the differences in intervals between two radiological images (Kim et al. 2019).

Amongst the most serious craniofacial deformities that cosmetic surgeons have come across is craniosynostosis (Johnson and Wilkie 2011). Both genetic and environmental factors contribute to the epidemiology of craniosynostosis. The chief drawback seems to be the lack of further evidence on the results of craniosynostosis cases. Researchers and surgeons are now capable of understanding both the genetic and environmental causes of this abnormality. Today, AI technologies are applied to combine the various pictures to support and enhance surgical preparation. This seems to be especially true in syndromic illnesses, where due to the pathophysiology of osteogenesis, recurring deformities may be more likely. Cosmetic surgeons can now employ artificial neural networks (ANNs) to anticipate postoperative difficulties following craniofacial surgery. Syndromic craniosynostosis may result in repeated cranial defects, and after corrective surgery, the bone may begin to develop abnormally. In many cases, AI and precision medicine may be used to boost surgical changes to maximize postoperative outcomes. Furthermore, Big Data picture processing can help configure cranial remodeling to better fit individual children (Kim et al. 2019).

1.13 Organ Transplantation

More than 100,000 organs are transplanted globally every day and yet more around the world are waiting for an organ transplant (Healio 2019). To tackle this challenge an AI-powered application

OrganSecure has been introduced, which seeks to connect organ donors with individuals who needs real-time organ transplantation. OrganSecure is now undergoing alpha testing and intends to overcome the two main problems in the organ donation ecosystem, i.e., having more people to become organ donors and providing the organs at the right time for those in need. The application starts by providing people with organ donation-related knowledge and allows them to sign up to become a patient, make them realize what organs they should donate depending on their medical records, and help them understand local regulations (Pradhan et al. 2020). Organ recipients would benefit from an AI-powered real-time rating on the donor list, and the timeframe required to move to the top of the list. Patients and their relatives will now be able to prepare themselves properly for the procedure with facts about anticipated prices, nearby organ banks, and other important data. Given the various criteria regulating organ donation, such as blood group and form of antigen, the software uses Azure Machine Learning to determine an organ match and approximate the rank and time needed for an expecting recipient (Pahl et al. 2020). If a potential donor has an injury or passes away, there is no convenient way to search the records to efficiently preserve the organs. Hospitals can check the donor's identification with OrganSecure before commencing the extraction process.

1.14 AI in Spinal Cord Injury

A 28 years old participant, Ian Burkhart, sustained a spinal cord injury from the diving incident (2010) and is still collaborating with researchers since 2014 on a program named NeuroLife (Cell Press 2020), aimed at returning sense to his right arm. The Ohio State University Wexner Medical Center and Battelle's research team announced that after spinal cord injury (SCI), paralyzed muscles can be resuscitated with a brain-computer interface (BCI) to improve motor control by itself. More importantly, the touching sensation is a central feature of motor activity (Ganzer et al. 2020). The device they created works with an electrode system on his skin and a silicon chip inserted in his motor cortex. The system fuses and improves synaptic impulses that are so tiny that they cannot be detected by artificial sensory input transmitted back to the user, resulting in extremely enhanced motor activity. According to Patrick Ganzer, a principal research scientist from Battelle, in patients with a "clinically complete" spinal cord injury, there are still some preserved flecks of the nerve fiber. Using haptic feedback, the sub-perceptual touch impulses are selectively brought back towards the participant. Mobile phone or gaming controller vibrations alert the user that it is working are the common examples of haptic feedback. The researchers are developing a next-generation sleeve that comprises the necessary electrodes and sensors and can be swiftly applied and withdrawn (Ganzer et al. 2020).

2 AI in COVID-19

In December 2019, some people were hospitalized with pneumonia of unknown origin, exhibiting symptoms such as fever, dry coughs, weariness, and some were also experiencing nasal congestion, runny nose, sore throat, pain, and diarrhea, resulting in the death of many in Wuhan, China. On the 31st of December, China reported this remorseless pneumonia to the WHO country office, and the outbreak was eventually declared a Public Health Emergency of International Concern (PHEIC) on January 31, 2020. On February 11, 2020, the International Committee on Taxonomy of Viruses (ICTV) labeled the virus causing pneumonia as "Severe Acute Respiratory Syndrome Coronavirus 2" (SARS-Cov-2), and the World Health Organization (WHO) called the condition "COVID-19".

One of the ways to deal with infectious diseases, particularly those that spread quickly, such as COVID-19, is to quickly identify the infection and ensure proper isolation. Although detecting a viral infection is time-consuming and needs perplexing techniques that are often performed in specialized laboratories. Diagnosis can take hours or days so, the time taken to determine the presence of an infectious agent can promote further spread of disease and delay the patient's care. For this reason, researchers in the United Kingdom are putting a new mobile diagnostic system to the test: a portable laboratory with chip devices that connect to the cloud via a smartphone. Studies show that even in remote areas, this can detect the early onset of infectious diseases. The connected smartphone application helps the user to undergo testing and diagnostics of the patient (Mashamba-Thompson and Crayton 2020). Sample from the patient is taken in a disposable cartridge that contains electrochemical sensors. After that, the cartridge is inserted into a portable device, which amplifies the sample by regulating the temperature until RNA and DNA are identified, which requires roughly 30 minutes. The device consists of a microcontroller that sends patient data to a smartphone application via Bluetooth. When the results are positive, location reports and related data are sent to the cloud for viewing on the Internet. New cartridges for new pathogens, such as the SARS-CoV-2 virus that causes COVID-19, can be developed in a week after the new DNA sequence is presented to the public. The cartridge can then be used to provide quick and easy access to the detection and controlling the outbreak. The Deep Learning Classifier examines the image for anomalies before segmenting it and applying large-scale texture extraction. AI can rapidly distinguish lungs from patients with frequent viral pneumonia or COVID-19 by counting the number and size of lesions and determining the severity of each case (McCall 2020; Santosh 2020; NYU 2020).

AI uses natural language processing which is capable to answer any questions related to COVID-19 (Cury et al. 2021), by providing authentic and true information, giving clear

recommendations, and offer multilingual virtual assistance, monitoring symptoms, and also recommending whether any people need to get hospitalize or self-quarantine at home. Apple Siri and Amazon Alexa respond at an advanced level to two out of seven questions related to "coronavirus" or "COVID-19" (Mehta et al. 2020).

Web scraping and data mining are technologies that play a significant role in collecting facts and minimizing the flow of erroneous information. This data helps medical practitioners to evaluate the success or failure and adjust policies according to that. Tools such as Healthmap and Johns Hopkins University dashboard are currently some of the most popular sources of information about the epidemic. They use network erosion, data mining, machine learning, and geographic information technology to gather information from a variety of sources, including local and national government hospitals and medical centers, news, chat rooms, forums, and more (Rennie et al. 2020).

Due to a new generation of uncooled infrared (IR) thermal sensors based on microbolometers, thermal infrared sensing technology has advanced dramatically during the last few decades. These detectors generate massive 2D arrays using microelectromechanical system (MEMS) techniques, which reduce costs while maintaining excellent sensitivity and image quality. To assess psychophysiological and emotional states, behavioral analysis and several Autonomic Nervous System (ANS) factors, such as skin conductance, the temperature of a hand palm, modulations of heartbeat, and peripheral vascular tone, have been measured (Cardone and Merla 2017). Thermal cameras are used to identify people with fever, but the disadvantage of the technology is that it requires an operator. Hence, AI-based multi-sensor cameras (Health Cam) are now used in airports, hospitals, and nursing homes. The Kogniz Health Cam is equipped with an installed optical camera, a thermal camera, and a high-resolution display screen that can be placed on a desk, or a wall. People's temperatures are measured in actual time whether they walk alone or in a crowd with high-precision infrared technology, allowing the image of anyone with a high temperature to be spotted. The Health Cam uses the latest AI to measure the temperature of a human close to his eyes to get the most precise reading. Real-time updates are forwarded via SMS and SlackTM. The live footage, including the temperature of an individual, is displayed on the Kogniz Health Cam monitor and is accessible via the Kogniz central smartphone app (Jiang et al. 2020).

AI can incur the costs of developing antibodies and vaccines against new coronaviruses that have been completely developed from scratch or by drug repurposing strategies. For example, Google DeepMind uses the AlphaFold system to create a structural model of virus-related proteins for a better understanding of the virus in the scientific community (Drori et al. 2019). However, the results have not been verified experimentally, and this is a positive step. A variety of research was done using AI to classify medications that have been created to fight certain diseases, but that may now be retrofitted to combat COVID-19. After analyzing the molecular structure of current drugs with AI, companies want to recognize which ones may compete with the manner COVID-19 works. Benevolent AI, a drug research company in London, is focusing on the issue of the COVID-19 pandemic utilizing its AI-powered information graph, which can store a large quantity of scientific literature and biomedical research to identify connections among disease genetic and biological characteristics and drug structure and behavior. An example is baricitinib, a drug introduced by Benevolent AI that prevents viral entrance by blocking endocytosis and is used to treat arthritis (Ghose et al. 2020). Researchers can use the knowledge they gain from other viruses with familiar qualities, check their functions, and then figure out whether the medications can be used to stop the virus.

The main areas in which robots can help are clinical, logistics, and information management, which solve tasks such as identifying infected people or adhering to quarantine or social distance requirements. In addition to the medical field, robots can preserve the economy and infrastructure by working for employees of important companies such as factories or waste management, or power generation. Robot nurses are available to give medicines and food to patients who have tested positive for the coronavirus, allowing doctors and nurses to restrict direct contact with the patient and reduce the risk of infection. An Indian startup Invento Robotics, Bengaluru, has developed a robot named "Mitra" for assisting patients to communicate with their relatives (Singh et al. 2021).

Authorities use drones to combat the deadly epidemic of coronavirus. The drones are used to spray disinfectants into areas such as hospitals, offices, and government buildings.

Wenzhou Central Hospital, New York University, and Cangnan People's Hospital scientists collaborated on designing Artificial Intelligence-based early warning systems, which can anticipate whether a patient will display SARS-Cov-2 symptoms later or not, especially senior citizens. The AI system measures various changes in three conditions – alanine rates of aminotransferase, recorded myalgia, and hemoglobin levels – which are the most reliable predictors of eventual extreme disease and may potentially determine the risk of SARS-Cov-2 virus. In Wenzhou, China, 53 patients were tested with this system, which proves that this system is around 80% accurate (Jiang et al. 2020). Another advantage is that AI-based test kits do not need blood or genetic material as it is

linked with the CT scan machines in Indonesia's government hospitals, and it can evaluate the test in just 10 seconds.

Presently, whoever is showing symptoms or whoever has encountered with novel coronavirus or has been hospitalized are required to be diagnosed first, the standard laboratory testing involves "RT-PCR", "molecular point-of-care", "paper-based tests", and "testing for antibodies in the blood", but all these required much time to give results with good accuracy. However, the specificity is good, but sensitivity differs greatly depending on different countries. So, an AI-based CT scan can be employed to reduce the workload on physicians. Research groups are exhibiting an AI-based algorithm that can help by detecting, quantifying, and tracking COVID-19 on chest CT scans (Li et al. 2020). Researchers are aiming to change and adapt current AI systems with basic clinical knowledge to quickly build AI-based methods to resolve the problem of COVID-19. The proposed program incorporates deep learning systems and clinical knowledge of 2D and 3D modeling and has been based on data from accessible foreign databases, as well as from infected areas in China. This can also help in distinguishing between pneumonia and coronavirus disease.

2.1 AI-based Voice Tool

In Mumbai, a team of three students and a professor from DY Patil Institute of Biotechnology and Bioinformatics have designed an Artificial Intelligence (AI)-based tool to diagnose COVID-19 via voice-based diagnosis on a smartphone. This Indian AI speech gadget is fully functioning and is already being used in Italy to identify COVID-19 patients. The students get access to a complete working platform that includes a huge patient database and safe samples. The University of Rome is presently using this technology to detect COVID-19 patients with 98 percent of precision. Each of our internal organs is a resonator, and it's expressed by our voice whenever we are having an issue with our heart or our lungs (The Hindu 2020). When they are healthy, the same person has one voice, and when they have a condition, the voice changes. Since this virus is destroying the lungs and airwaves, it impacts speech. To identify infected individuals, the subject should speak to the microphone, and the device will break down the speech into several attributes such as volume and amplification of the sound. These readings are compared to those of an ordinary person, and the patient's positive or negative status is determined using this technique (Almada and Maranhão 2021).

Conclusion

The medicine training with AI is evolving with the advancement of the Machine Learning algorithm. An effective AI system must have the ML component for handling databases such as EMR data, scanning images, genetic information, as well as the NLP component for unstructured text mining. Then, the advanced programs have to be tested by health care records which can help doctors with the diagnosis of illness and recommendations for treatment. These AI-based tools paired with advanced data science, are enhancing the validity and accuracy of the treatment and diagnosis among a wide range of professions. One noetic function is to build a powerful AI program. Such a program can be algorithmically built and can leave the human intellect far behind. The additional knowledge will help us eliminate illness, conflict, and misery with this creative data, and a strong AI can be developed that will be the biggest event in human history.

Here, we have discussed the role of AI and ML, and NLP in various fields of healthcare. Table 1 depicts some Artificial Intelligence applications deployed in the field of diagnosis, robotic surgeries, research, drug discovery, and disease management. As a result, we can conclude that AI can aid in the detection of abnormalities in various organs (colon, heart, brain, etc.) and surgeries (plastic surgery, spine surgery, retinal surgery, etc.). We also have virtual nursing assistants to answer any queries related to diseases and assist in finding a fair solution. Health records, genetic profiles, prescriptions, and environmental parameters are all examples of the information that AI may collect and evaluate, allowing more medical information to be retained, accessed, and analyzed.

The novel outbreak of coronavirus (COVID-19) was discovered in December of 2019 and requires special attention owing to its possible epidemics and worldwide threats. Since Artificial Intelligence (AI) proposes a modern technology for healthcare, it is utilized to understand data and make decisions. In addition to pharmaceuticals and clinical practices, numerous AI approaches and AI devices based on Machine Learning (ML) algorithms are used. This suggests that AI-based technologies would help doctors, and the existing physician-patient partnership would become stronger in the future. AI can help in decreasing the workload of healthcare professionals and provide better service to the people.

The limitation is the network's usage of multiple parameters to build its recommendation. If circumstances arise in which clinical characteristics are considered equally essential as all other variables, it may result in overfitting and overrepresentation of particular parameters. Since AI has been used to perform previously unimaginable tasks, awareness of the current revolution has yet to spread throughout the health sector for a variety of reasons, including a lack of evidence about the effectiveness, consistency, and safety of these tools, health sectors lacking AI regulations, and the ascription of liability in the event of an error. In any case, the outcome of ongoing machine-learning research will undoubtedly have an impact on longstanding disputes in cognitive science about the form and function of minds.

Table 1 Applications of AI deployed in the field of diagnosis, robotic surgeries, research, drug discovery & disease management

Domain	Sub-domain	Application	Reference/s
Diagnosis	Radiology	Colonoscopy	Hosny et al. 2018; Ciuti et al. 2020; Mori 2019
		Oncology	Esteva et al. 2017; Albarqouni et al. 2016
		Brain Imaging	Zaharchuk et al. 2018
		Cardiology	Pavlou et al. 2015; Kolek et al. 2016; Narula et al. 2016; Johnson et al. 2018
	Pathology	Lymphocyte morphology/cancer detection	Al-Kofahi et al. 2010; Ehteshami et al. 2017; Mohlman et al. 2020
		Cancer prognosis	Saltz et al. 2018; Corredor et al. 2019
Robot-Assisted Surgery	Cardiac Surgery	Mitral valve repair	Gillinov et al. 2018
	Orthopaedics	Hip arthroplasty and Spine Surgery	Lang et al. 2011; Hernandez et al. 2017
	Ophthalmic Surgery	Retinal surgery	Jensen et al. 1997; Dogangil et al. 2010; Urias et al. 2019
		Corneal surgery	Tsirbas et al. 2007; Pandey and Sharma 2019
	Plastic surgery	Prediction and clinical care	Ameer et al. 2013; Kim et al. 2019
Drug Discovery	Development	Identification and screening	Costa et al. 2010; Vamathevan et al. 2019
	Clinical Trial	Prediction of drug efficacy	Ferrero et al. 2017; Rouillard et al. 2018
Disease Management	Nursing	Virtual Nursing Assistant	Barrett et al. 2019
	Organ Transplantation	Finding organ match, rank and time	Pradhan et al. 2020
	Stroke management	Post stroke rehabilitation	Linder et al. 2015
		Clinical care	Krittanawong et al. 2017
	Training	Medical education	Paranjape et al. 2019

Conflict of Interest

The authors have no conflict of interest to declare.

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Journal of Experimental Biology and Agricultural Sciences, February - 2022; Volume – 10(1-BIONEXT-2021) page 227 – 247

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Leishmaniasis: Plants as a source of antileishmanial agents

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Plant derived antileishmanial compounds

Sandfly

Visceral *Leishmaniasis*

ABSTRACT

Leishmania infection causes a group of tropical diseases and has remained neglected for decades. It spreads by sandfly vector and is one of the most fatal protozoan diseases after malaria. Leishmaniases are a group of diseases caused by the infection of different Leishmania species and display clinically different forms like “Visceral leishmaniasis” (VL), “mucocutaneous leishmaniasis” and “cutaneous leishmaniasis” (CL). Approximately one billion people living in an endemic area are at high risk. Three hundred thousand cases of VL are reported annually and around twenty thousand people die every year, proving it as one of the most lethal forms of leishmaniasis. Until now, no effective vaccine could be made. There is an increase in drug resistance in the case of conventional drugs. New synthetic drugs are either too costly or have side effects. Requirements of new drugs are of utmost importance to control this situation. Plants provide a source of unlimited chemical diversity, which can be screened for antileishmanial activities. Moreover, their low cost and less or no side effects make them idle candidates in the search of new antileishmanial drugs.

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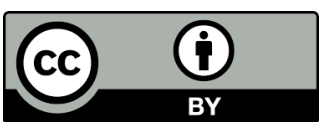


1 Introduction

Leishmaniases, a significant tropical disease is considered the 2nd most significant disease next to malaria (Ghorbani and Farhoudi 2017). The disease has been neglected for decades but now it has attained significant importance because of its geographic spread and growing cases of Leishmania-HIV coinfection (Conceição-Silva and Morgado 2019). Protozoan parasite of the genus Leishmania causes the disease leishmaniasis. Various clinical manifestations of the disease are presented, based on the type of Leishmania parasite infection. CL, VL, and mucocutaneous leishmaniasis (ML) are the major forms of leishmania (Burza et al. 2018). Sandflies transmit *Leishmania* parasites to the mammalian host, as they bite them. The promastigote (flagellated parasitic form) enters into the mammalian host (Figure 1). Inside the host, the parasites become immotile amastigote structures without flagella. Unfortunately, still, we have not been able to find one effective vaccine against leishmaniasis and most of the people depend on synthetic drugs (Ghorbani and Farhoudi 2017).

Pentavalent antimony-derived organic compounds are mostly used drugs. However, they cause stern side effects and also are not fully efficient against this lethal form of leishmaniasis (Guerin et al. 2002). Miltefosine, an available drug also develops resistance as per an *in-vitro* study (Perez et al. 2003). Though lipid formulation of Amphotericin B is an advanced drug, their high cost is a hurdle to make it available for poor people who are most affected (Ouellette et al. 2004; Croft et al. 2006)

Extracts/compounds from natural resources are a huge source of compounds for new drug discoveries of many tropical diseases which have remained neglected. Besides, because of minimum or no side effects and low price, the use of plant extracts as effective anti-leishmanial drugs is gaining importance.

We begin this chapter with the description of the disease-Leishmaniasis, which includes the history of Leishmaniasis, clinical aspects of the disease, and current treatments followed by the rationale behind the need for new plant-derived drugs and discussion on different classes of plant-derived entities which can be a promising antileishmanial agent.

2 Leishmaniasis: a brief history

Evidence suggests that the history of Leishmaniasis dated back to the 1stcentury AD. Many clay curios from Peru & Ecuador and of pre-Inca civilization displayed descriptions of lesions in the skin and facial deformities; these are typically showing mucocutaneous & cutaneous leishmaniasis. Later both Leishman and Donovan discovered the genus, *Leishmania* (Steverding 2017) while studying Kala-azar.

2.1 *Leishmania*/HIV co-infection

The prevalence of HIV/AIDS cases during the last few decades has changed the array of Leishmania infection in both epidemiological and clinical scenarios (Cruz et al. 2006). Since the first report of HIV/Leishmania co-infection was registered during the 1980s (de

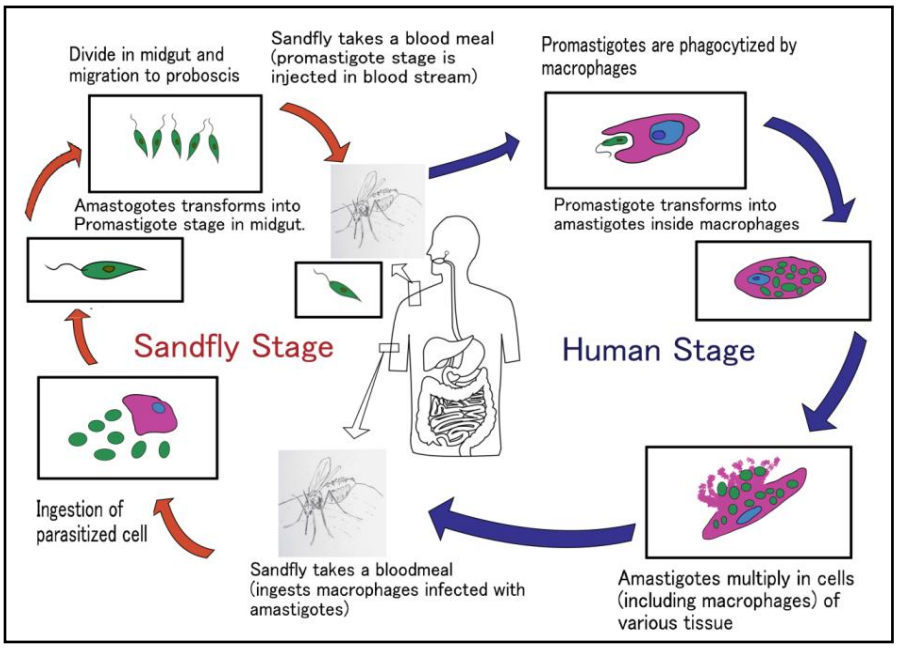


Figure 1 Leishmania life cycle: Sandflies transmit *Leishmania* parasites to the mammalian host, as they bite them. The promastigote (flagellated parasitic form) enters into the mammalian host. Inside the host, the parasites become immotile amastigote structures without flagella and cause the disease.

la Loma et al. 1985), there has seen a continuous surge and now about 35 countries in the world are combating *Leishmania*/HIV coinfection (Desjeux and Alvar 2003; Alvar et al. 2008) the greatest prevalence being in the Mediterranean region. The two diseases are mutually reinforcing. An HIV infection makes a patient more vulnerable to *Leishmania*, and subsequently, *Leishmaniasis* accelerates the replication of HIV and progression to AIDS. Till recently the global impact of HIV/*Leishmania* co-infection has remained underestimated because of the lack of an effective surveillance system. To monitor this, the "World Health Organization", in association with the "United Nations HIV/AIDS" program, has started an active surveillance system LEISHNET since 1998 to find out the actual spread of this problem and the report has been horrifying (Alvar et al. 2008; Cruz et al. 2006).

2.2 "Post Kala-azar dermal leishmaniasis (PKDL)"

The cutaneous expression of VL is represented in PKDL. The disease is related to lesions of skin and papules commonly appearing upon the face. The disease may usually appear 2-5 years of successful treatment of VL (Gedda et al. 2020). The disease is confined mainly to two regions – the Indian subcontinent and Sudan and its adjoining area. These two regions are also endemic to visceral leishmaniasis (Ganguly et al. 2010). In Sudanese PKDL, the disease gets cured spontaneously. But in India spontaneous healing does not take place and the disease needs to be treated with SAG (sodium antimony gluconate). However recently the increase in SAG resistance has caused the authorities to use newer drugs like Amphotericin B which are not resistant to *Leishmania*, to combat the disease (Das et al. 2009)

2.3 Zoonotic leishmaniasis

To date,15 well-known Leishmania species infect humans, among these 13 are known to have zoonotic nature. The disease is known to infect a wide range of animals including dogs, rodents, foxes wolves, jackals, and sloths (Gramiccia and Gradoni 2005)

3 Morphology of *Leishmania* parasites

3.1 *Leishmania* parasites have two Stages

3.1.1 Amastigote stage

The amastigote or non-flagellated stage occurs in vertebrate hosts, where the parasite resides in the reticuloendothelial system of man, dog, and hamster. The parasite is oval or round shaped, 2-4 µm long along the longitudinal axis with slight or no motility (figure 2 & 3).

3.1.2 Promastigote stage

It is an extracellular type of Leishmania parasite and is present in vectors (insects) only. However, it can be grown in cultures also. Fully developed promastigotes are 15-20 µm in length and have a long, slender spindle-shaped body. The nucleus lies at the center and the kinetoplastid is present transversely near the anterior end. A flagellum projecting from the front measuring the length of the parasite or even longer is also present (Figure 2 & 3).

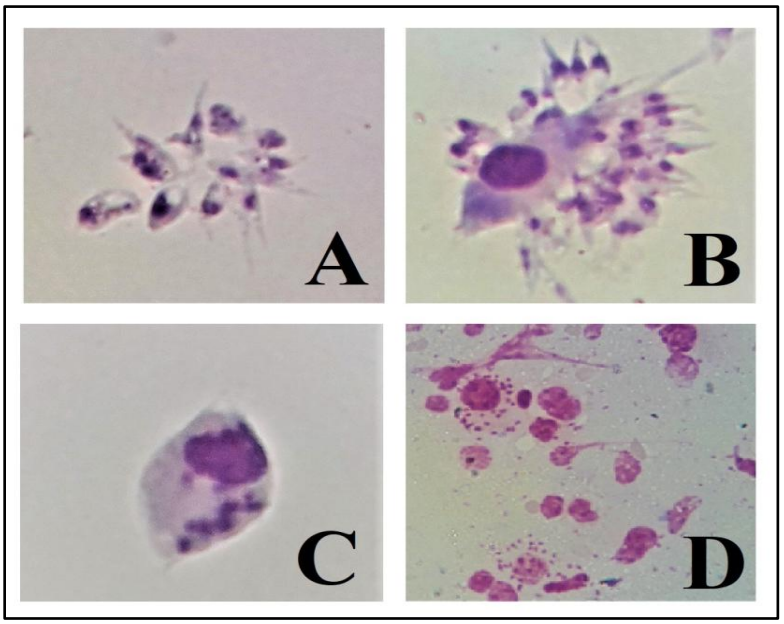


Figure 2 Promastigote stage of *Leishmania donovani* (100X) (A), Promastigotes infecting Mouse cell line (RAW 264.7) at 100X (B), Amastigote stage of *Leishmania donovani* parasites inside RAW 264.7 (100X) (C), *Leishmania donovani* infected liver cells of Balb/c mice model (100X) (D). Cells (A, B, C & D) are stained with Giemsa stain.

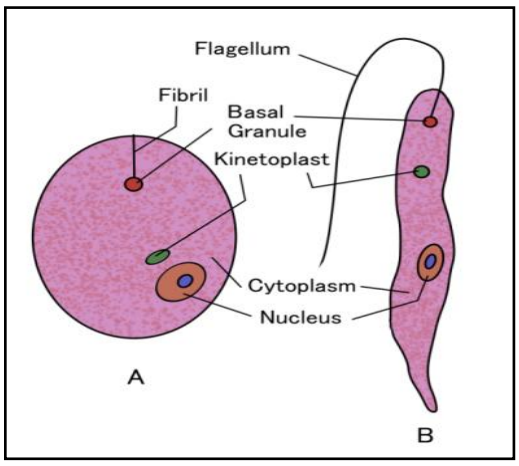


Figure 3 Diagrammatic representation Amastigote (A) and Promastigote (B) form of *Leishmania* parasite

4 Transmission of disease

The prime mode of infection of VL is by the bite of a female phlebotomine Sandfly. The parasite is transmitted by the bites of *phlebotomus* harboring *Leishmania* (Figure 1) in the old world and in the new world by the *Lutzomyia species* (Singh 2006). However other modes of congenital and parental transmission like blood transfusion, needle sharing, and laboratory accidents are also being reported (Herwaldt 1999).

5 Diagnosis

Preliminary diagnosis is dependent on the clinical signs and symptoms of visceral leishmaniasis such as hepatomegaly, splenomegaly, illness with prolonged irregular fever, and weight loss. However, the signs and symptoms may not necessarily represent VL and CL pathogenesis.

6 Parasite survival strategy: immune evasion mechanisms

Several factors are present that cause immunosuppression in host cells by *Leishmania* parasites as revealed during studies in animal models (hamster/mouse). Studies on mice models have revealed the role of T cells (Blackwell and Ulczak 1984), T-Helper II cells, and adherent cells as responsible for immunosuppression (Conceição-Silva and Morgado 2019). Reports say that *Leishmania* infection can cause the Suppression of superoxide production (Underhill and Ozinsky 2002; Pham et al. 2005; Lodge and Descoteaux 2006), decrease in NO (nitric oxide), production (Nandan and Reiner 1955; Blanchette et al. 1999; Wanderley et al. 2006), and inhibition of Interleukin-12 production (Rodriguez et al. 2001). *Leishmania* infection Delays apoptosis which is an adaptive mechanism and a defense strategy (Moore and Matlashewski 1994; Akarid et al. 2004; Donovan et al. 2009).

7 Control of leishmaniasis

There has been a lot of research for an effective drug or vaccine against leishmaniasis, but the search for a non-toxic, cost-effective and highly potent drug or vaccine is still going on. The first chemotherapeutic treatment of *Leishmaniasis* was developed in Germany by Ehrlich and his group in the late nineteenth century. Their novel approach of differential toxicity of the drugs towards pathogen and host is still the fundamental approach to all the drug development in modern-day also. It has been seen that in visceral leishmaniasis in India, the *Leishmania tropica* is the co-endemic mediator, this explains the reason behind the increase in the frequency of unresponsiveness towards therapy using sodium antimony gluconate. This further complicates the treatment in the Indian sub-continent. Thus, these compounds are slowly being replaced by newer formulations like the liposomal delivery of amphotericin B and other drugs like miltefosine and paromomycin (Chappuis et al. 2007).

7.1 Currently used therapeutic drugs

7.1.1 Antimonials

Macado and Vianna were the first to publish the use of trivalent antimonials (Sb^{III}) as an anti-*Leishmanial* chemotherapeutic agent in 1913 for CL. Later, in 1915 antimonials were introduced in India for VL treatment (Berman 1997).

7.1.2 Pentamidine isethionate

Pentamidine is used for 2nd line of treatment against VL. However, its exact working principle is not clear so far. Pentamidine is generally used as a competitive inhibitor of arginine-transport & noncompetitively inhibits spermidine and putrescine, it is speculated that its antileishmanial efficacy is probably facilitated via its ability to disrupt mitochondrial membrane potential and by effecting polyamine biosynthesis.

7.1.3 Amhotericin B

Amphotericin B, isolated from *Streptomyces nodosus* was initially an antifungal macrolide antibiotic and its antileishmanial activity was first shown in the early 1960s. Amphotericin B exhibits highly potent leishmanicidal activity. Because of growing Antimony ($Sb^{V)}$ unresponsiveness towards VL in the Indian subcontinent during the last couple of decades, amphotericin B has emerged as an alternate with high efficacy. 15 to 20 infusions, each having a dosage amount of 0.750 to 1 mg/kg bodyweight for either every other day or daily has reliably produced about 97 percent cure rates (Vertut et al. 1994).

7.1.4 Paromomycin

Paromomycin (identical to aminosidine), belonging to the class of aminocyclitol aminoglycosides is isolated from *Streptomyces rimosus* cultures and contains both antibacterial & antiprotozoal activity. The drug was made in the 1960s as an anti-leishmanial drug. But the compound remains neglected, until 1980 (Chunge et al. 1990). It provides a synergistic effect in combination with Sb^{v} as compared to Sb^{V} alone in several studies from India (Thakur et al. 1992, 1995)

7.1.5 Miltefosine

In 1987, Croft and Hogg (1988) described the activity of alkylphosphocholines, including the drug "miltefosine" to kill amastigotes of *L. donovani* under *in-vivo* (mice model) and *in-vitro* conditions (Croft et al. 1988). The introduction of miltefosine is a landmark in antileishmanial therapy as it is the first oral drug having satisfactory efficacy and toxicity. It has undergone phase I, II, and III trials and was found to be 94% successful (Sundar et al. 2002; Bryceson 2001).

7.1.6 Vaccine

Since the drug resistance developed due to chemotherapeutic treatment is a big health issue, exhaustive efforts have been taken towards the development of an effective vaccine. Increasing knowledge of immunological response and host-pathogen interactions involved in *Leishmania* infection has led to significant advancement in the search for vaccine candidates in *Leishmania* control.

Earlier studies in the search of suitable vaccine candidates included live as well as attenuated *Leishmania* parasites. Though live vaccines were effective these were discontinued because of problems associated with the virulence of effective vaccine (Gradoni 2001). DNA vaccines are more appealing newer generation vaccines but chances of DNA integration into host cell chromosomes and adverse immune response against DNA are some big hurdles (Watts and Kennedy 1999). Some vaccines, which have been tried exhaustively against *Leishmania*, are Leishmanisation (Tabbara et al. 2005) and Killed Vaccines (Russel and Alexander 1988; Handmanet et al. 1990; Olobo et al. 1995; Misra et al. 2001; Mohebali et al. 2004). Many other vaccine candidates like gp46/M2/Parasite Surface Antigen (Montgomary et al. 2000), LACK/p36 (Julia et al. 1996) dp72 and P0 (Rachamim and Jaffe 1993) PapLe22 (Fragaki et al. 2001), Amastigote cysteine proteases (Rafati et al. 2002) and Amastigote P4 and P8 (Soong et al. 1995) have been tried for their efficacy in different forms of *Leishmania*.

8 Natural products: source of antileishmanial Agent

Till now there is no effective vaccine against *Leishmania*. Most of the pentavalent antimony compounds had been developed as drugs to treat *Leishmania* infection before 1959. But increasing toxicity of these drugs, rising resistance, and various persistent side effects are still a matter of great concern. There has been an exhaustive exploration for antileishmanial agents and alternate therapy, like amphotericin B and pentamidine has been discovered but they also show unpleasant side effects (Brajtburg and Bolard, 1996; Sundar and Chakravarty 2015a; Hefnawy et al. 2018). Therefore, development for alternative drugs is urgently needed in this sector. Plants are a source of enormous chemical entities and the insistent require for alternative drugs has encouraged the researchers to find natural plant products for possible application in the treatment of leishmaniasis. Even, WHO has approved the usage of conventional medication in distant rural areas where primary health facilities are inaccessible (Chan-Bacab and Peña-Rodríguez 2001). Many people in rural areas, where appropriate health services are unavailable and people are unable to avail good medical treatment because of poverty and the absence of other agencies, people mostly rely on folk medicines which are generally extracted from natural resources like plants. The plant products occurring in nature are credible resources with an amazing variety and accessibility in their chemical composition. Many of the folk medicines which are being used for centuries have been scientifically proved to be potent. Data presented in Table 1 revealed the natural products which are a credible source of antileishmanial agents that have been explored extensively and many compounds are potent against different species of *Leishmania* (Vermelho et al. 2014). Improved drug designing and advancement against leishmaniasis is necessary at the current juncture if we want to continue the battle against the emerging drug-resistant variants of the deadly pathogen. New drug targets and the designing of novel compounds against the newly identified drug targets are necessary for clinical trials and toxicity studies (Table 2) (Hefnawy et al. 2017). Unfortunately, there has been little progress in developing alternative methods for managing *leishmaniasis* (Flórez et al. 2010; Pawar et al. 2014; Mol et al. 2015). Bioinformatic analysis can significantly reduce costs associated with the expensive clinical trials by identifying and analyzing drug candidates to the existing drug targets and also help us to identify new targets *in silico*. Bioinformatic analysis can facilitate characterization of the identified drug candidates and also predict their side effects and resistance. The analysis of the high-throughput genomics, proteomics, and metabolomics data through Bioinformatics may significantly contribute towards the discovery of new drugs against leishmaniasis (Xia 2017; Dos et al. 2018). Therefore, in this era of the constant onset of drug-resistant pathogens, bioinformatics can be used to accelerate the development of novel drugs against leishmaniasis.

Table 1 List of natural compounds showing antileishmanial activity

Family and Scientific name	Plant part	parasite tested*	References
Annonaceae			
Annona crassifora	Stem, root, bark	*LB, LD*	Brígido et al. 2000
Annona foetida	bark	*LB*	Brígido et al. 2000
Annona mucosa	Leaves, seed	*LB*	Brígido et al. 2000
Annona glabra	Leaves	*LD, LA*	Brígido et al. 2000
Annona purpurea	bark	*LD*	Brígido et al. 2000
Apocynaceae			
Aspidosperma spruceanum	Stem, bark, leaves	*LI*	Reina et al. 2014
Aspidosperma desmanthum	Stem, bark, leaves	*LI*	Reina et al. 2014
Peschiera australis	Stem	*LA*	Delorenzi et al. 2001
Pentalinon andrieuxii	Root	*LM*	Lezama-Dávila et al. 2014
Plumeria bicolor	Bark	*LD*	Sharma et al.2011
Himatanthus sucuuba	Bark	*LA*	Castillo et al. 2007
Altsonia scholaris	leaves	*LD*	Sharifi-Rad et al. 2018
Holarrhena curtisii	Leafe extract	*LD*	Kam et al. 1998
Peschiera australis	Stem, bark	*LA*	Delorenzi et al. 2001
Peschiera var. heurkii	Leaves, stem bark	*LA*	Muñoz et al. 1994
Picralima nitida	Seed	*LC*	Iwu et al. 1992
Tabernaemontana obliqua	Leaves	*LM, LA, LB,LB, LI, LD*	Weniger et al. 2001
Araliaceae			
Hedera helix	Leaves	*LM*	Hooshyar et al. 2014
Oreopanax floribundus	Leaves	*LP*	Cardona et al. 2020
Asclepiadaceae			
Gongronema latifolia	Leaves	*LC*	Iwu et al. 1992
Asteraceae			
Acanthospermum hispidum	Whole plant	*LA, LB*	Fournet et al. 1994b
Baccharis salicifolia	Leaves	*LB*	Fournet et al. 1994b
Chersodoma jodopappa	Leaves, Stem	*LA, LB, LD*	Fournet et al. 1994b
Cnicothamnus lorentzii	Leaves, Stem	*LA, LD, LB*	Fournet et al. 1994b
Echinacea purpurea	Whole plant	*Leishmaniasp.*	Panda and Luyten 2018
Munnozia fournetii	Leaves, Stem	*LD, LB, LA*	Fournet et al. 1994c
Neurolaena lobata	Leaves	*LM*	Berger et al. 2001
Ophryosporus piquerioides	Whole plant	*LA, LD, LB*	Fournet at al. 1994c
Perezia multiflora	Leaves	*LA, LD, LB*	Fournet at al. 1994c
Pterocaulona lopecuroideum	Whole plant	*LD, LB, LA*	Fournet at al. 1994c
Senecio clivicolus	Leaves, Stem	*LA, LD, LB*	Fournet at al. 1994c
Stevia yaconensis	Whole plant	*LD, LA, LB*	Fournet at al. 1994c

Family and Scientific name	Plant part	parasite tested*	References
Vernonia squamulosa	Stem	*LD, LB, LA*	Fournet at al. 1994c
Werneria nubigena	Leaves, Stem	*LB, LD, LA*	Fournet at al. 1994c
Xanthium catharticum	Root, Stem	*LB, LA, LD*	Fournet at al. 1994c
Berberidaceae			
Berberis vulgaris	Root	*LM, LT*	Mahmaudvand et al. 2014
Berberis bumeliaefolia	Bark	*LD, LB, LA*	Fournet et al. 1994b
Berberis cf. laurina	Stem	*LD, LB, LA*	Fournet et al. 1994b
Bombacaceae			
Hubero dendronpatino	Bark	*LP*	Weniger et al. 2001
Burseraceae			
Protium altsonii	leaves	*LA*	Santana et al. 2020
Protium hebetatum	leaves	*LA*	Santana et al. 2020
Celastraceae			
Maytenusi licifolia	Root bark	*LA*	Dos et al. 2013a
Clusiaceae			
Calophyllum brasiliense	Stem bark	*LI*	Da Silva et al. 2021
Crassulaceae			
Bryophyllum pinnatum (Lam.) Kurz	Leaves	*LA*	Rossi et al. 2000
Cucurbitaceae			
Coccinia grandis	Leaves	*LD*	Lahiry et al. 2018
Dilleniaceae			
Dillenia philippinensis	Stem	*LM*	Macahig et al. 2011
Euphorbiaceae			
Croton cajucara Benth	Bark	*LA*	Lima et al. 2015
Fabaceae			
Acacia nilotica	Bark	*LD*	Ali et al. 2021
Millettia richardiana	Stem bark	*LD*	Rajemiarimiraho et al.2014
Desmodium gangeticum L.	Whole plant	*LD*	Mishra et al. 2005
Gentianaceae			
Swertia chirata	Whole plant	*LD*	Medda et al. 1999
Lauraceae			
Endlicheria bracteolata	Leaves	*LA*	Rottini et al. 2019
Nectandra hihua	Stem bark, leaves	*LI*	Bosquiroli et al. 2017
Nectandra oppositifolia	Twig	*L. (L.) infantum chagasi*	Da Costa-Silva et al. 2019
Liliaceae			
Allium sativum L.	Bub	*LT*	Mahmoudvand et al. 2016
Melastomaceae			
Miconia langsdorffii	Ariel part	*LA*	Peixoto et al. 2011
Tibouchina paratropica	Ariel part	*LD*	Tracanna et al. 2015

Family and Scientific name	Plant part	parasite tested*	References
Meliaceae			
Azadirachta indica	Bark, Leaves, Seed	*LD*	Chouhan et al. 2015
Carapa guianensis	Seed oil	*LA*	Oliveira et al. 2018
Menispermaceae			
Cissampelos sympodialis Eichl.	Leaves	*LC*	Cavalcanti da Silva et al. 2012
Chondodendron tomentosum	Bark	*LI*	González-Coloma et al. 2012
Chasmanthera dependens	Stem, Bark	*LA*	Githinji et al. 2010
Tinospora sinensis	Stem	*LD*	Singh et al. 2008
Cissampelos sympodialis	Leaves	*LC*	Cavalcanti da Silva et al. 2015
Moraceae			
Pourouma guianensis	Leaves	*LA*	Torres-Santos et al. 2004
Moringaceae			
Moringa Oleifera	Flowers	*LD*	Singh et al. 2015
Myristicaceae			
Otoba novogranatensis	Leaves	*LA, LB, LI, LA, LB, LI*	Weniger et al. 2001
Otoba parvifolia	Bark	*LA, LB*	Weniger et al. 2001
Virola surinamensis	Leaves	*LD*	Barata et al. 2000
Myrsinaceae			
Maesabalansae	Leaves	*LD*	Maes et al. 2004
Papaveraceae			
Bocconia integrifolia	Leaves, Stem bark	*LA, LB, LD*	Fournet et al. 1994c
Bocconia pearcei	Leaves	*LA, LB, LD*	Fournet et al. 1994c
Bocconia pearcei	Fruit	*LM*	Fuchino et al. 2010
Phytolaccaceae			
Petiveriaalliaceae L	Leaves	*LA*	Garcia et al. 2017
Piperaceae			
Piper longum L	Ariel part	*LD*	Singh et al. 2011
Piper auritum	Ariel part	*LD, LM, LD, LB*	Monzote et al. 2010
Rubiaceae			
Corynanthe mayumbensis	Stem, Bark	*LI*	Lamidi et al. 2005
Rutaceae			
Citrus sinensis	Leaves	*LA*	Garcia et al. 2017
Galipea longiflora	Leaves, Root bark	*LD, LB, LA*	Fournet et al. 1994a
Swinglea glutinosa	Bark	*LA, LB, LI*	Weniger et al. 2001
Sapindaceae			
Dodonaea viscosa	Leaves	*LA*	Al-Sokari et al. 2015
Cupania dentate	Bark	*LM*	Peraza-Sánchez et al. 2007
Scrophulariaceae			
Conobea scoparioides	Leaves	*LA, LB*	Weniger et al. 2001

Family and Scientific name	Plant part	parasite tested*	References
	Solanaceae		
Brugmansia suaveolens	Flowers, Leaves	*LA*	Monzote et al. 2016
Brunfelsia cestroides	Leaves, Stem	*do*	Monzote et al. 2016
Capsicum annum.	Root	*do*	Monzote et al. 2016
Capsicum frutescens L.	Root	*do*	Monzote et al. 2016
Capsicum chinense	Root	*do*	Monzote et al. 2016
Cestrum nocturnum L.	Root	*do*	Monzote et al. 2016
Nicotiana rustica L.	Root	*do*	Monzote et al. 2016
Solanum americanum	Leaves	*do*	Monzote et al. 2016
Solanum lycopersicon	Fruits	*do*	Monzote et al. 2016
	Sterculiaceae		
Corchorus capsularis L.	Leaves	*do*	Pramanik et al. 2019
	Ulmaceae		
A. edentulaKuhlm	Stem bark	*LB*	Fournet et al. 1994b
	Verbenaceae		
Vitex heterophylla	Leaves	*LD*	Bhakuni et al. 1988

** LA (L. amazonensis), LC (L. chagasi), LB (L. braziliensis), LI (L. infantum), LD (L. donovani), LM (L. maxicana), LT (L.tropica), LM (L. panamensis)*

Table 2 Antileishmanial compounds and mechanism of action

Plant derived product	Mechanism of action	Reference
Quercetin	Inhibits *Leishmania amazonensis*arginase	Da-Silva et al. 2012
	Generation of reactive oxygen species and mitochondrial disruption of *L. amazonensis* promastigotes	Fonseca-Silva et al. 2011
	Inhibits topoisomerase II in leishmania sp.	Mittra et al.2000; Cortázar et al. 2007
Leteolin	Inhibits *L. donovani*topoisomerase II	Mittra et al. 2000
Licochalcone A	Disrupts the function and ultrastructure of leishmanial mitochondria	Zhai et al. 1995
	Inhibits leishmania Fumarate Reductase	Chen et al. 2001
leiocarpin	Disrupts mitochondrial membrane potential	Morais et al. 2020
Amarogentin	Inhibits L. donovani DNA-topoisomerase I	Ray et al. 1996
Plumbagin	Inhibits trypanothione reductase in leishmania	Sharma et al. 2012
Diphyllin	Inhibits parasite phagocytosis by macrophages	Di Giorgio et al. 2005
Artemisinin	Heme-triggered activation of Artemisinin	Geroldinger et al. 2020
Epigallocatechin 3-gallate	Inhibits *leishmania* arginase	Dos et al. 2013b; Khademvatan et al. 2019

Other natural compounds which have been explored are marine sources or microorganisms. For example, a protein containing carbohydrate moiety extracted from the sponge *Pachymatisma johnstonii*, demonstrated strong efficacy against *Leishmania braziliensis*, *Leishmania mexicana*, and *Leishmania donovani*, under *in-vitro* conditions. Another fungal metabolite, aphidicolin extracted from *Nigrospora sphaerica*, has also been found to suppress *L.donovani* amastigotes and promastigotes growth (Chan-Bacab and Peña-Rodríguez 2001). Nonetheless, plants have been the most explored natural source. The different classes of natural compounds explored have been discussed subsequently.

8.1 Flavonoids

The flavonoids, quercetin, and Luteolin isolated from a Polygonaceae *(Fagopyrum esculentum*) and a Verbenaceae (*Vitex*

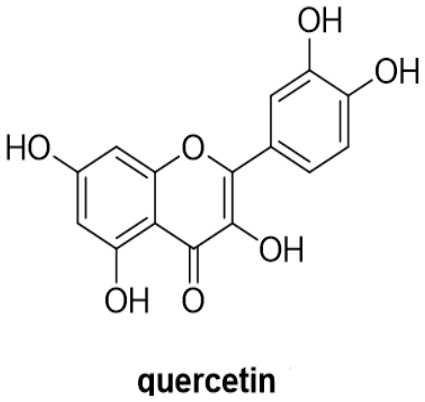
negundo) are effective antileishmanial compounds having IC50 as 12.5 micromoles and 14.5 micromoles respectively, against amastigote form of *Leishmania donovani*. Both these drugs are capable of stimulating topoisomerase II-dependent cleavage of kinetoplastid DNA in *Leishmania* donovani. Studies in animal models indicate that Luteolin gives protection of up to 80 percent in the *Leishmania* infected spleen when treated with 3.5mg/kg of body weight. Similarly, quercetin reduces parasite load up to ninety percent at a dosage of 14 mg/Kg weight of the body. Luteolin is nontoxic to human T-cells (Mittra et al. 2000). It is reported that quercetin and luteolin (figure 4) specifically inhibit a typical bi-subunit topoisomerase (topoisomerase I) present in *Leishmania* parasites (da Silva et al. 2012). The phenolic compound"5,7,4'-trihydroxyflavan" shows anti-amastigotes activity against *L. amazonensis* (Fonesca-Silva et al. 2016). The bioflavonoids "podocarpusflavone A", amentoflavone, and podocarpusflavone B, extracted from *C. maxicanum* leaves, show a mild action when used against *L. donovani* (Chang Bacab and Peña-Rodríguez 2001).

8.2 Chalcones

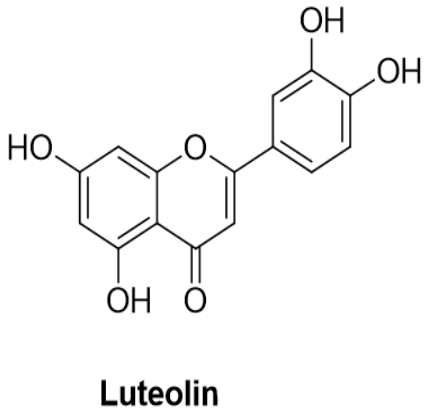
Licochalcone A (Figure 5) is an oxygenated chalcone that has been obtained from Chinese liquorice *Glycyrrhiza spp*. (Fabaceae), shows robust activity against leishmania, by preventing the proliferation of amastigote and promastigotes of *Leishmania donovani* and *Leishmania major* (Chen et al. 1993; Croft et al. 2006). A 96% reduction in the number of the parasite in the spleen and liver was seen under *in vivo* studies in hamsters (Chen et al. 1994; Croft et al. 2006). Licochalcone A and associated chalcones can destroy the mitochondrial ultrastructure of the *Leishmania* parasite. Moreover, the compounds can strongly inhibit Fumarate Reductase in *L. major* and trypanothione reductase in *L. donovani* (Chen et al. 2001; Ortalli et al. 2018). The chalcone-"(*E*)-1-[2,4-hydroxy-3-(3-methylbut-2-enyl)phenyl]-3-[4-hydroxy-3-(3-methylbut-2-enyl)phenyl]-prop-2-en-1-one" is toxic towards *Leishmania donovani* promastigotes, while "2',6'-dihydroxy-4'-methoxychalcone", obtained from flowers of "*Piper aduncum"*, is shown to exhibit a considerable *in vitro* action towards amastigotes and promastigotes of *L. amazonensis* (Chang Bacab and Peña-Rodríguez 2001)

8.3 Saponin

Mesabalides I-VI, all the six oleane tri-terpenoid saponins extracted from *Maesabalansae* (Myrsinaceae) has been reported to have potent *antileishmanial* action. Maesabalide III and IV (Figure 6) were most effective having IC(50) values against

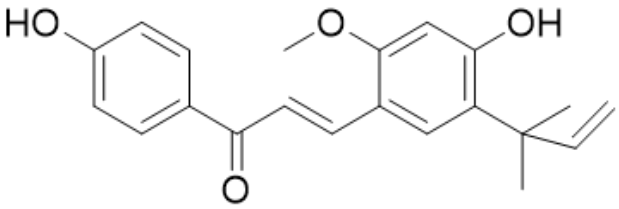


quercetin



Luteolin

Figure 4 Quercetin and Luteolin



Licochalcone A

Figure 5 Licochalcone A

amastigotes residing inside the macrophages at a dosage of 7ng/ml and 14 ng/ml respectively far less as compared to standard drug sodium stibogluconate (IC50, 5.6 µg/ml) for treating *Leishmania* infection (Germonprez et al. 2005). Similarly, a comparable efficiency of mesabalide III (0.8mg/kg for 1 day) to Amphotericin-B (5 mg/kg for one day) has been demonstrated in *L. donovani* infected hamsters (Maes et al. 2004). Racemoside A (Figure 6), a steroidal saponin derived from *Asparagus racemosus* (Liliaceae), was capable to cause apoptosis in *Leishmania donovani* amastigotes (Dutta et al. 2007). Asteroidal saponin extracted from bulbs of *Allium paradoxum* exhibited an anti-leishmanial effect by directly killing *L.major* promastigotes (Rezaee et al. 2018). These saponins decrease the parasitic membrane potential and inhibit the growth of promastigotes. However, the cytotoxic activity of some antileishmanial saponins on the host cell is quite concerning (Chan-Bacab and Peña-Rodríguez 2001).

8.4 Terpenes

Linalool (Figure 7), a monoterpene derived from a Euphorbiaceae, *Croton cajucara*, shows potent action against *L. amazonensis* (both promastigotes & amastigotes). Linalool was able to decrease the intracellular parasite burden by 50% in infected macrophages. It also increases NO production. *In vitro* mitochondrial swelling and destruction of chromatin and kinetoplastid was observed, ultimately undergoing cell lysis (do Sorocco et al. 2003). Another

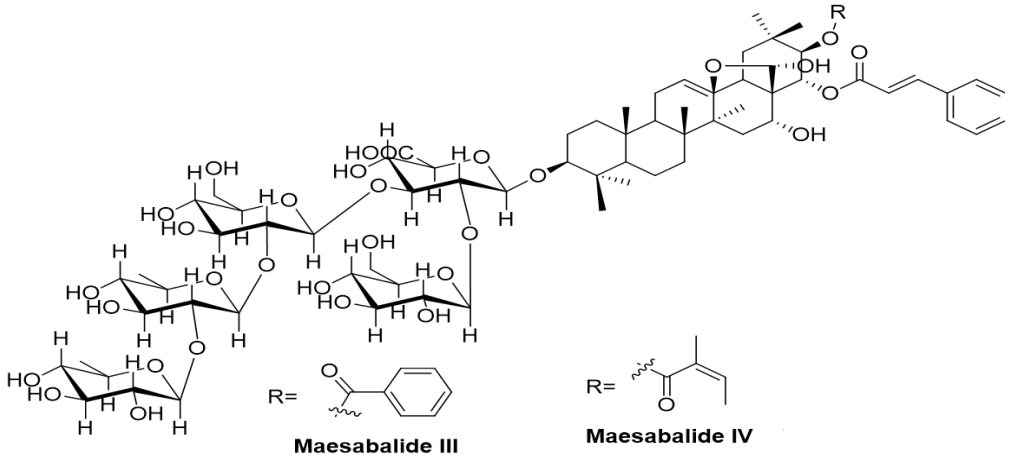


Figure 6 Maesabalide III, Maesabalide IV, Recemoside A

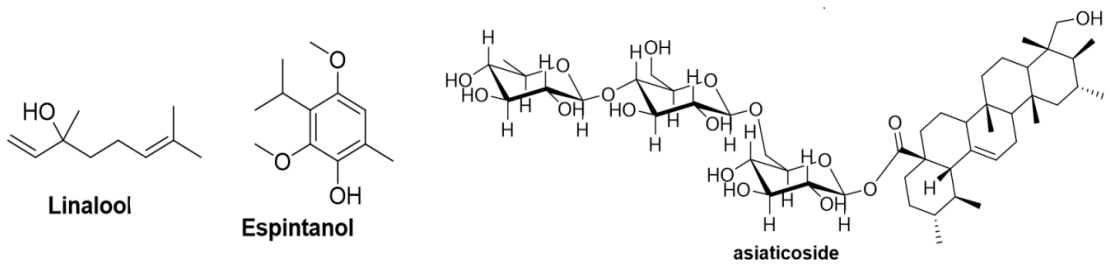


Figure 7 Linalool, Espintanol, Asiaticoside

monoterpenoid, Espintanol (Figure 7) obtained from *Oxandraespintana* (Annonaceae) bark, show strong efficacy against promastigotes of 12 different species of Leishmania. Nevertheless, *in-vivo* efficacy was very weak. Two monoterpenoid derivatives grifolin and piperogalin extracted from *Peperomia* completely lyses *L. donovani* and *L. braziliensis* promastigotes at an amount of 100 microgram/ml. Diterpenoids also show leishmanicidal activity. Jatrogrossidione and jatrophone, extracted from Euphorbiaceae species exhibit toxicity against different species of Leishmania parasite (*L. braziliensis, L. amazonensis, L. chagasi.)* promastigotes. 6-β-hydroxyrosenonolactone is another diterpene derived from *Holarrhena floribunda* (Apocynaceae) bark, showing moderate activity against *L. donovani*, and is less cytotoxic to host macrophage. Triterpenes are also another group of metabolites showing antileishmanial action, which consists of betulinaldehyde and ursolic acid, isolated from *Doliocarpus dentatus* (Dilleniaceae) stem and *Jacaranda copaia* bark, respectively. Both the compounds showed efficacy against *L. amazonensis* amastigotes. However, *in-vivo* results were not satisfactory and cytotoxicity was also a concern. Two new terpenoids named sugikurojin A and asiaticoside (figure 7) synthesized from *Cryptomeria japonica* and *Centellaasiatica* show antileishmanial activity against *L. infantum* (IC50 value-14.0 µM) and *Leishmania donovani* (IC50-11.2µM) (Bhaumik et al. 2012).

8.5 Iridoids

Amarogentin, a secoiridoid glycoside extracted from *Swertia chirata* (Gentianaceae) and the iridoid Molucidin (Figure 8) extracted from *M. lucida*, show strong *in-vivo* antileishmanial activity. Amarogentin potentially inhibits *Leishmania* topoisomerase-I (Ray et al. 1996). The non-ionic surfactant vesicle system i.e. niosomal formulation decreased the parasite numbers by ninety percent in the spleen after treating for six days with 2.5 mg per of the drug. Pathological studies, staining of histological slides, and the amount of certain liver enzymes show minimum cytotoxic activity.

8.6 Napthoquinone

Plumbagin, a naphthoquinone extracted from the Euphorbiaceae *"Perabenensis"*, inhibits the growth of intracellular amastigotes and promastigotes of *L. donovani*. Another bis-naphthoquinone, Diospyrin (Figure 9) (a semi-synthetic derivative) isolated from *Diospyros Montana* Roxb also shows potent activity against leishmania parasite under *in-vitro* and *in-vivo* studies (Hazra et al. 2013).

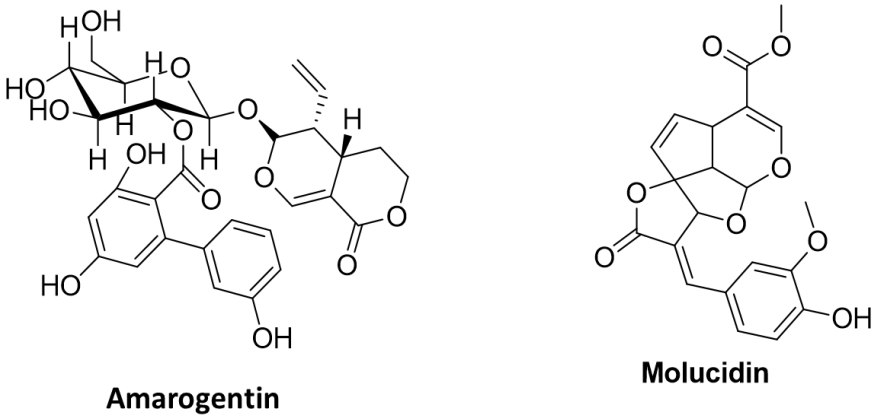


Figure 8 Amarogentin, Molucidin

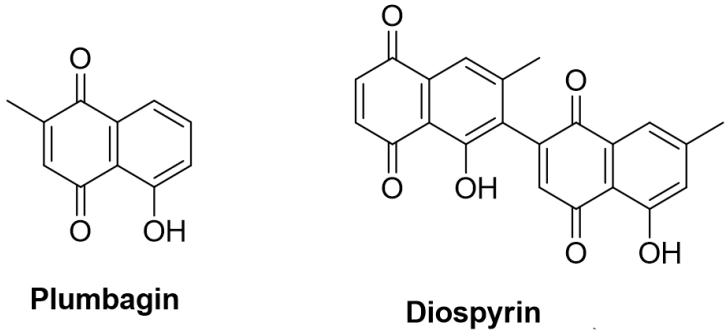


Figure 9 Plumbagin, Diosyrin

8.7 Quinoline Alkaloids

2- Substitute quinolines extracted from "*G. longiflora"* have been shown to exert potent activity against CL and VL. It is reported that China mine B (Figure 10) treatment at 50 mg/kg body reduced the lesion size by seventy-four percent and the leishmania burden by ninety percent in the cutaneous leishmaniasis mice model with a regime of 5 injections, each given at 4 days of interval (Fournet et al. 1994a). Treating with Chinamine D (Figure 10) resulted in eighty-seven percent hepatic parasite reduction (at a dose of 100 mg/kg of body weight for 5 days). "2-n-propylquinoline" lowers the parasite burden in the liver by 99.9 percent in an animal model of VL at a dose of 94 mg/kg body weight for ten days 10 days (Fournet et al. 1992). Recent studies with several derivatives of synthetic quinoline presented a notable decrease in the parasite number (80-90% in the lesions of *L. donovani* and *L.amazonensis* infected mice when treated orally with 25 mg/kg body weight for 10 days, twice daily (Coimbra et al. 2016).

8.8 Lignans

Diphyllin (Figure 11), a lignan derived from *H.bucharicum* (Rutaceae) showed leishmanicidal efficacy against *L. infantum* with IC50 values of 14.4 microgram/kg4.4 µg/ml and 0.2 µg/ml correspondingly. The derivative "s-ketosulfide (3,4-dimethoxy)-8-(40-methylthiophenoxy)- propiophenone of the neolignan 3,4,5-trimethoxy-8-[20,60-dimethoxy-40-(E)-propenylphenoxy]-phenylpropane" extracted from Myristicaceae (*Virolapavonis)* has shown moderate activity against the promastigote and amastigote form of *L. donovani* (Polonio and Efferth 2008). Another two important lignan glycosides (lyoniside and saracoside) and lignin derivative- niranthin kill *L. donovani* both under *in-vivo* and *in-vitro* conditions by inhibiting parasite-specific topoisomerases (Saha et al. 2013).

8.9 Toxoid

10-Deacetylbaccatin III, a toxoid isolated from the Taxaceae family (*T. baccata*) shows potent leishmanicidal efficacy against *L. donovani* amastigotes. It has been reported that 10-Deacetylbaccatin III is non-toxic to the host macrophages as much as a concentration of 5 µM. 10-deacetylbaccatin produces Nitric oxide to show antileishmanial activity (Polonio and Efferth 2008).

8.10 Sesquiterpenes

Artemisinin (Figure 12), an effective anti-malarial drug, extracted from *Artemisia annua* (Asteraceae) shows antileishmanial activity. The IC50 values are 22 µM against intracellular amastigotes and 160 µM against promastigotes. Dehydrozaluzanin C (Figure 12), a sesquiterpene lactone extracted from *Munnozia maronii* (Asteraceae) leaves, blocks the promastigote survival in 11 different *Leishmania* (at concentration raging 2.5 to 10 µg /ml). This has also been seen under *in-vivo* conditions through the reduction of lesion severity due to *L. amazonensis* infection in mice (Chan-Bacab and Peña-Rodríguez 2001; Polonio and Efferth 2008). In addition, Sesquiterpenes-rich compounds extracted from *Copaifera* spp. Showed leishmanicidal efficacy against *L. amazonensis* intracellular amastigotes (Soares et al. 2013).

Figure 10 ChimanineB, Chimanine D

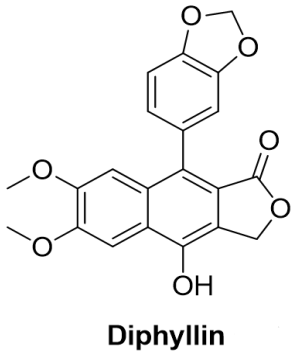


Figure 11 Diphyllin

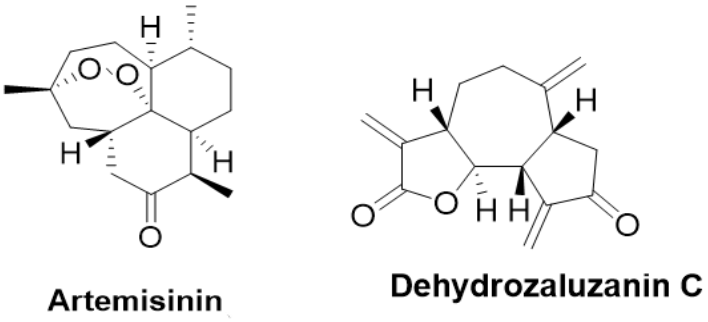


Figure 12 Artemisinin, Dehydrozaluzanin C

9 Investigational Drugs against Leishmaniasis

Among several natural products, which have shown potent anti-leishmanial activity, few have reached phase II trials. Two nitroimidazole compounds, PA-824, and fexinidazole have shown potent anti-leishmanial responses in pharmacokinetic studies in humans. Also, derivatives of quinoline scaffold, like Indolyl quinoline analogs and the Naphthoquinones derivatives like Buparvaquone currently have been listed for phase II trials (Sundar and Chakravarty 2015b).

Conclusion

There has been a lot of research for an effective drug or vaccine against *Leishmaniasis*, but the search for a non-toxic, cost-effective and highly potent drug or vaccine is still going on. Still, an effective vaccine against leishmania is lacking. Drugs based on Pentavalent antimony compounds are still the main course drugs. Nevertheless, the persistence of side effects and the growing drug resistance are still a matter of great concern. This advocates the urgent need of developing alternative drugs. Plant-based or plant-extracted materials may probably provide an important resource of new medicinal drugs, which could be used as alternative therapeutic strategies. So, research must be undertaken to screen natural products, especially, plant-derived products for probable use in *Leishmania* therapy. They have the advantage of having fewer side effects and low cost. Though initial studies are abundant, there is more need to further do research, isolate lead compounds and study the mechanism of action. Despite so many encouraging findings, these compounds have not been able to make it in the market or even in clinical trials. We advocate that the authorities should encourage more product-oriented initiatives along with R&D for this poor man's disease.

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Journal of Experimental Biology and Agricultural Sciences, February - 2022; Volume – 10(1-BIONEXT-2021) page 248 – 253

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Ancestry Specific variation in neuropsychological disorders among the South Asian population

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ABSTRACT

The enormous genetic diversity in South Asia resulting from a long and complex admixture history resulted in the emergence of variation in various traits and variations in disease susceptibility. Neuropsychological disorders are one such example that shows variation at the population level. In this study, we aimed at understanding the variation in neuropsychological disorders at the population level among South Asian populations by curating, comparing and contrasting single nucleotide polymorphisms (SNPs), known to be associated with the same. Whole-genome data comprising of 1662 South Asians, belonging to 241 distinct populations were obtained from the database of Dr. David Reich, Harvard Medical School, USA. Principal Component Analysis (PCA) revealed that the Ancestral Tibeto Burman (ATB) genomes form a distinct and distinguishable cluster for the SNPs known to be associated with neuropsychological disorders. Identical By Descent (IBD) analysis showed that out of the top seven populations in terms of IBD sharing, six are from Southern India indicating that these populations may have undergone a recent selective sweep for these SNPs. Further, out of the top ten genomes, according to the number of genomes fixed for the minor alleles, seven were from Southern India. Furthermore, several indigenous populations from South India depicted high F values (>0.25) for SNPs associated with neuropsychological disorders, indicating higher susceptibility for neuropsychological disorders among these South Indian populations. Interestingly, we found that most of the SNPs, fixed for the alternative alleles, were also found to be fixed among the ancient genomes from Indus Valley Civilization (IVC), indicating that these SNPs likely got transmitted to various modern-day South Indian populations from IVC.

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1 Introduction

South Asia, comprised of India, Sri Lanka, Pakistan, Bangladesh, Bhutan, and Nepal has a millennia-old history of mixing of gene-pools from various parts of the world including Southeast Asia, West Eurasia, and a hunter-gatherer lineage from South Asia who likely arrived here via 'southern exit route' from Africa (Shinde et al. 2019). These South Asian hunter-gatherers, referred to as Ancient Ancestral South Indians (AASI), have genetic similarities with present-day Andamanese people. Modern-day South Asian genomes largely belong to four ancestries: Ancestral South Indian (ASI), Ancestral North Indian (ANI), Ancestral Austro-Asiatic (AAA), and Ancestral Tibeto-Burman (ATB) comprising of various combinations of West Eurasian, Southeast Asian, and AASI ancestry fractions (Basu et al. 2016). The enormous genetic diversity has been maintained by the age-old practice of endogamy (Nakatsuka et al. 2017). Genetic admixture among various populations across the world, followed by the practice of endogamy has resulted in the emergence of variation in susceptibility towards various diseases and conditions among South Asian populations.

Neuropsychological disorders are one of the notable examples that show population-specific variation. More than 100 million people are suffering from various psychological disorders such as clinical depression and anxiety disorders in India (Sagar et al. 2020). Over the last decade, several studies have shown population-specific variation in neuropsychological disorders among South Asian populations. Dutta (2013) aimed at understanding the disproportionally higher number of cases of alcoholic cirrhosis among Indians compared to the Caucasian populations. He speculated the association of the 10109G allele of *PNPLA3* with the susceptibility towards alcoholic cirrhosis among North Indian populations.

While a handful of small-scale studies revealed that substance abuse is highly prevalent among Northeast Indians (Medhi et al. 2006; Mahanta et al. 2016), the association between the intrinsic genetic makeup and the substance use among the people from Northeastern states has remained understudied so far. A recent public health survey revealed that substance consumption and abuse are discernibly higher among males from Northeast India compared to the other parts of the country (Saikia and Debbarma 2020). The higher instances of substance abuse among these people have been linked to socioeconomic factors such as poverty and illiteracy. Another survey across the states of Northeast India depicted that >50% of adults consume some forms of tobacco in Meghalaya, Manipur, Tripura, and Mizoram (Ladusingh et al. 2017). Further, an increase in the abuse of illegal substances such as opium and heroin in many parts of Northeast India has also been reported despite strict legal restrictions (Chaturvedi 2003). Further, a cross-sectional study on samples from Global Adult Tobacco Survey in India, took place between 2009 and 10, comprising of more than 69,000 individuals, revealed that while the smoking prevalence is highest in Meghalaya, Mizoram has the highest prevalence of smoking among women in the country (Singh and Ladu singh 2014). Finally, smokeless tobacco usage is highest in Nagaland (49.9%) compared to other Northeastern states (Sinha et al. 2003).

In this study, we aimed at understanding population-specific variation in neuropsychological disorders among South Asian populations by curating and comparing the single nucleotide polymorphisms (SNPs), associated with the same.

2 Materials and Methods

2.1 Data Collection

A total of 4617 SNPs associated with various neuropsychological disorders such as alcohol dependence, Alzheimer's disease, mental health and depression, smoking, nicotine dependence, schizophrenia, and substance abuse were curated from the University of California Santa Cruz (UCSC) genome browser (http://genome.ucsc.edu/). The potential link between the curated SNPs with neuropsychological disorders was confirmed by annotating them using SNPnexus web-based server (Dayem Ullah et al. 2018). The genome-wide data (*IndiaHO_dataforrelease*) of 1662 South Asian individuals, from 241 distinct populations were obtained from the database of Dr. David Reich, Harvard Medical School, Harvard University, USA (Accessed on 14.11.2017, https://reich.hms.harvard.edu/datasets). PLINK v1.9 (Purcell et al. 2007) was employed for all file conversions and manipulations. SNPs related to neuropsychological disorders were extracted from *IndiaHO_dataforrelease* using the --extract command in PLINK. Out of 4617 curated SNPs, 4233 SNPs were absent in this genotype file. Therefore, our final dataset assessed 1662 South Asian genomes for 384 SNPs related to neuropsychological disorders.

Most South Asian genomes are genetic mosaics of Indus periphery and AASI related ancestries with variable ancestral fractions from Mid-Late Bronze Age steppe genomes (Steppe MLBA) (Shinde et al. 2019). A total of 22 Steppe MLBA and four Indus periphery genomes, including the only genome obtained from the Indus valley, were analyzed to investigate whether any of the 384 SNPs, employed in this study, were fixed for the alternative alleles among these ancient populations. These ancient genomes were also obtained from the aforesaid database of Dr. David Reich (Accessed on 21.08.2019).

2.2 Clustering of South Asians based on Neuropsychological SNPs

Fine structure within South Asian genomes, based on 384 SNPs associated with neuropsychological disorders, was discerned

employing Principal Component Analysis (PCA) implemented in PLINK v1.9. PC1 and PC2, the two most informative PCs, were plotted in Microsoft® Excel v16.42.

2.3 Identical by Descent (IBD) analysis

Populations with high IBD sharing can be under natural selection since the latter is presumed to promote longer IBD segments. IBD sharing was calculated in PLINK v1.9 using the Pi-Hat statistic (Proportion IBD: P(IBD=2) + 0.5*P(IBD=1)). A pie chart depicting the highest Pi-Hat values was plotted in GraphPad Prism v9.

2.4 Genomic Data analysis

Cluster-stratified (population-wise) Minor Allele Frequencies (MAF) defined by the family IDs were estimated in PLINK v1.9. Observed and expected homozygosity estimation and subsequent method-of-moments F coefficient calculation for each individual in the dataset was performed in PLINK. Further, the fixation indices (FST) were estimated separately for all 384 markers employed in this study in PLINK v1.9. The geographical affinity of the individual genomes was indicated by the family ID (FID).

2.5 Negative Control

To assess whether the population structure revealed by the SNPs associated with neuropsychological disorders is an artifact of the small number of SNPs under evaluation, we qualitatively compared the PCA plot produced by these SNPs with the ones generated by 100 SNP panels of randomly sampled 384 SNPs from the whole genome dataset.

3 Results

3.1 Clustering of populations from South Asia based on Neuropsychological SNPs

South Asians depicted an AAA –ASI –ANI cline along X-axis (PC1) with Juang, Koya, Gadaba, Ho, Santhal, Bondo, Oraon, Batudi, Kharia, Kondakamari, Asur, Porja, Kondh, Palliyar, Ulladan, and Irula genomes clustering at one end of the cline, and Ashkenazi Jews, Cochin Jews, Kamboj, Makrani and Kalash genomes clustering at the other end (Figure 1). The Ancestral Tibeto Burman (ATB) genomes including Magar, Sherpa, Chakeshanega, Kusunda, and Tharu populations formed a distinct cluster, differentiated from AAA and ASI along the Y-axis (PC2).

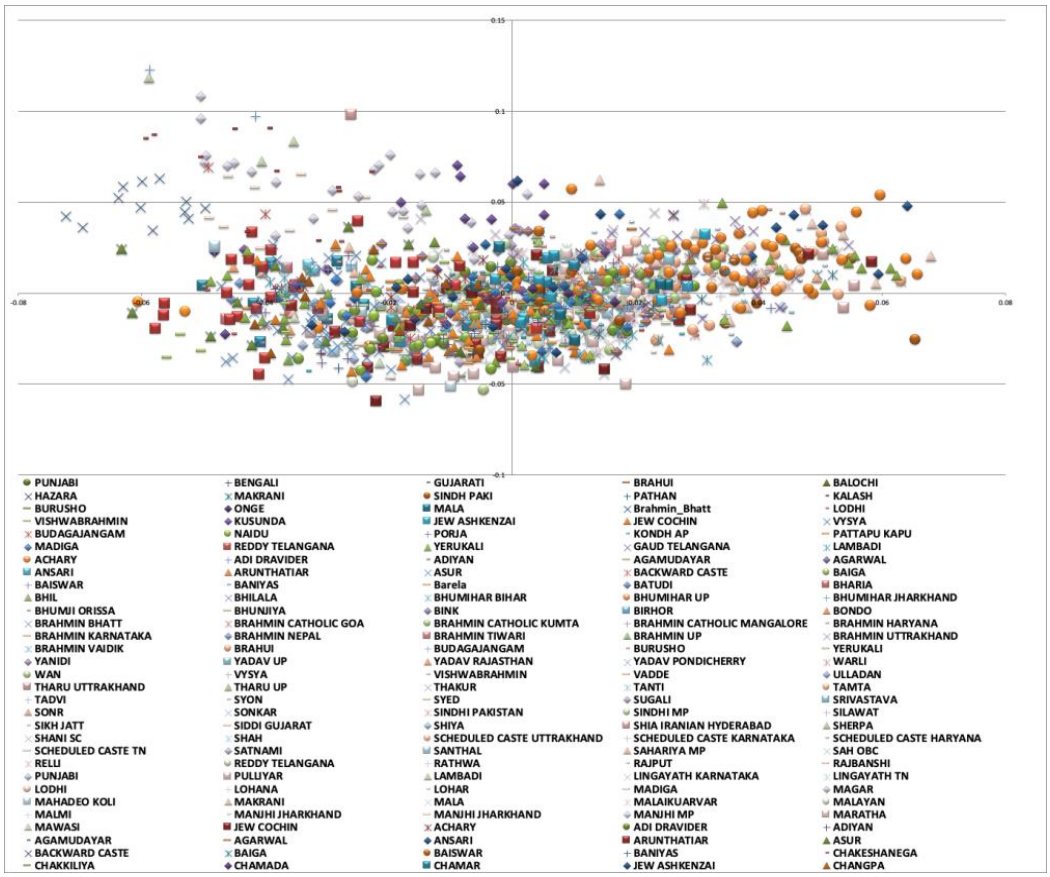

Figure 1 Principal Component Analysis (PCA) of South Asian populations based on SNPs, associated with neuropsychological disorders. PCA plot revealing the genetic differentiation among South Asian genomes based on 384 SNPs associated with neuropsychological disorders

Interestingly, Rajbanshi genomes from West Bengal formed a bridge between the ATB and AAA clusters for the study of SNPs associated with neuropsychological disorders.

To assess whether this AAA-ASI-ANI cline is unique for the SNPs associated with neuropsychological disorders or an artifact of a small sample size, we compared the PCA plot generated by these SNPs with those generated by 100 SNP panels of randomly sampled 384 SNPs from the whole genome dataset. No PCA plot generated by the random SNPs could recapitulate this cline and depicted random clustering of populations, irrespective of their ancestral associations. Therefore, we surmise that the AAA-ASI-ANI cline depicted by the SNPs associated with neuropsychological disorders is likely reflective of the clustering of South Asian genomes according to their propensity towards these diseases.

3.2 Identical by Descent (IBD) analysis

The highest Pi-Hat value obtained was 0.5276. For stringency, we made a cut-off of the Pi-Hat value at 0.3 and calculated the number of pairs sharing high Pi-Hat values in various populations under study. We found 19 populations sharing 10 or more high Pi-Hat values, indicating high IBD sharing. The highest IBD sharing was observed among Yerukali genomes from Andhra Pradesh and Telangana, closely followed by the native Burusho people from Pakistan, Yadavs from Pondicherry, and Vishwabrahmins from Southern India (Figure 2), a likely indication of a recent selective sweep in these SNPs, among the aforementioned populations. Notably, out of the top seven populations in terms of IBD sharing, six are from Southern India.

3.3 Genomic Data analysis

Out of 384 SNPs assessed, ≥35 was fixed for the alternate allele among Ho (N=40), Kondakamari (N=38), Malmi (N=38), Agamudayar (N=37), Kamsali (N=37), Gadaba (N=36), Gowli (N=36), Sonr (N=35) genomes respectively. Overall, we found 25 genomes with at least 10 alleles of MAF=1. To note, seven out of the top ten genomes according to the number of fixed alleles were from Southern India, and six out of ten were tribal groups.

Onge genomes due to their genetic distinctness, expectedly had the highest discrepancies between the observed and expected heterozygosities and thus had the highest F values (>0.25). Among others, Kusundas from Nepal, several indigenous populations, and tribal groups from South India including Palliyar, Ulladan, Narikuruvar, and Kallar depicted high F values (>0.25) for the study SNPs. Among these, Palliyars from Kerala had the maximum number of individuals with significant differences

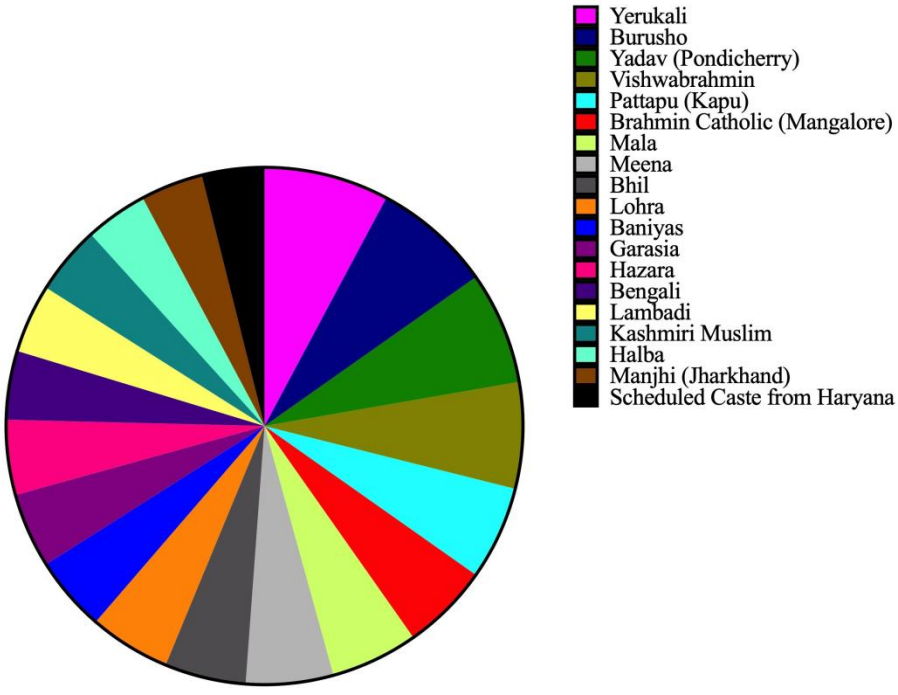

Figure 2 Identical by Descent (IBD) analysis of genomes from South Asia genomes.19 populations with strong IBD sharing (>10 high Pi-Hat values) are plotted. The pie chart indicates that maximum number of highest IBD scores was found among the Yerukali population from Andhra Pradesh (Pink), followed by the Burusho people from Pakistan (Navi blue) and Yadavs from Pondicherry (Dark green). The pie-chart was plotted in GraphPad Prism v9.

between observed and expected heterozygosities, indicating their genetic uniqueness for aforesaid SNPs.

The highest F_{st} (0.26) among the South Asian genomes assessed, was observed for rs11825659 at chromosome 11, which is associated with unipolar depression and alcohol dependence, followed by rs11038167 at chromosome 11 (F_{st} =0.13) and rs7683704 at chromosome 4 (F_{st} =0.08), which is associated with schizophrenia and alcoholism respectively (Shokraeian et al. 2019, Gizer et al. 2011). While none of the 384 SNPs were fixed for the alternative alleles among the Steppe MLBA genomes, 45 SNPs were fixed among the Indus Periphery genomes.

4 Discussions

In the current study, we aimed at understanding the variation in neuropsychological disorders at the population level among populations from South Asia. PCA on SNPs associated with neuropsychological disorders revealed a distinct cluster of Ancestral Tibeto Burman (ATB) genomes including Magar, Sherpa, Chakeshanega, Kusunda, and Tharu populations, significantly differentiated from the ANI, ASI, and AAA clusters (Figure 1). The genomic distinctness of ATB genomes for the study SNPs can be presumed to be indicative of their higher susceptibility to neuropsychological disorders. This echoes our previous study, where we showed that the SNPs related to neuropsychological disorders are under strong selection pressure among individuals from East Asia (Hande et al. 2021).

Besides ATB genomes, genomes of several tribal and indigenous populations from South India revealed a higher propensity towards neuropsychological disorders. For instance, out of the top seven populations in terms of IBD sharing, six were from Southern India hinting at a recent selective sweep in terms of the SNPs associated with neuropsychological disorders among these populations. Further, out of the top ten genomes fixed for the alternate allele seven were from Southern India and six were from tribal groups. Several indigenous populations from Southern India also revealed high F values, indicating their genetic higher propensity for neuropsychological disorders. Congruent with our findings, a recent comprehensive study carried out by the Indian state-level disease burden initiative revealed that Southern Indian states such as Telangana, Tamil Nadu, Kerala, Karnataka, and Andhra Pradesh revealed a discernibly high prevalence of adulthood mental disorders including depression and anxiety (Sagar et al. 2020).

Out of 384 SNPs assessed in this study, 45 were fixed for the alternative alleles among ancient Indus Periphery genomes. Interestingly, among the modern-day populations, these SNPs were found to be fixed among the tribal groups and indigenous populations from Southern India with high AASI ancestry. This indicates that these SNPs were likely fixed for the alternative alleles among people of AASI or Indus periphery ancestry and subsequently got transmitted to modern-day Indian populations, especially to those who had little or no Steppe MLBA admixture. To assess whether Indus Periphery genomes had signatures for susceptibility towards neuropsychological disorders, we annotated all SNPs of these genomes that were fixed for the alternative allele (MAF=1). A total of 90,781 SNPs were found to be fixed for the alternative allele, uniquely among the Indus Periphery genomes. Among them, 2273 are associated with various molecular pathways governing the neuronal system. It can be speculated that the residents of ancient Indus Valley likely had unique genetic signatures that made them more susceptible to neuropsychological disorders and might have propagated substance abuse and addiction among them. While not much is known about the substance abuse among individuals from the Indus valley civilization, Saraswat and Pokharia (2002) speculated that these people might have grown and used plants such as Ephedra and Datura for recreational purposes (Saraswat and Pokharia 2002).

Our study shines a light on the putative genetic signatures that may be associated with neuropsychological disorders among South Asian populations. The major limitation of our study is the genomic data employed for the analyses. This study was carried out using publicly available genomic data and therefore the disease status of these individuals is unknown. We did not recruit any patient genome in this study or perform any genome-wide association study (GWAS) to identify SNPs linked to neuropsychological disorders, both of which can be extremely crucial in understanding individual and/or population-specific variation in disease susceptibility. We surmise here that a more detailed understanding of this topic will require robust sequencing/microarray genotyping endeavors of patients suffering from neuropsychological disorders from varied ancestries and geographical locations.

Conclusion

Numerous admixture events among various populations across the globe have shaped the South Asian genome, which has been maintained by the century-old practice of intra-community marriage (endogamy). The socio-cultural and genetic diversity in South Asia, which is often reflected through the variation in different life-history traits and differential susceptibility towards various diseases and conditions. In the current study, we aimed at unraveling variation in susceptibility towards neuropsychological disorders among South Asian populations. We found that individuals from Northeast India, indigenous populations, and tribal groups from Southern India are genetically more susceptible to neuropsychological disorders compared to other populations in South Asia.

Overall, our study strongly advocates the adoption of pharmacogenomic approaches for developing population and individual specific therapeutics utilizing whole-genome sequencing data from people of diverse ancestries, performing GWAS on the same, and identifying population/individual specific disease loci. Adoption of a population genetics-driven strategy in neuropsychological disorders will potentially facilitate our understanding of patient genetic attributes that impact the variabilities in the disease presentations and help in the repurposing of existing drugs for the mitigation of neuropsychological disorders worldwide.

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Biosorption of Acid dye by Jackfruit Leaf Powder: Isotherm, kinetics and Response surface methodology studies

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ABSTRACT

A green adsorbent derived from Jackfruit leaf powder (JLP) was used to eliminate Acid Yellow 99 (AY 99) dye from an aqueous medium in this study. We checked the effect of pH, biomass dosage, and temperature (process parameters) on the adsorption potential of AY 99 was explored using the CCD model integrating the RSM approach. At a pH of 2.5, biosorbent dosage of 4 gL^{-1}, and a 30°C temperature, maximum removal was preferred. ANOVA was incorporated to observe the importance of experimental variables and their interactions. The solution pH (A) and biomass dose (C) had the greatest effects on the decolorization of AY 99, according to the findings. ANOVA was used to identify the most important factors, which included two independent variables (A and C) and two quadratic model terms (A^2 and C^2). The kinetic data were effectively interpreted using a pseudo 2^{nd} order with film diffusion model combination, indicating the chemisorptions phenomenon. Following the model of Langmuir isotherm, the utmost capacity for adsorption was determined to be 418.15 mg g^{-1} in terms of initial dye concentration. The findings of the maximum adsorption capacity showed that JLP could be employed as a useful adsorbent to eliminate AY 99 from its aqueous medium.

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1 Introduction

Water is an essential ecological resource for the survival of all living things on the planet. Pollution of water is a foremost worry in today's world (Wang et al. 2021). Acid Yellow 99, a negatively charged azo dye, is not biodegradable due to its complex organic structure (Khan et al. 2019). A large volume of dye is produced and used for mankind's benefit through various industrial processes, and wastewater contain an alarming amount of dye and harmful auxiliary compounds, all of which contribute to chemical oxygen demand (De Castro et al. 2018). The presence of these xenobiotic chemicals in natural water bodies has negative consequences for both the ecosystem and living creatures. Due to the limited penetration of sunlight, dye slows the growth of phototrophs and autotrophs in water (Lellis et al. 2019). As a result, dye-bearing wastewater must be treated before being discharged onto the earth's surface, as required by the BIS directive (Khan et al. 2019). However, many industries are still grappling with how to properly dispose of these wastes. Bio-adsorption has recently gained prominence in this area, owing to its superior benefits over traditional physicochemical procedures for dealing with dye effluents in an aqueous solution (Putri et al. 2021). Although bio-adsorption is most commonly utilized for metal binding, it can also be used to remove hazardous dyes as a cost-effective and alternative method for treating industrial wastewater and contributing to environmental remediation. The degree to which a pollutant is removed is determined by equilibrium and kinetics, both of which are influenced by experimental conditions. As a result, determining the bio-adsorption of a contaminant by biomass is an intricate task (Priyantha et al. 2018). The method primarily imports low-cost bio-based substances such as plant derivatives, microbial cells, and other bio-based substances with simple instrumental procedures at a low cost (Gupta et al. 2019). According to the survey, tons of agricultural wastes have been generated each year globally (Duque-Acevedo et al. 2020). The use of these low-cost, renewable wastes for water purification appears to be a significant step towards long-term sustainability.

The leaves of the jackfruit (*Artocarpus heterophyllus*) are attractive biosorbent materials that are low-cost and widely available in India and other areas of the world. This agricultural waste is made up of a variety of biomolecules, including proteins, lipids, and carbohydrates, all of which have active functional groups that are thought to be larger entities for dye interactions. (Das and Mishra 2019). The efficacy of biosorbents, on the other hand, is dependent not only on their physicochemical properties but also on the microenvironment of the target pollutant. Only a few studies have shown that raw Jackfruit leaf powder can be used to remove methylene blue (Uddin et al. 2009), crystal violet (Saha et al. 2012), Amido black 10B (Ojha and Bulasara 2015), and nickel (Ranasinghe et al. 2018). However, these techniques are still under development. There has been no research into the removal of Acid yellow 99 dye using such biosorbent (JLP) in its natural form to the best of our knowledge.

Despite these promising advantages, the design of the experiment (DOE) using RSM has been used to construct the most reliable batch adsorption system. The approach is especially well adapted to optimizing experimental findings, evaluating the effect of variables, and evaluating their interaction with a collection of least experiments (Bagheri et al. 2019; Naskar et al. 2020).

Considering the above background, the main objectives of this research are (1) to evaluate its potential for removing Acid Yellow 99 (AY 99) from the aqueous medium, (2) to investigate the process controlling parameters through RSM modelling in order to achieve the optimum ones with simultaneous variable interactions, and (3) to gain a better understanding of mechanisms during dye-biomass interactions.

2 Material and Methods

2.1 Reagents and chemicals

The Acid-Yellow (AY) 99 dye (C.I. 13900) was procured from Sigma-Aldrich, U.S. Other necessary chemicals required for completing the experiments were supplied by E-Merck, Germany.

2.2 Estimation and calibration of AY-99 dye solution

A UV-vis spectrophotometer (Perkin Elmer λ-25, Singapore) was used to calculate the dye concentration considering the calibration curve created from dye standards. The mass balance equation can be used to estimate the quantity of dye absorbed by the JLP biomass (mg g^{-1}) (Eq. 1) (Khan et al. 2011):

$$q_e = \frac{(C_0 - C_e)V}{1000W} \quad (1)$$

Here q_e depicts dye uptake (mg g^{-1}), C_0 denotes initial dye conc. (mg L^{-1}), and C_e highlights the dye conc. at equilibrium (mg L^{-1}), V indicates the working dye volume (in mL) and W is biomass weight used in each experiment (g)

2.3 Biosorbent: Jackfruit Leaf Powder (JLP)preparation

Jackfruit (*Artocarpus heterophyllus*) leaves were picked locally and washed well with tap water. Later on, these were further washed by double distilled water to eliminate redundant adhering dirt materials. The washed leaves were oven-dried at 70±5°C overnight to obtain the fine powder through the mechanical grinder.

2.4 Biosorption experiments through RSM modelling

RSM, a statistical technique was employed to examine the influence of essential physicochemical factors and to establish the optimum amount of variables for maximum dye decontamination by JLP. The three factors, along with their coded and real forms, were designed at three separate levels with the help of Design Expert (DE) version 12 (Stat-Ease, Inc., USA), as shown in Table 1. The influence of three independent variables affecting dye removals, such as starting pH (A), temperature (B), and biomass dose (C), was investigated using statistical models in this study. Various combinations of Six (6) axial points, Eight (8) factorial points, and Six (6) centre points were used in a set of 20 experimental runs (Table 2). The dye absorption (mg g^{-1}) was regarded as a system's dependent output response (Y). The quadratic model equation that was utilized to derive expected responses was (Eq. 2):

$$Y = \beta_o + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j + \sum \beta_{ii} x_i^2 \quad (2)$$

Here Y denotes predicted responses, β_o denotes the offset term, β_i and β_{ii} reflects the linear outcome and squared effect respectively. β_{ij} represent the interaction effect, x_i denotes ith independent variable, $x_i x_j$ indicates interaction term and x_i^2 highlights the quadratic terms.

The F-value and R^2 (regression coefficient) was used to evaluate the model's fittest and accuracy. This software was used to solve the regression equations, the three-dimensional (3D) responses for the different parameters to find the best conditions for dye uptake by the JLP.

Table 1 Experimental ranges with three different levels in the experimental design

Factor	Name	Units	Minimum	Maximum	Coded Low	Coded High	Mean
A	pH		2.00	3.00	-1 ↔ 2.00	+1 ↔ 3.00	2.50
B	Temperature	℃	20.00	40.00	-1 ↔ 20.00	+1 ↔ 40.00	30.00
C	Biomass dose	g L^{-1}	3.00	5.00	-1 ↔ 3.00	+1 ↔ 5.00	4.00

Table 2 CCD Design with the actual and predicted response

Run Order	Factor-A pH	Factor-B Temp (℃)	Factor-C Biomass dose (g/L)	Space Type	Actual Response (mg/g)	Predicted Response (mg/g)
1	3	40	5	Factorial	21.10	21.06
2	2.5	30	3	Axial	20.41	20.91
3	2.5	30	4	Center	23.69	23.56
4	2.5	40	4	Axial	23.81	23.99
5	3	30	4	Axial	19.34	19.83
6	2.5	30	4	Center	23.67	23.56
7	2	30	4	Axial	21.29	21.20
8	2.5	30	4	Center	23.70	23.56
9	2.5	30	5	Axial	24.01	23.92
10	3	40	3	Factorial	17.59	17.39
11	2	40	5	Factorial	21.75	21.84
12	2.5	30	4	Center	23.65	23.56
13	2	20	5	Factorial	21.43	21.53
14	2.5	30	4	Center	23.75	23.56
15	2.5	20	4	Axial	23.52	23.74
16	3	20	3	Factorial	17.40	17.21
17	2	40	3	Factorial	18.97	18.93
18	3	20	5	Factorial	20.38	20.32
19	2	20	3	Factorial	19.23	19.17
20	2.5	30	4	Center	23.71	23.56

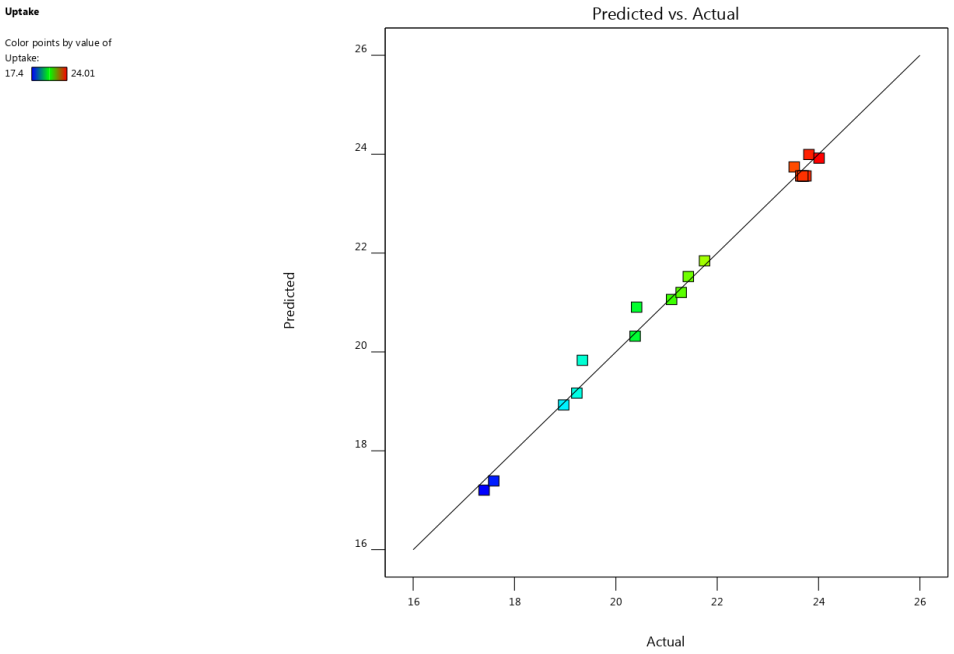

Figure 1 Actual versus predicted graph for adsorption of AY-99 by JLP biomass

2.5 Dye biosorption experiment

All biosorption experiments for decontaminating AY-99 by JLP were carried out in Erlenmeyer flasks (250 mL) with a 50 mL dye test solution of 100 mg L^{-1} at pH 2.5. The flasks were agitated well (120 rpm) in a temperature-controlled shaker incubator. After that, the biomass was removed from the solution using Millipore membrane vacuum filtrations, and the residual dye concentration was quantified using a UV-visible spectrophotometer.

For isotherm studies, the desired quantity of JLP was exposed to 50 mL of dye (concentrations ranging 50–5000 mg L^{-1}) of pH 2.5 at 30°C. Moreover, dye removal with respect to contact time was examined in Erlenmeyer flasks containing dye solution (100 mg L^{-1}) pH 2.5 with a fixed biomass dose of 4 g L^{-1}.

2.6 Statistical analysis

Using Design-Expert 12.0.0.6, each of the experiments was carried out for three times and the mean results were given in the result and discussion section, taking into account the p-value (0.05). The Origin 8.0 programme calculated the Chi-square analytical result and the experiment regression coefficient.

3 Results and Discussion

3.1 Central composite design and statistical evaluation

The experimental run is depicted in Table 2 along with a summary of the outcomes. The pragmatic association between independent factors and intended responses was discovered and stated by the following SOPE, according to the findings:

$$\text{Uptake} = +23.56 - 0.6860A + 0.1260B + 1.51C + 0.1063AB + 0.1887AC + 0.1388BC - 3.04A^2 + 0.3086B^2 - 1.15C^2$$

Where A, B, and C correspond to independent variables of pH, temperature (ºC), and biomass dose (g L^{-1}), respectively. Uptake is the response (Y) here. The equation with coded factors, on the other hand, may be used to predict response for different amounts of individual factors. In general, the factors' upper levels are recorded as +1, while -1 codes the lower levels of the factor. The coding equation can be used to calculate the relative significance of the factors compared with the coefficients of the factors. The coefficient provides the estimated change (response/unit change) in values of the factor while all other factors are kept constant. For orthogonal design, the intercept is the total average response in all runs. Experimental coefficients were adjustments, which depend on factor settings in the vicinity of the average. When the factors were orthogonal, the VIFs are 1; VIFs more than 1 denote multicollinearity, and higher this VIF reflects more extreme factor correlation. VIFs of less than ten are regarded as acceptable. The F-value of 129.88 for the model suggests that it is statistically significant. Due to noise, an F-value of this magnitude has a 0.01 percent probability of occurring. P-values < 0.0500 indicated that all model terms were significant. In the present scenario, two independent factors (A and C), as well as two quadratic model terms (A^2 and C^2), were found to be significant in affecting dye uptake (Table 3). If the value is more than 0.1000, the model terms

are irrelevant. The Adjusted R^2 of 0.9839 is reasonably fit to the Predicted R^2 of 0.9487. There only the difference is < 0.2. Alongside, the signal-to-noise ratio was calculated with sufficient precision. It is recommended to have a higher ratio than 4. In this experiment, the signal-to-noise ratio of 33.755 indicates that the signal is satisfactory. Accordingly, this model can be applied to the design process. Additional statistical parameters [mean (21.62), standard deviation (0.2844), and the coefficient of variance (1.32 %)] summarised in Table 4 also indicated the significance of our tested model. Moreover, with a high regression coefficient, the experimental outcomes were highly correlated to predicted responses, underlying the appropriate analysis for the datasets.

3.2 Biosorption of dye depends on pH and biomass dose: Response surface plots

We have chosen several key independent parameters in this investigation [A: pH, B: temperature (^oC), C: biomass dose (g L^{-1})]. The solution pH and the biomass dose had a substantial impact on the results. The surface charge of the biosorbent and the adsorbate behavior are both influenced by the pH of the solution in the dye adsorption process. At pH 2.5, the greatest adsorption capacity (24.01 mg g^{-1}) was recorded, and as the pH was increased, the adsorption capacity decreased. At pH 2.5, dye occurs as negatively charged species and is adsorbed on positively charged entities utilizing elevated electrostatic attraction, resulting in improved adsorption potential (Naskar and Majumder 2017). Increases in pH values from 2.5 to 6.0 resulted in proportional decreases in dye absorption capacity, as shown in Figure 2. The biomass surface becomes negative in charge as the pH rises (pH > 2.5), reducing dye interaction because of electrostatic repulsion. Our findings are also in line with those of other studies (Khan et al. 2011;

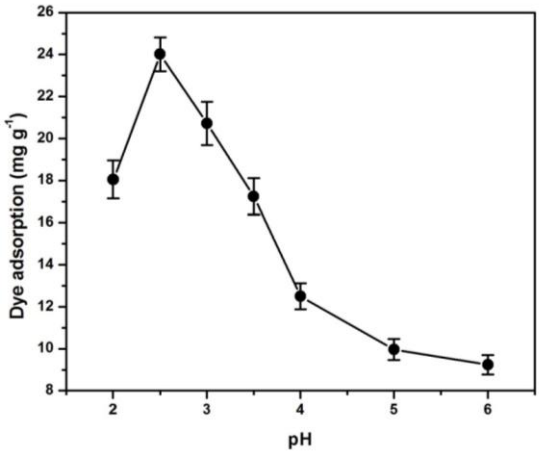

Figure 2 Effect of solution pH on AY-99 adsorption by JLP biomass (temperature: 30ºC, speed of agitation: 120 rpm and dye concentration: 100 mg L^{-1}): ±SD shown with error bars.

Table 3 ANOVA analysis regarding to AY 99 adsorption by JLP

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	94.57	9	10.51	129.88	< 0.0001	significant
A-pH	4.71	1	4.71	58.17	< 0.0001	
B-Temp	0.1588	1	0.1588	1.96	0.1915	
C-dose	22.71	1	22.71	280.72	< 0.0001	
AB	0.0903	1	0.0903	1.12	0.3156	
AC	0.2850	1	0.2850	3.52	0.0900	
BC	0.1540	1	0.1540	1.90	0.1977	
A^2	25.44	1	25.44	314.42	< 0.0001	
B^2	0.2620	1	0.2620	3.24	0.1021	
C^2	3.61	1	3.61	44.67	< 0.0001	
Residual	0.8090	10	0.0809			
Lack of Fit	0.8031	5	0.1606	134.97	< 0.0001	significant
Pure Error	0.0060	5	0.0012			
Core Total	95.37	19				

Table 4 Fit Statisticsregarding to AY 99 adsorption by JLP

Std. Dev.	0.2844	R^2	0.9915
Mean	21.62	Adjusted R^2	0.9839
C.V. %	1.32	Predicted R^2	0.9487
		Adeq Precision	33.7552

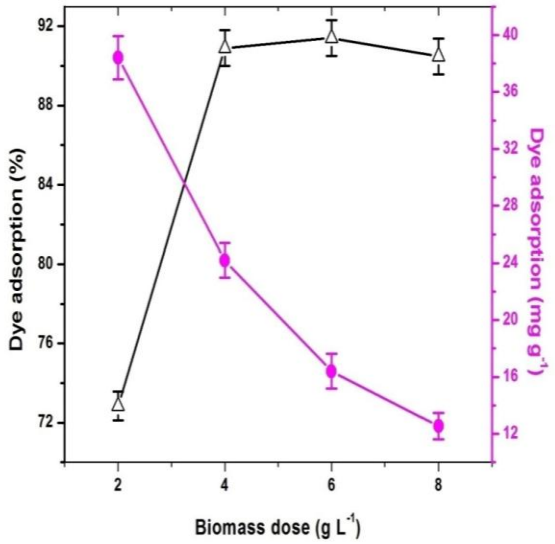

Figure 3 Influence of biomass dose on AY-99 adsorption by JLP

Khan et al. 2019). Another key aspect was investigated, and dye removal effectiveness rose progressively with increasing biomass dose, with 4 g L^{-1} biomass at pH 2.5 achieving optimum removal (~92 %). This could be owing to an increase in adsorbent particle contact surface due to increased biomass and more accessible binding sites for dye complexation (Cheruiyot et al. 2019). Further increased biomass dosage (6 g L^{-1}) has no discernible effect on dye decolourisation. Furthermore, the experimental results shown in Figure 3 reveal that the maximal dose (8 g L^{-1}) results in a little reduction (89%) in the dye removal pattern. The aggregation of active sites may explain this unequal adsorption behaviour, resulting in a modest decrease in dye removal (%). Furthermore, a larger dosage has a negative impact on biomass dye absorption capacity (mg g^{-1}). Other factors, such as temperature, had no significant impact on dye adsorption.

The perturbation graph of the 3 independent variable factors is depicted in Figure 4. According to this plot, the model's centre point, factors A (pH), exhibits a positive response at first, then a negative response moves away from that reference point. The plot clearly showed that the lower the pH, the more dye was removed, as our earlier research had demonstrated the same phenomenon (Khan et al. 2011; Naskar and Majumder 2017). The combined effects of pH-temperature (Figure 5), pH-biomass dose (Figure 6), and temperature-biomass dose (Figure 7) were demonstrated in this work using three response surface 3D plots. An elliptical 3D response surface plot regarding to pH and biomass dose demonstrated that the interactions between these factors were extremely significant, according to this finding. The following were the optimum levels of each variable in real values: pH = 2.5, temperature = 30 ºC, and biomass dose = 4 g L^{-1}, all of them fell inside the experimental series. The findings have corroborated with other research works (Khan et al. 2011; Naskar and Majumder 2017). Under these ideal conditions, the experimental response was 24.01 mg g^{-1}, which was very similar to the yield predicted by the statistical model (23.92 mg g^{-1}), with an R^2 value of 0.9915.

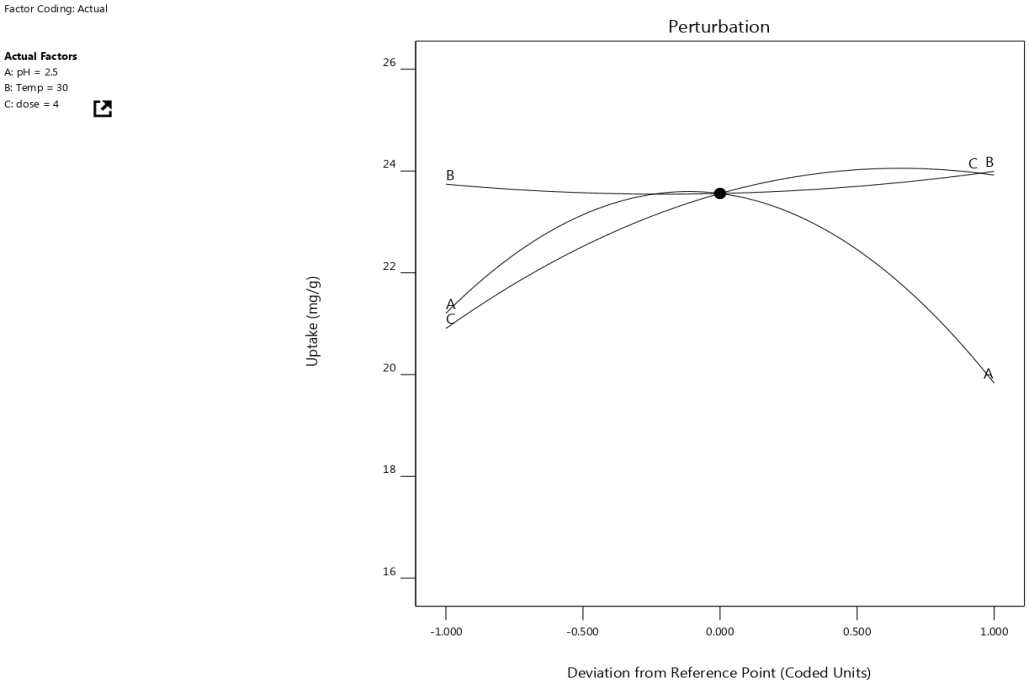

Figure 4 Perturbation plot on AY 99 adsorption by JLP biomass.

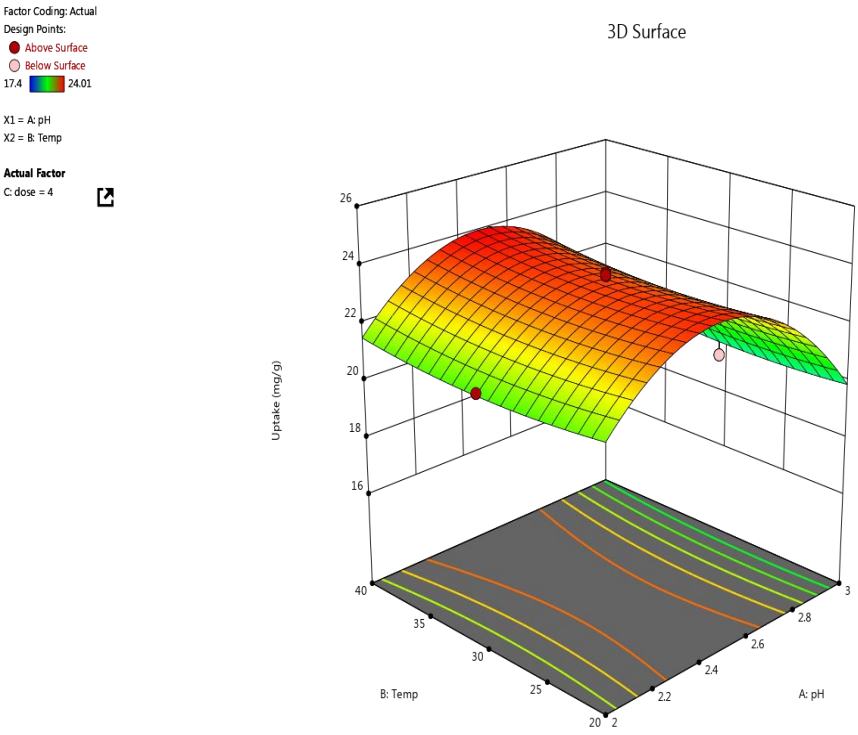

Figure 5 3Dimentional plot of pH versus temperature on AY 99 adsorption by JLP biomass.

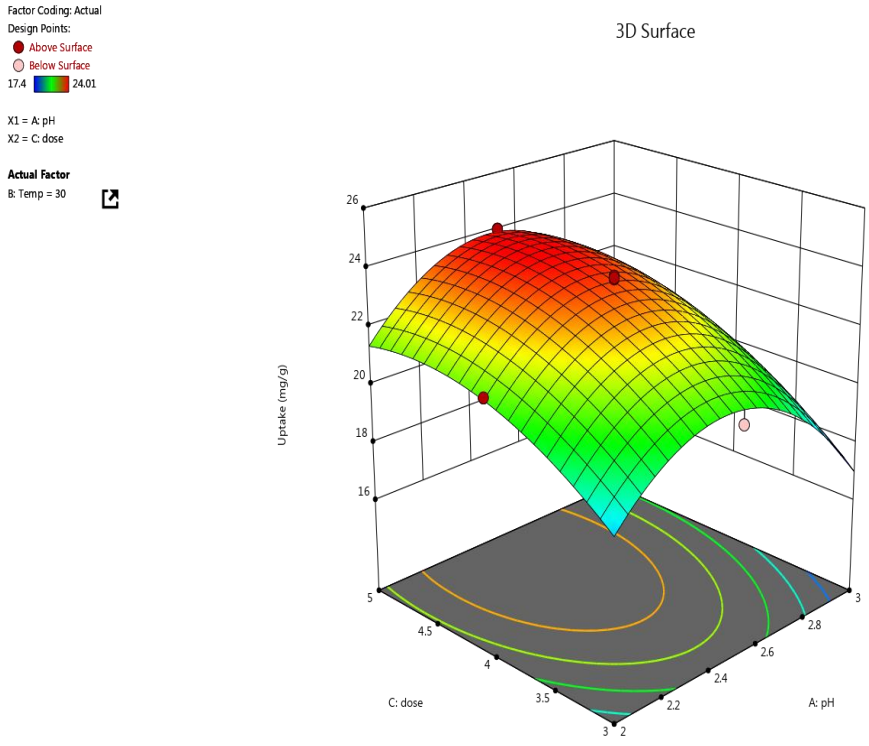

Figure 6 3D plot of pH versus biomass dose on AY 99 adsorption by JLP biomass.

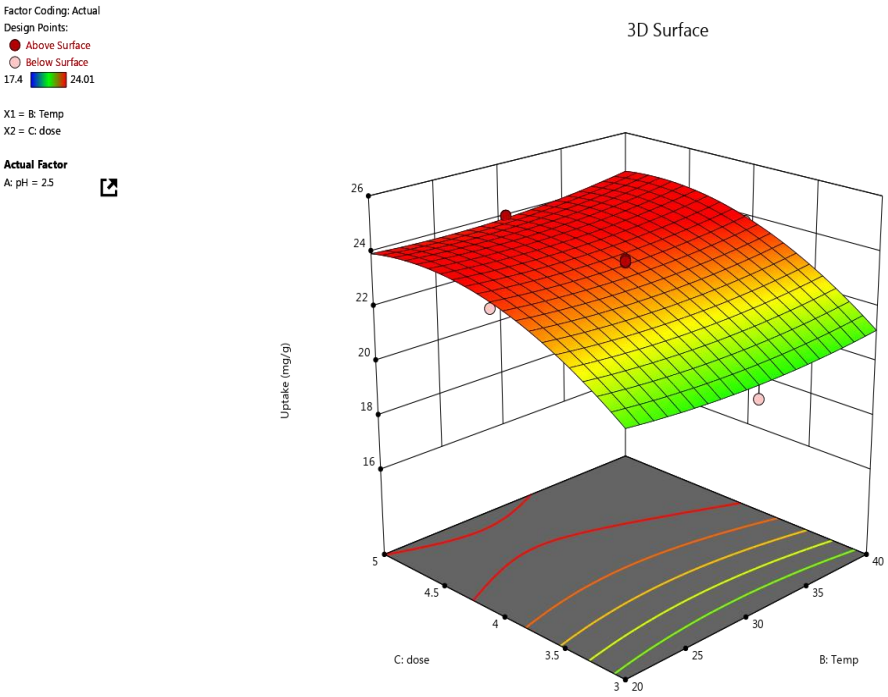

Figure 7 3D plot of biomass dose versus temperature on AY 99 adsorption by JLP biomass.

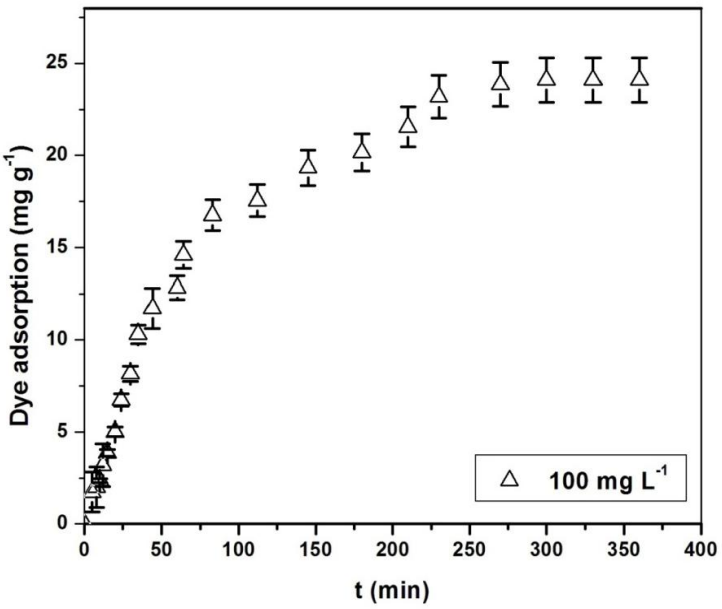

Figure 8 Influence of surface contact time on AY-99 dye adsorption by JLP biomass (b): ±SD shown with error bars.

3.3 Effect of SCT and kinetic study

The most crucial parameter is the contact time to forecast the rate of reaction and the mechanisms of dye removal by JLP (Saxena et al. 2020). The result revealed in Figure 8 speaks an initial rapid attachment of AY-99 with readily available active sites of JLP by electrostatic interaction or passive uptake by the physical force of attractions, followed by film mass transfer and finally accomplished in equilibrium state within 250 min by sluggish intra-particle diffusion (Basu et al. 2017). Similarly, Ozcan and Ozcan (2008) and Khan et al. (2011) reported this phenomenon earlier for the removal of AY-99 by DEDMA- sepiolite and coir

pith, respectively. This is noteworthy to mention that ~75% of the dye could be removed by JLP during the early 55 min thereby ensuring its prospect in the real process.

A step forward investigation was carried out by associating the experimental outcomes to widely accepted kinetic models and diffusion models to understand the mechanisms of mass transfer as well as the rate-controlling stage. The linear equation of (PFO) pseudo 1st order (Eq. 3) and (PSO) pseudo 2nd order (Eq. 4), intra-particle diffusion (IPD) (Eq. 5), and film diffusion (Eq. 6) model are as follows (Naskar and Bera 2018).

$$\ln(q_e - q_t) = \ln q_e - k_1 t \quad (3)$$

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \frac{t}{q_e} \quad (4)$$

$$q_t = k_p t^{1/2} + C \quad (5)$$

$$\ln(1-F) = -K_f t \quad (6)$$

Here q_e is the dye uptake in equilibrium (in mg g^{-1}) and q_t is dye uptake at time 't'(mg g^{-1}), the rate constants of PFO (min^{-1}), PSO (mg g^{-1} min^{-1}), IPD (mg g^{-1} $min^{-1/2}$) and film diffusion, were denoted by k_1, k_2, k_p and k_f, respectively, intercept (mg g^{-1}) is highlighted by C obtained from q_t vs $t^{1/2}$ plot, and F stands for the fractional achievement of equilibrium.

Table 5 shows the calculated findings of the PSO kinetic model (Figure 9) has a higher R^2 (correlation coefficient) than the PFO model (Figure 10). Alongside, the experimental adsorption capacity (24.06 mg g^{-1}) is near to the model calculated value (26.34 mg g^{-1}) for the pseudo 2nd order kinetic model. Therefore, under the observed experimental conditions, chemisorption may be considered for the rate limiting step in the removal of AY 99 by JLP biomass (Khan et al. 2011).

Because the preceding kinetic models are unable to describe the diffusion mechanism, the intraparticle diffusion and film diffusion models are used to further explore the experiment data (Majumder et al. 2017). Table 5 shows the experimental findings for the intraparticle diffusion model. A plot of q_t vs $t^{1/2}$ revealed a triphasic nature (Figure not shown). As a result, the curve and plot were nonlinear and did not intersect the origin, showing that IPD is

Table 5 The tabulated kinetic parameters for AY 99 removal by JLP biomass

Dye (mg L^{-1})	FOK			SOK			IPD			Film diffusion	
	k_1	q_e	R^2	k_2	q_e	R^2	k_p	C	R^2	K_f	R^2
100	0.013	15.89	0.949	6.9×10^{-4}	26.34	0.984	1.143	0.097	0.892	1.1×10^{-2}	0.979

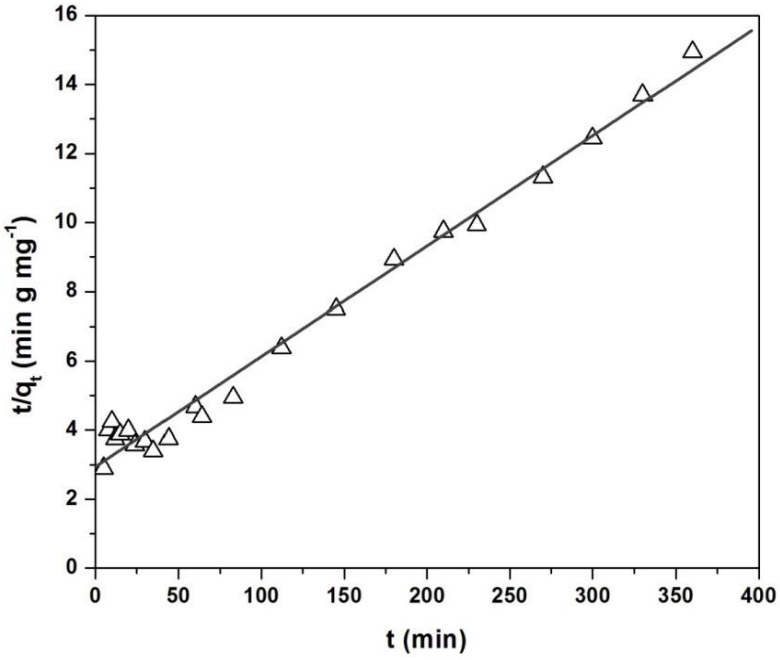

Figure 9 Plot of Pseudo 2ndorder kinetics on adsorption of AY 99 by JLP biomass.

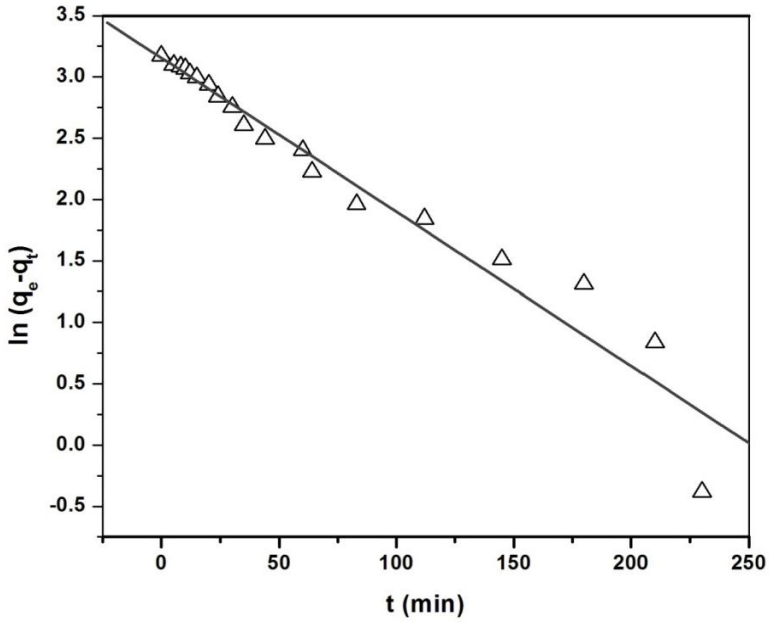

Figure 10 Pseudo-first order kinetics plot on adsorption of AY 99 by JLP biomass.

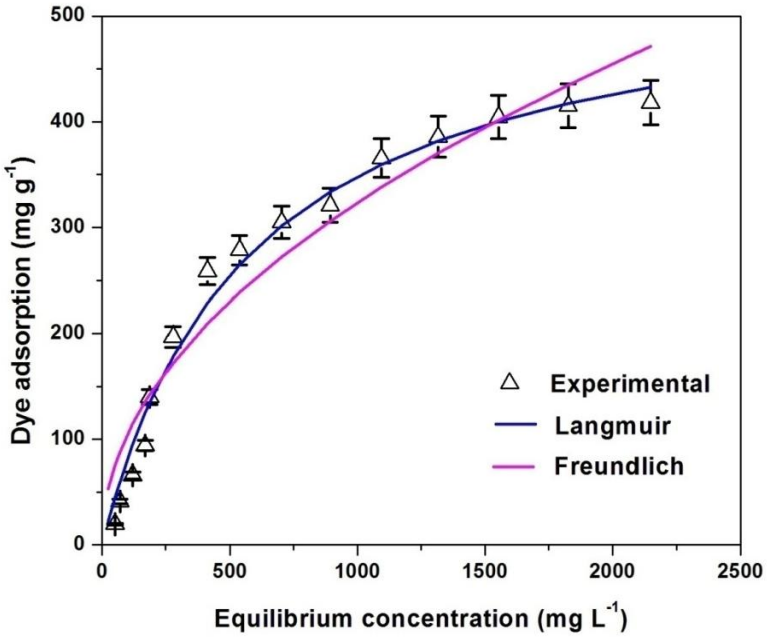

Figure 11 Adsorption isotherm of the dye (AY 99) on JLP biomass (pH: 2.5, temperature: 30ºC, and speed of agitation: 120 rpm)

significant but not only rate-determining phase in the overall dye elimination procedure. On the contrary, for the film diffusion-driven transport mechanism, the fitted straight line plots of ln (1 − F) vs.'t' revealed a high grade correlation coefficient (0.979). Furthermore, a little magnitude of intercept of 5.28×10^{-4} was found. As a result, the film-diffusion stage might be regarded as the rate limiting phase for the whole process.

3.4 Effect of dye concentration and isotherm study

The study of adsorption isotherms describes the affinity, adsorbent's surface properties, and understanding of the equilibrium nature of the process (De Castro et al. 2018). Equilibrium dye adsorption on JLP was carried out using different dye concentrations and outcomes are depicted in Figure 11. In fact,

Table 6 Isotherm parametersfor AY 99 removal by JLP biomass

Langmuir isotherm				Freundlich isotherm			
q_{max}	K_L	R^2	χ^2	K_F	n	R^2	$\chi 2$
449.01	1.74×10^{-3}	0.983	264.61	10.89	2.036	0.932	502.64

the higher the dye concentration, the greater the driving force for mass transfer, thereby increasing the adsorption of dye before reaching equilibrium as proposed by Majumder et al. (2017). This phenomenon was well reflected in this experiment, according to which the maximum adsorption of AY-99 on JLP was found to be 418.15 mg g^{-1}at equilibrium, which was remarkably higher in comparison with previous research reports (Ozcan and Ozcan 2008; Khan et al. 2011;).

The isotherm study provides further mechanistic insights into the dye removal process by fitting the experimental outcomes to widely applied Langmuir isotherm (LI) and Freundlich isotherm (FI) equation. The Langmuir isotherm illustrates the conception of monolayer adsorption along with the homogeneous allocation of surface active sites on adsorbent. In contrast, the Freundlich model highlights multilayer heterogeneous adsorption behaviour (Naskar et al. 2016). The equations of Langmuir (Eq. 7) and Freundlich (Eq. 8) model are as follows (Khan et al. 2019; Naskar and Majumder 2017):

$$q_e = \frac{q_{max} K_L C_e}{1 + K_L C_e} \quad (7)$$

$$q_e = K_F C_e^{1/n} \quad (8)$$

Where q_e and q_{max} are the equilibrium and maximum dye adsorption (mg/g), respectively, C_e stands for dye concentration at equilibrium (mg L^{-1}), Langmuir affinity constant is represented by K_L (L mg^{-1}), K_F is Freundlich equilibrium constant reflecting of dye uptake capacity and n determines intensity of the adsorption.

The derived values of LI and FI model constants are shown in Table 6. Because of the higher correlation coefficient (0.983) and lower Chi-square value (264.61), the isotherm data make a reasonably good fit for the Langmuir model as compared to the Freundlich model (Majumder et al. 2017; Priyantha et al. 2018). Additionally, the empirical relationship between the theoretical adsorption capability deduced from the Langmuir isotherm (449.01 mg g^{-1}) and the experimentally measured value demonstrates the proximity (418.15 mg g^{-1}).

4 Conclusions

The most effective biomass for AY 99 adsorption from its aqueous phase is determined to be JLP biomass. The adsorption process, as demonstrated by UV-visible spectroscopic analysis, is highly influenced by the pH of the solution, which is optimum at 2.5. The outcomes of the experiments were very close to the predicted responses (R^2= 0.9915), according to optimal physicochemical parameters calculated via response surface methodology (RSM). Two independent variables (A and C) and two quadratic model terms (A^2 and C^2) are found to be the significant factors. The LI, PSO model, and film diffusion, all fit the equilibrium adsorption data well, implying that the dye molecules may adsorb physically and chemically on the biomass surface. Hence, JLP has a strong ability to decontaminate AY 99 dye from its aqueous phase, indicating its real application potential. Chemical modification and/or processing of the JLP biomass to activate or enhance the number of sorption sites could boost the extent of AY 99 elimination even more.

Abbreviations

CCD: Central Composite Design; RSM: Response Surface Methodology; ANOVA: Analysis of Variance; SOPE: Second-order polynomial model equation; SCT: Surface contact time; PFO: Pseudo first order; PSO: Pseudo second order; IPD: Intra particle diffusion; LI: Langmuir isotherm; FI: Freundlich isotherm; FOK: First order kinetic; SOK: Second order kinetic

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Journal of Experimental Biology and Agricultural Sciences, February - 2022; Volume – 10(1-BIONEXT-2021) page 266 – 277

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A detailed investigation to study the pattern of the interplay of Cyclic AMP Receptor Protein (CRP) of *E. coli* with its different classes of promoters

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AR1

AR2

AR3

ABSTRACT

The activity of most of the promoters in *Escherichia coli*, involved in the metabolism of sugars other than glucose, is controlled by a CRP (cAMP receptor protein) or CAP (catabolite activator protein). CRP-dependent promoters are differentiated into various classes (Class I, Class II, and Class III) based on its cognate binding site's position on DNA. The promoters regulated by CAP are differentially regulated by this transcriptional factor and it is also imperative to mention that these promoters vary greatly in respect to the binding site of CAP to its cognate binding site, it has also been reported that either it overlaps with the binding site of RNA polymerase or it present upstream to it. In Class I CAP-dependent promoters, a particular CAP molecule makes protein-protein interaction for the start of transcription. In Class II CAP-dependent promoters, a particular CAP molecule makes multiple interactions for the start of transcription. At last, in Class III-CAP dependent promoters, more than one CAP molecule is involved and activation of transcription is done synergistically. It has also been documented that CAP shows a kind of biphasic behavior in some promoters. So, the main focus of this work is to find out whether this biphasic behavior is true for other *E. coli* promoters as well. Experiments have been performed to know more about this biphasic nature and the various patterns of interactions of catabolite activator protein (CAP) of *E. coli* with its different classes of promoters.

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1 Introduction

Gene regulation is important for all organisms and *E. coli* is the most suitable organism to study this gene regulation process. It has already been known to us that there are several transcription factors present that control the gene regulation and one of them is Cyclic AMP receptor protein (CRP) or Catabolite activator protein (CAP). The gene regulation of nearly 150 promoters is controlled by this DNA binding protein (de Crombrugghe et al. 1984; Busby and Ebright 1999; Lawson et al. 2004). As it is evident from its name, it is a cAMP (cyclic adenosine monophosphate) binding protein, which activates transcription only in the presence of this nucleotide. In the absence of this nucleotide, this transcriptional factor is inactive, but after binding of cAMP, the transcriptional factor undergoes a conformational change and activates transcription at various promoters specifically those which are involved in the metabolism of alternative carbohydrate sources like lactose, galactose, maltose, arabinose, etc. other than glucose (Lawson et al. 2004; Ebright 1993). Although CRP is a cAMP binding protein, this protein exhibits a clear biphasic dependence over the varying concentration of cAMP, and the cAMP level is more important rather than its bare existence.

The protein, *E. coli* CAP is homo-dimeric and its every subunit consists of 209 aa residues and each subunit has two domains. Each subunit is also consisting of an HTH motif responsible for DNA binding (Mckey and Steitz 1981). The binding of this transcriptional activator is fixed on the region of the promoter to activate or repress transcription. This site is a cognate 22 bp consensus sequence (5'-AAATGTGATCTAGATCACATTT – 3'), having a twofold sequence symmetry (Ebright et al. 1989; Ebright 1993). It has also been stated that the complex of this protein with DNA slightly bends the DNA that is about 90º in the crystalline state and about 80º to 180º in solution (Lawson et al. 2004).

To activate transcription, CAP needs to bind to its cognate site on the promoter DNA along with RNA polymerase (RNAP)[3]. The promoters, that are regulated by the CAP-cAMP complex are of various categories based on the appearance of CAP site present in the P/O region and also the pattern of interaction of CAP with RNAP. There are three different classes present, which are - Class I, Class II, and Class III. The binding site for CAP can be -41.5, -61.5, -71.5, -82.5, etc. In the case of Class I promoter the transcriptional activation involves interaction between the activating region 1 (AR1) of CAP and RNAP, whereas, in Class II, the mode of interaction is a little different and in this case the interaction mainly involves activating region 1 (AR1), activating region 2 (AR2) and activating region 3 (AR3) of CAP and RNAP. But, In the case of class III, two or more CAP molecules are involved and interaction with RNAP involves the mechanism of both Class I and Class II (Ebright 1993; Busby and Ebright 1997; Lawson et al. 2004).

In this paper, we have discussed briefly about CAP and its three different classes of promoters i.e., Class I, Class II, Class III promoters, and also we have done experiments to find out whether the biphasic character of cAMP is observed in all these classes of promoters or not.

1.1 What is CRP or CAP?

CRP or CAP is a global transcriptional protein that involves primarily in the regulation of transcription of promoters mainly involves in the metabolism of sugars other than glucose. As already mentioned, it has two subunits, and each subunit of the CAP has two domains, one N-terminal domain (amino acids 1-133), involved in cAMP binding, and a C-terminal domain (amino acids 139-209), involved in DNA binding (Tutar 2008). These domains are linked by a small hinge region consisting of amino acids 134-138 (Tutar 2008).

Energy conservation is crucial for all organisms and bacteria is also no exception to this rule and at certain metabolic conditions bacterial cells overexpress certain proteins where expressing other proteins at the basal level, as the presence of glucose in the medium inhibits the cAMP production and thus activation of CAP does not take place, but when glucose is exhausted in the medium, cAMP concentration increases and which in turn binds with CAP and thus CAP activates transcription of promoters (Kolb et al. 1993a; Sharma et al. 2009). Binding of this nucleotide results in a geometrical change in the protein from an inactive shape to an active shape. This active conformer can bind to its cognate sites on DNA and activates or repress transcription (Sharma et al. 2009; Saha et al. 2015). The binding of cAMP to the un-liganded CAP brings certain biochemical and biophysical changes in the protein and it has been observed that after cAMP binding (a) CAP becomes more sensitive to various proteases, (b) it also reduces the C178 (cysteine residue) accessibility and (c) also allows the intersubunit cross-linking at the C178 residue by a disulfide linkage, apart from other conformational changes as probed by various spectroscopic studies (Kolb et al. 1993a; Saha et al. 2015).

It has been already mentioned that CAP undergoes changes in shape upon cAMP binding, but since 2009, the exact molecular mechanism behind this conformational change remains elusive. Though several crystal structures of this protein were available, the crystal structure of un-liganded CAP or apo-CAP was not there. The apo-CAP (CAP without cAMP) structure was solved using X-ray crystallography technique and NMR spectroscopy technique in the year 2009 (Ryu et al. 1993; Sharma et al. 2009). Various structural changes have been observed between the structure of inactive form i.e. apo-CAP and active form i.e. ligand-bound CAP (cAMP-CAP) (Popovych et al. 2009). The changes are mentioned in Table 1.

Table 1 Changes within the inactive form of CAP (apo-CAP) and active form of CAP (cAMP-CAP).

Parts of CAP structure	Amino acids (Inactive form)	Amino acids (Active form)	Reference
D-Helix	135-152	139-152	Sharma et al. 2009
C-Helix	110-130	110-136	Saha et al. 2015
F-Helix	Buried inside DNA binding domain	Comes outside on the surface	Sharma et al. 2009
Hinge	130-134	136-139	Sharma et al. 2009

As already mentioned, in presence of cAMP, CAP becomes more susceptible towards various proteases and this particular change in protease sensitivity between the active and inactive form of CAP are due to these structural changes. Protease like chymotrypsin cleaves CAP at F136. The un-liganded form of CAP is resistant to protease because, in the inactive form of CAP, the F136 present within the rigid structure of D-helix and cAMP binding brings the F136 residue in the hinge region. For this reason, the active form of CAP becomes more susceptible to proteases (Garges and Adhya 1985; Sharma et al. 2009).

The most interesting character of CAP is its biphasic dependence over the varying concentration of cAMP. Previously it was thought that only 2 molecules of cAMP bind to CAP to its N terminal domain, but later on it was discovered that instead of 2, 4 molecules of cAMP can bind the protein at a higher cAMP concentration. Out of these 4 molecules, 2 bind to the NTD, and 2 bind to the CTD. It has also been noted that cAMP bind at the NTD in anti-conformation and these are the high-affinity sites, whereas the CTD binding sites are low-affinity sites, and cAMP bind in this domain in the syn-conformation (Passner and Steitz 1997; Mukhopadhyay et al. 1999). Actually, without cAMP the CAP is inactive and at a low level it becomes active, but again at higher concentration, it behaves like the un-liganded CAP, thus inactive. Biophysical experiments also proved that this structure is very much similar to un-liganded CAP (Heyduk and Lee 1989). This biphasic behavior of CAP has been proved by several experiments. When the sensitivity of CAP to proteases has been observed for various concentrations of cAMP then it was found that without cAMP the CAP is resistant to proteases, at low cAMP concentration the CAP is sensitive to proteases and at high concentration of cAMP, the CAP is again becoming resistant to proteases (Mukhopadhyay et al. 1999). Also, it has been found that the binding of cAMP decreases the accessibility of cysteine residues at 178 positions (C-178)[16]. The same kind of biphasic behavior can also be observed in the case of transcriptional activation of certain promoters as well (Mukhopadhyay et al. 1999).

1.2 Transcriptional regulation by cap

As already mentioned, CRP-dependent promoters are divided into three types- Class I CRP-dependent promoters, Class II CRP-dependent promoters, and Class III CRP-dependent promoters.

1.2.1 Class I CRP-dependent promoters

In Class I CRP-dependent promoters, the CRP binding site on double helix DNA is located in the upstream position of the RNA polymerase (RNAP) binding site (Ebright 1993; Lawson et al. 2004). An example of this promoter is *lac* promoter having CRP binding site at -61.5 position. *In vivo*, the other CRP binding sites in this promoter can be -72, -82, -92 positions (Ebright 1993; Busby and Ebright 1994, 1999; Lawson et al. 2004).

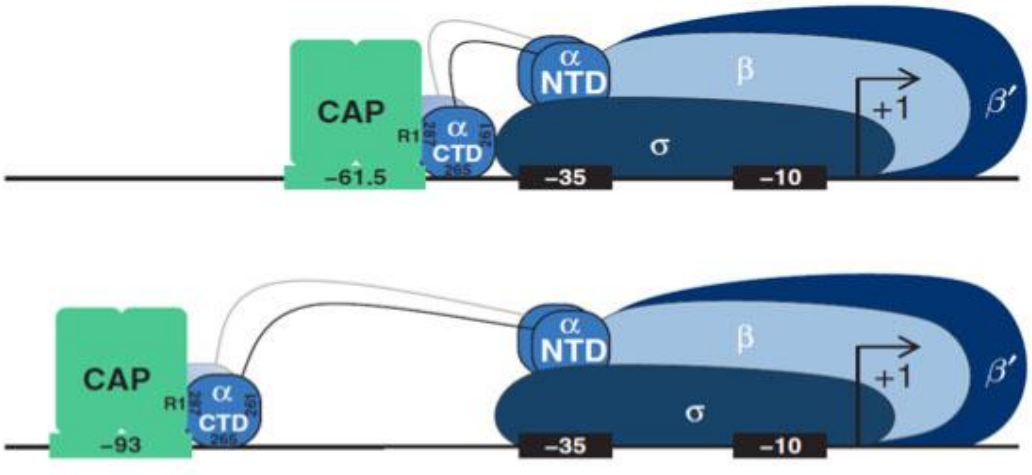

Figure 1 Transcription activation by Class I CAP-dependent promoter (adapted from Georis 2013)

Here, amino acid residues '156-164' of the downstream subunit of CRP denotes as 'activating region 1 (AR1)' and this region is mainly responsible for transcription activation in this promoter (Bell et al. 1990; Niu et al. 1994; Zhou et al. 1993). The AR1 interacts with the '287 determinant' of αCTD part of RNAP. This interaction allows binding of αCTD to DNA with its '265 determinant'. The αCTD binds with the σ^{70} subunit of RNAP with its '261 determinant'. Thus with these three determinants, transcription activation takes place and any mutation in these three determinants can cause defective transcription (Igarashi and Ishihama 1991; Murakami et al. 1996; Savery et al. 1998, 2002; Lawson et al. 2004).

There is a protein-protein interaction occurring in this Class I CRP-dependent transcription activation (Ebright 1993; Busby and Ebright 1999; Lawson et al. 2004) (Figure 1). Interaction occurring between CAP and RNAP makes RNAP-promoter DNA interaction stronger. When there is an increase in affinity, the binding constant, K_B also increases which forms the closed complex (RNAP-promoter) and thus initiation of transcription is also increased (Malan et al. 1984; Kolb et al. 1993b; Heyduk et al. 1993; Law et al. 1999).

1.2.2 Class II CRP-dependent promoters

In this promoter, the CRP binds on a region on double-helix DNA overlapping the binding site of RNA polymerase (RNAP). In vivo, -41.5 position on DNA strand denotes the site where CRP binds (Lawson et al. 2004). *GalP1* promoter is one of the best-known examples of Class II CRP-dependent promoters (Busby and Ebright 1997; Lawson et al. 2004).

It has been noted that all the three activating regions 'AR1', 'AR2', and 'AR3' are involved in this transcription activation.

AR1- This region is similar to Class I CRP-dependent promoter but the only difference is that in Class II CRP-dependent promoter AR1 is present in the upstream subunit of CRP (West el al. 1993; Zhou et al. 1994a, 1994b). The AR1 interacts with the '287 determinant' of one αCTD (Niu et al. 1996).

AR2- This region consists of residues "His19, His21, Glu96, and Lys101" and is present in the downstream subunit of CRP. The charge of AR2 is positive and it interacts with residues 162-165 of one αNTD (Niu et al. 1996; Rhodius et al. 1997).

AR3- This region is made by replacing Lys52 with an amino acid residue, which is mainly neutral or negatively charged. Residues 52-58 denote AR3 (Bell et al. 1990; Williams et al. 1991; West et al. 1993; Niu et al. 1996). AR3 is workable only in the downstream subunit of CAP (Williams et al. 1996). This AR3 interacts with the σ^{70} (residues 590-600) subunit of RNAP (Busby and Ebright 1997, 1999). Thus, there are three protein-protein interactions observed in this promoter (Figure 2).

1.2.3 Class III CRP-dependent promoters

In Class III CRP-dependent promoters, a single CRP dimer is not involved but rather two or more are involved. These CAP dimers collectively activate transcription in a few promoters. Here the distance between CAP binding sites on DNA is varied and the distance between CAP binding sites and RNAP binding sites are also different. The transcription activation in this class of promoters is really simple as it only involves the combination of Class I and Class II promoters (Busby and Ebright 1999).

If for example one CAP dimer is present at the -62 position and another CAP dimer is present at the -82 or -92 position then both can activate transcription synergistically through the Class I mechanism (Busby and Ebright 1999; Law et al. 1999). Similarly, if one CAP dimer is present at -62, -72, -82, or -92 position and another CAP dimer is present at -41.5 position then also both can activate transcription synergistically through the Class I mechanism is the upstream CAP subunit and Class II mechanism in the downstream subunit (Murakami et al. 1997; Busby and Ebright 1999, 1994) (Figure 3). In Class III CRP-dependent promoters both copies of αCTD are involved. In Class III CRP-dependent transcription activation both the CRP dimers make free contact with various surfaces of RNAP as well as no direct contact is required between CRP dimers (Busby and Ebright 1999).

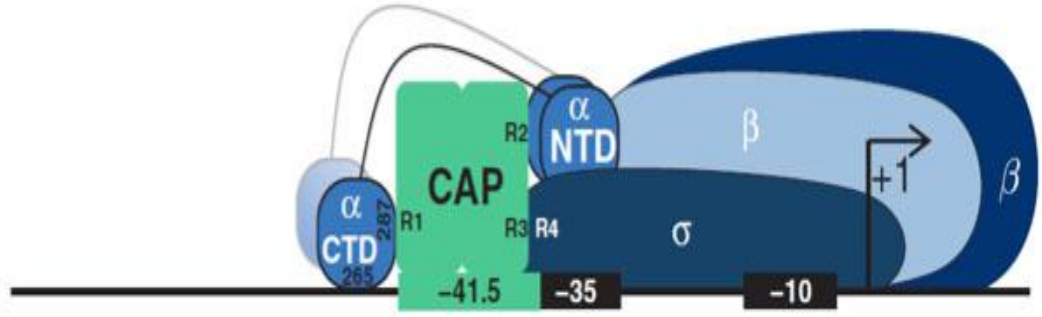

Figure 2 Transcription activation by Class II CAP-dependent promoter (adapted from Georis 2013)

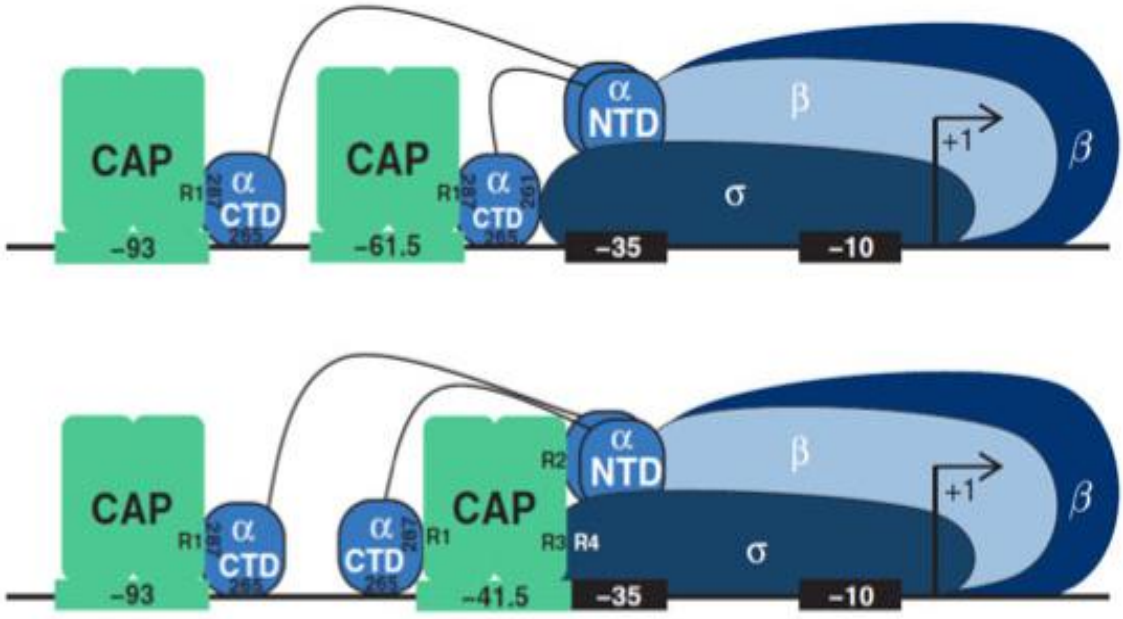

Figure 3 Transcription activation by Class III CAP-dependent promoter (adapted from Georis 2013)

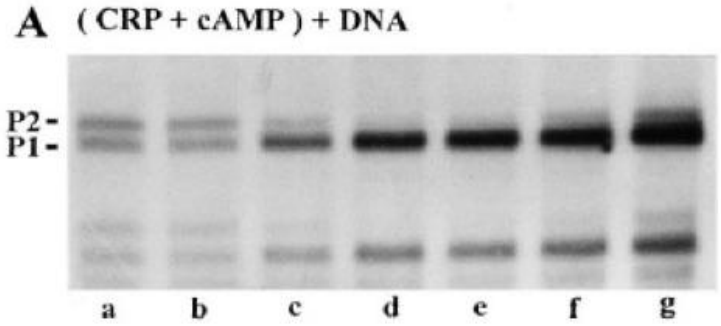

Figure 4 The effect of cAMP on *In vitro* transcription from *gal* promoters. Run-off transcripts from P1 and P2 promoters are indicated. cAMP concentrations used were 0, 0.2, 2, 20, 100, 200 and 400 μM (lanes a to g). At low level of cAMP, the transcript from P2 is less, but further addition of cAMP tends to reverse this effect.

2 Biphasic Behaviour of Cap

As it has been stated earlier that there is a clear biphasic behavior that CAP follows upon binding of cyclic AMP or cAMP. This biphasic behavior is very much evident and reported in Class II CRP-dependent promoters and more specifically in *gal* promoters (Mukhopadhyay et al. 1999). Our main objective of this work is to find out whether this biphasic character is observed only in *gal* promoters or is a general phenomenon for other promoters as well? To answer this question, *in vitro* transcription experiments and docking were done with other promoters of *E.coli*.

In the case of *gal* promoter, which is a Class II CAP-dependent promoter, the operon consists of two promoters P1 and P2 which overlap each other. It has been reported that when cAMP is absent, both the promoters produce transcripts at a basal level, there is no activation from either of the promoter but the addition of cAMP activates and initiate transcription, moreover, in the presence of a low level of cAMP, only P1 is activated and whereas the transcription from the other promoter i.e., P2 is repressed and thus transcripts are produced only from P1.So, at a low level of cAMP, gal P1 is activated and gal P2 is repressed but this pattern is only observed up to a certain concentration of cAMP (Figure 4). Moreover, further addition of cyclic AMP tends to alter this effect and further addition of cAMP tends to activate P2 whereas inhibits P1. This shows that *gal* promoter shows biphasic characteristics for the concentration of cAMP (Mukhopadhyay et al. 1999).

Table 2 Sequence of Primers used to generate a template for *In-vitro* Transcription

Name of the Primer	Sequence (5'-3')	Promoter region amplified
LacPF	CGCCCATATGGTTGGCCGATTCATTAATGC	*Lac*
LacPR	TTAGGGATCCATTACGCCAGCTGGCGAAAG	*Lac*
ABS 102	ATCGCCGCGCGTCACCATCG	*Cya*
ABS 103	TGATTCCGCCAACATCAACG	*Cya*

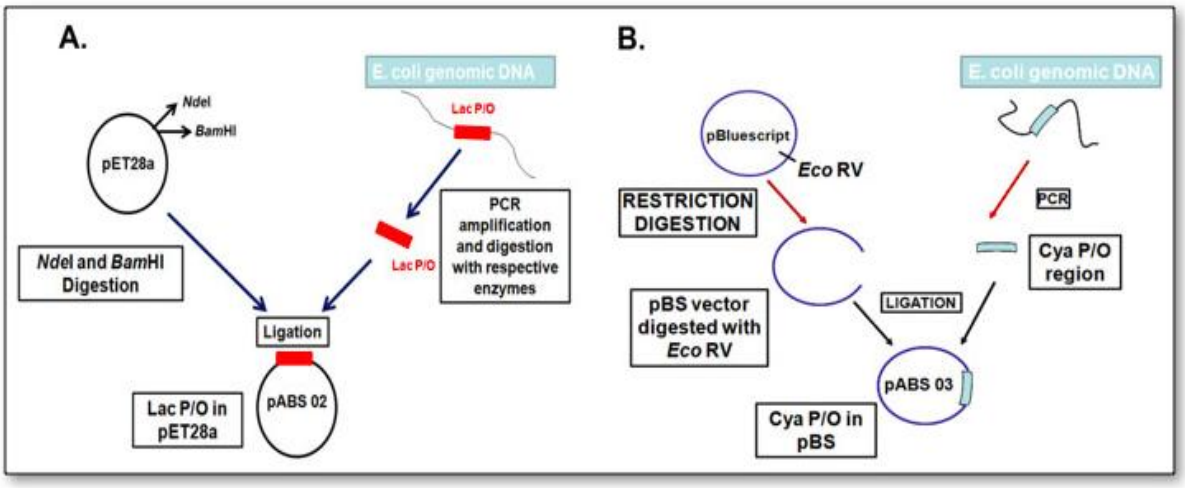

Figure 5 A schematic representation of cloning of lac (A) and cya (B) promoter region of *E. coli* into pET28a and pBluescript vector respectively.

3 Materials and Method

3.1 Preparation of DNA fragments for In vitro transcription experiment with the various promoter and operator regions of E. coli

For *in vitro* transcription experiment, 300 bp *lac* promoter operator region was amplified and cloned with suitable primers LacPF and LacPR (Table 2) as reported earlier (Saha et al. 2015). The –370 to +120 region of *E. coli cya* promoter was also amplified by PCR from *E. coli* genomic DNA employing suitable oligos (ABS 102 and ABS 103) and cloned into pBluescript vector at *Eco* RV site to obtain plasmid pABS 03. A schematic representation of cloning of lac and cya promoter regions is shown in Figure 5.

3.2 In vitro transcription

The IVT "*In vitro* transcription" reactions were done in 20 µl reaction volume with Lac P/O and Cya P/O region as described earlier The product was estimated by running a denaturing gel, and finally, the RNA product was estimated with a scanner system.

To investigate the biphasic cAMP dependence in other promoters, *in vitro* transcriptions reactions of CAP were also done in the presence of the varying amount of cAMP for both *lac* and *cya P/O* regions of *E. coli* as described above. The final concentrations of cAMP were 0, 0.3, 0.75, 2, 20, 50, 200, 400, 800 and 1000 µM respectively in case of *lac P/O* and 0, 50, 100, 200, 400, 800, 1000 µM respectively in case of *cya P/O*.

3.3 Docking of Lac and Gal promoters

The DNA sequences of the *Galactose* & *Lactose* promoter of *E. coli* were collected from the article (Kolb et al. 1983). Two 3D models (*Lac*&*Gal* promoter) were built with Avogadro: Molecular Editor and Visualization software (https://avogadro.cc/). After the 3D model was prepared then we have edited the structure by Text-Pad, we have changed the DNA two letters code to a three letters code for docking as the HADDOCK server accepts DNA three letters code.

The *E.coli* CAP-cAMP complex crystal structure (PDB:2GZW) was downloaded from online resources (Protein Data Bank). The protein has 4 chains (A,B,C,D) so, we have edited the structure in Text-Pad and cleave the chains Both C and D, and the final which we have made has two chains A and B.

The DNA-Protein Docking was carried out with HADDOCK 2.4 server (https://wenmr.science.uu.nl/haddock2.4/) (van Zundert et al. 2016). In the HADDOCK server two input molecule files were required that is DNA and Protein. So, we put the protein file which was the CRP-cAMP complex file in input molecule no.1, and the

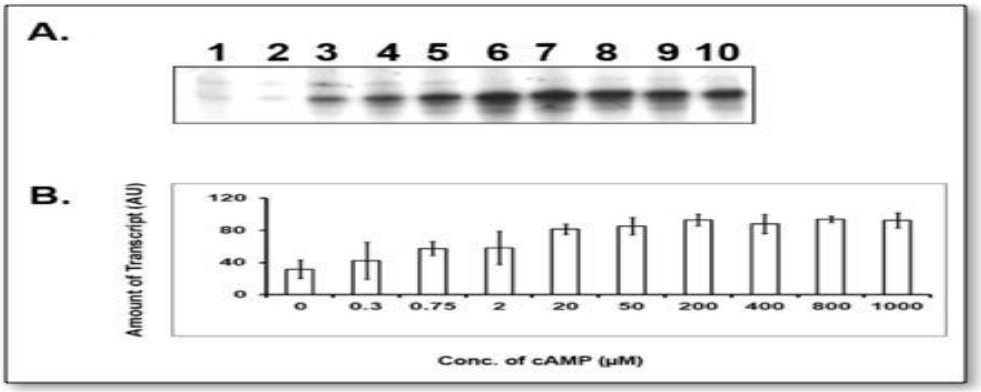

Figure 6 In vitro transcription of *lac* P/O with different concentrations of cAMP (A. CAP was preincubated with the indicated amount of cAMP on ice for 5 minutes, followed by addition to the reaction mixture containing 5 nM of template and 50 nM of RNA polymerase, followed by further incubation at 37^0C for 20 minutes. Transcription was initiated by the addition of the NTP mix, as described under Methods. After another 20 minutes, the reaction was stopped and the products were analyzed on a 10% polyacrylamide-7 M urea gel. Lanes 1-10, 0, 0.3, 0.75, 2, 20, 50, 200, 400, 800 and 1000 µM cAMP. B. Histogram of the above, showing the amounts of transcript produced as a function of cAMP concentration).

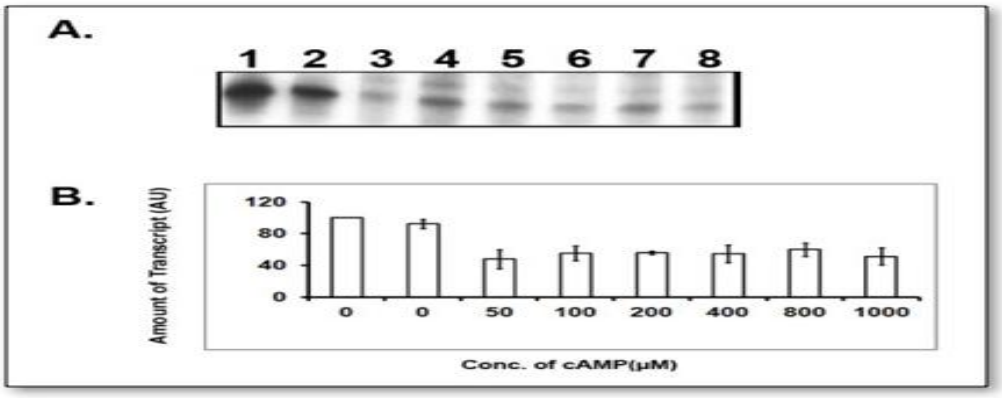

Figure 7 In vitro transcription of cya P/O with different concentrations of cAMP (A. The run off transcripts from *cya*p/o regions were analyzed on a 10% polyacrylamide-7 M urea gel. Lane 1, promoter alone; lanes 2-8, 100 nM CRP with 0, 50, 100, 200, 400, 800 and 1000 µM of cAMP. B. Histogram of the above, showing the amounts of transcript produced as a function of cAMP concentration)

DNA file which was the *Galactose* promoter file in input molecule no.2. The same process was followed in the case of Lactose promoters. After that, the parameter was given manually like the active residues and the chains which are involved in the interaction. At last, we selected the option in which the passive residues will be automatically taken.

After Docking the DNAproDB server (https://dnaprodb.usc.edu/index.html) was used to visualize the data (Sagendorf et al. 2020).

4 Results

4.1 In vitro transcription of CAP with lac and cya promoter regions at varying concentrations of cAMP

It has been suggested that two different shapes (conformers) of CAP present at low and high cAMP concentrations and these conformers behave differently regarding transcriptional regulation of *E. coli* gal promoters. To examine the probable function of these two conformers in the modulation of transcription in other promoters, we have done *in vitro* transcription experiments of CAP with *Lac* and *Cya* promoter/operator regions at a varying concentration of cAMP (Figure 6 and Figure 7).

In the case of the *lac* promoter/ operator region, the intensity of the promoter-specific transcript increased monotonously with varying concentrations of cAMP. Therefore, in the case of the *lac* promoter, clear monophasic transcription regulation by CRP with relation to cAMP concentrations is observed (Figure 6).

For *cya* promoter, the intensity of the promoter-specific transcript decreased monotonously with varying concentrations of cAMP (Figure 7). It has been reported earlier that transcription of the *E. coli ac* (adenylate cyclase) gene (i.e. *cya*) is regulated by the CAP-

Table 3 Docking parameter of CAP-cAMP with *gal* promoter and CAP-cAMP with *lac* promoter complex

Parameters	CAP-cAMP with *lac* promoter	CAP-cAMP with *gal* promoter
HADDOCK score	-92.1 +/- 1.7	-95.3 +/- 4.0
Size of the cluster	14	20
RMSD value (Lowest-energy structure)	31.3 +/- 0.1	29.9 +/- 0.4
V - W energy	-47.1 +/- 3.6	-47.9 +/- 4.0
Electrostatic energy	-483.1 +/- 18.0	-518.3 +/- 13.8
Desolvation energy	23.9 +/- 0.9	24.8 +/- 1.0
Restraints violation energy	277.0 +/- 48.4	313.9 +/- 21.8
Buried Surface Area	1783.6 +/- 89.2	1739.3 +/- 85.2
Z-Score	-1.3	-2.4

Table 4 Residues involved in interaction validated using DNAproDB web-based visualizing tool

Name	Interaction residues
CRP-cAMP with *lac* promoter	R180, E181, T182, G184, R185, V139, R385, T382, S179*
CRP-cAMP with *gal* promoter	R180, E181, T182, R185, V139, R385, S179*

Table 5 The docked complex structures are confirmed using DNA proDB (web-based) visualization tool (For CRP-cAMP with *lac* promoter)

DNA Entity ID	Pro. Chain ID	Pro. Chain Segments	Nuc-Res Interactions	Weak Nuc-Res Interactions	Total BASA [Å²]	Total H-bonds	Total vdW	Hydrophobicity Score (SAP)	Secondary Structure Composition
A1@A2	B	B1, B2	41	7	1010.126	20	98	-1.667	helix

Table 6 The docked complex structures are confirmed using DNA proDB(web-based) visualization tool (For CRP-cAMP with *gal* promoter)

DNA Entity ID	Pro. Chain ID	Pro. Chain Segments	Nuc-Res Interactions	Weak Nuc-Res Interactions	Total BASA [Å²]	Total H-bonds	Total vdW	Hydrophobicity Score (SAP)	Secondary Structure Composition
A1@A2	B	B1, B2	40	5	904.017	23	80	-1.815	helix

BASA= buried solvent accessible surface-area; vdW= V - W interaction; SAP= spatial aggregation propensity algorithm

cAMP complex in a negative manner. Therefore, it is clear from these two observations that CAP showed a monophasic behavior upon varying concentrations of cAMP, for the inducible promoter *lac* as well as the repressible promoter *cya* or in other words CAP does not show biphasic behavior upon varying cAMP concentrations universally in all promoters.

4.2 Docking of Class I and Class II Promoters with CRP

About 10 clusters were obtained after the docking and among them, the cluster having the lowest Z value and RMSD value were taken. The Z value, RMSD value, and restraints value of *gal* promoter is much less than that of the *lac* promoter. The best complex is also found out by using SASA (solvent accessible surface area) parameters for each molecule and also for the complex. All the parameters were mentioned in Table 3. The desolvation energy of the *gal* promoter is also more. CAP-cAMP complex interacting with *gal* promoter provided the most suitable complex structure with (-95.3 +/-4.0) HADDOCK score. It has also been observed that another parameter, the RMSD value from the lowest-energy structure is less in the case of *gal* promoter that's why *gal* promoter indicates a good stable structure.

Table 4 shows the residues of CAP-cAMP involved in the interaction with both the promoters. The interactions between these residues of CAP-cAMP with *lac* promoter (Figure 8) and with *gal* promoter (Figure 9) were also observed. The docked complex structures of *lac* and *gal* promoters are mentioned in Table 5 and 6. Lastly, through docking the structure of *lac* promoter with CAP-cAMP (Figure 10) and *gal* promoter with CAP-cAMP complex (Figure 11) were obtained.

It has been observed through this experiment that CRP-cAMP with *lac* promoter complex has two more interactions (G184, S382) other than CAP-cAMP with *gal* promoter. Moreover, it is imperative to note that *S179 residue interacts with the DNA loop (Table 4).

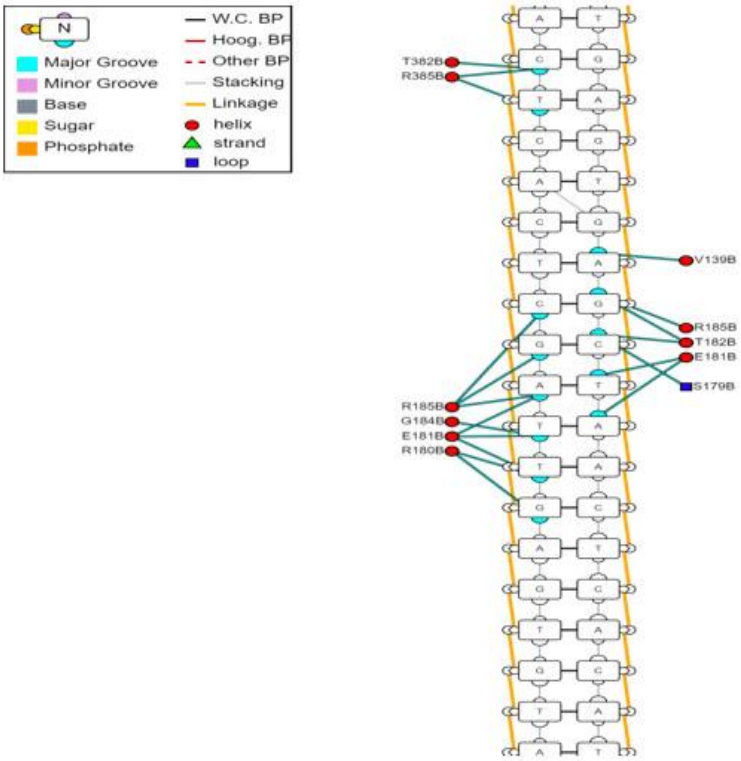

Figure 8 Interaction between CAP-cAMP and *lac* promoter

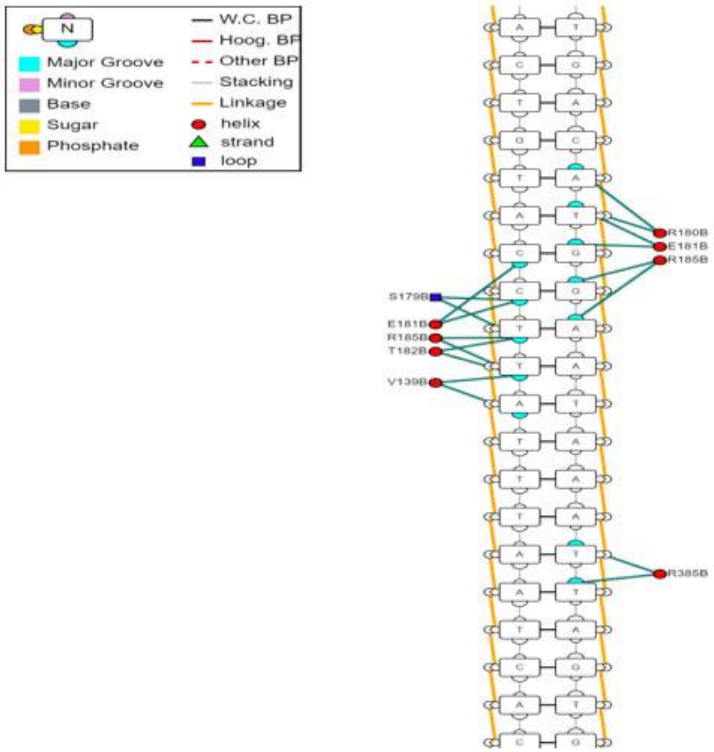

Figure 9 Interaction between CAP-cAMP and *gal* promoter

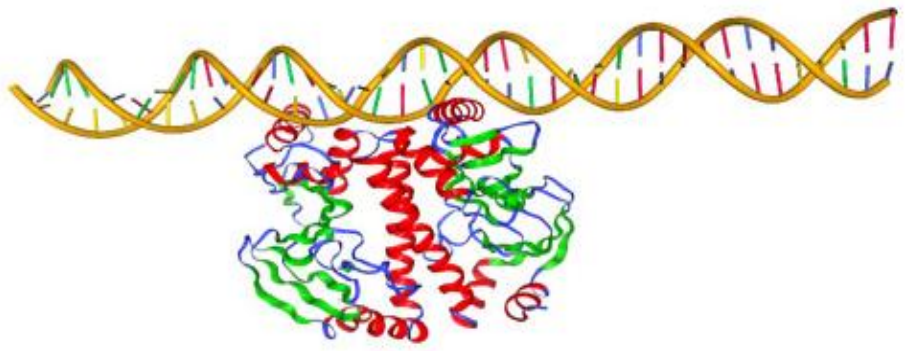

Figure 10 Docking complex of *lac* promoter and CAP-cAMP complex (The CAP-cAMP complex (red, green, blue) binds with the CAP binding site on the *lac* promoter DNA - yellow).

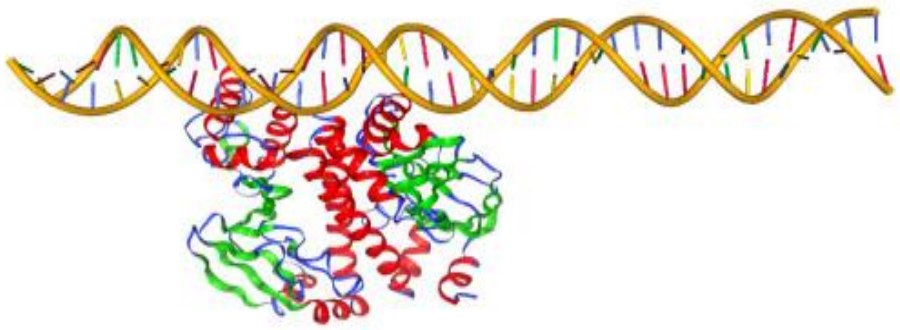

Figure 11 Docking complex of *gal* promoter and CAP-cAMP complex (The CAP-cAMP complex (red, green, blue) binds with the CAP binding site on the *gal* promoter DNA - yellow).

5 Discussions

So, it is clear from the result that the biphasic dependence of CAP with varying concentrations of cAMP might not be a general phenomenon. Though, the exact reason behind this difference was not clearly known but an assumption was made regarding this difference. It has been reported earlier that the residue Lys52 of CAP is important for transcriptional activation especially in Class II promoters and mutation of this residue affect Class II CAP-dependent transcription. cAMP binding site has a proximity towards Lys52. In the syn-cAMP complex, there is a conformational change of that part of the cAMP binding site which contains Lys52. If this CAP-cAMP complex bound to DNA interacts with any other protein, then the affinity of CAP for syn-cAMP will increase, and thus the conformation of CAP protein binding syn-cAMP will also get stabilized (Passner and Steitz 1997).

Abbreviations

cAMP- cyclic AMP; RNAP- RNA polymerase; αCTD- RNAP α subunit C-terminal domain; αNTD- RNAP α subunit N-terminal domain; AR1- activating region 1; AR2- activating region 2; AR3- activating region 3.

Conflict of Interest

There is no conflict of interest.

Author's Contribution

AS: conceptualization, investigation, writing original draft, supervision; SC: Investigation, writing, original draft, editing; SS: Computational investigation; RM: writing, editing; RM: conceptualization, supervision.

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