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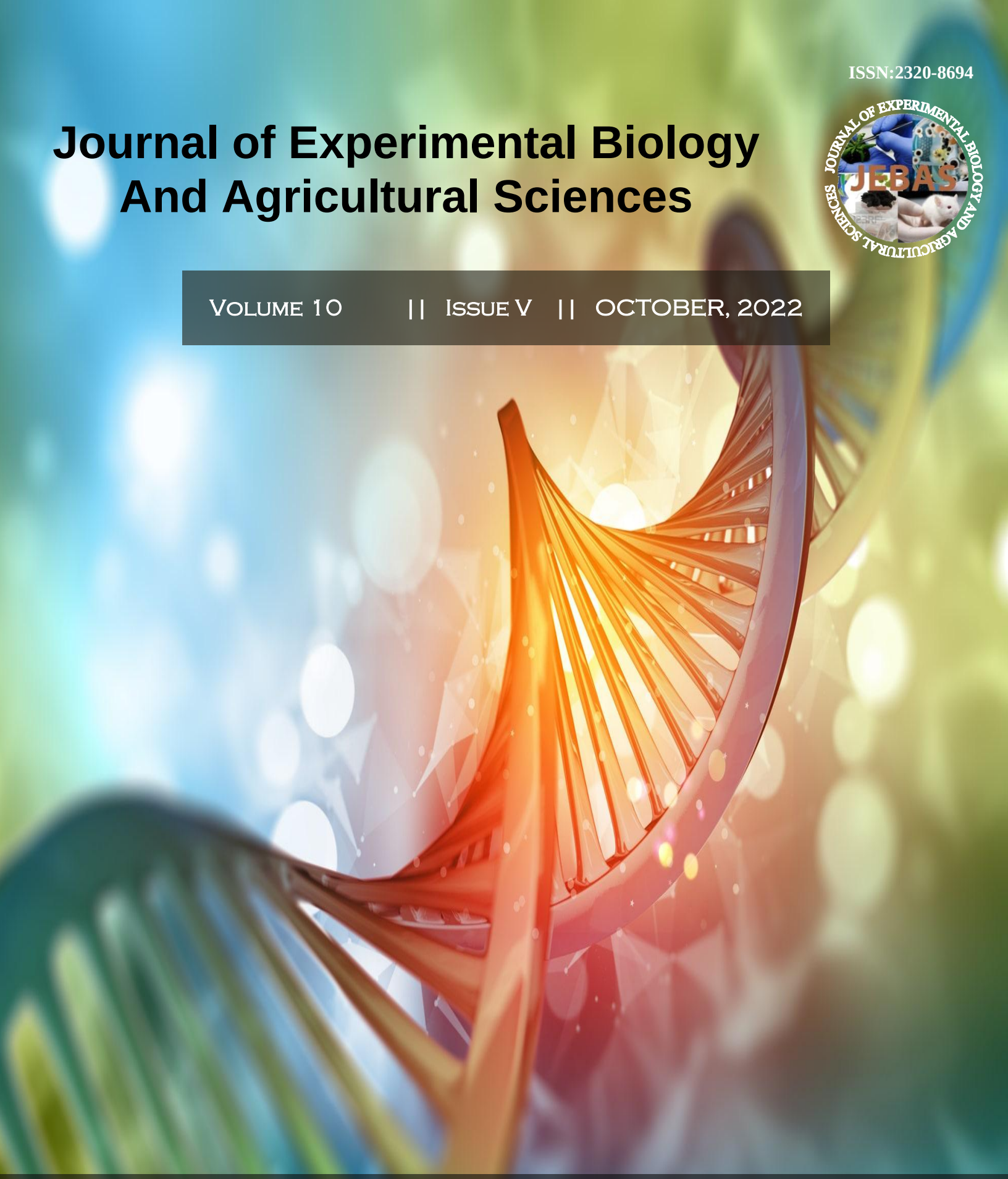

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Beneficial health effects of cumin (*Cuminum cyminum*) seeds upon incorporation as a potential feed additive in livestock and poultry: A mini-review

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ABSTRACT

Cumin (*Cuminum cyminum* Linn) is an annual plant of the family Umbelliferae, with its use dating back to ancient times when it was cultivated for its medicinal and culinary potential. Cumin seeds could contain a wide variety of phytochemicals, including alkaloids, coumarins, anthraquinones, flavonoids, glycosides, proteins, resins, saponins, tannins, and steroids. In particular, linoleic acid, one of the unsaturated fatty acids found in abundance in cumin oleoresin, is credited with promoting good health. Many of cumin's purported biological actions in livestock and poultry have been attributed to flavonoids such as apigenin, luteolin, and glycosides. Cumin has several healthful qualities, such as antibacterial, insecticidal, anti-inflammatory, analgesic, antioxidant, anticancer, anti-diabetic, anti-platelet aggregation, hypotensive, bronchodilatory, immunological, anti-amyloidogenic, and anti-osteoporotic properties. Cumin supplementation may improve milk production and reproductive function in dairy cows by altering the feeding pattern of bacteria in the rumen, encouraging the growth of beneficial microbes, or

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Medicinal herbs

stimulating the secretion of certain digestive enzymes. Because of the low price of cumin seed, it could be concluded that its inclusion in the diet might be beneficial to the commercial poultry industry and reduce the overall cost of egg and meat production. In recent years a rise in cumin's popularity has been seen as a result of the herbal movement spearheaded by naturopaths, yoga gurus, advocates of alternative medicine, and manufacturers of feed additives. Animal nutritionists are exploring the use of cumin for its potential to boost growth, improve nutrient usage efficiency, and reduce greenhouse gas emissions. This mini-review discusses how cumin could be used as a feed ingredient to boost productivity and ensure healthy animal reproduction.

1 Introduction

The ability of animals to treat themselves in the wild demonstrates the degree to which livestock and agricultural production have always been self-sufficient (Chandran et al. 2019; Patange et al. 2022a). Nonetheless, the shift in emphasis from agricultural production to livestock health rather than animal feeding is an intriguing new trend. This connection is based on the phytochemicals or bioactive components in plants and medicinal herbs that are good for animal and poultry health if employed in enough amounts (Dhama et al. 2015; Chandran and Arabi 2019; Deepak et al. 2020a; Abdelli et al. 2021; Hartady et al. 2021; Buttar et al. 2022; Seidavi et al. 2022). Plants' bioactive compounds have long served as a lifeline for both livestock and humans, providing them with vital nutrition, acting as potent immunomodulatory agents and healing remedies, and also suggested for adjunctive usage as an alternative to antibiotics in the era of emerging drug resistance as well as countering emerging pathogens (Dhama et al. 2014; Yadav et al. 2016; Dhama et al. 2018; Tiwari et al. 2018; Chandran and Radhakarishnan 2019; Chandran 2021a; Uddin et al. 2021; Kumar et al. 2022a; Kumari et al. 2022; Patange et al. 2022b). Wild animals and other scavenging species frequently self-medicate by eating plants known to have therapeutic effects. Farmers began utilizing antibiotics and vaccinations to combat disease-causing organisms as a result of intensification, which increased morbidity and mortality in animals (Chandran 2021a). However, antibiotic resistance emerged due to incorrect dosing, skipping the withdrawal phase, and prolonged antibiotic use. As a result of this trend, the World Health Organization has advocated for a complete prohibition on the use of all medically important/high priority antibiotics in livestock production below the therapeutic threshold (Chandran 2021b; Chandran et al. 2022; Prakash et al. 2021b). Alternative feeding practices are essential in contemporary animal nutrition, and especially so for young animals, who are particularly vulnerable to health problems. We may need to look at how animals thrive in the wild, without the aid of synthetic antibiotics, if we are to find a workable answer to this problem. Through the discovery and development of several phytomedicines, scientists have demonstrated that medicinal plants like cumin contains bioactive chemicals that are capable of improving health via its antibacterial, anti-parasitic, hematological, and other key health aspects (Chandran and Athulya 2021; Chandran 2021a; Kumari et al. 2022).

Therapeutic uses have long been associated with cumin (*C. cyminum*), an annual herb of the Umbelliferae family. It was thought to have been cultivated primarily in the Mediterranean region. India is both a major producer and consumer of cumin. India also exports many cumin seed value-added goods, such as cumin oil and cumin oleoresins (Chandran 2021a; Kumari et al. 2022). Cumin seeds are a common Ayurvedic remedy for a wide variety of mild to moderate conditions, including diarrhea, dyspepsia, flatulence, colic, abdominal distension, edema, bronchopulmonary disorders, puerperal disorders, analgesic, and cough. The effects of cumin are multifaceted, and it can improve eyesight, muscular endurance, and lactation (Prakash et al. 2021a). In traditional medicine, cumin seeds have been used to cure a variety of conditions, including toothache, dyspepsia, epilepsy, and jaundice. Pharmacological actions include anti-diabetic, immunologic, anti-tumor, and antibacterial functions. Essential oils extracted from cumin have been used successfully to treat a variety of medicinal issues, including epilepsy, indigestion, wounds, jaundice, and several respiratory and gastrointestinal disorders. It has been studied and found to have therapeutic uses, including antibacterial, antioxidant, anticancer, immunologic, and anti-diabetic properties. Therefore, it is evident that the aforementioned qualities of cumin make it an effective feed additive (Kumar et al. 2017; Prakash et al. 2021b). Toxic chemical residues, especially from antibiotics, have been related to their consumption of animal products and their use in animal farming. In recent years, the use of organic feed additives in animal nutrition has received a lot of attention due to concerns about food safety and human health (Kumar et al. 2022b). Cumin can improve the quantity and quality of milk produced by cattle. Cumin also helps greatly with the fermentation process in the rumen. Despite the availability of chemical additives, cumin seed is rising in popularity as a feed supplement for animals. This could be seen as a corrective action to mitigate the negative effects of chemical residue accumulation (Alizadeh et al. 2019). This review concentrates on the possibilities of using cumin seeds for boosting the comprehensive well-being of livestock.

2 Biochemical compositions of cumin seed

The nutritional composition of cumin seeds comprises carbohydrate (33 percent), fat (15 percent), protein (12 percent), starch (11 percent), fibre (11 percent), total ash (10 percent), moisture (7 percent), volatile oil (3-4 percent) and minerals (5.4-10.5 percent) (Gamal et al. 2021). Cumin seeds contain a plethora of essential oils, including saturated and unsaturated fatty acids such as petroselinic acid, oleic acids, and other fatty acids. Cumin's distinctive taste originates in its essential oils. Neutral lipids, glycolipids, and phospholipids make up 84.8 percent, 10.2 percent, and 5.1 percent of the dry weight of cumin seeds, respectively. Cuminaldehyde, p-cymene, cuminol, β-pinene, and terpenoids are the primary volatile components of cumin. Cumin has a distinctive strong flavour. It's warm aroma is due to its essential oil content. It's main constituents of aroma compounds are cuminaldehyde and cuminic alcohol. Other important aroma compounds of roasted cumin are the substituted pyrazines, 2-ethoxy-3-isopropylpyrazine, 2- methoxy-3-sec-butylpyrazine, and 2-methoxy-3- ethylpyrazine. Other components include γ-terpinene, safranal, p-cymene, and β-pinene. The oil content, quality, and nutritional composition have all been shown to vary, according to the research undertaken, depending on the country of origin and the growing conditions (Kumar et al. 2017). Figure 1 shows the biochemical makeup of cumin seeds.

3 Beneficial effects of incorporating cumin seeds in livestock feed

The beneficial effects of cumin seeds upon incorporation into livestock feed are depicted in Figure 2.

3.1 Influence of cumin seed on milk production and milk quality

For dairy farming to be sustainable, novel methods are needed to increase milk output while decreasing costs (Deepak et al. 2020b; Lejaniya et al. 2021a; Saleena et al. 2022a; Saleena et al. 2022b). Feedstuff prices, in particular, have been rising steadily in emerging countries like India, highlighting the urgent need for more efficient production processes. The demand for animal products is expanding because human beings depend prominently on these products to fulfilling their nutritional requirements (Chandran et al. 2021b; Lejaniya et al. 2021b; Sharun et al. 2021). The galactagogue herb *Payapro* has cumin as one of its main ingredients. Goat milk production was increased by 13 percent when cumin seed extract (1.27 percent of dry matter) was added compared to the control group (Chandran et al. 2021a). Miri et al. (2013) also experimented on lactating goats and found that cumin seed incorporation in the diet increased milk production by 13% in less-supplemented groups. The significant increase in monounsaturated and polyunsaturated fatty acids (PUFA) can also

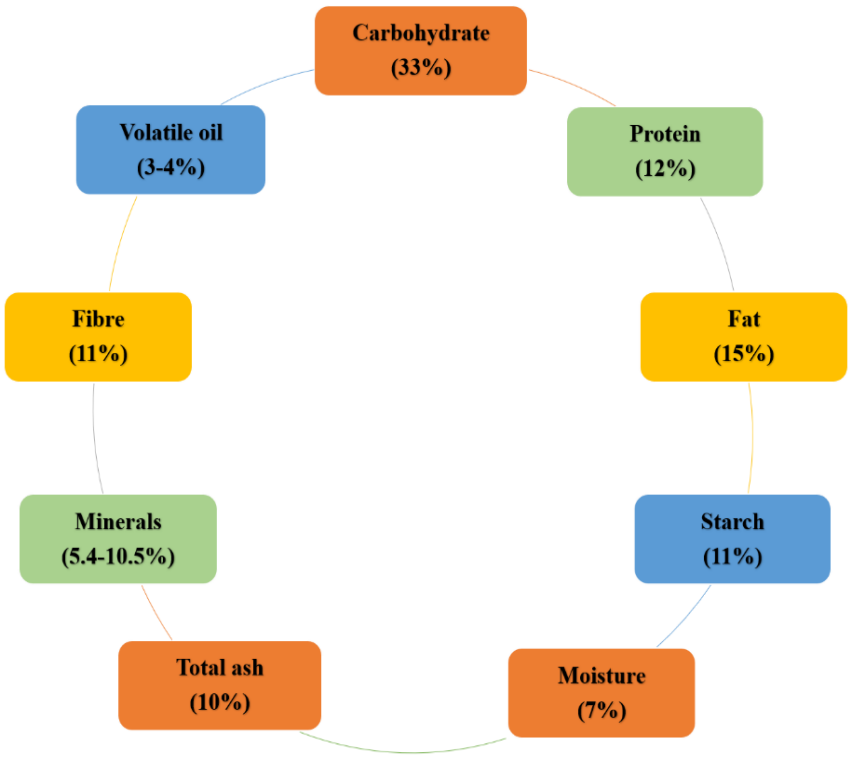

Figure 1 Biochemical composition of cumin seed

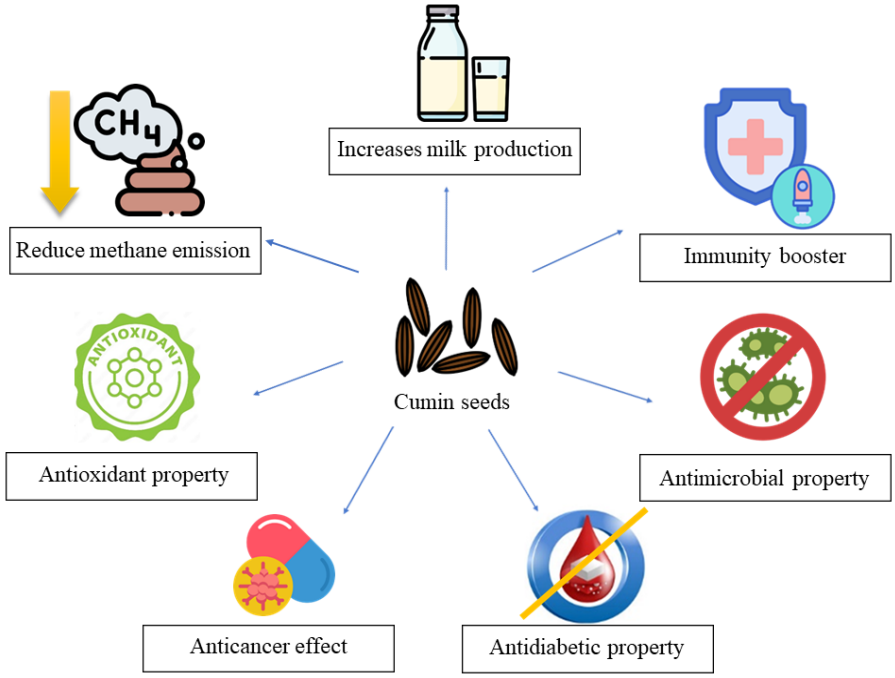

Figure 2 Beneficial health effects of cumin seed

be attributed to cumin seed supplementation in the diet. The ratio of PUFA to saturated fatty acid content in milk was well augmented, in addition to the higher concentration of conjugated linoleic acid (increased by 20 percent) and significant increase in δ-9 fatty acid desaturase (the enzyme responsible for the production of monounsaturated fatty acid) levels were significantly raised. This study demonstrates that the conjugated linoleic acid and PUFA content in livestock milk can be improved by the supplementation of cumin seed in their feed. Goats given 1g/L cumin seed extract had more linoleic acid and linolenic acid recovered in their milk. Tannins and other polyphenols can influence fatty acid metabolism at several points in the rumen's biohydrogenation process. Petroselinic acid (C18:1n-12), found in cumin, has been demonstrated to prevent the conversion of linoleic acid to arachidonic acid and may explain in part the rise in linoleic acid concentration in goat milk given cumin seed extract, as documented by (Bettaieb et al. 2011).

3.2 Role in enhancing nutrient utilization

Extensive studies show that feeding animals a diet that include cumin seeds increases their efficiency in digesting and using those nutrients (Kumar et al. 2017). Cumin seeds had no unfavorable effect on the palatability of the diet, as evidenced by no change in dry matter intake and milk composition (Heidarian et al. 2013). Broilers fed a meal supplemented with cumin oil gained considerably more weight than control animals. It has been also reported that including 2 percent cumin in the diet of heat-stressed broilers helped mitigate the detrimental effects of heat stress and improved growth performance. Increases in body weight gain, feed intake and feed conversion ratio were observed in broilers fed diets containing 0.75 and 1 percent cumin and turmeric mixture, respectively (Al-Kassi 2009). These results may be attributable to cumin's stimulant, carminative, digestive, antimicrobial, and gastric toxicity-preventative properties.

3.3 Effect on methane abatement

Fermentation in the hindgut of livestock produces methane, an essential component of greenhouse gases, and is one of the primary sources of greenhouse gases (Sorg 2022). When it comes to the greenhouse gases released into the atmosphere, agriculture and other land use practices account for about 24 percent of the total amount (Adegbeye et al. 2019). Cattle are responsible for 65% of total greenhouse gas emissions from the livestock sector, making it the largest contributor to global warming. Methane is predominantly created as a result of rumen fermentation in cattle; hence it is vital to lessen the methane production from animals, with feed additives playing a major role. Supplementing a goat's diet with cumin seed extract has been shown to reduce methane emissions by 11.8 percent *in vitro*, and by as much as 22 percent in buffered rumen fluid. Cumin seed extract may have an inhibiting effect on ruminal microbial biomass, which may explain this phenomenon. If the proliferation of gram-positive H_2-producing bacteria in the rumen is inhibited, less hydrogen is available for biohydrogenation and methanogenesis. It stands to reason that if protozoa are eradicated, the host animal will benefit from increased conjugated linoleic acid availability and methane emissions will

decrease. Herbal components including essential oils, saponins, and tannins have been found to act as rumen modifiers and reduce harmful emissions (Kuralkar and Kuralkar 2021). The rumen's gram-positive bacteria produce the hydrogen used in the process of methanogenesis. Therefore, cumin's inclusion in the diet has a deleterious effect on these species.

3.4 Antimicrobial action

The essential components of cumin seed essential oil include cumin aldehyde, ρ-mentdien-1,3-al-7, ρ-mentadien-1,4-al-7, ρ-cymene, β-pinene, and γ -terpinene (monoterpene carbohydrates), sabine hydrate, 1,8-cineole, caryophyllene, α-terpineol, and many other elements. The antibacterial properties of cumin essential oil are due to the presence of phenolic components such as cymene, limonene, and linalool (Teneva et al. 2016). It has been shown that essential oils can inhibit the growth of gram-positive and gram-negative bacteria and fungi. However, the effect can vary due to differences in essential oil strength and the types of bacteria that are being targeted (Alizadeh et al. 2019). Antimicrobial and antifungal properties were exhibited by cuminaldehyde against *Aspergillus flavus*, *A. niger*, *Penicillium chrysogenum*, *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus epidermidis*, and yeasts (*Saccharomyces cerevisiae* and *Candida albicans*). Its effect on the curb of certain diseases due to *P. aeruginosa* infections has been reported by Kumar et al. (2017). The larvicidal and antibacterial properties of cumin oil and cumin aldehyde were found to be quite potent. Cumin seeds were observed to reduce acid production and development of *Lactobacillus plantarum* in an *in vitro* investigation conducted by Singh et al. (2017). Extensive research shows that cumin oil is effective against a wide variety of food, soil, animal, and human infections (Kumar et al. 2017; Prakash et al. 2021b).

3.5 Antioxidant property

Unpaired electrons in the outermost shell of atoms make free radicals highly reactive species. An increase in the body's free radical production can result in oxidative stress, which can damage vital biomolecules and set off a cascade of adverse events that can lead to a variety of degenerative conditions. Antioxidant enzymes provide a natural defense against free radicals, or animals can supplement their diet with antioxidant vitamins and minerals. Research done in India has shown that the flavonoids in cumin seeds, especially apigenin and luteolin, provide the oil with a high level of antioxidant activity, which as a bonus, enables cumin seeds to function as an antioxidant. Essential oils derived from cumin seed include potent anti-oxidant properties. Cumin cultivars' phenolic contents in methanolic extracts varied from 4.1 to 53.6 mg/g dry weight. Cumin seed has a phenolic concentration of 9 mg/g (dry weight). Additionally, it was predicted that the methanolic extract of cumin seed had a higher antioxidant property than the aqueous extract (Nadeem and Riaz 2012). The phenolic components and antioxidant activity of 26 spice extracts, including cumin, were analyzed in a separate investigation conducted by Fatima et al. (2018). The trolox equivalent antioxidant capacity (TEAC; mmol trolox/100 g dry weight) was used to quantify the antioxidant activity. The total phenolic content of cumin was 0.23g of gallic acid equivalent per 100g of dry weight, and its trolox concentration was 6.61 mmol/100g. The antioxidant properties of cumin were assessed using several different techniques, including the DPPH (1-1 diphenyl-2-picrylhydrazyl) radical scavenging method, the soybean lipoxygenase-dependent lipid peroxidation methods, and the Fe^{2+} ascorbate induced rat liver microsomal lipid peroxidation assays. Cumin seed methanol extract inhibits rat liver microsomal lipid peroxidation with an IC 50 of 0.16±0.30. The IC 50 values for the DPPH radical scavenging method and the

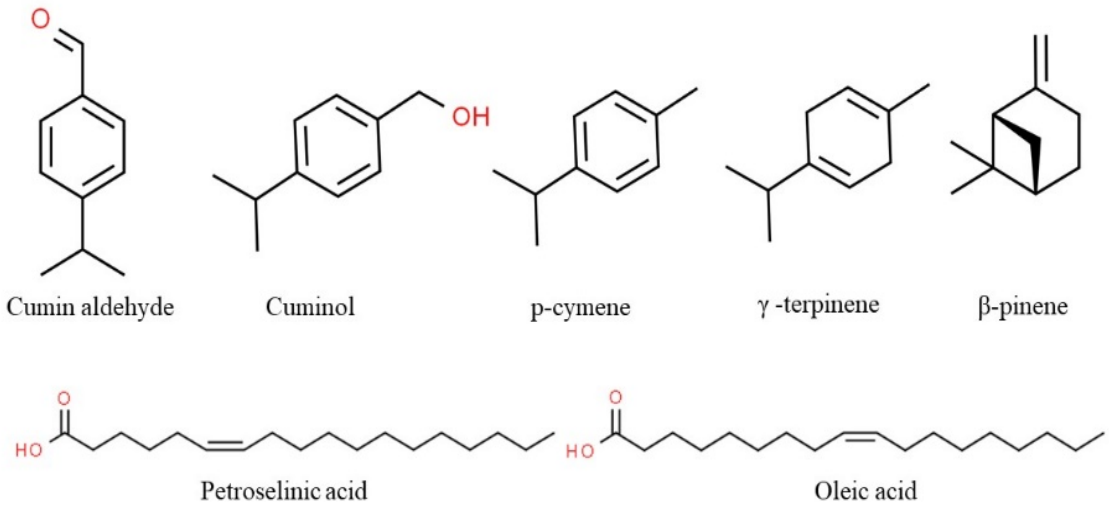

Figure 3 Chemical composition of cumin seed essential oil

lipoxygenase-dependent lipid peroxidation systems were 0.52±0.01 and 0.72±0.02 respectively (Wei et al. 2015). Cumin's ability as an antioxidant and its overall phenolic concentration are intertwined. This spice can be used as a natural antioxidant besides used as a flavoring agent (Nadeem and Riaz 2012). Superoxide dismutase, catalase, and reduced glutathione were all reported to be increased in mice fed cumin seed supplements; however, glutathione peroxidase and glutathione reductase activities were unaffected. It has also been shown that cuminaldehyde can scavenge the superoxide anion. The antioxidant activity of cumin seed extracts was validated by Juhainmi and Ghafoor (2013), who found that supplemented mice had higher DPPH radical scavenging activities than non-supplemented animals throughout a range of concentrations from 8.25 to 11.24 mg/mL. Cumin's phenolic component level was linked to its antioxidative capability, they said. A lower risk of cancer and cardiovascular and cerebrovascular problems may result from consuming foods high in bioactive substances with strong free radical scavenging properties.

3.6 Antidiabetic activity

A glucose tolerance test conducted in rabbits showed area expansion under the glucose tolerance curve and hyperglycaemic peak (Kumar et al. 2017). The inclusion of cumin seeds (at 1.25 percent) in the ration of streptozotocin-induced diabetic rats was found to be beneficial, as evidenced by lower levels of hyperglycemia and glucosuria, followed by an increase in body weight of diabetic animals. Diabetic mice also exhibited other metabolic alterations, including decreased blood urea levels and urea and creatinine excretions. Body weight, tissue and plasma cholesterol, free fatty acids, phospholipids, triglycerides, alkaline phosphate, γ-glutamyl transferase, and aspartate transaminase were all decreased in alloxan diabetic rats after oral administration of cumin seeds, possibly due to inhibition of aldose reductase and α-glucosidase (Singh et al. 2017).

3.7 Role in immunity development

Cumin plays a chief part in developing anti-inflammatory responses in livestock (Figure 4). Inflammation is a defense mechanism animals use to fight off infections and heal damaged tissues (Broom and Kogut 2018). Chauhan et al. (2010) evaluated the immunomodulatory properties of cumin seeds using flow cytometry and ELISA in immunosuppressed and normal animals. Cyclosporine-A was induced in Swiss albino mice and they were dosed orally with cumin seeds (25, 50, 100, and 200 mg/kg) on successive days. As a result, there was a dramatic rise in the population of T (CD4 and CD8) cells. These data support the hypothesis that cumin seeds help in the maturation of immunological responses in animals by modulating the expression of T cells. Cumin seeds decreased adrenal gland growth and reduced elevated corticosterone levels in immune-suppressed animals (Gamal et al. 2021). These findings provide credence to the idea of using cumin seed as an immunomodulator for those with compromised immune systems.

3.8 Role in mitigation of animal osteoporosis

Due to the presence of phytoestrogens, cumin seeds have estrogenic and anti-osteoporotic properties. Animals fed a diet supplemented with cumin seed extract had significantly lower urine calcium excretion, greater bone strength, and more calcium content. Improvements in bone ash density and microarchitecture were also documented, with no adverse effects such as weight gain or uterine atrophy (Chauhan et al. 2010).

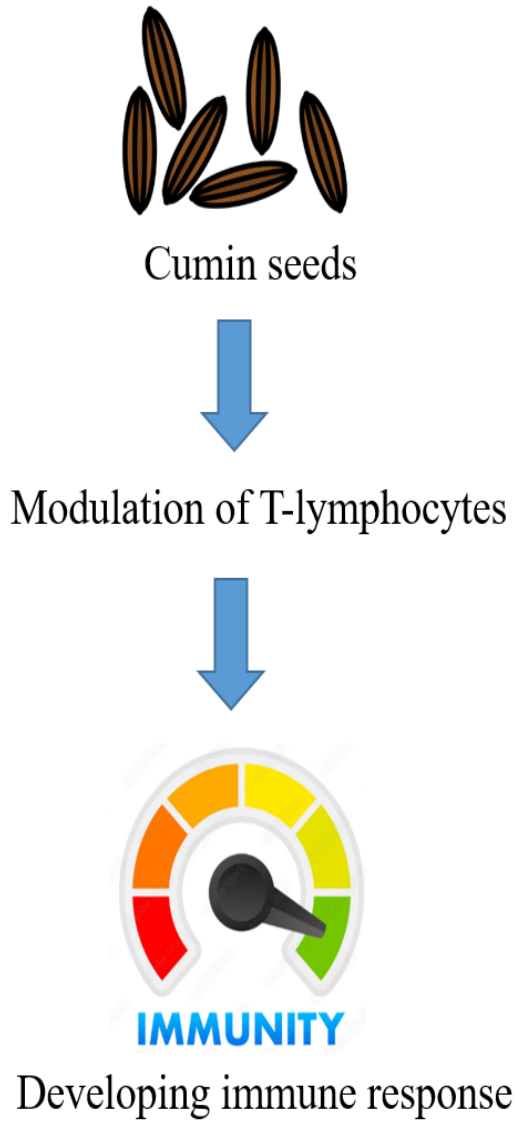

Figure 4 Immunogenic effect of cumin seeds

3.9 Anti-carcinogenic effect

Considered a necessary immune response to both ensnare invading pathogens and repair injured tissues; inflammation is at the forefront of modern immunology. On the other hand, inflammation is thought to be harmful because it causes nutrients to be redirected away from their productive uses. As a result, natural substances can be used as feed additives to better control tumor growth (Broom and Kogut 2018). Cumin is a blend of numerous herbs, several of which have been shown to have anticancer effects. Rats were fed cumin seeds in experiments designed to determine whether or not they affected preventing cancer. They exhibited resistance to colon cancer after being experimentally infected with the disease. Beta-glucuronidases activity and mucilage activity both decreased. It was found that uterine tumorigenesis which was induced by 3-methylcolanthrene, forestomach cancer tempted by benzopyrene, and hepatomas encouraged by dimethylaminoazobenzene in mice were prevented by the cumin provided in their diet. Metabolizing phase I enzyme and phase II enzyme present in cumin seeds are attributed to the modulation of cancerous mechanisms in mice (Kumar et al. 2017; Gamal et al. 2021).

3.10 Digestive stimulant action

Cumin seeds have been used in folk medicine and alternative medicine for centuries on the belief that they stimulate digestive enzymes; recent animal research by Fatima et al. (2018) confirmed this assertion. It has been studied extensively how both chronic feeding and single oral administration of cumin seeds affect the production of digestive enzymes in the pancreas and intestinal mucosa of rats. Lipase activity in the pancreas was inhibited by dietary cumin (1.25 percent), but trypsin, chymotrypsin, and amylase activity were all significantly increased. Cumin's ability to inhibit pancreatic lipase, amylase, trypsin, and chymotrypsin activity was demonstrated after a single oral dose was administered. Maltase activity in the small intestine was dramatically increased in rats fed cumin, while lactase and sucrose activity was unaltered (Abd El-Hack et al. 2016).

Although a single oral dose of cumin did not influence the rate of bile secretion, it did have a strong stimulatory effect on bile flow rate, increasing bile volume by 25 percent, as evidenced in the investigation of Shan et al (2005). Cumin had a significant impact on bile acid secretion (how much is secreted in a given amount of time), leading to a 70 percent increase compared to the control group. Cumin, when given orally, also significantly increased bile acid output after a single dose (Kumar et al. 2017). It stands to reason that cumin, which has a digestive stimulant action, might do so by stimulating the biliary secretion of bile acids, given that bile juice makes a significant contribution to the overall process of digestion and absorption, primarily by supplying bile acids required for micelle formation. The effect of the digestive stimulant spice cumin on the time it takes for food to leave the intestines of experimental rats has been investigated in a separate investigation. The use of cumin greatly reduced the time it took for the food to be delivered, by around a quarter of an hour. Cumin's positive effects on digestive enzymes and bile secretion roughly correlate with the shorter transit time it produces in the digestive tract (Fatima et al. 2018; Chandran 2021a).

3.11 Pulmonary-protective activity and anti-asthmatic effects

Research on the potential protective effects of cumin seed on experimental lung injury in rats following pulmonary aspiration revealed that cumin seed therapy suppresses inflammatory pulmonary responses. Additionally, it led to a notable decrease in iNOS activity and an increase in surfactant protein D in the lung tissue of various pulmonary aspiration models (Abdelli et al. 2021). The treatment with cumin seeds may help treat lung damage, which justifies prospective therapeutic application. Additionally, it has been found that cumin seed essential oil can help rats with hyperoxia-induced lung damage (Fatima et al. 2018).

Conclusion

One of the most widely used seed spices, cumin can be found in a wide variety of spice blends that are a regular part of the diet of animals and human beings. The chemical makeup of cumin included alkaloids, coumarins, anthraquinones, flavonoids, glycosides, proteins, resins, saponins, tannins, and steroids. Cumin oleoresin and its fatty acid composition are particularly high in linoleic acid, an unsaturated fatty acid with numerous positive health effects. Many of cumin's biological actions are attributed to flavonoids such as apigenin, luteolin, and glycosides. Cumin's nutritional profile revealed that it was rich in nutrients including thiamine, riboflavin, and niacin, in addition to the more commonly known carbohydrate, protein, and fat. It also contained iron and minerals in high concentrations, including iron and zinc, that are helpful for energy production, boosting the immune system, and treating skin diseases. The pharmaceutical and general applications of cumin are very extensive. Cumin is a popular and nutrient-rich spice, but it has recently been linked to several adverse effects, which suggests it should be used with caution in conjunction with therapeutic medications. Cumin seeds have been shown to have antimicrobial, insecticidal, inflammatory, analgesic, antioxidant, anticancer, anti-diabetic, anti-platelet aggregation, hypotensive, bronchodilatory, immunological, anti-amyloidogenic, and anti-osteoporotic effects. Based on the findings of this analysis, it can be concluded that using cumin as a feed additive in low doses has no negative effects on animal welfare and increases both nutrient consumption and production efficiency.

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Author's Contribution

All the authors contributed significantly.

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All authors declare that there exist no commercial or financial relationships that could, in any way, lead to a potential conflict of interest.

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Fungal and bacterial species in degrading carbamazepine: a metabolite perspective: Mini-review

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ABSTRACT

Carbamazepine (CBZ) is a ubiquitous pharmaceutical pollutant found in various water environments. This is due to the ineffective CBZ removal, despite employing advanced physiochemical treatment technologies in the current conventional wastewater treatment plants. Thus, bioremediation that utilizes enzymes in microorganisms' systems to bio-mineralize CBZ is suggested as an alternative or complementary technique to remove CBZ more effectively. However, information from published research on the biodegradation of CBZ, the toxicity of metabolites, or toxicity testing was rarely evaluated or assessed cohesively. This aspect is important because if bioremediation of CBZ produces toxic metabolites, it will defeat the main purpose of bioremediation. Thus, the focus of this review is to assess the effectiveness of fungi and bacteria in the biodegradation of CBZ, particularly by looking at the type of enzymes expressed, and the metabolites produced. In this review, information related to the fungal and bacterial species that were reported to degrade CBZ was collated from the published literature and analyzed. Results of the analysis showed that cytochrome P450, laccase, and manganese peroxidase were the common enzymes responsible to degrade CBZ. However, such enzymatic activities can sometimes produce epoxy-CBZ, which is a more toxic compound than the parent compound. Only the fungus *Pleurotus ostreatus* was able to oxidize epoxy-CBZ *via* the acridine pathway into acridone, the latter a metabolite that is susceptible to further biodegradation into nontoxic metabolites. However, the identity of the end metabolites is not reported nor characterized. Further, *Pseudomonas* spp. is the most promising bioremediating agent since it can metabolize CBZ into catechol, the latter can enter the carbon central pathways to generate energy for the bacterial cells.

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1 Introduction

Carbamazepine (CBZ) is an aromatic xenobiotic compound (Figure 1) that contains 98.0% of dibenzoazepine that carries a carbamoyl substituent at the azepine nitrogen (Alrashood 2016). CBZ is frequently detected in wastewater (Arye et al. 2011) because it does not undergo degradation during wastewater treatment (Clara et al. 2004), which leads to contamination of drinking water (Miao et al. 2005). According to a study conducted by Ternes et al. (2004), CBZ was detected in all 30 wastewater treatment plants and, also from 90% of the river water samples studied. Hospital and municipal effluents was the main contributor to CBZ found in the sewage treatment plants (Heberer and Feldmann 2005).

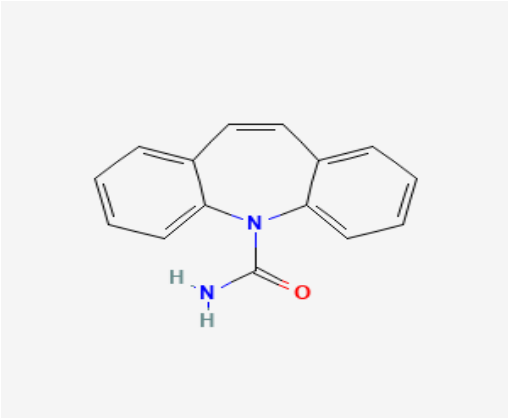

Figure 1 2D chemical structure of carbamazepine (PubChem 2021).

Physiochemical treatment technologies are commonly used to remove CBZ from wastewater (Table 1). The first two techniques which are well known to effectively remove most organic and inorganic compounds from water are nanofiltration and reverse osmosis (Radjenović et al. 2008). Even though nanofiltration and reverse osmosis show high organic and inorganic compounds removal efficiency from water, but the CBZ remains intact with the membranes which require further elimination action (Crini and Lichtfouse 2018). Physiochemical treatment that uses activated carbon also shows high CBZ removal efficiency from wastewater but the issue of disposing of the carbon matrix remains to persist with this (Crini and Lichtfouse 2018). The last type of physiochemical treatment is the advanced oxidation process (AOP) which utilizes the combination of chemical oxidation processes or ultraviolet (UV) irradiation on an added catalyst. This type of treatment can oxidize CBZ (Dai et al. 2012), but it is costly if it is operated on a large scale (Dai et al. 2012). Though the technologies mentioned in Table 1 show a high percentage of CBZ removal from water, the disappearance of CBZ provides only a partial indication of treatment efficiency (Kosjek et al. 2009). The most common transformation products of CBZ formed by such technologies belong to acridine and its derivatives, both being genotoxic (Bleeker et al. 1999). Moreover, the transformation products of CBZ from physicochemical treatments can become more resilient to further degradation (Kosjek et al. 2009). Thus, conventional wastewater treatment plants are found to be not wholly effective (Hai et al. 2018), and if such wastewater escaped into the environment it may cause serious ecological damage (Jos et al. 2003).

Numerous studies regarding the toxic effects of CBZ and its derivatives on aquatic organisms have been reported (Table 2). Jos et al. (2003) show the proliferation of aquatic algae *Chlorella vulgaris* was significantly inhibited within 48 hours of exposure to CBZ. Bivalve *Ruditapes philippinarum* was found to have its

Table 1 The efficiency of CBZ removal in water by various advanced physicochemical treatment technologies

Physicochemical Treatment	Treatment Type/ Systems	Starting Concentration of CBZ (ng/L)	Period of Treatment (min)	Removal Efficiency (%)	References
Pressure-driven membrane filtration technologies	Nanofiltration	84.5	-	98	Radjenović et al. 2008
	Reverse Osmosis	84.5	-	98	
Adsorption by activated carbon	Granular Activated Carbon	25	30	99	Park et al. 2007
	Powdered Activated Carbon	78	300	95	Snyder et al. 2007
Advanced oxidation processes	Ozonation	9	15	99	Huerta-Fontela et al. 2011
	UV	992×10^3	50	34	Dai et al. 2012
	UV/H_2O_2	210	20	74	Rosario-Ortiz et al. 2010
	UV/Cl_2	59	1.5	60	Sichel et al. 2011
	UV/TiO_2	992×10^3	10	10	Dai et al. 2012
	UV/Fenton	992×10^3	7	78	

Note: (-) means not stated.

Table 2 Toxicological effects of CBZ on aquatic organisms under different exposure conditions

Species	Starting Amount of Inoculum	Starting amount of CBZ (µg/L)	Period of Exposure (days)	EC50 (mg/L)	Effects	References
			Algae			
Chlorella vulgaris	1×10^6 cells/ml	-	2	3.66	Inhibited proliferation	Jos et al. 2003
Desmodesmus subspicatus	1×10^4 cells/ml	-	3	74	Inhibition of average growth rate	Cleuvers 2003
			Plankton			
Daphnia magna	10 neonates	-	1	112.23	Immobilized (Dead)	Jos et al. 2003
Daphnia similis	-	3	21	-	Inhibition of molting, delayed reproduction, and reduced fecundity	Chen et al. 2019
			Bivalve			
Ruditapes philippinarum	18 individuals	0.30 – 9.00	28	-	Inhibition of antioxidant enzymes in glycogen and electron transfer system	Almeida et al. 2015
Scrobicularia plana	10 individuals	0.30 - 9.00	28	-	Cell damage due to high lipid peroxidation	Freitas et al. 2015

Note: EC50 is the effective concentration that gives a half-maximal response; (-) means not available

Table 3 The efficiency of CBZ removal by various species of bacteria

Species	Enzyme Involved	Concentration of CBZ (mg/L)	Incubation Period (days)	Temperature (°C)	Shaking Speed (rpm)	pH	Removal Efficiency (%)	References
Labrys portucalensis F11	-	10.09	30	25	150	-	95.4	Bessa et al. 2019
Streptomyces MIUG 4.89	Laccase Phenoloxidase	0.2	7	25	150	7.2	35	Popa et al. 2014
Streptomyces SNA	Laccase	0.2	7	25	150	7.2	30	
Pseudomonas sp. CBZ-4	-	100	6	10	150	7	46.6	Li et al. 2013
Paraburkholderia xenovorans LB400	Biphenyl dioxygenase	10	1	25	100	7	100	Aukema et al. 2016
Pseudomonas sp. strain NCIB 9816-4	Naphthalene dioxygenase	10	1	25	100	7	>90	

Note: (-) means not stated.

metabolism inhibited when exposed to CBZ (Almeida et al. 2015; Almeida et al. 2021). Similarly, when the crustacean *Daphnia similis* was exposed to CBZ, it disrupts the endocrine system of *D. similis* (Chen et al. 2019). Since existing studies have reflected that CBZ can bioaccumulate, this indicates that the current wastewater treatments are not effective to remove CBZ, and a better approach to remediate CBZ is crucial to protect the organisms and environment.

Therefore, biological treatment processes such as biodegradation are available for the removal of CBZ and its metabolites in water. Biodegradation is a process whereby microorganisms are employed to degrade organic pollutants such as CBZ and convert them into less toxic or nontoxic forms of products (Zouboulis et al. 2019). Although biodegradation is an effective method to degrade CBZ, the achievement of a complete degradation varies within different types of organisms, particularly in between species (Li et al. 2013) of suitable organisms such as bacteria, fungi, algae, or plants that own the physiological abilities to degrade, detoxify, or render substrate of interest CBZ (Zouboulis et al. 2019).

To this concerning issue, enzymatic activities of microorganisms can be used to biodegrade CBZ more effectively (Singh et al. 2019). However, despite the wealth of information reported, the success to achieve a complete degradation varies within different types of organisms, between species and growth parameters (Li et al. 2013). Therefore, this review is to compare the efficacy of various species of fungi and bacteria in degrading CBZ, by investigating the various enzymes that degrade CBZ, and to determine which species can degrade CBZ into non-toxic metabolites.

In this current review, information n the enzyme expression and pathways involved in CBZ metabolism were summarized from different scientific databases including Google Scholar, PubMed, and Research Gate, etc., and collated in tabular for easy comparison. Available toxicity test studies on the metabolites generated by the enzymes of each species were assessed to achieve the aim of this review which is to determine the most efficacious species in degrading CBZ into non-toxic end metabolites in water.

2 Degradation of Carbamazepine by Bacteria

Table 3 summarizes the various CBZ-degrading bacterial species along with their diverse enzymes and operating parameters. Varying operating experimental conditions make comparison difficult when assessing which organism is the most efficient to degrade CBZ. Thus, it is best to investigate and compare the enzymes involved (Table 4) because different enzymes will produce different intermediate or end metabolites that might be toxic (Brusseau et al. 2019), which is the primary concern of this review. Furthermore, the different operating parameters will not affect the enzymes expressed, which makes the assessment more reliable.

2.1 *L. portucalensis*

It was reported that *L. portucalensis* degrades CBZ to produce OH-iminostilbene and epoxy-CBZ, catalyzed by the enzymes cytochrome 450 and MnP (Bessa et al. 2019). Following that, Epoxy-CBZ is proposed to be metabolized *via* the acridine pathway that resulted in a metabolite with molecular formula $C_{15}H_9NO_2$ and acridone. The latter has the potential to be degraded into nontoxic metabolites. However, a toxicity test conducted using the *Vibrio fischeri* luminescence (Jarque et al. 2016) resulted in an increase in toxicity which implies that the metabolites of CBZ degradation by *L. portucalensis* are more toxic than the parent compound CBZ itself.

2.2 *Streptomyces* spp.

Both strains of *Streptomyces* MIUG 4.98 and *Streptomyces* SNA expressed laccase during the degradation of CBZ (Table 4) to produce Epoxy-CBZ. In addition to laccase, *Streptomyces* MIUG 4.98 also expresses phenoloxidase (PO), although neither a conclusive study on this enzymatic pathway nor the resulting metabolites is available (Popa et al. 2014). Therefore, further identification of metabolites of CBZ degradation by *Streptomyces* spp. and the examination of toxicity after degradation are needed for better assessment.

2.3 *P. axenovorans*

P. axenovorans was shown to express a dioxygenase enzyme, Biphenyl-2,3-dioxygenase (BPDO) while degrading CBZ (Aukema et al. 2016). BPDO hydroxylates CBZ by oxygenating dihydrodiols which are cis-10,11-dihydroxy-10,11-dihydrocarbamazepine (diOH-CBZ), cis-2,3-dihydroxy-2,3-dihydrocarbamazepine, and carbamazepine-2,3-diol (Table 4). However, these dihydrodiols are toxic and highly reactive towards proteins, DNA, and lipids resulting in mutagenic and carcinogenic effects (Oesch-Bartlomowicz and Oesch 2007).

2.4 *Pseudomonas* spp.

The bacterial strain *Pseudomonas* sp. NCIB 9816-4 expressed a dioxygenase enzyme, Naphtahalene-1,2-dioxygenase (NDO) to degrade CBZ (Table 4) (Aukema et al. 2016). However, NDO's substrate specificity is to bind to naphthalene, a tricyclic aromatic ring compound unlike CBZ (Barry and Challis 2013). This suggests that *Pseudomonas* sp. degrades CBZ into naphthalene by other unidentified enzymes before the involvement of NDO.

Table 4 CBZ degradation by enzymes secreted from various species of bacteria

Species	Enzyme(s) Involved	End Metabolite	References
L. portucalensis F11	-	• $C_{14}H_{11}NO$ (OH iminostilbene) • $C_{15}H_9NO_2$ • $C_{13}H_9NO$ (acridone) • $C_7H_7NO_2$	Bessa et al. 2019
Streptomyces MIUG 4.89	Laccase, Phenoloxidase	-	Popa et al. 2014
Streptomyces SNA	Laccase	-	Popa et al. 2014
Pseudomonas sp. CBZ-4	-	-	Li et al. 2013
P. xenovorans LB400	Biphenyl-2,3-dioxygenases (BPDO)	• *cis*-10,11- dihydroxy-10,11-dihydrocarbamazepine (diOH-CBZ) • *cis*-2,3-dihydroxy-2,3 dihydrocarbamazepine • carbamazepine-2,3-diol	Aukema et al. 2016
Pseudomonas sp. strain NCIB 9816-4	Naphthalene-1,2-dioxygenase (NDO)	-	Aukema et al. 2016

Note: (-) means not stated.

Various species of *Pseudomonas* including *P. putida* (strains: NCIB 9816-4, G7, AK-5, PMD-1, and CSV86), *P. stutzeri* AN10 and *P. fluorescens* PC20 (Mahajan et al. 1994; Resnick et al. 1996; Annweiler et al. 2000; Basu and Phale 2008; Dennis and Zylstra 2004; Izmalkova et al. 2013) can metabolize naphthalene. The NDO catalyzes the oxidation of the aromatic rings of naphthalene into *cis*-dihydrodiol and later into catechol. Catechol is then cleaved to 2-hydroxymuconic semialdehyde following the *meta* route by catechol 2,3-dioxygenase (Yen et al. 1998). Lateroncatechol 2,3-dioxygenase is further hydrolyzed into pyruvic acid and acetaldehyde. Alternatively, catechol can be cleaved *via* the *ortho* route by catechol 1,2-oxygenase to yield *cis, cis*-muconic acid and further oxidized into succinyl-CoA and acetyl-CoA (Nozaki et al. 1968). Both routes produced non-toxic end metabolites that can furnish bacterial cells with energy.

3 Degradation of Carbamazepine by Fungi

Species of fungi that biodegrade CBZ are listed in Table 5. However, the percentages of CBZ's removal vary due to different operating parameters including starting concentration of CBZ, temperature, incubation period, shaking speed, and pH. The toxicity of the metabolites is not known, which defeats the purpose of bioremediation. Thus, a better comparison is by looking at the enzyme expression to deduce whether the metabolites produced are safe. Table 6 summarizes the identified enzymes involved in the degradation of CBZ and the corresponding metabolites in each fungal species.

3.1 *T. harzianum*

T. harzianum degrades CBZ by expressing cytochrome P450 system (Table 6). Cytochrome P450, a wide-ranging superfamily of monooxygenases, is one of the important intracellular enzymatic defense systems that protect fungi from toxic compounds including CBZ (Črešnar and Petrič 2011). However, the degradation of CBZ *via* cytochrome P450 might produce toxic daughter compounds (Buchicchio et al. 2016), such as 10,11-epoxy-carbamazepine (epoxy-CBZ) (Miao et al. 2005; Heye et al. 2016). Epoxides are common oxidation products that can inhibit glycosidase enzymes in the carbohydrate metabolism; or covalently bind to cellular proteins and nucleic acids resulting in mutations (Kallemeijn et al. 2014; Golan-Rozen et al. 2011; Di and Kerns 2016; Cajthaml et al. 2002).

However, a study by Buchicchio et al. (2016) showed that the toxic epoxy-CBZ is not present in the water samples treated by *T. harzianum*. This suggests that a variety of other enzymes including epoxidase within the cytochrome P450 system pathway (Olicón-Hernández et al. 2017) facilitated the degradation of epoxy-CBZ. However, the toxicity and the identity of the end metabolites are not known.

3.2 White Rot Fungi

White rot fungi (WRF) are known to biodegrade various types of aromatic pollutants (Gold and Alic 1993), including CBZ (Asif et al. 2017). Three WRFs with such potentials have been identified, namely *Trametes versicolor*, *Pleurotus ostreatus*, and *Phanerocheate chrysosporium*.

3.2.1 *T. versicolor*

Various enzymes including laccase (Rodríguez-Rodríguez et al. 2010), cytochrome P450 (Mir-Tutusaus et al. 2019), lignin peroxidase (LiP), and manganese peroxidase (MnP) are expressed

Table 5 The efficiency of CBZ removal by various species of fungi

Species	Enzyme(s) Involved	Concentration of CBZ	Incubation Period (days)	Temperature (°C)	Shaking Speed (rpm)	pH	Removal Efficiency (%)	References
Trichoderma harzianum	Cytochrome P450	4000 ng/L	15	25	100	7.6	72	Buchicchio et al. 2016
Pleurotus ostreatus	Cytochrome P450	4000 ng/L	15	25	100	7.6	68	
Trametes versicolor	Laccase	0.067 mg/g	2	25	135	4.5	57	Rodríguez-Rodríguez et al. 2010
Pleurotusostreatus strain PC9	Laccase, Manganese peroxidase, Cytochrome P450	0.025 mg/g	60	28	200	-	99	Golan-Rozen et al. 2015
Phanerochaete chrysosporium strain BKM-F-1767	Lignin Peroxidase, Manganese peroxidase	2×10^7 ng/L	7	30	90	4.5	62	Li et al. 2015

Note: (-) means not stated

Table 6 CBZ degradation by enzymes secreted from various species of fungi

Species	Enzyme(s) Involved	End Metabolite	References
T. harzianum	Cytochrome P450	-	Buchicchio et al. 2016
P. ostreatus	Cytochrome P450	-	Buchicchio et al. 2016
T. versicolor	Cytochrome P450, Laccase, Lignin peroxidase, Manganese peroxidase	• 2-hydroxycarbamazepine (2-OH-CBZ), • epoxy-carbamazepine (Epoxy-CBZ) • acridone, • acridine, • dihydroxycarbamazepine (diOH-CBZ)	Rodríguez-Rodríguez et al. 2010; Mir-Tutusaus et al. 2019; Jelic et al. 2012
Pleurotusostreatus strain PC9	Laccase, Manganese peroxidase, Cytochrome P450, Epoxide hydrolase, Aldehyde oxidase	• 10-methoxycarbamazepine (10-methoxy-CBZ) • TP 251 • Acridone • TP254 • 1-(2-Benzaldehyde)-(1H,3H)-quinazoline-2,4-one (BaQD) • TP 281 • TP 297 • TP 286 • 10-hydroxycarbamazepine (10-OH-CBZ) • TP 208	Golan-Rozen et al. 2015; Golan-Rozen et al. 2011
P. chrysosporium strain BKM-F-1767	Lignin peroxidase, Manganese peroxidase, Cytochrome P450	-	Li et al. 2015

Note: (-) means not stated. TP stands for unidentified transformation product.

by *T. versicolor* (Asif et al. 2017). The predominant degradation pathway taken by *T. Versicolor* is to degrade CBZ *via* laccase. Laccase enzyme oxidizes CBZ into Epoxy-CBZ and dihydroxycarbamazepine (diOH-CBZ), both are more toxic compared to CBZ (Naghdi et al. 2018). Both lignin peroxidase (LiP) and manganese peroxidase (MnP) are also known to oxidize CBZ into epoxy-CBZ (Asif et al. 2017).

In a field study, water polluted by CBZ was treated using *T. versicolor* and analyzed using a Microtox kit to assess toxicity in the water (Johnson 2005). Results of the study revealed that the treatment by *T. versicolor* reduces water toxicity by 50%. It was postulated the cytochrome P450 further metabolized the toxic epoxy-CBZ into non-toxic acridone (Golan-Rozen et al. 2015), although the reaction seems limited. Despite the wealth of enzymes expressed by *T. versicolor*, no conclusive study was shown that the species can biodegrade CBZ into a complete non-toxic metabolite.

3.2.2 *P. ostreatus*

In addition to cytochrome P450 and laccase, *P. ostreatus* was found to express MnP, epoxide hydrolase (EH), and aldehyde oxidase (AO) (Golan-Rozen et al. 2015). The extracellular enzyme MnPoxidizes can degrade CBZ into epoxy-CBZ or other aryls derivatives (Hildén and Mäkelä 2018). The epoxy-CBZ is then metabolized into acridine by EH *via* the acridine pathway to produce acridone (Golan-Rozen et al. 2015). The conversion of toxic acridine to acridone is mediated by AO (Kosjek et al. 2009). The acridone was found to be further metabolized into unidentified non-toxic end-metabolites (Golan-Rozen et al. 2015).

3.2.3 *P. chrysosporium*

Cytochrome P450 and MnP that can degrade CBZ into epoxy-CBZ are also expressed in *P. chrysosporium* (Table 6). Lignin peroxidase (LiP) is also present in *P. chrysosporium* (Li et al., 2015), and this enzyme metabolizes CBZ by a variety of reactions including benzylic alcohol oxidations, side chain cleavages, ring-opening reactions, demethoxylations, and oxidative de-chlorinations to producearyl compounds (Gold and Alic 1993). Unfortunately, there is no further data to suggest *P. chrysosporium* can degrade the toxic aryls formed *via* LiP into non-toxic metabolites. Aryls are highly oxidative and can react with macromolecules such as nucleic acids causing DNA mutations, deactivates proteins and enzymes such as cytochrome P450 and ribonucleotide reductase in DNA synthesis (Schweigert et al. 2001; Anku et al. 2017).

Conclusion

Despite the wealth of information, various gaps exist in terms of enzymatic pathways, metabolites, and toxicity evaluation as highlighted in this mini-review. So based on the existing information and data available, among all the investigated bacterial species, *Pseudomonas* spp. found most promising and has the highest CBZ degrading capability as it can generate biodegradable naphthalene, and the downstream product catechol than can enter

carbon central pathways to generate energy for the bacterial cells. The ability to degrade the toxic metabolites epoxy-CBZ and acridine into acridone *via* the acridine pathway mediated by EH and AO suggests that *P. ostreatus* is the best within the investigated fungal species. However, considering that CBZ degradation by *P. ostreatus* resulted in unidentified end metabolites, this will need further study to confirm the identity and characterization of such metabolites. Further studies on co-contaminant like heavy metals negatively impacting the enzymatic action and the metabolites should be investigated. This brings us to conclude that *Pseudomonas* spp. is the best candidate between the two species to treat CBZ-contaminated water without producing toxic waste.

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Conflict of Interest Statement

There are no conflicts of interest.

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Potential of lignocellulolytic biocatalysts of native and proposed genetically engineered microbial cell factories on jute fiber modification and jute waste recycling: A review

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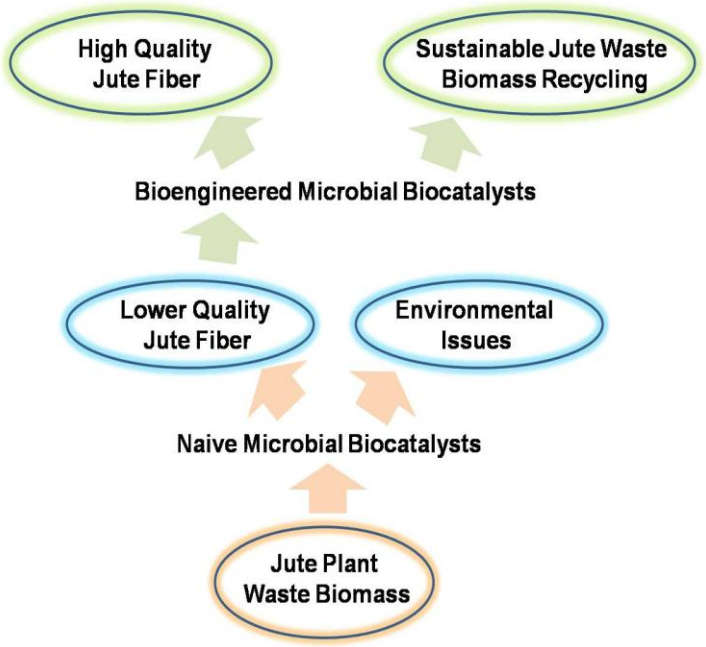
GRAPHICAL ABSTRACT



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ABSTRACT

The lignocellulolytic microbial systems from different parts of the world responsible for lignocellulosic biomass (LCB) like jute (*Corchorus* spp.) waste degradation, fiber modification, and bioenergy production are not limited to a specific prokaryotic or eukaryotic group. The industrial applications of these highly efficient bacterial, fungal and algal communities are related to the production of lignocellulolytic enzymes such as cellulase, hemicellulase, lignin-peroxidase, versatile peroxidase, laccase, thermostable oxidants, pectinase, etc. They are a blessing for the jute, dye, paper, pulp, and biofuel industries as they help to generate a sustainable ecosystem. The jute plant is lignocellulosic biomass so it can be utilized in various ways, from everyday goods to power generation. Jute industries generally use different physicochemical strategies to generate quality fiber and post-retting activities, but these approaches cannot produce desired products; hence microbial routes are best for quality fiber generation, waste remediation, and biofuel generation. To this end, this review summarizes the most important milestones of the development of the leading enzyme-producing cell factories and their engineering by genetic, metabolic, and synthetic biology approaches with the emergence of high throughput methods, such as site-directed mutagenesis and others that can analyze the relevant mutations to accelerate our understanding of lignocellulolytic enzymology.

1 Introduction

Jute resides in the genus of *Corchorus* under the family named *Tiliaceae* and is one of the most important and cheapest natural fibers as well as lignocellulosic biomass, having high sustainability and economic value (cash crop), just after cotton concerning its production and use in the South Asian countries like India, Bangladesh, etc. (Hossen et al. 2020; Jha et al. 2022). Retting defines the post-harvest operation that is the extraction of mature jute fiber from non-fibrous tissues and the woody part of the stem through various ways that helps to yield high-graded jute fiber. The Retting process can be carried out through different strategies like the conventional method of whole plant retting, chemical retting, microbial retting, mechano-microbial retting, in-situ retting with the microbial consortium, etc. (Majumdar et al. 2013). No single method can provide optimum results regarding retting time, fiber quality, cost, and environmental pollution. During microbial retting, microbial enzymes help to consume non-fibrous cementing materials like lignin, pectin, and hemicellulose (Manimekalai and Kavitha 2017). The Retting step is crucial as if improper retting takes place, it may lead to the generation of inferior quality fiber which ultimately results in a loss for the farmers. One problematic scenario observed every year in these countries, is the unavailability of a sufficient amount of free-flowing good, quality mild water during jute harvesting season for irregular climatic behavior that causes uncertainty of rainwater (Singh et al. 2019a). If water scarcity occurs, groundwater is used; as a result, the groundwater level goes down, and more water is required for soil saturation. The problem with chemical retting is that the fibers obtained after chemical treatment are rough, stiff, and coarser (Van sumere 1992). Van sumere (1992) reported that the bacterial retting process is much better than the chemical method as it provides better quality fiber and lower environmental pollution. In contrast, chemical retting is a high-energy process that generates costly waste. For that reason, different biological routes are used to get different microbial systems. It is known that the economic value of jute fiber depends on its fiber phenotypic and morphological characteristics like strength, weight, length, color, and luster of fibers (Islam et al. 2013). The jute retting period always varies with the thickness of the stem and the retting proceeds from top to bottom, but the base portion is highly complex, having to recalcitrate structure and thus very tough to ret. As a result, microorganisms attack most of the cambium portion and secondary phloem and cannot attack as well as ret the hardwood. The decomposition of the parenchymatous tissues proceeds due to microbial enzyme secretion (Haque et al. 2001). In recent years, microbial consortium approaches have been highly used to shorten the retting time and to upgrade the retting and fiber quality (Ghorai and Chakraborty 2020). Water absorption and liberation of soluble constituents like sugar, glucosides, and nitrogenous compounds from jute plants favor initial microbial growth. Further, these microbes utilize free sugars, pectin, hemicelluloses, and proteins of the plants as essential nutrients for their development, and multiplication occurs under favorable conditions (Ahmed and Akhter 2001). Microorganisms used for the single treatment or consortium approach are bacterial or fungal. Though extensive research has been done on different bacterial and fungal strains, there are still so many organisms that are untouched and can be used for jute retting enhancement as these organisms can produce the enzymes required for the breakdown of lignocellulosic biomass. Jute itself is a composition of lignocelluloses made by lignin, pectin, cellulose, and

hemicelluloses, and thus it can be retted by the ligninolytic enzyme-producing organisms (Hossain et al. 2021). Pectin is the cementing material that helps to stick the phloem fiber with the bark of the stem. It is the main target of most scientists as the breakdown of pectin without hampering the cellulosic domain (59-61%) improves the quality of the fiber, and on the other hand, hemicelluloses (21-24%) are the non-fibrous part of jute and its removal enhance the softness and the golden shiny nature of the fiber (Sfiligoj Smole et al. 2013). Hemicellulose is the structural backbone of the plant cell wall composed of a branched polymer of different sugars (i.e., hexose and pentose form). It is the second highest abundant polysaccharide in the plant cell wall. Based on the sugar residues present in the structural polymer as the backbone, hemicelluloses are classified likely into xylan, galacto-(gluco) mannans, and xyloglucans (Scheller and Ulvskov 2010). Jute fiber contains 12-14% of lignin which plays a major role in the photo-yellowing problem of fiber. This is an oxidative photochemical reaction caused by lignin photosensitization (Achwal and Sinkar 1994; Liew et al. 2017). Lignin loses its methoxyl groups due to light and degrades that form ortho-quinones which discolor the fiber (Cogulet et al. 2016). Fiber decolorization is another reason for strength loss. The removal of lignin resists discoloration of the fiber due to sunlight. Thus, pectinolytic, xylanolytic, and ligninolytic microbial agents are in much demand in the scientific community. In jute industries, many wastes are generated (Figure 1); jute root cuttings have not usually been utilized for jute product production and thus are dumped in the trash. This can lead to soil pollution. Effluents that are produced during the jute retting are used for irrigation purposes and have a very negative impact on seed germination of different types of plants and also in pisciculture (Sinha and Paul 2014). However, the effluents can be used in agricultural fields after their proper treatment by the microbial system that can break the organic loads in the wastewater effluents. Thus, the importance of microbial pathways has a tremendous impact on the jute fiber modification and treatment of the jute waste effluents, as we know that water scarcity is a grave problem in most South Asian countries. Thus, the utility of the treated effluent water is immense. In the current review article, an extensive literature survey has been done to find the bacterial and fungal systems already used in retting systems and are highly established. Another report is used to discover the microbiological systems that can break lignocellulosic biomass. One observable thing during these surveys is that most of the organisms used in the retting process are native and not genetically modified. When it comes to biofuel genesis, most organisms used for the breakdown of lignocellulosic biomass are genetically modified and metabolically engineered (Ghosh and Hallenbeck 2012; Chukwuma et al. 2021). Another thing is that genetic alteration in the genome of an organism modifies its substrate- binding affinity, which is determined by its Michaelis Menten constant, which is the Km value. In maximum cases, the Km value is reduced, which signifies the increased amount of substrate specificity of the enzymes (Ghosh and Das 2020). When the bacterial, fungal, and algal systems are modified,

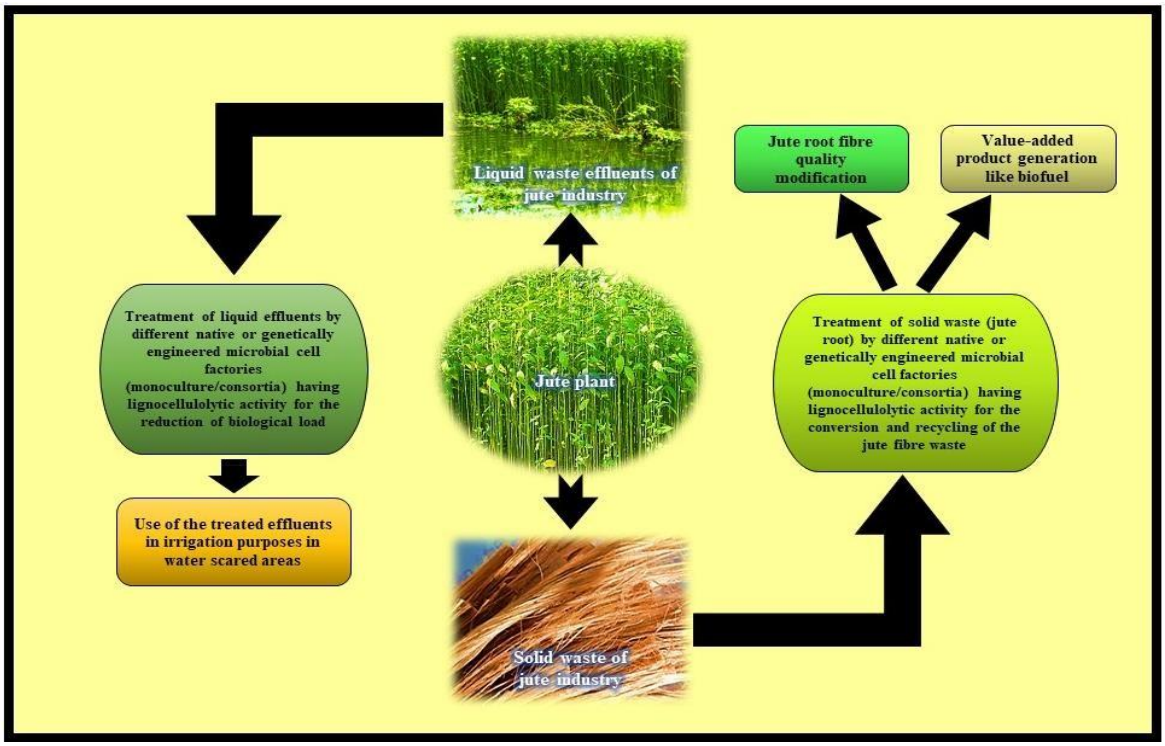

Figure1 Jute industry waste treatment, recycling and conversion

positive results have been generated. This review article emphasized the identification of native, genetically engineered, and consortia-based microbial systems that could gradually increase the product-producing rate and can enhance the jute fiber modification process, jute waste remediation, recycling, and biofuel generation.

2 Lignocellulosic biomass pretreatment process

Lignocellulose is a plant material and is highly recalcitrant. Its breakdown is very tough due to the presence of the protective layer of phenolic polymer lignin surrounding the lignocellulosic biomass. However, for any modification, conversion, or recycling, we need to break that rigid portion to get the polysaccharide part cellulose, hemicellulose, etc., that can be converted to value-added products (Islam et al. 2022a). Besides that, fiber modification also depends on the plant material's lignin content. The higher the lignin content, the lower the fiber quality, and vice-versa. Hence lignin breakdown is essential for any industry to generate economic products. Different physical, chemical, physicochemical, and biological strategies have been applied in different industries to get the treated lignocellulosic biomass that can be treated further by multiple microbial systems as well as consortium approaches to get the best value-added products starting from biofuel to decorative products (Shahinur et al. 2022; Ivanovska et al. 2022). In the acid pretreatment process (Chemical method), LCB has been treated with diluted or concentrated acids of 0.2 to 2.5 w/w% with vigorous mixing at 130°C to 210°C. This treatment has an expensive recovery process and costly equipment. Fermentation inhibitors like hydroxyl methyl furfural are produced during the process. Lignin is not removed properly (Naseeruddin et al. 2013; Harmsen et al. 2010). In the alkaline pretreatment process (chemical method), LCB has been soaked with alkaline solutions like sodium and ammonium hydroxide at an optimal temperature for a specific period. In this process, the total operational cost and the catalysts are also very expensive (Zhao et al. 2008). Organosolv is a chemical method in which LCB is treated with organic solvents like acetone, ethanol, methanol, or their mixture with water to remove lignin and hemicelluloses (Limayem and Ricke 2012). Oxidative Delignification is a chemical process that is performed by mixing LCB with ozone, oxygen, and hydrogen peroxide to convert lignin polymer into carboxylic acids (Sun and Chen 2007). Ionic Liquid treatment is a physicochemical method in which LCB is mixed with the ionic solvent at an optimal temperature between 90°C to 130°C and optimal pressure. Then the water added ends up forming precipitation of biomass and enhancing the accessibility of cellulose (Zhu et al. 2013; Cox and Ekerdt 2013). Wet oxidation is a physicochemical treatment process in which drying and milling of the LCB waste occur at 195 °C for 10 min to 20 min. After that, water is added along with Na_2CO_3. Then the mixture is aerated, which results in fractionated LCB. The sugar-yielding capacity of this process is deficient (Harmsen et al. 2010). Microwave heating with catalyst technology is a physicochemical treatment associated with heating LCB in the microwave between the temperatures of 100°C– 200°C. After heating, maleic acid is added in different concentrations, generating pentose sugar (Kim et al. 2012). The combinational culture approach is a biological pretreatment method in which combining combinational microbial inoculums has been used for the lignocellulosic biomass. However, it is a less productive pretreatment method (Katiyar et al. 2015). Physical pretreatment methods that can process LCB into more accessible materials include milling, microwave irradiation, ultrasound technology, mechanical extrusion, and pyrolysis. In the milling process, rotary cutters separate the material by varying the cutter direction on different axes, changing cutter speed and pressure. Milling processes are different, like ball milling, hammer milling, vibro-milling, colloid milling, two-roll milling, etc. Among all milling processes, ball milling can only be used for the treatment of dry as well as wet LCB wastes. However, it is incapable of removing lignin properly, as well as an energy-consuming process that makes it a less suitable physical pretreatment option. Microwave irradiation is the most common method of plant biomass pretreatment. This is an advantageous method as it is straightforward to conduct, has increased heating capacity, less processing time, fewer inhibitors, and low energy requirement. In extrusion pretreatment, LCB materials are mixed, heated, and sheared, which results in modification of the physical and chemical properties of the biomass. Low cost along with the best- controlled monitoring process makes extrusion pretreatment better than any other physical pretreatment method. Zero sugar degradation with good adaptable capacity makes it a perfect physical system more feasible for bioethanol production by pretreating lignocellulosic waste. Pyrolysis is a thermal breakdown method of lignocellulosic waste at a temperature ranging between 500 and 800°C, without any oxidizing agent generally used to produce bio-oil from LCB waste (Aftab et al. 2019). The ultrasound approach is a green technology that is novel as well as environmentally friendly. The ultrasound sonication technique reduces pretreatment time and the requirement for chemicals or enzymes. Lignocellulosic biomass fractionation takes ultrasonic waves with subsequent hydrolysis for biofuel generation (Subhedar and Gogate 2016; Patil et al. 2022).

3 Jute fiber modifications by different lignocellulolytic enzymes

Jute fiber is a lignocellulosic biomass. Hence its breakdown, conversion, and modification are possible using lignocellulolytic enzymes. The most treated enzymes for jute fiber modification are laccase, cellulase, and pectinase (Autore et al. 2009). In this review article, we will analyze the promising enzyme function and the physical and chemical modification of these enzymes (Figure 2).

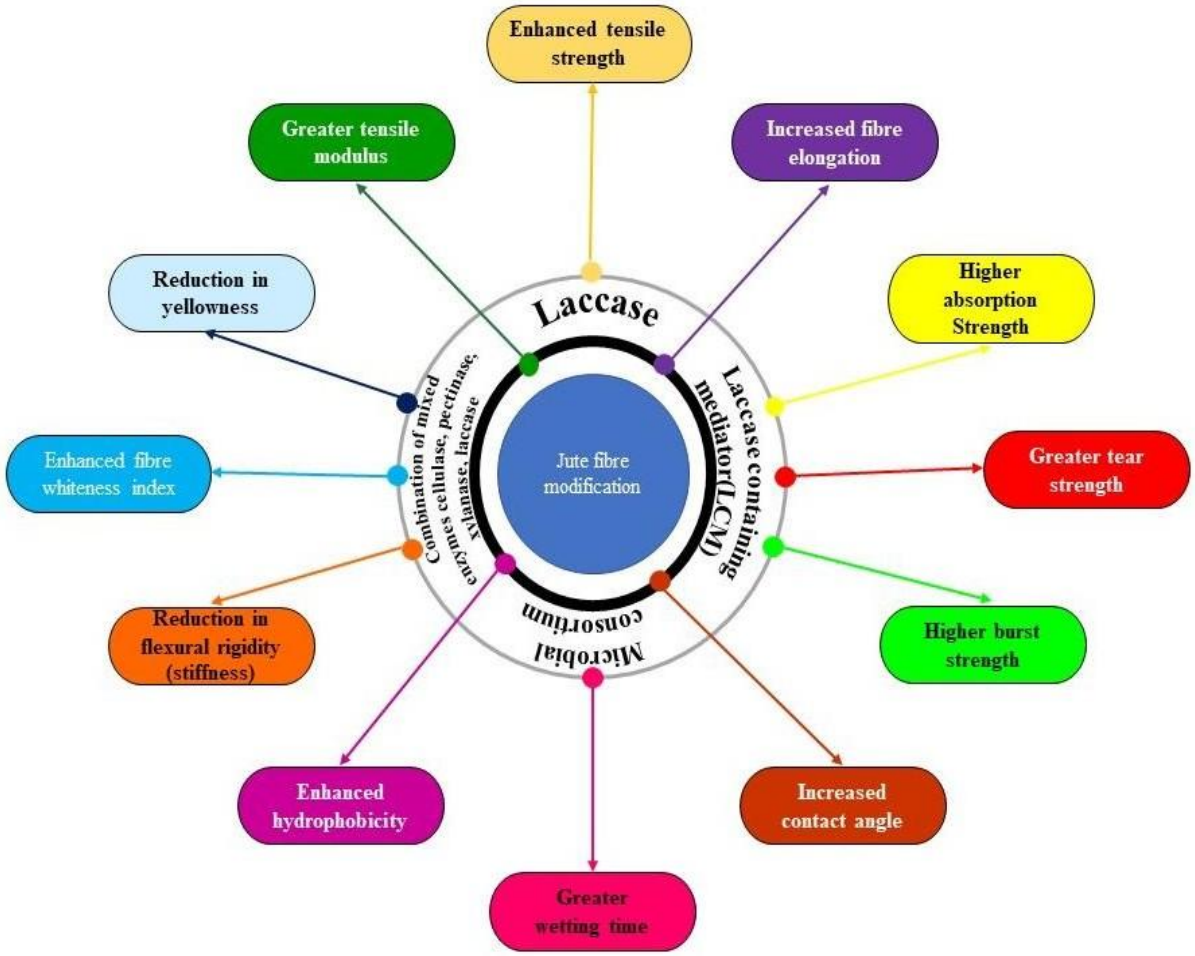

Figure 2 Jute fiber modification by laccase and combination of mixed enzymes (cellulase, pectinase, xylanase, and laccase)

3.1 Jute Fiber Modification by Laccase

Jute has gained massive attention because it is readily biodegradable and has significant mechanical properties, low cost, vast raw sources, etc. (Zhou et al. 2017; Wang et al. 2019). Due to the disadvantages of physical and chemical processing methods, using enzymes or enzyme technology to modify fiber draws the researcher's attention (Table 1). Laccase is a kind of oxidoreductase enzyme that causes the catalysis of jute lignin by oxidation and produces free radicals. It contributes to the formation of hydrophobic monomers. These glycoproteins oxidize phenols and aromatic or aliphatic amines, giving rise to reactive radicals where molecular oxygen is reduced to water in a simultaneous redox reaction (Riva 2006). The substrate containing cellulose, hemicellulose, and lignin is oxidized, which further continues for degradation and grafting (Table 3, 4). Grafting is the co-polymerization of the jute fiber with other chemicals like vinyl monomers, methacrylonitrile, and acrylonitrile that provide extra strength with enhanced thermal stability and modified surface smoothness to the jute fiber (Mondal et al. 2016). On the other hand, Jute fiber composite is an excellent alternative to synthetic fiber composite due to its easy availability, low weight and cost, non-toxicity, high flexural strength, and biodegradability (Song et al. 2021). Laccase oxidizes the phenolic hydroxyl groups in lignin, present in the jute fiber, leading toward the biogenesis of radicals of phenoxyl residues which have been coupled to end up with ether structures (Dong et al. 2018). Laccase-mediated reactions increase hydrophobicity on this fabric's surface and tensile properties (Kudanga et al. 2010; Zhou et al. 2013; Thakur et al. 2015; Nayab-Ul-Hossain et al. 2020). In the case of laccase/ mediator systems, laccase leads to the activation of synthetic mediators, including 2,2 - azino-bis- (3-ethylthiazoline-6-sulfonate) (ABTS) and 1-hydroxy benzotriazole and natural mediators like acetosyringone and syringaldehyde that result in the oxidization of the non-phenolic parts in the lignin structure (Reynaud et al. 2013; Witayakran and Ragauskas 2009). Jute fiber modification by using laccase after defibrillation is depicted in Table 2.

Table 1 Jute fiber modification by laccase and LCM (Laccase containing mediators)

Enzyme/ Enzyme plus mediator	Properties of jute fiber	Control	Modified result	Reference
Laccase	Tensile strength (N.m/g)	6.3±0.2	8.8±0.1	Dong et al. 2016
	Elongation percentage	0.40±0.04	0.60±0.04	
	Absorption Strength (mJ/g)	15.7±0.9	30.8±1.5	
	Tear strength (mN.m^2/g)	1.4±0.3	1.8±0.2	
	Burst strength (kPa.m^2/g)	1.038±0.003	1.098±0.003	
	Contact Angle	-	95.48°	
	Wetting time	-	3058s	
Laccase and ABTS	Tensile strength (N.m/g)	6.3±0.2	Decrease by 17%	
Laccase and 2,6-dimethoxyphenol (DMP)	Tensile strength (N.m/g)	6.3±0.2	Decrease by 9.1%	
Laccase and Alkali lignin (AL)	Tensile strength (N.m/g)	6.3±0.2	Increased by 13.6%	
Laccase and AL	Elongation percentage	0.40±0.04	0.60±0.04%	
	Absorption Strength (mJ/g)	15.7±0.9	35.9±2.2	
	Tensile strength (N.m/g)	1.4±0.3	2.0±0.2	
	Burst strength (kPa.m^2/g)	1.038±0.003	1.194±0.005	
	Contact Angle	-	68.69°	
	Wetting time	-	1438s	
Laccase and Guaiacol (G)	Tensile strength (N.m/g)	6.3±0.2	Increased by 14.8%	
Laccase and G	Elongation percentage	0.40±0.04	0.60±0.02	
	Absorption Strength (mJ/g)	15.7±0.9	36.6±2.3	
	Tear strength (mN.m^2/g)	1.4±0.3	2.0±0.1	
	Burst strength (kPa.m^2/g)	1.038±0.003	1.202±0.003	
	Contact Angle	-	106.26°	
	Wetting time	-	4199s	

Table 2 Jute fiber modification by using laccase after defibrillation

Laccase plus, defibrillation				
Enzyme	Properties of jute fiber	Control	Modified result	Reference
Laccase plus, defibrillation	Contact Angle	-	Increased to 95.48°	Dong et al. 2016
	Wetting time	-	3058s	
	Tensile strength (N.m/g)	-	Increased by 39.7%	
	Tear strength (mN.m^2/g)		Increased by 28.6%	
	Burst strength (kPa.m^2/g)	-	Increased by 5.8%	

Table 3 Jute fiber modification by Grafting process

Jute/polypropylene Composites with laccase	Tensile strength (MPa)	Tensile modulus (MPa)	Reference
Control jute/pp	34.58±1.74	2669.57±99.66	Ni et al. 2015
PG-grafted jute/PP	36.81±2.02	2757.71±100.69	
OG-grafted jute/PP	37.06±1.44	2925.37±105.48	
DG-grafted jute/PP	40.30±0.36	3139.43±44.45	
Retention of tensile strength and modulus of different jute fiber composites after water immersion of 216 hours			
Control jute/pp	86.58±1.56	55.75±0.63	Ni et al. 2015
PG-grafted jute/PP	89.89±1.35	56.85±1.05	
OG-grafted jute/PP	90.21±0.97	58.43±1.62	
DG-grafted jute/PP	92.93±0.74	60.23±2.15	

Table 4 Enhancement in mechanical properties of jute fibers by grafting process

Composite type	Properties of jute fiber	Modified result	Reference
Grafted Dodecyl gallate (DG) onto jute fabric by laccase for the enhancement of surface hydrophobicity of jute fiber	Water contact angle (°)	Increased to 133.01	Ni et al. 2015
	Water absorption	Increased by 22.21 %	
	Thickness swelling	Increased by 17.05%	
	Tensile strength (MPa)	Increased by 16.54%	
	Tensile modulus (MPa)	Increased by 17.60%	
	Elongation at break (%)	2.78%	
	Loss of modulus (E")	Higher than control	
Grafted Octal gallate (OG) onto jute fabric by laccase for the enhancement of surface hydrophobicity of jute fiber	Water contact angle (°)	Increased to 121.70°	
	Tensile strength (MPa)	Increased by 70.17%	
	Tensile modulus (MPa)	Increased by 9.58%	
	Elongation at break (%)	2.57%	
Grafted Propyl gallate (PG) onto jute fabric by laccase for the enhancement of surface hydrophobicity of jute fiber	Water contact angle (°)	Increased to 117.54°	
	Tensile strength (MPa)	Increased by 6.45%	
	Tenacity loss	Increased by 3.30%	
	Elongation at break (%)	2.51%	

3.2 Jute Fiber Modification by Cellulase

Jute has been considered as one of the most primeval cash crops in South Asian countries. Several disadvantages of jute fiber include harshness, stiffness, and complex structural properties because of the presence of lignin (Vigneswaran and Jayapriya 2010). Jute contains 58-63% of cellulose, 21-24% of hemicellulose, 12-14% of lignin, 0.2-0.5% of pectin, and other compounds including wax (0.4-0.8%), protein (0.8- 2.5%), mineral matter (0.6-1.2%). Nevertheless, it has been reported that cellulase has significant potential in softening the jute fiber and working with other enzymes (Table 5, 6, 7) (Samanta et al. 2005, 2006). However, pretreatment methods, i.e., mixed enzyme treatment and amino silicone treatments, have been executed by Basu et al. (2008). The enzymatic intervention has been executed involving a mixture of cellulase, pectinase, and xylanase in different ratios. Though -CHO group of oxidated cellulose or hemicellulose of jute induces a reaction with the -NH_2 group of amino silicone substance and leads to the formation of an inter-fiber bond and further durable film coverage on the surface of jute. Treatment with the mixed enzyme at 55°C for 2 hrs converts the fiber into more improved quality in fineness, brightness, and softness and lowers bundle tenacity (Chakrabarti and Sinha 2001).

Table 5 Action of cellulase in jute fiber modification

Jute fiber	Cellulase concentration	Modified result	Application	Reference
Tenacity loss	2%	9.05%	Loss of fiber strength	Vigneswaran and Jayapriya 2010
	3%	14.76%		
	4%	19.83%		
Improved fiber elongation	2%	0.96%	Improved elongation	
	3%	3.87%		
	4%	5.26%		
Reduction in flexural rigidity (stiffness)	2%	20.49%	Enhanced suppleness and softness in fibers	
	3%	25.47%		
	4%	34.67%		
Enhanced whiteness index	2%	12.35%	Improved whiteness index	
	3%	16.02%		
	4%	3.47%		
Reduction in yellowness	2%	54.90%	Enhanced brightness	
	3%	58.02%		
	4%	54.97%		

Table 6 Jute fiber modification by mixed enzymes

Mixed enzymes treatment of jute fiber				
Enzymes	Properties of jute fiber	Control	Modified result	Reference
Xylanase/Laccase	Contact Angle	0.00°	96.17±0.12°	Dong et al. 2016
	Wetting time (s)	664.0±45.5	3454.4±58.9	
	Tensile strength (N.m/g)	6.3±0.2	Increased by 10.2%	
	Tear strength ($mN.m^2/g$)	1.4±0.3	1.9±0.2	
	Burst strength ($kPa.m^2/g$)	1.038±0.003	1.106±0.005	
Cellulase/Laccase	Contact Angle	0.00°	97.53±0.29°	
	Wetting time (s)	664.0±45.5	3655.6±39.5	
	Tensile strength (N.m/g)	6.3±0.2	Increased by 26.1%	
	Tear strength ($mN.m^2/g$)	1.4±0.3	2.2±0.2	
	Burst strength ($kPa.m^2/g$)	1.038±0.003	1.182±0.003	

3.3 Jute Fiber Modification by Pectinase

Pectinases are the enzymes that catalyze the degradation of complex molecules called pectin. Depolymerization or de-esterification reactions are to be performed by this enzyme (Singh et al. 2019b). Three types of pectinases are generally found; i.e., (1) pectin methylesterases, (2) Polygalacturonases, and (3) pectin transaminases (Ramos and Malcata 2011). When commercial pectinases or xylanases are added, it shows the ability to loosen the protruding fiber bundle of jute in an enzyme treatment under 50°C (Sreenath et al. 1996). Interest in Bio degumming is growing day by day because of its low-cost, environment-friendly, and high efficiency (Banik et al. 2007; Biswas et al. 2013). Non-Cellulosic components like pectin, lignin, and hemicelluloses in jute are broken down by the activity of enzymes produced by microorganisms (Kozlowski et al. 2006). *Pectobacterium* sp. DCE-01 has the potential to secrete pectinase along with other enzymes (Duan et al. 2016). A *Micrococcus* sp. strain exhibits the ability to accomplish the jute degumming in 6 days (Haque et al. 2003). Several bacteria such as *Bacillus pumilus*, *B. subtilis*, *B. cereus*, and *B. licheniformis* are used in fiber retting, whereas *B. pumilus* has been reported to produce exo- pectinase (Tepe and Dursun

Table 7 Jute fiber modification by mixed enzymes (mixture of cellulase, xylanase, and pectinase)

Jute fiber parameter	Mixed enzyme concentration	Modified result	Application	Reference
Tenacity loss	2%	11.96%	Loss of fiber strength	Vigneswaran and Jayapriya 2010
	3%	13.47%		
	4%	23.81%		
Improved fiber Elongation	2%	3.44%	Improved elongation	
	3%	5.00%		
	4%	5.48%		
Reduction in flexural rigidity (stiffness)	2%	13.61%	Enhanced suppleness and softness in fibers	
	3%	29.87%		
	4%	36.97%		
Enhanced whiteness index	2%	14.66%	Improved whiteness index	
	3%	15.49%		
	4%	26.37%		
Reduction in yellowness	2%	52.84%	Enhanced brightness	
	3%	55.58%		
	4%	58.02%		

2014; Liang et al. 2015). Bacterial consortia consisting of *P. aeruginosa*, *Bacillus* sp, *B. subtilis*, and *Enterococcus* sp. show enzymatic potential by producing pectinase, mannase, and xylanase (Tamburini et al. 2003; Hossain et al. 2021).

4 Native bacterial communities involved in jute fiber modification

Lignocellulosic biomass like jute fiber is cemented to adjacent cells inside the stem with pectin extracted through retting, also known as the degumming process. Different pectinolytic anaerobic bacteria are responsible for water retting treatment that helps to decompose pectic substances from jute stems and produce high-quality fiber. In this process, pectin is depolymerized by a different group of pectinolytic enzymes: Polygalacturonases, pectin lyase, pectate lyase, and pectin esterase. Additionally, xylanase selectively removes the non-fibrous hemicelluloses without affecting cellulosic fiber resulting in soft fiber generation. Hence Pectinolytic microorganisms having xylanase activity and negative cellulase activity is of great importance. Another substance called lignin is a phenolic polymer that protects the outermost layer of jute and is one of the most critical restriction generators for improving fiber quality. Without breaking the lignin polymer, we cannot get the economic jute fiber (Barai et al. 2020). Thus pectinase, xylanase, and ligninase are the three most critical enzymatic factors contributing to the science behind jute fiber improvement. Water-based microbiological retting is the most economical and promising avenue that mainly involves bacterial communities along with different types of fungal, protozoan, algal, and diatom-based biological systems. Bacterial regimes can be classified as aerobic and anaerobic. The most crucial aerobic retting bacteria belonging to the genus *Bacillus* are *B. subtilis, B. polymyxa, B. mesenteric, B. pumilus, B. cereus, B. megaterium, and B. macerans*. Some other gram-negative genera including *Erwinia* and *Pseudomonas* are involved in microbial retting. During the post-retting period, some anaerobic bacteria like *Clostridium acetobutylicum, C. stercorarium*, and *C. tertium* are playing a vital role in the retting process. The best bacterial strains for degumming comprise *Bacillus cereus, B. megaterium, B. subtilis, B. koreensis, B. xiamenensis, Proteus mirabilis, Enterobacter tabaci, Kosakonia oryzae, Serratia nematodiphila* and *Aeromonas jandaei*. Further, *B. megaterium* has the highest pectinolytic and xylanolytic activity (Hasan et al. 2020). *Micrococcus* spp. has been identified as an accelerator of jute retting. Referring to the recent advancements made in isolating completed/partial genes controlling desirable traits, it is suggested to use modern molecular techniques to improve not only the quality of jute fibers but also to bioengineer microbial flora to further reduce the retting time without sacrificing fiber qualities (Haque et al. 2003). On the other hand, several aerobic bacteria like *Bacillus subtilis, B. polymyxa, B. mesenteric, B. maserans, C. tertium, C. aurantibutyricum, C. felsinium*, etc. have been isolated from the retting water. These aerobic microbial systems grow first, utilize dissolved oxygen, and promote the growth of aerobes. It has been stated that a more significant part of decomposition is carried out by aerobic species (Hasan et al. 2020). In the last decade, genetic engineering approaches have been used extensively to enhance the productivity of desired enzymes. Some of the modifications have been depicted in Table 8.

Table 8 Most promising genetically engineered bacterial systems that can be used in jute fiber modification and jute waste recycling

Bacterial Strain	Mutated Strain	Mutation Strategy	Engineered Enzyme	Substrate	Reduction % of Km Or fold of enzyme activity	Application	Reference
Rhodococcus jostii	*R. jostii* N246A	Oligonucleotide directed mutagenesis	versatile peroxidase	H_2O_2	44.44	Fiber modification, Dye decolorization, Biofuel Production	Roberts et al. 2011
Paenibac illus polymyxa Z6	*P. polymyxa* Z6 H218D	Site-directed mutagenesis	Pectinase	Linear 1,5-alpha-L-arabinan	--		Wang et al. 2014
Bacillus pumilus	*B. pumilis* L386Q/G4 17I	Site- directed mutagenesis	Laccase	guaiacol	77.14		Ihssen et al. 2017
Bacillus subtilis	*B. subtilis* M502F	Site-directed mutagenesis	Laccase	ABTS	90.80		Durão et al. 2006
Pyrococcus horikoshii	*P. horikoshii* C106A/C159A	Site-directed mutagenesis	Hyperthermophilic beta-1, 4 endoglucanase (EGPh)	Carboxymethyl cellulose	1.7 fold higher		Kang et al. 2007
	P. horikoshii C106A/C1 59A/C372A/C4 12A	Site-directed mutagenesis	Hyperthermophilic beta-1, 4 endoglucanase (EGPh)	p- nitrophenyl cellobiose	2.1 fold higher		
	P. horikoshii C372/AC4 12A	Site-directed mutagenesis	Hyper thermophilic beta-1, 4 endoglucanase (EGPh)	p- nitrophenyl cellobiose	1.6 fold higher		
	P. horikoshii E201, H297, H299 and E342	Site-directed mutagenesis (alanine scanning method)	Endogluc anase	p- nitrophen yl cellobiose	Enhanced enzyme activity		Kim et al. 2007
Clostridi um cellsulov orans	*C. cellsulovo rans* K94R, S365P, K9 4R-S365P	Site-directed mutagenesis	Mesophilic endogluca nase (EngZ)	carboxym ethyl cellulose	Enhanced enzyme activity		Kim et al. 2009
Bacillus subtilis JA18	*B. subtilis* JA18 Egl330	Truncation of the cellulose binding domain (CBD)	Endo- beta-1, 4- glucanase	CMC	78% higher catalytic efficiency		Wang et al. 2009

5 Native fungal communities involved in jute fiber modification

Wide ranges of fungi from different origins are capable of retting different wet and dry ribbons of jute under controlled laboratory conditions. Post-retting treatments of jute fiber using fungal cultures can be used to minimize the negative effects of jute root by removing the hard bottom part. Many fungal species are beneficial in improving the fiber quality of jute. During and after retting, the microbial load per ml of retting water increment occurs. Different methods like ribbon, dry and chemical retting can be an alternative system to conventional retting that can overcome the scarcity of retting water. Multiple attempts have already been made to isolate and screen different fungal systems like *Aspergillus niger*, *Macrophomina faciolina*, *Mucor*, *Chaetomium* sp., *Phoma* sp., *and Penicillium* sp., etc. All these systems have been good retting agents. The microbial population varies from place to place in the jute-growing areas in Asian countries. Diverse research has found that post-retting water contains a higher fungal load. In the in-vitro test, the addition of post-retting fungal strains increases the amount of retting in a significant way and also reduces the time required for the retting technique. The retting period becomes almost half after using post-retting water. Isolated fungi of *Aspergillus clavatus*, *Rhizopus* sp., *Zygorinchous* sp., *Sporotrichum* sp., *Trichoderma* sp., *Penicillium* sp., *Curvularia* sp. has examined for the retting efficacy of green jute ribbons. In laboratory and field conditions, *Sporotrichum* sp. retted the jute material in 7 days, whereas *Trichoderma* sp. and *Curvularia* sp. retted the green ribbon in 11 days. In the case of retting by *Sporotrichum* sp., no adverse effect on the fiber bundle strength and fiber yield has been observed, and according to the Pressley index, fiber strength is found to be 10.82 lbs/mg, and fiber yield is about 2.8kg out of 40kg green ribbons (Haque et al. 2003). Other multi-diverse fungal communities can produce multiple enzymes like ligninase, laccase, cellulase, and hemicellulase that can convert lignocellulosic biomass like jute fiber into value-added products like bioethanol, etc. That means these ligninolytic microbial systems can be able to ret jute as well. Some of these fungal communities with their pivotal impacts have been discussed here. *Trichoderma reesei* is a mesophilic filamentous fungus capable of secreting many cellulolytic enzymes like endoglucanase, exoglucanase, and β-glucosidase that have huge industrial applications in substrate conversion, that is, cellulose which is a major ingredient of jute plant biomass (Bayram Akcapinar et al. 2015). In contrast, strain *T. harzianum* contains only endoglucanase, exoglucanase and lacks β-glucosidase for generation of six carbon sugar. *Pestalotiopsis* is an ascomycete fungus with pathogenic activity against plants and can grow in aerobic and anaerobic situations on polyurethane synthetic polymer as the sole carbon source, hence helps in bioremediation (Sánchez 2009; Elgamsy et al. 2022). Enzymes produced by this microbial system are endoglucanase and exoglucanase to generate value-added products from cellulose-containing jute plant waste biomass (Islam et al. 2022b). Anaerobic fungus like *Neocallimastix frontalis*, which lives in the rumen, have been captive to producing endoglucanase and exoglucanase and has a high demand in the cellulose conversion industry. *Rhizopus oryzae* is a well-known micro-fungus having heterothallic filamentous construction that is found as a saprotrophic in soil and rotting vegetation and has an efficiency of cellulose breakdown by producing industrially important 1,3-β-D-Glucosidase. *Fomitopsis palustris* is a polypore fungus that causes brown rot disease by the enzymatic breakdown of the woody part cellulose. Further, *F. palustris* possess three different cellulase enzymes i.e. EG-II, exoglucanase, and β-glucosidase, which assist in loosening cell-wall structural polysaccharide network by disassembling hemicellulose portion joined with cellulose. Similarly, *Phanerochaete chrysosporium* is the most studied white rot fungus that possesses a Secretome and secretes an array of peroxidases, oxidases, and cellulases. Endoglucanase, exoglucanase, and β-glucosidase are the enzymes of this fungus that can help to convert cellulose into glucose to produce energy-producing components (Sánchez 2009). *Penicillium brefeldianum* can produce 1,6-β-D-Glucosidase, whereas *Myceliophthora* sp., *Humicola* sp., *Fusarium oxysporum*, and *Eichhornia crassipes* produce different types of cellulases (Sánchez 2009; Dashtban et al. 2009). *Aspergillus niger* is a fungus that causes "black mold" disease on certain fruits and vegetables and is responsible for the production of multiple enzymes like α-L Arabino-furanosidase, a feruloyl esterase, Exo-β-1,4-mannosidase, endogalactanase, endo- α-1,5-arabinanase for detachment and breakdown of hemicellulose. Another species that can break hemicellulose is *A. nidulans* which is a potentially resourceful organism having the capability of the production of naïve and different heterologous enzymes for industrial applications like Exo-1,4-β- Xylosidase, endogalactanase for the breakdown of hemicellulose. *Neocallimastix* sp. is a type of fibrolytic fungi that causes colonization and breakdown of the structural polysaccharides of plants by producing the enzyme p-Coumaroyl Esterase. *T. longibrachiatum* is responsible for large-scale commercial production of hemicellulases like Endo- 1,4-β-xylanase, xylan α-1,2-glucuronosidaseetc (Sánchez 2009). *Sclerotium rolfsii* is a necrotrophic fungal plant pathogenic community that acquires hemicellulolytic enzymes like Endo-β-1,4- mannanase. Other fungal origins responsible for hemicellulose detaching enzymes are *A. fumigatus* (Xylan α-1,2-glucuronosidase), *Humicola insolvens* (β-Glucosidase), *T. reesei* (Acetyl esterase, acetyl xylan esterase, glucuronyl methyl esterase), *Phanerochaete chrysosporium* (Glucuronyl methyl Esterase), *Acremonium alcalophilum* (Glucuronyl methyl Esterase) (Sánchez 2009). Lignin, the phenolic polymer in lignocellulosic biomass, is the toughest part of the plant's structural organization. Hence different chemical, physical and physicochemical processes have been done to separate lignin from lignobiomass to produce six

Table 9 Most promising genetically engineered fungal systems that can be used in jute fiber modification and jute waste recycling

Fungal Strain	Mutated Strain	Mutation Strategy	Mutated Enzyme	Substrate	Reduction % of Km Or enzyme activity	Application	Reference
Trichoderma reesei	*T. ressei* Q126F, K272F, Q274V	Site directed mutagenesis	Endoglucanase I	cellulosic material	Enhanced enzyme activity	Fiber modification, Dye Decolourisation, Biofuel Product ion	Roberts et al. 2011
Thermotoga maritime	*T. maritime mutant*	Inverse polymerase chain reaction (IPCR)	Endoglucanase Cell 12B	Carboxy methyl cellulose	Enhanced enzyme activity		Zhang et al. 2015
Aspergillus awamori	*A. awamori* D71I, D77N, and D77I	Site-directed mutagenesis	Feruloyl esterase- A	alpha- naphthyl butyrate	12.12/ 45.45/54.54		Koseki et al. 2005
Aspergillus niger CIB 423.1	*A. niger* CIB 423.1 D93G	Site-directed mutagenesis	Feruloyl esterase	furalate	10.625		Zhang and Wu 2011
Phanerete chrysosporium	*P. chrysosporium* P106R/Q10H/L211V/ A243R/F255L	Alteration of amino acid sequences	Lignin peroxidase	H_2O_2	Enhanced enzyme activity		Ryu et al. 2008a, b.
	P. chrysosporium A140G/S190P/P193A /S196F/E20Q	Alteration of amino acid sequences	Lignin peroxidase	2,4-dichlorophenol	Enhanced enzyme activity		
Pleurotus eryngii	*P. eryngii* A260F and R257A	Site-directed mutagenesis	versatile peroxidase	lignin	20-to-50-fold higher enzyme activity		Ruiz-Duenas et al. 2008
Pleurotus ostreatus	*P. ostreatus* W170A, R263N, Q266F, and V166/168L	homologous gene expression system	Laccase	H_2O_2	Enhanced enzyme activity		Tsukihara et al. 2008
	P. ostreatus Mutant POXA1bDEL TA16/P OXA1bDELT A4	Site-directed mutagenesis	Laccase	Syringalda zine	Enhanced enzyme activity		Autore et al. 2009
Melanocarpus albomyces	*M. albomyces Mutant L559A*	Site-directed mutagenesis	Laccase	Syringalda zine	Enhanced enzyme activity		

carbon sugars that can be used for different value-added products generation. In jute industries, the multi-faced physicochemical system has been used to generate quality fiber, but the problem is that not a single system can generate grade one fiber. Hence microbial communities like fungal systems became very popular for their large-scale enzyme production like lignin peroxidase and laccases. Fungal toxicity is a barrier in this system. However, *Phanerochaete chrysosporium* is a saprophytic fungus that breaks the woody part of dead plants by using the enzymes lignin peroxidase, glyoxylate oxidase, manganese peroxidase, horseradish peroxidase, cellobiose dehydrogenase (Fujian et al. 2001; Sánchez 2009). On the other hand, *Neurospora crassa* can produce a special type of lignin-breaking enzyme, laccase, a powerful enzymatic system among all other ligninolytic enzymes. *Aspergillus sclerotiorum* is a genetically identified fungal system indexed in the Environmental Relative Moldiness Index (ERMI) that releases lignin peroxidase and manganese peroxidase for the breakdown of lignin in woody plants. *Cladosporium* species are ubiquitous and represent isolated airborne fungi that can release lignin peroxidase and manganese peroxidase for uncovering the lignocellulosic biomass. *Mucor racemosus* is a fast-growing mold that has a global distribution and capable of producing enzymes like lignin peroxidase and manganese peroxidase, responsible for the generation of sugary materials by breaking down the lignin segment from lignocellulosic biomass (Tsukihara et al. 2006; Sánchez 2009; Bonugli-Santoset et al. 2010; Rathner et al. 2017; Frommhagen et al. 2017). No single fungal community can produce all lignocellulolytic enzymes needed to break down waste lignocellulosic biomass. This is because the structural integrity of the biomass is very complex and has very high physicochemical

constraints. To meet the industrial need, various efforts have been carried out to develop effective genetic engineering approaches that can increase biomass accessibility (Table 9).

6 Native and genetically engineered promising algal systems that can be used for jute fiber modification

The jute plant is a complex mixture of cellulose, hemicellulose, pectin, and lignin, and a network of carbon sugar monomers links these components. The most critical factors to be considered in managing jute wastes (solid and liquid) are the reduction of organic loads present in jute industry effluents used in agricultural fields near jute industries and water scars areas (Das and Ghosh 2021). Using solid jute waste as cheaper feedstocks could help to produce biofuels (Ochoa- Chacón et al. 2022). Two types of biomasses are present in nature: traditional and modern. The former refers to the plant residue utilized for heating and cooking. In contrast, the latter refers to the waste biomass used for transportation fuels and electricity generation. Waste biomass refers to the lower-value by-product of various industrial sectors such as agriculture, forestry, etc. Energy crops can be used as raw materials for second-generation biofuel production. Jute, textile, paper, and pulp industrial waste conversion to value-added products is carried out through three sequential steps (i) lignocellulosic waste pretreatment, (ii) Physico-chemical as well as biological lysis of polymeric sugar, (iii) separation and purification (Ghosh and Talukdar 2020; Ghosh and Das 2021; Chares Subash and Muthiah 2021). The increasing popularity of alternative fuel sources and lignocellulosic waste conversion has prompted scientists to explore the potential of bioconversion of lignocellulose. The main challenge is the delignification of lignocellulose for its recalcitrance composition and toxicity. Multiple biological regimes have disintegrated lignocellulosic biomass like bacteria, fungi, etc. However, analyzing algal systems for producing ligninolytic enzymes is a newly emerging field of research for biofuel and high-quality jute fiber generation. To this end, the different algal system has been introduced that can produce required biocatalysts for the breakdown of lignocellulosic biomass like lignin peroxidase, laccase, thermostable oxidants, etc. Laccases are multi-copper oxidases commonly found in fungi, bacteria, and higher plants. They also show their presence in the algal system of *Tetracystis aeria*, which was first observed by Otto et al. (2010). This organism can produce the extracellular laccase-like enzyme. The strain has been characterized by its ability to biodegrade various xenobiotics, such as phenanthrene and azo dyes. Its ability to effectively convert lignocellulosic solid waste into lignin has been identified. It has been assumed that algae can convert phenolic compounds into complex polymers by a ring cleavage mechanism and these can be oxidized indirectly through redox mediators. Lignin biosynthesis and degradation is a natural function of laccase in bioremediation. Genus *Tetracystis* is a green alga that inhabits the soil and can be studied for its laccase-like enzymes. This model could support the study of different phenolic compounds released from lignocellulosic waste. Purification of *T. aeria* laccase has been done by Otto and Schlosser (2014), which contains two polypeptides of molecular weight of 110kDa and 71 kDa. High substrate specificity has been observed in purified laccase. Chlorophyceae algae like *T. aeria* can show ABTS oxidizing properties. *Scenedesmus* clade can oxidize phenolic constituents by its thermostable low-molecular-weight enzyme. True laccase has been secreted by *Chlamydomonas moewusii* that optimally act at neutral to alkaline pH. Oxidation of 17α-ethinylestradiol, bisphenol A, nonylphenol, and triclosan are carried out by laccase of *Tetracystis aeria* in the presence of ABTS, which is a redox mediator. Green algal regimes can help in the bioremediation of the ecosystem by breaking down the phenolic pollutants found in contaminated industrial surface waters (Otto et al. 2010; Otto and Schlosser 2014; Otto et al. 2015). *Spirulina platensis* CFTRI, a cyanobacterial strain, can produce an extracellular thermostable laccase of 66 kDa that efficiently shows ligninolytic action in the presence of ABTS at alkaline pH. Enhanced laccase activity has been observed in the presence of Cu^{+2}, Zn^{+2}, and Mn^{+2}. It shows dye decolorization activity that can help bioremediation (Afreen et al. 2017). Seven microalgal species that have been isolated and screened by Abd Ellatif et al. (2021) can show dye decolorization of orange G, crystal violet, malachite green, brazilwood, Naphthol Green B dyes, etc. These are *Nostoc humifusum*, *N. muscorum*, *Oscillatoria* sp., *A. oryzae*, *S. platensis*, *Chlorella vulgaris* and *W. saccata*. Ligninolytic activity has been determined in all strains by ligninase assay. *C. vulgaris* shows maximum lignin peroxidase activity, whereas *A. oryzae* and *W. saccata* show maximum laccase activity. Optimum decolorization by *C. vulgaris*, *A. oryzae*, and *W. saccata* indicates their potentiality for breaking down lignocellulosic waste like jute root waste biomass and its conversion efficacy into value-added products (Abd Ellatif et al. 2021). Jute waste degradation and biofuel generation not only depend on ligninolytic enzymes but also on cellulase and hemicellulase enzymes that are being produced by genetically modified marine algal systems like *Chlamydomonas* sp. and *Dunaliella* sp. that help in algal biofuel genesis and can be used as waste converting tools into value-added products (Subhadra 2010; Georgianna and Mayfield 2012; Arora et al. 2019). Algal Xylanase is capable of breaking the hemicellulosic part of lignocellulosic biomass. Photosynthesis is the most promising property of marine algae and hence can fix carbon dioxide in the presence of solar energy. Metabolic engineering and synthetic biology strategies help us to convert chloroplasts in *C. reinhardtii* and *Dunaliella tertiolecta* by forming recombinant proteins inside the algal system. Growth in long pH ranges and different brine densities make these systems a true candidate for biofuel production and waste remediation. Chloroplast transformation of *D. tertiolecta* has been done by homologous

recombination. Erythromycin has been used in the antibiotic resistance selection process. Five recombinant enzymes, i.e., phosphate- hydrolase, α-galactosidase, β-mannanase, xylanase, and phytase, have been produced in the plastids of *D. tertiolecta* and *C. reinhardtii*. The particle bombardment method has been used to transform the plastid using 5′UTR, psbD promoter, psbA terminator, and 3′-UTR in the algal genetic systems (Georgianna et al. 2013). In recent years, genetic and metabolic alteration of the algal system opened the opportunity for generating recombinant proteins like xylanase, which is being used for the depolymerization of hemicelluloses, cellulase for the disintegration of plant structural polysaccharides, etc. (Subhadra and Grinson-George 2011). Hence, the enzymes required for the fragmentation of lignocellulosic biomass are now available from both naive and genetically and metabolically engineered algal strains. So, a mono algal culture or a hypothetical algal consortium with all the required enzymes for bioremediation of a lignocellulosic waste, jute retting, or bioethanol production would be the environmentally sustainable key player that will provide growth in both the jute and fuel industry from the economic aspect. These activities are interrelated with lignocellulosic biomass breakdown as well as fiber modification. Besides the native algal system, some genetically engineered marine algae can produce different cellulolytic enzymes like hemicellulase, cellulase, mannase, xylanase, glucosidase, etc. A hypothetical concept depicts that an algal consortium may act better than individual treatment for good-quality fiber and biofuel genesis. The consortium approach is a mixed naïve and genetically altered algal system capable of faster retting and stable process mechanism instead of monoculture.

7 Proposed microbial Consortium that can be used in jute fiber modification

Jute fiber modification can be enriched by the hub of multiple lignocellulolytic symbiotic microbial strains due to the presence of lignocellulose degrading genes in the consortia. The enrichment culture technique can help to make the consortia with specific and mixed enzymatic properties by maintaining functional diversity, distribution, environmental relationships, and abundance of the participating co-members. The fascinating microbial consortia responsible for the breakdown of lignocellulose is XDC-2 composed by Guo et al. (2010) from the compost, which was studied further by Hui et al. (2013). It has been reported that the consortium cultures can efficiently degrade the lignocellulose rapidly and 17.6% of unpretreated corn stalks can be degraded by this consortium, along with cellulose, hemicellulose, and lignin degradation in 10.4%, 16.5%, and 9.6% ratios, respectively (Hui et al. 2013). Zhang et al. (2015) produced a microbial consortium TMC7, which can degrade 79.7% of rice straw at 65˚C within 15 days. Similarly, Lu et al. (2019) produced a thermophilic consortium TC-Y, which can degrade 49.5% of corn stalks in just 20 days. A synthetic fungal-bacterial mixed consortium has been designed by Hu et al. (2017) that can efficiently improve the activity of the lignocellulolytic enzyme. Their consortium analysis has made it clear that the bacterial members are more critical than the fungi for lignocellulolytic enzyme activity (Hu et al. 2017). Liang et al. (2018) also produced a consortium OEM2, which can degrade 41.5% rice straw in 9 days and 85% hemicelluloses in 12 days (Liang et al. 2018). Cortes-Tolalpa et al. (2018) have constructed a salt-tolerant lignocellulosic-biomass breaking microbial consortium system by enriching a halo-marsh soil microbiome with carbon and energy source, i.e., wheat straw and the consortia results in higher lignin as well as cellulose breakdown. Results indicate that compared to fungi, the bacterial system shows the primary role in the degradation of the recalcitrant substrate under salt conditions (Cortes-Tolalpa et al. 2018). It has been observed that pretreated substrates can be easily hydrolyzed, which is not cost-friendly as the pretreatment process is responsible for additional expense and pollution. Hence, the industries highly need a consortium with the capability of breaking down the unpretreated jute fiber.

8 Conclusion and Future Outlook

Jute fiber and jute waste (lignocellulosic waste biomass) are cheaper substrates and can be used to produce different types of value-added products and biofuel. Hence, historical evidence of jute-based research outcomes by the industry and by different academic research organizations is glorious and triumphant. Along with the positive side, jute fiber has some lacuna like lower flax than other natural fibers, sensitivity to water absorption, poor bonding capacity with different matrices, non-uniform fiber, limited fiber volume, etc. The various steps of the lignocellulosic jute waste treatment or jute fiber modification process are economically unfeasible and adversely affect the environment. In addition, the use of industrial waste effluent harms human health and the environment. All these gaps can be filled by the proper biological treatment methods for generating various kinds of necessary products for humankind. The monoculture retting method is now old. The microbial consortia-based retting process is in high demand as it can provide the best economic product. Nevertheless, shortly, mixed microbial consortia and genetically modified microbial consortia will be the workhorse for generating valuable lignocellulolytic enzymes required for jute retting. The rapid emergence and improvement of lignocellulolytic enzymes through protein engineering have ameliorated the laccase, lignin-peroxidase, pectinase, and cellulase/hemicellulase production rates. This process is regulated through various factors such as substrate-binding affinities, transcriptional regulatory factors, and enzyme-specific activities. A paradigm shift has been observed during genetically modified microbes are developed and used in the consortia-based approach. However, this paradigm shift requires

compromises in terms of time consumption. Metabolic engineering is a suitable key for generating cell factories but identifying the ideal targeted gene cluster and alteration in metabolic pathways is challenging; hence, sophisticated genetic tool-box and target-oriented methodologies are needed to improve the efficacy of lignocellulosic waste recycling and jute fiber modification. A rational design of a synthetic metabolic pathway could be utilized to improve the lignocellulosic waste biomass for bioenergy and high-grade fiber generation. An accelerated enzymatic degradation approach can be used to improve the utilization of lignocellulosic waste. Systems biology, metabolic engineering, and synthetic biology could play critical roles in mass LCB degradation and minimize the toxicity of lignocellulosic waste. On the other hand, the genetic consortium can be the key player in producing all the required lignocellulolytic enzymes in a single microbial host that can change the line of limitation.

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Author's contribution

All the authors contributed equally.

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Host-delivered-RNAi-mediated resistance in bananas against biotic stresses

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ABSTRACT

Both the biotic and abiotic stressors restrict the yield potential of many crops, including bananas. Bananas belong to the genus *Musa* and are the world's most popular and widely produced fruit for their nutritional and industrial importance. The demand for bananas is growing each day worldwide. However, different pest infestations are hampering the production of bananas, making it a matter of concern for global food security. Several biotechnological tools and applications including RNA interference (RNAi) have been employed to enhance the biotic stress resistance in plants. The capacity to silence targeted genes at transcriptional and post-transcriptional levels makes the RNAi technique a popular choice for gene knock-down and functional genomics studies in crops. Silencing of different suppressor molecule coding genes through RNAi helps crops to combat the detrimental effects of plant pathogens. The host-induced gene silencing (HIGS) technology, also known as the host-delivered RNAi (HD-RNAi), is nowadays gaining popularity due to its ability to target an array of pathogens, comprising bacteria, nematodes, fungi, viruses, and insects. This methodology is employed to manage disease pest outbreaks in a diverse range of crop species, including bananas. Besides HIGS, virus-induced and spray-induced gene silencing (VIGS and SIGS, respectively) are the potential approaches where RNAi technology is exploited to control plant-pathogenic diseases. The current review emphasizes the different kinds of diseases of bananas and the potential of HD-RNAi, a new-age and promising technology to build a barrier against significant crop and economic loss.

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1 Introduction

Biotic and abiotic stresses are the major threats to global food security due to their potential to cause severe yield loss in crops. Banana is a major fruit crop worldwide, covering over 136 subtropical and tropical countries, and it provides food security to over 500 million people (Pillay et al. 2012; FAOSTAT 2018; Jekayinoluwa et al. 2020a). India secures 1st position worldwide in terms of area and production of bananas (FAOSTAT 2018). Bananas and plantains are popular crops due to their year-round availability, high nutritious value, and economic importance. The yield of banana and plantains are drastically affected by numerous biotic and abiotic stressors. Biotic stressors like bacteria, nematodes, fungi, viruses, and insects can cause complete yield loss in banana cultivation. Most of the popular banana cultivars are propagated through vegetative propagation, restricting the chances of generation of genetic variation in the banana gene pool (Ghag and Ganapathi 2019). The most devastating diseases of bananas are fungal diseases like Sigatoka disease and *Fusarium* wilt, viral diseases like Banana Bunchy Top Disease (BBTD), and weevil infestation (Figure 1). The breeding approaches to develop disease resistance in bananas are very difficult due to the triploid genetics of bananas. Extensive use of inorganic pesticides, herbicides, and insecticides is impacting detrimental effects on the environment as well as living organisms.

Biotechnological approaches are the alternative to imposing pathogen resistance in bananas in a sustainable way. One such approach is the utilization of RNA interference to combat different biotic stresses. RNAi is not only used as a popular reverse genetic tool but also explored to confer resistance against disease pest infestation in crops due to its capability to alter gene expression for desired traits (Younis et al. 2014). The biological mechanism, RNAi employs dsRNA molecules to inhibit protein synthesis by sequence-specific targeting of complementary mRNA (Taning et al. 2021). The RNAi mechanism can be activated through the endogenous production of micro-RNA (mi-RNA) or the exogenous introduction of single-interfering RNA (siRNA). Dicer is an enzyme posing RNase-III-like activity, which cleaves the dsRNA for the formation of single-interfering RNA (siRNA) duplexes (19–24 bp). This siRNA duplex uses a single strand incorporated into the argonaute (AGO) protein complex, creating a RISC (RNA-induced silencing complex). The target mRNA is then degraded through cleavage or the translational suppression is seen as a result of the RISC binding to the target mRNA in a sequence-specific way (Figure 2).

HD-RNAi and HIGS exploit the RNAi mechanism for the production of dsRNA that targets pathogenesis-related genes. At the time of a pathogen attack, the dsRNA moves inside the pathogen cell and is converted into siRNAs (Bharat et al. 2021). The siRNAs are taken up by pathogens during infection, and they activate the RNAi machinery and suppress the target gene transcription in pathogens (Bharat et al. 2021). The pathogenesis-related genes and suppressor molecules are taken as the target genes for designing HD-RNAi constructs (Artificial Pre-miRNA, hairpin RNA, sense or antisense RNA) that are introduced into the host plant. At the time of pathogen infection, the HD-RNAi

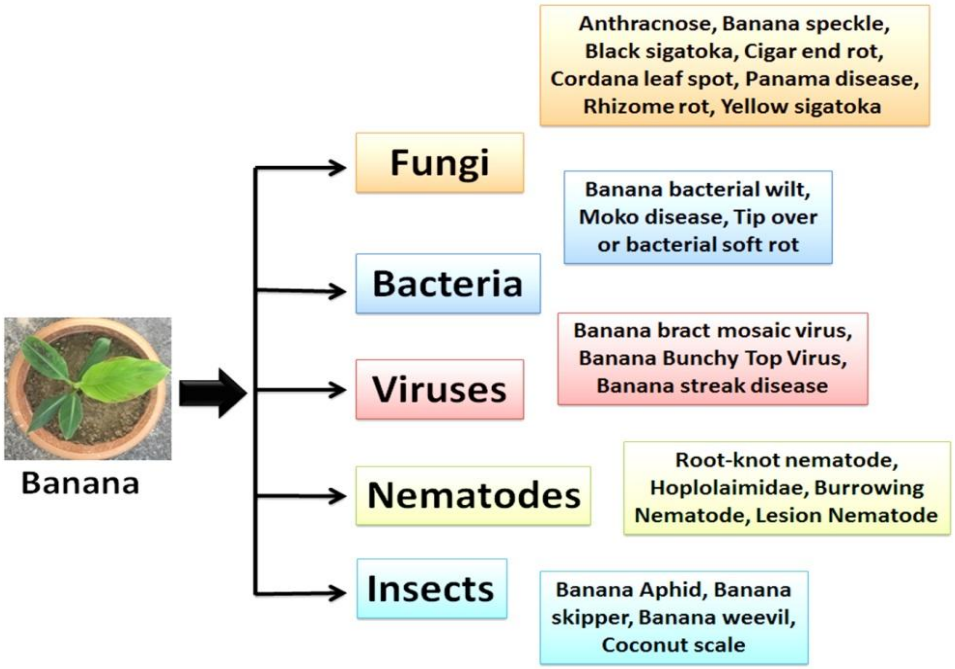

Figure 1 The most devastating biotic stressors of banana crops

constructs are converted to small RNAs by Dicer-like nucleases (DCLs) in the host cell, and one strand of the small RNA is incorporated with AGO to form the RISC complex. In the next step, the mRNA of the target gene interacts with the RISC complex by complementary binding and gets disintegrated, resulting in pathogenesis inhibition (Figure 3). The HD-RNAi approach has been employed against numerous pathogens, including fungi, viruses, bacteria, nematodes, and herbivorous insects to protect crops from yield loss. HD-RNAi technology is gaining popularity day by day for providing disease pest resistance to the banana.

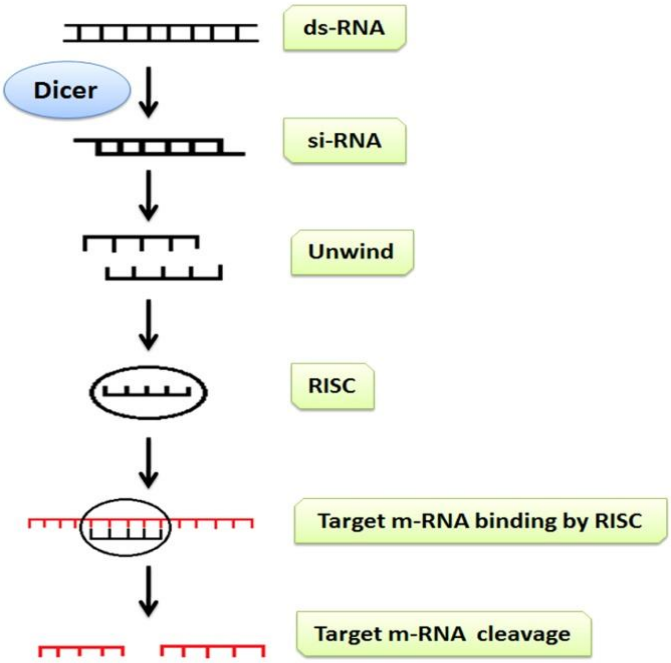

Figure 2 A simple conceptual representation of RNAi mechanism in plants

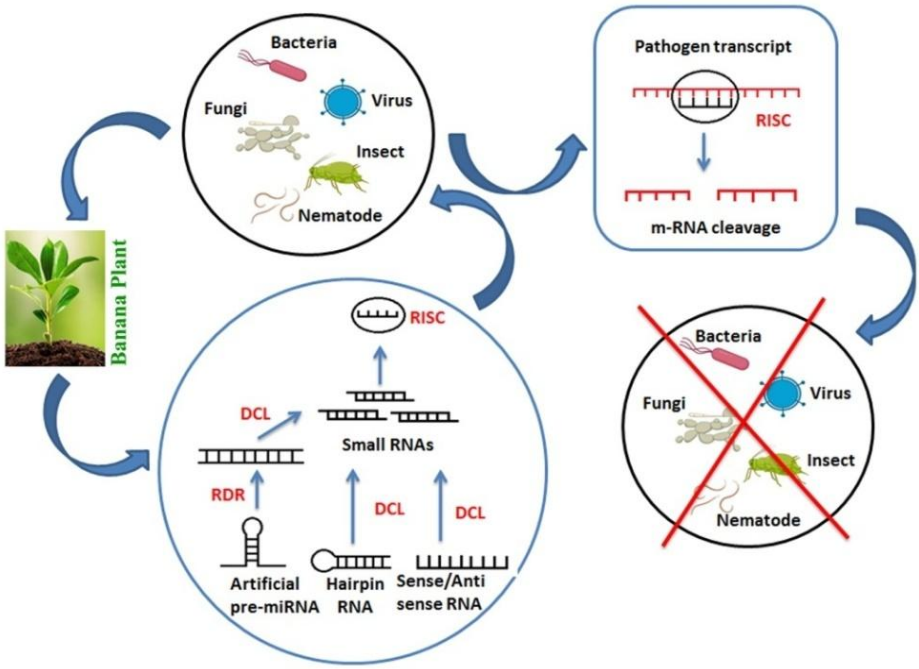

Figure 3 A pictorial representation of the HD-RNAi-mediated defense in banana plants against biotic stressors.

Table 1 List of major pathogens in banana crop and the method of RNAi implemented for their control

Pathogens	Resistance strategies employed	Reference
Mycosphaerella fijiensis	ds-RNA	Mumbanza et al. (2013)
Pseudocercospora fijiensis	RNAi	Thomas et al. (2021)
	ds-RNA	Mumbanza et al. (2013)
	ds-RNA	Fei et al. (2016)
Fusarium oxysporum	HIGS	Duo et al. (2020)
	HIGS	Ghag et al. (2015)
	HIGS	Ghag et al. (2014)
	RNAi	Elayabalan et al. (2013)
Banana Bunchy Top Virus	RNAi	Krishna et al. (2011)
	RNAi	Shekhawat et al. (2012)
Pentalonia nigronervosa	RNAi	Jekayinoluwa et al. (2020a)
Cosmopolites sordidus	ds-RNA	Mwaka et al. (2021)
Meloidogyne incognita	hd-RNAi	Kakrana et al. (2017)

2 HD-RNAi mediated resistance in banana

Banana is highly susceptible to disease-pest infestation (Table 1). Biotitic stressors like fungi, bacteria, viruses, insects, and nematodes cause huge yield loss in banana cultivation. HD-RNAi has the potential to combat biotic stressors.

2.1 Resistance to fungi

Resistance against fungal pathogens can be achieved via RNAi technology. Among the diseases caused by fungi, Sigatoka disease, Panama wilt, and anthracnose are the main threat to banana cultivation. The production quality and quantity of bananas are severely affected due to fungal infection. Rasthali a banana cultivar susceptible to *Fusarium* wilt, showed resistance against the *Foc* Race 1 infection when *Fusarium transcription factor* 1 (*ftf1)* of the fungal race was targeted through HIGS (Ghag et al. 2014). By targeting the *ERG6* (*C*-24 sterol methyltransferase), and *ERG11* "(cytochrome P450 lanosterol *C*-14α-demethylase) genes of *Foc* TR4 through HD-RNAi approach, resistance against the fungal strain has been developed (Duo et al. 2020). RNAi-mediated silencing of polyketide synthase genes like *PKS8-2* and *PKS10-2* conserved genes of *Pseudocercospora fijiensis* showed a reduction of pathogenicity in bananas (Thomas et al. 2021). Targeting four genes, such as, *Gene A*, *Gene B*, *Gene C*, and *Gene D* of *Fusarium oxysporum* f. sp. cubense (FOC) through the HD-RNAi approach, developed resistance against the soil-borne fungal infection in bananas (Fei et al. 2016).

2.2 Resistance to bacteria

The harmful interactions between pathogenic bacteria and plants lead to disease development by using virulence factors and effector molecules (Ronald and Joe 2018). Bacterial diseases like Banana *Xanthomonas* wilt (causative organism: *X. campestris* pv. *musacearum*, moko and bugtok (causative organism: *Ralstonia solanacearum*), and bacterial head rot (causative organism: *Erwinia spp.*) are the most devastating diseases resulting in huge yield loss in banana cultivation (Blomme et al. 2017). Genetic engineering and genome editing are the most important tools of modern biotechnology to impose resistance against bacterial diseases in numerous crop species, including bananas. Targeting the pathogenesis-related genes or expressing the *R* (Resistance) genes, antimicrobial genes, and defense-related genes in the host plant lead to combating pathogenesis (Mohandas and Ravishankar 2016). Transgenic plants bearing the *plant ferredoxin-like protein* (*Pflp*) and *hypersensitive response assisting protein* genes (*Hrap*) of chili showed resistance against numerous plant-pathogenic bacteria, such as *Xanthomonas spp*, *Pseudomonas*, *Erwinia*, and *Ralstonia* (Tripathi et al. 2017). Transgenic bananas of the cultivar 'Sukali Ndiizi' (AAB) and 'Nakinyika' (AAA) having *Hrap* or *Pflp* gene insertion, showed resistance to *Xanthomonas* wilt disease without compromising the architecture or yield of banana plants (Tripathi et al. 2010; Namukwaya et al. 2012; Tripathi et al. 2014a). Transgenic banana-bearing pattern recognition receptor (PRR) XA21 from rice exhibited improved response to *Xanthomona campestris pv. Musacearum* in greenhouse conditions (Tripathi et al. 2014b). In rice, the knockdown of *OsSSI2* through RNAi enhanced the resistance against *X. oryzae* attacks (Jiang et al. 2009). The utilization of siRNA to silence oncogenesin *Arabidopsis*, *Nicotiana*, and *Lycopersicum* conferred resistance against *Agrobacterium tumefaciens* (Escobar et al, 2001). RNAi mechanism can be used to target bacterial pathogens by designing dsRNA to suppress the virulence factors and the effector molecules of bacteria. HIGS can

be employed to achieve bacterial disease resistance in bananas through genetic engineering.

2.3 Resistance to viruses

In HIGS, host plants are engineered to incorporate an inverted repeat sequence of a gene of interest inside the plant genome. At the time of pathogen attacks, the inverted repeat sequence will be converted to ds-RNA first and subsequently to s-RNA, leading towards the activation of RNAi machinery. The success story of applying RNAi mechanism to provide plant viral disease resistance has been enlisted in numerous cases of papaya ring spot virus, mung bean yellow mosaic virus, cucumber mosaic virus, cassava mosaic virus, and soybean mosaic virus (Jekayinoluwa et al. 2020b). Among the plant pathogenic viruses of bananas, the Banana Bunchy Top Virus (BBTV), Banana Streak Virus (BSV), and Banana Bract Mosaic Virus (BBrMV) are the main constrain for the cultivation and production of bananas. BBTV is a multipartite virus with six genomic components. By designing RNAi construct against the BBTV DNA-*R* gene, BBTD resistance has been developed in transgenic Grand Nain banana (Elayabalan et al. 2013; Elayabalan et al. 2017). Genetically engineered bananas with ihp-RNA construct targeting DNA-R (replication initiation gene) component showed improved resistance against BBTV (Shekhawat et al. 2012). The utilization HD-RNAi, targeting the *replicase* gene of BBrMV, protected from Banana Bract Mosaic Virus (Rs et al. 2021).

2.4 Resistance to insects and nematodes

Numerous major diseases of bananas are transmitted through insect vectors. The most destructive insects that cause severe yield loss to bananas are banana aphids, banana skippers, banana weevils, and coconut scales. Thus, the transmission of disease can be manipulated by either interfering with the aphid-pathogen interaction or controlling the aphid population. RNAi-mediated targeting of *RR-1* genes in *Pentalonia nigronervosa* and DNA-N of BBTV can open up a potential path to control the spreading of BBTV through banana aphids. RNAi-mediated silencing of *snf7*, *rps13*, and *mad1* genes of banana weevil (*Cosmopolites sordidus*) were successful in controlling the insect population (Mwaka et al. 2021). Application of RNAi technology by designing dsRNA against the acetylcholinesterase (AChE) gene of banana aphids, showed a significant reduction in the aphid population (Jekayinoluwa et al. 2021). RNAi-mediated suppression of the *ubiquitin E2* gene caused 100% mortality in banana weevils (Ocimati et al. 2014).

The most devastating plant-pathogenic nematodes of bananas are *Pratylenchus goodeyi*, *Radopholus similis*, *Helicotylenchus multicinctus*, *Meloidogyne incognita, and Pratylenchus coffeae*. The expressions of plant cystatins and peptides are seen to develop resistance against *Radopholus similis* and *Helicotylenchus multicinctus* (Atkinson et al. 2004; Tripathi et al. 2015). Bt endotoxin genes and lectins have the potential for suppressing *Meloidogyne* species (Yu et al. 2015). RNA interference-based defenses are being developed against *Meloidogyne* species (Papolu et al. 2013), *Pratylenchus* species (Tan et al. 2013), and *R. similis* (Li et al. 2015). Further, siRNA or hairpin RNA construct containing genetically engineered banana plants can be utilized as a potential weapon against banana insects and nematode infestations.

3 Conclusion and Future prospects

RNAi has the potential to control disease pest infestation sustainably. HIGS or HD-RNAi approach has revolutionized the way of developing pathogen resistance. Identification of target gene sequence through genomics or transcriptomics analysis is a prerequisite for designing of HD-RNAi construct. To avoid ectopic expression, tissue-specific promoters can be added while designing the construct. Targeting the intronic regions that are poorly conserved across species for HIGS, can minimize the off-target effect. The advances in the field of biological science have made it possible to utilize the HD-RNAi approach to pave the path toward precision agriculture. The production quality and quantity of bananas can be maintained properly by exploiting the HD-RNAi approach.

Production problems, particularly disease-pest infestation, represent a threat to global food security. The uncontrollable and extensive use of synthetic pesticides is the most preferred method for protecting crops from pathogens. Some of these substances have been in use for about a decade and impose hazardous effects on the environment as well as living beings. Consequently, it is necessary to develop advanced technologies that are more eco-friendly and sustainable. A unique and powerful strategy called RNA interference (RNAi) is gaining popularity nowadays as a way to combat disease-pest infestations in many commercially significant crops. Natural RNAi pathways have an impact on antiviral defense, cross-kingdom communication between species, and gene regulation in plants. Meanwhile, numerous labs have been successful in taking advantage of the RNAi mechanism to generate significant changes for promoting plant disease resistance by manipulating the components involved in RNAi-mediated gene silencing. Against various disease-pest infestation, transgenic and non-transgenic plant-based RNAi methods have shown promising results, and significant opportunities to pave the pathway toward new horizons. Despite a few drawbacks, numerous studies have demonstrated that the use of RNAi to enhance disease resistance in plants, is anticipated to be the most effective and significant strategy moving forward. The designing and generation of exogenous dsRNA targeting the pathogenesis-related gene is an essential tool for employing RNAi strategy against pathogens. The

review concentrated on focusing on the utilization of RNAi technology for combating the most destructive pathogens of bananas to secure the yield potential. By targeting the intronic regions and using tissue-specific promoters, the off-target effect and ectopic expression due to the RNAi strategy can be overcome. The root-specific promoter can be used while designing the HD-RNAi construct targeting root pathogens, *Fusarium* in bananas to avoid the expression of that construct in other plant parts. When it comes to controlling pests and pathogens, RNAi technology will be incredibly effective for not only bananas but also all types of crops.

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Angiotensin Converting Enzyme 2 (ACE2) - A macromolecule and its impact on human reproduction during COVID-19 pandemic

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ABSTRACT

Coronavirus disease 2019 (COVID 19) is caused by severe acute respiratory syndrome novel coronavirus 2 (SARS-nCoV-2). It has been declared a pandemic by the World Health Organization (WHO) on March 11, 2020. Since then, several researchers have worked/ are working on this virus by a multifactorial approach to finding out the mechanism of entry, transmission route, post-infection replication process, survival, and post-recovery utilities. As we know, SARS, MERS, and Zika viruses have affected human reproductive potentials, consequently, COVID 19 also can affect both men's and women's reproductive potential through ACE2 macromolecule. This study aimed to summarize the role of ACE2- macromolecule in COVID 19 entry and further processes in the reproductive path of both men and women. Research articles were searched in NCBI-NLM, Google Scholar, and Scopus databases. We searched based on the phrase “COVID 19”, “ACE2”, “ACE2 in testes”, “ACE2 in the female reproductive tract”, “ACE2 during pregnancy”, “ACE2 during early embryo”, “COVID 19 and impact in human reproduction” and selected the articles for summarizing this article. Most recent articles and the mechanism of COVID 19 were selected for our understanding. The results of the study revealed that COVID 19 impacts the reproductive potential of both men and women. Testes are the most vulnerable organ prone to infection in men, and vaginal fluid and the uterus could be the choice of infection in the female. Till now, COVID 19 has not been directly detected in semen samples and vaginal fluid. Results of the study can be concluded that ACE2 plays a major role in COVID 19 infection, ACE2 expression could

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be more in the testes, ovary, uterus, and vagina. COVID 19 could impact more on human reproduction and lead to a loss of fertility status for a while. All antiviral treatments could pose a negative impact on human reproduction. Further research should be carried out on the already existing theoretical hypothesis of SARS-Co-V-2 on human reproduction.

1 Introduction

Severe acute respiratory syndrome (SARS) coronavirus 2 (SARS-Co V-2) was first identified in 2019, December in Wuhan, China (Huntley et al. 2020). Since then, the viral infection has spread across the entire globe victimizing people from more than 150 countries (Ali and Alharbi 2020). This novel coronavirus causes acute respiratory syndrome in patients. World health organization (WHO) declared the infection a pandemic in March 2020 (Jebril 2020) and named this disease COVID 19 (Coronavirus disease 2019). As per the world data, the virus has infected 25.3 million people worldwide and has been the cause of 848 thousand deaths, as of August 2020 end. SARS-CoV and SARS-CoV-2 share 76% sequence similarity and SARS-CoV-2 utilizes almost the same mechanism as SARS before infecting the host (Rabaan et al. 2020). This infection shows very mild symptoms such as cold, cough, and fever in nearly 80% of the infected population (Mohapatra et al. 2020). While people with good immune systems remained asymptomatic on contracting the virus, 20 % of the infected population showed severe symptoms leading to hospitalization (Long et al. 2020). Of the symptomatic 20% cases, only 4% developed acute respiratory distress syndrome (ARDS) and need ventilator support for artificial breathing during treatment (Ma et al. 2020; Granados-Bolivar et al. 2022; Tran et al. 2022; Matin et al. 2022; Mirsaliyev et al. 2022).

Also, COVID 19 shows gender differences and it affects men more widely than women, the phenomenon of occurrence of which is still unknown (Blaskó et al. 2020; Islam et al. 2022; Rabiul Islam et al. 2022). This gender disparity is extended to the fatality rate with higher fatalities in men compared to women (Blaskó et al. 2020). ACE 2 (angiotensin converting enzyme 2) plays a major role in SARS-CoV-2 pathogenesis, it aids in direct host cell damage (Yan et al.2020). Until today, it remains very unclear how COVID 19 disrupts the innate immune system of the hosts. Immune deregulation termed as “cytokine storm” is highly associated with acute respiratory problems (Rabaan et al. 2021; Chau et al. 2021). Many researchers have confirmed the presence of proinflammatory cytokines in the COVID 19 patients. Various research has also confirmed the innate immune response against COVID 19 from the presence of inflammatory chemockines in the bronchoalveolar fluid of COVID patients (Merad and Martin 2020; Feys et al. 2022; Margiana et al. 2022). Other research suggests a significant upregulation of genes of interferon, those directly involved in the antiviral activity (Holzinger et al. 2007). These could be due to the innate immune response against COVID 19 in mildly symptomatic patients. COVID 19 infection results in the upregulation of various chemokines associated with an increase in several inflammatory cytokines like IL-6 (Interleukin-6), leading to the damage of tissue (Azkur et al. 2020; Karimabad et al. 2022). Another mechanism COVID 19 triggered various IFN (interferons) responses, which leads to the upregulation of proinflammatory genes in the lungs (Zhou et al. 2020; Chakraborty et al. 2022).

The binding of COVID 19 to the ACE2 receptor mediates the viral entry and replication process. Tissues with higher ACE 2 expression levels serve as the potential target for SARS-CoV-2 infection (Zhang et al. 2020a). When compared to other body tissues, testes are found to have more expression of ACE2 that might be prone to infection. The expression of ACE2 could be found more in the testes when compared with the lungs as well (Hikmet et al. 2020). Spermatogonia, seminiferous duct, Sertoli cells, and Leydig cells are the major cell types that have ACE2 expression. Moreover, the expression of ACE2 could be very high when compared to ovarian cells (Malki 2022). The involvement of ACE2 in SARS-CoV2 transmission coupled with data on higher expression of ACE2 in male testicular tissues lead to speculation on the potential SARS-CoV-2 impact on male gonadal functions. Until today, there is no clear mechanism of how ACE2-mediated SARS-CoV-2 infection affects the reproductive potential of both men and women (Verma et al. 2020; Sadeghi et al. 2022). During the epidemic of SARS, in 2002, Orchitis studied the infected patients and found that SARS-CoV-2 could affect spermatogenesis, and germ cell damage and further lead to poor semen quality (Payne et al. 2020). Also, previous studies revealed the presence of inflammatory infiltrates in the seminiferous tubules (Li et al. 2020b). SARS-CoV was found in the semen of infected patients; however, there are no reports of traces of COVID 19 in semen samples of infected patients to date (Guo et al. 2020; Donders et al. 2022). The inflammatory responses and immune responses take a major role in COVID-19-mediated testicular damage. ACE2 expression level could be based on the age group. Hence, only young male patients could be the target for COVID 19 in testicular damage (Jin et al. 2020; Pallotti et al. 2022).

TMPRSS2 cleaves the ACE2 receptor and this mechanism helps the entry of the virus into the host cell. Like other infections, COVID 19 also utilizes the host machinery for its growth, reproduction, and survival during infection (Perico et al. 2020;

Sultan et al. 2022). Always the viral host and protein-protein interactions can be utilized effectively for evaluating the mode of transmission, mechanism of infection, the route follows up, and, a probable drug against infection (Ji et al. 2020). The infectivity rate can be higher in case a combinatorial mutation occurs at ACE2 and S-priming residues. Previously antiviral targets were the treatment methods for viral infections like SARS, MERS, Zika, and Ebola virus during their epidemics in various countries (Tse et al. 2020).

All the antiviral drugs potentially target the reproductive functions in both men and women. SARS-CoV-2 infection mediated by ACE2 receptors causes direct testicular damage, in some cases affecting only the inflammatory and immunological responses (Bourgonje et al. 2020; Delli Muti et al. 2022). Since male and female fertility is already in decline worldwide, further frequent viral epidemics and pandemics will pose a major threat to humankind in the case of fertility and reproductive functions. In this review, we analyzed exhaustively, how ACE2 receptors help the foreign body COVID 19 with its entry, replication, and further processes at reproductive junctions (Rogers et al.2020).

We analyzed and summarized the role of ACE2 and other genes, and macromolecules involved in the COVID infection on the reproductive path, we elucidated the role of ACE2 in both men's and women's reproductive paths. We included the ACE2 role and its expression in testes cells especially Leydig and Sertoli cells, and the role of ACE2 in the prostate, epididymis, seminal vesicles, and male reproductive tract. This study also summarized the ACE2-mediated infection in the ovary, uterus, and vagina for analyzing the female reproductive potential. We also summarized the role of ACE2 during pregnancy and the developing embryo. We also summarized the impact of COVID 19 on both male and female infertility during the global pandemic.

2 Presence of ACE2 in Testes- Role in Leydig and Sertoli Cells

Many recent studies revealed the importance and potential routes of SARS-CoV2 entry and infection in the cardiovascular, digestive, respiratory, urinary, and reproductive systems (Zhang et al. 2020b; Shen et al. 2022). Reports on SARS-infected patients presented thickened membranes in the testes, destroyed germ cells, and, absence of active sperm cells in the seminiferous tubules (Vishvkarma and Rajender 2020). However, the available data on SARS-CoV-2 infection has failed to give clues on the effect of COVID 19 on the reproductive system. The single-cell resolution method is the choice for estimating the RNA expression level of ACE 2 in adult testes. This could enhance further ideas about ACE2 and its impact on the testes as well as human reproduction (Calicchio et al. 2014; Nayar et al. 2022). Researchers also revealed that the presence and expression of ACE2 are predominant in Leydig and Sertoli cells (Salonia et al. 2021). Positive ACE2 cells showed more transcripts associated with transmission and viral reproduction (Douglas et al. 2004; Temena and Acar 2022). Genes responsible for viral entry transmission and reproduction were abundantly found in ACE2-positive spermatogonia when compared to negative ones. COVID 19 also shares the same receptor as SARS disease; collectively, these observations lead to speculation on the possibility of COVID 19 to have testes as the route of infection (Leal et al. 2009). Differentiation of spermatogonial stem cells is controlled and monitored by testicle seminiferous tubules. Leydig cells help in producing testosterone that supports spermatogenic sperm cell differentiation. Any problem that arises with male germ cells or somatic cells may cause male infertility and or male reproductive system failure (Corona et al. 2020). Positive Sertoli and Leydig cells at many times express higher genes involved in cell-to-cell junction and immunity. All such findings reveal the risk of testes vulnerability through SARS-CoV2 infection that might lead to failure of spermatogenesis (Corona et al. 2020).

A complete analysis of ACE2 is mandatory for exploring the route of infection and transmission. Nonetheless, the testes are the key organ for the male reproductive system, and expression of SARS CoV2 in the testes has been revealed by many researchers. However, the detection of SARS CoV2 antibodies in testicular tissue is tough and non-specific (Parra-Medina et al. 2021; Masterson et al. 2022). In recent times, single-cell RNA sequencing can help us to effectively outline the cell types. This will enable the specificity of ACE2 expression levels. The analysis of ACE2 expression was compared between ACE2 in healthy and also in infertile men for concluding remarks and a similar tendency was noticed in both groups (McClelland et al. 2015). The expression of ACE2 can be possibly correlated with reproductive disorders. Testicle tissue damage is reported in infected patients. Further, IgG was highly expressed in Sertoli and Leydig cells (McClelland et al. 2015). The overall mechanism and theme of the article are explained in figure 1.

The biological process of ACE2 includes but is not limited to angiotensin maturation, regulatory functions in amino acid transport, positive regulation in cardiac muscle contraction, activating the receptor-mediated virion attachment to the host cell, blood vessel diameter regulation, regulation of germ cell proliferation, regulation of spermatogonia proliferation, and differentiation, regulation of cytokine production, inflammatory response regulation, transport of tryptophan and viral entry into the host cell (Sorour et al. 2020; Thakur et al. 2022). The major molecular functions of ACE2 are carboxylation, endopeptidase activity, protein binding, Zn binding, and virus receptor activity (McClelland et al. 2015).

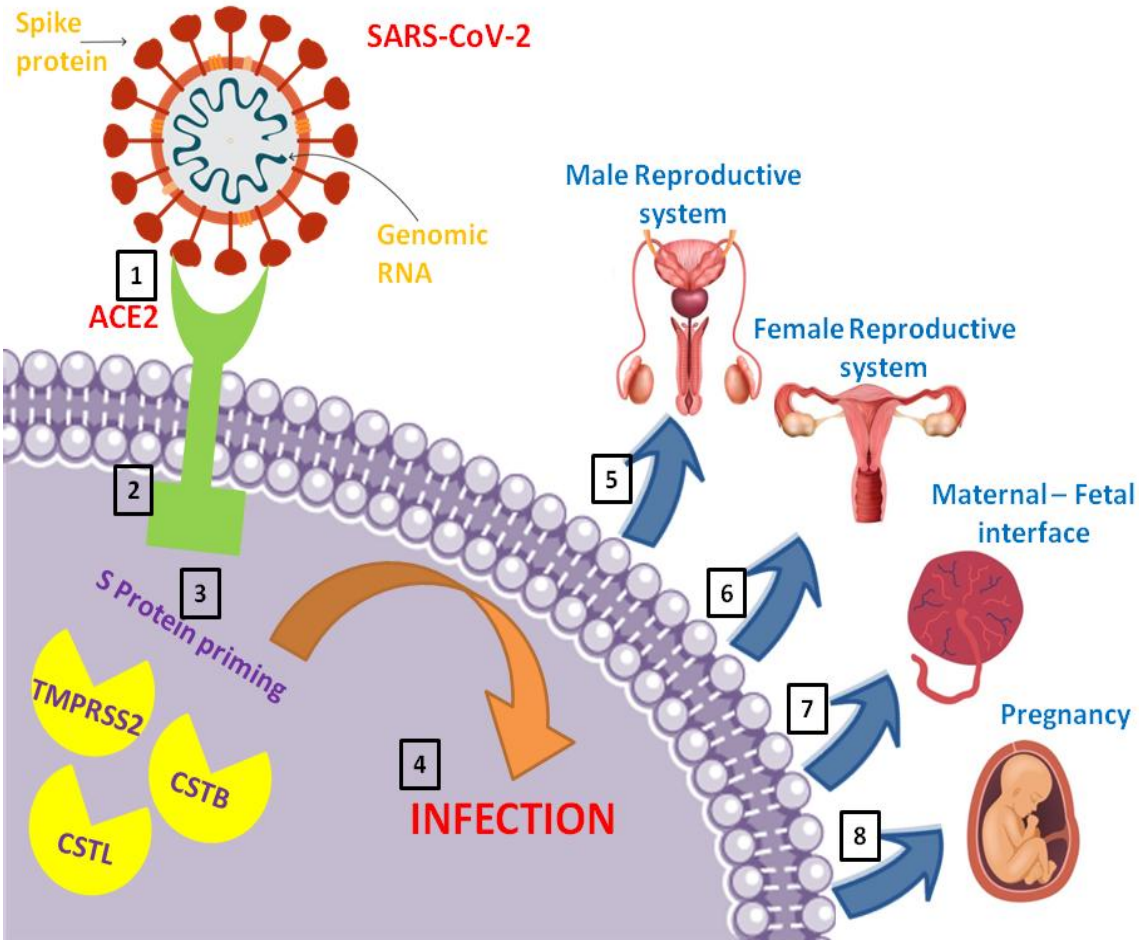


Figure 1 Illustration of the mechanism of SARS-CoV-2 infection and its effect on human reproduction; 1. Binding of S protein (Spike protein) of SARS-CoV-2 and ACE receptor; 2 Entry of the virus; 3 S priming by TMPRSS2, CSTB, and CSTL; 4 Release of genomic RNA causing COVID 19 infection; This mechanism may affect; 5 Testes and prostate of the male reproductive system; 6 Uterus, vagina and ovary of the female reproductive system; 7 Placenta and umbilical cord in the maternal-fetal interface, and 8 the developing embryo and breastfeeding during pregnancy

3 Expression of mRNA of ACE2 in testes

SARS-CoV2 showed variation in the case of infection percentage, way of abnormality, and death rates between age groups (Shen et al. 2020; Barletta 2022). In the case of healthy men in the reproductive age group, ACE2 expression showed the highest percentage with 2.8%, the percentage was 1.39 in the case of youngsters the age of 20 or less, and around 0.88 % in the case of 60 year age group (Harmer et al. 2002).

4 Expression of ACE2 in the prostate

Many on-going and completed research works on ACE2 and its expression studies on the prostate shows that the prostate is most susceptible to COVID 19 infection (Song et al. 2020; Abdolmaleki et al. 2022). The double-positive cells with both ACE2 and TMPRSS2 expression can act as a medium or reservoir of COVID 19 infection (Singh et al. 2020; Tanaka et al. 2022). This, in turn, damages the prostate gland to some extent. As we know, the prostate gland secrets Zn abundantly into the seminal plasma for sperm protection. In this case, damage in the prostate gland due to COVID 19 infection can stop the essential need or energy supply to sperm cells (Mollica et al. 2020).

This hypothesis further supports the correlation between COVID infection and human male infertility. Further, the common genetic signature shared between prostate club cell and lung club cell strengthen the hypothesis (Bhowmick et al. 2020). The similar club cells in the lung and prostate for ACE2 and TMPRSS2 expression were compared and found that 0.62% of prostate cells exhibit the co-expression. This implies that the COVID 19 infiltration in the prostate gland is through club cells (Zhang et al. 2020c). The mechanism behind this study is unclear and further clinical studies need to be conducted to warrant the hypothesis (Bhowmick et al. 2020).

5 ACE2 expression in Non-obstructive azoospermia

Many authors are investigating ACE2 and its impact on COVID 19 transmission, particularly on SARS-CoV-2 process-related genes. This will provide enormous ideas about virus transmission and reproduction (Liu et al. 2020a; Ezechukwu et al. 2022). The single-cell RNA sequencing method helps the researchers to tabulate all the data required. The sequence of Sertoli cells of non-obstructive azoospermia patients can help us in understanding the current state of the disease. To analyze the effect of COVID 19 on testes, the expression pattern of ACE2 and functions of ACE-positive cells between healthy men and non-obstructive azoospermia will give a clue for COVID 19 infection and its reproductive potential (Reis et al. 2010). TMPRSS2, CTSL, CTSB, and BSG are possible COVID-19 transmission-related genes that are expressed in testes. TMPRSS2 plays a major role apart from ACE2 in infecting the host through transmission (Liu et al. 2020b).

Both ACE2 and TMPRSS2 were compared and unfortunately, the pattern of expression was different. ACE2 expression is more in Sertoli cells and less in spermatogenic stem cells and vice versa for TMPRSS2 (Matsuyama et al. 2020; Harb et al. 2022). Hence, for the invasion of COVID 19, the combined action of ACE2 and TMPRSS2 is necessary. The interaction and expression studies of CTSL (Darbani 2020) and CTSB (Brann et al. 2020) show that both are needed for the COVID 19 infection transmission via S protein priming (McKee et al. 2020), and this phenomenon exists at all four stages of spermatid. The spermatogenic cells of patients with Non-obstructive azoospermia could be the potential target identification since mature sperms are absent. Researchers compared the ACE expression pattern between healthy men and NOA donors for the identification of diseased conditions (Khawar et al. 2019; Wu et al. 2022). Both ACE-positive cells and ACE expression levels in both these groups were compared. The ACE2 expression level was significantly reduced in the case of NOA donors when compared to healthy men. Rennin angiotensin system (RAS) also correlates with human fertility through various sperm functions (Bernie et al. 2015). ACE2 expression levels showed a significant difference in the dataset of NOA patients when compared with Sertoli cells. The effect of ACE2 on human male infertility and sperm functions can be elucidated with more functional clinical studies (Riordan 2003).

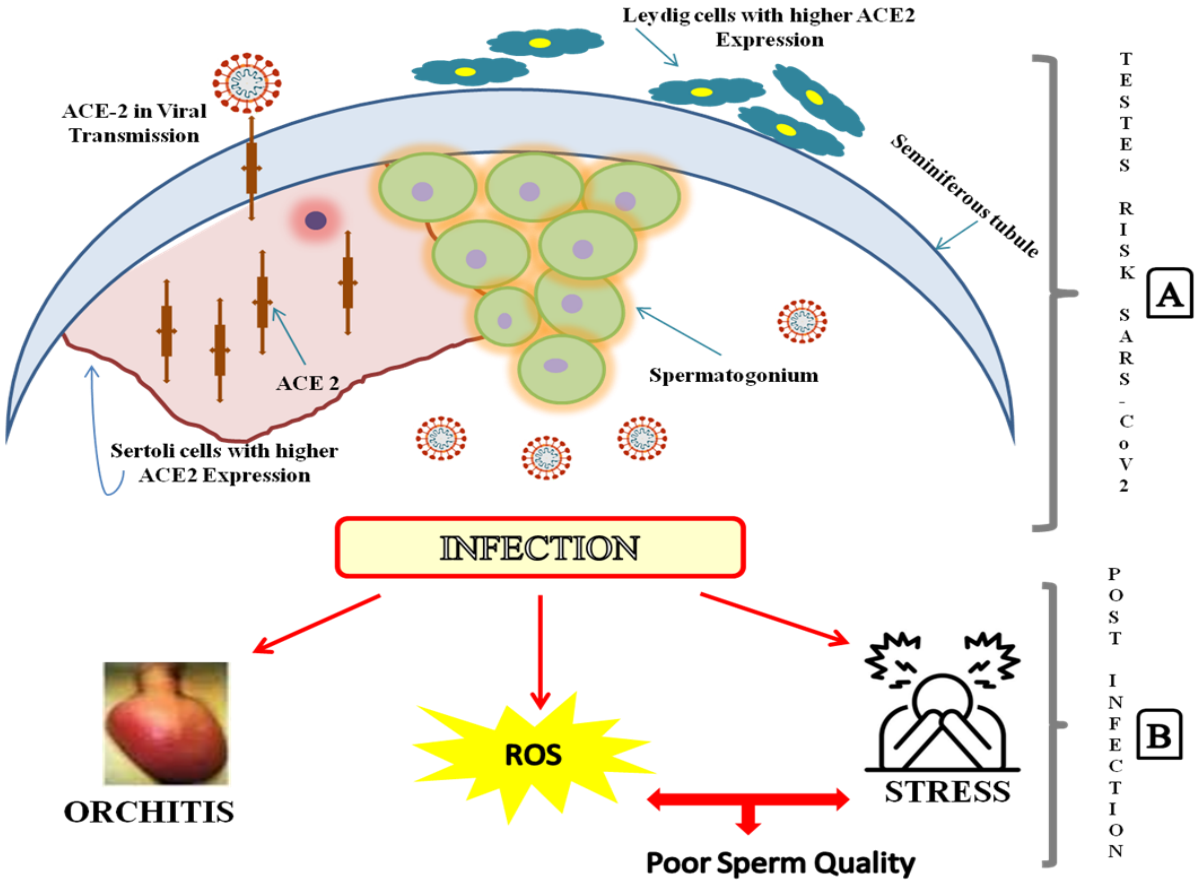
6 ACE2 mediated SARS-CoV-2 male infertility

COVID 19 can disturb the male reproductive function through multiple mechanisms. Oxidative stress (OS) is the major mechanism on which many normal functions of male reproduction rely (Menezo et al. 2016; Delli Muti et al. 2022). It can be hypothesized that COVID 19 virus activates pathways mediated by inflammatory responses. The viral entry could be because of ACE2 activation and expression in male reproductive organs (Tay et al. 2020). Oxidative stress mediates male infertility in numerous ways and this mechanism is documented and supported by data to a large extent (Smith et al. 2006; Xue et al. 2022).

Oxidative stress affects sperm quality, especially motility and morphology. Cellular oxidation damages the spermatozoa and DNA and leads to lipid peroxidation of the sperm membrane (Smith et al. 2006). SARS-2002 infection affects the male fertility status by inducing more ROS production in the semen leading to poor sperm quality and DNA damage (De Iuliis et al. 2009). On the contrary, the release of cytokines also mediated by OS leads to inflammatory responses. COVID 19 may cause orchitis which is one of the causes of oxidative stress. Sometimes, the treatment procedures for COVID 19 infection cause psychological symptoms leading to oxidative stress and in turn loss of fertility status (Rihayat et al. 2019). The treatment of COVID 19 uses ribavirin and other antiviral drugs that have negative effects on men's fertility status (Vellingiri et al. 2020). These medications lead to the induction of oxidative stress, decreased amount of testosterone, impaired spermatogenesis, and other sperm malfunctions. The available animal studies support these data (Asadi et al. 2017).

COVID 19 may result in acute hypogonadism, and such androgenic reduction action can lead to very fatal conditions. Hypogonadism increases the inflammatory cytokines which in turn act as an important mediator for COVID 19 pathophysiology (Gemmati et al. 2020; Mintziori et al. 2022). Researchers confirmed the suppressive activity of the hypothalamic-pituitary axis if critical inflammatory conditions exist along with COVID 19 (Coronel-Restrepo et al. 2017). This causes hormonal imbalance by reducing LH, FSH, and testosterone levels in the body. These changes terminate in male infertility. COVID 19 infection and intonation of sex hormones could be the point where the research should focus on in the future since there are no supporting documents for the effect of COVID 19 infection on sex hormones (Hanscom et al. 2020; Kokkinaki and Hatzidaki 2022). A possible hypothesis explaining the effect of SARS-CoV2 infection on male infertility was shown in figure 2.

During the SARS epidemic, the use of antiviral medications led to a reduction in sperm count, impaired spermatogenesis, and sperm DNA damage or fragmentation (Sengupta and Dutta 2020; de Albuquerque et al. 2022). All these changes were sustained for a minimum of 8 months after recovery by treatment. Previously, it was discussed how COVID 19 damages the testes, and from the male infertility point of view, the testes might be the potential organ target for SARS-CoV-2 (Zheng et al. 2021). Primarily, COVID 19 affects the testes and causes direct testicular damage mediated by ACE2 receptors, and in some cases, secondary inflammatory responses (Cheng et al. 2020).



Possible hypothesis explaining the effect of SARS-CoV2 infection on male fertility
A: Higher expression levels of ACE2 genes in Leydig and Sertoli cells increases the risk of SARS-CoV2 transmission in testicular tissues
B: Increased Reactive oxygen species (ROS) levels , Orchitis and Psychological stress due to SARS-CoV2 infection affects spermatogenesis and results in poor sperm quality.

Figure 2 Possible hypothesis explaining the effect of SARS-CoV2 infection on male infertility

Male infertility term cannot be missed during COVID 19 pandemic since fertility is already going down worldwide due to various abnormalities and this infection further decreases the number (Tufvesson et al. 2022). But at the same time, a decline in sperm quality may be temporary in many cases and we cannot consider the count as infertility during this period (Purvis and Christiansen 1993). Till now there is no track record of men for male fertility status and follow-up, as only 8 months have passed over after the pandemic. More data and follow-up are needed for the patients who have recovered from COVID 19, concerning their reproductive functions (Qiu et al. 2020). Also, the data for reproductive functions during treatment can be taken. These all coherently help the andrologists and clinicians to come up with a new hypothesis for male infertility mediated by ACE2 and COVID 19 (Pan et al. 2020a; Ocanas 2022).

7 Expression of ACE2 in the uterus and vagina

Research has confirmed the expression of ACE2 in the uterus and vagina of females. As already discussed, the ACE2 expression is more in epithelial cells as compared to stroma cells (Chadchan et al. 2021; Saadedine et al. 2022). Although COVID 19 can infect the vaginal tract, the infection through sexual transmission to the partner is not yet elucidated clearly (Qin et al. 2013). ACE2 expression is very important for the endometrium maintenance and menstruation cycle (Abhari and Kawwass 2020). Many researchers proved the role of ACE2 in the regular menstrual cycle, and the absence of ACE2 in the vagina and uterus may lead to endometrial carcinoma (Cui et al. 2020). Hence, although ACE2 is an important protein in the female reproductive system, it can behave as a potential target for virus transmission as well (Stanley et al. 2020).

8 ACE2 expression in the human ovary

ACE presence and its expression were studied in the human ovary and were found that ACE2 activity is more in the case of postmenopausal women's ovary when compared with pre and menopausal women (Liu et al. 2020b; Carp-Veliscu et al. 2022). At the serum level, significant ACE2 activity in the ovary was not recorded and there was not much difference in the ACE2 activity during pre and postovulatory phases for human beings (Dominska 2020). ACE2 expression was also found in the luteinized granulose (La Vignera et al. 2020). The presence of ACE2 in the ovary has many physiological functions but the involvement of ACE2 in ovary-related diseases has been confirmed by many researchers like PCOS (polycystic ovary syndrome), ovarian hyperstimulation, and ovarian cancer. Further findings also indicate FSH mediates the ovarian RAS launch (Palumbo et al. 2016). Studies have confirmed that ACE2 plays a major role in inhibiting cancer cell proliferation and differentiation in the ovary (Kobayashi et al. 2009; Nagappan et al. 2022). Any changes in ACE2 activity and its expression can lead to ovarian dysfunction and sometimes ovarian cancer (Kajihara et al. 2010).

9 Expression of ACE2 in the human maternal-fetal interface

The specific expression pattern of ACE2 is very less in maternal-interface cells (Li et al. 2020c; Miller et al. 2022). The cells where maximum ACE2 expression can be identified are perivascular cells cluster 1, syncytiotrophoblast, stromal and decidual cells. Researchers also confirmed that SARS-CoV-2 is not evolved properly enough to capture perivascular cells cluster 1 for its transmission (Chen et al. 2020a). This could be the reason behind the low risk of early maternal-fetal interface for COVID 19 infection (Liu et al. 2020c; Sufriyana et al. 2022). In the case of the Zika virus and MERS, there were chances of vertical transmission of infection from mother to fetus, but in the case of COVID 19, no reported vertical transmission is reported yet (Schwartz 2020).

From December 2019 to March 2020, we don't have much data to support COVID 19 infection in the male reproductive system. But previous infections like SARS and MERS (epidemic diseases) have shown the impact of infection on male reproductive functions (Adhikari et al. 2020). During SARS, many researchers worked on the impact of the reproductive system and proved that there exists a negative correlation with reproductive functions (Knez 2013). The fertility status of men belonging to the reproductive group declined in SARS survivors. As we know SARS-CoV and SARS-CoV-2 share 76% sequence similarity and since both use ACE2 as a receptor for transmission, it is necessary to investigate the reproductive status of men after COVID 19 recovery (de Souza Silva et al. 2020). Researchers also investigated a group of men and their semen samples for COVID 19 virus presence, nearly 16% of patients' semen samples showed the presence of COVID 19. This raises an alarm about the high chance of sexually transmitting the COVID 19 to the partner (Paoli et al. 2021). We have already discussed the expression of ACE2 in the vagina, so when COVID 19 enters via semen during intercourse, the vaginal fluid supports the expression of ACE2 receptor activation (Hoffman et al. 2020). Although the acidic nature of vaginal fluid can curtail virus survival, the presence of the ACE2 receptor overcomes this block. The antibacterial activity of seminal plasma (Bourgeon et al. 2004) might kill the lactobacilli present in the vaginal tract, so that the acidic medium may not exist for long durations. The occurrence of such a situation is a hypothesis and if it happens, then it is easy for COVID 19 to infect via the female reproductive system and start reproducing on its own (Korber et al. 2020; Saadedine et al. 2022). Although the SARS-CoV-2 presence was identified in semen, no further studies on sperm quality including motility and morphology, or even the immunological response of semen samples were studied (Payne et al. 2020).

10 ACE2 in developing embryo

The present scenario necessitates 1 in 6 couples to rely on IVF methods to conceive a child (Inhorn and Patrizi 2015). COVID 19 infections were found to be asymptomatic in many cases, some or many of the infected individuals have already been conceived or some even tried to get conceived during the pandemic period (Schwartz 2020; Shams et al. 2022). In all these cases, a careful watch on the mechanism of COVID 19 infection risk in the developing embryo is needed (Chen et al. 2020b). Researchers analyzed the dataset of developing embryos for the expression level of ACE2, BSG, CTSL, and other genes involved (Colaco et al. 2020).

ACE2 expression was checked in gametes, morula, zygotes, and other predominant stages. ACE2 expression was more in the blastocyst embryos and very low in compact morula (Cremades et al. 2004). We know ACE2 could be essential for the COVID 19 infection, but the viral infectivity and promotion are majorly done by TMPRSS2. In another study done by the researcher, 80% of the cells exhibit ACE2 expression, but none of them show expression of TMPRSS2 in ICM (later stage of the embryo) (Chanana et al.2020). Whereas, in the case of epiblast and trophectodermal cells, ACE2 and TMPRSS2 were co-expressed (Singh et al. 2018).

This indicates that early embryonic cells could be vulnerable to COVID 19 infection as a mode of entry (Sungnak et al. 2020; Andrews et al. 2022). In the absence of ACE2 in particular organs, an extracellular metalloproteinase enhancer, CD147 (Tang et al. 2004) has been found to have the capacity to biding both COVID 19 and SARS. This shows that the promotion of viral entry could be possible independent of ACE2 and TMPRSS2. In addition, BSG and CTSL need to be co-expressed alongside the entry of the virus, similar to the co-expression of ACE2 and TMPRSS2

(Menon et al. 2020). BSG and CTSL were found to be co-expressed in almost all the cells of the developing embryo. In the case of trophectodermal cells, both ACE2 and CD147 mediate the viral entry and require cathepsin L for COVID 19 infection (Colaco et al. 2020; Louis et al. 2022). It is well known that the entry of viruses by various mechanisms, but what happens after the virus enters the cells? The next step is the replication of the virus, which needs the interaction of viral proteins and the host proteins (Akhtar and Shukla 2009). In the embryos, the blastocyst shows the expression of host proteins that leads to viral replication. Through the available data, researchers also suggest that various genes might be involved in endocytosis and replication of the virus, and are expressed in a majority of the cells of developing embryos (Villalba et al. 2016). Cells of trophectoderm and epiblast have been found with genes of endocytosis and replication even in the absence of ACE2 and TMPRSS2 (Hoffmann et al. 2020). In human embryos, many proteins might play role in interacting with COVID19 in absence of ACE2 (Datta et al. 2020). Both epiblast and trophectoderm undergo further gastrula and placental stage and any damage to these cells will cause lethality in the later embryo development (Stephens et al. 1995). Epiblast retains the pluripotent capacity for a longer time and self-renewal is also observed. A lipid profile is mandatory for optimal viral production and further replication. Enriched lysosomes in epiblast prove the process of viral replication in the host (Rambhatla and Carpenter 2007).

11 ACE2 expression during pregnancy and COVID 19 infection

Many researchers proved the presence of ACE2 and its expression in the placenta region, especially in cytotrophoblast (Hecht et al. 2020; Abdolrazaghnejad and Miraj 2022). During maternity, in the maternal stroma (Meteeb and Al-Dhalimy 2020), the ACE2 is expressed at higher levels at the intravascular trophoblast. The next major part of ACE2 expression is found in the umbilical cord, and its expression is more in the early gestation period (Goolam et al. 2020). With the available data sources, it was proven that the expression of ACE2 is more in the placenta than in the lung (Hikmet et al. 2020). This shows the possibility of COVID 19 infection at the placenta. There is no proven information or data existing for intrauterine infection. But when analyzing the COVID

Table 1 Important studies with COVID-19 and human fertility status

Author studied	Year	Concluding remarks	Link to Human reproduction with relevance to COVID 19
Segars et al. 2020; Gizzi et al. 2022	2020, 2022	COVID 19 infection leads to severe alternation in female pregnant women and affects the offspring too	COVID 19 affects both male and female sex gametes
Cavalcante et al. 2020	2020	The affinity of COVID 19 towards ACE2 in female reproductive organs were explored and ACE2 acts as a source of entry for COVID 19	COVID 19 enters via the female reproductive system and results in female infertility, still more research is needed to support the data
Anifandis et al. 2020	2020	COVID 19 affects the IVF outcomes	COVID 19 affects both sperm functions and egg performance and leads to give more stress on the IVF patients
Espinola et al. 2021; Minich et al. 2022	2021, 2022	Supplementation of Vitamin D and Myo-inosital during COVID 19 pandemic will act as a preventive measure for pregnant women and women who undergoing IVF	COVID 19 pandemic affects IVF outcomes severely in many countries
Dutta and Sengupta 2021	2021	Theoretical prediction proved that the testes could be the primary target of SARS CoV-2, which severely damages the testes and further results in male infertility	COVID 19 virus severely affects the testes and affects male reproduction further leading to male infertility
Rennu et al. 2020	2020	The disturbed IL-4 decreases the level of ACE-2 with the inflammation	COVID 19 infection leads to male infertility via Th2 cells and JAK-STAT signaling.
Younis et al. 2020	2020	ACE2 is found more abundant in testes and it acts as a receptor for COVID 19 entry	COVID 19 infection affects the process of spermatogenesis and leads to male infertility
Olaniyan et al. 2020; Balawender et al. 2022	2020, 2022	SARS-CoV-2 can also affect the urogenital tract	Role of ACE2 receptors in promoting SARS-CoV-2-induced blood-testis/epididymal barrier infiltration and testicular dysfunction.
Aitken 2020	2020	ACE2 receptors mediate SARS-CoV-2-induced blood-testis/epididymal barrier infiltration and testicular dysfunction	COVID 19 indirectly correlates the male sexual dysfunction
Li et al. 2020a	2020	Autopsied testicular and epididymal specimens of COVID-19 showed the presence of interstitial edema, congestion, and red blood cell exudation in testes, and epididymides.	Impairment of spermatogenesis was observed in COVID-19 patients leading to male infertility

19 infection history, it has affected newborns in many countries. The first such case was filed at Wuhan hospital, later many countries reported positive COVID newborn cases (Pan et al. 2020b).

Many researchers postulate that COVID 19 may infect the fetus in the early gestation itself and hence diagnose positive for the newborn (Prochaska et al. 2020). Also, other available databases show the presence of ACE2 in the female breast. There is no proven data for the presence of COVID 19 in breast milk, but this can probably act as a medium of transmission (Contini et al. 2020). There are no conclusive reports available for COVID 19 infection and breast milk or breastfeeding (Thomas et al. 2022). Even though no active virus is present in breast milk, it could affect newborn that is fed with breast milk with infections (Williams et al. 2020). Hence, newborn babies that generally have less immunity can avoid breastfeeding from affected mothers. So far, the functions of ACE2 during pregnancy are regulating the blood pressure & the fetus development, stimulating trophoblast invasion, and acting as a paracrine regulator during the entire pregnancy term (Shoemaker et al. 2019).

The major function of ACE2 is exhibited as a balance in maintaining hydro-salinity during the pregnancy term. COVID 19 infection and its spread created a threat to both pregnant women and babies. It can cause fetal distress, premature birth, and rupture of the foetus membrane (Karimi-Zarchi et al. 2020). Also, the renal and kidneys express ACE2, and researchers commented on the presence of higher ACE2 in renal tubules of pregnancy. Research and survey show that COVID 19 had very low maternal cases and fatality than other previously existing viral respiratory disorders like SARS, and MERS. There are no special symptoms of pregnant women when compared with non-pregnant women. In case of severe illness due to COVID 19, it results in premature labor pain and or early delivery. The important studies on COVID 19 and its impact on human reproduction were tabulated in table 1.

12 Conclusions

Based on our exhaustive review of the published articles, ACE2 expression is found to be more in the testes, ovary, and vagina. ACE2 can be the key to COVID 19 infection alongside other important genes associated. Based on the available reports, SARS-nCoV-2 targets testes because of its potential ACE2 expression level and other genes favoring the virus transmission and replication inside the host cell. COVID 19 infection spreads among men widely when compared to women and the fatality is more in the case of men. A suggested theoretical hypothesis is that SARS-CoV-2 may lead to testicular damage and further to male infertility and poor sperm quality. Similar to the ovary, uterus could be the target for SARS-CoV-2 in the female. ACE2 receptors mediate and cause direct damage to testes or infect through secondary inflammatory and immunological responses. SARS-Co-V-2 can be a setback in human reproduction for a while. However, available data ensures there will not be a lifelong threatening factor for SARS-CoV-2 in terms of fertility and the threat will be only for a minimum of 8 months to 1 year post-recovery from COVID 19 for both men and women.

13 Future Prospective

From December 2019 to date, limited data is available on the impact of SARS-Co-V-2 on human reproduction and fertility status. Previously, we have data on SARS Co-V and its presence in semen, but we don't have any data for n-Co V 2019 presence in a semen sample and vaginal fluid. However, scientific hypotheses suggest the impact of SARS-Co-V-2 on human reproduction in both men and women. The mechanism of ACE2 and its expression level can be studied in vivo in the testes, epididymis, seminal vesicles, ovary, uterus, and vagina, and during early embryonic development. Though, clinicians and physicians aim at controlling and treating COVID 19, andrologists and researchers look at the impact of COVID 19 on human reproduction. COVID 19 can have either an immediate or delayed impact on male and female fertility and extensive research in this area is required to prove the hypothesis. So far we don't have data on men and women who have recovered from COVID 19 and their track record on reproductive functions. Since the infertility rate is already on the rise globally, these types of infections may worsen the situation. We suggest more research initiatives for understanding the concepts of COVID 19 and its impact on human reproduction.

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Conflict of Interest

The authors declare that there are no such conflicts

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Impact of Metallic Nanoparticles on the Nutritional Values of *Spirulina*

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ABSTRACT

Spirulina has high nutritional values and anti-oxidative properties. It is a staple diet due to its easy cultivation and greater nutritional values in biological macromolecules (proteins, lipids, and carbohydrates), pigments (chlorophyll, carotenoids, phycobiliproteins) vitamins, minerals, phenolic compounds, and amino acids. *Spirulina* also has been used as a nutraceutical to treat numerous diseases and disorders due to its promising therapeutic values. However, extensive anthropogenic activities cause the discharge of metals and metallic nanoparticles into the environment that might cause toxicity to marine and freshwater microalgae due to bioaccumulation. The presence of metals in the environment beyond the normal range does not only affect the growth but also the nutritional values of microalgae. The nutritional properties and usage of *Spirulina* along with the harmful effects of metals and metallic nanoparticles on *Spirulina* are highlighted and summarized in this paper.

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1 Introduction

Spirulina has been consumed for nearly centuries and is the most consumed microalgae species. World Health Organization (WHO) coined *Spirulina* as a "superfood" concerning its higher nutritional values and anti-oxidative properties (Liang et al. 2021). It is among one of the 35 wild species that are commonly found in lakes with extreme alkalinity (Raj et al. 2020). *Spirulina platensis* (*Arthrospira platensis*), *S. maxima* (*Arthrospira maxima*), and *S. fusiformis* (*Arthrospira fusiformis*) are widely studied due to their edible properties and nutritional values in addition to their promising therapeutic values (Beheshtipour et al. 2012; Michael et al. 2018). The Mexican population had been using *S. platensis* as a source of food since the Aztec civilization (Farrar 1966). It is majorly commercialized as a staple diet due to its greater nutritional value, easy cultivation, and low cost (Molino et al. 2018). However, recent anthropogenic effects such as urbanization, and current industrial and agricultural activities have led to the contamination of soil and aquatic environments (Ekubo and Abowei 2011). The massive industrial usage of engineered metal nanoparticles (MNPs) such as zinc, silver, titanium, and copper nanoparticles in industrial applications results in the intentional and accidental release of these nanoparticles into the aquatic environment (Praveena et al. 2010) which can affect the growth and the nutritional values of microalgae growing in the polluted water bodies through physiological and biochemical alterations (Navarro et al. 2008; Morais et al. 2009). This review presents the nutritional properties and usage of *Spirulina*, along with the effects of metals and metal nanoparticles on *Spirulina*.

2 Nutritional Values osf *Spirulina*

Spirulina species are highly nutritious and contain a significant amount of proteins, lipids, carbohydrates, beta-carotene, vitamins, amino acids, and minerals (Khan et al. 2005). *Spirulina* species generally contain 55.0 - 70.0% proteins, 15.0 - 25.0% carbohydrates, 5.0 - 6.0% lipids, 6.0 - 13.0% nucleic acids, and 22.0 - 48.0% minerals (Kulshreshtha et al. 2008). The major percentage constituent level of protein in *Spirulina* is the main reason for it to be chosen as a food source. Carbohydrate content usually acts as a secondary role when the microalga is used as protein-rich food (Becker 2013). The structure of branched polysaccharides in *S. platensis* is similar to glycogen (Wan et al. 2016). Further, the lipid contents in this microalga average vary from 1.0 - 40.0% and, up to 85.0% of the dry weight under certain conditions. The lipids formed in *S. platensis* can be either saturated or unsaturated fatty acids (Becker 2013). *S. platensis* species also contain lots of vitamins such as a variety of vitamin B complexes which are B_1, B_2, B_3, B_6, B_9, and B_{12}, and minerals like calcium, copper, iron, magnesium, phosphate, sodium and zinc, and photosynthetic pigments (Houston 2002).

Microalga like *S. platensis* is rich in antioxidants due to the presence of various types of phytochemicals (Al-Dhabi and Valan Arasu 2016). These antioxidants are generated to nullify the oxidative stress (Xia et al. 2015) caused by reactive oxygen species (ROS) (Djearamane et al. 2020). The environment in that *S. platensis* thrives generally contributes to the such occurrence (Siddiqui and Prasad 2017; Hussein et al. 2019). Thus, with the accumulation of antioxidants, the effect of ROS is suppressed (Riss et al. 2007). Its properties as antioxidants are beneficial regardless of being used for humans or in animal feed (Wu et al. 2016). Two main varieties of antioxidants i.e. enzymatic antioxidants and non-enzymatic antioxidants are found in *S. platensis* (Carlsson 1978). The non-enzymatic antioxidants are the most significant ones consisting of chlorophyll, tocopherols, carotenoids, flavonoids, phycocyanin, phenolics, phycobiliproteins (PBPs), and polyunsaturated fatty acids (PUFAs) (Richmond 2004). Flavonoids known as phenolic acids are predominant in *Spirulina* with the ability to be antioxidants in suppressing various diseases such as infamous cancer and cardiovascular-related diseases as well as its scavenging ability towards free radicals (Wali et al. 2015). Further, phycocyanin, with phycoerythrin masks the pigmentation of chlorophyll (Karimi and Moradi 2015). These two photosynthetic pigments give *Spirulina* blue-green colour and hence it is known as the blue-green microalga (Desai and Sivakami 2004). Tables 1, 2, and 3 show the macronutrient composition of *Spirulina* and comparison with common human food sources. Based on Table 1, *S.platensis* has a well-balanced derived composition of various bioactive nutrients (Liang et al. 2020) varying from vitamins (inclusive of vitamin K, vitamin A, vitamin E, Thiamine B1, Riboflavin B2, Niacin B3, Vitamin B6, Vitamin B12, Provitamin A, Riboflavin B2, Folic Acid, Panthothenic acid and inositol (Koru et al. 2008)), minerals (8%), proteins (60-70%), phenolic compounds, γ-linolenic acid (GLA) (49%), phycocyanins (15%), and constituent of phytochemicals (Jaime et al. 2005; Stanic-Vucinic et al. 2018). In addition, it is also well known for its higher content of carotenoids (456.00 mg/100g of dry weight) (tetraterpenoids) including about 80% of beta-carotene and 20% of secondary carotenoids like xanthophyll which is typically higher than found in carrots (Siddiqui and Prasad 2017; Shao et al. 2019). Most importantly, *S. platensis* is rich in complete essential amino acids (EAA) and gamma linoleic acid (GLA) (Koyande et al. 2019). These attributes commercialized *S. platensis* as a nutritional food for human consumption despite the ongoing validation against the claims of its adversities which are yet to be claimed by scientific research. International and national quality standardization have been established to ensure safety and to avoid public health issues due to toxic blooms as well as other toxic issues from microalgae for safe consumption (Henrikson 2010).

Table 1 Comparison of macronutrients of *Spirulina* with the common human food sources

Sample	Average content in Percentage (% DW)			Reference(s)
	Proteins	Lipids	Carbohydrates	
Milk	26	28	38	(Becker 2013)
Rice	8	2	77	(Becker 2013)
Soybean	34-40	16-20	19-35	(Becker 2013)
Egg	45- 47	41	4	(Gouveia et al. 2008)
Cheese	30- 36	-	-	(Koru 2012)
Chicken	31	-	-	(Koru 2012)
Fish	15–22	-	-	(Koru 2012)
Baker's yeast	39	1	38	(Becker 2013)
S. fusiformis	62.3	8.2	19.3	(Rafiqul et al. 2003)
S. maxima	68-77	4-14	8-16	(Hoseini et al. 2013; Matassa et al. 2016)
S. platensis	46-74	6-13	8-25	(Hoseini et al. 2013; Matassa et al. 2016)

3 Therapeutic Effects of *Spirulina*

Various studies and trials have elucidated the therapeutic values of *S. platensis* (Al-Harbi 2008; Moor et al. 2017; Al-Qahtani and Binobead 2019; Shamsudin et al. 2019; Raj et al. 2020). *S. platensis* cell walls are made of liposaccharides that can induce the innate immune response through the toll-like receptor 4 (TLR-4) (McCarty et al. 2010). Although *S. platensis* is a Gram-negative bacterium, it does not exhibit an antagonistic reaction towards TLR-4 due to its nature as a cyanobacterium (Okuyama et al. 2017; DiNicolantonio et al. 2019). However, other Gram-negative bacteria such as *Salmonella* do not tolerate TLR-4 reactions (Lima et al. 2017).

The lack of cellulose in its walls enables digestibility up to 90%, which is relatively equivalent to the digestibility of casein (Mao et al. 2005). Even the absorption of iron is more than 50% higher than the conventional iron supplements. *S. platensis* richness in micronutrients puts it on the frontline for vegans and vegetarians especially when its iron content is thrice than found in meat as well as comparable with micronutrients found in milk (Bensehaila et al. 2015). Furthermore, it has been reported that *S. platensis* liposaccharides can inhibit the development and growth of tumors. Furthermore, C-phycocyanin inhibits the enzyme cyclooxygenase-2 (Cox-2) involved in the biosynthesis of prostaglandins (PGs). This Cox-2 is highly expressed in cancerous cells to promote PGs as inflammatory mediators (Fournier and Gordon 2000).

S. platensis is also an excellent free radical scavenger and immunological stimulant as proven by various *in vivo* and *in vitro* studies (Assaye et al. 2018). *Spirulina* is considered as a non-toxic dietary supplement and safe to be consumed at normal consumption levels (Raj et al. 2020). There is still a grey area in the possible reaction of *Spirulina* with therapeutic compounds or other dietary supplements (Hoseini et al. 2013), and some shortcomings are also associated with the consumption of *Spirulina*. Mazokopakis et al. (2008) reported that the consumption of *Spirulina* may induce headache, stomach ache, flushing of the face, and muscle pain effects. Severe side effects of hepatotoxicity and rhabdomyolysis are also reported due to the consumption of *Spirulina* tablets as dietary supplements (Iwasa et al. 2002). Papapetropoulos (2007) suggested that patients with phenylketonuria and autoimmune diseases should avoid taking *Spirulina*. Despite *S. platensis* is generally recognized as safe and highly nutritional with various constituents of nutrients and micronutrients, its intrinsic properties are fundamentally dependent on its cultivated conditions and environments (Belal and El-Hais 2012) which certainly affect the *S. platensis* nutrient bioavailability (Falquet and Hurni 1997).

4 Effects of Heavy Metals and Metallic Nanoparticles on *Spirulina*

The development of technology and nanotechnology (Castro-Bugallo et al 2014), human activity, and natural earth processes are leading to the release of metals and metallic nanoparticles (MNPs) into the environment (Tsao et al. 2011). Heavy metals are categorized as metals that have an atomic density higher than water or at least 4 grams per centimeter cube (Hawkes 1997). The presence of heavy metals amplified during the ore mining era which was the main source of accumulation of heavy metal in the environment besides the natural occurrence of heavy metals through leaching and the weathering effect of rocks filled with ore.

Studies on heavy metals mainly assessed the ecological risk. Most of the heavy metal accumulations are due to anthropogenic effects

as the main contributors (Dubey 2021). Heavy metals are transported through water bodies like rivers and estuaries which act as a sink for these toxic matters to accumulate and influence the heavy metals accumulation in a particular area (Yap and Al-Mutairi 2021). The heavy metals flowing from upstream of rivers into the ocean accumulate in a huge amounts together before releasing into the ocean. Heavy metals are found in parent rock which dissipates and enters the environment through hydrological effects, weathering effects, and also by erosions (Mushtaq et al. 2020).

Recent industrialization with massive utilization MNPs such as silver, zinc, titanium, and copper NPs in numerous industrial and consumer products, and the subsequent release of these MNPs in the industrial and domestic wastewaters brings the threat of nano pollution of water bodies. Numerous studies have been conducted to analyze the effect of metals and metallic nanoparticles on microalgae, especially in the aspect of the growth rate and biochemical composition (Djearamane et al. 2019; Liang et al. 2021; Thenarasu et al. 2022). Studies by Saçan et al. (2007) and El-Sheekh et al. (2003) have shown various stimulatory effects of metals such as lead, aluminum, and cobalt at low concentrations in microalgae species. However, at levels surpassing the optimal level of metals and MNPs activate the inhibitory mechanisms in microalgae due to oxidative stress (Suman et al. 2015). The toxicity mechanisms of MNPs on microalgae are illustrated in Figure 1. The mechanism involves various pathways and the main mechanism is the formation of ROS due to the induction of oxidative stress by MNPs. MNPs induce oxidative stress in the cells by developing ROS. The ROS produced degrades and destroys the affected cell biomolecules such as proteins, and lipids as well as disrupts the biological activities as a response to the MNPs (Liang et al. 2020). The biochemical properties of *S. platensis* are affected when exposed to metals and MNPs due to the accumulation of reactive oxygen species (ROS), which results in the reduction of growth and photosynthetic rate as well as the degradation of proteins, phycobiliproteins, and carotenoids. Studies from El-Sheekh et al. (2003) and Zinicovscaia et al. (2017) have reported the degradation of biomass, and phenolic compounds, and decreased production of carbohydrates when microalgae were exposed to MNPs at high concentrations.

The formation of oxidative stress causes growth inhibition, reduction in proteins, lipids (Casazza et al. 2015), carbohydrates (Shilpi et al. 2014), chlorophyll (Deniz et al. 2011), carotenoids (Lone et al. 2013) and phenolic compounds (Comotto et al. 2014) of microalgae. The main components of *S. platensis* such as proteins, carbohydrates, and lipids are reported to be reduced significantly when exposed to selenium from 24 to 72 h (Zinicovscaia et al. 2017). Generally, metals and MNPs cause the inhibition of growth rate and also the production of biological macromolecules, pigments, and polyphenols. Table 2 shows the effect of metals and MNPs on *S. platensis*, while Table 3 reveals the effects of metals on *S. maxima.*

A study by Zinicovscaia et al. (2017) reported a reduction in *S. platensis* phycobiliproteins, especially phycocyanin (65.0%) and carbohydrates (76.0%) at 72 h after its exposure to selenium. Further, 80% reduction in biomass was found at 72 hrs due to the oxidation of the structural and functional components of *S. platensis*. Notably, *S. platensis* has a high efficiency to absorb metal ions from the environment through its various biochemical metabolic pathways especially due to its high ratio of surface area to volume (Shao et al. 2019). This is applicable in both viable and nonviable types of algae as the viable ones sequester metals compared to the non-viable ones where the carboxyl groups are

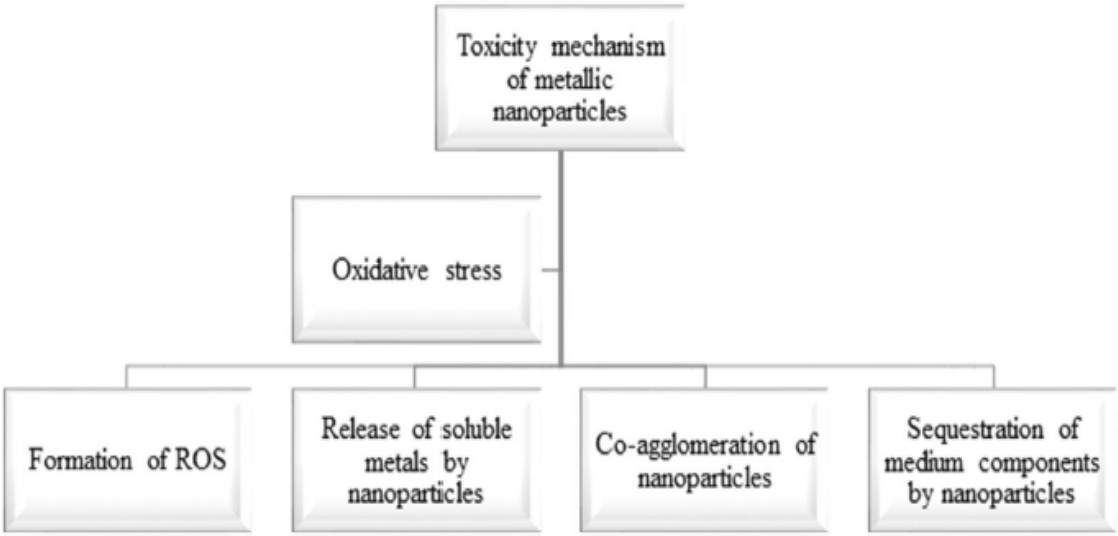

Figure 1 Toxicity mechanisms of Metallic Nanoparticles

Table 2 Effect of metals and metallic nanoparticles on *S. platensis*

Metals	Time (h)	Conc. (mg/L)	Effect	Reference(s)
Cr	216	10	62% reduction in biomass yield 64% reduction in chlorophyll-a 65% reduction in proteins 62% reduction in carbohydrates	(Shilpi et al. 2014)
Cu (II)	168	1	50% reduction in biomass yield 27% reduction in chlorophyll-a 20% reduction in carotenoids 13% increment in malondialdehyde	(Deniz et al. 2011)
Se	72	100	69% reduction in proteins 80% reduction in lipids 76% reduction in carbohydrates 90% reduction in phycocyanin	(Zinicovscaia et al. 2017)
TiO_2 NPs	504	100	74% reduction in biomass yield 24% reduction in phenolic compounds	(Comotto et al.2014)
TiO_2 NPs	120	500	66% reduction in lipids 12% reduction in polyphenols	(Casazza et al. 2015)
ZnO NPs	240	10	41% reduction in biomass yield 93% reduction in chlorophyll-a 50% reduction in carotenoids 79% reduction in proteins 78% reduction in nitrate reductase activity	(Lone et al. 2013)
ZnO NPs	96	200	76% reduction in biomass yield 76% reduction in carotenoids 74% reduction in phycocyanin	(Djearamane et al. 2018)

Table 3 Effect of metals on *S. maxima*

Metals	Time (h)	Conc. (mg/L)	Effect	Reference
Cd	504	1.2	30% reduction in biomass yield	(Augusto de Costa and de Franca 1998)
Cr(VI)	96	11.16	50% reduction in biomass yield	(Chen et al. 2003)
Zn	312	0.01mM	17% reduction in biomass yield	(Balaji et al. 2013)
Ni	312	0.01mM	33% reduction in biomass yield	(Balaji et al. 2013)

bound to the metal ions (Govindaraju et al. 2008). *S. platensis* sequesters metals in the aquatic environment while phytochelatin synthesis assists in the uptake of metals in microalgae (Rajamani et al. 2007). *S. platensis* binds to metals, especially polyvalent metals by its high binding capacity together with various functional groups assisting this function (Da Silva Vaz et al. 2016).

Zinc metal showed a 17% reduction in biomass yield after 312 hours of being exposed to *S. maxima*. Zinc oxide after 240 hours showed a report of a 79 % reduction in proteins of *S. platensis.* Most of the heavy metals such as chromium, copper, selenium, titanium oxide, etc., that were exposed to *S. platensis* caused a reduction in various parameters like biomass, chlorophyll-a, carbohydrates, carotenoids, phycocyanin and the phenolic compounds which ranged between 20% to 90%. Further, a study by Upasani et al. (2001) reported an increase in malondialdehyde upon treatment with copper indicating lipid peroxidation. An increase in lipid peroxidation is due to the accumulation of free radicals in the cell and destroys algal cells.

Conclusion and Future Recommendations

Algal cells have the potential to absorb metals and MNPs as the algae cell wall has the affinity in binding the metals, which explains the ability of *S. platensis* to absorb and retain metals and MNPs in the natural environment or bioremediation processes. The accumulation of certain metals in *S. platensis* is a potential risk for humans. Thus, proper evaluation of *S. platensis* on its contents is essential to avoid risks to human health. Metals that are found in the aquatic environment are usually more than one type, which could pose a synergistic effect causing a higher toxicity effect.

As metal contamination is becoming a global challenge, farmers are advised to check the quality of the water source used for the cultivation of *Spirulina*. The presence of metal pollutants does not only slow down the development of the *Spirulina*, the build-up of the metals in the cell can be a threat to human health. Although most of the studies found the metal pollutants contained in

Spirulina are still within the safety level for human consumption, the issue should be kept in check to limit the presence of the contaminants in the future.

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Sustainable Livelihood: Guidelines for Human Capital Access of Goat Farmers in the Upper Northern Region of Thailand: A case study

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ABSTRACT

This article aimed to explain and present guidelines for the human capital development of goat farmers in the upper northern region of Thailand. To analyze the whole point of development towards sustainability, there was a field visit to explore the basic information of the basic goat farming context. In-depth interviews and non-participant observations were used as tools for conducting the research using the narrative method, and the story of goat farmers who have a high experience in raising goats was analyzed and conveyed. The information was obtained from farmers of 3 cases (Information–Rich Case) in Chiang Mai, Lamphun, and Lampang provinces and examined in a triangular way from visiting the area, and the results of the study were analyzed, summarized, and described. The results of the study revealed that **"**Human Capital**"** is the most valuable factor in production among other factors of production because, in addition to acting as a mechanism for carrying out various activities, it is also a factor that must be developed for the development of sustainable practices. Sustainable livelihoods will be available in the future for farmers as well, but before human capital can be a quality factor, it has to be learned to acquire skills, experience, and what human beings have accumulated, forged, and merged. That is to say, the development of the human capital of goat farmers for sustainable livelihoods is the development of knowledge of specific professional skills that are applied to ensure the stability of the farmer sector along with continuous support from the government and academic departments.

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1 Introduction

Agriculture has been associated with the life of Thai people for a long time, whether in the form of farming, gardening, or raising animals. It is in parallel with cultivation, which has been the main occupation of most Thai people in the past (Kamluangng et al. 2019). Besides, Thailand is known as an agricultural country since it is located in a terrain that is suitable for agriculture. Most of the country's population has always been engaged in agriculture or in some ways related to agricultural activities. Despite an effort to become an industrialized country, Thais are still strongly reliant on agriculture like other developed countries. The evolution and development of Thai agriculture have not only changed throughout time but have also been corresponding to the ever-changing trends of the world (Thongmeethip 2021). Presently, specifically in the northern region, the main occupations are agriculture and livestock which are relevant to the people's way of life in the upper northern region, and the new popular type of occupation that has just emerged is "Goat Farming" (Raksasiri et al 2019).

The primary reason for this form of livestock is the suitable climate in the upper north as it is mostly hot and humid alternating with dry weather (Agriculture Extension and Development Office at six 2017). Therefore, this kind of weather is suitable for producing many types of crops. Thus, this reinforces the fact that a career in goat farming became popular. Although goat farming in the area accounts for only about 10 percent of the country's total goat farming (Moonmanee 2020), the number of goat farmers has increased steadily over the past three years (2017-2020). There are currently 824 individuals who are now working in goat farming (Department of Livestock Development 2020), and the trends are positively promising. However, the fact that the number keeps rising cannot guarantee that the farmers' job is secured. Besides, each farmer does not come from the same background and some may lack an essential quality: "Human Capital" which is related to knowledge development that farmers should be provided. In other words, people lack life sustainability. This study aimed to provide information regarding the development and risk assessment of the physical capital of goat farmers in the Upper North of Thailand.

The researcher has studied the concepts and theories that are pertinent to the subject matter to employ them as theoretical frameworks and to analyze and discuss the result of the study. Therefore, the literature review related to this paper can be divided into approaches and concepts.

1.1 Sustainable Livelihoods Approach

In the mid-1980s, the concept of sustainable livelihoods, or rural livelihood, emerged in development academia in the West. The academics Robert Chambers and Gordon Conway were the ones defining the term, "Sustainable Livelihoods." This concept opens up rural people's perspectives and offers alternatives to livelihoods beyond being tied to the land. In Chambers and Conway's view, livelihoods consist of the competence to own assets and activities necessary for the means of living (Chamber and Conway 1992).

A sustainable Livelihood Study has a purpose which is to understand the living system which supports opportunities for improvement to reduce poverty. To understand Sustainable Livelihoods, there are six main concepts which are as follows: (i) People-Centered: starting from the analysis of farmers' lives and methods changed over time. The impact of policy changes on organizations, people, property ownership, and the dimension of

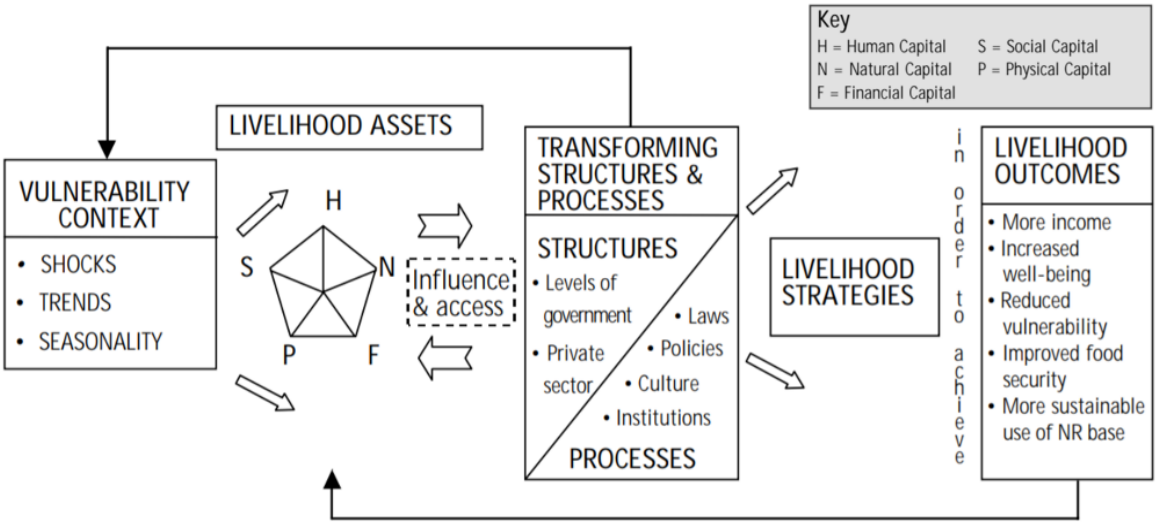

Figure 1 Sustainable Livelihoods Analytical Framework (Adopted from Department for International Development (2001) Sustainable Livelihood Guidance Sheets)

poverty is well recognized. Working towards the goal emphasizes the importance of policy and institutional management and influences on the poverty agenda. This serves to support people to achieve their goals of living. Poverty is also reduced when external support works following the livelihoods approach, environment, society, and the ability of people in the community for use in each locality; (ii) "Holistics" approach suggested that everything is interconnected and is not segregated by geography and social groups. This is to be aware of the various influences on humans, to find out, and to understand the relationship between these influences and the impact they have on livelihoods. The awareness of a variety of duties means accepting different ways of living and seeking to lead to various outcomes of living; (iii) "Dynamics" seeking to understand and learn from change to be able to support positive outcomes and mitigate the negative effects that will arise from external effects; (iv) "Building on strength" the key principle is to start an analysis of strength over needs; (v) "Macro-micro linking" in the study of sustainable livelihoods, the link between communities and individuals to the policy and institutional level; and (6) "Sustainability" an assessment that is based on four main components**:** society, economy, institutions and the environment (Department for International Development 2000).

According to Figure 1, the Sustainable Livelihood Study discusses the relationship between the five elements. What leads to the livelihood goals of the target audience includes (1) the contextual component of vulnerability and uncertainty; (2) the capital of livelihood which is the most important; (3) the structure and processes that cause change; (4) the livelihood strategy; and (5) the results of the activities which is the relationship between five elements that lead to the goals of the farmers**'** livelihoods and describes the elements and contexts relevant to livelihoods within each context where people live and make a living (Department for International Development 2000).

As mentioned above, the **"**Capital of Livelihoods**"** is the most important element that farmers use in their livelihood, which is positively correlated with outcomes and affects the opportunity to choose a way of life that is directly influenced by the vulnerability context and the structural and institutional changes. Five main types of the capital of Livelihoods are shown in figure 2.

The first is Human Capital; this is not just about individuals as resources, but it also covers their energy, health and well-being, knowledge and skills, motivations, and emotions. The second is Natural Capital; this represents the environmental and ecological resources that are needed to produce goods or deliver services. This consists of energy, water, fuels, raw materials, and other natural resources, as well as the ecosystems from which they are taken. The third one is Social Capital; this describes the way that people interact in the various departments within the organization. It is also about how people relate through other networks, partnerships, and less formal groups. The fourth is Physical Capital; this covers material goods and infrastructures used by an organization to generate products and services, but they are not part of the delivered output. This includes buildings, machines, tools, communications networks, IT systems, etc. The last one is Financial Capital; these are assets that exist in a currency form, including cash, shares, bonds, and loans (Porritt 2007).

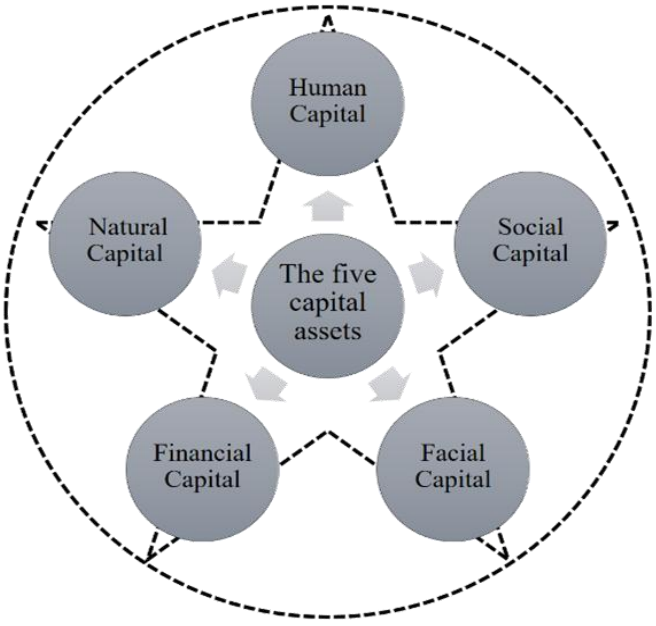

Figure 2 Five capital assets for Sustainable Development

1.2 Sustainable Development Concept

A sustainable development concept emphasizes the change that has been done or has been planned, toward a better direction. Although the changes do not improve the current condition, it is still called "Development". According to the dictionary of the Royal Institute, the word "Development" means to grow which corresponds to its English counterpart since in English the word "Development" means any change that can lead to expansion, growth, or improvement of a situation (Thongmeethip 2019). In other words, sustainable development is a development guideline that meets the needs of the current generation and does not fail to consider the needs of future generations (Brundtl and Report 1987). There are three key components to achieving sustainable development: economic growth, social inclusion, and environmental protection, and all these are aimed at the Sustainable Development Goals 17 (SDGs) which strengthen and revitalize global partnerships. To that end, the goals can be categorized according to five interconnected factors (5P): (1) people development, focusing on eliminating poverty and hunger, and reducing inequality in society; (2) environmental development reinforcing the protection and preservation of natural resources, environment, and climate for next generations; (3) economy and prosperity promoting individuals' well-being and harmony with nature; (4) peace and justice upholding the principles of peaceful coexistence for a harmonious, and inclusive society; and (5) development partnerships, a cooperation of all sectors in driving the sustainable development agenda (Blewitt 2018).

In this research, the authors employed two concepts as a ground used to compare the data obtained from field trips on the topic, "Sustainable Livelihoods: approaches to human capital development of goat farmers in the upper north of Thailand.". The researchers have put forward a partial literature review of each case study to both describe and present specific aspects of the local context such as "Human Capital" which is a part of the five Capitals, or a framework for sustainability. The idea contributes to the development that is related knowledge and skills that can be devised and applied so that people can reap the most benefits it. There are three significant attributes to this. The first one is intellect or tacit knowledge, which consists of knowledge, an ability to learn, specialized skills, and experience individuals have accumulated throughout their lifetime. The second characteristic is social, or a network of relationships. Lastly, the third part is emotions which include features such as self-awareness, integrity, and resilience (Wedchayanon 2008). Therefore, as far as the matter of the sustainable development concept under the sustainable development goals (SDGs) framework is concerned, the second goal of SDG aims to terminate the hunger of the people, enhance food security, elevate nutritional levels, and ensure agricultural sustainability. Hence, if people understand human capital, they will be able to increase their productivity as well as the income of small business entrepreneurs especially those whose occupations concern farming, livestock farming, and fishery, not to mention the accessibility to land, resources, factors of production, knowledge, financial services and marketing, and opportunities to add value to their work and to be recruited to work outside of their farm sphere stably and fairly.

2 Materials and Methods

The research was conducted at Chiang Mai, Lamphun, and Lampang Provinces, which are pilot areas to develop goat farmers, and has an important aim to explain and present a sustainable approach to human capital development of goat farmers in the northern region of Thailand (Figure 3). For sampling the Information-Rich Cases, there is a person who has very vast experience and knowledge about goat farming has been chosen. With a target audience for collecting data from 3 main sectors and using a qualitative approach, including observations and in-depth interviews, the data was examined in a triangular way after visiting the area, and the results of the study were analyzed and summarized by description.

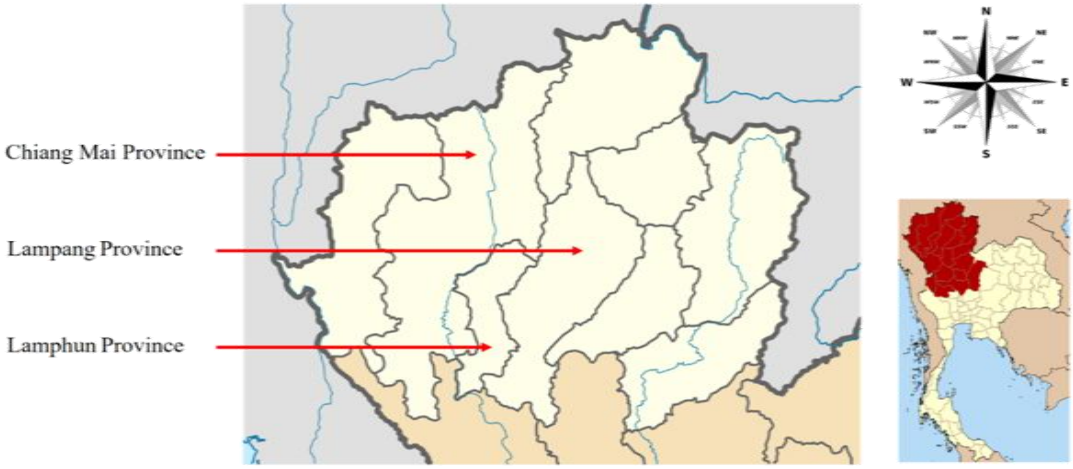

Figure 3 A pilot area for promoting goat farming in the northern region of Thailand

3 Results and Discussion

3.1 The Risks and Certainty of Life with Goat Farming

The results of the study showed that the people of the northern region of Thailand have been raising goats for decades, especially among the rural area people who prefer to raise them for two purposes**:** to consume and sell to foreign countries. In general, farmers do not raise goats as their main occupation, but rather as a secondary occupation that is blended with other agricultural occupations and as an additional money-making venture since goats are highly tolerant of hot and dry climates, and better at foraging than other types of farm animals. Besides, they also require a shorter time to raise, take up less space, and their entire body can be sold. All these are the distinctive features of goat farming. Despite these benefits, the number of goat farming in the upper northern area is still relatively small compared to that of other animals. It is only popular among certain groups of people, and, unfortunately, goats have become a low-prioritized economic animal.

However, with the government programs that promote goat farming over the past period and the pilot areas for raising, which are Chiang Mai, Lamphun, and Lampang Provinces, goats have gradually become an important economic animal that farmers in all regions pay more attention to as well (Figure 3). Some farmers hold goat farming as their main occupation or supplementary venture, which can generate a lucrative income for their family. This is because goat meat products started to be in demand both domestically and internationally. Thus, goat farming is predicted to expand more and more.

As mentioned above, the northern region of Thailand still has a small number of goat farming. This may be due to some limitations such as; local people do not generally consume goat meat, or they may simply lack the knowledge of where to distribute and sell goat meat products. Goat farmers do not know where and how they can gain the most benefits out of the goats, which can be a primary problem or obstacle that hinders people from farming goats. Therefore, it is inevitable that goat farmers should learn about human capital so that they can gain fruitful knowledge and some important skills about goat farming. If farmers are well aware of this knowledge, they can prevent any potential risks, which can arise from any economic approach that does not view capital as just money but categorizes capital into many aspects, such as natural capital, social capital, etc. At present, human capital is widely accepted both in the agriculture and non-agriculture sector, which is an important factor driving various activities that can contribute to the goals that have been initially set effectively.

3.2 Human Capital and Sustainable Development of Goat Farmers

Human capital is one part of the concept of **"**Sustainable Livelihood**"** which is equivalent to the foundation of individualism. This consists of knowledge, abilities, skills or expertise, and experiences individuals have accumulated within themselves, and all those combined can become a potential or an essential, valuable resource. This can be advantageous in many circumstances and various contexts. Therefore, for one to be able to manage or find a way to make use of his potential or knowledge to the greatest extent, one must understand the qualities of **"**Human Capital**"** that are different from other resources. That is, people are **"**intangible assets" without depreciation like other assets; people are **"**human capital**"** that can build **"**added value**"** (Thosuwonchinda 2005). In other words, goat farming can be viewed as similar to planting trees. If people keep watering, cultivating, fertilizing, and paying attention, the plants will bloom and thrive. They will have stronger roots than those neglected ones. If goat farmers have competence in human capital, they will be prepared to deal with the risks mentioned above. Consequently, goat farmers in the upper north of Thailand can have a sustainable life and secure occupation.

The results of the interviews with goat farmers revealed that nowadays, goat farmers rely only on the prior knowledge of goat farming that comes from individuals' experiences. However, this is not enough because each goat farmer has a different experience in raising goats. Therefore, goat farmers must seek further knowledge and training to increase their body of knowledge as a guideline for goat-raising management along with building skills by practicing until they become experts in goat farming. This is evident and consistent with the concept of human capital that human capital is a body of knowledge and specific professional skills that are applied to benefit (Kamluang 2019) and under what Nigro (2007) said, human capital development is a process undertaken to achieve the development of human capital so that its potential is ready for the fulfillment of the mission assigned to it by the organization. By the tools used for the development of effective and accepted human capital that is human resource development, the new and old methods are used to obtain the results and performance both quantitatively and qualitatively. The determination of the target of human resource management, the basis according to the concept of "RDMU", has the following goals**:** 1) Recruitment and Selection 2) Development 3) Maintenance, and 4) Utilization.

Moreover, the non-participation survey of the observed areas revealed that government agencies in the areas have given importance to the development of goat farming potential with farmers to organize promotional activities and transfer or pass on knowledge about goat farming in the community. In addition, access to support and assistance in the development of goat potential from the academic department is always delivered. It operates in line with the sustainable development concept under the sustainable development goals (SDGs) framework of Goal 2 on

Figure 4 Development of goat farming potential with farmers to organize extensional activities

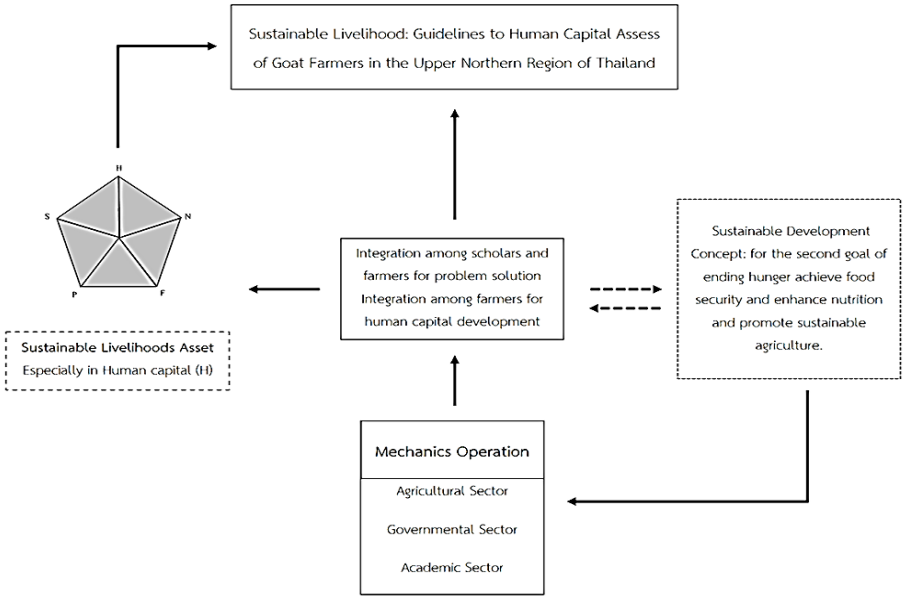

Figure 5 Guideline for human capital development for goat farmers

the issue of promoting sustainable agriculture to increase not only agricultural productivity and income of small food producers, indigenous people, farmers' households, goat farmers and fishermen, but also access to land resources and inputs, knowledge, financial services, markets and opportunities for value-adding and non-farm employment fairly and equally (Figure 4).

4 Conclusion

4.1 Approaches to Human Capital Development of Goat Farmers

In the conclusion of the article, sustainable livelihood: guidelines for human capital access of goat farmers in the upper northern region of Thailand, the study found that sustainable livelihood is based on the stable and sustainable development of "human capital". Also, it is responsible for future activities but before humans can become a factor of quality, and possibility, investments must be made to obtain skills, experience, and things that humans have accumulated, forged, and merged until we become who we are today. Then, we will call it "Human Capital." Together with intellectual knowledge and the ability to learn, people have accumulated specializations, skills, and experiences. Moreover, an important development mechanism is social, which consists of a network of relationships between the farmer, government, and academic sectors which can help drive and fulfill the sustainable development of human capital. The last aspect is emotions which consist of attributes such as self-awareness, integrity, as well as individual resilience (Figure 5).

4.2 Farmer and the importance of "Human Capital"

Finally, the researcher concluded that it could be seen that the "human capital" of the farmers is very important. Therefore, ensuring the sustainability of the development of farmers in each period is considered very significant for the livelihood of farmers as well. In other words, the more the farmers develop in the dimensions of specific knowledge and professional skills, the more the livelihood and quality of life of farmers will improve.

In addition, the government must be the center of learning to increase the capacity of farmers to develop quality, as it should be under support of the partnership, from the farmers, the community, and the academic sectors to achieve genuine development in the upper northern area and Thailand's livestock.

5 Recommendation

The development of goat farming in the northern region of Thailand–despite continuous development according to the potential of farmers, occupation, and assistance from some agencies–is still not acceptable as it should be. "Goat farming" is a profession that requires a certain level of knowledge and understanding in the field of livestock for one to have a successful career. Therefore, the search for such development paths is focused on the development principle, which is, first, the need for continuous development planning in goat farming. Secondly, there should be measures to increase the efficiency and quality of goat production to increase marketing channels, support innovation, and use various technologies, including agricultural management. Thirdly, costs from both the public and private sectors for the development of goat farming careers should be combined. Lastly, the self-reliance of farmers as the main goal should be promoted so that farmers can help themselves.

Conflicts of interest

The authors affirm that they do not have any conflict of interest

Ethics approval and consent to participate

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Authors' contributions

All authors discussed and designed the experiments:Thongmeethip K., Phayakka N., Sreshthaputra S. and Limnirankul B. conducted the main experiments and data analysis. All authors wrote, read, and agreed on the final manuscript.

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In Silico Targeting of influenza virus haemagglutinin receptor protein using Diosmetin, Tangeritin, and Anthocyanidins as potential drugs

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ABSTRACT

Influenza viruses cause acute respiratory illnesses in birds, humans, and other mammals, and are a major public health concern around the world. Pandemic flu could be caused by an unforeseen human adaptation of an influenza subtype or strain rather than currently circulating influenza viruses. The need for plant metabolites-based new anti-influenza drugs appears to be urgent. Blocking Haemeagglutinin (HA) protein is one of the most appealing drug targets to halt the growth of the virus. The influenza virus can acquire resistance to currently existing therapies, therefore necessitating the development of new medications. The plant's bioactive metabolites, flavanoids are having potential medicinal efficacy. The current study aimed to identify certain flavonoids (Diosmetin, Tangeritin, and Anthocyanidins) that might interact with the HA protein of the influenza virus and help in inhibiting its growth. We used PyRx v0.8 for virtual screening and docking studies. The highest binding affinity docked structures were analyzed using PyMOL and Discovery Studio Visualizer. The present study revealed that these naturally occurring compounds interacted with HA protein, resulting in the minimization of energy in the range

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of -5.2 to -7.0 kcal/mol. Diosmetin showed the best binding affinity of -7.0Kcal/mol. The molecular binding studies revealed that Diosmetin, Tangeritin, and Anthocyanidins are potential compounds to test against HA protein and can be used to develop effective anti-influenza agents.

1 Introduction

Influenza A (H1N1) virus causes acute respiratory illnesses in birds, humans, and other mammals, and is a serious public health issue globally (Kapoor and Dhama 2014; Bonilla-Aldana et al. 2020; Philippon et al. 2020; Kessler et al. 2021)**.** The influenza A virus (IAV) is known to spread by wild birds. Strain avians can adapt to their human host through acquired mutations and attain human-to-human transfer (Das 2012; Dhama et al. 2013; Yeo and Gan 2021). Rather than currently circulating influenza viruses, a pandemic flu could be triggered by an unanticipated human adaptation of an influenza subtype or strain. It is seasonal and kills between 2,50,000 and 5,00,000 people (Wu et al. 2017). The Spanish flu (1918 H1N1), Asian flu (1957 H2N2), Hong Kong flu (1968 H3N2), and swine flu (2009 H1N1) pandemics demonstrate the devastating socioeconomic and public health consequences of pandemic flu and keep us alert for the next one (Dhama et al. 2012; Joseph et al. 2017; Kessler et al. 2021;)**.** Approximately 50,000 people die each year from seasonal flu. Because of its high mortality (about 60%) and contagious nature, the H5N1 type IAV, infected 18 patients in Hong Kong and in 1997 six patients were killed, posing a major threat to human health in the coming time (Yang et al. 2013). The life cycle of the influenza virus has so far been thoroughly studied and various treatment targets have been validated. Thus, the need for new anti-influenza drugs appears to be pressing. Blocking Haemeagglutinin (HA) protein is one of the most appealing among them. HA is a homotrimer protein whose inactive monomer is HA0 which contains 566 amino acids, plus signal peptide is broken down into two subunits i.e. HA1 subunit contains 325 amino acids and HA2 contains 222 amino acids in the extracellular medium. Sialic acids on the surface of infected cells are recognized by the antigenic sites of the global head of the HA1 subunit. Because of the fusion peptide, the HA2 subunit is organized as a helix stem and is heavily involved in the membrane fusion process. There are now 18 subtypes of HA, which can be further split into 5 clades and 2 groupings. The flexible nature of HA makes rational medication creation extremely difficult. Both antigenic drift and shift may also help to increase the diversity of HA (Wilson et al. 1981; Stevens et al. 2006)**.** The current study discussed how HA protein can be a potential target in the influenza virus by using various flavonoids.

When virus influenza enters the endosome, the endosome's acidic pH i.e. about 5 causes a conformational modification in which the HA1 subunits separate from each other and the HA2 subunit goes through the spring-loaded process and converts into a linear helix, hence membrane fusion begins when fusion peptide enters the endosome membrane, allowing the influenza genome to be integrated into the host's cell. The previous study discovered invariant residues and SLs group in this loop zone, indicating that this location could be a promising target for avoiding therapeutic evasion (Lao and Vanet 2017). Nine residues make up this possible binding site. The residues E392, E397, E401, F393, N394, N405, and N402 are in variants, while SLs residues are K395 and K398. The influenza virus can acquire resistance to currently existing therapies, necessitating the development of new medications such as Zanamivir and Lanamivir (Chavan et al. 2014). Researchers showed that leaf extracts of *Jatropha curcas* contain many flavanoids, tannins and saponins that inhibit the hemagglutinin protein of the H1N1 virus (Patil et al. 2013). Liu et al. (2018) also conduct a study on other surface receptors such as Neuraminidase, of the influenza virus and concluded that herbal extracts of some medicinal plants have inhibitory action against the receptor and inhibit the growth of the influenza virus. The authors also revealed that *Allium sativum* and *Plumbago indica* inhibit the synthesis of viral nucleoprotein and polymerase activity which inhibits (H1N1) pdm09 virus (Chavan et al. 2016). He et al (2011) demonstrated the role of dandelion, a traditional Chinese medicine (TCM) against, human A/PR/8/34, influenza virus type A, and WSN (H1N1) and showed a negative effect against virus growth (He et al. 2011). Chavan et al (2014) used an *in-silico* approach against Neuraminidase, Hemagglutinin, and M2 protein channel of the H1N1/A/2009 virus by using Allicin and Plumbagin as a potential multidrug target. Therefore, the current study was designed to identify the potential utility of phytochemicals to interact with viral protein receptors i.e. haemagglutinin.

2 Materials and Methodology

2.1 Retrieval of three-dimensional receptor structure

3-D crystal structure of influenza haemagglutin in with PDB ID: 1HTM with the resolution of 2.50 Å was retrieved from the online database RCSB-PDB (Research Collaboratory Structural Bioinformatics-Protein Data Bank) (Figure 1) (Bullough et al. 1994). For docking studies, water molecules and heteroatoms were removed from the 3-D structure of the target protein.

2.2 Preparation of ligand's preparation and analysis of ADME properties

Three phytochemicals were selected as ligands for virtual screening and their 3-D structures were downloaded in sdf format

from PubChem. The Open Babel was run to convert df format of ligand structures into pdb format (Figure 1). The unfavorable absorption, distribution, metabolism, and elimination (ADME), the online tool was used to determine the profiling of studied ligands (pH 7) (Jayaram et al. 2012). Lipinski's rule of five, i.e. physiochemical properties of ligand viz. LogP (<5), H-bond acceptor (<10), molar refractivity, H-bond donor (5), molecular weight (<500 Da), and drug likeliness were considered (Lipinski 2004) (Table 1).

2.3 Molecular docking of phytochemicals with Influenza Haemagglutinin

PyRx v0.8 was used for virtual screening and docking studies. For energy minimization of ligands Universal force field (UFF) was applied and then ligands structures were converted into pdbqt by OpenBabel (O'Boyle et al. 2011). The highest binding affinity docked structures were visualized using Discovery Studio Visualizer and PyMOL.

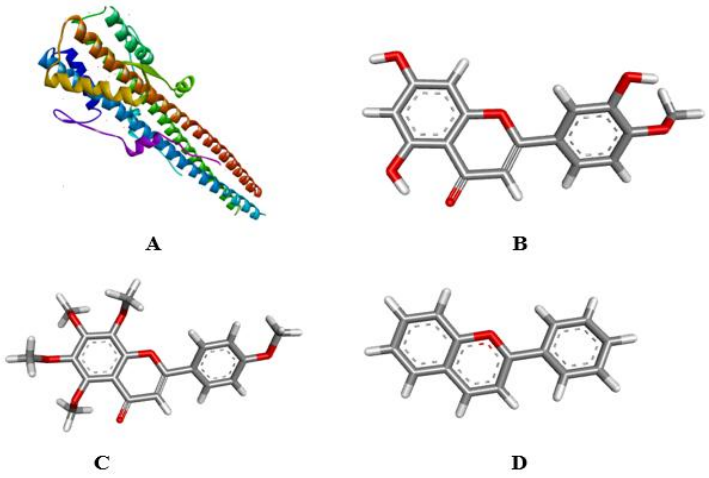

Figure 1 3D view of the receptor (A) Chemical structures of Diosmetin (B), Tangeritin (C), Anthocyanidins (D)

Table 1 Physio-chemical or ADME Properties of ligands

S. No.	Ligands (Pub Chem CID)	ADME Properties (Lipinski's Rule of Five)		Drug Likeliness
		Properties	Values	
1	Diosmetin (5281612)	Molecular weight (<500 Da)	300	Yes
		LogP (<5)	2.4	
		H-bond donor (5)	3	
		H-bond acceptor (<10)	6	
		Molar Refractivity	77.3	
2	Tangeritin (68077)	Molecular weight (<500 Da)	372	Yes
		LogP (<5)	3.3	
		H-bond donor (5)	0	
		H-bond acceptor (<10)	7	
		Molar Refractivity	98.5	
3	Anthocyanidins (145858)	Molecular weight (<500 Da)	20	Yes
		LogP (<5)	4.1	
		H-bond donor (5)	0	
		H-bond acceptor (<10)	1	
		Molar Refractivity	65.3	

Table 2 Binding affinity and RMSD value of Diosmetin with Influenza Haemagglutinin

Ligand	Binding Affinity (Kcal/mol)	rmsd/ub	rmsd/lb
Diosmetin_uff_E=178.56	-7.0	0	0
Diosmetin_uff_E=178.57	-6.9	6.096	1.183
Diosmetin_uff_E=178.58	-6.7	6.052	0.846
Diosmetin_uff_E=178.59	-6.4	5.988	1.019
Diosmetin_uff_E=178.60	-6.3	14.311	12.878
Diosmetin_uff_E=178.61	-6.3	14.277	12.886
Diosmetin_uff_E=178.62	-6.1	3.212	1.99
Diosmetin_uff_E=178.63	6.0	2.235	1.892
Diosmetin_uff_E=178.64	-6.0	2.408	1.517

Table 3 Binding affinity and RMSD value of Anthocyanidins with Influenza Haemagglutinin

Ligand	Binding Affinity (Kcal/mol)	rmsd/ub	rmsd/lb
Anthocyanidins_uff_E=324.34	-6.7	0	0
Anthocyanidins_uff_E=324.35	-6.4	7.495	1.799
Anthocyanidins_uff_E=324.36	-5.8	8.769	3.186
Anthocyanidins_uff_E=324.37	-5.7	8.218	2.242
Anthocyanidins_uff_E=324.38	-5.5	15.664	14.21
Anthocyanidins_uff_E=324.39	-5.4	15.886	14.356
Anthocyanidins_uff_E=324.40	-5.3	16.488	15.01
Anthocyanidins_uff_E=324.41	-5.3	18.023	15.401
Anthocyanidins_uff_E=324.42	-5.1	18.24	15.615

Table 4 Binding affinity and RMSD value of Tangeritin with Influenza Haemagglutinin

Ligand	Binding Affinity (Kcal/mol)	rmsd/ub	rmsd/lb
Tangeritin_uff_E=556.89	-5.7	0	0
Tangeritin_uff_E=556.90	-5.4	9.108	3.393
Tangeritin_uff_E=556.91	5.3	7.884	2.187
Tangeritin_uff_E=556.92	-4.9	33.138	29.191
Tangeritin_uff_E=556.93	-4.9	7.958	2.521
Tangeritin_uff_E=556.94	-4.9	33.723	29.442
Tangeritin_uff_E=556.95	-4.8	34.562	32.078
Tangeritin_uff_E=556.96	-4.8	16.347	14.342
Tangeritin_uff_E=556.97	-4.8	15.063	13.877

3 Results and Discussion

Nowadays *in silico* molecular docking is considered as an important method to find potential drug candidature. Computational screening offers the advantage of rapid, convenient, and cost-effective testing. Virtual screening suggested a mechanism of binding receptor proteins to molecules (Skariyachan et al. 2020). In virtual docking, the binding affinity score is calculated. Docked structures with the least binding energy and highest binding affinity were considered as the most stable structures. Current study results revealed that Diosmetin, Tangeritin, and Anthocyanidins showed binding affinity in the range of -5.2 to -7.0 kcal/mol with the target molecule (Tables 2, 3, and 4). Molecular docking studies revealed that among studied ligands diosmetin exhibited the highest binding affinity of -7.0 kcal/mol. Diosmetin interacts with Arg123, Phe138, Ser93, Ala96,

and Leu126 residues of influenza haemagglutinin. While anthocyanidin interacts via Leu126, Ala96, Val100, and Phe119.136 residues of target protein whereas tangeritin formed interactions with Ileu140, Val100, Arg123, and Leu126 of haemagglutinin. The docked pose of ligand and haemagglutinin (PDB ID: 1HTM) receptor has been shown in Figures 2, 3, and 4. Du et al. (2021) investigated the anti-influenza activity of an imidazopyridine based compound to inhibit the activity of group 2 IAVs and suggested it as a promising drug candidate. They also conducted *in silico* molecular docking interactions of their novel compound against hemagglutinins protein receptors (Du et al. 2021). In addition, Makau et al. (2017) explored the utility of 4-hydroxyquinolinone compound to inhibit the replication of the influenza virus and docked the said molecule against NP monomer (PDB ID: 2IQH) receptor protein (Makau et al. 2017). Results of the current study are also in agreement with an earlier study by Liu et al. (2015) who reported that quercetin, chlorogenic acid, oleanolic acid, and baicalein were potential inhibitors for neuraminidase of H7N9. Further, Behera et al. (2012) reported that B-Sialic acid and O-Sialic acid potentially inhibited both Hemagglutinin and Neuraminidase receptors from Influenza. Paramivir, Aramivire, Munimivire, and Anamivire were also reported as inhibitors for neuraminidase of H7N9 (Khan et al. 2017). Moreover, Sadati et al. (2019) reported that some

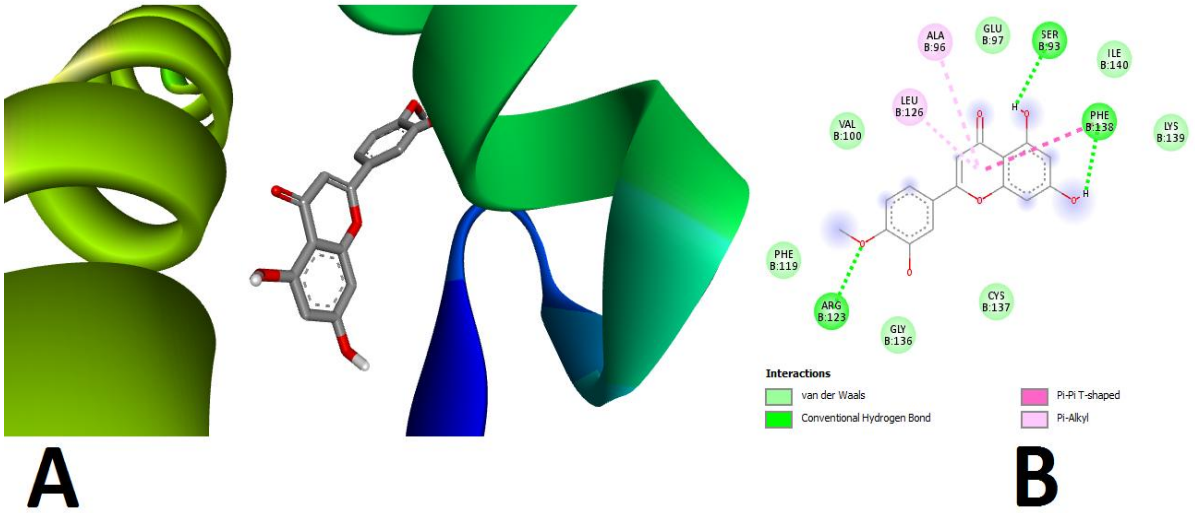

Figure 2 The molecular docking of Influenza Haemagglutinin and Diosmetin (A) Best binding mode in the pocket of protein; (B) The interacting amino acid of the target with ligand

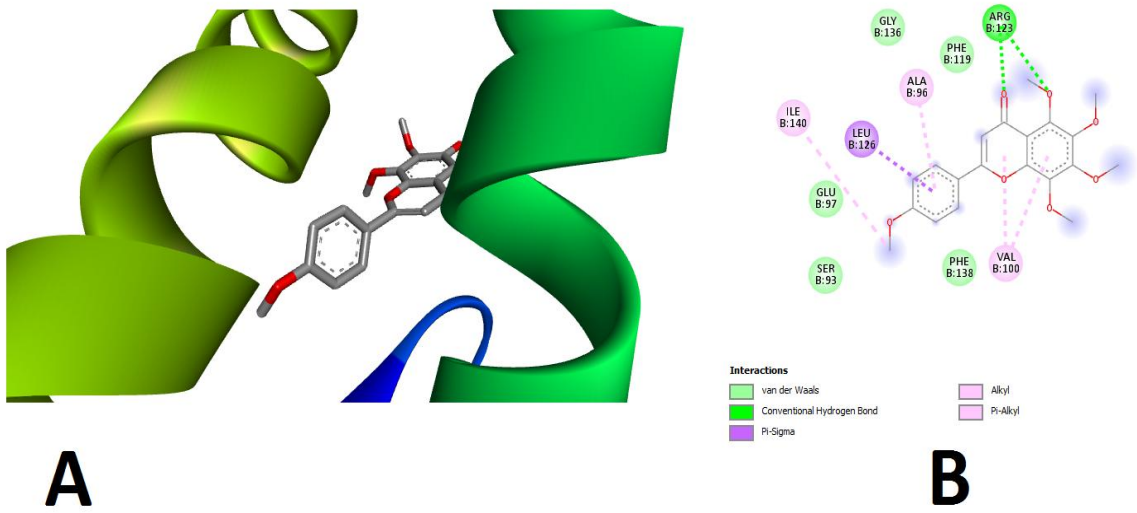

Figure 3 The molecular docking of Influenza Haemagglutinin and Tangeritin (A) Best binding mode in the pocket of protein; (B) The interacting amino acid of the target with ligand

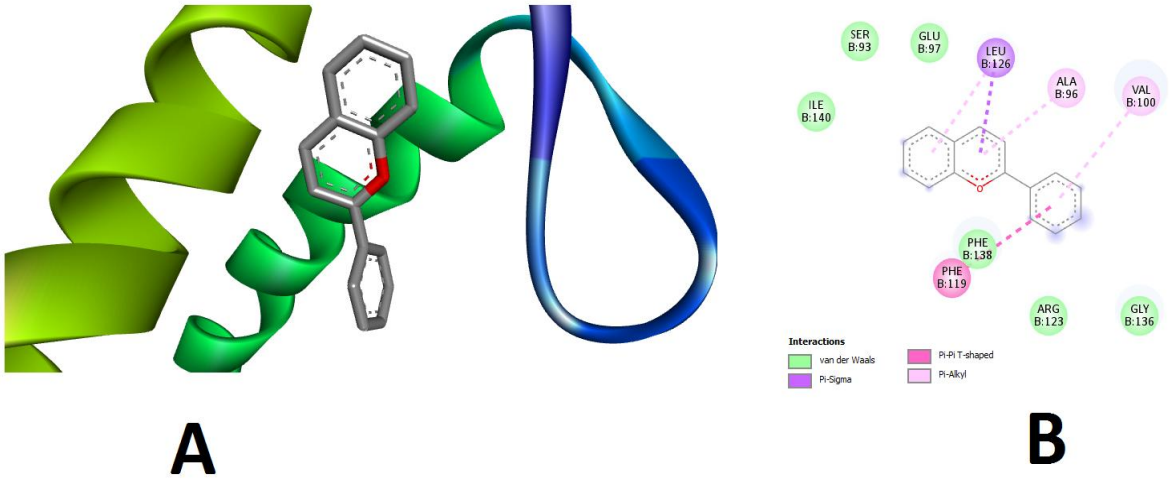

Figure 4 The molecular docking of Influenza Haemagglutinin and Anthocyanidins (A) Best binding mode in the pocket of protein; (B) The interacting amino acid of the target with the ligand

Table 5 Best binding affinity and interacting residues of studied ligands with Influenza Haemagglutinin

S. N.	Ligands	Binding Affinity (kcal/mol)	Interacting residues
1	Diosmetin	-7.0	Ser93, Phe138, Ala96, Leu126, Arg123
2	Tangeritin	-5.7	Arg123, Ala96, Leu126, Ile148, Val100
3	Anthocyanidins	-6.7	Leu126, Ala96, Val100, Phe138

flavonoids i.e. kaempferol, quercetin, luteolin, catechin, luteolin, hispidulin, chrysin, and vitexinmay act as anti-influenza agents. Recently Bui et al. (2022) reported natural alkaloids i.e. Berberine, lycorine, hemanthamine, aloperin, and dendrobine act as anti-influenza agents. Similarly, in silico docking studies of compounds as anti-influenza agents were reported in the literature (Hariyono et al., 2021; Mtambo and Kumalo 2022; Abdullahi et al., 2022a, b). Table 5 represented an overview of the current study and highlights top binding affinities and majorly interacting amino acid residues.

Conclusion

Influenza is considered to be an emerging viral infection in birds, humans, and various other mammals. Recent mutations in the structure of the virus further result in the development of drug resistance. Therefore, such drug resistance-based incidents are always to known initiate novel therapeutic agents with promising health benefits. The results of the present study explored molecular docking interactions between ligands (Diosmetin, Tangeritin, and Anthocyanidins) and the HA protein of IAV. In the present study, molecular interactions between HA protein and chosen phytochemicals were found in the range of -5.2 to -7.0 Kcal/mol. Therefore, naturally occurring metabolites can be studied in the future to design novel anti-IAV agents

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Effectiveness of Quercetin and Its Derivatives Against SARS CoV2 -*In silico* Approach

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ABSTRACT

The COVID-19 pandemic that erupted in November 2019 is continuing, with no effective antiviral agent to date. Synthetic antiviral agents have limitations such as a narrow range of therapeutic effectiveness of the activity, toxicity, and resistant viral strains and traditional antiviral medicines at large seem not to have these limitations. Here, some of the existing phytochemicals are cherry-picked for repurposing against the enzyme or protein targets of SARS CoV2, by the principles of structure-based drug design based on molecular docking studies. The most important drug targets of SARS CoV2 namely, Mpro protease (6LU7), RdRp polymerase (7BTF), and Spike glycoprotein of SARS CoV2(6VSB) were employed for docking analysis with chosen phytochemicals and binding affinity was calculated using PRODIGY software and docking sites determined using Chimera software. For docking studies, 160 phytochemicals were selected from a large pool of phytochemicals. Based on the binding affinity values, 61 phytoconstituents were selected for further in-silico screening which resulted in 15 phytochemicals, with higher binding affinity to spike glycoprotein of SARS CoV2. Moreover, Guaijaverin, Quercetin, Quercitrin, Quinic acid, and spiraeoside binds both to the spike glycoprotein of SARS Cov2 and the host receptor of human ACE2. Hence these compounds may serve as two-pronged drug candidates for SARS CoV2. In nutshell, we present a few phytochemical candidates with higher binding affinity to the Spike protein of SARS CoV2, which needs to be further optimized by in vitro studies to minimize the cytotoxicity and increase or retain the binding affinity, towards an effective antiviral drug against COVID 19.

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1 Introduction

The COVID-19 pandemic was recorded to have originated in the Chinese city of Wuhan around November 2019, caused by a novel zoonotic beta-coronavirus closely related to bat coronaviruses. The virus was named as Novel Corona Virus and later renamed SARS CoV2 due to its similarities with SARS (Severe Acute Respiratory Ryndrome) coronavirus first discovered in 2003 (Chatterjee et al. 2020; Dhama et al. 2020; Zheng, 2020). Globally now, more than 261 million people were affected by this pandemic and the death toll is more than 5 million. Unusual characteristics of SARS CoV2 infections are comparable to that of SARS and MERS and they share symptoms such as fever, fatigue, dry cough, and dyspnoea, leading to acute respiratory distress syndrome (ARDS) (Sharma et al. 2020). SARS CoV2 is highly transmissible and spreads through fomites, cough, and cold droplets, and human contact. Globally accepted prevention strategies such as hand sanitizing, wearing a mask, and keeping social distance are still counted as crucial while vaccine and drug research are proceeding at war footing (Chiu et al. 2020).

By now, many prophylactic vaccines are available against COVID 19 and a few chemical compounds such as chloroquine, remdesivir, lopinavir, molnupiravir, paxlovid, and ritonavir have shown favorable results *Invitro* and clinical studies. The SARS CoV2 genome has about ten ORFs (open reading frames). ORF-1 which covers a major region of viral RNA encodes 16 NSPs (non-structural proteins). RNA-dependent RNA Polymerase (RdRp) main protease (M pro) and Papain-like protease (PLpro) are the major NSPs while spike (S), envelope (E), nucleo-capsid (N), and membrane (M) proteins are the four main structural proteins (Luk et al. 2019; Fahmi et al. 2020). The SARS CoV2 replication cycle mainly includes virus entry, germination of virions, genome replication, and assembly. M-pro, RdRp, and spike glycoproteins are the most important viral proteins aiding the spreading of the virus. The Spike glycoprotein region of Covid-19 is considered as the region responsible for transmission by binding with angiotensin-converting enzyme 2 (ACE2) (Cagiliani et al. 2020; Letko et al. 2020; Lu et al. 2020; Luan et al. 2020). M-pro cleaves polyprotein towards forming of replication-transcriptase complex. The NSP12 region which is otherwise known as the RdRp region binds with NSP-7 and NSP-8 regions of the whole genome for the initiation of the replication of the virus (Romano et al. 2020). Interruption of any stage of viral entry or replication cycle is expected to be a potential strategy for the development of antiviral agents (V'Kovski et al. 2021).

An effective strategy for developing antiviral agents will be by interrupting any stage of viral entry or replication cycle against SARS CoV2. We screened a vast number of phytochemicals, reported to have significant antiviral activity and or extensively used as traditional medicines of plant origin. Synthetic antiviral agents have limitations such as a narrow spectrum of activity, limited therapeutic usefulness, toxicity, and resistant viral strains, and traditional antiviral medicines at large seem not to have these limitations. Whereas, phytochemicals from traditional medicine represent a vast repertoire of pharmacologically active substances with less toxicity and some of them are well researched lately. Here, some of the existing phytochemicals are cherry-picked for repurposing against the enzyme or protein targets of SARS CoV2, by the principles of structure-based drug design based on molecular docking studies. Such a computation-based approach will not only hasten drug discovery but will also lead toward specific antiviral agents. The current study aimed to deduce a library of potential antiviral agents against SARS CoV2 by *in silico* approach.

2 Materials and Methods

2.1 Selection of phytoconstituents and protein

From previous reports and traditional knowledge (Ethnopharmacology), based on reported anti-inflammatory as well as antiviral activities total of 160 phytoconstituents were selected (Mukhtar et al. 2008; Pushpa et al. 2013; Lin et al. 2014; Ma et al. 2015; Domitrovic and Potocnjak 2016; Bachar et al. 2021; Idrees et al. 2021; Ambrose et al. 2022; Sharma et al. 2022). The SDF files of the phytoconstituents were redeemed from the database of chemical molecules named PubChem (https://pubchem.ncbi.nlm.nih). Mol2 and PDB structures of selected phytoconstituents were deduced using Open babel software (O'Boyle et al. 2011). M protease, RdRp polymerase, spike glycoprotein, and ACE2 human receptor are the four targets selected for interaction studies. All the corresponding structures were redeemed from the databank of protein – RCSB PDB, 6LU7 for Mprotease, 7BTF for RdRP polymerase, 6VSB for SARS CoV2 Spike glycoprotein and 6M17 for Human ACE2 receptor (www.rcsb.org).

2.2 Docking analysis

The selected phytoconstituents were screened individually as in figure 1 by measuring the interactivity with targeted proteins using the software tool Auto Dock Vina (Trott and Olson 2010). For further analysis, the structures obtained using docking were redeemed in the PDB format and affinity values for binding were determined using PRODIGY software (Xue et al. 2016). The Chimera software (version.1.13.1) was used for the analysis of docked structures for calculating the possible bond distances obtained from intra as well as inter-hydrogen bonding (Pettersen et al. 2004). The chemical structures of selected phytoconstituents are redeemed from EMBL-EBI (www.ebi.ac.uk/chebi /).

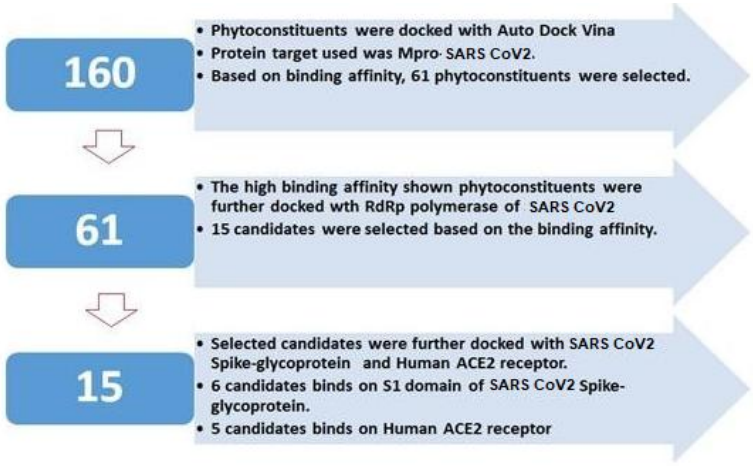

Figure 1 Flowchart represents the screening strategy for the selection of anti-SARS CoV2 phytoconstituents

2.3 ADMET and drug-likeness evaluation

The SMILE (canonical simplified molecular input line entry systems) of selected 15 phytoconstituents were considered for the molecular as well as pharmacokinetic evaluation- *in-silico* in the Swiss ADME tool. The adsorption, desorption, metabolism, and excretion (ADME) predictions were computed in the Swiss ADME tool for blood-brain barrier permeability, Log Kp of skin permeation value, gastro-intestine absorption, P-gp substrate and cytochrome-P inhibitors (Daina et al. 2017). For understanding the toxicological behavior of the selected phytoconstituents, the Pro Tox II was used for evaluating immunotoxicity, carcinogenicity, mutagenicity, cytotoxicity, LD50, etc. and Osiris property explorer was used for determining irritant properties (Banerjee et al. 2018).

3 Results and Discussion

3.1 Selection and screening of phytoconstituents

The most important drug targets of SARS CoV2 namely, Mpro protease (6LU7), RdRp polymerase (7BTF), and Spike glycoprotein of SARS CoV2 (6VSB) were employed for docking analysis with the chosen phytochemicals. From the docked structures, binding affinity was calculated using PRODIGY software and docking sites were determined using Chimera software. For docking studies, 160 phytochemicals were selected from a large pool of phytochemicals known to have ethnopharmacological indications and or reported antiviral activity and were docked with Mpro (6LU7) protease and the binding energy

Table 1 Comparison of binding affinities to some phytochemical constituents and control drug on Mpro protease (6LU7) and RdRp Polymerase (7BTF)

S. N.	Name of the Ligand	ChemID	Ligand Source	Binding affinity Mpro Protease: 6LU7 (KCal/Mol)	Binding affinity RdRp polymerase: 7BTF (KCal/Mol)
1	Apigenin	CHEBI:18388	*Ficus mucuso*	-7.8	-7.6
2	Ursolic acid	CHEBI:9908	*Malus domestica,* *Radermachera boniana* *Rubia yunnanensis* *Symplocos lancifolia* *Ficus mucuso* *Prunus domestica* *Terminalia catappa* *Rhododendron ferrugineum* *Rosa laevigata*	-7.6	-7.7
3	Oleanolic acid	CHEBI:37659	*Radermachera boniana* *Symplocos lancifolia* *Diospyros kaki* *Juglans sinensis* *Rhododendron ferrugineum*	-7.8	-7.6
4	Vasicinolone	CID: 13970119	*Adathoda vassica*	-6.15	-8.04
5	Vasicol	CID:92470596	*Adathoda vassica*	-6.27	-7.8

S. N.	Name of the Ligand	ChemID	Ligand Source	Binding affinity Mpro Protease: 6LU7 (KCal/Mol)	Binding affinity RdRp polymerase: 7BTF (KCal/Mol)
6	Anisotine	CHEBI:2738	*Adathoda vassica*	-6.87	-9.08
7	Quinic Acid	CHEBI:17521	*Klenia grandiflora* *Quercus pedunculata* *Arabidopsis thaliana*	-7.86	-8.81
8	Avicequinone C	CID:10563004	*Glycosmos pentaphylla*	-7.20	-7.00
9	Guaijaverin	CID:5481224	*Psidium guajava*	-8.60	-8.00
10	Marmeleide/ Imperatorin	CID:10212	*Aaglemarmelos*	-7.10	-7.50
11	Quercetin	CHEBI:16243	*Mimosa diplotricha* *Ophioglossum pedunculosum* *Lepisorususs uriensis*	-8.00	-7.90
12	Saponin	CHEBI:26605	*Asparagus racemosus* *Randia spinosa* *Gymnema sylestre* *Bacopa monnieri* *Ficus hispida* *Clerodendrum serratum* *Mimusops elengi* *Coscinium fenestratum* *Achyranthes aspera* *Putranjiva roxburghii* *Saraca asoca* *Symplocos racemosa* *Hemidesmus indicus* *Terminalia arjuna*	-8.10	-9.50
13	Isorhamnetin	CID:5281654	*Trigonella foenum graecum*	-7.30	-8.00
14	Kaempferol	CHEBI:28499	*Pittocaulon velatum* *Ficus mucuso*	-7.50	-8.20
15	Ellagic Acid	CHEBI:4775	*Myrciaria jaboticaba*	-8.30	-7.90
16	Carpaine	CHEBI:3433	*Carica papaya* *Trigonella foenum graecum*	-7.70	-8.80
17	Graecunin E	CID:156783	*Trigonella foenum graecum*	-8.40	8.80
18	Fenugreekine	CID:444170	*Trigonella foenum graecum*	-8.20	-8.80
19	Yamogenin	CHEBI:10086	*Trigonella foenum graecum*	-7.40	-7.10
20	Diosgenin	CHEBI:4629	*Dioscorea bulbifera* *Trigonella foenum graecum*	-7.40	-7.00
21	Smilagenin	CID:91439	*Trigonella foenum graecum*	-7.50	-7.00
22	Sarasaspogenin	CID:92095	*Trigonella foenum graecum*	-7.50	-7.00
23	Tigogenin	CHEBI:9595	*Trigonella foenum graecum*	-7.50	-7.00
24	Neotigogenin	CHEBI:80752	*Trigonella foenum graecum*	-7.30	-7.00
25	Gitogenin	CHEBI:5363	*Trigonella foenum graecum*	-7.50	-7.10
26	Neogitogenin	CHEBI:80854	*Trigonella foenum graecum*	-7.50	-7.00
27	Yuccagenin	CID:3083608	*Trigonella foenum graecum*	-7.40	-7.00

S. N.	Name of the Ligand	ChemID	Ligand Source	Binding affinity Mpro Protease: 6LU7 (KCal/Mol)	Binding affinity RdRp polymerase: 7BTF (KCal/Mol)
28	Rutin	CHEBI:28527	*Physalis longifolia* *Trigonella foenum graecum*	-8.10	-9.10
29	Vitexin	CHEBI:16954	*Eminium spiculatum* *Trigonella foenum graecum*	-8.20	-7.70
30	Isovitexin	CHEBI:18330	*Ficus deltoidea* *Trigonella foenum graecum*	-7.50	-7.10
31	Sigmastadienol	CID:129636643	*Vermonia anthelmitia*	-7.80	-7.00
32	Stigmasterol	CHEBI:28824	*Vermonia anthelmitia*	-7.60	-7.80
33	Luteolin	CHEBI:15864	*Cynaldon dactylon*	-7.90	-7.50
34	Orientin	CHEBI:7781	*Sonchus arvensis* *Cynaldon dactylon*	-8.20	-8.60
35	Neoxanthin	CHEBI:25501	*Cynaldon dactylon*	-7.80	-7.20
36	Violaxanthin	CHEBI:27295	*Cynaldon dactylon*	-7.60	-7.30
37	Asiaticoside	CHEBI:79928	*Centella asiatica*	-8.00	-9.90
38	Madecassoside	CHEBI:66651	*Centella asiatica*	-8.90	-8.50
39	Narcissin	CID:5481663	*Aerva lanatta*	-9.10	-8.50
40	Sitogluside	CID:5742590	*Aerva lanatta*	-7.40	-7.80
41	Solanine	CHEBI:9188	*Solanum tuberosum* *Solanum lycopersicum* *Solanum melongena* *Aerva lanatta*	-8.00	-9.50
42	Chaconine	CID:4115417	*Aerva lanatta*	-9.20	-9.30
43	Kaempferol-3-α-D Glucoside	CID:44258798	*Aerva lanatta*	-8.10	-8.00
44	Mangiferrin	CID:5281647	*Mangifera indica*	-7.50	-8.10
45	Artemisinin	CHEBI:223316	*Artemisia annua*	-6.80	-6.70
46	Hyperoside	CHEBI:67486	*Quercetin derivative*	-7.80	-8.20
47	Isoquercitrin	CID:5280804	*Quercetin derivative*	-8.50	-8.10
48	Spiraeoside	CID:5320844	*Quercetin derivative*	-7.90	-8.10
49	Quercitrin	CHEBI:17558	*Quercetin derivative*	-8.20	-8.10
50	Avicularin	CHEBI:65460	*Juglans regia* *Foeniculum vulgare* *Quercetin derivative*	-8.50	-7.60
51	Protocatechuic acid	CID:72	*Hibiscus sabdariffa*	-5.40	-6.20
52	Caffeic acid	CHE BI:36281	*Eucalyptus globulus*	-5.70	-5.60
53	Liquiritin	CHE BI:80845	*Polygonum aviculare* *Artemisia capillaris*	-8.00	-7.80
54	Hesperidin	CHEBI:28775	*Citrus aurantium*	-8.80	-9.50
55	Apigetrin	CHEBI:11595	*Teucrium gnaphalodes*	-7.80	-8.20

S. N.	Name of the Ligand	ChemID	Ligand Source	Binding affinity Mpro Protease: 6LU7 (KCal/Mol)	Binding affinity RdRp polymerase: 7BTF (KCal/Mol)
56	Rosmarininc acid	CID:5281792	*Salvia rosmarinus* *Perilla frutescens* *Salvia officinalis* *Mentha arvense* *Ocimum basilicum.*	-7.60	-7.50
57	Oxypeucedaninhydrate	CID:5281792	*Ferulago sylvatica*	-7.30	-7.30
58	Byakangelicin	CHEBI:3250	*Murraya koenigii* *Triphasia trifolia*	-7.00	-7.50
59	Glycyrrhizin	CID:128229	*Glycyrrhiza uralensis* *Glycyrrhiza inflata*	-7.10	-9.60
60	Nobiletin	CHEBI:7602	*Citrus tankan*	-6.60	-6.50
61	6-Gingerol	CID:442793	*Illicium verum* *Piper nigrum*	-5.80	-5.50
62	Ramdesivir	CID:121304016	*Control-Drug*	-7.20	
63	Saquinavir	CID:441243	*Control-drug*		-9.2

exhibited a range of -3.50 to 9.20 Kcal /Mol (Mukhtar et al. 2008; Pushpa et al. 2013; Lin et al.2014; Domitrovic and Potocnjak 2016; Ma et al. 2015;). As a standard for the analysis Mpro was also docked with the known antiviral agent Remdesivir and the corresponding binding affinity of -7.20Kcal/Mol, was considered as the cut-off value for the initial screening. Based on the binding affinity values, 61 phytoconstituents were selected for further in-silico screening.

RdRp polymerase (7BTF) is responsible for the replication of the virus and so the selected phytochemicals were docked with 7BTF and a binding affinity ranging from -5.7 to -10.20 Kcal/Mol was observed, while saquinavir, an accepted antiviral drug taken as control, had an affinity of -9.2 Kcal/Mol with RdRp polymerase. Table 1 represents the binding affinity of selected candidates towards Mpro and RdRp polymerase. Based on the binding affinity towards Mpro and RdRP polymerase, 15 phytochemicals were shortlisted for further analysis as mentioned in figure 1.

3.2 Protein-ligand interaction study: With spike glycoprotein of SARS CoV2

Spike, a class 1 fusion protein is the surface glycoprotein of SARS CoV2 and is responsible for viral attachment with human ACE2 (Angiotensin-converting enzyme 2) receptor and its consecutive fusion with the host cells, which follows the S1 subunit of the protein attached to the ACE2 receptor via its RBD region, the receptor binding domain and protein changes its conformation to a post-fusion form. The key amino acids of the S1 subunit, responsible for viral attachment, are reported as LEU455, PHE486, GLN493, SER494, ASP501, and TYR505, present in the ACE2 receptor binding region (333-527 residues) (Yuan et al. 2020). The S2 subunit is composed of a HR1, HR2, FP, TM domain, and cytoplasmic domain fusion (CT). S2 is responsible for viral fusion and entry but not actively involved in viral attachment and after binding, the protein changes to a post-fusion form. In addition to that the interacting regions of TMPRSS2- transmembrane protease serine 2 of the viral spike proteins at 685-ARG / 686-SER and 815-ARG / 816-SER.

Table 2 and figure 2 represent the protein-ligand interaction studies of 15 phytochemicals, among them higher binding affinity showing phytoconstituents are Asiaticoside (-12.04 Kcal/mol), Fenugreekine (-15.05 Kcal/mol) and Graecunin E (-18.81 kcal/mol), which were observed to bind with the S2 cavity region of spike glycoprotein. This could potentially contribute to the high binding affinities of these compounds. But Guaijaverin derived from *Psidium guajava* shows a binding affinity of -11.22 Kcal/mol and forms hydrogen bond in the S1 domain of spike glycoprotein, especially at the ACE2 receptor binding region, i.e., R466 and R355. In other words, other phytochemicals binding to the S1 subunit, such as Avicequinone C, hyperoside, quercetin, spiraeoside, 9 benzyl 2 fluoro 9 hpurin 6amine show considerably less binding affinity than Guaijaverin, but binds near RBD with binding amino acids positions such as avicequinone C-R355, hyperoside -N544, quercetin-S514 &R355, spiraeoside-I312, 9 benzyl 2 fluoro 9 hpurin 6 amine-T478. An interesting fact is that Guaijaverin, hyperoside and spiraeoside are quercetin derivatives. Another quercetin derivative, quercitrin binds to the R815 of the S2 domain, the TMPRSS2 binding site of spike glycoprotein. The interaction of TMPRSS2 to the spike protein is a crucial port for viral entry. The druggability will be more for the compound that interacts with the TMPRSS2 region that interacts with the C-terminal

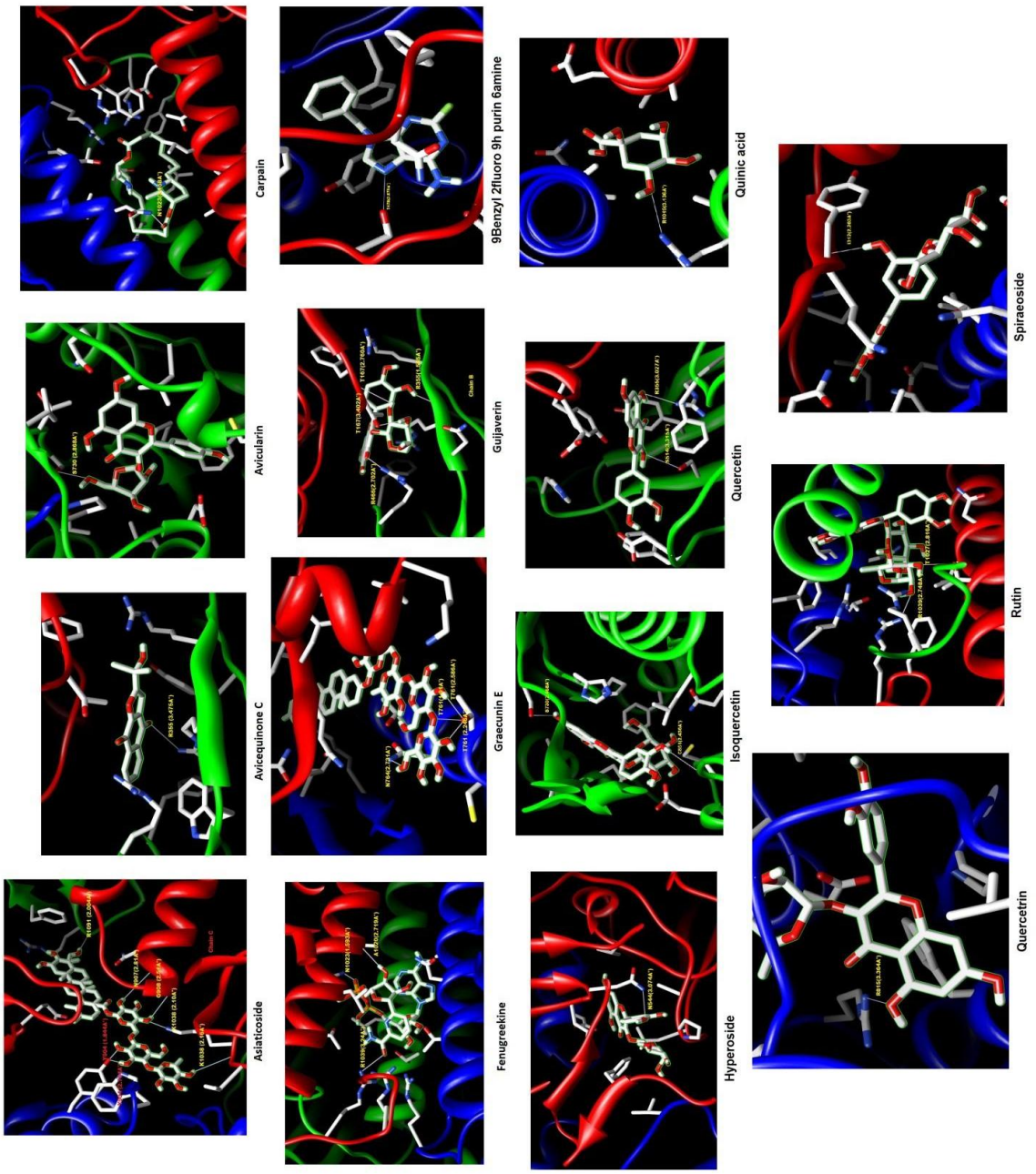

Figure 2 Binding regions of selected phytoconstituents with SARS CoV2spike glycoprotein

cleavage site of the S2 domain (Arg815/Ser816) of SARS-CoV-2 spike protein than in the N-terminal cleavage site observed in S2 domain (Arg685/Ser686) (Hussain et al. 2020).

3.3 Protein-ligand interaction study with ACE2, as the viral receptor

ACE2 receptor plays a pertinent role in the spreading of the disease by acting as a host receptor for the SARS CoV2. The residues 24-GLN, 82-MET, 79-ILE, 31-LYS, 34-HIS, 37-GLU, 354-GLY, 325-GLN, 38-ASP, 330-ASN, 329-GLU, 42-GLN and 45-LEU of ACE2 interact with RBD region of spike glycoprotein of SARS CoV2 (Vardhan and Sahoo 2020). So, from the present study, among the 15 phytochemicals that bind to the spike glycoprotein, Guaijaverin (ASN210), Quercetin (ASN210, GLU564), Quercitrin (ASN210), Quinic Acid (ALA296) and Spiraeoside (GLU495) binds to the ACE2 receptor.

Table 2 Binding affinities of the compounds on the Spike glycoprotein (6VSB) and their interaction with the binding site

Sl No	Name of the ligand	Binding region Spike protein	Binding Affinity with Spike glycoprotein-6VSB (Kcal/Mol)	Hydrogen bonding interactions with residues & Bond length (A°)
1	Asiaticoside	S2	-12.04	K1038 (2.16A°) Chain C K1038 (2.10A°) Chain C G908 (2.51A°) Chain C N907 (2.81A°) Chain C R1091 (2.004 A°) Chain B Y904 (2.764A°) Chain A Y904 (1.844 A°) Chain A
2	Avicequinone C	S1	-9.76	R355(3.475A°) Chain B
3	Avicularin	S2	-7.2	S730(2.868A°) Chain B
4	Carpaine	S2	-10.31	N1023 (3.158A°) Chain A
5	Fenugreekine	S2	-15.05	R1039 (3.24A°) Chain B A1020 (2.719A°) Chain C N1023 (1.593A°) Chain C
6	Graecunin E	S2	-18.81	N764 (2.731A°) Chain A T761 (2.246A°) Chain A T761 (1.914 A°) Chain A T761 (2.586 A°) Chain A
7	Guaijaverin	S1	-11.22	R466 (2.702A°) Chain B R355 (1.545 A°) Chain B T167 (3.402A°) Chain C T167 (2.760 A°) Chain C
8	Hyperoside	S1	-7.6	N544 (3.074A°) Chain B
9	Isoquercitrin	S2	-7.8	C851 (2.436A°) Chain B S730 (2.468A°) Chain B
10	Quercetin	S1	-7.91	S514 (3.315 A°) Chain B R355 (3.027A°) Chain B
11	Quercitrin	S2	-7.8	R815 (3.364A°) Chain B
12	Rutin	S2	-8.97	R1039 (2.748A°) Chain B R1029 (2.816A°) Chain B
13	Spiraeoside	S1	-7.6	I312 (2.203A°) Chain B
14	9Benzyl 2fluoro 9h purine 6amine	S1	-5.12	T478(2.875A°) Chain B
15	Quinic Acid	S2	-6.47	R1019 (3.138A°) Chain B

Table 3 The selected compounds interact with binding sites of ACE2 receptor molecules (6M17) and their bond distances

S. N.	Name of the ligand	Hydrogen bonding interactions with ACE2 receptor (6M17) residues & Bond length (A°)
1	Guaijaverin	N 210 (1.417 A°) Chain B
2	Quercetin	N 210 (2.884 A°) Chain B E 564 (2.134 A°) Chain B
3	Quercitrin	N 210 (3.434 A°) Chain B
4	Quinic acid	A 396 (2.696 A°) Chain B
5	Spiraeoside	E 495 (2.880 A°) Chain B

The phytochemical candidates which possess affinity towards spike binding regions of the ACE2 receptor may inhibit the interaction of spike glycoprotein to the ACE2 receptor (Table 3 and figure 3) (Vardhan and Sahoo 2020; Sahoo et al. 2020). Hence these compounds may serve as two-pronged drug candidates for SARS CoV2 and must be tested in cell culture models of SARS CoV2 infection.

3.4 Insilico Drug likeness and Toxicity analysis

The effectiveness of drug candidates was evaluated using ADME / Tox properties. Using the Swiss ADME, selected fifteen phytoconstituents were analyzed and the obtained results were compared with Ramdesivir and Saquinavir for establishing the drug-likeness nature of the candidates (Daina et al. 2017). Lipinski's rule

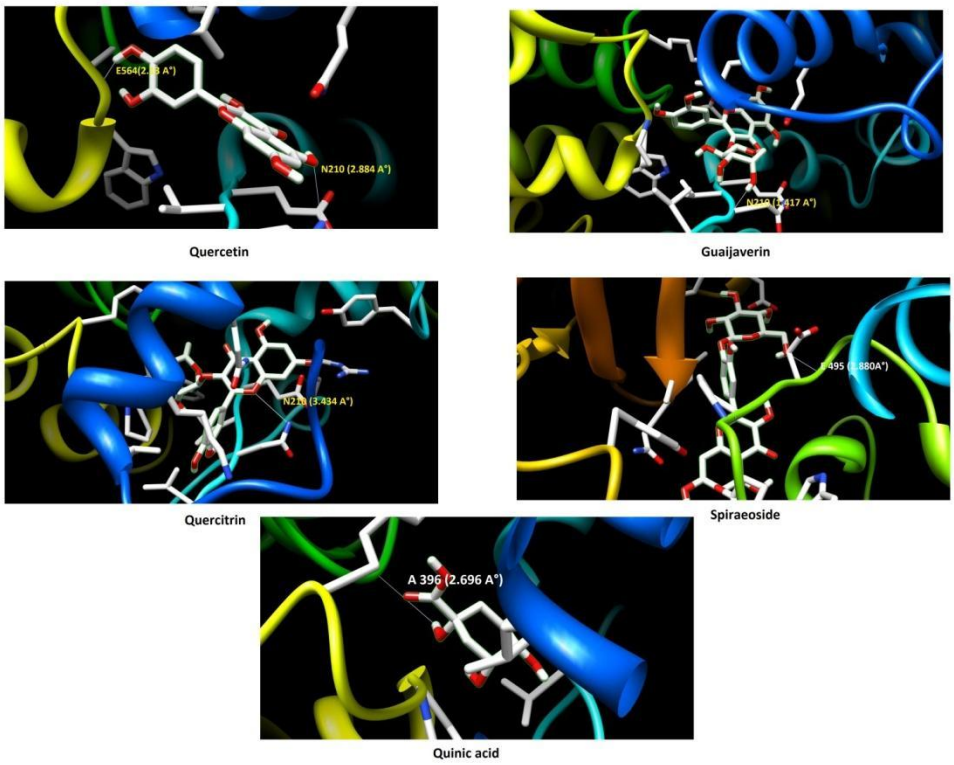

Figure 3 Binding regions of selected phytoconstituents with Human ACE2 receptor.

Table 4 Drug Likeness prediction of Compounds

Molecule	Formula	MW	NRB	NHA	NHD	TPSA	iLOGP	Lipinski rule of five violations
Asiaticoside	$C_{48}H_{78}O_{19}$	959.12	10	19	12	315.21	2.5	3
9 Benzyl 2 fluoro 9h purin 6 amine	$C_{12}H_{10}FN_5$	243.24	2	4	1	69.62	2.01	0
Avicularin	$C_{20}H_{18}O_{11}$	434.35	4	11	7	190.28	1.86	2
Avicequinone C	$C_{15}H_{12}O_4$	256.25	1	4	1	67.51	2.13	0
Carpaine	$C_{28}H_{50}N_2O_4$	478.71	0	6	2	76.66	4.4	0
Fenugreekine	$C_{21}H_{27}N_7O_{14}P_2$	663.43	11	18	8	346.89	0.24	3
GraecuninE	$C_{51}H_{82}O_{22}$	1047.18	11	22	12	335.06	3.78	3
Guaijaverin	$C_{20}H_{18}O_{11}$	434.35	3	11	7	190.28	1.61	2
Hyperoside	$C_{21}H_{20}O_{12}$	464.38	4	12	8	210.51	2.11	2
Isoquercitrin	$C_{21}H_{20}O_{12}$	464.38	4	12	8	210.51	0.94	2
Quercetin	$C_{15}H_{10}O_7$	302.24	1	7	5	131.36	1.63	0
Quercitrin	$C_{21}H_{20}O_{11}$	448.38	3	11	7	190.28	1.27	2
Quinic acid	$C_7H_{12}O_6$	192.17	1	6	5	118.22	-0.12	0
Rutin	$C_{27}H_{30}O_{16}$	610.52	6	16	10	269.43	2.43	3
Spiraeoside	$C_{21}H_{20}O_{12}$	464.38	4	12	8	210.51	1.45	2
Ramdesivir	$C_{27}H_{35}N_6O_8P$	602.58	14	12	4	213.36	3.24	2
Saquinavir	$C_{38}H_{50}N_6O_5$	670.84	16	7	5	166.75	3.66	2

MW - Molecular weight, NHD - Number of Hydrogen Donor, NRB - Number of rotatable bonds, NHA - Number of Hydrogen Acceptor, TPSA - Total polar surface area

Table 5 ADME Predictions of selected phytoconstituents

Molecule	Formula	GI absorption	BBB permeability	Pgp substrate	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor	log Kp (cm/s)
Asiaticoside	$C_{48}H_{78}O_{19}$	Low	No	Yes	No	No	No	No	No	-12.08
9Benzyl 2fluoro 9h purin 6amine	$C_{12}H_{10}FN_5$	High	Yes	No	Yes	No	No	Yes	Yes	-6.27
Avicularin	$C_{20}H_{18}O_{11}$	Low	No	No	No	No	No	No	No	-8.25
Avicequinone C	$C_{15}H_{12}O_4$	High	Yes	No	Yes	No	No	No	Yes	-6.44
Carpaine	$C_{28}H_{50}N_2O_4$	High	No	Yes	No	No	No	No	No	-4.75
Fenugreekine	$C_{21}H_{27}N_7O_{14}P_2$	Low	No	No	No	No	No	No	No	-14.55
GraecuninE	$C_{51}H_{82}O_{22}$	Low	No	Yes	No	No	No	No	No	-13.62
Guaijaverin	$C_{20}H_{18}O_{11}$	Low	No	No	No	No	No	No	No	-8.64
Hyperoside	$C_{21}H_{20}O_{12}$	Low	No	No	No	No	No	No	No	-8.88
Isoquercitrin	$C_{21}H_{20}O_{12}$	Low	No	No	No	No	No	No	No	-8.88
Quercetin	$C_{15}H_{10}O_7$	High	No	No	Yes	No	No	Yes	Yes	-7.05
Quercitrin	$C_{21}H_{20}O_{11}$	Low	No	No	No	No	No	No	No	-8.42
Quinic acid	$C_7H_{12}O_6$	Low	No	Yes	No	No	No	No	No	-9.15
Rutin	$C_{27}H_{30}O_{16}$	Low	No	Yes	No	No	No	No	No	-10.26
Spiraeoside	$C_{21}H_{20}O_{12}$	Low	No	Yes	No	No	No	No	No	-8.2
Ramdesivir	$C_{27}H_{35}N_6O_8P$	Low	No	Yes	No	No	No	No	Yes	-8.62
Saquinavir	$C_{38}H_{50}N_6O_5$	Low	No	Yes	No	No	No	No	Yes	-7.38

Log Kp - skin permeation value; GI - gastro-intestinal; BBB - blood–brain barrier; P-gp - P-glycoprotein; CYP - cytochrome-P

of five violations gives insight into recommending molecules as orally administrable drug candidates. Two or more violations lead to non-recommendation, and among fifteen candidates Asiaticoside, Fenugreekine, Graecunin E and Rutin show a violation of the rule and are considered to be non-recommendable for oral administration (Table 4). From the Swiss ADME analysis, 9 benzyl 2 fluoro 9H purin 6 amine, Avicequinone C, Carpaine, and quercetin shows high gastrointestinal (GI) absorption while 9 benzyl 2 chloro 9h purine 6 amine, Avicequinone C shows blood-barrier (BBB) permeation (Table 5). Like Ramdesivir and Saquinavir, Asiaticoside, Carpaine, Graecunin E, Quinic acid, Rutin, and Spiraeoside may act as substrates of permeability glycoprotein (Pgp). The CYP inhibition of candidates may lead to toxic or unwanted adverse effects. Asiaticoside, Avicularin, Carpaine, Fenugreekine, Graecunin E, Guaijaverin, Hyperoside, Isoquercitrin, Quercitrin, Quinic acid, Rutin and Spiraeoside are potential non-inhibitors for CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4 while remdesivir and saquinavir are potential inhibitors of CYP3A4. In this sense, the above-mentioned phytochemical compounds are safer for human administration than remdesivir and saquinavir.

LD_{50} values indicate the acute toxicity and toxicity classification 1(toxic) and 6(non-toxic) of phytoconstituents. Based on LD_{50}, only Graecunin E (55mg/Kg) and quercetin (159 mg/Kg) have shown higher acute toxicity than Ramdesivir (1000 mg/Kg) which is class 4 (Table 6). From the toxicology prediction data obtained from Pro ToxII, Carpaine, Fenugreekine, and Quinic acid act as non-hepatotoxic, non-carcinogenic, non-Immunotoxic, non-mutagenic, non-cytotoxic and non-irritant along with Ramdesivir and Saquinavir (Banerjee et al. 2018). Whereas, Asiaticoside, Avicularin, Guaijaverin, Hyperoside, Isoquercitrin Rutin, and Spiraeoside have shown some degree of immunotoxicity.

Conclusion

To sum up, out of the fifteen chosen phytochemicals which have high binding efficiency to spike protein, Asiaticoside, Avicularin, Guaijaverin, Hyperoside, Isoquercitrin, Rutin and Spiraeoside exhibit higher toxicity values compared to remdesivir and saquinavir. Quercitrin, a phytochemical that binds to R 815 of S2, most likely has an implication in the activity of TMPRSS2 which is a crucial transmembrane molecule involved in viral entry to the cell. However, further *Invitro* studies are essential to analyze the extent of antiviral and toxicity effects of these phytochemicals. Appropriate chemical derivatization by retaining the binding affinity to the corresponding amino acid positions needs to be explored, to moderate the toxic effect. Guaijaverin (ASN210), Quercetin

Table 6 Prediction of Toxicity of phytoconstituents

Molecule	Formula	LD_{50} (mg/Kg)	Toxicity Class	Hepatotoxicity	Carcinogenicity	Immunotoxicity	Mutagenicity	Cytotoxicity	Irritant
Asiaticoside	$C_{48}H_{78}O_{19}$	4000	5	No	No	Yes	No	No	No
9Benzyl 2fluoro 9h purin 6amine	$C_{12}H_{10}FN_5$	1190	4	Yes	No	Yes	No	No	No
Avicularin	$C_{20}H_{18}O_{11}$	5000	5	No	No	Yes	No	No	No
Avicequinone C	$C_{15}H_{12}O_4$	1500	4	No	Yes	No	No	No	No
Carpaine	$C_{28}H_{50}N_2O_4$	500	4	No	No	No	No	No	No
Fenugreekine	$C_{21}H_{27}N_7O_{14}P_2$	7000	6	No	No	No	No	No	No
GraecuninE	$C_{51}H_{82}O_{22}$	55	3	No	No	Yes	No	Yes	No
Guaijaverin	$C_{20}H_{18}O_{11}$	5000	5	No	No	Yes	No	No	No
Hyperoside	$C_{21}H_{20}O_{12}$	5000	5	No	No	Yes	No	No	No
Isoquercitrin	$C_{21}H_{20}O_{12}$	5000	5	No	No	Yes	No	No	No
Quercetin	$C_{15}H_{10}O_7$	159	3	No	Yes	No	Yes	No	No
Quercitrin	$C_{21}H_{20}O_{11}$	5000	5	No	Yes	Yes	No	No	No
Quinic acid	$C_7H_{12}O_6$	9800	6	No	No	No	No	No	No
Rutin	$C_{27}H_{30}O_{16}$	5000	5	No	No	Yes	No	No	No
Spiraeoside	$C_{21}H_{20}O_{12}$	5000	5	No	No	Yes	No	No	No
Ramdesivir	$C_{27}H_{35}N_6O_8P$	1000	4	No	No	No	No	No	No
Saquinavir	$C_{38}H_{50}N_6O_5$	2000	4	No	No	No	No	No	No

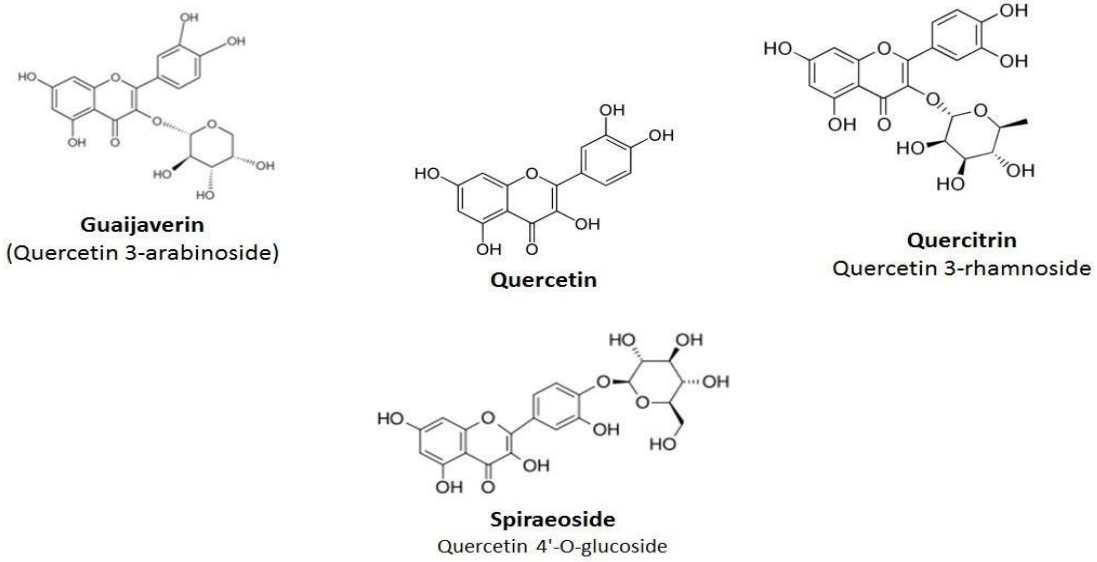

Figure 4 Chemical Structures of Quercetin and Quercetin Derivatives

(ASN210, GLU564), Quercitrin (ASN210), Quinic acid (ALA296), and spiraeoside (GLU495) can bind to the spike protein of the virus and ACE2 receptor of the host. Hence these compounds may serve as two-pronged drug candidates for SARS CoV2. Interestingly, the four among these are quercetin derivatives and are shown in figure 4. A few of the phytochemicals explored here such as Carpaine, Fenugreek, and Quinic acid, and their chemical modifications may increase the binding energy to spike protein. Further, synthetic combination molecules with high affinity and low toxicity moieties can be derived sooner than later which might inhibit the SARS CoV2 viral entry via spike protein to the human and animal hosts. We have a handful of very promising antiviral phytochemical moieties which may lead toward an effective antiviral drug against SARS CoV2.

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In-silico designing of an inhibitor against mTOR FRB domain: Therapeutic implications against breast cancer

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FRB domain

mTOR/P13K/AKT pathway

Therapeutics

Structure Inhibitors

ABSTRACT

Worldwide breast cancer causes significant fatalities in women. The effective therapeutic solution for treating the disease is using new and probable antagonistic biologically available ligands as anticancer drugs. To identify a successful therapeutic approach, the scientific community is now interested in creating novel ligands that in the future may be used as anticancer drugs. The mechanistic target of rapamycin (mTOR) is a protein kinase connected to several processes governing immunity, metabolism, cell development, and survival. The proliferation and metastasis of tumors have both been linked to the activation of the mTOR pathway. Female breast cancer represents about 15.3% of all new cancer cases in the U.S. alone and is frequently diagnosed among women aged 55 to 69 years. Given that the P13K/AKT/mTOR pathway is one of the most often activated in cancer, much attention has been paid to its resistance as a novel oncological treatment approach. mTOR/FRB Domain's recruitment cleft as, well as substrate recruitment mechanism, was targeted using a structural-based approach. A series of selective inhibitory small molecules have been designed and screened for the best inhibiting target binding triad of the FRB Domain with better ADME and no detectable toxic effects.

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1 Introduction

The cancer cells transfuse and are transported via the blood to other parts of the body, causing Breast cancer. One in eight women will develop breast cancer during their lifetime. The past several years have seen dramatic changes in anticancer drug development. More specifically, improvements have been seen in the production of anticancer drugs during the last few years. Even though various inhibitors have been discovered in recent times, and quite a few have been efficaciously developed to cure breast cancer, there has been more emphasis on the discovery of value-added anticancer drugs (Sharma et al. 2022a). The FRB domain with its rapamycin-binding site functions as a sentinel. By inhibiting substrate recruitment and further restricting active-site access, rapamycin-FKBP12 inhibits the kinase. By directly preventing substrate recruitment and further restricting active-site access, rapamycin-FKBP12 inhibits the kinase. PI3K/AKT/mTOR pathway is the most promising target for cancer treatment, although clinical trials are still being conducted (Sharma et al., 2017). PI3K/Akt/mTOR pathway being a complex intracellular pathway is known to result in cell growth and tumor proliferation, and thus it could act as a striking target for anticancer drug development (Sharma, 2020, 2022). Growth factors binding to the receptors activate PI3K, resulting in phosphorylation of the PI3-K-dependent kinases, which trigger AKT (Sharma et al. 2021b). AKT further triggers mTOR by phosphorylation and inhibition of TSC2, which negatively regulates mTOR. Further, p70S6 kinase upon phosphorylation by mTORC1 phosphorylates the 4E-BP1 and S6 (a ribosomal protein) which leads to protein translation. mTORC1 and mTORC2 are the two main complexes of mTOR. The components of mTOR Complex 1 comprise MTOR, RAPTOR, MLST8, PRAS40 , and DEPTOR (Kim and Guan 2019), while the Complexes of mTORC2 consist of MTOR, RICTOR, MLST8, and mSIN1. At the C-terminus, MTOR has four different domains for catalytic activity; FAT, FKBP12-rapamycin-binding domain (FRB), the kinase domain, and the FATC domain. At the N-terminus, MTOR contains 20 HEAT (Huntingtin elongation factor 3) repeats (Singh et al. 2022). The cleft is shaped like a deep V, thanks to the FRB or FKBP12-rapamycin-binding domain, which also limits access to the substrate-binding region (Ram et al. 2020). When rapamycin-FKBP12 binds to the FRB (Raghav et al. 2022), it effectively blocks all access to the substrate-binding site (Sharma et al. 2010). Besides aiding in a restriction to the active site, FRB also facilitates S6K1 (a ribosomal protein kinase) access to the active site, yielding increased kinase activity (Raghav et al. 2020). mTOR binds to the FK506-binding protein, FKBP-12, which upon binding, performs mTOR reticence (Sharma et al. 2020). mTOR upon inhibition further reduces phosphorylation of both the downstream targets, 4E-BP1 and S6K(Sharma et al. 2019a), inhibiting protein synthesis (Sun et al. 2020; Wakchaure and Ganguly 2021). Current investigation has proposed de-novo generated new lead molecule targeting FRB domain of mTOR, one of the key molecules in PI3K/AKT/mTOR pathway using the structure-based designing approach (Sehrawat et al. 2020). The current study has proposed a de-novo generated new lead molecule targeting the mTOR FRB domain, using a structure-based designing approach in the PI3K/AKT/mTOR pathway (Sharma et al. 2019b).

2 Materials and Methods

The energy-refined models of mTOR were engendered through iterative threading assembly refinement (I-TASSER) (Yang et al. 2015). PROCHECK was used for cross-checking the predicted models for energy distribution and stereochemistry. The substrate recruiting triad was then picked as the site of investigation, and in this complete study, we questioned the complementation of the same site. The substrate recruitment triad was then chosen as the site of inquiry, and we addressed the complementation of the same site in our complete analysis. Natural ligand search, as a seed molecule of 4JSP, revealed Benzoxazepine. Benzoxazepine was found in 4JSP's natural ligand quest. Its natural tendency to bind with class AKT (Sharma et al. 2021a) kinase and be a small molecular structure (molecular weight ~145.16) fits over accepted rules of the SBDD approach (Sharma and Sharma 2021). Thus Benzoxazepine as a whole was picked as a starting point for the de-novo generation of ligands. Benzoxazepine clean 3D structure PDB file was generated using ACD Labs freeware Chemsketch (Samanta et al. 2010). It was then used in the Schrodinger Maestro suite as an input file. Using “Maestro” and site-directed approach, we then placed seed molecule Benzoxazepine in available search space close to the binding triad. The seed molecule was placed such that it would interact well with the location (Figure 1). Utilizing an internal library of organic fragments, the growth strategy of Ligbuilder (v1.2genetic) algorithm was employed to populate more than 500000 molecules. Binding affinities were calculated by an empirical scoring function (Wang et al. 2000). Chemical stability, synthesis feasibility, and toxicity were also considered by defining "forbidden structure" libraries. It is also reasonable to consider chemical stability, synthesis feasibility, and toxicity by identifying libraries of "forbidden structure". Lipinski's parameters were used for screening and processing the population of produced compounds, and a total of 20 different fragment combinations were ultimately chosen for research (Sjöholm and Sandler 2019).

Then, using MGL and Autodock tools, the binding energies of generated ligands and mTOR were examined. The binding energy of developed ligands was further assessed by MGL and Autodock tools (Huey et al. 2012). The docking experiment was completed using the Lamarckian Genetic algorithm and the default Local Search parameters. With a decreased RMSD from the initial

conformation, Gibbs free energy (G) demonstrated strong interaction between produced ligands and mTOR (Table 1). The examined ligands' activity concentration (IC50) was also acceptable within the micro-molar range (Table 2). Biosafety and Bioavailability studies were then carried out through different web tools, and a comparison of candidates for the arrest of the mTOR FRB domain-mediated substrate recruitment mechanism revealed the best-fit candidate (Sharma et al. 2022b). To better understand the conformational dynamics of docked complexes, GROMACS was used to conduct molecular dynamics simulations (Berendsen 1995). Newton's equation of motion was solved by using the all-atoms simulation technique. Protein topology was created using GROMACS pdb2gmx modules. In addition, the topology of the ligand was created by PRODRG 2.5, an online tool (Adams et al. 2010). The complex was positioned in a dodecahedron box with a 10 Å distance from the edges using the edit conf module. The system was solvated using the specific point charges water model to keep equilibrium{SPC216}. The solvated system was then minimized with a cut-off of 50,000 steps at a maximum force of 1000.0 KJ/mol/nm. The system was also approaching equilibration at a temperature of 300K for the ensemble of the system's number of atoms, volume, and temperature (NVT) (Sharma et al. 2021a). For 100 ps, NVT and NPT was calcuted to keep the system in equilibrium. The system was put through a 1ns molecular dynamics simulation to test the complex stability. The structural analyses (Rg, RMSF andRMSD) was done and graphs were produced using the Graphing, Advanced Computation and Exploration application Xmgrace (Bansal et al. 2022).

3 Results and Discussion

Computer-aided drug design (CADD) methodologies are potentially promising and are playing an ever-increasing role in the drug discovery process, which is a cost-effective and suitable path provider for wet lab experiments. Moreover, CADD has been widely employed for the identification of promising drug candidates. This study designed a novel lead molecule, which has the best possible characteristic targeting properties, especially targeting the FRB domain. The same has exhibited significant overall goodness (G factor) and substantial core residual value (80.3%) of the modelled structures. Upon structure modelling, Model-1 has proven to be the best model. The results generated are in agreement with other studies reported in the literature, which prove the geometry and stereochemistry checks also along with the energy distribution using PROCHECK (Sharma et al. 2021b). The values obtained using the tool are following the studies reported in the literature.

Model 1, with 82.3 percent core residues, was found to be the most stable structure after projected models were reevaluated for energy distribution and stereochemistry using PROCHECK (Aruleba et al. 2018). Cross-validation of the shown models was done using a

Table 1 Models evaluated in terms of c-Score, Z-score, and Goodness factor values

Model	C-score	Core Value	G-factors	Z-Score
Model 1	-5.00	82.3% core	-0.39	-11.42
Model 2	-2.66	76.3% core	-0.52	-10.48
Model 3	-3.00	78.2% core	-0.49	-9.34
Model 4	-5.00	77.8% core	-0.32	-10.4
Model 5	-5.00	76.8% core	-0.40	-12

Table 2 Analysis of Various ligands based on their best conformations attained, ΔG (kcal/mol) value, RMSD values, and inhibition constant values.

Ligand Name	Best Conformation	ΔG (kcal/mol)	Ref RMSD	Inhibition Constant
Ligvar 2	2	-3.53	2.6	3.8 mM (millimolar)
Ligvar 21	3	-3.24	2.83	4.15 mM (millimolar)
Ligvar 22	5	-3.25	3.05	4.76 mM (millimolar)
Ligvar 23	4	-5.86	4.26	6.17 uM (micromolar)
Ligvar 26	7	-4.17	1.2	3.79 mM (millimolar)
Ligvar 37	2	-2.48	1.2	3.80 mM (millimolar)
Ligvar 38	8	-4.03	2.65	5.15 mM (millimolar)
Ligvar 39	9	-3.25	2.26	4.32 mM (millimolar)

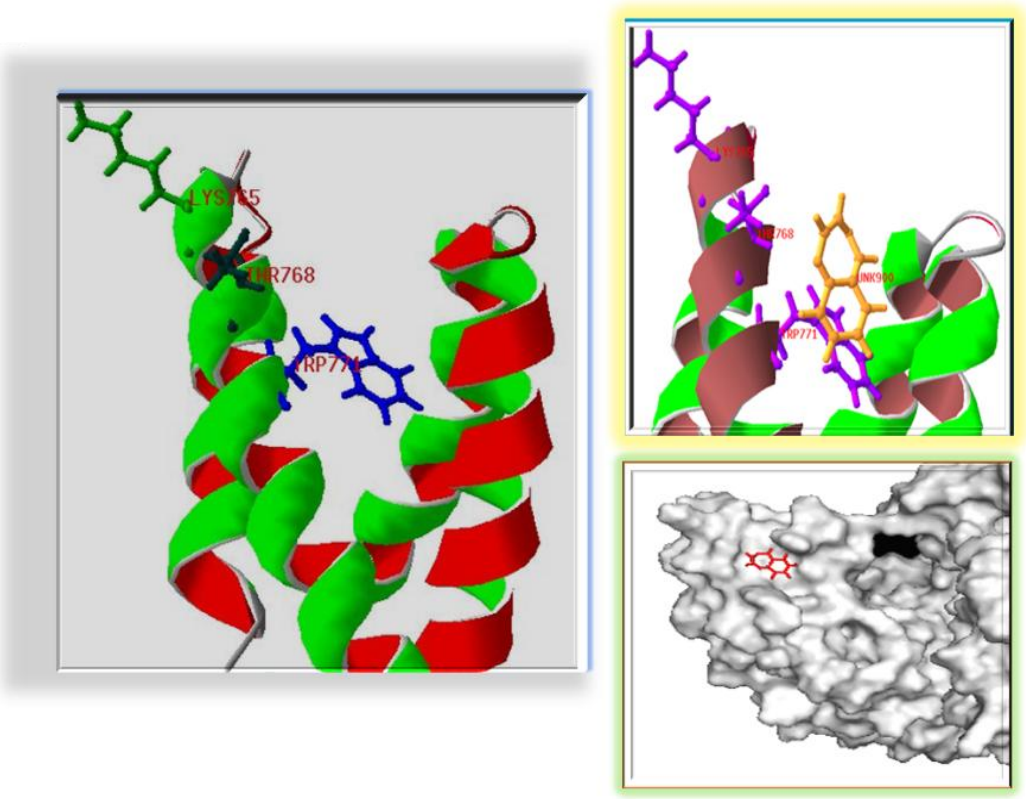

Figure 1 Benzozapine and its best bound position A. Ribbon view B. Surface view

comprehensive examination of model quality (Z-Score), and the findings showed that model 1 was the best fit (Table 1). FRB domain analysis and literature search showed that the active triad (Lysine (K) 765, Threonine (T) 768, and Tryptophan (W) 768) (Figure 1) plays a crucial role in substrate recruitment mechanism. The Ligsitemeta server also predicted enough active space around these three residues. Which have been reported in the literature as well.

Three amino acids viz. K-765, T-768, and W-771 at the specified positions were the primary focus of our study. Benzoxazepine was considerably used for the growth strategy, with which a population of binding fragments was generated, with pKd values ranging from 5-7 molars (Chaube et al. 2016). The designed ligands were subjected to molecular docking analysis to determine their binding energies with the protein of interest, which revealed that amongst all the designed ligands, Ligvar23 suggested positive affinity i.e ΔG -5.86 Kcal/mole around the catalytic triad position. Previous studies have convincingly shown that the active triad of K-765, T-768, and W-771 is capable enough in the fulfillment of substrate recruitment mechanism, which blocks the disease-causing path in the FRB domain. After performing docking studies, Ligand 23 displayed nominal bonding with Threonine-768. For MD simulations, water was added into the periodic box along with three chlorine ions by using the genion module of GROMACS. The system's volume was found 632.22 nm^3 while the density of the system was observed at 1003.04 (g/l). A total of 19285 solvent (water) molecules were added (Lemkul 2018). The potential energy graphical representation displayed a sharp drop in the system's PE at the beginning, after which it was stabilized. The system's PE was accomplished at 884 EM steps (Figure 2). Protein was in a dynamic state at 300K and constant pressure after NVT and NPT ensemble (Figures 3 and 4). Throughout the course, density fluctuated from 1017 to 1022 kg/m^3, with an average value close to 1020 kg/m^3 (Figure 5). MD was then run for a total of 1ns. In addition, the RMSD value was observed over time. Higher values suggest protein misbehavior during simulation, while changes in the range of 1-3 Å are acceptable (Lemkul 2018).

While the complex was under simulation, it was perceived that it passes through minute changes, with a modest rise in RMSD (Cabeza de Vaca et al. 2018). Red denotes the equilibrated protein backbone, which remains in the acceptable range compared to the black line (crystal structure) (Figure 6). RMSF was computed to demonstrate the undulation of amino acid residues of the complex during the MD run. The peak depicts the residues that fluctuate the most during the simulation (Figure 7).

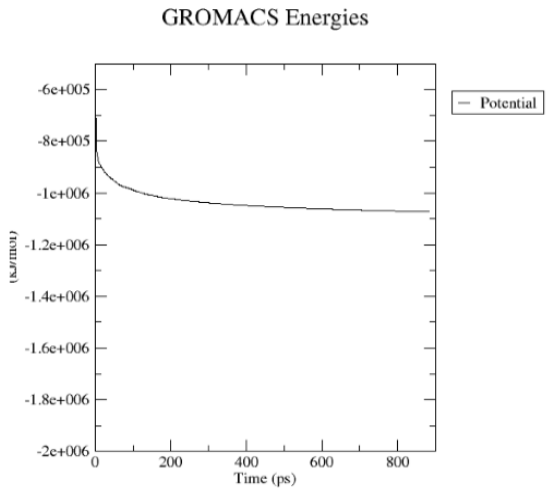

Figure 2 System PE minimization was accomplished after 884 EM steps

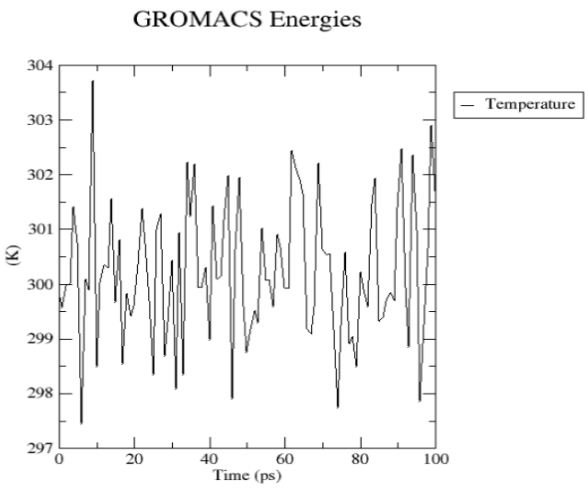

Figure 3 NVT of the system for 100 picoseconds

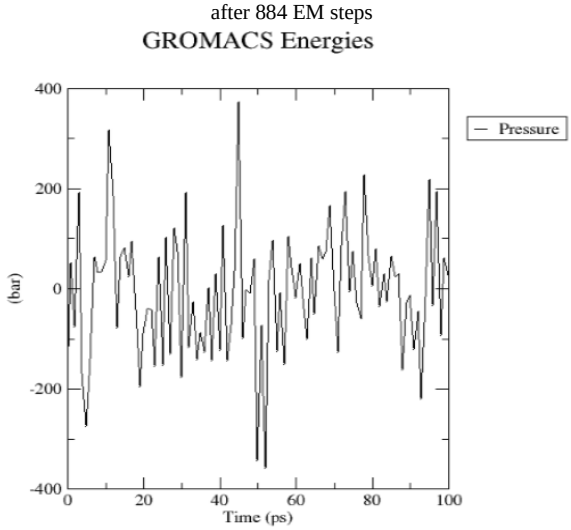

Figure 4 NPT of the system for 100 picoseconds

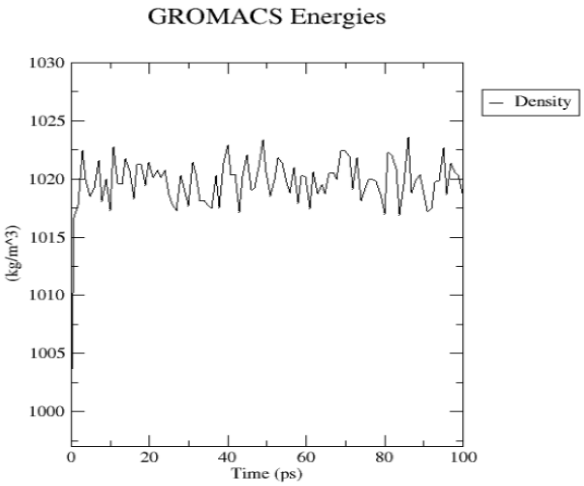

Figure 5 System density observed during the study which fluctuates around 1020 kg/m^3

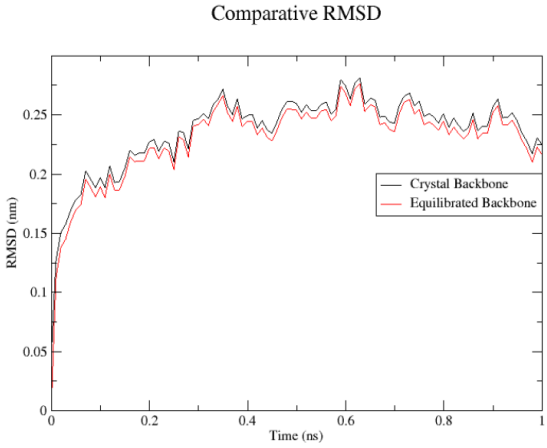

Figure 6 Comparative RMSD of the system. Crystal backbone vs. equilibrated backbone

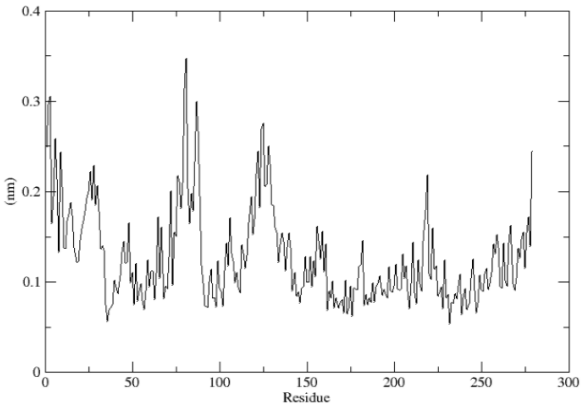

Figure 7 System RMSF shows fluctuation over all residues of the protein

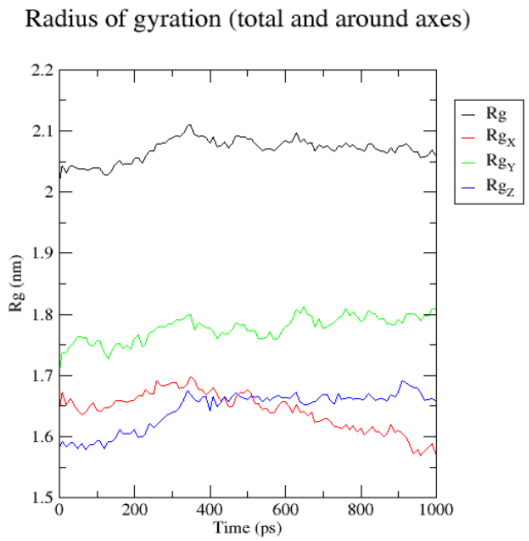

Figure 8 Radius of gyration of the system for 1000 Ps

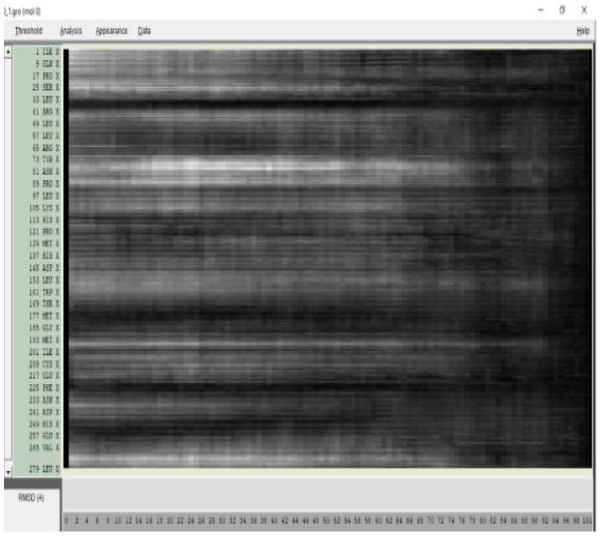

Figure 9 Heat map of RMSD over 101 frames. Encircled area showing highest RMSD

A minute change in radius of gyration having a value of 0.05nm was observed over 1000ps, indicating that protein remains stable during MD simulations (Figures 8 and 9). In terms of bioavailability and biosafety parameters, ligand Ligvar 23 displayed enzyme inhibition properties and could also act as the best candidate due to its non-toxic fragments. The present MD study based on Equilibrated NVT-NPT, PEM, RMSD, RMSF, and Radius of Gyration revealed that the complex remains stable throughout the observation period which was validated previously as well (Valdés-Tresanco et al. 2021). The findings are certainly significant from a therapeutic perspective against breast cancer especially targeting the mTOR/FRB domain.

Conclusions

The FRB domain of mTOR protein was targeted in the present study for which a library of designed molecules was constructed. After performing an extensive screening based on binding affinities, binding energy, bioavailability, toxicity, and biosafety parametric calculations, Ligvar 23 was found to be the most promising with the best binding affinity along with the best-fitted biosafety parameters, having the capability to act as potent drug molecules. The MD study based on Equilibrated NVT-NPT, PEM, RMSD, RMSF, and Radius of Gyration revealed that the complex remained stable throughout the study, and the complex was proven to be a bioavailable and the most suitable candidate.

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Conflict of Interest

Regarding the publication of this paper, there are no conflicts of interest among the authors.

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Physicochemical and Biological Characteristics of Shrimp Pond Sludge in the Thua Thien Hue Province, Vietnam

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Microorganisms

Biogas

Shrimp sludge

White-leg shrimp systems

Aquaculture wastewater

ABSTRACT

This study has been carried out to analyze the physical and biological indicators of shrimp pond sludge samples obtained from the Phu Vang and Phu Loc districts of the Thua Thien Hue Province, Vietnam. All standard methodologies have been used to analyze the selected parameters like pH, organic carbon, total nitrogen, total phosphate, and microbial density. The results of the study revealed that the sludge was characterized by a neutral to alkaline pH (6.9 - 7.5), and the total organic carbon content was in the range of 103.8–173.5 mg/kg. The sludge was rich in organic matter (17.8–29.9%), total nitrogen (13.5–32.5 g/kg), and total phosphate (7.9–20.1 g/kg). Further, in the case of the microbial density of pathogenic microorganisms, the density of total bacteria, *coliform*, *E.coli*, *Salmonella* spp., *Vibrio* spp., and *Clostridium* spp. was also estimated at two opposing weather conditions (spring, February to March; summer, June to July). The microbial community increased rapidly during the cool spring months. The total bacterial levels were recorded as 8.77 log10 CFU/mL in the Phu Loc district and 9.11 log10 CFU/mL in the Phu Vang district. The levels decreased during the hot summer months, and the level of total bacteria, *Coliform*, *E.coli*, *Salmonella* spp., and *Vibrio* was reported 2.57, 1.49, 1.06, 0.56, and l2.54 log10 CFU/mL respectively from the Phu Loc district of Vietnam. The results obtained using the anaerobic decomposition model showed that on the 60th day, the amount of CH_4 generated at the high output value for the Phu Vang district was 22385 ppm. The results reported here revealed that CH_4 gas can be potentially produced from shrimp waste sludge in this province.

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1 Introduction

Whiteleg shrimp (*Litopenaeus vannamei*) farming is a profitable industry for Vietnamese farmers (Van Nguyen et al. 2021). Currently, Whiteleg shrimp are widely grown in Vietnam. Shrimp farming is concentrated mainly in the Phong Dien, Phu Vang, Quang Dien, and Phu Loc districts within the Thua Thien Hue province. In general, the farm areas are in the range of 5–30 hectares. In some areas, the areas of the shrimp farms are in the range of 50–100 hectares. However, the development of aquaculture also leads to many difficulties. Unregulated construction of farms, use of non-standard disease-preventing chemicals, and direct discharge of water and sludge from ponds into waterways are some important issues that are directly associated with aquaculture development and caused server environmental pollution (Lich et al. 2022). It is highly profitable to farm Whiteleg shrimps as these shrimps have high protein value and are in demand for human consumption. Many reports have indicated that Whiteleg shrimp is sensitive to disease factors as well as environmental changes, and these factors lead to financial risks for farmers. The severity of diseases in shrimp has increased with the development of shrimp farming.

Recently Janecko et al. (2021) and Kumar et al. (2021) reported an association of the bacterial pathogen *Vibrio* spp. with Asian and Mexican shrimp aquaculture which causes an acute hepatopancreatic necrosis disease (AHPND). This pathogen causes an annual loss amounting to USD 1 billion and shrimp mortality exceeds 70% (Han et al. 2017). The occurrence of this disease is associated with various factors, including pond-water pollution. According to the assessment of the Ministry of Agriculture and Rural Development of Vietnam, wastewater and waste from intensive farming facilities contain excess feed, feces, other excreta, drug residues, chemicals, and garbage and it is increasing with each passing year.

Further, the sewage sludge formed during the aquaculture process contains decomposing residual food sources, chemicals, antibiotics, minerals, diatomite, dolomite, sulfur deposits, and toxic Fe^{2+}, Fe^{3+}, Al^{3+}, SO_4^{2-} species, and this mud layer is 0.1–0.3 m-thick. Toxic decomposition products, such as H_2S, NH_3, CH_4, and mercaptan, are formed during anaerobic digestion, and these are discharged into the surrounding environment, affecting the quality of farmed aquatic products. The sludge obtained under conditions of industrial shrimp farming contains Si (27,842 mg/kg), Ca (13,256 mg/kg), K (5,642 mg/kg), Fe (11,210 mg/kg), H_2S (8.3 mg/kg), NH_3 (36.1 mg/kg), NO_3 (0.3 mg/kg), NO_2 (0.1 mg/kg), and PO_4 (1.8 mg/kg), and taken together, they amount to 29.5% of the total mass. The total organic carbon (TOC) content for the bottom sludge of the Catfish pond (pH range: 4.37–5.39) is in the range of 1.56–1.89%, and it contains approximately 24% nitrogen and 24% phosphorus. Among these, nitrogen and phosphorus cause serious environmental pollution that needs to be treated effectively (Anh et al. 2010). However, most shrimp farming facilities are not equipped with an effective sludge treatment system.

The risk of infection attributable to pathogenic microorganisms is also a concern in the ponds if the ponds are not handled carefully after each cycle of shrimp culture. Alfiansah et al. (2018) studied the microbiome density in an industrial shrimp pond system through 16s rRNA sequencing and reported that the dominating microbial groups in shrimp ponds sediment are *Gammaproteobacteria*, *Alphaproteobacteria*, *Flavobacteria*, *Bacillus* spp., and *Actinobacteria*, belonging to the Halomonas and Psychrobacter groups. In this context, microorganisms can be divided into two main groups' i.e. organic compound decomposers and pathogenic microorganisms.

To date, a comprehensive study on microbial contamination in shrimp ponds in the Thua Thien Hue province has not been conducted. Therefore, the current study has been carried out to analyze the physical and biological indicators of shrimp pond sludge samples obtained from the Phu Vang and Phu Loc districts of the Thua Thien Hue Province, Vietnam. These samples were collected at two different times in a year (spring and summer), and an analysis of changes in the population of pathogenic microorganisms and the physical and biological characteristics of the sludge samples were carried out. The results provide an assessment of the contamination level of disease-causing microbial populations, which helps in mapping out strategies to prevent and treat pollution.

2 Materials and methods

2.1 Study site and sample collection

A total of 12 bottom sludge samples were collected from Whiteleg shrimp ponds located at Phu Loc (6 samples) and Phu Vang (6 samples) in different months (February and March, representing spring, and June and July, representing summer) (Figure 1). These samples were collected using a sterilized bucket and were transferred to a 2 L sterilized screw-capped bottle. Subsequently, the samples were transported to the laboratory in an icebox within 4 hrs after collection for microbiological analysis. In addition, on-site analysis of temperature, electrical conductivity, redox potential, and sludge sampling period was carried out. The sampling buckets were sterilized and flushed with water for 10 min before sampling. All samples were transported and stored at 4 °C and processed within 24 hrs.

2.2 Physical and organic characterization of the sludge

The pH, total organic carbon (TOC), organic matter (OM), total phosphate (TP), and total nitro (TN) of the collected sludge were determined by following standard methods.

Figure 1 Map of the study area (sampling locations are denoted by blue stars)

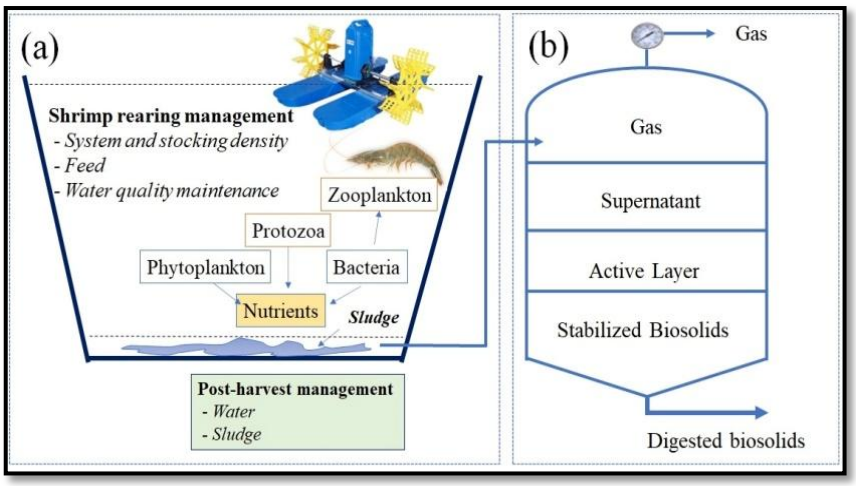

Figure 2 (a) Model of shrimp farming, and (b) model of the CH_4 gas generation process in the shrimp pond sludge

2.3 Isolation and identification of microbial strains

The compositions of the solid wastes obtained from the collected samples are shown in Tables 1 and 2. To isolate the microbial content, 1 mL of the waste obtained from shrimp pond sludge was mixed with 9 mL of sterile distilled water. The sample was then serially diluted to 10^{-4}. One hundred microliters of the sample from the last dilution were placed on the surface of casein agar in a Petri dish (1% casein and 2% agar), and the prepared sample was incubated at 35 °C for 72 hrs (Lich et al. 2022). The bacterial strains with strong protease activity were identified by molecular techniques.

The rate of flow of biogas and accumulated gas content, including CH_4 and H_2S, was also determined by using the gas chromatography (GC, GC-14 Gas Chromatograph, Shimadzu, Japan) technique. The machine was fitted with a flame ionization detector (FID), and the sample was quantified against a standard.

2.4 Statistical analysis

All experiments were performed in triplicate. Absorbances were recorded three times, and the average values were used for statistical analysis. Data were analyzed using Statgraphics 19, and the results are presented as mean ± standard deviation (SD). The difference between means was assessed by ANOVA, and Duncan's test was used to compare data from different samples. $P< 0.05$ was considered to be statistically significant.

3 Results and Discussion

Shrimp pond farming in Thua Thien Hue Province proceeds over several phases: pond preparation, shrimp fry selection, shrimp rearing management, and post-harvest management (Figures 2a and 2b). Environmental factors shape the structure and function of microbial communities, and research has shown that the succession of microbial communities was influenced by combinations of

Table 1 Characteristics of sludge obtained from different areas in the Thua Thien Hue district (spring season)

Name of sampling point	pH	TOC (mg/kg)	OM (%)	TP (g/kg)	TN (g/kg)
Phu Loc	6.9 ± 0.2	130.0 ± 6.7	22.4 ± 1.1	20.1 ± 0.2	32.5 ± 1.6
Phu Vang	7.3 ± 0.1	173.5 ± 5.3	29.9 ± 0.9	7.9 ± 0.1	24.1 ± 0.9

Table 2 Characteristics of sludge obtained fromdifferent areas of theThuaThien Hue district (summer season)

Name of sampling point	pH	TOC (mg/kg)	OM (%)	TP (g/kg)	TN (g/kg)
Phu Loc	7.5 ± 0.1	103.8 ± 4.2	17.8 ± 0.7	12.7 ± 0.1	16.6 ± 1.2
Phu Vang	7.5 ± 0.1	107.8 ± 1.7	18.6 ± 0.3	17.4 ± 0.2	13.5 ± 0.8

available organic matter, TN, TP, chemical oxygen demand, pH, and feed sources of the ponds (Alfiansah et al., 2018; Liu et al., 2020). Recently, researchers have studied the impact of pH on the cultured shrimp digestion process. The changes in intestinal activities were reported with the changes in the pH of the pond water. It was reported that the suitable pH for Whiteleg shrimp farming ranged from 7.8 to 8.5. The characteristics of the sludge collected from different areas of Thua Thien Hue during the spring and summer seasons are presented in Tables 1 and 2. The results of the study suggested that the pH of the sludge obtained from the two districts was different, and the pH values varied with the seasons. In spring, the mean pH of the sewage sludge collected from the Phu Vang district was 7.3, while the pH of the sludge samples collected from the Phu Loc district was 6.9. A small increase in the pH value was reported during summer, and the pH of the sewage sludge collected from the Phu Vang and Phu Loc districts was reported to be 7.5. The National technical regulation QCVN 02–19:2014/BNNPTNT was outlined by the Ministry of Agriculture and Rural Development of Vietnam for brackish shrimp farm conditions to ensure veterinary hygiene, environmental protection, and food safety. It has been reported that the optimal pH ranges between 5.5 and 9. During aquaculture, the pH of the sludge can change easily, which directly affects the physical, chemical, and biological conditions of the water environment of the pond. The change in pH also affects the cultured shrimp. The pH of the system can be controlled by controlling the number of algae and the amount of carbon oxide (CO_2) released by shrimps during respiration. The amount of CO_2 produced depends on the density and weight of the cultured shrimp. The respiration process of shrimps reduces the pH, as, during respiration, organisms consume dissolved oxygen and release carbon dioxide. If oxygen is deficient in shrimps, the sludge at the bottom of the pond is acidified. In addition, under anaerobic conditions, the sludge undergoes anaerobic fermentation, resulting in a reduction in the sulfate (SO_4^{2-}) content and the production of hydrogen sulfide (H_2S), which is toxic to shrimps. Similarly, Hung et al. (2015) reported the average pH of the sludge samples collected from Ha Noi, Vietnam, was in the range of neutral to alkaline (7.04–7.4). In contrast to this, Selamawit and Agizew (2022) reported that the pH values of the sludge samples obtained from wastewater collected from the sewerage system and exchange stations were 5.1 and 6.9, respectively. Sudiartha et al. (2022) reported that the pH of the aerated sludge sample was 7.09, and this value increases as the temperature increases. The pH increases to 7.64 at 42 °C. In contrast, the pH of anaerobically stabilized sludge varies between 6.7 and 7.8 (Michalska et al. 2022). The findings of the current study are consistent with previously reported results (Table 2). An increase in the pH was reported during the summer months, which can be potentially attributed to water heating in the presence of sunlight. This results in an electrochemical reaction that splits water into OH^- and H^+. As the concentration of the H^+ ions in the pond increases, the pH also increases. As the pH increases with temperature, polymerization and condensation times associated with the reduction of acid content increases (Gascó et al. 2005).

The TOC content corresponding to the sludge samples collected from different areas of Thua Thien Hue during the spring and summer months ranged from 103.8 ±4.2 to 173.5 ±5.3 mg/kg (Tables 1 and 2). As shown in figure 3a, the TOC corresponding to the sludge sample collected from the Phu Vang district is higher than that collected from Phu Loc. The TOC corresponding to these samples is higher in spring than in summer. The maximum average TOC was recorded for the sludge sample collected from the Phu Vang district during spring (173.5 ±5.3 mg/kg), and the minimum value was recorded for the sludge sample collected from the Phu Loc district in the summer months (103.8 ±4.2 mg/kg). Further, figure 3b showed the organic matter (OM) content of the sludges obtained from the two districts in the spring and summer seasons. The OM content does not greatly vary between the different shrimp farming areas in the Thua Thien Hue province in the summer, and it was reported in the range of 17.8 ±0.7–18.6 ±0.3%. However, the OM value corresponding to the Phu Vang district increased to 29.9 ±0.9%in spring. These results are consistent with the study reported by Azzouz et al. (2017). Nutrients are mineralized during the decomposition of OM and the level of C, N, and cation exchange increases with the increase of the OM. Chemical properties such as pH, electrical conductivity, and redox potential of the soil vary with seasons (Clapp et al. 1986). Azzouz et al. (2017) recorded the value of OM in the sewage sludge

collected from the Guarchia wastewater treatment plant, in Benghazi, Libya, and reported that the maximum OM value was reached in February (24.20%) and the minimum was recorded in March (17.83%). Silva et al. (2018) reported that the OM obtained during the acidogenic stage was efficiently used to produce CH_4. Recent studies have suggested that the OM content in sewage sludge can reach 29 to 39% (Michalska et al. 2022).

The TP content in shrimp pond sludge samples is presented in Figure 3c. The minimum TP concentration was recorded from the sample collected from the Phu Vang district in the spring (7.9 ±0.1g/kg)), and the maximum TP was also recorded during the spring from the two locations of the Phu Loc district (20.1 ±0.2 g/kg). The variations in the TP between districts can be potentially attributed to the differences in the farming method, farming time, pond size, the density of shrimp farming, and the shrimp farming habits in the two areas. The accumulation of excess food and shrimp feces at the bottom of the pond increased the phosphorus content in the sludge. Burzyńska (2019) suggested that the maximum amount of dissolved phosphorus in the sludge solution is present in the top layer. Phosphorous from the top layer may migrate with surface runoff, increasing the risk of eutrophication. Figure 3d shows that the average TN content in the collected sludge samples did not differ significantly between seasons. The maximum average TN content (32.5 ±1.6 g/kg) was found for the sludge sample collected from Phu Loc in spring, while the minimum (13.5 ±0.8 g/kg) was recorded for the sample collected from Phu Vang in the summer. These values are below the maximum level specified in Vietnam's technical regulations on industrial wastewater. The factor influencing the release of P and N from sludge and the migration of these nutrients into the sludge-water environment affects the mineralization process associated with the OM in sludge (Burzyńska 2019). Scientists have suggested that shrimps incorporate 24 to 37% of nitrogen and 11 to 20% of phosphorus from the feed into their bodies. The presence of leftovers will result in changes in pH, dissolved oxygen in the water and pond sediment, and the extent of proliferation of bacteria and plankton (Alfiansah et al. 2018; Liu et al. 2020).

Shrimp intended for export must meet the heavy-metal content and bacteriological standards of the importing countries. The microbial density of pathogenic microorganisms in the selected ponds was also studied under two different sets of weather i.e. cold season/spring (Figure 4) and the hot season/summer (Figure 5) for two different locations of the Thua Thien Hue province to elucidate the presence of microorganisms in the collected sludges.

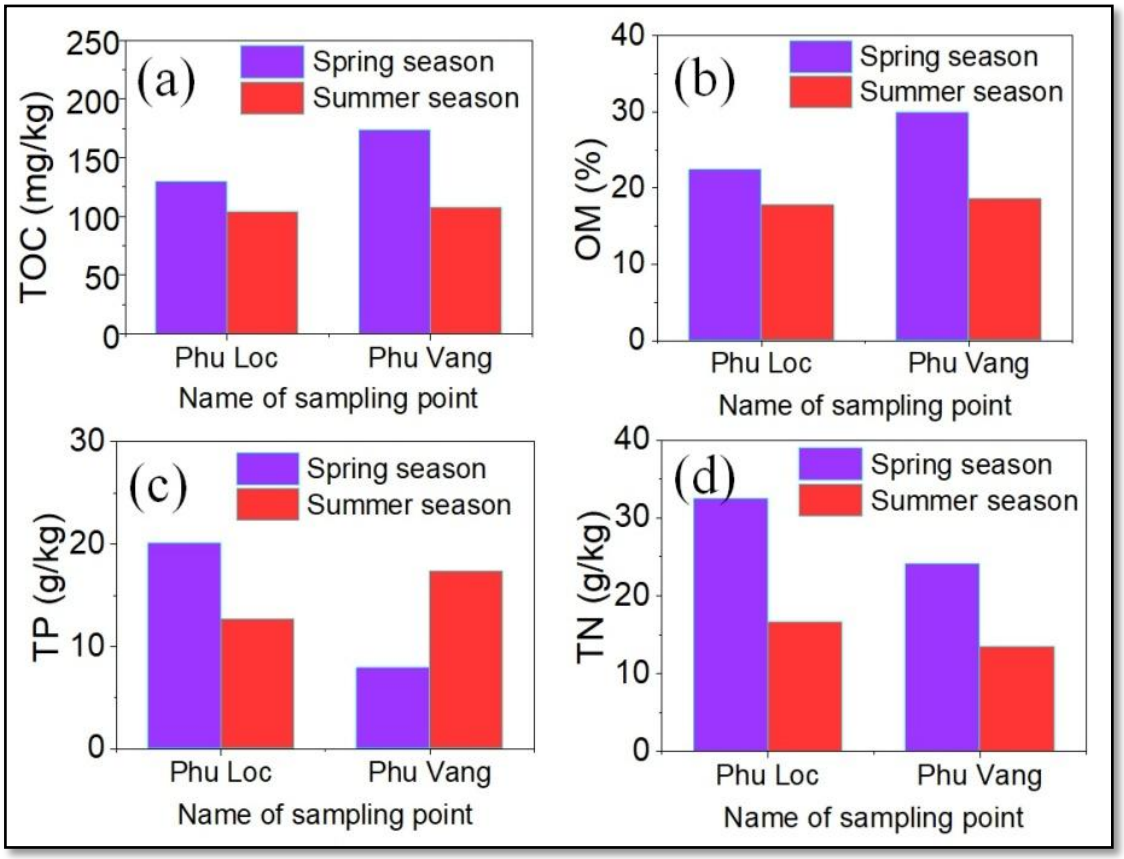

Figure 3 Physicochemical characteristics of sludge samples obtained from different locations of Thua Thien Hue

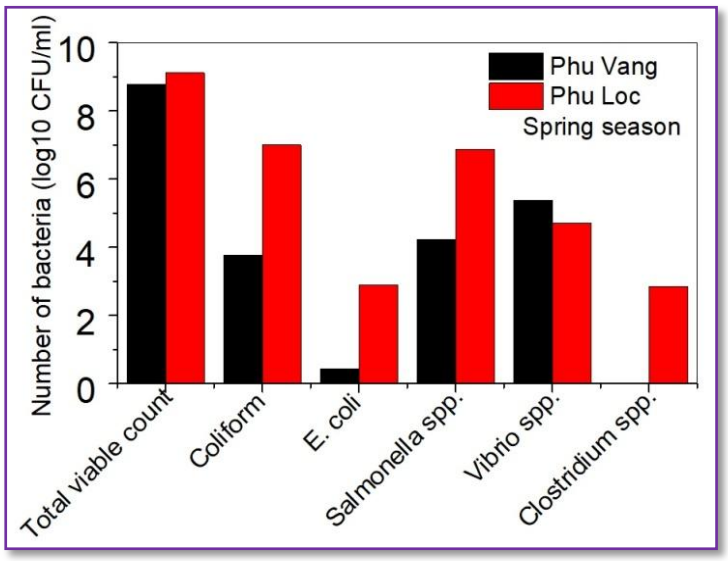

Figure 4 Microbial density in pond sludge samples obtained from different areas of the Thua Thien Hue district (spring season)

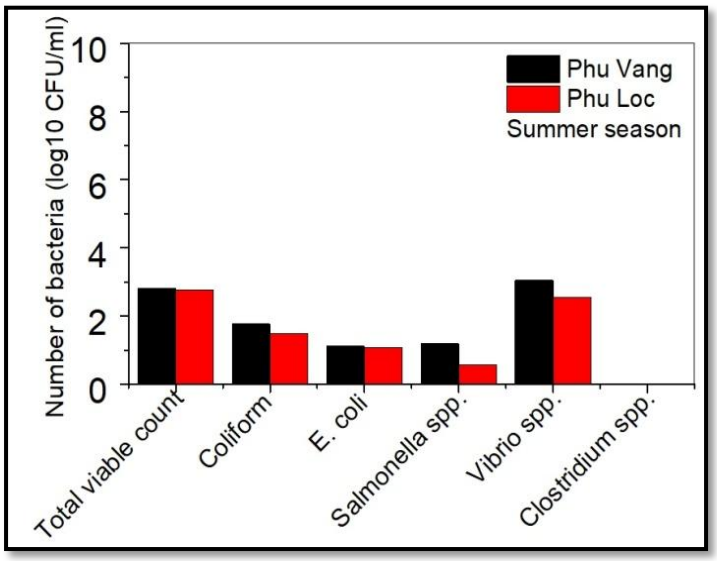

Figure 5 Microbial counts in pond sludge samples obtained from different areas of the Thua Thien Hue district (summer season)

Results presented in Figure 4 revealed the counts of total bacteria, *Coliform*, *E.coli*, *Salmonella* spp., and *Vibrio* spp. in the sludge of shrimp ponds in spring. Further, the presence of *Clostridium* spp. was reported in sludge samples collected from the Phu Loc District, while it was absent in the samples collected from the ponds of the Phu Vang District. The analyzed results showed that the number of bacteria detected in the sludge obtained from both districts in spring is quite high, and values ranged from 8.8 to 9.11 log 10CFU/mL. The bacterial content in the sludge samples collected from the Phu Loc districts tended to be higher than that of the Phu Vang districts (Figure 4). The relatively high counts of *Coliform* (6.99 log10 CFU/mL), *Salmonella* spp. (6.78 log10 CFU/mL), and *Vibrio* spp. (4.7 log10 CFU/mL) in the ponds of the Phu Loc District, in particular, indicate the need for water treatment methods for these bacteria in the Phu Loc District. Further, *Salmonella* spp. and *Vibrio* spp. are recognized as important food-borne pathogens, and most importing countries do not accept their presence in raw frozen shrimp (Yen et al. 2020). Thus, the farm owners need to carefully treat the pond bottom so that pathogens have no opportunity to enter the shrimp. In summer, the *total bacteria*, *Coliform*, *E.coli*, *Salmonella* spp., and *Vibrio* spp. counts decreased, while *Clostridium* spp. disappeared altogether (Figure 5). In general, for a given location, the incidence of bacteria recorded for the sludge samples obtained from shrimp ponds during spring was higher than those recorded during the summer seasons (Figure 6).

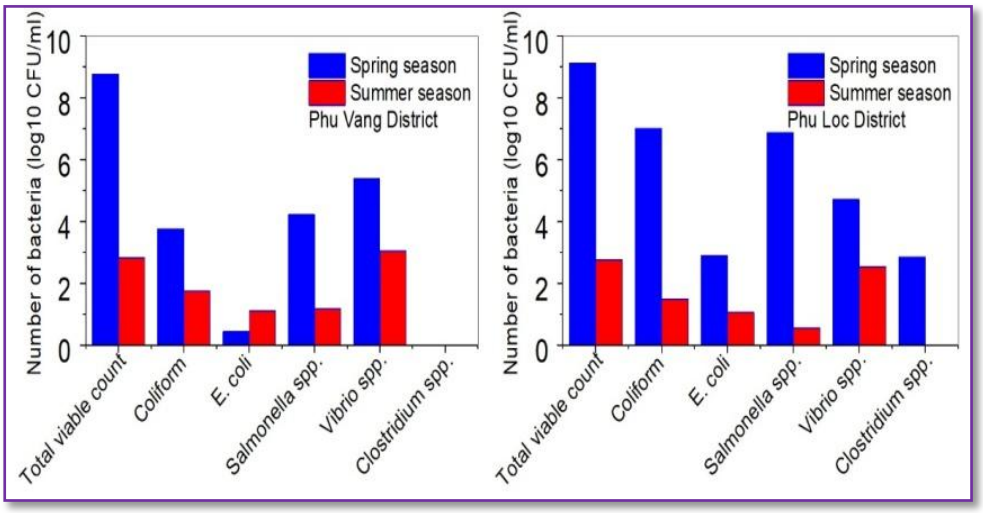

Figure 6 Microbial counts in pond sludge samples obtained from (a) Phu Vang and (b) Phu Loc

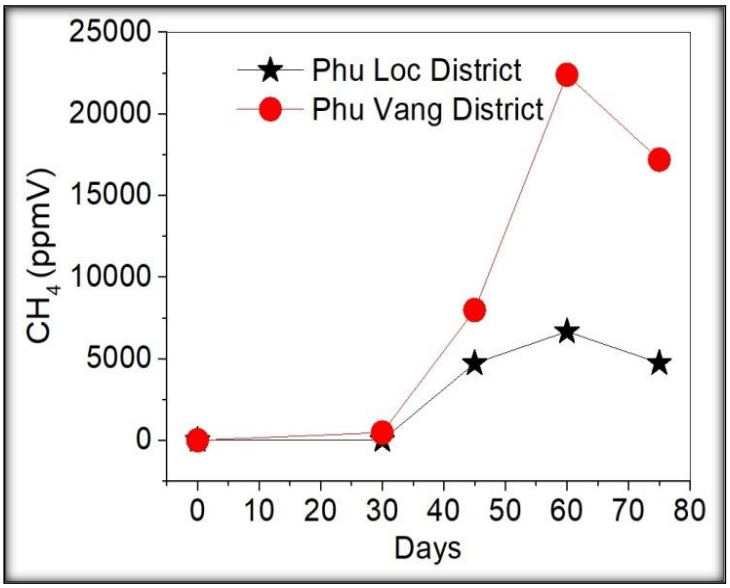

Figure 7 CH_4 production across 75 days of anaerobic digestion using shrimp pond sludge samples obtained from the Phu Vang and Phu Loc districts

In "Industry 4.0", renewable resources will play a crucial role. Biogas from waste, residues, and energy crops can be used as a replacement for fossil fuels in power and heat production. Sewage sludge can be treated using biochemical, thermochemical, or mechanical methods to generate biogas. Recently, researchers have suggested that sludge has a lot of potential for gas production (Selamawit and Agizew 2022). According to a review report, the sludge of shrimp ponds contains particulate and dissolved organics such as microorganisms, leftover shrimp food, shrimp manure, and other organic substances. Thus, it can be potentially used for generating gas. This is confirmed by the use of an anaerobic digester that was used to investigate the biodegradability of sludge through the conversion of biomass energy into biogas (Figure 7).

The amount of CH_4 produced gradually increased with the increase of the reaction time for the shrimp pond sludge samples collected from both the Phu Vang and Phu Loc Districts. After the 30th day of sludge incubation, the amount of CH_4 gas increased sharply, and it reached its maximum value on the 60th day. Following this, the CH_4 gas content began to decrease. For Phu Loc, on the 60th day of the experiment, the amount of CH_4 generated from the sludge was 6664 ppm, and it shows an increase of 70 times compared to the 95 ppm recorded on the 30th day. The amount of CH_4 gas generated from the shrimp pond sludge samples collected from Phu Vang was even higher, and it reached 22385 ppm on the 60th day of the experiment. Through theoretical and experimental studies Schaum et al. (2015) reported that at 37 °C, the content of dissolved

methane in digested sludge was approximately 22 ppm. According to studies, OMs decompose, and the grain size reduces within an anaerobic digester system. This improves the solubilization of organic waste, and the increased bacterial activity results in the release of hydrolytic enzymes, which significantly increases the extent of biogas production realized (Selamawit and Agizew 2022). It can be predicted that the CH_4 yield depends on substrate origin and composition. It also depends on the operating conditions (temperature and strains of bacteria). The fact that the extent of CH_4 gas production peaked on the 60^{th} day of sludge incubation revealed that this is the best condition for gas generation. However, when the OM content in the sludge samples was completely decomposed, the amount of CH_4 gas produced began to decrease. Similar results were reported by Sudiartha et al. (2022), who investigated CH_4 production in a two-stage anaerobic digestion system by co-digesting food waste, sewage sludge, and glycerol. According to another study, an increase in CH_4 gas production was accompanied by an increase in the *Methanoculleus* content at 48 °C (Sudiartha et al. 2022). It has also been reported that sludge can be used to produce CH4 gas effectively. Silva et al. (2018) investigated the process of CH_4 production using a mixture of food waste, anaerobic sewage sludge, and glycerol. The maximum yield of CH_4 obtained from this mixture was 342.0 mL CH_4·g. The results herein revealed that in addition to economic benefits, shrimp farming promotes CH_4 production under anaerobic digestion conditions. These results allow prospects of the production of CH_4 gas from shrimp waste sludge in Thua Thien Hue province. In addition, an effective method that can improve the production of CH_4 gas from shrimp sludge using *Stenotrophomonas rhizophila* MT1 bacterial isolated from sludge samples of shrimp ponds (Lich et al. 2022) which can be a topic that will open the door of future research.

Conclusions

Results of the current study revealed the physical and biological indicators of shrimp pond sludge samples taken from the Phu Vang and Phu Loc districts of the Thua Thien Hue Province, Vietnam. The results can be concluded that the sludge samples of the study area are characterized by neutral to alkaline pH (6.9–7.5), the TOC, OM, TN, and TP ranged from 103.8 to 173.5 mg/kg, 17.8 to 29.9%, 13.5 to 32.5 g/kg, and 7.9 to 20.1 g/kg respectively. These parameters vary differently between regions and seasons. Further, the microbial density of pathogenic microorganisms such as total bacteria, *Coliform*, *E.coli*, *Salmonella* spp., *Vibrio* spp., and *Clostridium* spp. was also determined in the collected sludge sample under the conditions of two opposing sets of weather (cool spring and hot summer season). The microbial count was found to increase rapidly during spring and reached 8.77 log10 CFU/mL in the Phu Loc district, and this value was recorded at 9.11 log 10CFU/mL for the Phu Vang district. This microbial count was decreased during the summer seasons. During the summer months, the total bacterial, *Coliform*, *E.coli*, *Salmonella* spp., and *Vibrio* count was recorded 2.57, 1.49 1.06, 0.56, and 12.54 log10 CFU/mL respectively in the Phu Loc district. Later on, the *Clostridium* population fell to zero. Results from experiments carried out with an anaerobic decomposition model system showed that on the 60^{th} day of the experiment, the amount of CH_4 generated at the highest output value was 22385 ppm and 6664 ppm for the Phu Vang and Phu Loc regions, respectively. These results revealed that CH_4 gas can be potentially produced from shrimp waste sludge obtained from the Thua Thien Hue province.

Acknowledgments

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Conflicts of interest

All authors declare no conflicts of interest.

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Effect of Monosodium Glutamate on the Digestibility of Different Nutrients Using Standardized Static In vitro Digestion Model

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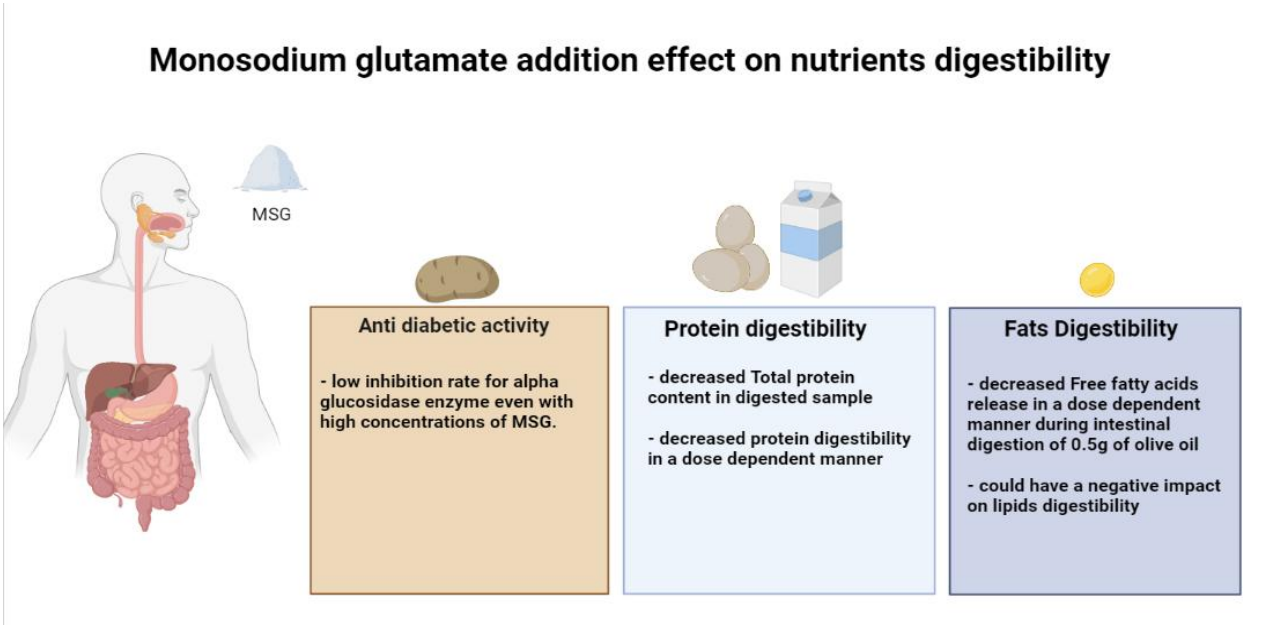
GRAPHICAL ABSTRACT



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ABSTRACT

Monosodium glutamate (MSG) is a flavor enhancer and food additive with a unique umami taste. Due to its widespread use in humans, this study focused on the cytotoxicity, anti-diabetic effect, and interaction with protein digestion by performing a standardized static in vitro digestion model and lipid digestion by estimating free fatty acids released from 0.5 g of olive oil during intestinal lipolysis. The study showed that monosodium glutamate has an apparent cytotoxic effect on the Caco-2 cell line in a dose-dependent manner. MSG glutamate also showed low inhibitory activity on alpha-glucosidase enzyme even at high concentrations (16.3 % at 1800 ppm). By performing simulated in vitro digestion to study the interaction between MSG and protein digestion, followed by MTT study, total protein determination, and pH drop method, all results concluded that MSG affected proteolysis. Finally, the impact of MSG on lipolysis was studied through a free fatty acid release test. The results of the study demonstrated that MSG harmed fat digestibility in a concentration-dependent manner. As a result, it is essential to conduct further studies, especially in vivo studies, to determine the potential negative effects of MSG on human health.

1 Introduction

A food additive is any substance added to processed food to improve its taste, quality, chemical properties such as alkalinity or acidity, and physical properties, including consistency, texture, and color. Moreover, food additives are used as antioxidants, flavorings and coloring agents, preservatives, sweeteners, and thickeners (Wu et al. 2021). MSG is a widely used flavor enhancer. According to European legislation, it is also known as E621 and is available in a crystalline powder form that can easily dissolve in water (Hajihasani et al. 2020). MSG's specific taste is called umami. Umami is a meaty flavor and is one of the five basic tastes besides salty, sweet, sour, and bitter (Kurihara 2015). There is considerable controversy around MSG safety. However, the European Food Safety Association (EFSA), the World Health Organization (WHO), and the Food and Drug Administration (FDA) considered MSG to be safe, with 30 mg per kilogram of body weight per day as an acceptable daily intake (ADI), although many studies revealed its toxicity and harmful effects on human health (Henry-Unaeze 2017; Zanfirescu et al. 2019). Moreover, numerous studies have linked MSG to obesity and metabolic disorders such as insulin resistance, diabetes, and high blood sugar (Araujo et al. 2017; Niaz et al. 2018). MSG could cause obesity by increasing the food's pleasant taste and disturbing the leptin-mediated hypothalamus signaling cascade (He et al. 2011). The concern is that the studies and data are inconsistent; for example, some recent studies have suggested that umami taste reduces hunger's post-ingestive recovery and suppresses obesity (Stańska and Krzeski 2016).

Previous studies have demonstrated inconsistent results regarding MSG's impact on brain health. The concern about the effect of MSG on brain health was raised because glutamate in MSG and the body are identical, and it is crucial in brain function as an essential neurotransmitter (Kazmi et al. 2017; Chakraborty 2019). Many studies have reported that MSG is neurotoxic at neonatal administration and hypothalamus neurons destruction in rats, which causes metabolic abnormalities such as pseudo-obesity, growth disturbances, depressive-like behaviors, hypogonadism, metabolic dysfunctions, and chronic inflammation (Bodnár et al. 2001; Perelló et al. 2003; Rosa et al., 2015; Göbel et al. 2017). Other studies have stated that MSG can also contribute to neurodegenerative diseases such as Parkinson's and Alzheimer's (Appaiah, 2010). MSG also negatively affects rats' memory and cognitive skills. A recent study reported that MSG reduced learning capabilities and shortened memory in rats, even with low doses (Abdel Moneim et al. 2018). Further, Ali et al. (2000) reported that MSG, even at low doses, could affect cognition during early childhood, a period in which the brain is accessible and vulnerable due to the blood-brain barrier's high permeability to small and large molecules (Ali et al. 2000). Several reports have indicated that MSG is genotoxic, and reactive oxygen species (ROS) and oxidative stress are crucial in MSG-induced genotoxicity and cytotoxicity in rats' brains, kidneys, and livers (Farombi and Onyema 2006).

In an in vitro study on human peripheral blood lymphocytes, it has been reported that MSG can cause DNA damage and genotoxic effects on the exposed cells (Ataseven et al. 2016). Ismail (2012) suggested that MSG could harm male reproductive health, causing testicular harm and infertility. On the other hand, many researchers believe that MSG is safe and causes no genotoxicity (Shibata et al. 1995; Rogers 2016). Oxidative stress occurs when reactive oxygen species are elevated. It leads to lipids, carbohydrates, proteins, nucleic acid damage, cellular metabolism disruption, and apoptosis (Saeidnia and Abdollahi 2013), which is the fundamental reason for MSG hepatotoxicity. A study by Onyema et al. (2006) on rats reported that a dose of 0.6 mg/g body weight of MSG induced

hepatotoxicity and oxidative stress by decreasing reduced glutathione (GSH) level, inducing lipid peroxidation (LPO), and increasing the activities of catalase, superoxide dismutase (SOD), and glutathione-s-transferase (GST) in the liver of the rats. In addition, liver enzymes, including alanine aminotransferase (ALT), ϒ-glutamyl transferase (GGT), and aspartate aminotransferase (AST), were significantly elevated in blood serum (Onyema et al. 2006). Another study on rats by Elshafey et al. (2017) stated that exposing rats to a dose of 4 mg/kg MSG for 90 days reduced antioxidant enzymes and increased lipid peroxidation and fibrosis. Some people can experience adverse effects after consuming MSG. This condition is called the MSG symptom complex, which includes flushing, dizziness, difficulty breathing, headache, weakness, numbness, muscle tightness, and even loss of consciousness (Zanfirescu et al. 2019). According to the Federation of American Societies for Experimental Biology (FASEB) and FDA reports, some mild short-term, transient symptoms, such as tingling, headache, palpitations, numbness, flushing, and drowsiness, may happen in some sensitive people who consume 3 g or more of MSG without food (Food & Administration, 2012). This study aimed to determine the MSG's physicochemical properties, cytotoxic effect on the Caco-2 cell line, and antioxidant behavior and to assess its interaction with the digestion process using a standardized in vitro static digestion model.

2 Materials and Methods

2.1 Chemicals and reagents

During this study, 0.05% Trypsin-EDTA (Himedia, India), Dulbecco's modified Eagle's medium (DMEM) (Himedia, India), DMSO (Lobalohemia, India), Phosphate buffered saline (PBS) (Serox, Germany), Fetal bovine serum (FBS) (Biochrom), DPPH (Himedia, India), methanol (Elnasr, Egypt), NaCl (Supelce, Germany), $MgCl_2 \cdot 6H_2O$ (LOBA, India), $(NH_4)_2CO3$(LOBA, India), KCl (Supelco, Germany), KH_2PO_4 (Supelco, Germany), $NaHCO_3$ (Supelco, Germany), MSG (Loba Chemie, India), HCl Hydrochloric acid (2N) (Samchun chemicals), Sodium hydroxide (Carl Roth), pepsin (Loba Chemie, India), pancreatin (LOBA Chemie, India), $CaCl_2 \cdot H_2O$ (Chem lobnu Belgium), and bile (LOBA Chemie, India) were used without additional purification. Caco-2 cell lines were collected from Vacsera, Giza, Egypt. Powdered milk, olive oil, and hen eggs were purchased from the local market, and whey protein isolates were obtained from a gym supplement shop. Olive oil was used for free fatty acid release tests.

2.2 Physiochemical characterization

X'Pert PRO-PAN analytical diffractometer was used to perform X-ray diffraction (XRD) measurements, with Cu-Kα radiation (λ= 1.54056 A˚) at 40 kV and 30 mA to examine the polycrystalline nature of MSG to determine crystallites size using the Scherer equation (Mele et al. 2022).

$$D = \frac{0.9\lambda}{\beta \cos\theta} \quad (1)$$

Where λ is the wavelength in A˚, *K* is the shape constant (~ 0.9), β is the observed peak width at half-maximum height in red, θ is the Bragg angle in degrees, and D is the crystallite diameter in A˚. The functional groups were pinpointed using FTIR-4100 type A in the 349.053 -7800.65 cm^{-1} range, with a resolution of 4 cm^{-1} at room temperature.

2.3 Cytotoxicity of MSG (MTT assay)

The MTT assay was performed using Caco-2 cells according to the Van Meerloo protocol (Van Meerloo et al. 2011). In brief, MSG was dissolved in water with different concentrations (200, 100, 50, 25, and 12.5 mM). Caco-2 cells were cultured at 37 °C and 5% CO_2 for 24 h, then exposed to different concentrations of MSG solutions. After 24 hrs of exposure, PBS was used to wash the cells, and 50 µL of MTT solution and 50 µL of serum-free media were added into each well. Finally, after finalizing the procedure, the absorbance was measured at a wavelength of 590 nm. The values were calculated three times and the means of three replicates were used as the final results. The percent cytotoxicity was calculated by equation 2 (Van Meerloo et al. 2011):

$$\text{Cell viability\%} = \frac{\text{Mean OD treated well } [-\text{blank}]}{\text{Mean OD control well } [-\text{blank}]} \times 100 \quad (2)$$

Where OD is the optical density.

2.4 Antioxidant activity of MSG

2.4.1 DPPH assay

Antioxidant activity was determined by the DPPH assay (Boly et al. 2016). By dissolving in water, five different MSG solution concentrations were prepared; 20, 16, 12, 8, 4, and 2 g/mL. Finally, color intensity was measured at 517 nm UV-visible light using a spectrophotometer (SPECORD 200 PLUS, Analytik Jena, Germany). The data were obtained by equation 3 and represented as means ± SD.

$$\text{RSA \%} = \left(\frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}\right) \times 100 \quad (3)$$

Where RSA is radical scavenging activity, and Abs is absorbance.

2.4.2 Iron chelation assay

The assay was carried out according to the method of Santos et al. (2017), with minor modifications. In 96 well plates, 50 µL of the

sample (MSG solution with a final concentration of 5000 µg/mL) was mixed with 20 µL of the freshly prepared ferrous sulfate (0.3 mM). At the end of the incubation period, the intensity of the produced color was measured at 562 nm. The values were represented as means ± SD according to equation 4:

$$\text{Inhibition (\%)} = \frac{\text{Abs}_{\text{Blank}} - \text{Abs}_{\text{Sample}}}{\text{Abs}_{\text{Blank}}} \times 100 \qquad (4)$$

The results were recorded using a FluoStar Omega microplate reader.

2.4.3 ABTS assay

The ABTS assay was performed according to the method of Arnao (2000) with minor modifications. A solution of MSG dissolved in water was prepared at a 100 μg/mL final concentration. At the end of incubation time, which was 30 min in the dark at room temperature, the change in color intensity of ABTS was measured at 734 nm, and the values were represented as means ± SD according to equation 5:

$$\text{Inhibition (\%)} = \frac{\text{Abs}_{\text{Blank}} - \text{Abs}_{\text{Sample}}}{\text{Abs}_{\text{Blank}}} \times 100 \qquad (5)$$

Where Abs is the absorbance.

The results were recorded using a FluoStar Omega microplate reader.

2.5 The effect of MSG on carbohydrates digestion

2.5.1 Anti-diabetic activity assay (α -Glucosidase inhibition)

To evaluate the anti-diabetic activity of MSG, an α-Glucosidase inhibition assay was performed as described in previous studies (Elya et al., 2012; Qaisar et al. 2014). Finally, α-Glucosidase activity was calculated using a spectrophotometer at 405 nm by measuring the released yellow p-nitrophenol quantity. The MSG sample concentration ranged between 1800 and 23.8 µg/mL and the positive control was acarbose. The blank replaced the enzyme by adding nitrophenyl α-D-glucopyranoside with buffer solution instead. The inhibitory activity was expressed as percentage inhibition (%) using equation 6:

$$\text{Inhibition (\%)} = \left(1 - \frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}\right) \times 100 \qquad (6)$$

2.6 The effect of MSG on protein digestibility

2.6.1 In vitro digestion model

This study used the standardized static in vitro digestion model (Minekus et al. 2014). Three different phases of digestion were stimulated in the in vitro digestion models: the salivary, gastric, and intestinal phases. Each phase had different enzyme compositions and electrolytes, simulated as described in the protocol of Minekus et al. (2014).

To assess the behavior and interactions of MSG with protein digestion, two digested samples were prepared, and among these, one contained egg white protein only, while the other contained egg white protein with MSG. Then, the digested samples were taken to investigate protein digestibility in both samples using SDS-PAGE and total protein determination.

2.6.2 SDS-PAGE

SDS–PAGE was performed on the digested samples at the Unit of Analytical Chemistry, Faculty of Science, Assiut University, Egypt, using Biometra according to Laemmli's procedure (LAEMMLI, 1970).

2.6.3 Total protein determination

Total protein determination was performed for samples at the Unit of Analytical Chemistry, Faculty of Science, Assiut University, by the Bradford method (Kruger, 2009), using Spector UV-VIS double beam PC scanning spectrophotometer UVD-2950 Labomed.

2.6.4 pH drop method

The method of pH drop was performed as described by Hsu et al. (1977). 50 mL of protein solutions (6.25 mg protein source/mL) were prepared and adjusted to pH 8. A pancreatin solution was also prepared and adjusted to pH 8, and the protein solution was stirred at 37 °C. 5 mL of the solution was dropped on the protein solution, and the pH drop was recorded each minute for 10 minutes. For this regression, equation (7) was used**.**

$$Y = 210.464 - 18.103X \qquad (7)$$

Where Y is protein digestibility %, and X is the final pH at 10 minutes of digestion in the multi-enzymatic medium. The procedure was repeated for each protein source alone, and protein source besides adding 1 g and 2 g MSG; all values are means of triplicated records.

2.7 Fats digestibility

2.7.1 Fat digestibility (Free fatty acids release %)

The digestion activity of lipase for fats was measured by determining the release of free fatty acids from 0.5 g of olive oil during 30 minutes of lipolysis using a titration method mentioned in previous studies (Ji et al. 2019; Li et al. 2011). A lipase solution was prepared by dissolving 500 mg of lipase powder in 50 mL of simulated intestinal fluid, and a final concentration of 10 mg/mL

was prepared under stirring at 37 °C. Then, 100 µL of bile salt solution (160 mM) and 20 µL of $CaCl_2$ were added to 5 mL of previously prepared simulated intestinal fluid solution containing lipase enzyme while stirring. Furthermore, 0.5 g of olive oil was added to the solution. The mixture was left under stirring for 5, 10, 20, and 30 minutes. At the end of lipolysis, 10 mL of (95%) ethanol was added to the mixture to stop lipase enzyme activity, and 1% (w/v) phenolphthalein was used as an indicator. A direct titration with 0.1 N NaOH to a phenolphthalein endpoint was performed using a burette. All the mentioned steps were repeated by adding 50 mg and 100 mg of MSG to the 5 mL of simulated intestinal fluid containing lipase. The FFA% release was calculated using the equation reported in previous studies (Li and McClements 2010).

2.8 Statistical analysis

The data were represented as mean±SD. The data were analyzed using *Microsoft Excel*®, and the IC_{50} value was calculated using *Graphpad Prism 6*® by converting the concentrations to their logarithmic value and selecting a non-linear inhibitor regression equation (log (inhibitor) vs. normalized response – variable slope equation).

3 Results and discussion

3.1 Physiochemical properties of MSG

3.1.1 XRD analysis

The XRD pattern of MSG is presented in Figure 1. The diffraction pattern reflected the polycrystalline nature of MSG with a characteristic peak at 2θ (10.018, 20.027, 25.525, 38.241, 46.474, and 51.380), which agreed with the XRD reported by Saeidnia and Abdollahi (2013). The calculated crystal size of MSG from the Scherer equation was 40.13 ± 12.6 nm, which reflected the nano nature of MSG. This smaller size might be responsible for the cytotoxic effect of MSG as it facilitated its uptake through cells.

3.1.2 FTIR spectrum of MSG

The FTIR spectra of MSG were characterized by several vibrational bands, as shown in Figure 2. The vibrational bands observed in the 3000 ~ 3600 cm^{-1} corresponded to O-H stretching vibration in the molecule, which was formed due to hydrogen bonds. The vibrational band observed at 2900 cm^{-1} was due to C-H stretching vibration, while C=O stretching vibration appeared at 1687 cm^{-1}. The band observed at 1604 cm^{-1} was due to N-H stretching vibration. The bands at 1528 cm^{-1} and 1404 cm^{-1} correspond to C=C and –COO stretching vibrations, respectively. The absorption bands at 1104, 613, and 524 cm^{-1} were attributed to the stretching vibration of -COOH, wagging vibration of $(COO)^-$ and the deformation of HOCC, respectively (Onyema et al. 2006).

3.2 Cytotoxicity of MSG (MTT assay)

The number of viable cells of Caco-2 cells after exposure to different concentrations of MSG (12.5, 25, 50, 100, and 200 mM) was estimated by the MTT assay. Figure 3 demonstrates the cell viability curve as estimated from the MTT assay. A gradual decrease in the cell viability was observed from 136% to 90% as the concentration of MSG increased from 12.5 to 50 mM. However, at the higher concentrations, a slight decrease in the cell viability from 90% to 81%, with an increase in MSG concentration from 50 to 200 mM, was reported. These results revealed the cytotoxic effect of MSG, which was consistent with many previous studies (Elshafey et al. 2017). However, a few studies showed that MSG was not cytotoxic on RGA and H295R cell lines (Shannon et al. 2019).

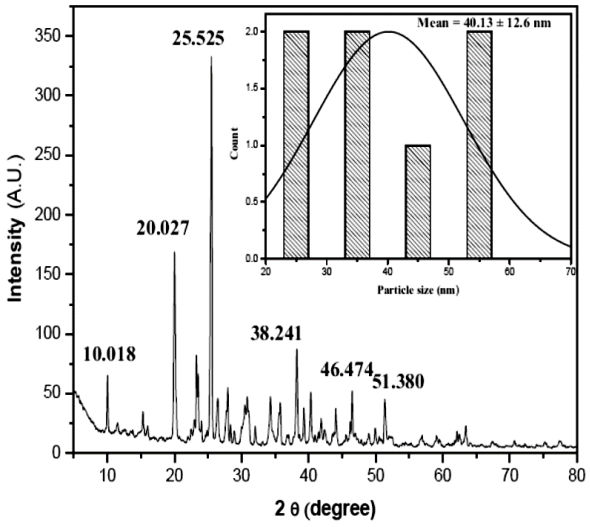

Figure 1 XRD pattern of MSG.

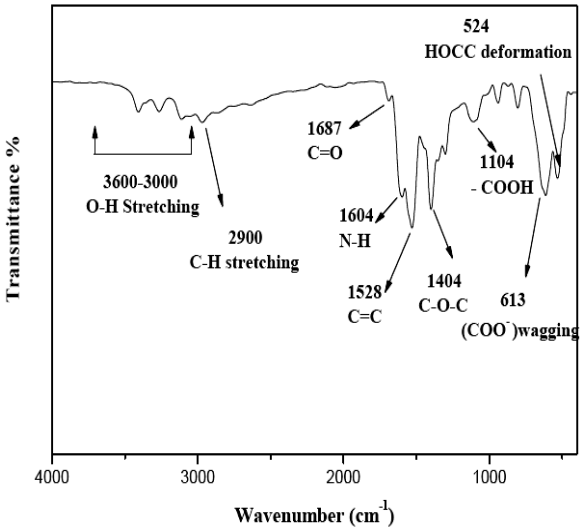

Figure 2 FTIR pattern of MSG.

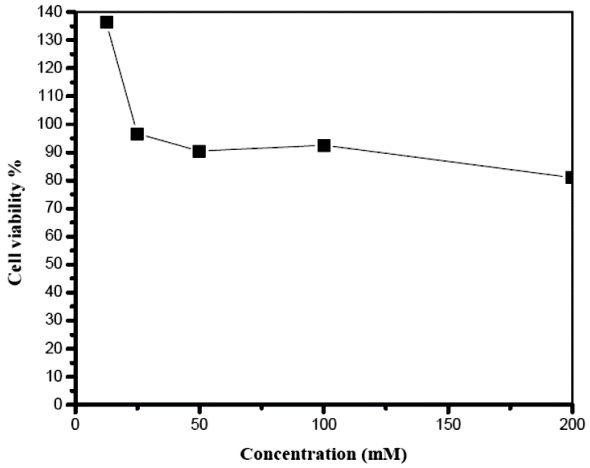

Figure 3 Cell viability of Caco-2 cell line after exposure to MSG with different concentrations (200, 100, 50, 25, and 12.5 mM).

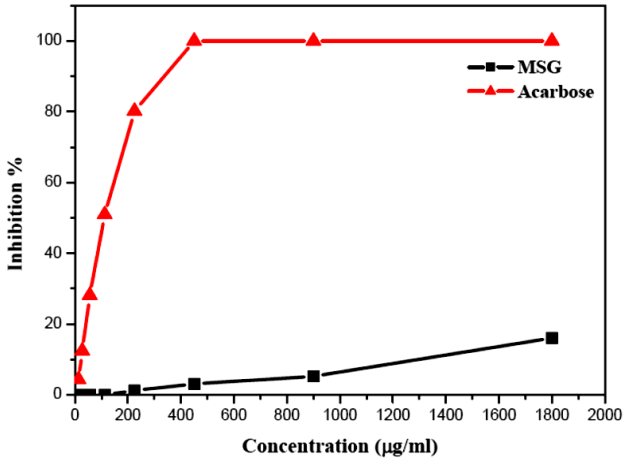

Figure 4 The alpha-glucosidase inhibition rate of MSG compared to acarbose.

Table 1 The inhibition rate of alpha-glucosidase by MSG compared to acarbose

Sample	LC_{50} (µg/mL)	LC_{90} (µg/mL)	Inhibition%
MSG	-------	-------	16.3% at 1800ppm
Acarbose	125±3	245±2.7	100% at 1800ppm

Table 2 Total protein content in the digested egg white protein only and egg white protein with 1 gm of MSG after the whole digestion process

Egg white protein only	5100 mg/l
Egg white protein + MSG	4349 mg/l

3.3 Antioxidant activity of MSG

3.3.1 DPPH assay

No action was detected, which meant it had no scavenging activity.

3.3.2 Iron chelation assay

No action was detected, which meant it had no iron chelation activity.

3.3.3 ABTS assay

No action was detected even with escalating the MSG concentration up to 100 µg/mL, assuring that MSG has no scavenging activity.

3.4 The effect of MSG on carbohydrate digestibility

3.4.1 Alpha-Glucosidase inhibition - anti-diabetic activity assay

The alpha-glucosidase inhibition assay in Figure 4 showed that MSG has a low inhibition effect on the alpha-glucosidase enzyme, even at the highest concentration. As illustrated in Table 1, the inhibition percentage reached only 16.3% by MSG at 1800 ppm, while in the case of acarbose, which served as a standard, it reached 100% inhibition at 450 ppm; the IC_{50} of acarbose was 125±3 ppm, and IC_{90} was 245±2.7 ppm.

3.5 The effect of MSG on protein digestibility

3.5.1 SDS-PAGE

SDS-PAGE was performed to assess the in vitro digestion of egg white protein only and egg white protein with MSG. As shown in Figure 5, all unique bands of egg white protein digested samples disappeared, which revealed its susceptibility to proteolytic enzymes in gastric and intestinal phases. A light aggregation was detected in the case of egg white protein with MSG-digested samples at 17 kDa, likely for lysozyme (Wang et al. 2018). Our results showed that MSG could alter protein digestibility.

3.5.2 Total protein determination

We applied the Bradford method for protein determination in digested egg white protein samples with and without MSG addition. Table 2 represents the results of total protein content after digestion. The digested protein content in the sample containing only digested egg white protein was 5100 mg/L, which was significantly higher than the digested protein content in the egg white protein with MSG digested sample (4340 mg/L). Our results suggest

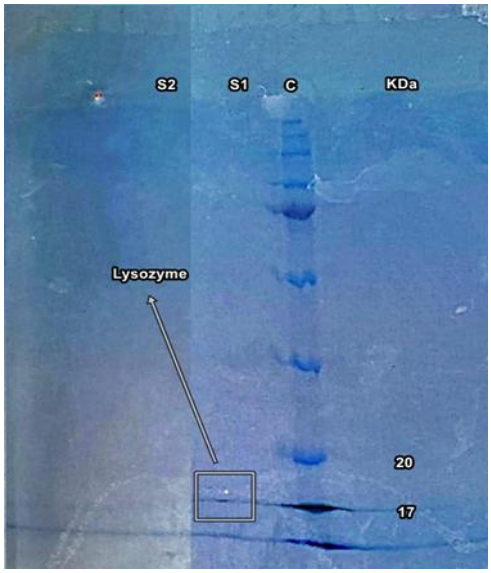

Figure 5 SDS-PAGE for digested egg white protein only (S2) and digested egg white protein with MSG (S1). The markers of standard Mw are in the right lane (c).

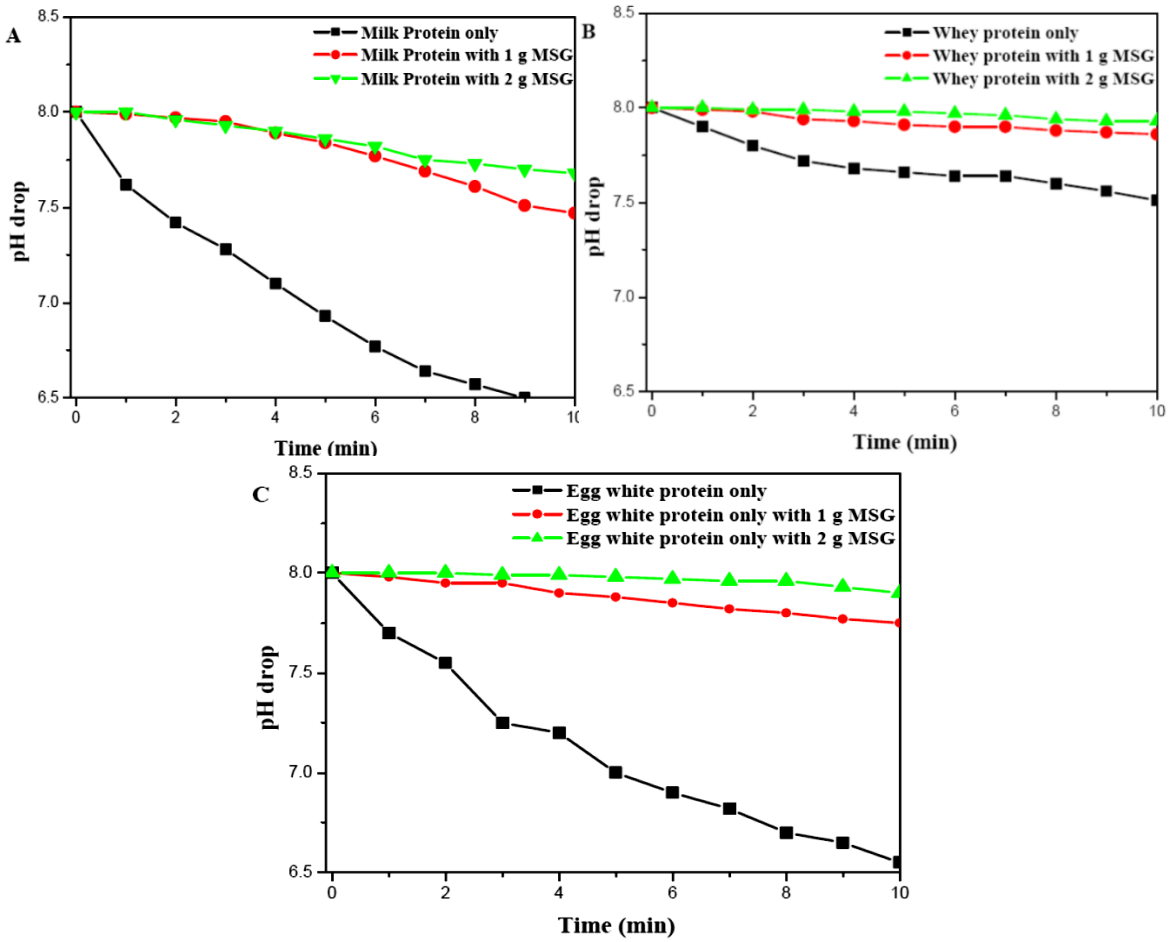

Figure 6 pH drop curves over 10 minutes of digestion with different concentrations of MSG (1 and 2 g) for powdered milk protein (A), whey protein isolate (B), and egg white protein only and with the addition of 1 g and 2 g MSG (C).

that MSG suppresses protein digestion while no previous studies have dealt with this effect to the best of our knowledge.

3.5.3 pH drop method and protein digestibility

Figure 6 describes the pH drop method for the three types of proteins used in this study in the presence of pancreatic supports protein *in-vitro* digestion results were mentioned before. It was observed that protein digestibility was suppressed by adding 1 g and 2 g MSG to each protein solution during digestion in a dose-dependent manner. In addition, Figure 7 represents the protein digestibility as calculated from regression equation 6. The protein digestibility of powdered milk proteins after 10 minutes of digestion was 93.89%, decreased to 75.25 % with adding 1 g MSG and dropped to 71.45% with adding 2 g MSG. Whey protein showed low digestibility. In the case of whey protein isolate alone, protein digestibility after 10 minutes of digestion was 74.53. Its digestibility decreased to 68.19% with adding 1 g MSG and dropped to 66.93% with adding 2 g MSG. Egg white protein digestion after 10 minutes of digestion was 91.91%. Its digestibility decreased to 70.19% with adding 1 g MSG and dropped to 67.47% with adding 2 g MSG.

3.6 The effect of MSG on fat digestibility (FFA release %)

The FFA of percentage release represented in Figure 8 showed a negative effect of MSG presence in the intestinal phase digestion of lipids over 30 minutes in a dose-dependent manner. The olive oil digestion FFA release percentage reached 41.47%. FFA percentage release decreased by adding 50 mg of MSG to 27.64%

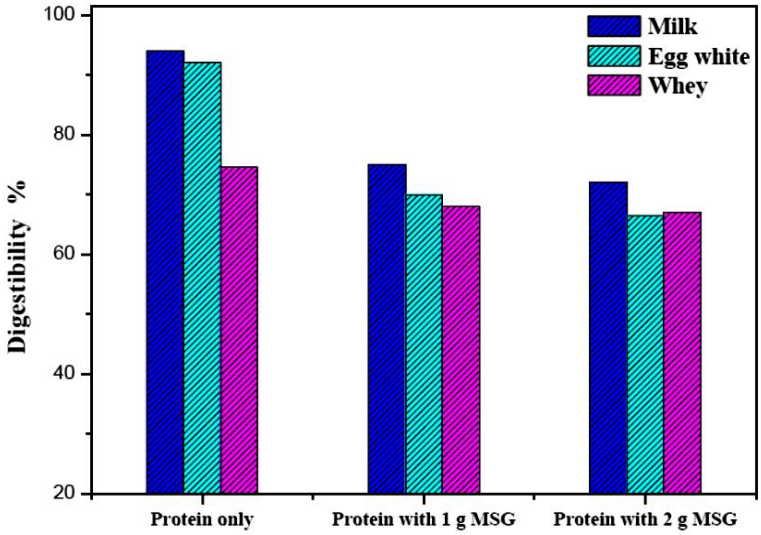

Figure 7 Protein digestibility of powdered milk protein, egg white protein, and whey protein isolate alone and in addition of 1 and 2 g MSG.

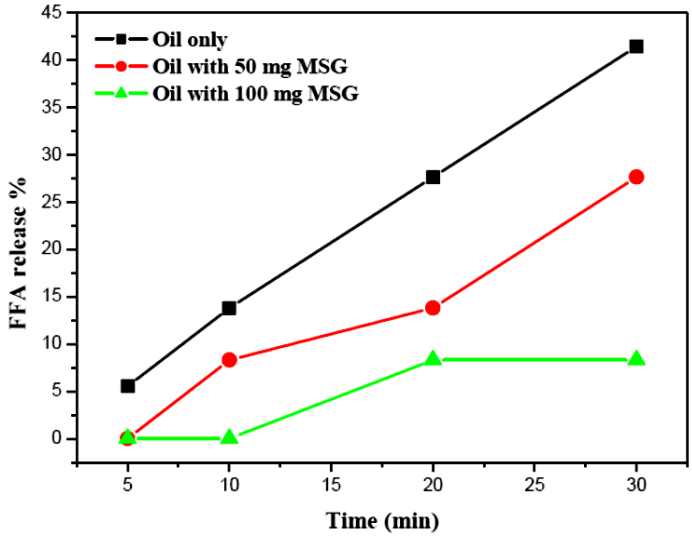

Figure 8 FFA releases % from 0.5 mg of olive oil with and without adding MSG.

and by 8.34% when adding 100 mg of MSG to the digestion media. The current results are novel due to the lack of previous studies concerned about the inhibitory effect of MSG on intestinal lipolysis. To the best of our knowledge, we found only an in vivo study that hypothesized that MSG might alter the lipid lipolysis processes in the small intestine's lumen (Kohan et al. 2016). Another study suggested that MSG administration in rats caused a significant decrease in lipolysis but not in adipose tissue (Dolnikoff et al. 2001).

Conclusion

MSG is a ubiquitous food additive categorized as a flavor enhancer. This study shows that MSG can have a cytotoxic effect on the Caco-2 cell line. MSG's antioxidant activity demonstrates that it has no antioxidant activity at all. In contrast, it leads to oxidative stress through reactive oxygen species (ROS). MSG has a very low inhibition effect on one of the main enzymes contributing to carbohydrate metabolism, alpha-glucosidase. MSG also negatively affects protein and lipid digestion in the gastrointestinal tract (GIT). This study recommends and stresses the significance of more investigations into MSG's interaction with digestive enzymes and nutrient digestion to declare its safety.

Authors' contributions

Alaa Hassan Said wrote the original manuscript and analyzed the data. M. Yasser Alsedfy did the experimental part. A.A. Ebnalwaled and Mona Moustafa revised the original manuscript. The original manuscript was approved and revised by all authors.

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Not applicable.

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Antibacterial Effect of Green Synthesized Silver Nanoparticles using *Cineraria maritima*

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ABSTRACT

Nanoparticles display entirely novel physicochemical characteristics for specific applications because of their exceptional size and shape. Owing to the present study, we reported biosynthesis, characterization and antibacterial properties of *Cineraria maritima* (*Cm*) assisted silver nanoparticles (Ag NPs). The surface plasmon vibration, crystalline structure, surface morphology, elemental composition, and possible functional molecules vibration of prepared *Cm*-Ag NPs were characterized by different instrumentation techniques. The spectrum of UV-Vis of *Cm*-Ag NPs showed maximum plasma intensity occurred around 425nm. XRD spectrum showed the face-centred cubic (FCC) nature of *Cm*-Ag NPs. The SEM image of the *Cm*-Ag NPs demonstrated a predominantly spherical shape with cluster formation of small particles to large particles with sizes ranging from 21.57 nm to 39.16 nm. EDS spectrum indicated the existence of Ag elements in *Cm*-Ag NPs. FTIR intense peaks of *Cm*-Ag NPs showed the different functional molecules such as phenol, alkene, aldehydes, and a carbonyl group. In addition, *Cm*-Ag NPs coated textile cotton fabric sample showed substantial anti-bacterial properties against a tested bacterial pathogen.

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1 Introduction

Nano-technology is one of the most actively explored areas in the biomedical sector. Generally, the properties of nanoparticles can be different due to their morphology and size (Tamulya et al. 2013; Shanmugavadivu et al. 2014). Silver (Ag) is recognized as a potential antimicrobial agent that inhibits the growth of pathogenic microbes. Many studies indicated that the Ag NPs coated with textile and medical devices to control pathogenic infection. However, for the scale-up process, commercially viable, environmentally friendly with enhanced anti-microbial properties of NPs, novel approaches in the production of silver nanoparticles must be developed (Ranjithkumar et al. 2013; Roua et al. 2021). Recent reports showed that preparation of metal and metal oxide NPs using microorganisms (extracellular and intercellular) plant extract (leaf, stem, root, fruit, seeds, peel, etc.) had proven to be eco-friendly and low-cost methods (Mittal et al. 2013; Joud et al. 2021; Das et al. 2022). In comparison with other biological routes that have been developed so far using bacteria, fungi, and algae, the plant extract-assisted Ag NPs have several advantages including scaled-up, low cost, and reaction time (Selvaraj et al.2014; Paulkumar et al. 2021; Kavitha et al. 2021).

The mechanism of biosynthesis of NPs mostly involves bioreduction using microbial intracellular and extracellular enzymes or the plant phyto-compounds present in leaves, stems, flowers, seeds, fruits, or peels (Figure 1). So far, different medicinal plants extracts such as *Cymbopogan citrates*, *Aloe vera*, *Adiantum capillus-veneris*, *Azadirachta indica*, *Memecylon edule*, *Magnolia kobus*, *Rhinacanthus nasutus*, *Morinda tinctori*, *Phyllanthus emblica*, *Malus domestica*, etc., had been used to synthesize Ag NPs and these NPs showed significant biomedical applications (Chandran et al. 2006; Elavazhagan and Arunachalam 2011; Masurkar et al. 2011; Lee et al. 2013; Lalitha et al. 2013; Pasupuleti et al. 2013; Saini et al.2013; Santhoshkumar and Nagarajan 2014; Roy et al. 2014; Vanaja et al. 2014; Rihab et al. 2021; Netra et al. 2022). For centuries, *Cineraria maritima* has been used in the homeopathic system to treat cataracts and other severe eye-related conditions such as conjunctivitis, opacity, and corneal clouding. It is an Asteraceae family annual exotic medicinal shrub (Durgapal et al. 2021). There have been very few studies on the use of *C. maritima* whole plant extract for the biosynthesis of nanoparticles. Hence, this study was carried out to biosynthesize Ag NPs using whole plant powder extract of *C. maritima* and to evaluate the antibacterial properties of these nanoparticles.

2 Materials and Methods

2.1 Preparation of whole plant extract

Fresh plant of *C. maritime* powder was obtained from Homeopathic Research Institute at Emerald, South India. About 10 g of whole plant powder of *C. maritime* was mixed with 100 mL of deionized water and boiled for 30 minutes and the obtained crude *C. maritime* extract. After that, the crude extract of *C. maritime* was filtered using Whatman No.1 filter paper to obtain the extract of *C. maritima*.

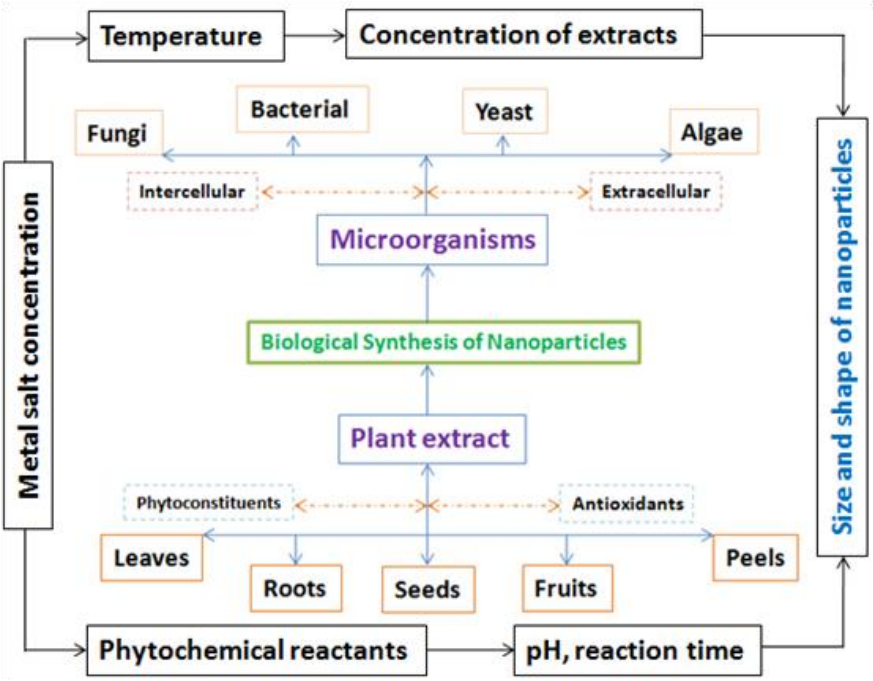

Figure 1 Source of biological substance for NPs synthesis

2.2 Biosynthesis of *Cm*-Ag NPs

About 1mM aqueous solution of 90 mL $AgNO_3$ was added into 10 mL of crude extract of *C. maritima* and the reaction vessel was kept in a dark condition for one day. After that, the reaction mixture was centrifugation for 30 minutes at 6000 rpm to collect *Cm*-Ag NPs. Pellets were washed with deionized water and then dried and stored for further processing.

2.3 Characterization of *Cm*-Ag NPs

The reduction of *Cm*-Ag NPs ions was observed by measuring the surface plasmon resonance (SPR) by UV-2450, Shimadzu UV-Vis analysis. The crystalline structure of the prepared *Cm*-AgNPs was examined by X-ray diffractometer (XRD) spectrum. The surface topology of biosynthesized *Cm*-Ag NPs was obtained by S-4500-SEM (Scanning electron microscopy) and the elemental composition of *Cm*-Ag NPs was performed by energy dispersive spectroscopy (EDS) attached with a scanning electron microscope (SEM). The functional groups of *Cm*-AgNPs were investigated using Fourier Transform infrared (FTIR) spectroscopy (Nicolet Avatar 660, USA).

2.4 Anti-bacterial activity of *Cm*-Ag NPs

The bacteria inoculum (*Escherichia coli*) was swabbed uniformly into culture plates prepared using MHA agar. Round cotton fabrics were cut with a diameter of 1.8 - 2.0 cm and the prepared *Cm*-Ag NPs (1mg/ mL) were treated on the fabric by standard dip dry methods. During dipping, fabrics were submerged in a solution containing *Cm*-Ag NPs (100 µL), pulled out of the bath, squeezed, and then air-dried. A fabric with *Cm*-Ag NPs was placed on top of the culture plates and incubated for 24 hours at 37°C. The zone of inhibition was recorded in millimeters (mm).

3 Results and Discussion

Crude aqueous extract *C. maritime* was used as a biological reducing agent in the production of *Cm*-Ag NPs in this investigation. The reaction mixture turned brown after 24 hours of incubation, which indicated that *Cm*-Ag NPs were being biosynthesized (Figure 2A). The biosynthesis of Ag NPs in the reaction mixture is shown by the SPR peak, which showed at 425-430 nm (Figure 2B). In general, in the surface plasma resonance phenomena, Ag NPs in an aqueous solution have a dark yellowish-

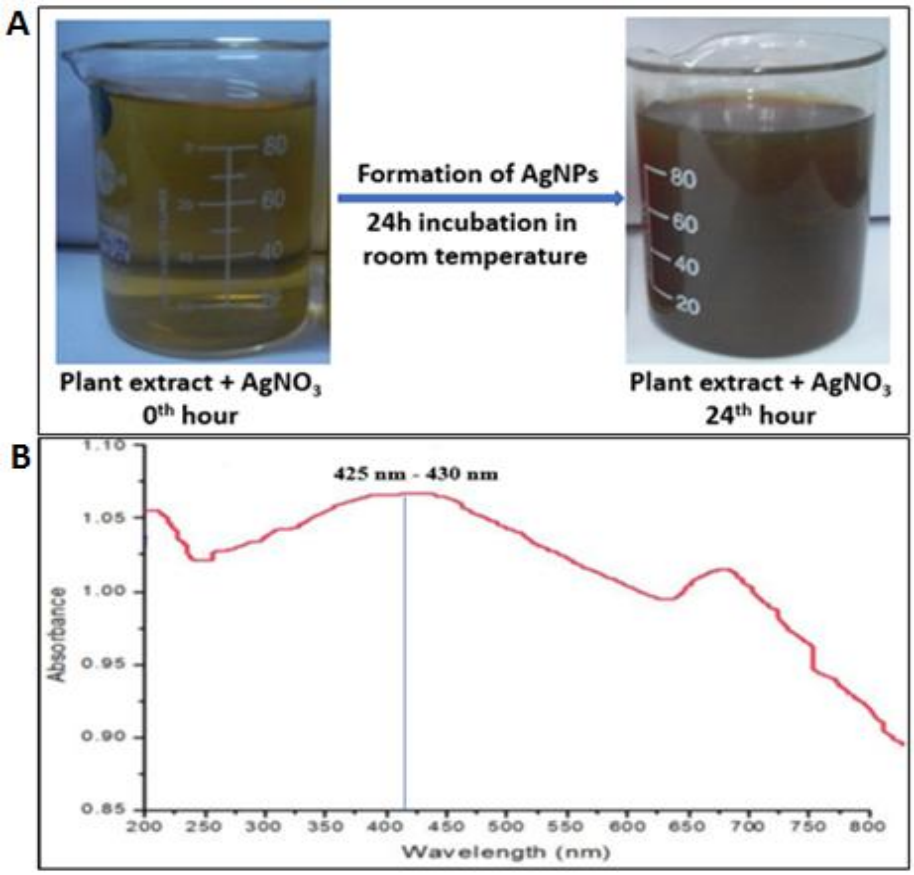

Figure 2 Biosynthesis of *Cm*-Ag NPs (A) Colour change of *Cm*-AgNPs aqueous solution, (B) Optical spectrum of colloidal solution of *Cm*-Ag NPs using UV-Vis analysis

brown color. Plant extract-assisted biogenesis Ag NPs colloidal solution was validated by UV-Vis spectrum analysis, with intensity peaks around 400 nm-450 nm (Krishnaraj et al. 2010; Chhangte et al. 2011;). Similarly, in the current study the SPR peak of *Cm*-Ag NPs was around 430 nm.

The XRD spectrum of *Cm*-Ag NPs revealed prominent peaks that corresponded to the Miller indices [hkl] of silver (111), (200), (211), (220), and (311), indicating that *Cm*-Ag NPs are face-centered cubic (FCC). In addition, the XRD pattern revealed silver oxide Miller indices [hkl] of (100) and (110), orientation to JCPDS card no 04-0783, and the observed Miller indices [hkl], the observed peaks at 2θ = 38.79°, 46.48°, 64.68°, and 85.13° location suggest a synthesis of silver. In addition, regarding JCPDS file no 00-076-1393, the observed peak at 2θ = 27.32°, 32.33°, 57.49°, and 61.51° indicate the formation of silver oxide (Figure 3).

In general, the plant has a variety of phytochemicals that are responsible for NPs production through the biosynthesis method. Earlier reports suggested that the plant extract-assisted biogenesis Ag and Ag_2O exhibited X-Ray diffraction intensity at 2θ of 27°, 32°, 38°, 44°, 49°, 57°, 61°, 64° and 72° (Vijaya Raj et al. 2012; Awwad et al. 2013; Rufen et al. 2019). Correspondingly, the Braggs reflections pattern of XRD of the current study confirmed the presence of silver in prepared *Cm*-Ag NPs. The XRD image with additional peaks (*) may be due to the crystallization of the plant phytochemicals phase. Through the XRD pattern using the Scherrer equation (Madhan et al. 2021), the prepared *Cm*-Ag NPs revealed a particle size of around 18.36 nm to 36.15 nm.

The surface morphology of the prepared *Cm*-Ag NPs was visualized using a SEM image (Figure 4A). The topology images of *Cm*-Ag NPs revealed that the surface morphology was not well defined; however, the prepared *Cm*-Ag NPs are predominantly spherical with cluster formation of small particles to large particles with particles size of *Cm*-Ag NPs around 21.57 nm to 39.16 nm.

The surface topology such as the size and shape of NPs showed crucial functions in the applications of NPs in different sectors (Sharmila et al. 2019; Mitchell et al. 2021). The elemental composition of *Cm*-Ag NPs was determined using their corresponding EDS spectra in this investigation, the obtained *Cm*-Ag NPs showed a peak at 3.27 keV corresponding to the binding energies of Ag ions (Figure 4B). There were also additional small peaks in the EDS spectra, indicating the presence of trace element precipitates in the *C. maritima* whole plant extract. The weight percentage of *Cm*-Ag NPs from the whole plant extract *C. maritima* was reported around 67.60 %.

FTIR analysis of *Cm*-Ag NPs shows many peaks, which indicate different functional molecules involved in the surface of the prepared sample (Figure 5). The bands observed at 3869.20 cm^{-1}, 3726.27 cm^{-1}, 3606.89 cm^{-1}, 2879.72 cm^{-1}, 1514.12 cm^{-1}, 1463.97 cm^{-1},999.13 $cm^{-1,}$ and 678.94 cm^{-1} were allotted to the vibrations of N-O, C=O, C=C, O-H, C-H, and N-H, corresponding to functional groups of carbonyl groups, cis-disubstituted alkene, phenol, carboxylic acids, aldehydes, alkene, hydroxyl, secondary amide, and alcohol. The existence of C-H, O-H, N-H, and C-C vibrations of carboxylic, hydroxyl, amine, and aromatic groups in the plant extract mediated Ag NPs was shown by FTIR intense peaks at 3422cm^{-1}, 2921 cm^{-1}, 1743 cm^{-1}, 1631 cm^{-1}, 1240 cm^{-1} and 1043 cm^{-1} (Kumari et al. 2016) and these phytochemicals act as a reduction, capping and stabilization agents.

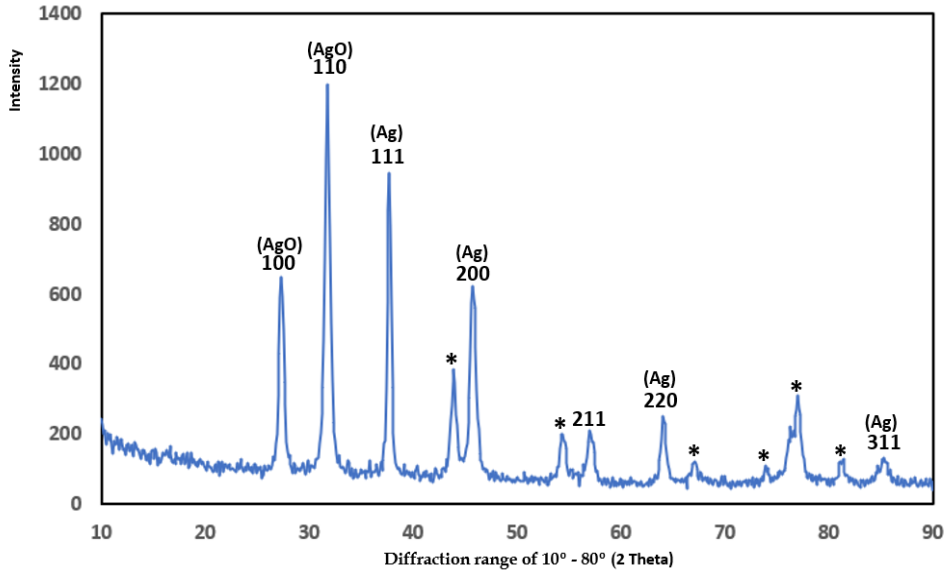

Figure 3 XRD spectrum of *Cm*-AgNPs

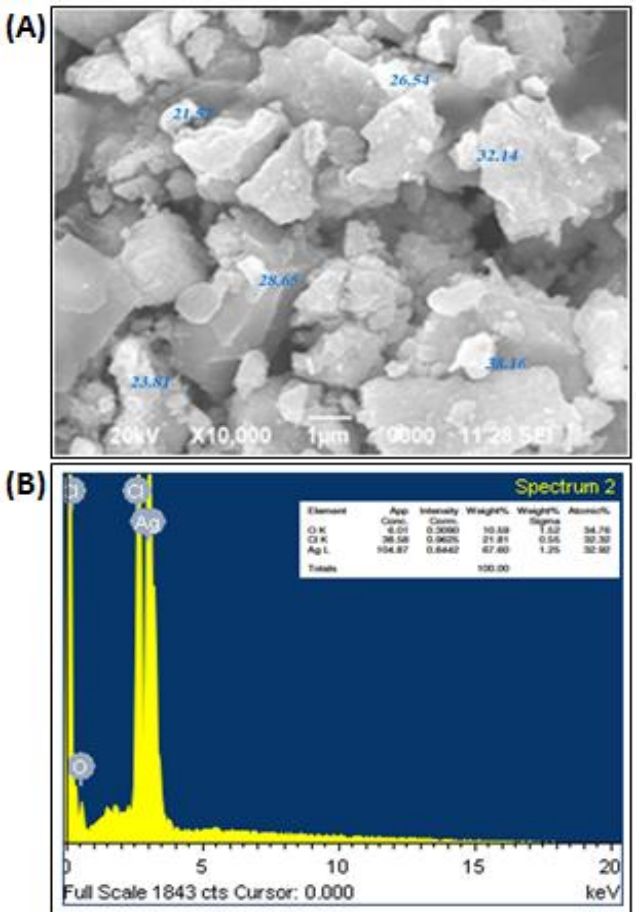

Figure 4 SEM-EDS analysis of *Cm*-AgNPs

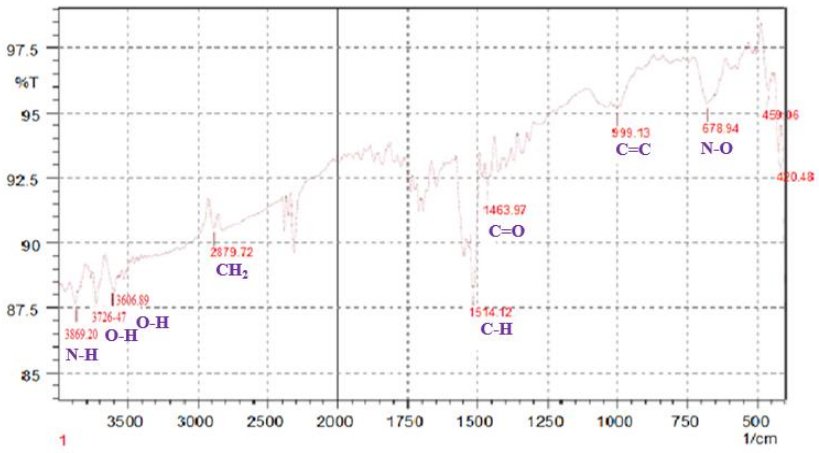

Figure 5 FTIR spectrum of *Cm*-Ag NPs

The FTIR spectrum revealed that the prepared *Cm*- Ag NPs contain different types of functional groups in the whole plant aqueous extract of *C. maritima* (Table 1). The phytochemicals found in the plant extract are the reason for the reduction of silver nitrate into *Cm*-Ag NPs. Ag NPs are the most effective anti-bacterial agents when compared to another metal oxide NPs (Liao et al. 2019; Urnukhsaikhan et al. 2021). The anti-bactericidal activity of *Cm*-Ag NPs fabricated fabric sample was demonstrated in this study, which showed considerable growth inhibition properties against the test pathogen

Table 1 Functional groups from FTIR spectrum of Cm-Ag NPs

Absorption (cm^{-1})	Molecular motion	Functional group
3869→3606	O-H with N-H stretching modes	Alcohol, secondary amide, hydroxyl
2879	CH_2 vibration	Alkene, aldehydes
1514→1463	C-H, C=O stretching	Carboxylic acids, phenol
999→678	C-H, C=O stretching	cis disubstituted alkene carbonyl group

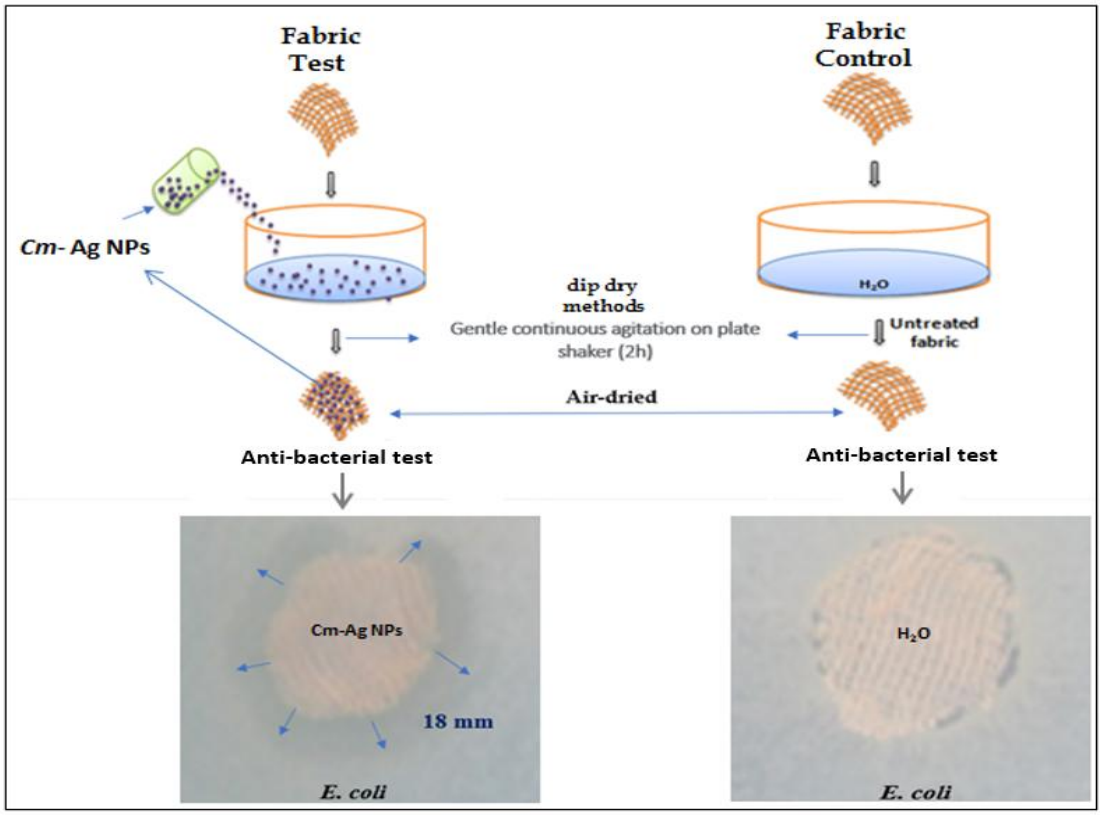

Figure 6 Antibacterial Activity of AgNPs coated fabric

of *E. coli*. Figure 6 shows the anti-bacterial activity of *Cm*-Ag NPs treated and untreated cotton fabric samples. An earlier study on the anti-bacterial properties of spherical-shaped Ag NPs produced from leaf extract of *Psidium guajavu* with particle size around 55 nm exhibited significant results against *S. aureus* and *E. coli* (Sharmila et al. 2018).

Different processes could be involved in the anti-bacterial properties of NPs such as the interaction of NPs on the surface of the bacteria cell membrane, ribosome disassembly, inactivation of essential enzymes, which are involved in the protein synthesis, NPs interact with cell signaling, etc. Nevertheless, the anti-bacterial effects of NPs are mostly determined by the size, shape, and kind of capping agent present on the NP's surface (Francis et al. 2020; Gupta et al. 2021). In the present study, the prepared *Cm*-Ag NPs coated fabric sample demonstrated a significant anti-bacterial effect on *E. coli*, the zone of inhibition was 18 mm, whereas, the uncoated fabric sample had no inhibition zone and hence it can be used to treat and manage a wide range of microbial infections.

Conclusion

The presence of diverse active phyto-molecules in the prepared C. *Maritime* extracts resulted in the biogenesis *Cm*-Ag NPs which exhibited a brown colour in an aqueous solution and revealed an optical density peak of about 430 nm, indicating the formation of *Cm*-Ag NPs in aqueous solution. XRD spectrum indicated FCC configuration of *Cm*- Ag NPs and silver oxide NPs, and the silver oxide nanoparticles were further reduced to silver nanoparticles. The SEM image showed that the prepared *Cm*-Ag NPs have predominantly spherical surface morphology with the particle size

around 21.57 nm to 38.16 nm. EDS spectrum revealed the presence of Ag in the prepared sample. The FTIR spectrum disclosed the functional groups of alcohol, secondary amide, hydroxyl, alkene, aldehydes, carboxylic acids, phenol, cis-disubstituted alkene, and carbonyl groups. The bioreduction of *Cm*-Ag NPs may be due to active phytochemicals found in the aqueous extract of *C. maritima*. The findings show that the predominantly spherical shape *Cm*-Ag NPs had significant anti-bacterial efficacy against *E. coli*.

Conflicts of interest

The authors affirm that they do not have any conflict of interest.

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Partial Purification of Extracellular Amylase From Halotolerant Actinomycetes *Streptomyces brasiliensis* MML2028

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ABSTRACT

Amylase is considered as an industrially important enzyme as it occupies the most important function in the food, paper, and pharmaceutical industries. The present study is concerned with the optimization, production and partial purification of halotolerant amylase from newly isolated *Streptomyces brasiliensis* MML2028, from Kelambakkam salt pan, Tamil Nadu, India. The primary screening was carried out by well diffusion assay to find the zone of lysis. The assay was observed for each media optimization by measuring the release of reducing sugar (RS) by the 3,5 dinitro salicylic acid (DNS) method and expressed in the international unit (UI). Ammonium sulphate precipitation was used to partially purify the enzyme and then lyophilized. SDS-PAGE was performed to identify the molecular weight. The production medium was optimized with 1% of the starch substrate, 3% of NaCl at 24 °C and pH 9, and incubation of 9 days. The total activity of the partially purified α-amylase was observed to be 1806.9U/mL. The partially purified enzyme was more active with 3% NaCl, pH 8, and 24 °C which is known to be a halotolerant alkaline α-amylase. The enzyme showed tolerance towards magnesium, manganese ions, Triton x-100, and urea. De-inking of α-amylase showed good results proving that the enzyme activity is more efficient. Hence, the alkaliphilic amylase from Halotolerant actinomycetes *S. Brasiliensis* MML2028 could be a better microbial source that can be used in many industries, especially in paper and textiles.

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1 Introduction

The thrust on industrially important enzymes has become more important due to their eco-friendly nature (Asrat and Girma 2018). The amylolytic enzymes function as a major part of the carbon cycle. They can be divided into three groups namely α-amylase, β-amylase, and glucoamylase, which are capable of hydrolyzing starch and glycogen. These enzymes cleave the starch and starch linked polymers to give up products. In the beginning, amylase was used to hydrolyze the α-1, 4- glucosidic bonds of amylose, amylopectin, glycogen, and their degradation products (Mohamed et al. 2021). Extreme environments are measured as a flourishing resource for extremophiles that can produce dynamic enzymes that have numerous industrial applications and are appropriate for harsh surroundings to evaluate their counterpart. Alpha amylase can be produced by micro and macro organisms (Simair et al. 2017) and is also used as an add-on in detergents owing to their elevated alkaline pH stability which is now needed for industries as the alkaline nature of groundwater (Asad et al. 2011). At present, there is a demand for alkaline amylase production in the worldwide market (Abdullah et al. 2014).

Extremophiles from the extreme environment can produce enzymes that are useful for the industrial process (Zhu et al. 2020). Alkaline environments have drawn attention to the isolation of alkaliphilic bacteria, which are capable of producing alkaline enzymes. The industrial demand for enzymes, with appropriate specificity and stability to pH, temperature, metal ions, surfactants, and organic solvents continues to stimulate the search for new enzyme sources. Enzymes with high activity and stability at higher temperatures are interesting for bioengineering and biotechnological applications (Arauza et al. 2009). Fabric, paper, food, and fermentation are the industries where amylases have a large range of applications (Haq et al. 2010).

The previous studies carried out at Biocontrol and Microbial Metabolites Lab, Centre for Advance Studies in the Botany Department of Madras University identified 30 halotolerant actinomycetes isolated from Kelambakkam salt pans that could produce a huge amount of industrially important extracellular enzymes. Therefore, the present study aimed to investigate these halotolerant actinomycetes for the production of amylase enzymes. So far, no report was available on amylase production from halotolerant actinomycetes *S. brasiliensis.* In addition to screening, partial purification and characterization of the amylase enzyme were also carried out. Furthermore, because of the increasing importance of amylase enzymes in biotechnological applications, the present study also indented to conclude the effect of pH, thermostability, and application of the partially purified amylase produced by the halotolerant actinomycetes *S. brasiliensis* MML 2028.

2 Materials and Methods

2.1 Preliminary screening for amylase production

In the current study, 30 halotolerant actinomycete isolates were screened for amylase production by plating the isolates on Nutrient agar amended with 1% of starch and incubated at 28°C for 4 days. After incubation, the plates were flooded with an indicator containing 2% of iodine and 1% potassium iodide and the zone of clearance was observed and measured the zone in diameter (mm). The maximum zone-produced culture was selected for further studies.

2.2 Identification of halotolerant actinomycete MML2028

The potential isolate with significant activity was further identified based on morphological, physiological, biochemical, and molecular characterization (Buchanan and Gibbons 1974).

2.2.1 Colony morphology

Halophilic actinomycetes MML2028 was grown on ISP-2 medium and incubated for eight days and the colony morphology, pigmentation, sporulation, etc. were recorded (Krishnakumar et al. 2015).

2.2.2 Cover-slip culture technique

This technique was performed to study the morphology of the actinomycetes. Sterile SCA medium was poured into Petri plates and 3-4 sterile square coverslips were inserted into the medium at an angle of 45°C. The broth culture of actinomycetes was slowly released over the coverslip at the intersection of the medium and the coverslip. The plates were incubated at 28°C for 4-8 days. The coverslips were removed and stained with Lactophenol cotton blue and then observed under a Light microscope (45X) (Jagan Mohan et al. 2014).

2.2.3 Determination of cell wall amino acid

A yeast extract medium was prepared and the culture was inoculated into the sterile medium. After 4 days of incubation, the culture was centrifuged and washed with methanol. The upper layer was removed and the precipitate was washed and it was then freeze-dried. From this, 20g of the freeze-dried pellet was dissolved in 5 mL of 6N HCl at 100°C for 18 hours and evaporated to remove HCl. After evaporation, the pellet was subjected to thin-layer chromatography (TLC). The solvent system contained methanol: H_2O: 10 M HCl: Pyridine in the ratio of 80:26:25:20 by volume (Becker et al.1964). The bands were viewed by spraying ninhydrin (0.1%) solution.

2.2.4 Molecular identification of Halotolerant actinomycetes

The DNA was extracted by the phenol-chloroform method. The extracted DNA sample was amplified by PCR technique consisting of initial denaturation (90s at 94ºC), 30 cycles (denaturation for 15s at 94ºC, annealing for 10s at 60°*C*; extension for 30s at 72ºC), and final extension for 4 min at 72ºC. Primers used were 5AGAGTTTGATCCTGGCTCAG-3 and 5'-ACGGCTACCTTGT TACGACTT-3'(Weisburg et al. 1991). Then the PCR product was subjected to sequencing. Nucleotide sequence data were obtained from DNA sequencing software of ABI 3730xl DNA analyzer (Model 373, Forster, CA, USA) (Monciardini et al. 2002; Li et al. 2016; Chen et al. 2016). Multiple sequence alignment was performed using CLUSTAL W and then the sequences were submitted to NCBI (GenBank).

2. 3 Selection of amylase production medium

Six different optimized media for actinomycetes from various articles were screened for enzyme production, which included: Amylase production medium 1 (APM 1) (Chao-Hsun and Wen-Hsiung 2004), Amylase production medium 2 (APM 2) (Poornima et al. 2008), Amylase production medium 3 (APM 3) (Ray and Kar 2009), Amylase production medium 4 (APM 4) (Prabavathy et al. 2006), Amylase production medium 5 (APM 5) (Suman and Ramesh, 2010) and Amylase production medium 6 (APM 6) (Stamford et al. 2001). The culture was grown in different production media (APM 1 - 6) and incubated at 24°C for eight days, after incubation the enzyme activity was visualized by a zone of lysis by adding the indicator. Protein concentration was determined by a dye-binding method of Bradford using Bovine Serum Albumin as the standard protein **(**Bradford 1976**).**

2.3.1 DNS assay method

The culture supernatant was used for amylase activity and the activity was estimated by a modified DNS assay method. The assay was carried out at 50°C using 1% starch as a substrate. The substrate was prepared in 100 mM Glycine NaOH buffer (pH10.5). About 0.5 ml of starch buffer solution was pre-incubated at room temperature for 5min. The reaction was initiated by adding 1 ml of the enzyme. After incubation for 60 min at 5°C, the reaction was terminated by adding 2 ml of 1% dinitrosalicylic acid (DNS) reagent. The content was incubated for 15 min at 50°C. Further, 1 ml of 40% Rochell's salt solution (Potassium sodium tartrate) was added and the sample absorbance was read at 540 nm. One unit of enzyme activity was defined as 1µg of glucose released per min (Ashabil et al. 2008).

$$\text{Enzyme activity} = \frac{\text{Amount of glucose liberated } \left(\frac{\text{mg}}{\text{ml}}\right) \text{ x Total assay volume}}{\text{Volume of enzyme x Time of incubation x Volume in cuvette}}$$

2.4 Optimization of the amylase production medium for increased enzyme production

The culture was grown in different time intervals (1 to 9 days), different starch concentrations (0.5% to 3.5%), different temperatures (4°C to 55°C), different NaCl concentrations (3% to 7 %), and different pH levels (pH 5 to11) to get a suitable medium for maximum amylase production. The enzyme activity was visualized as a zone of clearance by adding the indicator and measure zone in diameter (mm) (Balakrishnan et al. 2021)

2.5 Mass production in optimized amylase production medium

The culture MML2028 was grown in an optimized production medium at 28°C for 8 days. Then, the culture broth was centrifuged at 10,000 rpm for 20min and the supernatant containing the crude enzyme was collected.

2.6 Partial purification of amylase produced by Halotolerant actinomycete MML2028

All the steps in partial purification were carried out at 4°C. The actinomycetes culture supernatant liquid containing the extracellular enzyme was lyophilized to perform the further assay. The lyophilized supernatant was added with ammonium sulphate (Green and Hughes 1955) with nonstop overnight stirring and removed into the following saturation ranges: 0–20, 20–40, 40–60, and 60–80%. Then it was centrifuged at 12,000*g* for 15 min and the pellet was collected. The pellet was dissolved in 0.1 M citrate-phosphate buffer, pH 5.0. Dialysis was carried out with the same buffer for 12 hours many times to remove the salt content (Plumer 1978).

2.7 Quantitative and Qualitative assay of a partially purified amylase

The lyophilized sample was qualitatively assayed for amylase production. The amylase production medium was poured into Petri plates and after solidifications, 8 mm diameter wells were made using a cork borer, 100µL of culture filtrate, and 100 µL of dialyzed protein sample were inoculated in a well. After 24 h of incubation, the enzyme activity was visualized as a clear zone with the addition of an indicator. The zone was determined in diameter (mm). Protein and DNS assays were performed on the lyophilized sample.

2.8 Effect of pH, temperature, salt concentration, and metal ions on amylase activity and stability

To find the temperature stability of the partially purified enzyme was pre-incubated at 16°C to 55°C up to 45 min at the optimum pH and the remaining activity was determined under standard enzyme assay conditions. For pH stability, the enzyme was pre-incubated at pH between 4 to 10 at 24°C for one and two hours. NaCl concentration up to 3 to 7% was used according to the standard enzyme assay conditions.

The effects of metal ions ($CaCl_2$, $MgSo_4$, $CoCl_2$, $MnSo_4$, $ZnS0_4$) and chemical reagents including chelating agents and inhibitors (EDTA, Triton, Beta Mercaptoethanol, Urea, SDS) on partially purified enzyme activity were studied by pre-incubating the enzyme in the presence of substances with a concentration of 3 to 5 mM for 45 min at 24°C, and then continue the assay in the presence of the same substances at the optimum temperature. The melts ions used for this study were in chloride form.

2.9 SDS - Polyacrylamide gel electrophoresis

The protein content of the lyophilized crude was examined by SDS-PAGE. The sample was resuspended in 1 ml of Phosphate buffer (0.2M, pH 7) and used as the source of protein. Sodium dodecyl sulphate is a very strong anionic detergent. It is an amphiphatic molecule that consists of a non-polar hydrophilic region and a strong polar anionic group. In the presence of SDS and reducing agents such as β-mercaptoethanol, oligomeric proteins are dissociated into their constituent polypeptide chains. These polypeptide chains were shown to migrate in SDS gels of the correct porosity according to their molecular weights. The size of polypeptide chains of given proteins can be determined by comparing its electrophoresis mobility in SDS gels with mobility marker proteins of known molecular weights (Laemmli 1970).

2.10 Application for Amylase

2.10.1 Deinking with Alpha-Amylase

Ink jet-printed paper from an HP printer was pulped by soaking in hot water for 2 hours and macerated in a domestic mixer after adding 0.1 % Tween 80, a non-ionic surfactant. The macerated pulp was oven-dried at 50°C and stored in a sterile container under refrigeration. Immediately before use, the pulp was soaked in water for 30 min. The pulp was sterilized at a consistency of 3-6% (3-6 g pulp in 100 ml of half-strength seawater) by autoclaving. After cooling, it was incubated with the enzyme at room temperature. After 3 to 4 days the decolorized pulp was washed thoroughly with tap water. The washed pulp was filtered to obtain the pulp in the form of hand sheets. These sheets were pressed flat between two stainless steel plates and oven-dried at 50°C for 12 h. They were gently pressed with a steam iron to get uniform thickness (Saxena and Singh Chauhan 2017)

2.10.2 Desizing with α-amylase

A stiff piece of grey fabric having maximum starch was used in the present study. An equal size (5×5 inch) fabric piece was weighed on an electric balance. The cloth strip was then dipped in 100 ml of enzymatic solution (pH 6.5) and then placed in an incubator at 60°C for 1 hr. The cloth strip was washed with tap water and then oven-dried. After drying, the cloth strip was weighed again (Chimata et al. 2011). The % removal of starch was calculated by applying the following formula:

$$\% = \frac{\text{Wt. of starch removed by enzyme}}{\text{Total starch present on the fabric strip}} \text{ x } 100$$

2.11 Statistical analysis

The amylase activity was done in a triplicate manner, and data existing in figures and tables are the mean of three experiments. Statistical assessment for important differences between average values was performed using one-way ANOVA software.

3 Results

3.1 Preliminary screening of amylase enzyme

Cultures for screening amylase activity were obtained from the culture collection of Biocontrol and Microbial Metabolite Lab (MML2001 to MML2030). Among them, halotolerant actinomycete MML2028 showed good amylolytic activity by producing a maximum clear zone of clearance (Table 1).

Table 1 Preliminary screening for amylase production

S. N	Culture	Results	S. N	Culture	Result
1.	MML2001	++	16.	MML2016	+
2.	MML2002	_	17.	MML2017	++
3.	MML2003	++	18.	MML2018	+
4.	MML2004	_	19.	MML2019	-
5.	MML2005	_	20.	MML2020	-
6.	MML2006	++	21.	MML2021	+
7.	MML2007	_	22.	MML2022	+
8.	MML2008	++	23.	MML2023	+
9.	MML2009	_	24.	MML2024	++
10.	MML2010	_	25.	MML2025	++
11.	MML2011	_	26.	MML2026	-
12.	MML2012	+	27.	MML2027	-
13.	MML2013	_	28.	MML2028	+++
14.	MML2014	+	29.	MML2029	+
15.	MML2015	+	30.	MML2030	-

3.2 Identification of haloterant actinomyceteMML2028

3.2.1 Colony morphology

Colonies were raised, wrinkled, opaque, pink coloured, earthy odour, and non-motile. These morphological characters are found to coincide with the characters of *Streptomyces* sp. based on Bergey's manual of systematic bacteriology (Table 2).

Table 2 Colony morphology of halotolerant actinomycetes MML2028

Colony Morphology	Characteristics
Elevation	Raised
Surface	Wrinkled
Density	Opaque
Size	Very Long Rods
Shape	Rods
Pigment and Odor	Pink, Earthy
Spore and Sporangia	Positive
Mycelium	Aerial
Spore Motility	Non Motile
Spore Surface	Smooth and Hairy

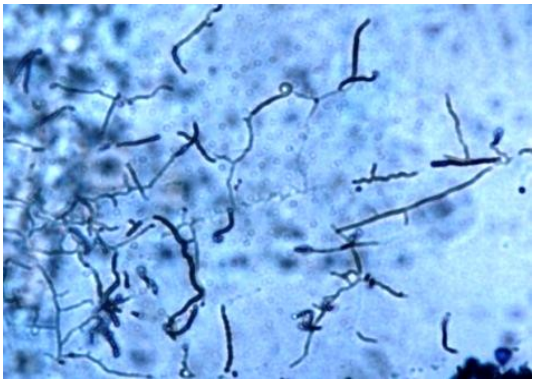


Figure 1 Morphological view of MML 2028 on Cover-slip culture technique

3.2.2 Cover-slip culture technique

Coverslip culture of MML2028 was observed periodically under a phase contrast microscope from day 2 onwards up to seven days. The development of dense substrate mycelium was observed after 2 days, which later gave rise to highly branched aerial mycelium with a typical spiral branching pattern (Figure 1).

3.2.3 Determination of cell wall amino acid

The cell wall amino acid analysis of MML2028on TLC revealed the presence of LL-diaminopimelic acid (LL-DAP), which was visualized as a yellow spot with R_f value of 0.71. The same R_f value was also determined for the LL-DAP of the reference strain, *S. avermitilis*. In addition to the above yellow LL-DAP spot, glycine was observed as a pink spot (cell wall type I) (Figure 2).

3.2.4 Molecular identification of halophilic actinomycetes MML 2028

DNA was isolated and it was amplified by PCR technique. The PCR products were sequenced and the sequences were compared with 16S rRNA gene sequences available in the NCBI database by BLASTn search. The sequence showed 97% of similarity to *S. brasiliensis.* The GEN bank accession Number is KF542679.

3.3 Selection of amylase production medium

Among the six amylase production medium used, APM1 was found as an efficient production medium for the production of amylase enzyme, protein estimation, and DNS enzyme assay was recorded and tabulated (Figure 3a to f).

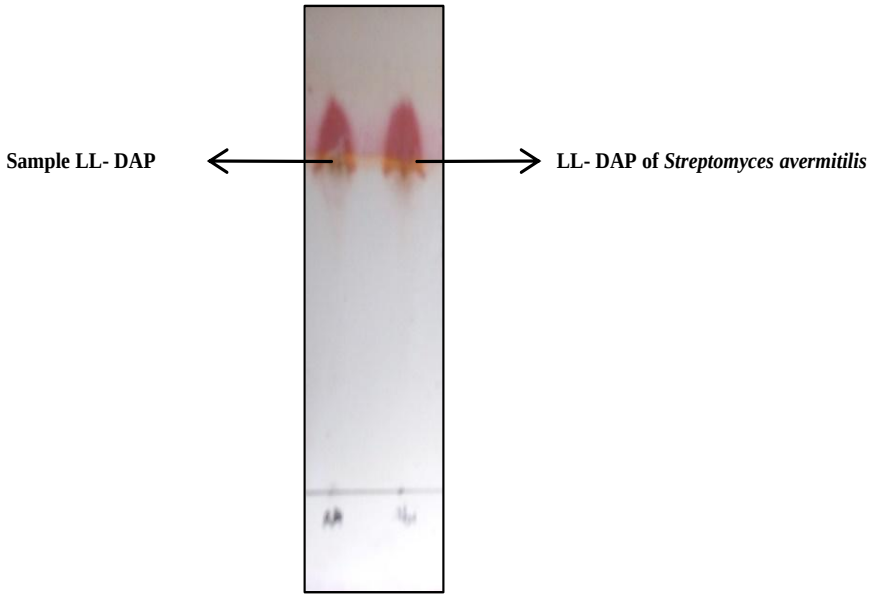


Figure 2 Determination of cell wall amino acid

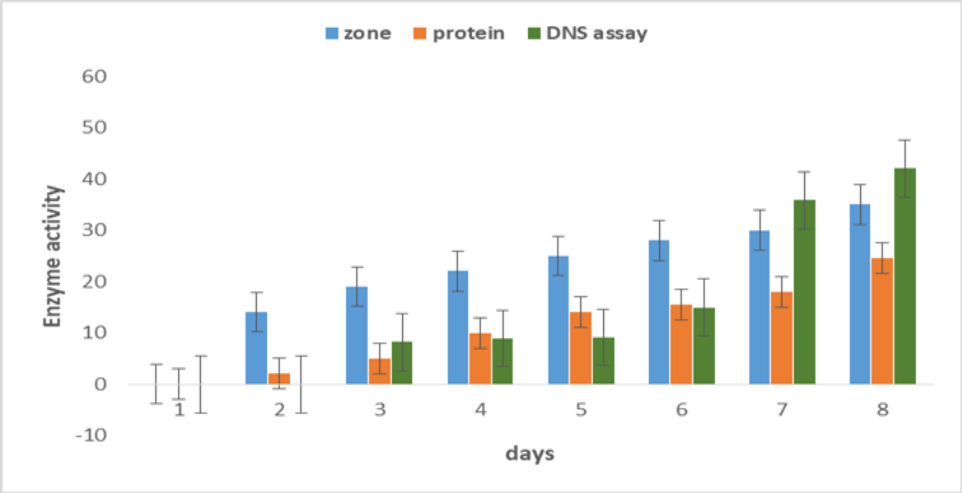


Figure 3a Screening of Halotolerant *S. brasiliensis* MML2028 in APM 1 for amylase production

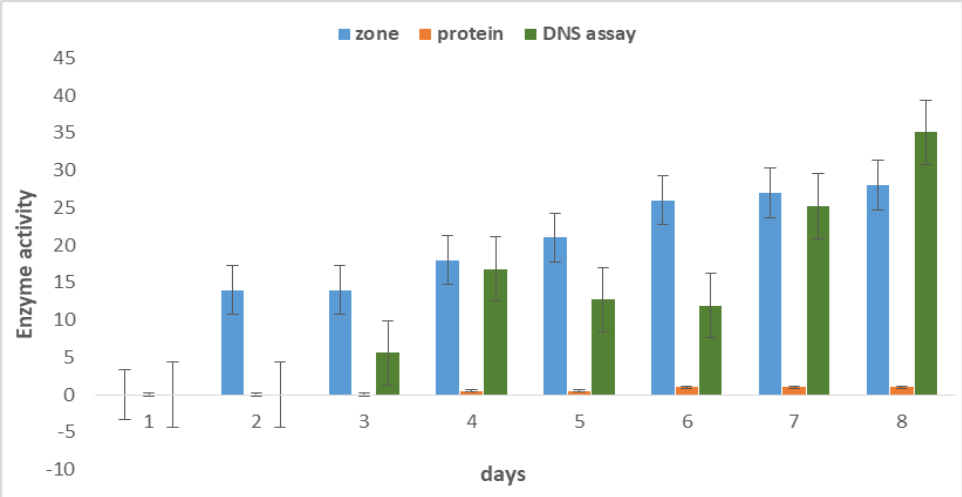


Figure 3b Screening of Halotolerant *S. brasiliensis* MML2028 in APM 2 for amylase production

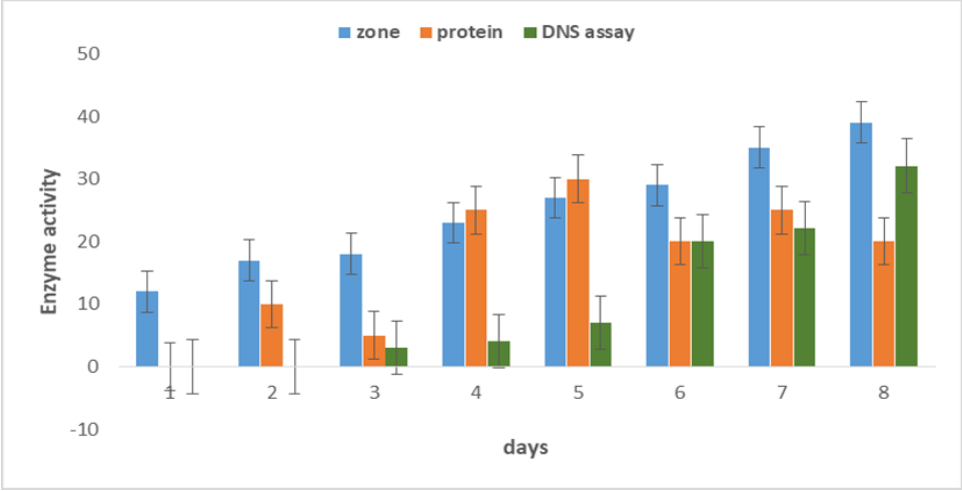


Figure 3c Screening of Halotolerant *S. brasiliensis* MML2028 in APM 3 for amylase production

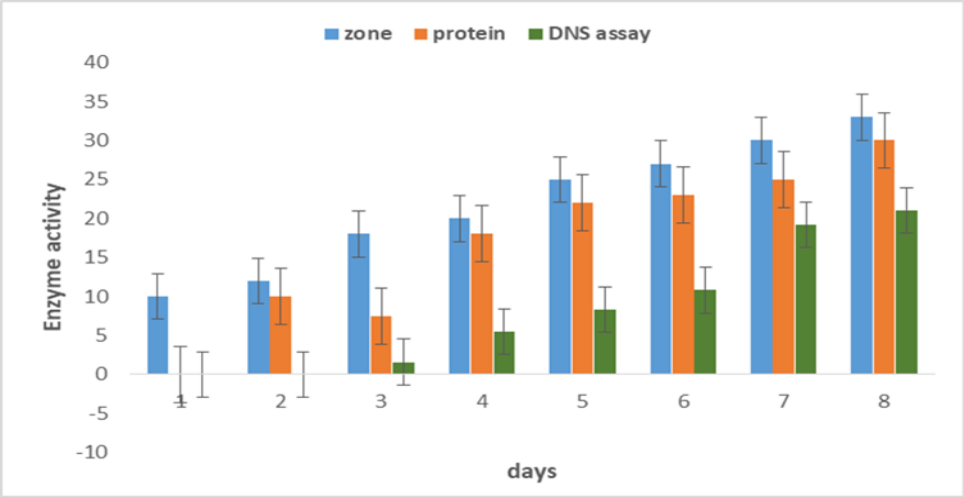


Figure 3d Screening of Halotolerant *S. brasiliensis* MML2028 in APM 4 for amylase production

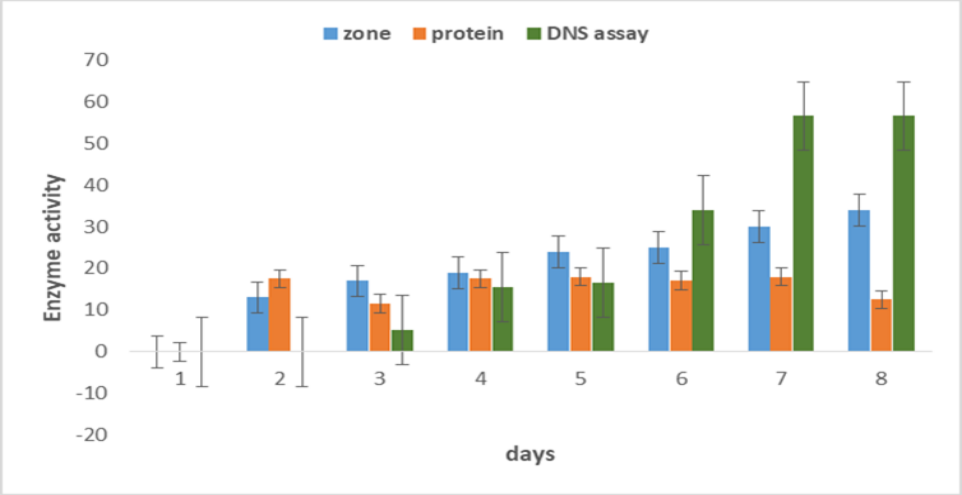


Figure 3e Screening of Halotolerant *S. brasiliensis* MML2028 in APM 5 for amylase production

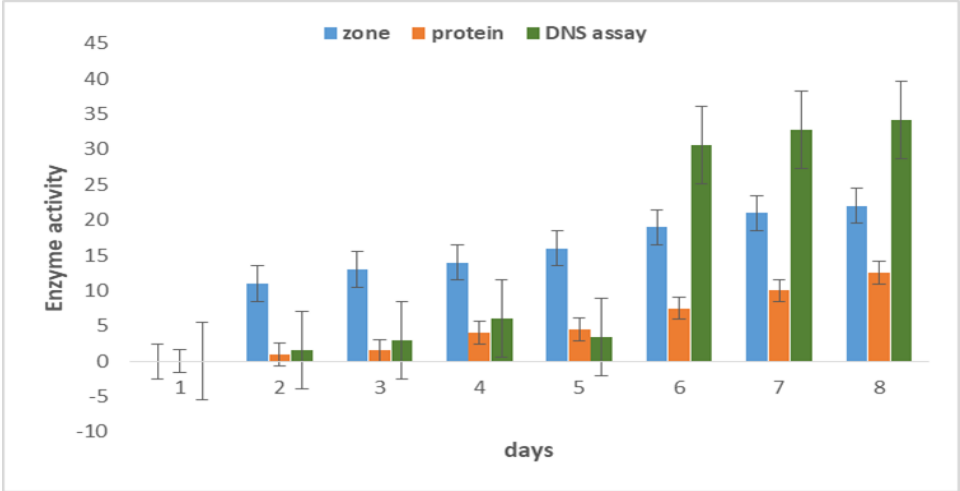


Figure 3f Screening of Halotolerant *S. brasiliensis* MML2028 in APM 6 for amylase production

3.4 Optimization of amylase production medium for increased enzyme production

Maximum production of amylase was recorded in 1% starch concentration and 21 mm in well diffusion assay. In different levels of pH, the maximum zone was visualized at pH 9 with 36mm. In the case of different temperatures (4°C, 16°C, 24°C, 37°C, 45°Cand 55°C) the optimum temperature for enzyme production was observed 24°C and the maximum zone of clearance was observed to be 31mm. Moreover, the enzyme production was found to be maximum at 3% NaCl, with a 30 mm zone. The culture was grown in different time intervals (1 to 9 days) in an APM1 medium to determine the suitable age for maximum amylase production. The maximum growth and amylase production were observed at 8 days (Figures 4a to d).

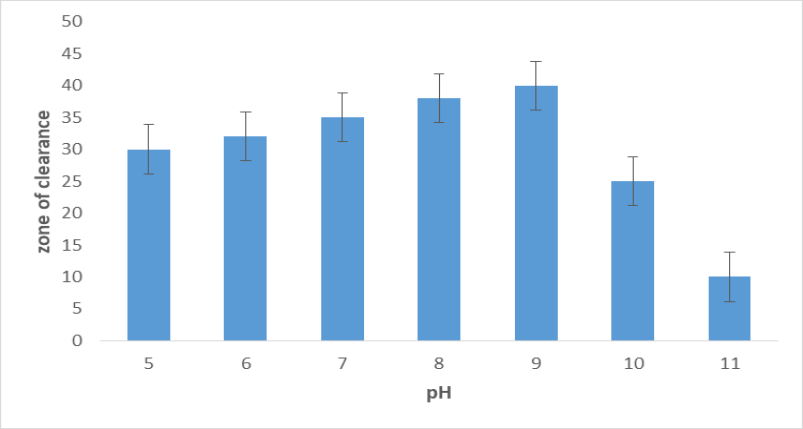


Figure 4a Optimization of different Starch concentrations

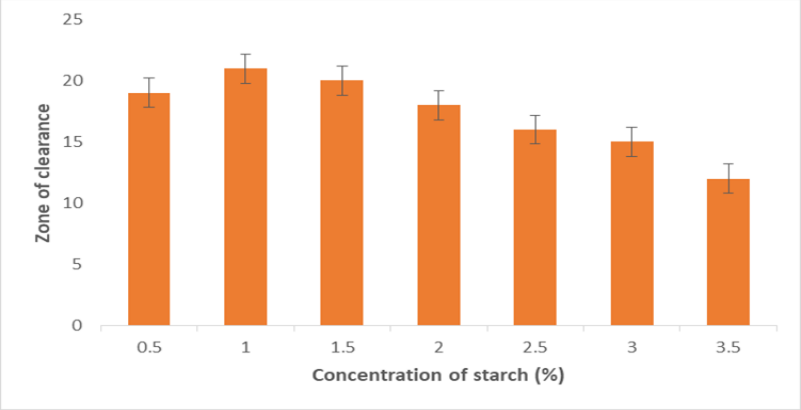


Figure 4b Optimization of different pH

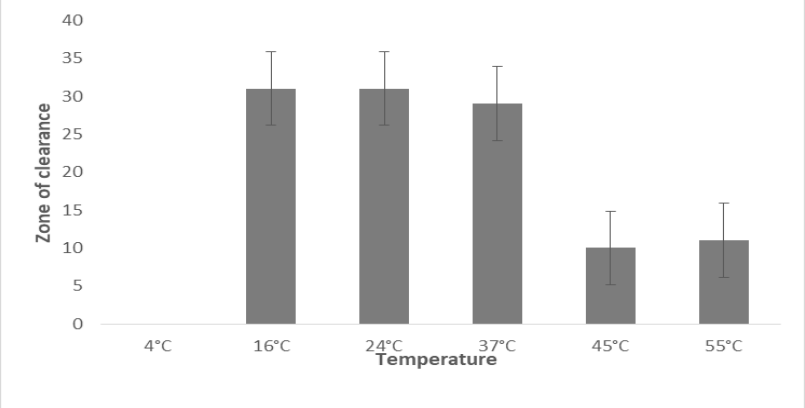


Figure 4c Optimization of different temperatures

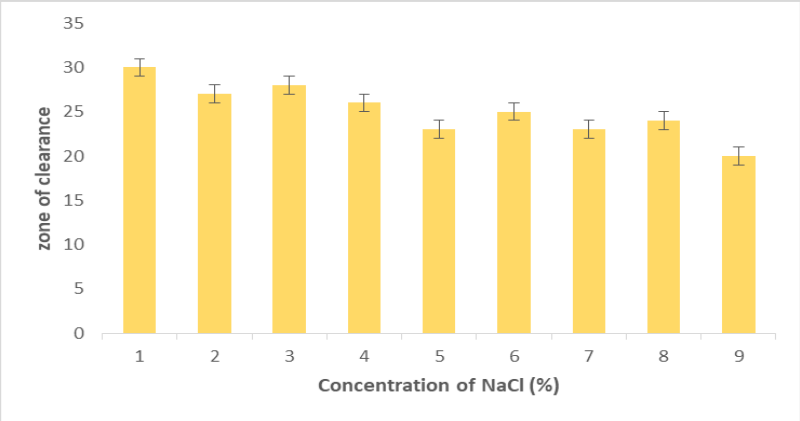


Figure 4d Optimization of different NaCl

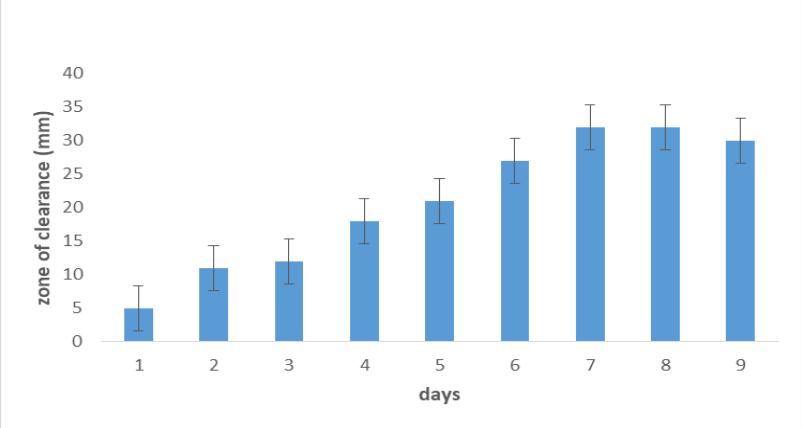


Figure 4e Optimization of different days

3.5 Mass production in optimized amylase production medium

Optimized APM1 medium was prepared for 5 liters and autoclaved. Then the medium was inoculated with 100 ml of 3 days old seed culture of *S. brasiliensis* MML2028and kept in a rotary shaker at 24°Cfor 8 days.

3.6 Partial purification

After 8 days the culture was harvested, centrifuged at 10,000 rpm for 15 min and then the supernatant was collected. The protein was precipitated using 70% ammonium sulphate. Once again, the content was centrifuged and then the pellet was collected and dissolved in 0.2 M phosphate buffer (pH 7.5). The above sample was dialyzed and this partially purified sample was lyophilized which yielded 1.387 g of enzyme sample which was analyzed quantitatively and qualitatively.

3.7 Quantitative and Qualitative assay of a partially purified amylase

The partially purified sample was analyzed by well diffusion method by addition of the sample (50, 100, 150 µl) in the starch abundant medium. The clear zone was visualized by the addition of iodine (indicator) measuring up to 4 mm, 10 mm, and 20 mm respectively as shown in Figure 5. The protein content was estimated by Bradford's method **(**Bradford 1976**).** The reaction mixture consisted of 1 ml sample + 5 ml of CBB-G250 which showed 35 µg/ml. The amount of protein was calculated using BSA standard graph (Figure 5). The lyophilized sample was quantified by DNS (di nitro salicylic acid) assay method as detailed below.

Total activity =

$$\frac{\text{Amount of glucose liberated} \times \text{total assay volume}}{\text{Volume of enzyme} \times \text{Time of incubation} \times \text{volume in cuvette}} = \frac{1.508 \times 5}{0.5 \times 60 \times 2}$$

= 1809.6 U/mL

Parameter	Optical density (O.D)
Live (enzyme + substrate)	1.508
Heat killed (enzyme killed+ substrate)	1.267
Enzyme blank	0.007
Substrate blank	0.029

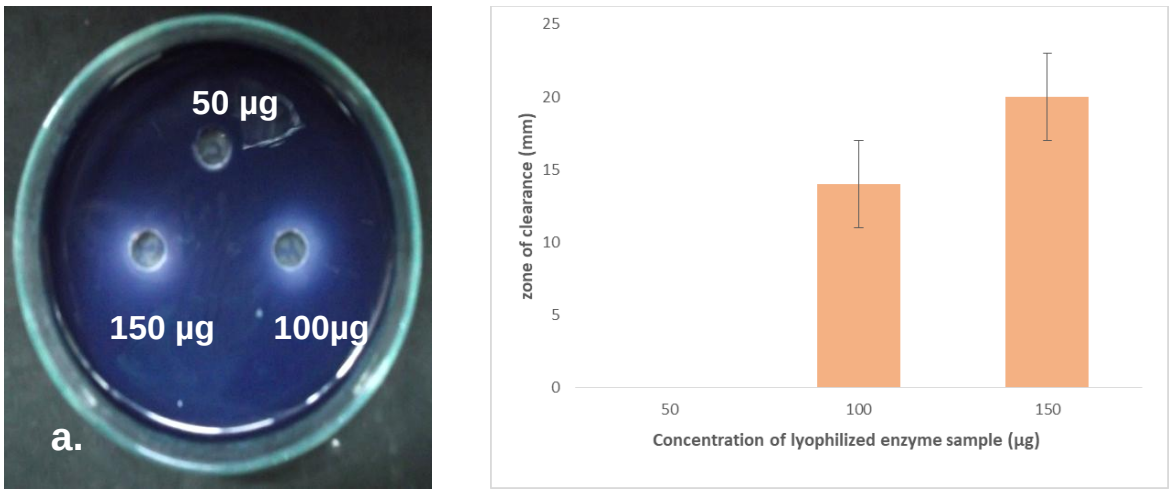


Figure 5 Well diffusion assay of partially purified amylase enzyme

3.8 Effect of pH, temperature, salt concentration, and metal ions on amylase activity and stability

The optimum pH was determined using four different buffer systems. Through the protein and plate assays, the activity and stability of the enzyme was found to be maximum at pH 8. For the thermal stability estimation, the enzyme was pre-incubated at a temperature between 4°C and 55°C from 15 min to 60 min at the optimum pH, and it was found that the enzyme was stable up to 24°C. The optimal salt concentration for maximum activity was 3% of NaCl. Although the enzyme activity increased in the presence of 3% and 5% of NaCl, the activity was observed to be lost in the presence of 7% NaCl. The enzyme was noted to be stable in Mg^{2+} and Zn^{2+}ions and was lost with metals like Fe^{3+}, Co^{2+}, $Ca^{2+,}$ and Mn^{2+}. Among the tested inhibitors, EDTA and SDS (5 mM) was the most inhibitory in which the enzyme lost its activity (Figure 6a to 6e).

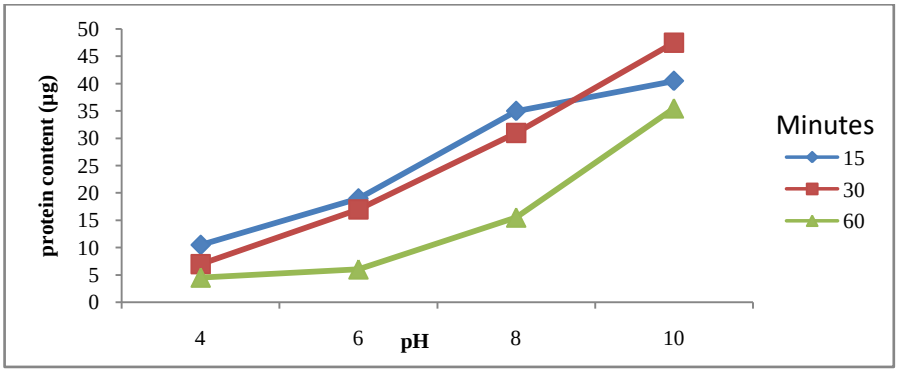


Figure 6a Effect of pH on activity and stability of amylase enzyme

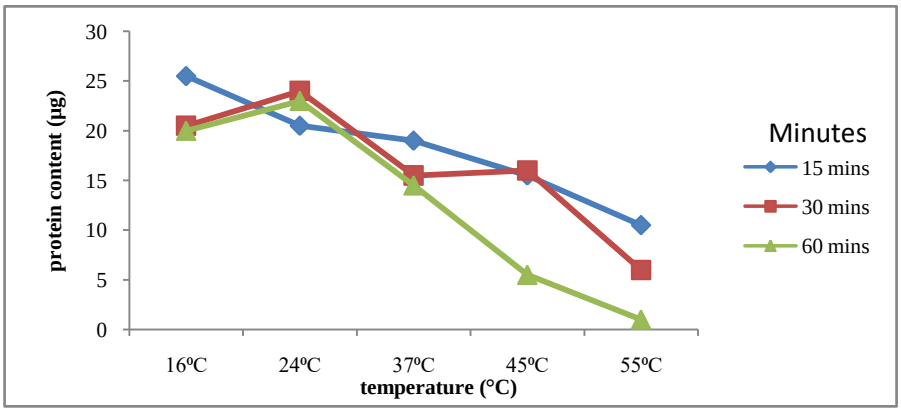


Figure 6b Effect of temperature on activity and stability of amylase enzyme

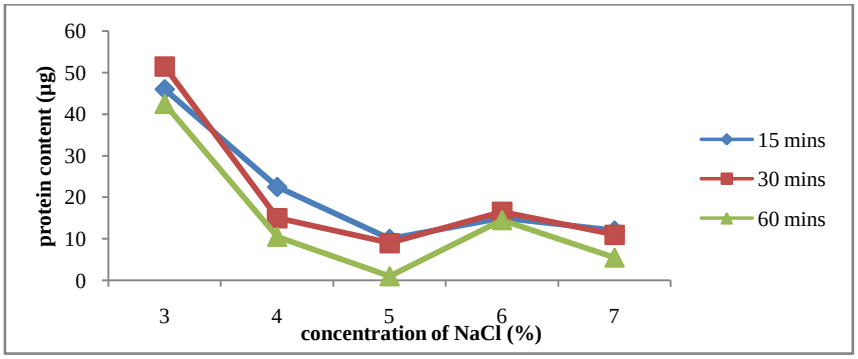


Figure 6c Effect of NaCl on activity and stability of amylase enzyme

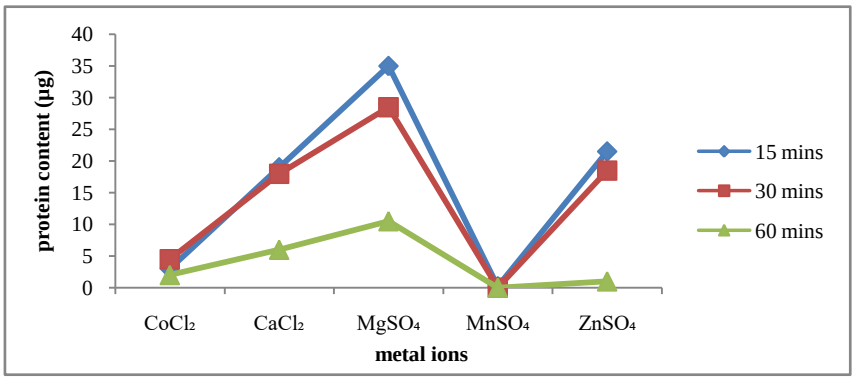


Figure 6d Effect of metal ions on activity and stability of amylase enzyme

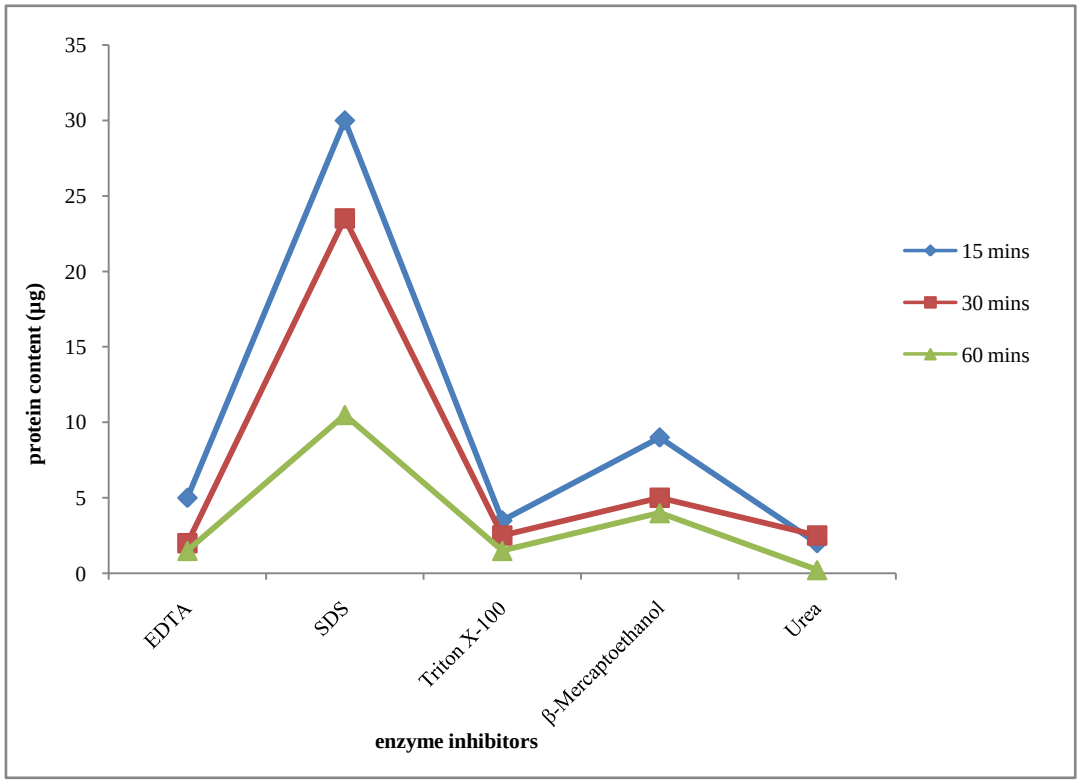
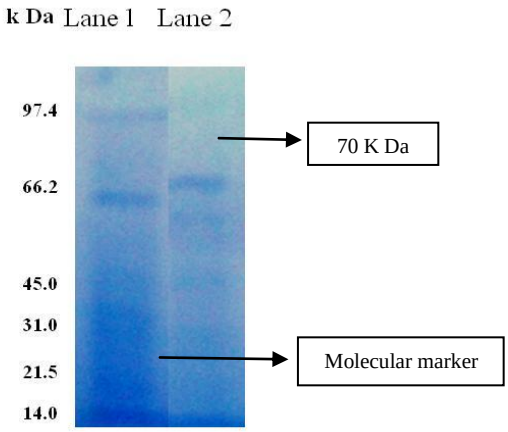


Figure 6e Effect of metal ions on activity and stability of amylase enzyme



Lane 1 - Protein marker; Lane 2 - Enzyme sample

Figure 7 Protein analysis of amylase by SDS – PAGE

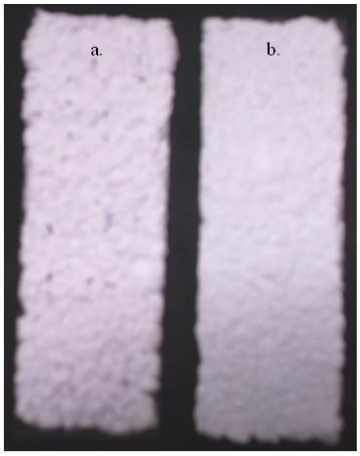
3.9 SDS - polyacrylamide gel electrophoresis

Proteins after the partial purification followed by lyophilization were separated using 12% acrylamide in SDS - polyacrylamide gel electrophoresis. Protein bands were visualized by staining in CBB-R250 which revealed a prominent band estimated to be 70 kDa (Figure 7).

3.10 Applications of Amylase

3.10.1 Deinking

The amylase treated paper showed far better results while compared with the control. The effective removal of ink from the paper pulp was clearly visualized. The enzyme is good enough to be applied in paper industry for deinking (Figure 8a).

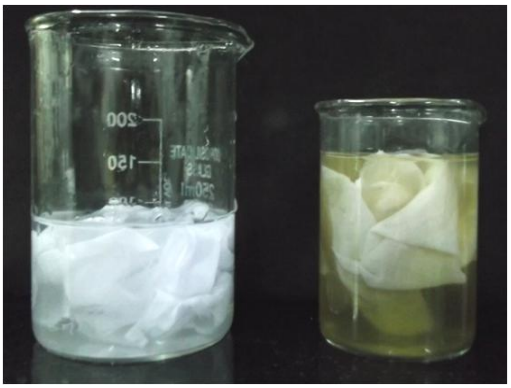


a. control, b. enzyme treated paper

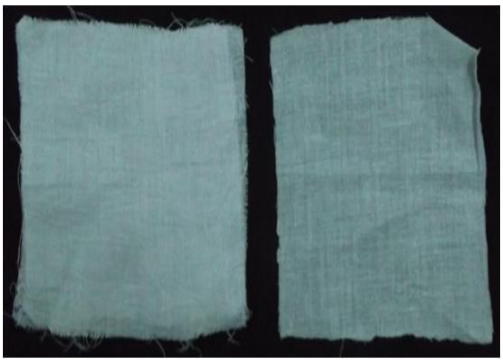
Figure 8a Deinking of paper

3.10.2 Desizing

The desizing of starch from the fabric by α-amylase was calculated as 81%. This shows the effective removal of starch by the enzyme and is hence considered to have an effective role in the textile industry (Figure 8b).



a. Starch de-sizing in water



a. Control; b. Test

Figure 8b Starch de-sizing in amylase enzyme

4 Discussions

In this work, an attempt has been made to produce stable amylase from halotolerant actinomycetes, isolated from the soil sample collected from the Kelambakkam salt pan. *S. brasiliensis* MML2028 was found to be the best producer of amylase enzyme, among the 30 actinomycetes cultures. *Streptomyces sp.* was reported to grow well on starch casein agar (SCA) as reported by earlier researchers (Laidi et al. 2008; Valsalam et al. 2019). Hence, SCA media enriched with 30 days old 100% natural seawater was used for the isolation and subculturing of actinomycetes (Bhanu et al. 2021).

The morphology of the colonies was elevated with wrinkled surface and solid density was similar to other studies on *Streptomyces sp.* (Djebbah et al. 2021). *Streptomyces* is characterized by dry, tiny compact, soft to powdery colonies constantly attached to the medium forming aerial and substrate mycelium with an earthy smell (Sathi et al. 2001; Yanti et al. 2019). The developed mycelia are colourless to white, chalky red, or grey olive (Oskay et al. 2004). The conversion of spore colour from gray to dark brown is a trait of the genus *Streptomyces*. The unique property of the genus *Streptomyces* is the formation of spira and retinaculliaperti spores (Li et al. 2016). The colour of the *Streptomyces* is due to pigment production. Some of the pigments are phenazines, phenoxazinones, and prodiginines as reported by Abdur Rahman et al. (2000). These were sporangia and spore-forming in nature and spores were non-motile with sporulating nature in the surfaces (You et al. 2005).

The cell wall amino acid assay also confirmed the test organism as *Streptomyces.* Similarly, strain AE-19 contains LL-Diaminopmelic (LL-DAP) and glycine on the cell wall. This indicates that the *Streptomyces* belongs to chemo type-I. *Streptomyces*, *Streptoverticillium*, *Chainia*, *Actinopycnidium*, *Actinosporangium*, *Elyptrosporangium*, *Microellbosporia*, *Sporichthya*, and *Intrasporangium* are examples of the chemotype-I cell wall (Lechevalier and Lechevalier 1970). Polyphasic taxonomy for the identification of the culture revealed that it was *S.brasiliensis* which can able to tolerate and grow in NaCl.

The six optimized amylase production media screened for enhanced production of amylase showed good results, whereas, amylase production in medium 1 showed constant enzyme production even in an alkaline pH. Similarly, Singh et al. (2017) used three production media for amylase production. Roy et al. (2012) observed the maximum amylase activity at pH 6 while Demirkan et al. (2017**)** reported the maximum enzyme activity at pH 7. Sodhi et al. (2005) found that the enzyme activity was higher at pH 6.5. However, in the current study, the maximum activity was reported at pH 9 and these results are in agreement with the findings of Saxena et al. (2007). Since alkaline amylases are considered to be efficient, especially in the detergent industry, the amylase produced by this alkaline medium is considered to be an alkali-stable amylase, and therefore, this production medium was chosen for mass production.

Molecular weights (MW) of α-amylases differ between 10 to 210 kDa. The least MW was found = 10 kDa in *Bacillus caldolyticus* (Grootegoed et al. 1973) while the highest MW was recorded 210 kDa in *Chloroflexus aurantiacus* (Ratanakhanokchai et al. 1992). Further, the molecular weight of the partially purified amylase enzyme was 70 KDa. Similarly, 70 K Da was reported in *T. harzianum* amylase A3 (Mohamed et al. 2011).

The temperature for the actinomycete of the present study was optimized as 24°C which indicates that the enzyme is not thermostable, likewise, the strain AE-19 showed maximum amylase activity at the temperature of 45°C which further indicates that the enzyme is not thermostable. In contrast, Stamford et al. (2001); Nipkow et al. (1989); Kundu (2006) reported that the as optimum temperature for the production of amylase was found to be 70°C, 50°C, and 60°C respectively. This discloses that the optimum conditions for amylase enzyme production differ from species to species (Eman et al. 2018).

The partial purification of the enzyme includes ammonium sulphate precipitation and Dialysis in phosphate buffer pH 7.5. The dialyzed sample was lyophilized for future use. The maximum enzyme activity was found to be 1809.6 U/mLwas observed in AMP1 medium. Similarly, the maximum production was found 2000 U/mL in soil microorganisms (Vidyalakshmi et al. 2009; Singh et al. 2017).

The partially purified enzyme is studied for its effect on different concentrations of NaCl, temperature, pH, metal ions, and inhibitors, and their results proved stability with NaCl at 3%, temperature at 24°C, and pH 8. The enzyme was stable in Urea and Triton – X. Similar results were reported by Roy et al. (2014).

Conclusion

In the present study, it was concluded that the soil samples of the Kelambakkam salt pan are a good source of materials for the isolation of potential actinomycetes. It also revealed that the tentatively identified species, *S. brasiliensis* MML2028 isolated from the soil possesses good α-amylase activity. The study has also standardized the growth parameters of the actinomycetes for maximum enzyme production, which can be effectively used in the large-scale production of enzymes for commercial purposes. The halotolerant and alkaliphilic quality of the amylase enzyme can be used in many industries, especially in paper and textiles.

Conflict of interest

The author and the co-authors declare no conflict of interest.

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Antibacterial Efficacy of Zinc oxide nanoparticles against *Serratia marcescens* (ATCC 43862) and *Enterococcus faecalis (*ATCC 29121)

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Enterococcus faecalis

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ABSTRACT

Zinc oxide nanoparticles (ZnO NPs) are a novel and alternative biomaterial for active biomedical applications among all metal and metallic oxide nanoparticles due to less toxicity and biocompatibility with human cells. In this study, we studied the growth curve of *Serratia marcescens* and *Enterococcus faecalis* to identify the mid-log phase of the bacterial growth to perform the exposure with ZnO NPs for investigating the antibacterial efficacy. The INT assay was used to determine the anti-bactericidal efficiency of ZnO NPs against *S. marcescens* and *E. faecalis.* The results showed that both the test bacteria attained the mid-log phase at the 5th hour. The determination of minimum inhibitory concentration (MIC) demonstrated a higher efficacy of ZnO NPs on the Gram-positive bacterium *E. faecalis* compared to the Gram-negative bacterium *S. marcescens*. The present study reports a higher susceptibility of Gram-positive bacterium over Gram-negative bacterium to the treatment of ZnO NPs.

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1 Introduction

Antibiotics have been used for over 70 years to combat bacterial infections around the world. High numbers of pathogenic bacteria are becoming resistant to antibiotics; it is well known that more than 50 years ago the bacteria were resistant to antibiotics. According to Stapleton and Taylor (2002), most of the *Staphylococcus aureus* isolates were resistant to penicillin. The development of new antibiotics, such as vancomycin and methicillin, leads to the emergence of antibiotic-resistant bacteria (Aslam et al. 2018). However, bacteria developed resistance mechanisms against many antibiotics and in 2019, World Health Organization considered antimicrobial resistance as one of the top ten human global health threats (Mancuso et al. 2021). This is a serious concern, which can result in up to a 25% mortality rate among patients with severe infections (Oerther and Oerther 2020; Boucher 2020). In general, nanotechnology is a tool that uses science at the nanoscale level to manipulate materials. The combination of biology and nanotechnology revolutionizes biomedical research using new phenomena and properties (physical, chemical, and biological) of materials present at the nanoscale and by the direct application of nanomaterials with biological targets (Menaa 2011). As it grows more sophisticatedly, it has found numerous applications in medical technology such as diagnostics, drug delivery, patient monitoring, and treatment (Zhang et al.2008).

Metal oxide nanoparticles (MONPs) and carbon-based 2D nanostructures are considered to be potential antiviral/antibacterial agents (Ray and Bandyopadhyay 2021). Thus, with the emergence of drug resistance in the treatment of microbial diseases, the development of new antibacterial materials is essential. Currently, metal oxide NPs (Ag NPs, ZnO NPs, CuO NPs, FeO NPs, etc.,) can be used in various biomedical applications (Jaison et al. 2022). ZnO NPs are extensively applied as an effective anti-bacterial agent against pathogenic bacteria**.** Thus, nanoparticles might serve as a promising candidate against multidrug-resistant bacterial infections and also as a novel antimicrobial agent of their unique target-specific binding property. This can be achieved by developing nanomaterials as implants and wound dressing complexes in clinical practices (Siemer et al. 2019; Gao and Zhang 2021). The emerging area is autonomous nano-objects that enhance the effect of antibiotics or impart bactericidal activity without the use of antibiotics. Another new area is nanostructured surfaces that prevent bacterial adhesion or kill bacteria through physical and mechanical interactions with bacteria (Shaikh et al. 2020).

In particular, a large number of studies have shown that ZnO NPs have high anti-bacterial potential against pathogenic bacteria due to the biological activity of zinc ions and the anti-inflammatory effects of ZnO NPs. Due to excellent broad-spectrum antibacterial properties, long-term effects, and excellent biocompatibility, ZnO nanomaterials can become a suitable effective alternative to traditional antibiotics (Silva et al. 2019). However, the antibacterial effects of these nanoparticles are depending on the cell wall assembly of the target organism. Based on this characteristic feature, ZnO NPs exhibit an extensive range of antibacterial activities against a variety of microorganisms, including *Escherichia coli*, *Bacillus subtilis*, etc. (Alam 2021). Zinc ions are responsible for the antibacterial activity caused by ZnO and also particles of these metal oxide pass through the cell membrane and cause oxidation of the cells by the excess production of ROS, which results in cell death (Djearamane et al. 2020). Further, ZnO NPs also have a wide range of photo-catalytic as well as high light absorption capacity in the UVA (315-400 nm) and UVB (280-315 nm) regions, which help in inducing bacterial toxicity (Lakshmipriya and Gopinath 2021). ZnO NPs are superior to compare with other MONPs due to their biocompatibility and excellent antibacterial potential. Hence, the present study focused on finding the antibacterial activity of ZnO NPs against *Serratia marcescens* and *Enterococcus faecalis*.

2 Materials and Methods

2.1 Chemicals and Nanoparticles

Zinc Oxide Nanoparticles (ZnO NPs) powder was obtained from Sigma- Aldrich, USA. The antibiotics Chloramphenicol and Ampicillin used as positive control were purchased from Bio Basic Inc, Canada. Luria Bertani (LB) medium was purchased from Laboratories CONDA, Spain.

2.2 Characterization of ZnO NPs

The previous publication of the same author (Djearamane et al. 2022) studied the surface topology of ZnO NPs using scanning electron microscopy and reported it as the mixed rod and spherical-shaped particles with an average size of 59.1 nm.

2.3 Bacteria Culture

The pure culture of Gram-negative bacterium *S. marcescens* (ATCC 43862) and Gram-positive bacterium *E. faecalis (*ATCC 29121) were obtained from the Faculty of Science, Universiti Tunku Abdul Rahman and subcultured to mid-log phase in LB broth for further utilization.

2.4 Preparation of ZnO NPs Suspension

A stock solution of ZnO NPs (320 µg/mL) was prepared by suspending ZnO NPs powder in Luria Bertani broth, mixed homogenously by vortex, and prepared the required concentrations of 5, 10, 20, 40, 80, and 160 µg/ mL of ZnO NPs by adding extra amount of LB Medium.

2.5 Growth Curve of Bacteria

The growth curves were plotted to identify the mid-log phase for both the test bacteria *S. marcescens* and *E. faecalis.* The bacteria were grown in Luria Bertani broth in 15 mL falcon tubes and incubated at 37 ^{O}C for *S. marcescens* and 35°C for *E. Faecalis* without shaking. The turbidity of bacteria was determined by spectrophotometer at 600 nm for every 1 hr interval from 0 to 8 hr, and then from 24 to 72 hr.

2.6 Growth Inhibition Test

2.6.1 INT Assay

The INT dye was used to determine the bacteriostatic concentration of ZnO NPs against *S. Marcescens* and *E. faecalis*. Upon reaching the mid-log phase at the 5th hour, the bacteria were incubated with different concentrations of mixed-shaped ZnO NPs (5, 10, 20, 40, 80, and 160 µg/ mL) for 24 hours, 1 mL of test bacteria suspension from each concentration, positive control (*S. marcescens* and *E. faecalis* after treating with 0.08 mg/ mL of chloramphenicol and 1.0 µg / mL of ampicillin respectively) and negative control (*S. marcescens* and *E. faecalis* without any treatment) were added separately to the microcentrifuge tubes and washed twice with 1 mL of 1x PBS at 6000g for 10 min and resuspended with 1 mL of 1x PBS. After that, 100 µL of the washed bacterial suspension was added with 20 µL of INT dye in the 96 wells plate and incubated for 20 min to observe colour change. The INT assay was done in a dark condition since INT dye is light-sensitive.

3 Results and Discussion

3.1 Growth Curve of Bacteria

The bacteria growth curve of *S. marcescens* was plotted to identify the growth pattern of the bacterial cells over time. According to figure 1, the lag phase was observed at the first two hours, while the log phase was found between 3rd to 7th hours, hence, the mid-log phase of *S. marcescens* was identified to be at 5th hour. The turbidity continued to increase after 8 hours of incubation until it came to the death phase at 48 hours of incubation.

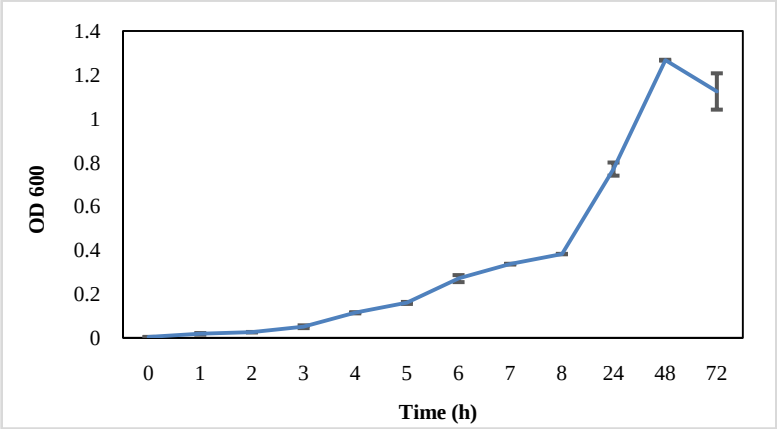

Figure 1 Growth curve of *S. marcescens* in Luria Bertani broth incubated at 37^{O} C

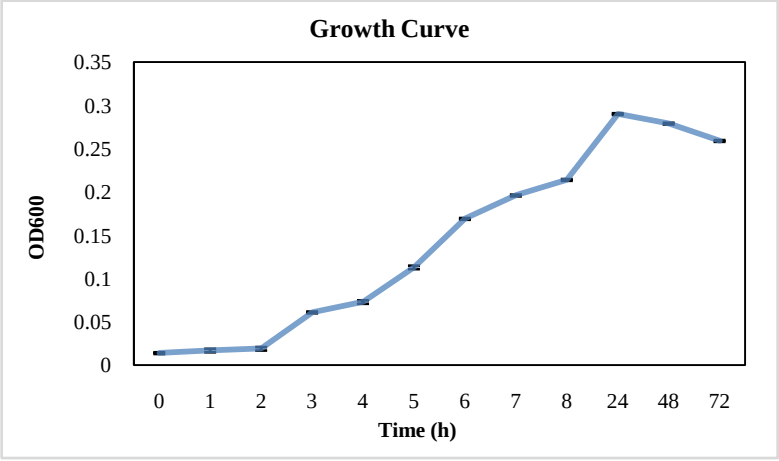

Figure 2 Growth curve of *E. faecalis* in Luria Bertani broth (LB) at 35°C

Similarly, the growth curve of *E. faecalis* indicated that the log phase of *E. faecalis* began at 2 hours and lasted until 7 hours (Figure 2). The mid-log phase of *E. faecalis* was then identified at 5 h. The growth of *E. faecalis* started to decline from 24 to 72 hrs indicating the decline phase of the growth curve. During the lag phase, the bacterial cells do not increase in number and also on the size as they take this duration to adapt to the new environment. Whereas the bacterial cells multiply actively in the log phase resulting in many folds increase in the cell population, this phase continues until the availability of favorable conditions and adequate nutrients. At the death phase, the cells lose their capacity for cell division and progress to death. The antibacterial studies are conducted during the mid-log phase of bacterial growth since they are most active in metabolism and cell division during this stage (Brazas and Hancock 2005; Kathleen and Arthur 2012; Dhanasegaran et al. 2021).

3.2 INT Assay

INT dye was used to identify the minimum inhibitory concentration (MIC) which is defined as the lowest concentration of an antibacterial substance that can inhibit bacteria growth (Dzotam et al. 2018). Iodonitrotetrazolium (INT) is an indicator dye that turns pink/purple to indicate the reduction of the dye by viable bacteria by the activity of dehydrogenases. The wells which show the purple or pink colour indicate the viable bacterial cells. The lowest concentration of ZnO NP displaying no visible purple or pink colour was recorded as the MIC value. According to figure 3, pink colour was observed until 160 µg/mL of ZnO NPs for *S. marcescens*. On the other hand, as shown in Figure 4, no pink colour formation was observed at 160 µg/mL of ZnO NPs after incubating with INT dye for 20 minutes indicating that 160 µg/mL as the MIC that can inhibit the growth of *E. faecalis*. Further, it is also visually observed that the intensity of the pink colour is decreased as the concentration of ZnO NPs increased for both the tested bacteria (Figure 3 & 4) indicating the dose-dependent bacteriostatic effect of ZnO NPs against *S. marcescens* and *E. faecalis.* In line with these findings, our earlier publication reported a dose-dependent growth inhibition of ZnO NPs on *S. marcescens* and *E. faecalis* with more percentage of growth inhibition on *E.* faecalis with the reported values of 5.42, 16.69, 23.74, 32.07, 54.73, and 63.50 % compared with 1.34, 5.65, 16.03, 21.73, 32.1 and 51.27 % of growth inhibition on *S. marcescens* for 5, 10, 20, 40, 80, and 160 µg/ml of ZnO NPs respectively at 24 hours (Djearamane et al. 2022).

Antibacterial agents are more effective when their MIC value is lower. In the present study, ZnO NPs showed a MIC value of >160 µg/ mL against the Gram-negative bacterium *S. marcescens* and MIC of 160 µg/ mL against Gram-positive bacterium *E. faecalis*, evidencing the higher sensitivity of Gram-positive bacterium towards ZnO NPs treatment compared with Gram-negative bacterium. Similar results on the higher sensitivity of Gram-positive bacterium to ZnO NPs were reported by the earlier studies, and this might be due to the presence of the liposaccharide in the outer membrane (Sukri et al. 2019; Demissie et al. 2020).

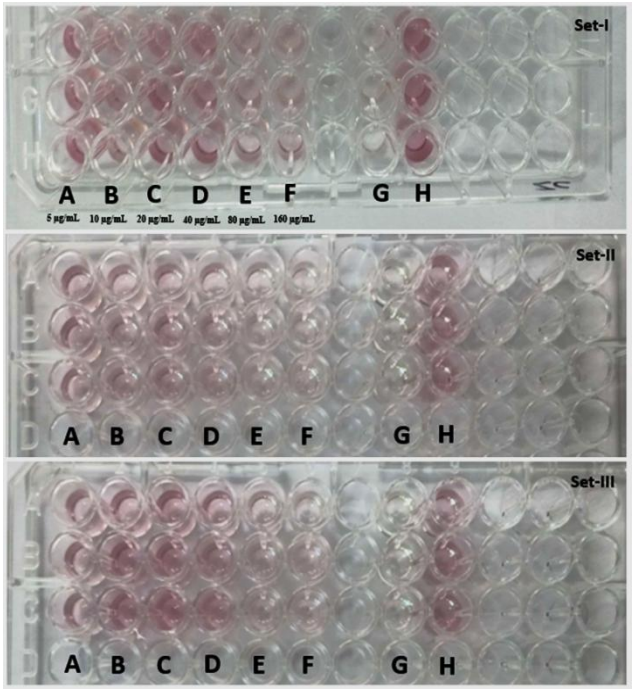

Figure 3 The colour changes of *S. marcescens* upon treating with ZnO NPs with (A) 5 µg/ mL; (B) 10 µg/ mL; (C) 20 µg/ mL; (D) 40 µg/ mL; (E) 80 µg/ mL; (F) 160 µg/ mL as compared to (G) positive control and (H) negative control

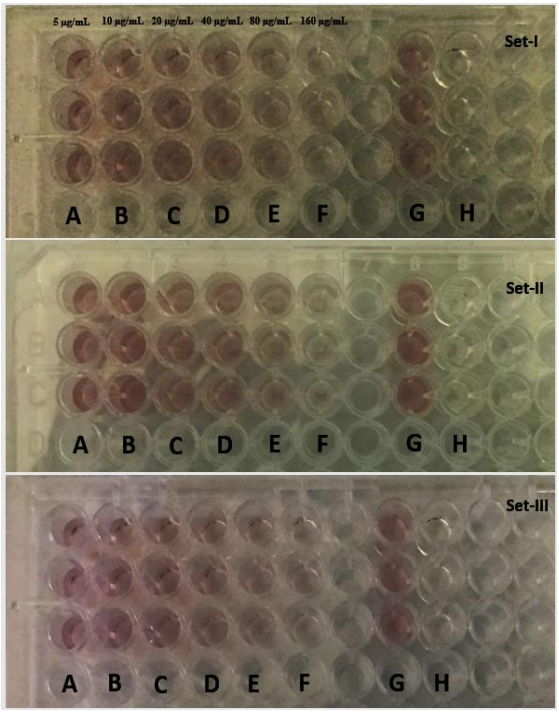

Figure 4 The colour changes of *E. faecalis* after being treated with (A) 5 µg/mL; (B) 10 µg/mL; (C) 20 µg/mL (D) 40 µg/mL; (E) 80 µg/mL; (F) 160 µg/mL of ZnO NPs as compared to the negative control (G) and positive control (H)

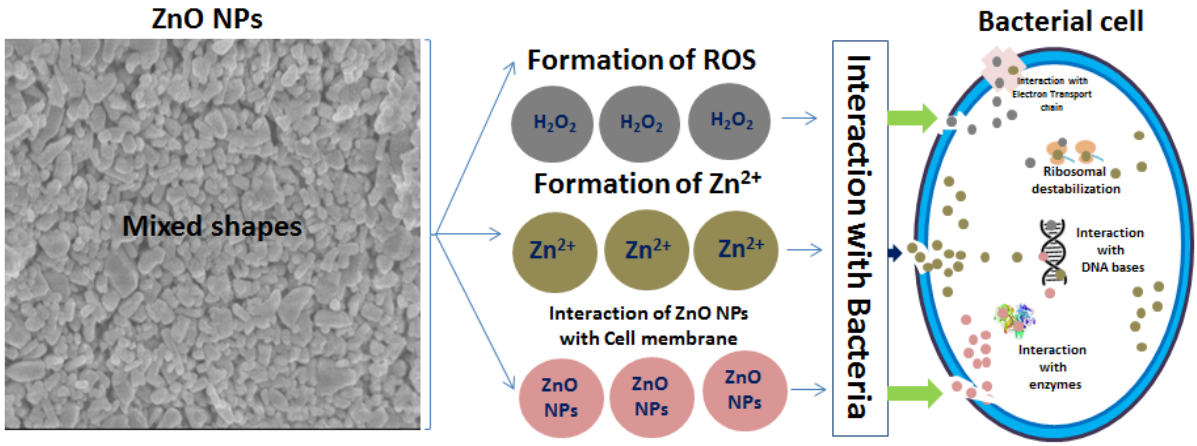

Figure 5 Mechanisms of antibacterial action of ZnO NPs

Earlier studies on the antibacterial properties of ZnO NPs reported good antibacterial efficacy against antibiotic-resistant Gram-negative bacillus of *Acinetobacter baumannii* in growth kinetics and disc diffusion assay (Tiwari et al. 2018). About 20nm in size and spherical shape ZnO NPs revealed significant antibacterial properties against *Staphylococcus aureus* and *Salmonella typhimurium*, and this might be due to the surface topological modifications by ZnO NPs which made bacterial cells pitted and distorted (Akbar et al. 2019). Similarly, Gupta et al. (2018) demonstrated the hexagonal wurtzite shapes of ZnO NPs synthesized from leaf extract of *Catharanthus roseus* caused a significant antibacterial effect against *Escherichia coli* MTCC (40), *S. pyogenes* (MTCC 1926), *Proteus mirabilis* (MTCC 3310), *P. aeruginosa* (MTCC 424), *S. aureus* (MTCC 9760) and *Bacillus cereus* (MTCC 430). The size, shape, and concentration of ZnO NPs have a big impact on their antibacterial activity. Metal ions created by NPs bind to active enzymes in the respiratory chain, causing ROS to develop and damage the cell wall membrane, and DNA, and disturb protein synthesis which eventually result in bacterial cell death (Dimapilis et al. 2017; Roberta et al. 2019; Liang et al. 2020; Gudkov et al. 2021). Figure 5 depicts the probable antibacterial mechanisms of ZnO NPs.

Conclusion

The present study identified the existence of a mid-log phase for *S.marcescens* (ATCC 43862) and *E. faecalis* (ATCC 29121) at the 5th hour. Based on the MIC values reported from the INT assay, it is observed that the Gram-positive bacterium *E. faecalis* showed higher sensitivity to the treatment of ZnO NPs compared to the Gram-negative bacterium *S. marcescens*. Further, the visual examination of the INT assay evidenced a dose dependent decrease in the viable cells of both the tested bacteria as the concentration of ZnO NPs increased. Hence, the findings of this study indicate the potential of ZnO NPs as an antibacterial agent.

Conflicts of interest

The authors affirm that they do not have any conflict of interest.

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Bio Characterization via FTIR and GCMS Analysis of *Cucurbita* variety (Yellow and White Pumpkin)

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KEYWORDS

Cucurbita pepo L.

Peel

White pumpkin

Yellow pumpkin

Ethyl acetate

GC-MS

ABSTRACT

The current study aimed to conduct phytochemical screening, FTIR, and GCMS analysis in squash (*Cucurbita pepo* L.,) also known as a yellow and white selected pumpkin. It's one of the dicotyledonous vegetables consumed in daily diets that imparts high inhibitor properties of inflammation, cancer, and diabetes. Traditionally it is used as an anti-helminthic remedy. The phytochemical characterization can facilitate seeking out the substance with a therapeutic property. The peel, flesh, and seed sample of each pumpkin variety were used as sources and extracted consecutively with ethyl acetate and acetonitrile using the maceration method. Phytochemical screening and quantification were carried out by standard analytical methods. The functional groups of the sample extracts were analyzed using FT-IR methods. Further, phytochemical profiling was carried out utilizing the GCMS technique to identify the therapeutically important chemicals contained in the sample. Phytochemical analysis of ethyl acetate and acetonitrile extracts showed the presence of major components like alkaloids, phenol, carbohydrate, and proteins. The farthest alkaloid, phenol, carbohydrate, and protein varied consequently for different parts like peel, flesh, and seed. The FT-IR analysis of each extract in the peel, flesh, and seed revealed that the ethyl acetate extract had the most functional groups. The major peak was characterized at wavelength 3004.24 to 3421.05 nm which indicates O-H functional group. Further quantification and GC-MS analysis were performed in ethyl acetate extract. Remarkably, GC-MS analysis of yellow and white pumpkin ethyl acetate extracts showed the utmost 6 - 8 compounds within the flesh part. Further, employing these compounds for anti-inflammatory and anti-microbial assays may aid in the discovery of new drugs for therapeutic applications.

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1 Introduction

Fruits and vegetables are vital components of a healthy daily diet because they provide a well-balanced plate of natural antioxidants. As a result, there has been an increased interest in screening natural antioxidants, preferably from fruits (Chandrasekara and Shahidi 2010, 2011; Deng et al. 2012; Li et al. 2007, 2008; Stangeland et al. 2009). Cucurbits are fruit and vegetable crops that belong to one of the most genetically diverse plant families (Bisognin 2002). It is a tropical and subtropical plant found worldwide. It is a member of the Cucurbitaceae family, which includes approximately 110 genera and 650–850 species (Pandey 1969). While the growth habitats above the ground level and at the root are comparable in grown cucurbits, they have a wide range of fruit characteristics. The fruits have dined as baked, pickled, candied, salads, or desserts. Members of the Cucurbitaceae family have always been thought about as a theme of analysis because of their anti-fungal, anti-bacterial, anti-viral, anti-diabetic, anti-tumour, and anti-AIDS activities (Mukherjee et al. 2022).

Cucurbita, commonly known as white and yellow pumpkin, is a popular vegetable in Tamilnadu due to its medicinal properties. The cytotoxicity, hepatoprotective, anti-inflammatory, and cardiovascular effects of the Curcurbitaceae family have been widely researched, and utilized in many pharmacopeias for millennia (Kaushik et al. 2015). Cucurbits are one of the most nutritious dietary for a healthy diet as they contain vitamins A, B1, B6, and C, a large number of alternative nutrients, and 96% of water. Due to its ethnopharmacological properties such as anti-inflammatory, anti-viral, analgesic, anti-ulcer, anti-diabetic, and anti-oxidant, *Cucurbita pepo* (yellow pumpkin) is one of the world's oldest cultivated species, according to the literature review (Smith 1997; Wang et al. 2001). Earlier phytochemical analysis on fruits of eighteen cultivars of Cucurbita found to possess low phenolic content (Kostecka-Gugała et al. 2020). At the same time seeds of this species are known to have an expensive supply of natural edible oil (fatty acid) reported to have a significantly high level of linoleic acid (Shelenga et al. 2020)

Cucurbita maxima (White pumpkin) which are grown in warm countries are popularly acknowledged vegetable crop for its nutritional and medicinal purposes. Peel and fruits of pumpkins contain sugars, volatile oils, flavonoids, glycosides, saccharides, proteins, carotenes, vitamins, and minerals (Marquez Cardazo et al. 2021). Although numerous reports on phytoconstituents and their healthful properties are available the current investigation was to unveil the constituents present via solvent potency in every peel, flesh, and seed part of yellow and white pumpkin, individually.

2 Materials and Methods

2.1 Plant Material

The whole fruit of pumpkin white and yellow varieties was purchased from the general market located in Saidapet, Chennai. To remove the dirt and its specks of dirt, the fruits were vigorously washed with water followed by distilled water wash.

2.2 Sample Preparation and Extraction

From the cleaned sample, peel, flesh, and seed were separated from each species individually and left to shade dry to get rid of the equilibrium wet from the sample. The samples were ground to powder form and stored for further study. Extraction was carried out through the maceration method as described by Muralidharan et al. (2018). About 5 g of peel, flesh and the seed of each species on an individual basis was extracted with 100 ml of ethyl acetate and acetonitrile on a shaker with 150 rpm at 30°C for 24 hours. The extracts were filtered using Whatman No. 1 filter paper.

2.3 Qualitative Phytochemical Screening

Qualitatively phytochemicals screening was carried out according to the method described by Morsy (2014).

2.4 Metabolites Quantification

2.4.1 Total Carbohydrate Determination

Hedge and Hofreiter (1962) described a method for estimating carbohydrate content. In a boiling water bath, 100 mg of the sample was hydrolyzed for 3 hrs with 5.0 mL of 2.5N HCl and neutralized with solid sodium carbonate until the effervescence has stopped. It was made up to 100 mL with distilled water and centrifuged at 5000 rpm for 15 min and the supernatant was collected. About 0.2 mL of supernatant of each sample, 0.8 mL of distilled water, and 4.0 mL of anthrone reagent were mixed. The reaction mixture was heated for 8 min, in a boiling water bath and cooled rapidly. Using a spectrophotometer, the colour intensity was measured at 630 nm. Standard glucose was prepared by dissolving 100 mg in 100 mL of distilled water. Further working standard was prepared from 10 mL of stock diluted to 100 mL with distilled water. The glucose content was measured using D-glucose as a standard and expressed as (µg/mL).

Amount of carbohydrate present in 100 mg of the sample

$$= \frac{\text{mg of glucose x 100}}{\text{Volume of test sample}}$$

2.4.2 Total Protein Determination

The total protein was determined using Lowry's method (1951). About 4.5 mL of reagent I having 96 mL of solution A (2 %

Content	Test	Observation	Inference
Alkaloid	**Mayer's Test** A few drops of Mayer's reagent were applied to a 2 mL extract along the test tube's side	Appearance of white creamy precipitate	Presence of alkaloid
Protein	1 mL concentrated H_2SO_4 and a few drops of concentrated Nitric acid were added to 1ml of extract	Formation of yellow colour	Presence of protein
Flavonoid	1 mL of 10% lead tetra acetate was added to 1 mL of extract	Indication of yellow hue	Presence of Flavonoid
Coumarins	A total of 3 mL of 10% sodium hydroxide was added to a 2 mL extract	Yellow colour formation	Presence of Coumarins
Carbohydrate	**Fehling's Test** 5 mL Fehling's A reagent was added to 1 mL extract, and the mixture was boiled for 5 minutes in a boiling water bath	Shift in yellow colour to red precipitate	Presence of carbohydrate
Glycosides	**Liebermann's Test** 2 mL ferric chloride and 2 mL H_2SO_4 were added to a 2 mL extract	Brown ring formation	Presence of glycosides
Steroid's	**Salkowski Test** 2 mL acetic anhydride was added to a 2 ml extract, and 2-3 drops of concentrated H_2SO_4 were added slowly along the sides of the test tube	Change in colour from violet to blue to green	Presence of Steroid
Terpenoid's	2 mL acetic anhydride was added to a 2 mL extract, and 2-3 drops of concentrated H_2SO_4 were added slowly along the sides of the test tube	Indication of deep red hue	Presence of Terpenoid
Saponin's	**Emulsion Test** Few drops of olive oil were added to a 5 mL of extract	Formation of emulsion	Presence of Saponin
Tannin	**Braymer's Test** 2 mL extract was dissolved in 2 mL distilled water, and 2-3 drops of ferric chloride (5%) were added	Presence of green precipitate	Presence of Tannin
Phlobatannin	**Precipitate Test** 2 mL extract and 2 mL of 1% HCl, heated in a boiling water bath for 5 minutes	Indication of red colour precipitate	Presence of Phlobatannins
Emodins	2 mL of 0.25% Ninhydrin, and 3 mL of benzene were added to a 2 mL of extract, which was then boiled for a few minutes	Formation of blue colour	Presence of Emodins
Anthraquinones	**Borntrager's Test** 3 mL of benzene and 5 mL of 10 % ammonia were added to 3 mL of extract.	Indication of pink, violet, or red coloration in the ammonical layer.	Presence of Anthraquinones
Anthocyanin	Add 2 mL of 2N HCl and a few drops of ammonia to 2 mL of extract	Colour change from pinkish red to bluish violet	Presence of Anthocyanin
Leucoanthocyanin	Add 5 mL of Isoamyl alcohol to 5 mL of extract	Change of organic layer into red	Presence of Leucoanthocyanin
Phenol	**Ferrichloride Test** In 5 mL of distilled water, the extract (50 mg) is dissolved. A few drops of a neutral 5% ferric chloride solution were added to this	Dark green colour formation	Presence of Phenol

Na_2CO_3 in 0.1N NaOH) + 2.0 mL of solution B (1% NaK Tartarise in H_2O) + 2.0 mL of solution C (0.5 % $CuSO_4$ in H_2O) was added to 1.0 mL of sample extract and incubated in a boiling water bath for 10 min. After 30 min of incubation, 0.5 mL of reagent II (Folin Phenol) was added and incubated in the dark for 30 min. At 660 nm, the intensity of the colour was measured. From the standard graph, the amount of protein in the samples was calculated.

2.4.3 Total Alkaloid Determination

The alkaloid content was determined using the Shamsa et al. (2007) method. Tannic acid was tested at different concentrations, with results expressed as mg tannic acid equivalents/g dry matter (TAE/g DM). The dried samples of about 1 mg each part were dissolved with 1 mL of 2N HCl and filtered. The filtered samples were transferred to a separating funnel; to this 5.0 mL of phosphate

buffer (pH4.7) and 5.0 mL of bromocresol green solution were added. The mixture was shaken by sequentially adding 1 mL, 2 mL, 3 mL, 4 mL, and 5 mL of Chloroform. The chloroform layer was collected in a 10 mL volumetric flask and diluted with chloroform up to the mark. The samples were absorbed against blank at 470 nm.

2.4.4 Total Phenol Determination

The total phenol content of the extract was determined using the Folin-Ciocalteau reagent, according to Bhuvaneshwari et al. (2016). The Folin-Ciocalteau reagent (500 µL) was added to both 100 µL of phenol standard solutions (Gallic acid was tested at various concentrations and the result was expressed as mg Gallic acid equivalents/g dry matter – GAE/g DM). To achieve a total volume of 1.5 mL, distilled water was added to each tube. After 8 minutes of incubation, 2.5 mL of 20% sodium carbonate was added to each test tube. After thoroughly shaking the mixture, the total phenolic content was measured at 765 nm against a blank.

2.5 Fourier Transform-Infrared (FT-IR) Spectroscopy

FT-IR is a useful tool for the identification and characterization of functional groups (chemical bonds) in a compound. Furthermore, FT-IR spectra are unique in that they are similar to a molecular "fingerprint." Between the cells, the drop creates a thin layer of film. Solid samples can be milled with potassium bromide (KBr) and then compressed into a thin pellet using a hydraulic press before being analyzed. FTIR spectroscopy IR-affinity was performed on a yellow pumpkin sample extracted with ethyl acetate and acetonitrile extracts (Shimadzu, Japan). The samples were run in the infrared region between 400 nm and 4000 nm, and a standard DLATGS detector with a mirror speed of 2.8 mm/sec was used.

2.6 Gas Chromatography-Mass Spectroscopy (GC-MS)

A Perkin Elmer Turbo Mass Spectrophotometer (Norwalk, CTO6859, and USA) with a Perkin Elmer Auto sampler XLGC was used for the GC-MS analysis. Perkin Elmer Elite - 5 capillary columns measuring 30 m x 0.25 mm with a film thickness of 0.25 mm composed of 95 % dimethylpolysiloxane were used. Helium was used as the carrier gas, with a flow rate of 0.5 mL/min. And a sample injection volume of 1 µL was used. The inlet temperature was maintained constant at 250°C. And the oven temperature was set to 110°C for 4 min and then increased to 240°C. Temperatures were then programmed to rise to 280°C at a rate of 20°C per minute for 5 min. The total running time was 90 min. The temperature of the MS transfer line was kept at 200°C. The source temperature was kept constant at 180°C. For compound identification and quantification, GC-MS was analyzed using electron impact ionization at 70eV, and data were analyzed using total ion count (TIC). The spectrums of the components were compared to a database of known component spectrums stored in the GC-MS library (Seemakkani and Thangapandian 2012).

2.7 Statistical Analysis

The mean and standard deviation of triplicate determinations was calculated using Microsoft Excel.

Table 1 Phytochemical constituents in ethyl acetate extract

S. No	Constituents	Yellow Pumpkin			White Pumpkin		
		Peel	Flesh	Seed	Peel	Flesh	Seed
1.	Tannins	+	-	-	+	-	-
2.	Flavanoids	+	+	+	+	-	-
3.	Terpenoids	-	+	+	-	-	+
4.	Saponins	-	-	-	-	-	-
5.	Steroids	+	++	+	++	+	+
6.	Phlobatannins	+	+	+	-	-	-
7.	Carbohydrates	++	++	++	++	+	+
8.	Glycosides	++	+	+	+	-	+
9.	Coumarins	+	+	+	-	-	-
10.	Alkaloids	+	+	+	+	-	-
11.	Proteins	+	+	+	+	-	-
12.	Emodins	-	-	-	-	-	-
13.	Anthraquinones	-	-	-	-	-	-
14.	Anthocyanins	-	-	-	-	-	-
15,	Leucoanthocyanins	-	-	-	-	-	-
16.	Phenol	+	-	-	+	-	-

Here ++ indicate: Highly presence, + indicate: Presence, - indicate: absence

Table 2 Phytochemical constituents in acetonitrile extract

S. No	Constituents	Yellow Pumpkin			White Pumpkin		
		Peel	Flesh	Seed	Peel	Flesh	Seed
1.	Tannins	+	-	+	+	-	-
2.	Flavanoids	+	+	+	+	-	-
3.	Terpenoids	-	-	+	+	-	-
4.	Saponins	+	+	+	+	++	+
5.	Steroids	+	-	+++	+	+++	-
6.	Phlobatannins	-	-	+	-	-	-
7.	Carbohydrates	+	+	+	+	+	+
8.	Glycosides	+	+	+	+	-	+
9.	Coumarins	+	+	+	-	-	-
10.	Alkaloids	+	+	+	+	+	-
11.	Proteins	+	+	+	+	-	-
12.	Emodins	-	-	-	-	-	-
13.	Anthraquinones	-	-	-	-	-	-
14.	Anthocyanins	-	-	-	-	-	-
15,	Leucoanthocyanins	-	-	-	-	-	-
16.	Phenol	+	-	+	+	-	-

Here ++ indicate: Highly presence, + indicate: Presence, - indicate: absence

Table 3 Quantitative analysis of primary metabolites in ethyl acetate extract

Primary Metabolites	Yellow Pumpkin			White Pumpkin		
	Peel	Flesh	Seed	Peel	Flesh	Seed
Total Carbohydrate (µg/ml)	1.3±0.1	2.2±0.2	2.1±0.1	2.6±0.2	3.1±0.2	3.23±0.2
Total Protein (µg/ml)	10.4±0.3	13.2±0.2	15.4±0.4	8.4±0.3	6.7±0.4	11.3±0.4

3 Results

3.1 Qualitative Phytochemical Screening

The phytochemical screening in the yellow and white pumpkin variety was carried out using acetonitrile and ethyl acetate extracts. The screening test disclosed the presence of constituents in varied proportions within the peel, flesh, and seed as described in Tables 1 and 2. The result showed that tannin, flavonoid, steroid, saponin, carbohydrate, protein, alkaloid, and phenol were present in each extract of the two pumpkin varieties. The acetonitrile extract of each part of both kinds had a substantial presence of metabolites, but the ethyl acetate extract had a lower number of metabolites. Among the fruit parts studied, flesh showed a reduced range of metabolites like saponin, steroid, carbohydrate, and alkaloid, compared to peel and seed parts. Though the samples were analyzed by polarity-based different solvents, ethyl acetate extract of each yellow and white pumpkin variety showed an inflated range of metabolite presence, therefore for additional study ethyl acetate extract samples were solely analyzed.

3.2 Determination of Primary Metabolite

Plant growth requires the presence of primary metabolites, which are essential components and play important roles in pharmaceutical compounds like antipsychotic drugs as precursors or pharmacologically active metabolites (Jayaraman 1981). The results of the measurement of primary and secondary metabolites observed in the study are shown in Table 3. The carbohydrate content was found to be high within the flesh part compared to the peel and seed part, with an amount of 2.2±0.2 and 3.1±0.2 µg/mL in the yellow and white variety respectively. Whereas a lesser amount of carbohydrate was found in the yellow peel part compared to other parts and peel parts of the white variety. Consequently, total protein analysis showed the extreme presence of 15.4±0.4 µg/mL and 11.3±0.4 µg/mL in seed parts of yellow and white varieties respectively. Whilst a low amount of 6.7±0.4 µg/mL was shown in white flesh extract. The amount of protein in the yellow variety parts was found to be higher than in the white variety parts.

Table 4 Quantitative analysis of secondary metabolites in ethyl acetate extract

Secondary Metabolites	Yellow Pumpkin			White Pumpkin		
	Peel	Flesh	Seed	Peel	Flesh	Seed
Total Alkaloid (µg/ml)	2.8±0.3	4.9±0.2	4.8±0.3	7.4±0.3	2.0±0.2	2.0±0.3
Total Phenol (µg/ml)	39.1±0.7	54.0±0.5	23.2±0.4	22.2±0.4	43.6± 0.8	26.6±0.5

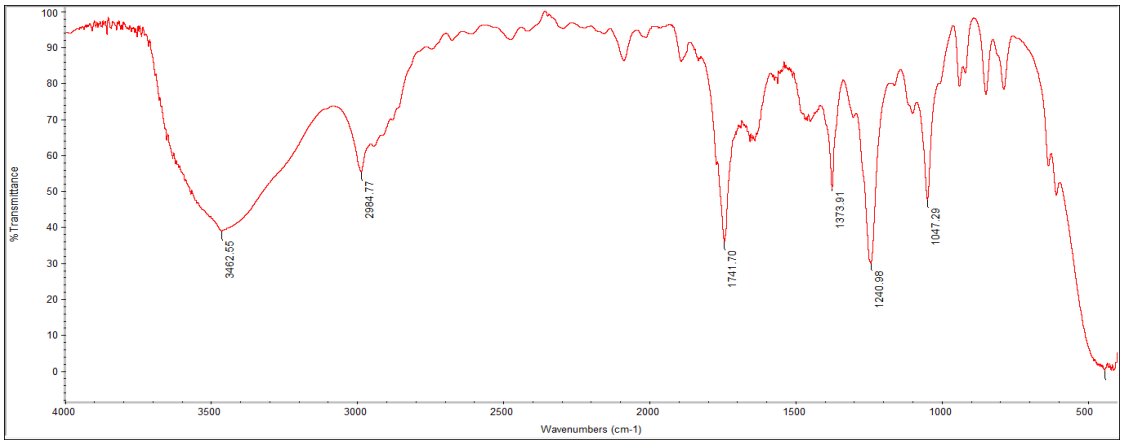

Figure 1 FTIR spectrum of Yellow pumpkin variety peel

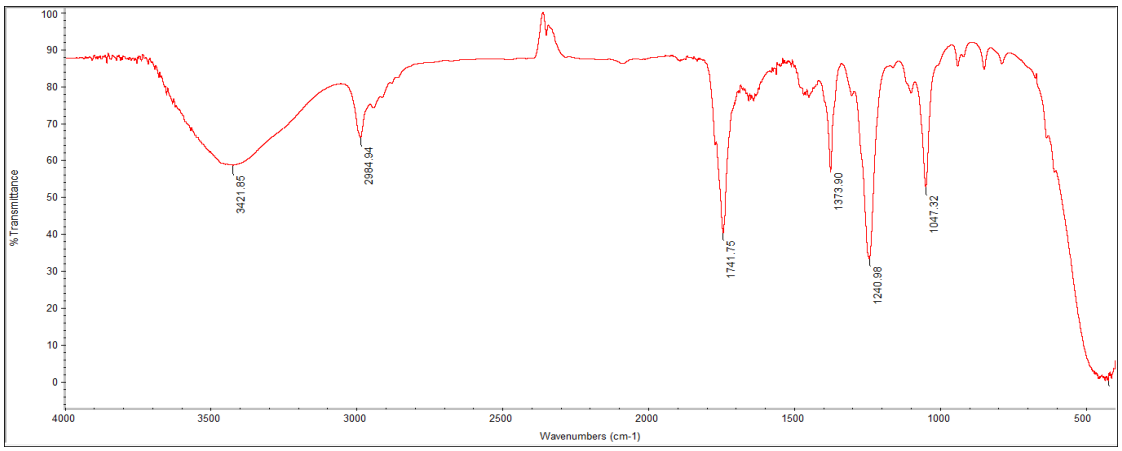

Figure 2 FTIR spectrum of Yellow pumpkin variety flesh

3.3 Determination of Secondary Metabolite

Plant secondary metabolites are important to treat various diseases and pharmacological applications in recent decades. It conjointly plays a task as a troubleshooter in the ecological interaction between plants and their environment. White variety peel had a significantly higher alkaloid concentration (7.4±0.3 g/mL) compared to its seed which showed lower alkaloid concentrations. While in the case of the yellow variety, the amount of alkaloid was found to be similar in flesh and seed (2.0±0.2 µg/mL) and the peel showed 2.8±0.3 µg/mL**.** Subsequently, the analysis of phenol content in flesh parts showed a considerably higher amount of 54.0±0.5 µg/mL and 43.6±0.8 µg/mL in yellow and white pumpkin varieties (Table 4).

3.4 Fourier Transmittance-Infrared Spectrometry (FT-IR)

The functional groups in ethyl acetate extract of yellow and white variety peel, flesh, and seed, were separated based on absorption using an infrared spectrum and are displayed in Figures 1 to 6. The results of the FTIR spectrum helped to spot the active components within the extract-supported magnitude relation within the region of IR radiation. The FTIR result analysis showed the absorption peaks of alcohol, amine, alkane, esters, nitro compounds, carboxylic acid and phosphines are the major functional groups observed in both varieties of pumpkin ethyl acetate extract. FTIR spectrometry proved to be a reliable and sensitive methodology for detecting biomolecular composition based on the functional groups present (Tables 5 and 6).

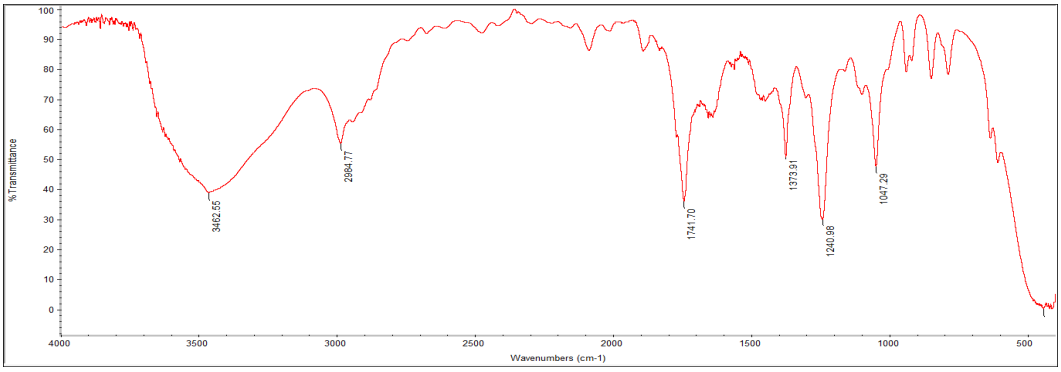

Figure 3 FTIR spectrum of Yellow Pumpkin variety seed

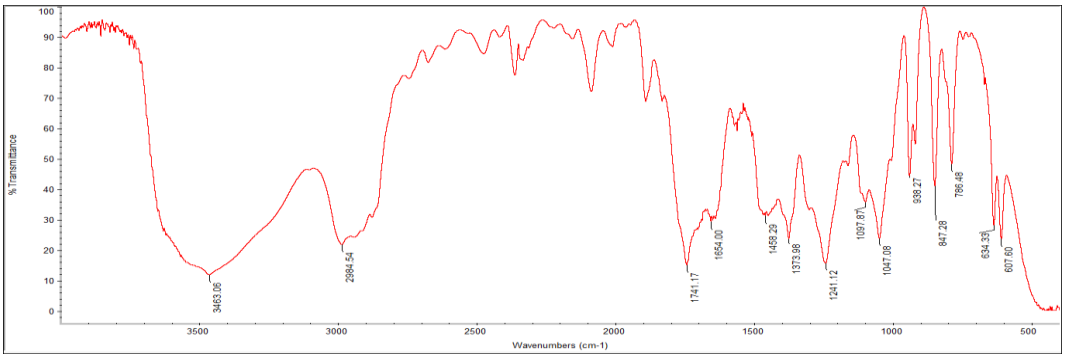

Figure 4 FTIR spectrum of White Pumpkin variety peel

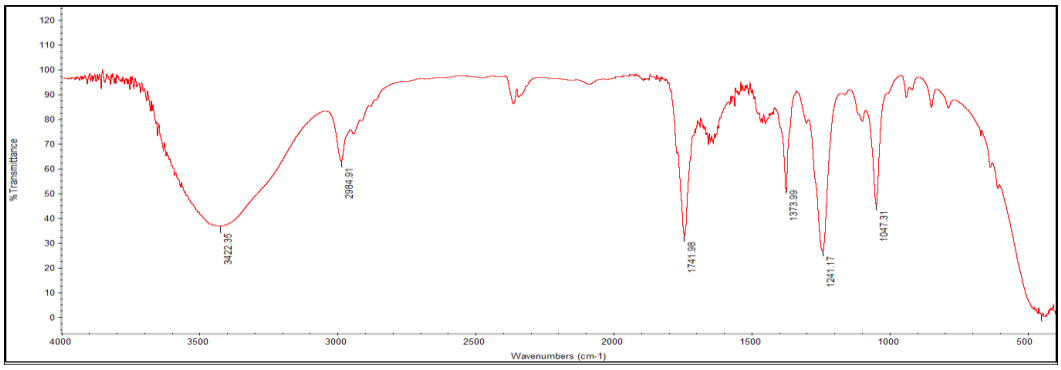

Figure 5 FTIR spectrum of White pumpkin variety flesh

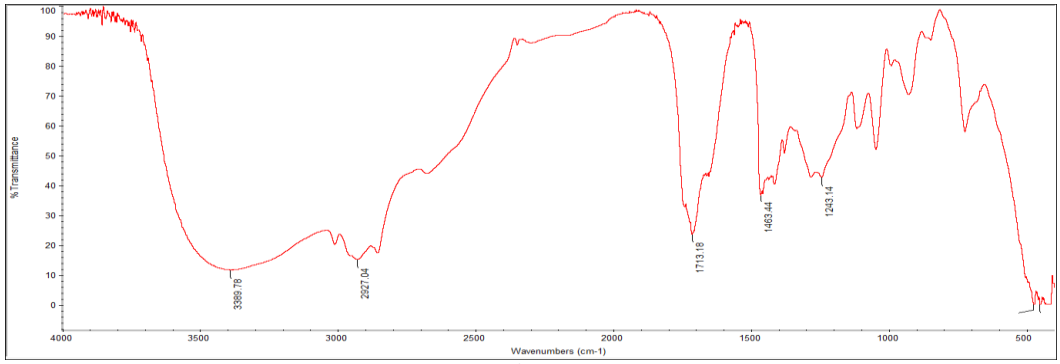

Figure 6 FTIR spectrum of White pumpkin variety seed

Table 5 Peak value, bond type, and functional group for FTIR spectra ethyl acetate extract of Yellow Pumpkin Variety

Sample Analysed	Frequency Cm^{-1}	Functional Group	Class
Peel	3402.55	O-H	Alcohol
		N-H	amine
	2984.77	C-H	Alkane
	1741.70	C=O	Esters
	1373.91	N=O	Nitro compounds
	1240.98	C-O	Carboxylic acids
		C-N	Amines
		P-H	Phospines
		P=O	Phosphonate, phosphoramide
		$Si-CH_3$	Silane
	1047.29	O-C	Anhydrides
		C-N	Amines
		S=O	Sulfoxide
		P-OR	Esters
		Si-OR	Silane
Flesh	2984.94	C-H	Alkane
	1741.75	C=O	Esters
	1373.90	N=O	Nitro
		S=O	Sulfate
	1240.98	C-O	Carboxylic acid
		C-N	Amines
		N-O	Aromatics
		P=O	Phosphonate, Phosphoramide
		$Si-CH_3$	Silane
	1047.32	O-C	Anhydride
		C-N	Amines
		S=O	sulfoxide
Seed	3420.07	OH	Alcohol
		N-H	Amines
	1646.93	C=C	Alkene
		C=O	Amines
		NH_2	Amines
	1241.15	C-O	Carboxylic acid
		C-N	Amines
		P-H	Phosphines
		P=O	Phosphonate, Phosphoramide
		$Si-CH_3$	Silane
	668.47	NH_2 & N-H	Amines

Table 6 Peak value, bond type, and functional group for FTIR spectra ethyl acetate extract of White Pumpkin Variety

Sample Analysed	Wavelength Cm^{-1}	Functional Group	Class
Peel	2984.54	C-H	Alkane
	1741.17	C=O	Esters
	1654.00	C=C	Alkene
		C=N	Oxime (Oxidized Nitrogen)
		C=O	Amides
	1458.29	C=C	Aromatic
		N=O	Nitoso, Nitro
	1373.98	N=O	Nitro
		S=O	Sulfate
	1241.12	C-O	Carboxylic Acids
		C-N	Amines
		N-O	Aromatic
		P=O	Phosphonate, Phosphoramide
		$Si-CH_3$	Silane
	1097.87	C-O	Alcohol
		O-C	Anhydrides
		C-N	Amines
		C=S	Thio carbonyl
	1047.08	O-C	Anhydrides
		C-N	Amines
		S=O	Sulfoxide
		Si-OR	Sulfoxide
	938.27	C-C	Alkane
		P-OR	Esters
		N-O	Oxime
	847.28	C-C	Alkane
		NH_2& N-H	Amines
		S-OR	Esters
	786.48	NH_2-NH	Amines
		S-OR	Esters
Flesh	2984.91	C-H	Alkane
	1741.17	C=O	Esters
	1373.99	N=O	Nitro compounds
	1241.17	C-O	Carboxylic
		C-N	Amines
		P-H	Phosphines
		P=O	Phosphonate
		P=O	Phosphoramide
		$Si-CH_3$	Silane
	1047.31	O-C	Anhydrides
		C-N	Amines
		S=O	Sulfoxide
		P-OR	Esters
		Si-OR	Silanes

Sample Analysed	Wavelength Cm^{-1}	Functional Group	Class
Seed	2927.04	C-H	Alkane
		O-H	Carboxylic Acids
	1713.18	C=O	Carboxylic Acids
		C=O	Ketone
	1463.44	C=C	Aromatics
	1243.14	$Si-CH_3$	Silane
		P=O	Phosphoramide, Phosphonate
		P-H	Phosphine
		N-O	Aromatic
		C-N	Amines

Table 7 Phytocomponents identified in ethyl acetate extracts of peel, flesh, and seed of Yellow Pumpkin var. by GC-MS peak

S. No	Sample Name	RT (Min)	Mol. wt	Mol. formula	Name of the compound	Compound Nature	Biological activity
1	*Peel*	20.44	280	$C_{18}H_{32}O_2$	9,12-Octadecadienoic acid (z,z)	Polyunsaturated fatty acid (Linoleic acid)	Anti-inflammatory, Nematicide, Insectifuge, Hypocholesterolemic, Cancer Preventive, Hepatoprotective, Antihistaminic, Antiacne, Antiarthritic, Antieczemic
		22.95	390	$C_{24}H_{38}O_4$	Di-N-Octylphthalate	1,2-Benzenedicarboxylic acid, dioctyl ester	Antifouling, Antimicrobial
		24.95	474	$C_{30}H_{50}O_4$	1,2-Benzenedicarboxylic acid, Diundecyl ester	Phthalic acid	Antioxidant, Anemiagenic
		29.34	468	$C_{32}H_{52}O_2$	Lup-20(29)-E,N-3-ol, Acetate,3-Beta	Lupeol acetate	Antinoceptive, Anti-inflammatory
		30.03	468	$C_{29}H_{40}O_5$	Phthalic acid, 3-methoxybenzyl tridecyl ester	Phthalate	Antioxidant, Anemiagenic
2	Flesh	19.81	280	$C_{20}H_{40}$	9-eicosene – E	Essential oil	Antimicrobial and Cytotoxic properties
		22.23	418	$C_{26}H_{42}O_4$	Phthalic acid, Bis(7-methyloctyl) ester	Plasticizer compound	Antimicrobial, Antifouling
		22.56	334	$C_{16}H_{31}BrO_2$	Pentadecanoic acid, 15-bromo-, methyl ester	Fatty acid esters	Antifungal, antibacterial, antimicrobial, emulsifier,
		22.80	278	$C_{16}H_{22}O_4$	1,2-benzene dicarboxylic acid, BIS (2-methyl propyl) ester	Isobutyl phthalate	Antimicrobial, antifouling
		24.66	222	$C_{12}H_{14}O_4$	1,2-benzene dicarboxylic acid, diethyl ester	Diethyl phthalate	Cosmetics, insecticides, plasticizers
		24.88	446	$C_{24}H_{38}O_4$	1,2-benzene dicarboxylic acid, diisodecyl	Diisoctyl phthalate	Antimicrobial antifouling
		29.51	426	$C_{30}H_{50}O$	Lupeol	Triterpenoids	Anti-inflammatory, anticancer, antiprotozoal, chemopreventive
	Seed	18.40	256	$C_{17}H_{36}O$	n-Heptadecanol-1	Fatty alcohol	Antiarthritis, skin diseases
		20.18	213	$C_{12}H_{23}NO_2$	1-Hexyl-2-Nitrocyclo hexane	Ketone	Neuroactive, anti-inflammatory, analgesic properties
		22.84	390	$C_8H_6O_4$	1,2-benzene dicarboxylic acid	Phthalic acid	Antioxidant, antimicrobial, antifouling

3.5 Gas Chromatography-Mass Spectroscopy Analysis (GC-MS)

The key to revealing the constituents of volatile matter, long-chain, branched-chain hydrocarbons, alcohols, acids, esters, and so on is GC-MS. Yellow and white variety pumpkins to date are used for various medicative functions to grasp the constituents present within the peel, flesh, and seed, therefore ethyl acetate extract of plant sample was analyzed. The compound was analyzed based on the retention time present in the total ionic chromatogram. Results presented in Tables 7 and 8 revealed the list of active principle compounds along with their retention time, molecular formula, chemical name, and common name.

The active principle compounds identified in our present study within the peel, flesh, and seed parts of both yellow and white varieties (Figures 7 and 8) are 9,12-octadecadienoic acid (z,z), 1,2-benzene dicarboxylic acid, Di-undecyl ester, 9-eicosene–E, 1,2-benzene dicarboxylic acid, diethyl ester, lupeol, and eicosane.

Table 8 Phytocomponents identified in ethyl acetate extracts of peel, flesh, and seed of White Pumpkin variety by GC-MS peak

S. No	Sample Name	RT (Min)	Mol wt	Mol. formula	Name of the compound	Compound Nature	Biological activity
1.	Peel	20.12	213	$C_{12}H_{23}NO_2$	1,-Hexyl-2-Nitrocyclohexane	Ketone	Neuroactive, anti-inflammatory, analgesic properties
		22.78	390	$C_8H_6O_4$	1,2-benzene dicarboxylic acid	Phthalic acid	Antioxidant, antimicrobial, antifouling
		24.65	390	$C_{24}H_{38}O_4$	1,2-benzene dicarboxylic acid, diisodecyl	Diisoctyl phthalate	Antimicrobial antifouling
		28.93	470	$C_{32}H_{54}O_2$	9,19-cyclonostan-3-ol,acetate, (3,-Beta)	Cycloartenol	Steroid precursor
		29.31	290	$C_{20}H_{34}O$	Thunbergol	Diterpene alcohol	Antibacterial
		30.15	282	$C_{20}H_{42}$	Eicosane	Alkane	Antifungal, antitumor, antibacterial, larvicidal, antimicrobial, cytotoxic
2	Flesh	19.18	280	$C_{20}H_{40}$	9-eicosene – E	Essential oil	Antimicrobial and Cytotoxic properties
		20.41	278	$C_{16}H_{22}O_4$	1,2-benzene dicarboxylic acid, BIS (2-methyl propyl) ester	Isobutyl phthalate	Antimicrobial, antifouling
		22.69	390	$C_8H_6O_4$	1,2-benzene dicarboxylic acid	Phthalic acid	Antioxidant, antimicrobial, antifouling
		22.71	390	$C_{24}H_{38}O_4$	1,2-benzene dicarboxylic acid, diisodecyl	Diisoctyl phthalate	Antimicrobial antifouling
		22.74	278	$C_{16}H_{22}O_4$	1,2-benzene dicarboxylic acid, BIS (2-methyl propyl) ester	Isobutyl phthalate	Antimicrobial, antifouling
		19.18	280	$C_{18}H_{32}O_2$	9,12-Octadecadienoic acid (z,z)	Polyunsaturated fatty acid (Linoleic acid)	Anti-inflammatory, Nematicide, Insectifuge, Hypocholesterolemic, Cancer Preventive, Hepatoprotective, Antihistaminic, Antiacne, Antiarthritic, Antieczemic
3	Seed	18.11	256	$C_{17}H_{36}O$	n-Heptadecanol-1	Fatty alcohol	Antiarthritis, skin diseases
		19.75	138	C_9H_{14}	Isophorone	Cyclic ketone	Anti-platelet
		19.81	248	$C_{12}H_{25}Br$	2-Bromo dodecane	Alkane	Antibacterial activity

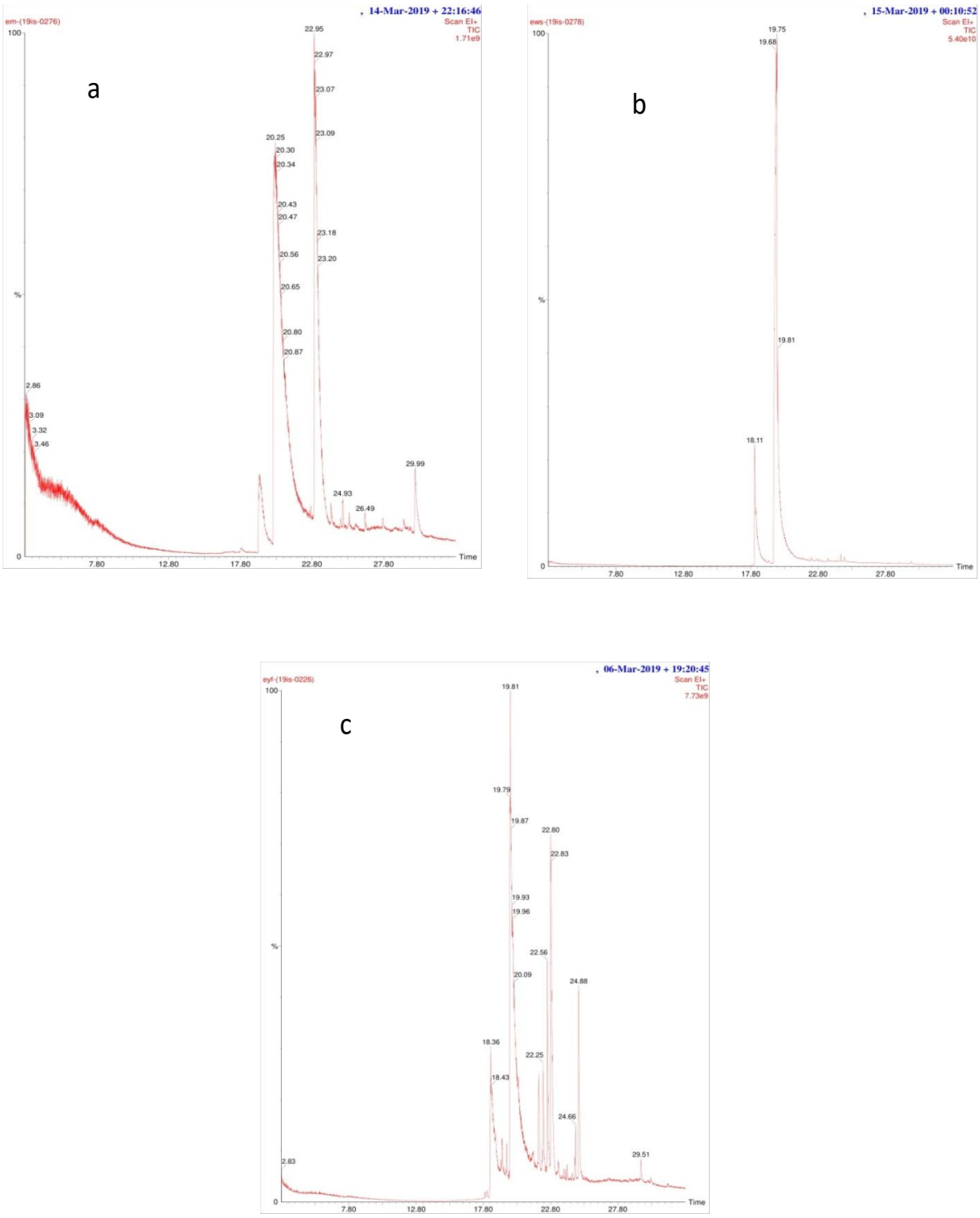

Figure 7 TIC of GC-MS analysis of Yellow pumpkin var. Ethylacetate Extract a: Peel; b: Flesh; c: Seed

Following our study, similar results are ascertained in some medicative plants (Banakar and Jayaraj 2018; Naz et al. 2020). The results indicated the ethyl acetate extract's capacity to extract more active components, which may be responsible for a variety of biological functions when comparing the biological activity of the compounds to literature data from previous research on medicinal plants (Swamy et al. 2015; Velmurugan and Anand 2017; Banakar and Jayaraj 2018; Rajadurai et al. 2018; Naz et al. 2020).

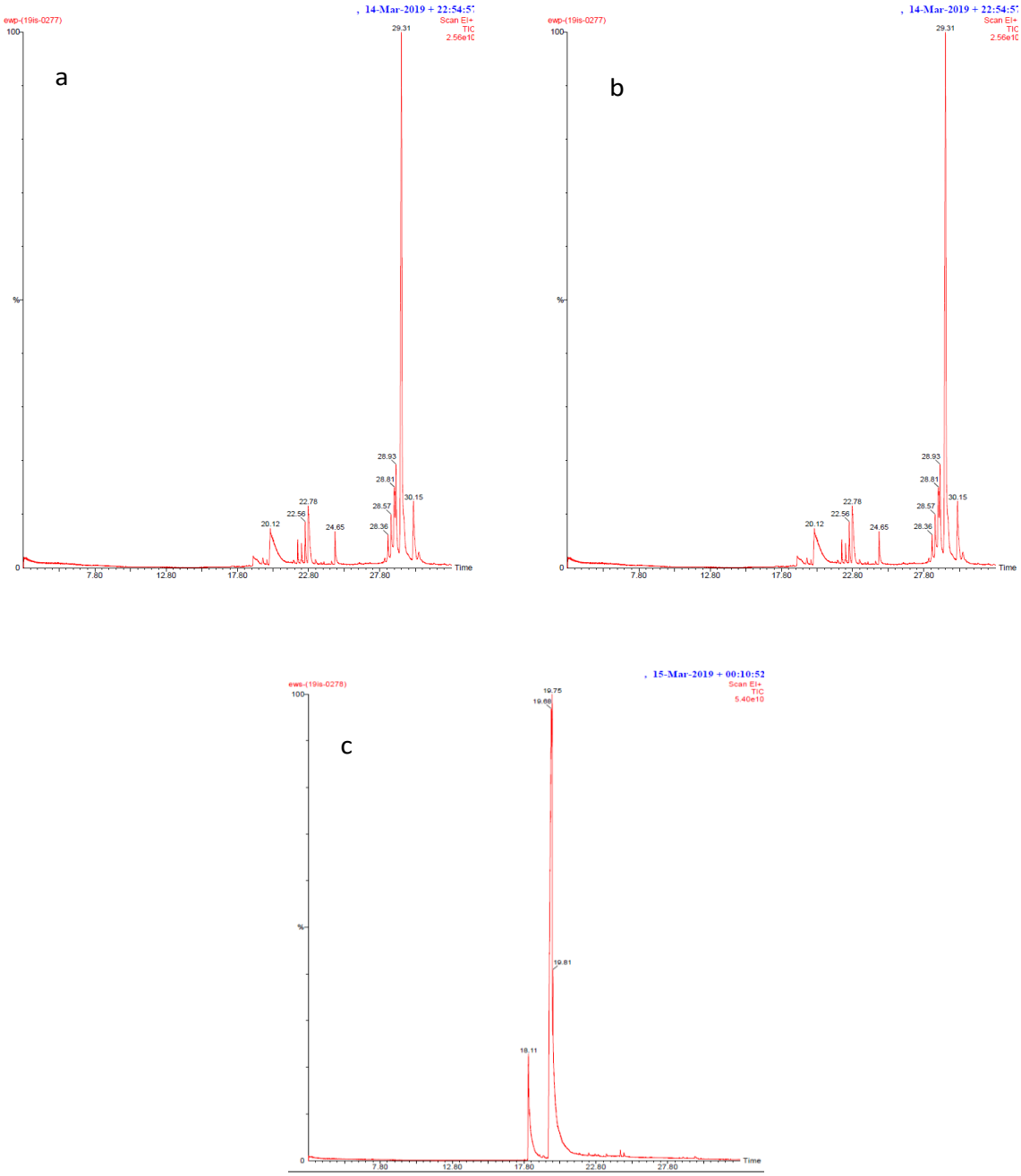

Figure 8 TIC of GC-MS Analysis of White pumpkin var. Ethylacetate extract a: Peel; b: Flesh; and c: Seed

4 Discussions

4.1 Qualitative Phytochemical Screening

Preliminary phytochemical screening aids in the development of new drugs (Doss 2009). In the present study, tannins are found to be lacking in the flesh and seed parts in both varieties. Similar to the previous studies on Cucurbita varieties, our study showed the presence of saponin, quinones, glycosides, phenol, alkaloids, and terpenoids in seed extract (Sumathi and Janarthanam 2016). The phytochemicals reported in the ethyl acetate extract of white variety fruit was similar to the study report of Vijayalakshmi (2014). In this study, researchers identified significant differences in the presence of phytochemical constituents in the peel, flesh, and seed of both varieties. The variation within the phytochemical constituent might be due to the solvent utilized for the extraction, location, and genetic variation (Wadood et al. 2013).

4.2 Determination of Primary Metabolite and Secondary Metabolite

In general, traditional or ayurvedic medicines are made from a single plant or a mixture of plants. The knowledge of taxonomic features revealed the potency of plant parts and the biological property of medicinal plants that are found to be dependent on the presence of primary and secondary metabolites, according to Vinoth et al. (2011). Primary metabolites play a direct role in the growth, whereas secondary metabolites appear to serve a secondary role and are only used as an accelerator. Following the above phytochemical screening in yellow and white varieties, ethyl acetate extract of peel, flesh, and seed revealed maximum numbers of metabolites, thus further ethyl acetate extract alone was employed for the quantification of major metabolites.

The current study on the estimation of primary metabolites (total soluble carbohydrate and total protein) and secondary metabolites (alkaloid and phenol) present in ethyl acetate extract of peel, flesh, and the seed of yellow and white varieties aids in understanding the source for isolation of therapeutically and industrially necessary compounds. The quantitative analysis of primary metabolites given in Table 3 showed that the carbohydrate content was found to be high in yellow flesh (2.2±0.2 µg/mL) and white variety seed (3.2±0.2 µg/mL). Subsequently, protein content was found to be high in seed parts of yellow (15.4±0.4 µg/mL) and white (11.3±0.4 µg/mL) varieties compared to the flesh and peel of each species. Carbohydrates are one of the building blocks of the cell, exploited for potential energy supplements. Similarly, proteins are most significantly important for maintaining the structure or function of all life and for growth development. The presence of higher protein levels in fruit parts of our current study has the potential to increase food value or to be used in the future for the isolation of protein-based bioactive compounds (Thomsen et al. 1991). Plant sugars are used as synthetic sweeteners and can even help patients with diabetes by assisting the body in its rebuilding process (Freeze 1998). Raj et al. (2018) performed similar work on the quantification of primary metabolites within the peel, flesh, and seed of yellow and white varieties and concluded that a sample with low carbohydrate content is appropriate for diabetic and hypertensive patients requiring a low-sugar diet. Likewise compared to our study alkaloid and phenol content has been analyzed in white and yellow variety peel, flesh and seed have been reported (Raj et al. 2018). Young et al. (2002) suggested that including or excluding polyphenol-enriched or fortified foods may have unfavorable effects. Due to their analgesic, spasmolytic, and disinfectant properties, pure isolated alkaloids or synthetic derivatives are used as basic health agents (Stray 1998).

4.3 Fourier Transmittance-Infrared Spectrometry (FT-IR)

FT-IR spectrum confirmed the presence of alcohols, alkene, aromatic, ketone, carboxylic, aromatic amine, nitro compounds, silane, and phosphine. The presence of aromatic and aliphatic amine has been reported on the aqueous extract of *Gymnema sylvestre* FT-IR analysis (Sangeetha et al. 2014). Similarly, OH-group potential expression was observed in different solvent extracts like petroleum ether, chloroform, methanol, and ethyl acetate in FT-IR analysis of four medicinal plants (Ashok Kumar and Ramaswamy 2014).

4.4 Gas Chromatography-Mass Spectroscopy Analysis (GC-MS)

The total ionic chromatogram of GC-MS analysis in peel, flesh, and seed ethyl acetate extract of yellow variety revealed a total of 15 compounds (Figure 7) that might contribute to the medicinal property of this particular species. Further, the total ionic chromatogram of ethyl acetate extract of white variety peel, flesh, and seed showed 14 compounds (Figure 8). The bioactive compounds known by GC-MS analysis in the present study are medicinally vital as they possess a novel structure with specific biological activities. Lupeol is one of the pharmacologically active triterpenoids that has been identified in yellow flesh and has been extensively studied for its anti-inflammatory property. Lupeol is abundant in vegetables such as white cabbage, pepper, cucumber, and tomato (Saleem 2009). Another study on a lupeol-rich extract of the *Pimenta racemosa* plant exhibited significantly high anti-inflammatory activity in animal models (Lima et al. 2007). Moreover, lupeol has a complicated pharmacology in humans, with anti-protozoal, anti-microbial, anti-inflammatory, anti-tumor, and chemopreventive properties (Gallo and Sarachine 2009; Saleem 2009). Thunbergol is another major metabolite that has been observed in the peel of white variety, which has been previously reported to be found in flower buds of tobacco (Xu et al. 2015) and tuberous roots of *Ampelocissus latifolia* (Theng and Korpenwar 2015). Similar to our study, Di-2-ethylhexyl phthalate had been reported in the fruit of the white variety (Du et al. 2011). GC-MS analysis revealed the presence of polyunsaturated fatty acids such as linoleic acid in both yellow and white varieties. Linoleic acid, a precursor of arachidonic acid, is important in the inflammatory cascade (Wendt 2005). The compounds identified in the different parts of both yellow and white variety ethyl acetate extracts support the potential for many medicinal applications of this vegetable or fruit. In the present study, the biological activity of the compounds detected was analyzed from Duke's phytochemical and Ethno botanical database. The identification of these compounds in various parts would serve as the foundation for determining the potential health benefits of this vegetable.

Conclusion

Yellow and white pumpkin varieties are commonly used vegetables in Tamilnadu. Due to the change in lifestyle in recent years, people get affected by many diseases; hence they turned towards healthy diets. Although both the species of our study are used as a food source, the understanding of these species is less known. Though many studies on these species have been reported, the goal of this study was to learn about the biologically significant elements of the plant part as a nutritional source. In general, the whole fruit of pumpkin is taken as a dietary source, hence to reveal the biological potential of each part of the present study was carried. Simultaneously, the solvent efficiency was also analyzed. From the qualitative analysis, it has been observed that ethyl acetate solvents are efficient in extracting a maximum number of principal compounds in different parts of the study plant material. Flesh parts are found to have linoleic acid, and essential oil, which are essential sources of cancer-preventive, anti-inflammatory, and antimicrobial properties.

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Conflict of Interest

The authors declare that they do not have any conflict of interest.

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Inflammatory mediator responses of *Vaccinium corymbosum* extracts on the streptokinase induced acute glomerulonephritis in rats

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Blueberry extract

ABSTRACT

Blueberry (*Vaccinium corymbosum*) has many health benefits including anti-inflammatory and antioxidant activities. Glomerulonephritis is a commonly found kidney disease in companion animals that is characterized by glomerular proliferation and inflammation likes characteristics. The present study was carried out to evaluate the potential of blueberry against inflammatory response in the kidney of acute glomerulonephritis (AGN) in animal models. For this, twenty male Wistar rats were randomized into five groups i.e. A - E (n=4). Among these Group A has four healthy individuals administrated with aqua dest (negative control), group B individuals have streptokinase (6000IU/rat) induced acute glomerulonephritis rats treated with aqua dest (positive control) while group C-E has streptokinase (6000IU/rat) induced acute glomerulonephritis rats treated with different concentrations of blueberry extract (500, 1000, and 1500 mg/kg body weight) for 14 days, respectively. After 14 days, kidney samples were harvested for histology and immunohistochemistry examinations. One-way ANOVA followed by the Tukey test was used for statistical analysis ($P< 0.05$). The blueberry extract treated AGN rats showed a significantly decreased in IL-1beta expression and inflammatory cell numbers compared to negative and positive control rats and 1500 mg/kg of the blueberry extract was found as the optimal dose. Results of the study can be concluded that blueberry extract has a strong anti-inflammatory effect that could depress the inflammatory responses in acute glomerulonephritis rat animal models.

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1 Introduction

Glomerulonephritis is a kidney disorder that is characterized by an inflammation in the glomerular capillary and expanded permeability of the glomerular filtration barrier (GFB) (Medica et al. 2021). Post-streptococcal glomerulonephritis is the most common acute glomerulonephritis caused by β-hemolytic streptococcal nephritogenic strains group A (VanDeVoorde 2015). In animals, the incidence of acute post-streptococcal glomerulonephritis is common in dogs and cats, and about 55% of male dogs with an age of 4-8 years, and 75% of cats with an average age of 3-4 years are susceptible to this disease (Brown 2016).

Acute post-streptococcal glomerulonephritis occurs due to a complicated process that involves antibodies and antigens that interact in the blood and make immune complexes (Noris and Remuzzi 2013). Streptokinase is an extracellular protein derived from Streptococcus β-hemolytic (Shilpi et al. 2013) which converts free plasminogen into plasmin (an active enzyme) by breaking the peptide chain. This plasmin will bind to the blood vessels and degrade the fibrin polymer into small fragments, and this fibrin degradation increases vascular permeability (Brodsky and Nadasdy 2017). This process causes kidney damage and activates C3a and C5a complements (Keragala et al., 2018). This activation of the C3 and C5a complement increases the renal capillary permeability and increases IL-1β levels. IL-1β is a proinflammatory cytokine that is involved in fighting infection and functions as a neutrophil chemotactic factor, which will cause the release of neutrophils following the migration of eosinophils, basophils, and macrophages or inflammatory cell infiltration to the location of complimentary activation (Akdis et al. 2016). Treatment of glomerulonephritis can be performed by giving immunosuppressive drugs (cyclophosphamide or cyclosporine), diuretic drugs to elixir proteinuria, anti-inflammatory medications, and Angiotensin Converting Enzyme (ACE) inhibitors (for example, benazepril, enalapril, ramipril) (Brown, 2016). However, the side effect of the chemical drugs should be a concern and it could worsen the condition of kidney damage.

Blueberry (*Vaccinium corymbosum*) has 86% water content, 9.7% carbohydrates, 0.6% protein, and 0.4% fat. Moreover, blueberry has various antioxidant active ingredients such as ascorbic acid, polyphenols, and anthocyanins (Khoo et al. 2017). The anthocyanin contents in blueberries can be reached up to 495mg / 100g, including avidin, delphinidin, petunidin, cyanidin, and peonidin (Michalska and Łysiak 2015). Antioxidant contents protect body cells from free radicals by binding to free radicals to prevent inflammation in the cells (Alkhalf and Khalifa 2018). Ahmet et al. (2009) suggested that blueberry could protect the heart in ischemic conditions, and it could improve renal function (Nair et al. 2014). However, available information about how blueberry can inhibit the inflammation of acute glomerulonephritis is limited. Therefore, this study was carried out to determine the effect of blueberry extract on the proinflammatory cytokine of IL-1beta and total inflammatory cells in glomerulonephritis animal models induced by streptokinase.

2 Material and Methods

2.1 Animal experiment

In this study, six to eight weeks old (200±20g weight) twenty male Wistar rats were used as experimental animals. Before starting the experiment, ethical clearance was received from the university under approval no.1025-KEP-Universitas Brawijaya. These rats were randomly divided into 5 groups i.e. group "A" negative control (healthy individuals administrated with only aqua dest), group "B" positive control (AGN individuals treated by aqua dest), group "C" AGN individuals treated by 500 mg/kg of *Vaccinium sp.* extract, group "D" AGN individuals treated by 1000 mg/kg of *Vaccinium sp.* extract, and group "E" AGN individuals treated by 1500 mg/kg of *Vaccinium sp.* extract. All the individuals of Group B, C, D, and E were injected with streptokinase @ 6000 IU/rat to create an AGN animal model. After five weeks, all animals were euthanized and the kidney was collected for further analysis.

2.2 Streptokinase induced acute glomerulonephritis (AGN)

The dose of streptokinase given to experimental animals was 6000 IU/rat. Streptokinase vial (1,500,000 IU) (Fibrion, Dexa Medica, Indonesia) powder was diluted with aqua pro injection, afterward homogenized with vortex. At the end of the acclimatization period, streptokinase injection was given through the coccygeal vein. The streptokinase injection was given three times at five-day intervals on the 8th, 13th, and 18th days.

2.3 Blueberry Extract Therapy Administration

The Blueberry extract was prepared by the maceration method using 70% ethanol. Blueberry therapy was given to the C, D, and E groups for 14 days, gradually using oral gavage according to each group dose and each rat received 2 mL extract once a day.

2.4 Euthanization Method

Experimental animals were euthanized with 100mg/kg ketamine and 10mg/kg xylazine, followed by intracardial blood collection (Shomer et al. 2020). Rats were fixed in dorsal recumbency. Dissection was achieved by an abdomen incision, and subsequently, kidneys were collected. The kidney samples were washed with PBS and stored in an organ container filled with 10% formalin.

2.5 Immunohistochemistry for IL-1β

The whole sample was made from kidney tissues of all rats. Kidney tissues were fixed in original paraffin blocks and 4-µm

sections were cut from these blocks. The slide organs were washed with pH 7.4 PBS for 3 times for 5 minutes. Followed by the dripping of 2% H_2O_2 and the slide was left for 20 minutes. Afterward, it was soaked in 5% BSA in PBS for 30 minutes and subsequently washed 3 times for 5 minutes. The slide organs were fixed with primary antibodies (Anti rat IL-1β) overnight at 4°C and then washed with PBS. The slide organs were incubated with secondary antibodies labeled biotin (Anti Rabbit IgG biotin-labeled) for an hour at room temperature and washed with PBS. SA-HRP was dropped into a slide for 40 minutes and washed with PBS. Diaminobenzidine (DAB) and substrate solution were given and incubated for 10 minutes and counterstaining was performed using Mayer's hematoxylin for five minutes at 27 °C, and then the slides were washed with water, dried, and mounted using entellant.

2.6 Quantification of IL-1β expression

The *IL-1β* immunopositive cells were determined over at least five random histological fields under 400 x magnifications. The brown stained area on the slide would be calculated as a percentage (%) of the expression area using the ImmunoRatio image analysis software.

2.7 Inflammatory Cells Count

The histological examination was performed on kidney tissue samples with hematoxylin-eosin (HE) staining. Under the microscope, the kidney micrographs were evaluated by counting inflammatory cells over five visual fields, equalling one mm^2. The results counted as cells/mm^2 (Alzahrani et al. 2021).

2.8 Data Analysis

The data analysis was conducted quantitatively applying one-way ANOVA (analysis of variance) continued by Tukey's post hoc test using the SPSS software with a 95% confidential value.

3 Result and Discussion

3.1 Effect of Blueberry Extract on Interleukin 1 Beta Expression

To examine the influence of blueberry extract on AGN, IL-1β expression was measured as one of cytokine pro-inflammatory. The results of the present study revealed that streptokinase induced an inflammatory response in form of IL-1β, which can be marked as brown stained in all groups except the control

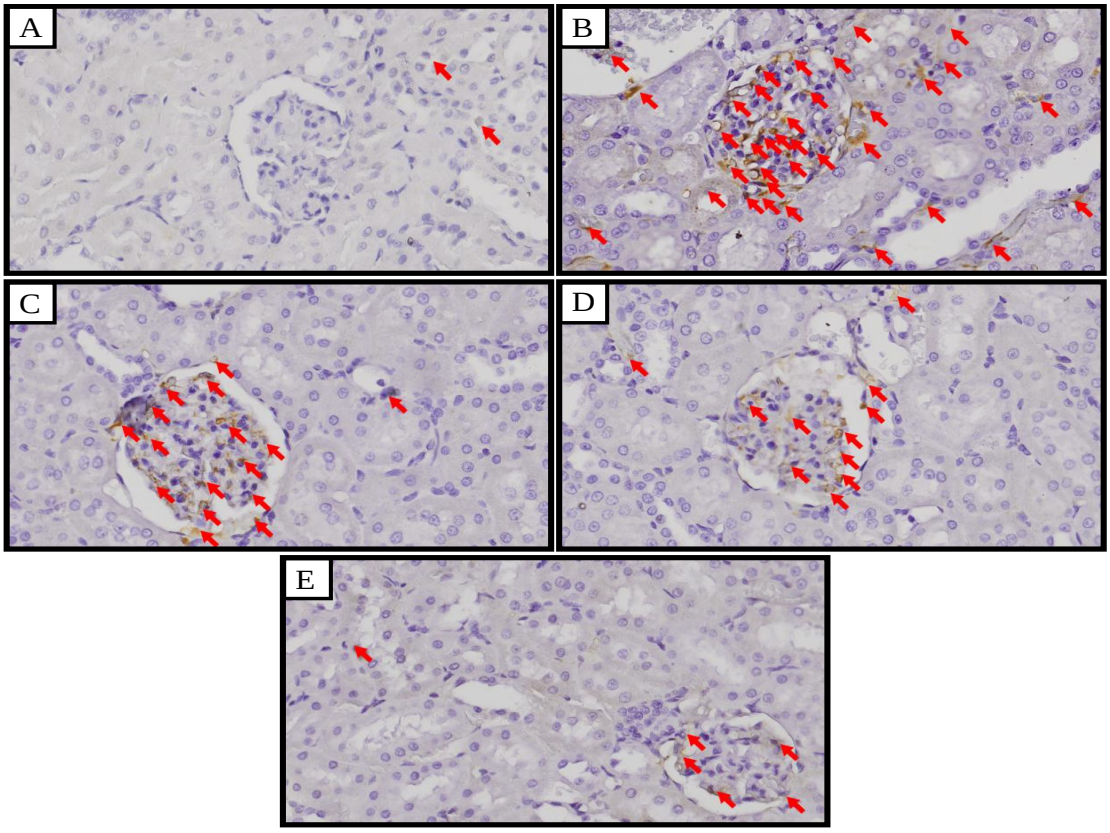

Figure 1 Blueberry therapy inhibit interleukin 1β (IL-1β) expression in the kidney of glomerulonephritis rat model (Immunohistochemistry: magnification of 400x), here A= Negative control; B= Positive control (glomerulonephritis/AGN); C= AGN with Blueberry extract dose 500mg/kgBW; D= AGN with Blueberry extract dose 1000mg/kgBW; E= AGN with Blueberry extract dose1500mg/kg BW; Red arrow shows the expression of IL-1β marked by brown color on cells

Table 1 The mean percentage of IL-1β expression in rat's kidney

Groups	Expression of IL-1β (% area) Mean ± SD	Percentage Decreases against Positive Control (%)
A (Negative control)	0.52 ± 0.11^{a}	-
B (AGN Positive control)	$12.12 \pm 0,37^{e}$	-
C (500 mg/kg)	8.18 ± 0.51^{d}	32.50
D(1000 mg/kg)	6.00 ± 0.16^{c}	50.49
E(1500 mg/kg)	3.42 ± 0.26^{b}	71.78

The Values given are the average of four replicates; mean ± SD value followed by the different letters in the same vertical column are significantly different ($p < 0.05$)

group "A" (Figure 1 A-E). Further, significant inhibition of AGN occurrence was demonstrated in the group treated with the blueberry extract (Table 1). Streptokinase converts plasminogen into plasmin, activating the complement reaction (C3 and C5a), increasing renal capillary permeability, and triggering an increase in IL-1β level. Damaged tissue during inflammation also causes the activation of macrophages and the formation of ROS that triggers the release of proinflammatory cytokines, one of which is IL-1β (Soderholm et al. 2018). Proinflammatory cytokine production can continuously lead to a prolonged inflammatory phase and recovery time.

The IL-1β expression in group A showed the lowest percentage in all groups, while group B showed the highest percentage. The expression of IL-1β significantly decreased in the blueberry therapy groups as compared to the positive (B) control group ($p<0.05$). Among blueberry groups, group E have significantly lower IL-1β expression compared with group C and D ($p<0.05$); however, the percentage of IL-1β expression was still significantly higher compared with the negative control group (A) ($p<0.05$).

Decreased IL-1β levels in the blueberry therapy groups were proportional to the increasing dose of blueberry extract. Blueberry extract therapy doses of 500 mg/kg, 1000 mg/kg, and 1500 mg/kg were able to decrease the IL-1β expression by 32.50%, 50.49%, and 71.78%, respectively as compared to the positive control. Findings of the study indicated that blueberry extract has a positive effect on preventing inflammatory response of AGN. The blueberry anthocyanin content has been reported as 487 mg/100g, which is the highest among berry fruits (Kalt et al. 2020). Antioxidant and anti-inflammatory effects of berry fruits are related to the concentration of anthocyanins. The role of blueberry in the treatment of cardiovascular (Zhu et al. 2013) and kidney disease (Elks et al. 2011) was also well established. Berry fruit acts as an anti-inflammatory compound by inhibiting the conversion of arachidonic acid into prostaglandin (PG) E2 through the synthesis of cyclooxygenase (COX) enzymes and also by suppressing the formation of antigen-antibody complexes occurring in the glomerulus (Denis et al. 2015). Thus, it can suppress complement activation and release of IL-1β cytokines.

3.2 Effect of Blueberry extract against Inflammatory Cells

The morphological evaluation of the rat's kidney showed abnormal structure due to streptokinase induction (Figure 2 B-E). The microscopic analysis of the kidney showed inflammatory cell infiltration, tubular epithelial erosion, interstitial hemorrhage, and glomerular hypertrophy. Nevertheless, compared to streptokinase-administered rats, the blueberry post-treated animals exhibited improvement in histological changes (Figure 2 C-E). Further, in the blueberry therapy groups, Bowman's space becomes wider, impairment in epithelial tubular and glomerular cells, and the presence of few inflammatory cells have been also reported. Glomerulonephritis was characterized by the increasing cell inflammation and the addition of endothelial and epithelial cells in the glomerulus that cause glomerular enlargement and narrowing of Bowman's space (Pirozzi et al. 2018).

The pathogenic mechanism of streptokinase-induced acute glomerulonephritis is started with an accumulation of streptokinase antigen in the glomeruli leading to complement activation, deposition of C3 and IgG, mobilization of immune cells, and following aggravation of glomerular inflammation (Nordstrand et al. 2009). Leukocytes, as one of the immune cells, patrol normal glomerulus. Meanwhile, neutrophil cells become the first responders against inflammation in the glomerular. Furthermore, other immune cells i.e. CD4+ and CD8+ T-cells, macrophages, and dendritic cells, alleviate local inflammation and systemic immunity (Richard Kitching and Hutton, 2016).

A quantitative assessment of inflammatory cell density was achieved by calculating the number of inflammatory cell occurrences on the kidney micrograph. The highest total of inflammatory cells was in group B, and these are significantly different from the negative control group (A) and blueberry therapy group (Table 2 C-E). The injection of streptokinase triggers the release of proinflammatory cytokines as inflammatory cell chemotactic which resulted in infiltration of inflammatory cells, i.e., migration from monocyte and macrophages to the place of complement activation. Damaged tissue during inflammation also causes the activation of macrophages (Soderholm et al. 2018).

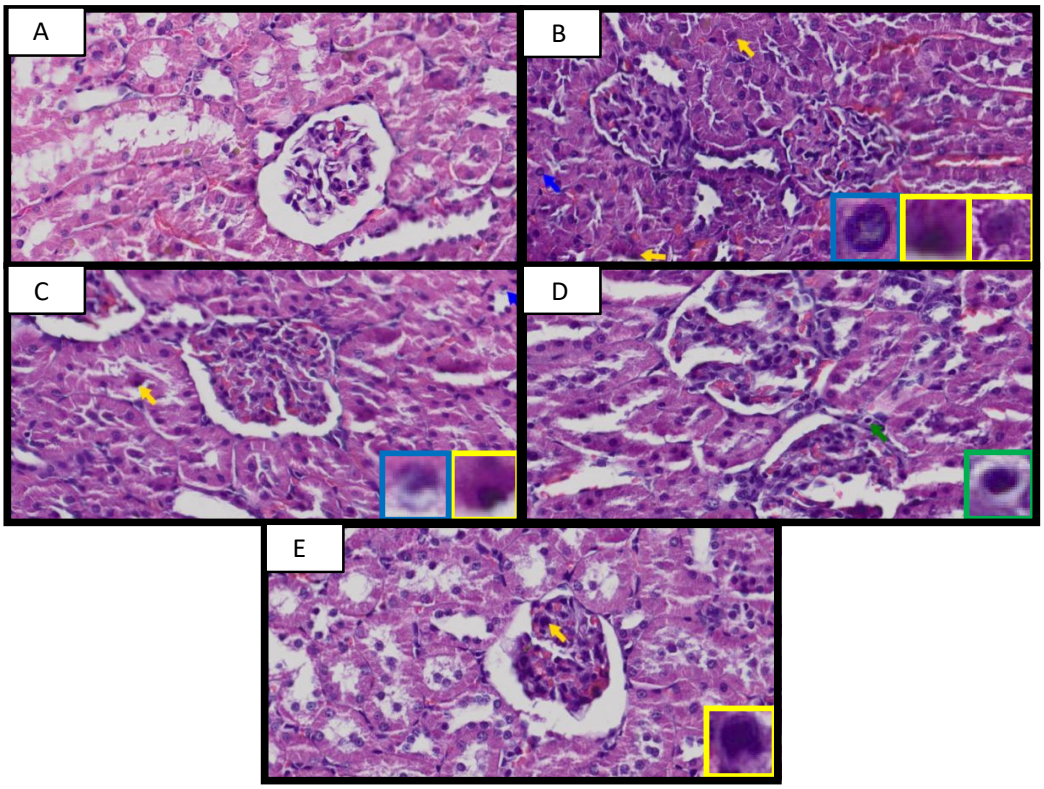

Figure 2 Blueberry therapy impairment renal tissue damaged due to streptokinase-induced glomerulonephritis model in the rat (H&E staining: magnification of 400x); here Yellow arrow and yellow box show the macrophage; Blue arrow and blue box show the neutrophil; Green arrow and green box shows the lymphocyte, Negative control group (A); Positive control group (B); Blueberry extract dose 500mg/kg (C); Blueberry extract dose 1000mg/kg (D); Blueberry extract dose 1500mg/kg (E)

Table 2 Mean of total inflammatory cell count on rat's kidney histopathology

Group	Total of Inflammatory Cell (cell/mm^2)	Percentage Decreases against Positive Control (%)
A (Negative control)	2.25 ± 0.50^a	-
B (Positive control)	7.25 ± 2.06^b	-
C (BB 500 mg/kg)	4.25 ± 0.95^a	41.38
D (BB 1000 mg/kg)	3.50 ± 1.29^a	51.72
E (BB 1500 mg/kg)	3.25 ± 0.95^a	55.17

The Values given are the average of four replicates; mean ± SD values followed by the different letters in the same vertical column are significantly different ($p < 0.05$)

The total of inflammatory cells in the blueberry therapy group gradually declined following the increased dose of the blueberry extract but this difference is not statistically significant ($p>0.05$). Blueberries are rich in anthocyanidins and anthocyanins content and the anti-inflammatory properties of blueberries might be due to the presence of these chemicals (Pervin et al. 2016; Khoo et al. 2017; Fauzi et al. 2021). The result obtained in this study suggests that blueberry extract has potential anti-inflammatory activities against AGN, and abolishes inflammatory cell infiltration. According to Subarnas and Wagner (2000), a higher concentration of anthocyanins had anti-inflammatory activity by restraining cyclooxygenases and cytokines' pro-inflammation production. Further, anthocyanin inhibited the change of arachidonic acid into prostaglandin (PG) E2 through a synthesis of the cyclooxygenase (COX) enzyme. It suppressed the formation of antigen-antibody complexes that occur in the glomerulus (Matout et al. 2019). It complements the activation and release of cytokines. Inhibition of the release of cytokines causes infiltration of inflammatory cells so that the reduced inflammatory response can make cells functional again. The role of other phenolic content cannot be denied because they might also involve in the anti-inflammatory mechanisms.

Conclusion

Results of the study can be concluded that the extract of Blueberry has a significant anti-inflammatory effect on acute glomerulonephritis (AGN) animal model with an optimum dose was 1500 mg/kg. Furthermore, the results of this study encourage the use of blueberry extract as alternative supplementation therapy for coping with acute glomerulonephritis (AGN). Future analysis of the bioactive *V. corymbosum* is essential to prove its mechanism in kidney injury.

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Conflict of Interest

The authors declare that there is no conflict of interest.

Authors Contribution

AF, FSP: designed the conceptualization, investigation, and manuscript writing; AN, AFP: conducted investigation, data curation, and writing draft preparation. NT: Validating, editing, and reviewing. The manuscript's final version was approved by all the authors.

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Seroprevalence and Risk Factors of Infectious Bovine Rhinotracheitis in Dairy Cattle of Chitwan, Nawalpur and Rupandehi Districts of Nepal

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KEYWORDS

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Rhinotracheitis

Seroprevalence

Risk factors

Dairy cattle

ABSTRACT

The cross-sectional study from July 2018 to September 2018 was conducted to determine the seroprevalence and risk factors of Infectious Bovine Rhinotracheitis (IBR) in cattle of the Chitwan, Nawalpur, and Rupandehi districts of Nepal. The existence of antibodies against IBR was investigated in 92 serum samples obtained systematically from 55 cattle herds using Indirect-ELISA. A questionnaire interview was done to collect individual and herd-level data. The association between categorical variables and the outcome variable (seropositive) was assessed by bivariate analysis and multivariate logistic regression analysis in SPSS version 19.0. The seroprevalence of IBR was 18.48% (95% CI: 11.1-27.9), and district, breed, and herd size were identified as potential risk factors for IBR seropositivity. Significantly higher risk for IBR was found in Chitwan (Percentage-Positive "PP" = 36.37%; Odd ratio "OR" = 5.211; p = 0.008) than in Nawalpur (PP = 9.38%; OR = 0.931) and Rupandehi (PP = 10.00%). PP of IBR was significantly higher in Jersey crosses (PP = 30.00%; OR = 2.893; p = 0.048) than Holstein Friesian crosses (PP = 12.90%). Similarly, herds with more than 10 cattle (PP = 33.33%; OR = 4.167; p = 0.042) were found significantly at higher odds for seropositivity

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than herds having less than 10 cattle (PP = 10.71%). Due to the moderate prevalence of IBR among cattle in Nepal, this study recommends conducting additional planned research on IBR at the national level to safeguard the country's dairy businesses from potential financial losses.

1 Introduction

In recent years, decreased fertility in dairy cows has become an international issue. The reproductive process of dairy cows is hampered by several factors. The main contributor to infertility in dairy cattle is infectious agents such as bacteria, viruses, and parasites (Prakash et al. 2021a; Prakash et al. 2021b; Chandran et al. 2021a; Chandran et al. 2021b; Sharun et al. 2021; Tiwari et al. 2022). These agents can lead to estrus, repeated pregnancy, early embryonic death, retention of placenta, delayed return to estrus, early embryonic death, and abortion. These will eventually trigger calving and a decrease in milk production in dairy farms (Chandran et al. 2019; Sonaa et al. 2021; Kumari et al. 2022).

Bovine herpes virus-1 (BoHV-1) causes infectious bovine rhinotracheitis (IBR), also known as a red nose or necrotic rhinitis, which is a highly contagious, infectious viral disease of cattle (Muylkens et al. 2007; Nandi et al. 2009), resulting in significant economic losses for the dairy and beef industries due to abortions, metritis, retention of placenta, repeat breeding, death of animals, loss of production and trade restrictions (Verma et al. 2014; Chandran and Arabi 2019; Chandran 2021a; George et al. 2021; Anand et al. 2022). While bovines are the most common hosts for BoHV-1, the virus has been found in other Artiodactyls (including goats, sheep, water buffaloes, and camelids) as well (Biswas et al. 2013). The main route of transmission is by direct contact with nasal, ocular, and genital secretions from infected animals (Ackermann et al. 1990; Nuotio et al. 2007), or indirectly through contaminated material and air-borne particles (Wentink et al. 1993). The disease is clinically manifested by respiratory signs like serous nasal discharge, salivation, conjunctivitis, fever, anorexia, and depression (Ackermann et al. 1990; Miller 1991; van Oirschot et al. 1996). Pustular vulvovaginitis and balanoposthitis are two genital infections that commonly occur in areas where natural mating is common (OIE 2017). Most of the infections have a very moderate or subclinical course, but some can result in the abortion-inducing evacuation of a dead fetus and bloody fluid, as well as reproductive abnormalities such as repeat breeding, metritis, and retention of the placenta (van Oirschot et al. 1993; Chandran 2021a; Chandran 2021b; Lejaniya et al. 2021a; Lejaniya et al. 2021b).

Trigeminal ganglia after a respiratory infection and sacral ganglia following genital infection are the primary sites of latency in IBR; other non-neural locations include lymph nodes in the pharynx, cervical region, retropharynx, inguinal, and tonsils (Winkler et al. 2000), as well as peripheral blood (Fuchs et al. 1999). Reactivation of the latent infection can occur under stressful settings such as transport and parturition and through the use of corticosteroids, and viral DNA persists in ganglion neurons, likely for the lifetime of the host (Muylkens et al. 2007). Consequently, the virus may switch between a latent and lytic infection that can be identified through the detection of antibodies against BHV-1 in serum (Lemaire et al. 2000).

IBR is OIE listed notifiable disease and emerging disease of cattle (OIE 2017; Chandran 2021a), and it was first recorded in 1841 in Germany by Buchner and Reimann in the form of infectious pustular vulvo-vaginitis (IPV) in cattle (Biswas et al. 2013), IBR is now widespread all over the world except few countries like Austria, Denmark, Finland, Sweden, and Switzerland (Ackermann and Engels 2005). In Nepal, researchers have confirmed the seroprevalence of IBR (Jha 2005; Dyson et al. 2000). Out of 118 serum samples from cattle having reproductive problems like abortion, anestrous, and repeat breeding 60 samples i.e. 50.8%, were found seropositive for IBR in Nepal (Jha 2005). It was also discovered by Dyson et al. (2000) through research conducted in Nepal that animals exhibiting reproductive problems have antibodies against IBR. However, there is a lack of data in the study area because of the paucity of studies on the seroprevalence and risk factors related to IBR in cattle across the country. The purpose of this research was to assess the incidence of IBR and identify the factors that put cattle at risk for developing the disease in the farms of Nepal's Chitwan, Nawalpur, and Rupandehi districts, specifically among the population of improved cattle that has a documented history of reproductive issues (including abortion, placental retention, and repeated breeding).

2 Materials and Methods

2.1 Study area, design, and experimental animals

A cross-sectional descriptive study was conducted from July 2018 to September 2018 in 55 herds of improved dairy cattle from 20 different areas of Chitwan, Nawalpur, and Rupandehi districts of the mid-tropical region of Nepal (Figure 1), having an estimated cattle population of 71864, 61086 and 98384 cattle respectively (MoLD 2017). These districts possess a similar geographical background, climatic conditions, population density, vegetation, and biodiversity. People living in these areas are mainly involved in agriculture and animal husbandry. These districts are well-known in the country for dairy cattle farming and production. Thus, the areas were selected purposively as these areas have a larger number of cattle and commercial dairy farms in the country (Chandran 2021a; Chandran 2021b).

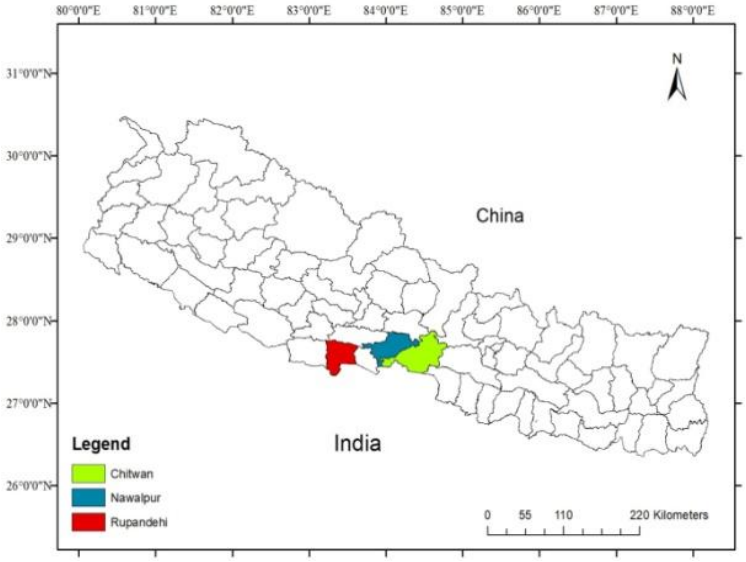

Figure 1 Map of Nepal showing the study area

The required sample size was 382, calculated from the EpiTools epidemiological calculator by Ausvet with an assumption of 50.8% prevalence of IBR in improved and crossbreed cattle of Nepal as reported by Jha (2005), keeping expected precision of 5% and 95% confidence level. However, due to the unavailability of an ELISA kit, only 92 samples were taken into consideration.

The animals used in the study were female dairy cattle of more than 2 years of age having a history of reproductive problems like abortion, retention of placenta, and repeat breeding. The animals were selected for sample collection by a purposive two-stage sampling procedure. In the first stage, pocket areas from each district (7 areas from Chitwan, 6 areas from Nawalpur, and 7 areas from Rupandehi) were selected by convenience sampling procedure which was taken as the primary sampling unit (PSU). In the second stage, blood samples of cows with a history of reproductive disorders were collected, by visiting the farms purposively in the preselected pocket areas, which were taken as a secondary sampling unit (SSU).

2.2 Variables

2.2.1 Dependent variable: Seropositivity for IBR

2.2.2 Independent variable

- Age: categories of age 2-5 years and ≥5 years
- Herd size: categories of ≤10 cattle and >10 cattle
- Breed: Holstein Friesian Cross and Jersey Cross
- History of reproductive problems like abortion, ROP, and repeat breeding.

2.3 Epidemiological Data

Epidemiological data were collected by using a structured questionnaire having both open-ended and close-ended questions for individual animal data as well as herd-level data. Individual level data included age, breed, pregnancy status and history of abortion, retention of placenta, and repeat breeding. Herd-level data include husbandry practices, vaccination, housing system, feeding system, interaction with other animals, the introduction of a new member, and breeding practice. The questionnaire was completed through face-to-face interviews with the farm owner.

2.4 Blood Samples

Selected animals were properly restrained, and 5 ml of blood was collected in a sterile SSGT (serum separating gel tube), properly labeled and immediately kept in a cool box. Then, the blood samples were brought to the laboratory of the National Cattle Research Program (NCRP), Chitwan, Nepal, where sera were extracted by centrifugation at 3000 rpm for 10 minutes. Extracted sera were then transferred into Eppendorf tubes, labeled, and stored in a deep freezer at -20˚C until laboratory analysis was performed.

2.5 Laboratory Analysis

Serum samples were tested by Indirect ELISA manufactured by IDvet, France (ID Screen® IBR Indirect) for the detection of anti-BOHV-1 antibodies. The test was conducted in the laboratory of the National Cattle Research Program (NCRP), Rampur, Chitwan, Nepal as per the protocol and procedures provided by the kit manufacturer.

2.6 Statistical Data Analysis

Data were first coded in the MS Excel spreadsheet (MS Office 2013) and then imported into SPSS version 19.0 for statistical analysis. The overall prevalence rate and district-wise prevalence and 95% confidence interval for prevalence were calculated from SPSS. Similarly, the association between categorical variables and the outcome variable (seropositive) was assessed by bivariate analysis (Pearson chi-square test) in SPSS. Finally, the multiple effects between predicted variables and outcome variables were analyzed by the forward likelihood ratio (Forward LR) method in a logistic regression model. For all analyses, a p-value of less than 0.05 at 95% CI was considered statistically significant. Finally, tables were used to present the results generated from SPSS.

2.7 Ethical Consideration

The study was conducted following the Declaration of Helsinki and approved by the Internship Advisory Committee of Veterinary Teaching Hospital, Institute of Agriculture and Animal Science, Tribhuvan University, Nepal. Oral consent was sought from farmers before commencing blood sampling from each farm or herd. The pain was kept to a minimum during blood collection.

3 Results

3.1 Overall seroprevalence

As shown in table 1, out of 92 serum samples tested, 17 samples were found positive for IBR with an overall seroprevalence of 18.48% (95% CI: 11.1-27.9%**)**. Among them, 11, 3 and 3 samples were positive from Chitwan, Nawalpur and Rupandehi out of 30, 32 and 30 samples collected, resulting seroprevalence of 36.37% (95% CI: 19.9-56.1%**)**, 9.38% (95% CI: 2-25%**)** and 10.00% (95% CI: 2.1-26.5%**)** respectively (Table 1).

3.2 Risk factors associated with IBR Seropositivity

Bivariate analysis (using Pearson chi-square test) between categorical variables (risk factors) and the outcome variable (IBR seropositivity) (Table 2) revealed a statistically significant association of IBR with location, breed, and herd size (P<0.05),

Table 1 Overall Seroprevalence of IBR in Cattle of Chitwan, Nawalpur, and Rupandehi District based on SPSS

Test Assay	District (Total Samples)	Classification	Prevalence %	95% CI
ELISA	Chitwan (30)	Positive:11 Negative:19	36.7	19.9-56.1
	Nawalpur (32)	Positive:3 Negative:29	9.4	2-25
	Rupandehi (30)	Positive:3 Negative:27	10.00	2.1-26.5
Total	92	Positive:17 Negative:75	18.5	11.1-27.9

Table 2 Results of Bivariate Analysis of Risk factors associated with seroprevalence of antibodies of IBR

Factors	Categories	N	Seropositive N (%)	χ^2 Value	P-Value	Odd's ratio (95% CI)
Location	Chitwan	30	11(36.37)	9.780	0.008*	5.211(1.278-21.237)
	Nawalpur	32	3(9.38)			0.931(0.173-5.015)
	Rupandehi	30	3(10)			Ref.
Breed	HF Cross	62	8(12.90)	3.923	0.048*	2.893(0.985-8.498)
	Jersey Cross	30	9(30.00)			
Herd size	>10	27	9(33.33)	4.123	0.042*	4.167(0.987-17.592)
	≤10	28	3(10.71)			
Age	2-5	39	4(10.26)	3.038	0.081	2.844(0.849-9.527)
	≥5	53	13(24.53)			
Abortion history	Yes	26	5(19.23)	Fisher's exact test	1.000	
	No	66	12(18.18)			
Retention of placenta history	Yes	24	2(8.33)	Fisher's exact test	0.221	
	No	68	15(22.06)			
Repeat breeding history	Yes	62	11(17.74)	0.068	0.794	
	No	30	6(20.00)			

*: Statistically significant; CI: Confidence Interval

Table 3 Results of Logistic Regression analysis for IBR seropositivity

Variables	*N*	Odd's Ratio	95% CI	p-value
Location	92			
Chitwan	30	4.209	0.929-19.077	0.009
Nawalpur	32	0.398	0.062-2.546	
Rupandehi	30	Ref	-	
Herd Size (> 10/≤10)	92	6.615	1.506-29.061	0.012
Breed (HFC/JC)	92	0.256	0.070-0.935	0.039

CI: Confidence Interval; HFC: Holstein Friesian Cross; JC: Jersey Cross

whereas age and history of reproductive disorders like Abortion, ROP and Repeat breeding were not significantly associated (P>0.05). Again, in the logistic regression model using the Forward LR method with 95% CI, the association of the seroprevalence of BoHV-1infection was analyzed for the predicted variables (variables with P<0.20 in bivariate analysis), which identified location (P=0.009), herd size (P=0.012) and breed (P=0.039) as risk factors (Table 3).

3.3 Breed-wise seroprevalence

Out of 30 samples from the Jersey cross (JC) and 62 samples from the Holstein Friesian cross (HFC) 9 (30.00%) and 8 (12.90%) samples were found positive respectively. Seroprevalence of IBR between JC and HFC breeds was found significant (P<0.05) where Jersey cross-breed cattle had higher odds to be seropositive than HF cross breeds (OR=2.893; 95% CI: 0.985-8.498) (Table 2).

3.4 Herd-wise seroprevalence

Among 55 herds tested, 28 herds were having cattle ≤10 and 27 herds were having cattle >10, out of which 3 (10.71%) and 9 (33.33%) herds were found seropositive for IBR respectively (Table 2). The seropositive herd was the herd with at least one seropositive animal. Seroprevalence of IBR between herd sizes of ≤10 and >10 was significant (P=0.042, i.e. P≤0.05), showing higher odds in herds having more than 10 cattle to the ones having less than or equal to 10 cattle (OR=4.167; 95% CI: 0.987-17.592)

3.5 Age-wise seroprevalence

Cattle below 2 years of age were not included in this study. Four samples out of 39 samples from age groups 2-5 years and 13 samples out of 53 samples from ≥ 5 years of age were found positive (Table 2) with a seropositive percentage of 10.26% and 24.53% respectively. There was no significant difference between seropositivity and age categories of 2-5 years and ≥ 5 years (P>0.05).

3.6 Seroprevalence by reproductive problems

The medical history of the abortion, retention of placenta, and repeat breeding were found in 28%, 26%, and 67% of the total cattle sampled respectively. Five samples out of 26 samples had abortion history, 2 samples out of 24 samples had retention of placenta (ROP) history, and 11 samples out of 62 samples having repeat breeding history were found positive for IBR (Table 2) with seropositivity percentage of 19.23%, 8.33%, and 17.74% respectively. There was no significant association between the seroprevalence of IBR and any of the history of reproductive problems like abortion, ROP, and repeat breeding (P>0.05).

4 Discussion

In the present study, the overall prevalence was found to be 18.48% (Total samples=92) which is similar to the prevalence found in Meru, Kenya (17.4%) (Kipyego et al. 2020), Karnataka (21%) (Koppad et al. 2007), Uttaranchal (10.75%) (Jain et al. 2006), Kerala (14.88%) (Rajesh et al. 2003) and West Bengal was (22%) (Ganguly et al. 2008), but, lower than the previous finding reported in Nepal by Jha (2005) i.e., 50.8% (Total samples=118) and study in Turkey (39.53%) (Ince and Şevik 2022). Tests conducted on animals in Nepal that were exhibiting reproductive abnormalities revealed the existence of antibodies against IBR, as was previously reported by Dyson et al. (2000). According to these studies, the BoHV-1 virus is widespread in Nepalese cattle farms. However, since there is no evidence of vaccination practice against IBR in Nepal as of yet, the circulating antibodies are likely only the result of natural infection of the virus already present within the herd or from newly introduced infected cattle without proper screening (Kampa et al. 2004). However, the low seroprevalence of IBR in this study may be mainly because of two reasons: first, a fall of immune response below detection after some years (OIE, 2017) as self-clearance of the virus may occur over time by replacement of infected and imported animals which is further potentiated by probably low intensive production systems i.e. with a low level of stress to animals (Kampa et al. 2004), and second is due to small but significant seasonal association of IBR (winter is a high risk than summer for IBR), detected by Woodbine et al. (2009) and supported by Sayers et al. (2015), and the present study was done in summer (July to September is summer in Nepal). Also, in contrast to our result, higher seroprevalence was found in different areas like 14%–60% in Africa and 36%–48% in Central and South America (Straub, 1990), 36% in China (Yan et al., 2008), 43% in England (Woodbine et al., 2009), and 63%–86% in

Eygpt (Mahmoud et al., 2009). Similarly, in Great Britain, among unvaccinated herds of cattle, the prevalence was found to be 62% by the bulk milk tank test (Martina et al. 2017). According to Kampa et al. (2004), variation in the prevalence rate of IBR at various farms is likely caused not only by the reactivation of dormant BoHV-1 infection under natural conditions but also by variances in management and/or geographical variables. Also, a study that was based on medical history concluded that non-vaccination, intensive rearing, the purchase and mixing of animals without IBR screening in all of the farms, and natural insemination from unscreened bulls in one farm are thought to be the primary causes of the high prevalence (Patil et al. 2015).

In location-wise analysis, Chitwan district has significantly higher seroprevalence (36.37%; $p<0.05$) as compared to Nawalpur (9.38%) and Rupandehi (10.00%) in this study. The reason behind this may be the differences in management and population of cattle as Chitwan has almost double the number of improved cattle (24744) as compared to Nawalpur (12092) and Rupandehi (13175). A high density of dairy cows and intensive management promote the viral spread and increase the chances that healthy susceptible animals will encounter infected animals (Chandranaik et al. 2014). To a similar extent, there is a greater likelihood of migration of cattle from one herd to another in Chitwan, which may play a role in the transmission and consequently results in a higher prevalence of the IBR antibody among cattle in that region.

Out of 30 samples from the Jersey cross (JC) and 62 samples from the Holstein Friesian cross (HFC), 9 (30.00%) and 8 (12.90%) samples were found positive respectively. JC breeds showed significantly higher odds for seroprevalence of IBR in comparison to HFC breeds ($P<0.05$; OD=2.893; 95% CI: 0.985-8.498) which is in contrast with Singh et al. (1985), who concluded that the prevalence was higher in Holstein-Friesian (50.35 %) than in Jersey (35.48 %) while screening a total of 506 cattle, and Sarmah et al. (2015), who screened only 51 samples of breeding bulls. The plausible explanation seems to be that most of the exotic cattle breeds reared in Nepal are Jersey Crossbreed as compared to Holstein Friesian which might reflect more transmission contact, thus higher prevalence.

Seroprevalence of IBR between herd size with cattle ≤10 (10.71%) and >10 (33.33%) was statistically significant ($P<0.05$) in this study which is in agreement with the previous finding by Woodbine et al. (2009) and Boelaert et al. (2005). Higher rates of seropositive animals in larger herds may be attributable to an increase in opportunities for disease transmission, both within and across herds (through, for example, veterinarians, technicians, workers, other farmers, and acquired cattle) (Woobine et al. 2009; Boelaert et al. 2005; van Schaik et al. 1998). Reactivation of latent infections is also more likely to occur in larger herds due to the added stress of having more animals and maybe introducing new calves into the herd (Singh and Sinha 2006).

For the age-wise analysis, blood samples from reproductively mature cattle (≥ 2 years of age) were sampled and tested for IBR. There was no significant difference between seropositivity and age categories of 2-5 years and ≥5 years ($P>0.05$). However, 4 samples out of 39 samples from age group 2-5 years and 13 samples out of 53 samples from ≥ 5 years of age were found positive with seropositive percentages of 10.26% and 24.53% respectively. Increasing seropositivity with an increase in age was observed which is in accordance with the findings of Singh and Sinha (2006), Ganguly et al. (2008), Woodbine et al. (2009), Bandyopadhyay et al. (2009), Verma et al. (2014), Patil et al. (2015) and Samrath et al. (2016). Possible causes include animals becoming more susceptible to disease as they become older; recurring, low-grade infections with the same virus that keep the antibody titer high enough to be identified; or a combination of lowered immunity and greater stress that activates dormant viruses (Singh and Sinha 2006; Samrath et al. 2016).

Furthermore, the current study showed that 17(18.48%) among 92 samples with a history of reproductive disorders were seropositive for IBR antibodies. However, there was no significant difference between the seroprevalence of IBR and reproductive problems history like abortion, ROP and repeat breeding. Further, 5 out of 26 samples with abortion history, 2 out of 24 samples with ROP history, and 11 out of 62 sam repeat breeding history were found positive for IBR with seropositivity percentages of 19.23%, 8.33%, and 17.74% respectively. Seropositivity for IBR was discovered in 83% of abortion cases, 83% of repeat breeding cases, and 65% of retention of placenta cases, according to Patil et al. (2017), which is corroborated by studies conducted by Nandi et al. (2009) and Bera et al. (2015).

Conclusion

In conclusion, this research study revealed that IBR is moderately prevalent among dairy cattle in Nepal. Statistical modeling revealed that geographic regions, breed, and herd size are the strongest indicators of IBR occurrence. Seroprevalence estimated in this study provides the basis for future monitoring and surveillance of disease with an urge for further planned research on IBR at the national level and to introduce the vaccination practice to protect our dairy industries from potential economic losses due to the infectious disease, despite the limitations of small sample size and a purposive sampling procedure.

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Conflict of Interest

No authors declare a conflict of interest.

Data Availability Statement

The datasets generated during and/or analyzed during the study are available from the corresponding author upon reasonable request.

Author's Contribution

SP, and MPA conceptualize the study. SP, SS and MPA designed the methodology. SP, DS, and SS performed the field and laboratory work. SP, SS and DS wrote the original draft. SP, DS, MPA, DC, KD reviewed and edited the final manuscript. All authors read and approved the final manuscript.

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Infertility Impact of Cotton Seeds (*Ceiba pentandra*) and Eggplant Cepoka (*Solanum torvum*) on the Nfkb Expression and Seminiferous Tubules Diameter

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ABSTRACT

The presence of the solasodine and gossypol compounds in Kapok seeds (*Ceiba pentandra*) and cepoka eggplant (*Solanum torvum*) suggest the use of these two as an herbal contraceptive ingredient in male animal fertility. This study aimed to determine the effect of cepoka eggplant extract (*S. torvum*) and kapok seed (*C. pentandra*) on infertility in rats (*Rattus norvegicus*) through NFkB expression and seminiferous tubule diameter. The study was carried out on 75 to 90 days old male Wistar rats having 150-200 grams of body weight. The experimental design used in this study was a completely randomized design (CRD) with three treatment groups i.e. C (control), KP1, and KP2 having six rats in each group. Here the rats of group C were not treated with gossypol or solasodine while the rats of group KP1 were treated with solasodine compound at a dose of 1g/kg BW and rats of group KP2 were treated with gossypol induction at a dose of 0.1 g/kg BW. Kapok seed extract and eggplant cepoka extract were extracted by the maceration method with 70% ethanol solvent. NFkB expression was examined using the immunohistochemical (IHK) method, which was analyzed with immunoratio software. Histopathological preparations (HE) of seminiferous tubule diameter were analyzed using raster image software 3. All the obtained data were analyzed by the One Way ANOVA test and Tukey's follow-up test with a 95% confidence level ($\alpha = 0.05$). The results showed that the administration of cepoka eggplant extract could significantly increase the expression of NFkB ($p<0.05$) with an average amount of 87.22 ± 6.89 with a dose of 1 g/kg BW. Treatment with Kapok seed extract can reduce the diameter of the seminiferous tubules (STs) by an average of 0.31 ± 0.016 at a dose of 0.1 g/kg BW. The results of the study can be concluded that the extract of cepoka eggplant and kapok seeds can be used as candidates for herbal contraceptives.

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1 Introduction

The explosion of the dog population in areas prone to zoonotic diseases can cause unrest in the community because it can trigger the occurrence of rabies and other diseases. The population of stray dogs in big cities such as East Denpasar, Indonesia has a ratio of 13.2: 1, with 96 dogs on a human population of 1272, and this has highly increased from 2008-2009 (Puja et al. 2012). The wild stray dog population can cause the risk of disease transmission in fellow animals or from animals to humans. The methods that have been frequently used for controlling the stray dog population include surgery, ovariohysterectomy, and castration. These methods are ineffective due to the time-consuming, expensive, and limited specialties. Elimination of wild animals no longer follows the principle of animal welfare, so it is necessary to develop a population control method using herbal compounds that are more effective.

Previous literature suggested that eggplant cepoka (*S. torvum*) and kapok seed (*C. pentandra*) can affect fertility and especially have a notable effect on male fertility. Fruit extract of eggplant cepoka contains alkaloid solasodine and steroids which are thought to have contraceptive potential and can be used as a male contraceptive as it decreased the number and motility of spermatozoa. Further, it has been reported that solasodine can suppress the functioning of the anterior pituitary and can reduce the secretion of FSH and LH. The decrease in LH causes a decrease in the production of testosterone in Leydig cells (Astrid 2012; Rafiqa et al. 2013).

Gossypol is obtained from kapok extract and can cause disturbances in the reproductive system; besides it can interfere with transcription factors (p53, p50, and p65) also, thereby it inhibiting the formation of hormones needed for reproduction (Moon et al. 2011). Cunha et al. (2012) reported that giving kapok extract in sheep feed can reduce the number of Leydig cells and make the testes smaller. Further, gossypol compounds found in the blood caused damage to the testes tissue. Damage to the testes tissue triggers an inflammatory mechanism and increases cell apoptosis which is characterized by the expression of Nuclear Factor kappa-B (NFκB) found in Sertoli cells. NFκB (Nuclear Factor kappa B) has an essential role in activating the inflammatory response and cell proliferation. Inflammation occurs in Sertoli cells and Leydig cells activate the expression of NFκB, which increases the cell apoptosis, and reduces the rate of spermatogenesis in Sertoli cells which results in infertility in males. Based on the background above, a study was conducted on the effect of kapok seed extract and eggplant cepoka as contraceptive candidates in male rats (*Rattus norvegicus*) through NFκB expression and seminiferous tubule diameter.

2 Materials and Methods

The study was carried out on 75 to 90 days old male Wistar rats having 150-200 grams of body weight. Other used materials are rat food and drink, 95% albumin vaseline, alcohol (70%, 80%, 90%, 95%, 100%), physiological NaCl 0.9%, PBS pH 7.4, H_2O_2 3%, object-glass, formalin buffer 70%, NFκB antibody, extract of kapok seed, extract of eggplant cepoka, Hematoxylin Eosin (HE) dye, distilled water, paraffin wax, ether 70%, xylol, and chloroform.

This is a laboratory study that was carried out in Completely Randomized Design (CRD). The experimental rats used in this study were first acclimatized for seven days with regular feeding and drinking water ad libitum. A total of eighteen male rats were randomly divided into three groups, and each group consisted of 6 rats. The treatment groups in this study are (i) C (control) - rats of this group were given standard feed and drinking water ad libitum, and not induced by gossypol and solasodine extract with ethanol as solvent, (ii) KP1 - rats of this group were given standard feed and drinking water ad libitum and orally given solasodine extract (eggplant cepoka extract) at a dose of 1 g/kg BW and (iii) KP2 - rats of this group were given standard feed and drinking water ad libitum and orally induced by gossypol extract (kapok extract) at a dose of 0.1 g/kg BW. Predefined doses of the kapok seed extract (0.1 g/kg body weight) and eggplant cepoka extract (1 g/kg body weight) were given orally once a day in the morning before the mice were fed. Euthanization of rats was carried out on the 11th day of rearing using the chloroform inhalation method. After euthanization, surgery was performed with an incision in the linea abdominis, then a pair of testes were collected. The 0.9% physiological NaCl was selected to wash the organ before putting them into a 10% formalin buffer. The diameter of the seminiferous tubules was calculated by observing the stained histology preparations under a microscope in five fields of view with a magnification of 100x. The measurement of the diameter of the seminiferous tubules was carried out using image raster three macros software with units of millimeters (mm).

Quantitative data were analyzed by one-way ANOVA with an accuracy of 95%, provided that the data were homogeneous and normally distributed. Tukey's test was also used to identify the difference between treatment groups ($\alpha = 5\%$).

3 Results

The comparative results of giving cepoka eggplant and kapok seed extract on male rats' infertility through NFκB expression and seminiferous tubule diameter were observed using the immunohistochemical method (IHK). The results of the testicular CPI were indicated by the expression of NFκB in male rats.

Table 1 Treatments result on NFκB expression

Groups	The average expression of NFκB(%) ± SD
Control	64.38 ± 13.84[a]
KP 1 (1 g/kg BW)	87.22 ± 6.89[b]
KP 2 (0.1 g/kg BW)	85.52 ± 6.94[b]

Value given are the average of four replicates; mean ± SE value followed by the different letters in the same vertical column are significantly different (p <0.05)

Table 2 The diameter of the STs in rats

Groups	Average diameter of the STs± SD
Control	0.44 ± 0.016[a]
KP 1 (1 g/kg BW)	0.40 ± 0.033[b]
KP 2 (0.1 g/kg BW)	0.31 ± 0.016[c]

Value given are the average of four replicates; mean ± SE value followed by the different letters in the same vertical column are significantly different (p <0.05)

Observations were made using a 400x magnification microscope with five times the field of view in the seminiferous tubule area. A brown color indicated nFκB expression in the tissue due to the binding between the primary anti-rat NFκB antibody and a secondary antibody labeled biotin, anti-Rabbit IgG biotin-labeled with the addition of DAB substrate (Diaminobenziddine). NFκB observations were carried out in all treatment groups, namely the control group (C), KP1, and KP2.

The accumulation of NFκB expression was statistically analyzed using SPSS software with the one-way ANOVA test. The results of the study showed a significant difference (P<0.05) between the control and the treatment group. Among the treatment group, group KP1 had a higher average NFκB expression than the negative control and KP2.

Results presented in table 1 suggested that the control group (C) had the lowest mean NFκB expression compared to the other treatment groups (64.38 ± 13.84). It might be due to the rats used in the control group being only given food and drink ad-libitum and did not get exposure to toxic compounds as inflammatory mediators. Some NFκB expression was also found in normal rats and it might be because of germ cell apoptosis which occurred to eliminate defective germ cells and carry DNA mutations (Pentikainen 2002). Further, among the treatment groups, group KP1 has the highest average NFκB (87.22 ± 6.89) followed by the KP2 (85 .52 ± 6.94), and these treatments showed a 26.18 and 24.71% increase from the control group (C), respectively.

The quantitative data of the diameter of the STs were analyzed using SPSS software with the one-way ANOVA method. The results showed a significant difference (P<0.05) between the control group (C), treatment group KP1, and KP2. Results of the study suggested that giving cepoka eggplant extract at a dose of 1 g/kg BW and kapok seeds at a dose of 0.1 g/kg BW can reduce the diameter of the STs in rats (Table 2).

4 Discussion

This study proved that treatment group KP1 could inhibit the process of spermatogenesis and increase the expression of NFκB in testicular tissue. The results also showed the presence of NFκB expression in treatment group KP2 and this is marked by brown color in the testicular tissue (Figure 1). These results are in line with previous research conducted by Susilo and Akbar (2016), who found a decrease in spermatozoa motility when treated with 1 g/kg BW cepoka extract and also reduced the number of spermatozoa by 20.33 million/ml. Cepoka has solasodine as an active ingredient which is thought to be used to increase infertility in male rats. Previous studies suggested that solasodine compounds have activities that can suppress the function of the anterior pituitary and reduce the secretion of FSH and LH hormones through negative feedback to the Hypothalamus.

The results of this study revealed that treatment group KP2 had a lower NFκB mean (85.52 ± 6.94) but this is not significantly different (P>0.05) from the treatment group KP1 (Table 1). The lower NFκB mean of KP2 might be due to the lower concentration of gossypol compounds in kapok extract. The results of this study are in agreement with Gadelha et al. (2014) who suggested that gossypol could act as an antifertility, and anti steroidogenic. Further, gossypol can cause changes in the structure of the epididymis, the diameter of the seminiferous tubules of the testes, damage to the plasma membrane, and cause regression in Sertoli and Leydig cells. Gossypol compounds have a cytotoxic and reactive effect that can disrupt and damage cellular activities. Further, gossypol can bind to the protein connexin 43 (Cx-43) on Sertoli cells and inhibit the production of gonadotropin hormones by interfering with the action of FSH and LH hormones in the anterior pituitary. Gossypol also affects transcription factors (NFκB, p53, p50, and p65), and interferes in the formation of hormones and enzymes needed in reproduction (Moon et al. 2011). Yurekli et al. (2009) suggested that gossypol interferes in steroidogenesis by inhibiting the activity of the cytochrome enzymes p450, 3β–hydroxysteroid dehydrogenase (3β–HSD), 5α–reductase, and aromatase. Barriers to the mechanism of action of these enzymes cause the steroid hormone produced to decrease so that spermatogenesis is disrupted.

This study indicates that eggplant cepoka extract with a dose of 1 g/kg BW increased the expression of NFκB, and this extract has a more significant effect on seminiferous tubules by increasing apoptosis and inhibiting the process of spermatogenesis in male rats.

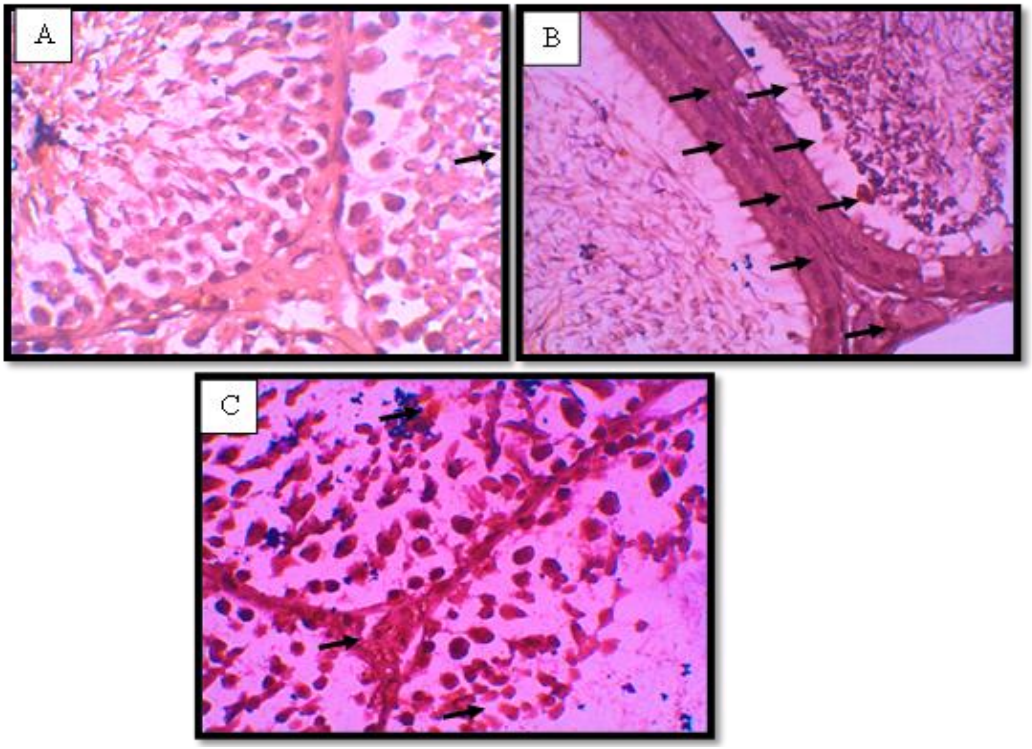

Figure 1 Immunohistochemistry of Testicular Tissue (400x magnification); Black arrows indicate NFκB expression
A = Negative Control Group; B = KP1; C = KP2

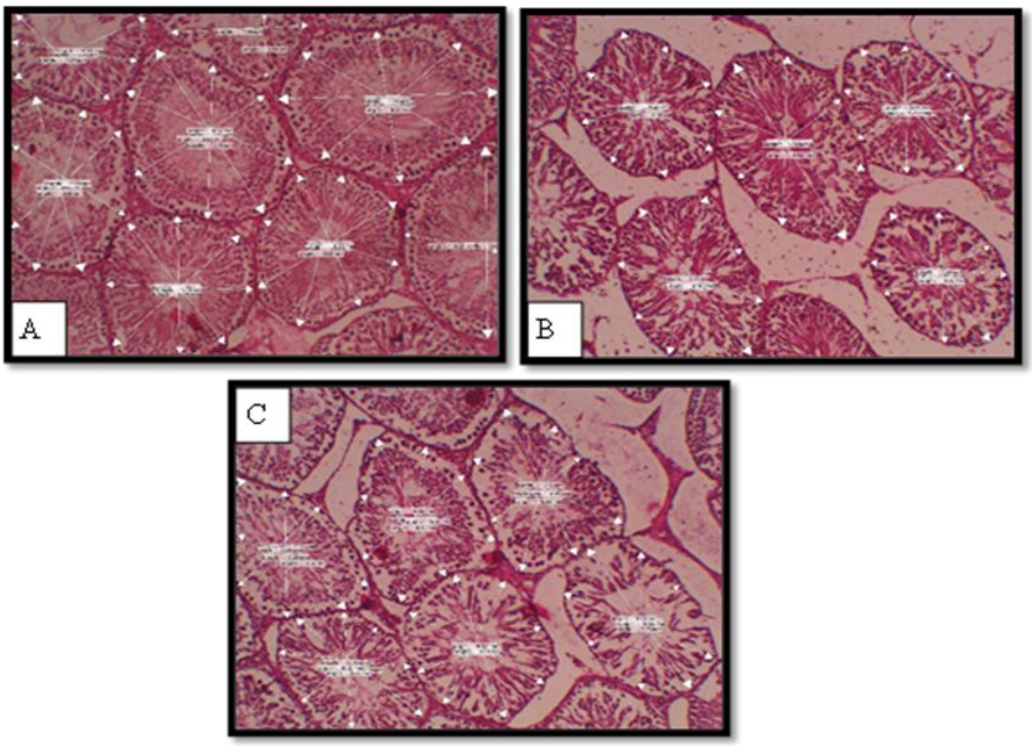

Figure 2 Histology of rat seminiferous tubules (100x magnification); A = cross section of control group testicle (C); B = cross section of KP1; C = B = cross section of KP2 here white arrows indicate the diameter of the STs

Nuclear Factor kappa B (NFκB) is the part of transcription factors that regulate the expression of gene encoding cytokines, chemokines, and several acute-phase proteins in health, which have an essential role in inducing the regulation of various genes in the inflammatory response, and cell proliferation. Generally, NFκB is in an inactive state because in the cytoplasm it has bonding with IkB inhibitors (IkB) (Riawan and Samodijanti 2006). Treatments with cepoka eggplant and kapok seed extracts act as inflammatory mediators and cause the activation of the Toll-Like Receptor (TLR), which is used to activate NFκB and trigger apoptosis in germ cells.

The result of Hematoxylin Eosin (HE) staining shows the presence of normal seminiferous tubule tissue (Figure 2). The histological examination of the seminiferous tubules revealed the presence of a basement membrane structure with a dense and tight arrangement of spermatogonia cells. Spermatogonia cells are still attached to the basement membrane of the seminiferous tubules. Further, in treatment group KP1, a decrease in thickness, separation of spermatogonia cells from the basement membrane, and widening of the lumen were observed in the seminiferous tubules (Figure 2). Treatment group KP2 also experienced similar symptoms and reported a decrease in the thickening, change in the histological shape of seminiferous tubules that were no longer reported, and also experienced atrophy (Figure 2). The atrophic testicular morphology shows the absence of spermatogenic cell development, thinning of the basement membrane epithelial layer, and a decrease in the number of interstitial cells in the testes (Epstein 2012).

The measurement of the diameter of the STs showed a 9.1% decrease in the average tubular diameter in a treatment group KP1 as compared to the control group. The control group had a mean and standard deviation of 0.44 ± 0.016, while it was reported 0.40 ± 0.033 in the treatment group KP1. The results of this tubular diameter measurement proven that the extract of cepoka eggplant and kapok seed can cause a significant decrease in the diameter of the STs and cause disturbances in the process of spermatogenesis. The histology of seminiferous tubules in the control group was thicker as compared to both treatment groups. Ismail (2018) suggested that regular feeding did not suppress intratesticular testosterone levels, which functioned to develop spermatogenic cells.

Histological description of seminiferous tubules after induction of cepoka eggplant extract in treatment group KP1 showed a decrease in the number of spermatogenic cells in the basement membrane, this might be due to the presence of solasodine compounds and steroid content in cepoka eggplant which suppressed the testosterone levels and caused detrimental effects. The development of spermatogenic cells, causes the thickness of the seminiferous tubules to become smaller (Ismail 2018). The content of solasodine compounds in cepoka eggplant results in an increase in testosterone concentration in the blood resulting in a negative feedback effect on the pituitary gland. It has an impact on inhibiting the secretion of Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) (Hidayati and Nofianti 2014).

Treatment group KP2 has the highest effect in reducing the diameter of the STs with a decreasing percentage of 29.5% and an average of 0.31 ± 0.016. Histology of the tubules in the treatment group KP2 experienced a decrease in diameter, indicated by the histological shape of the seminiferous tubules that were no longer round and atrophied (Figure 2 C). Several other studies have stated that gossypol compounds disrupt the process of spermatogenesis, resulting in a decrease in sperm motility and the number of spermatozoa as a result of degeneration of testicular tissue. Disruption of the process of spermatogenesis will increase the percentage of abnormal sperm. The increase in abnormal sperm and degeneration of testicular tissue by gossypol compounds are probably caused by damage to mitochondria in the tail of the sperm and damage to the germinal epithelium in the seminiferous tubules.

Conclusion

According to the results of the study, it can be concluded that the administration of cepoka eggplant extract with kapok seeds at a dose of 1 g/kg BW and 0.1 g/kg BW orally can increase the expression of NFκB in the seminiferous tubules of white rats, with the best dose of 1 g/kg BW. Oral administration of cepoka eggplant extract with kapok seed decreased the diameter of the STs of white rats, with the best dose of kapok seed extract at 0.1 g/kg BW.

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Effects of protein levels of commercial diets on the growth performance and survival rate of rabbitfish (*Siganus guttatus*) at the nursing stage

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Rabbitfish

Protein levels

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ABSTRACT

This study aimed to determine the effect of a commercial diet's protein level on the fry-to-fingerling stage. Thirty days-old fries having the initial length and weight of 18.25 ± 0.15 mm fish^{-1} and 0.036 ± 0.50 g fish^{-1} respectively have been used in this study. Diet having three protein levels i.e. 30% (trial 1 as control), 35% (trial 2), 40% (trial 3), and 45% (trial 4), respectively, have been used to evaluate the effect of protein, and each trial has been repeated three times. During the study, stocking density was allocated to 1000 fish per composite tank with a volume of 1 m^3. After 30 days of rearing, the weight of fingerlings in trial 1 reached up to 1.50 ± 0.02 g fish^{-1} and it was recorded as 1.52 ± 0.01g for trial 2, these two were lower than that of trials 3 and 4, where fingerling weight was reported 1.69 ± 0.01 and 1.58g fish^{-1} respectively and obtained the best weight compared to others. The length of fingerlings at the end of the experimental period was also changed in different trials and it was recorded 47.12; 46.92; 50.97; and 48.89 mm fish^{-1} for trail 1, 2, 3, and 4 respectively, among the tested combinations lower fingerlings length was recorded for trial 2 (35% CP), but it is not significantly different for trial 1 and 2 and a significant difference ($P < 0.05$) was reported for trail 2, 3, and 4. The survival rate of fingerlings ranged from 67.27 to 72.33%. Meanwhile, the herd distribution coefficient variation (CVW) in the treatment using 40% protein (trial 3) was the highest at 72.33% ($p < 0.05$). The results of the study can be concluded that the level of protein has a significant effect on the various growth parameters of fingerlings.

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1 Introduction

Rabbitfish is a brackish water cultured fish species with a high economic value in Tam Giang - Cau Hai lagoon systems, Vietnam. According to Tram and Ngoan (2013), rabbitfish has a digestible protein ratio that is distinct in the different types of feed (e.g., whole soybean meal, shrimp head, peanut meal, and oil meal), which reached more than 45% in practice, farmers usually used the protein diet around 35% protein for a commercial culture of this species (Hieu et al. 2018) but they found it is not suitable for the better survival and growth performance. The species has a comprehensive food plan and can use a variety of foods such as seaweed, phytoplankton, large algae, zooplankton, Copepoda, organic residues, and other low-energy feed (Parazo 1990). However, under farming conditions, rabbitfish and other species (*S. canaliculatus and S. spinus*) can accept many types of food (Carumbana and Luchavez 1979). At the fingerling stage, rabbitfish requires a high amount of protein that is approximately 30 - 55%, and an energy level of 3,832 - 4,000 kcal/kg, (Parazo 1990); therefore, species that have dietary protein and lipid levels of 40% and 6%, respectively resulted in the best growth performance and feed utilization efficiency (Salem et al. 2021). In aquaculture, the feed cost reached up to 70 - 80% of the total input cost because of the high protein requirements, and due to this more investment is needed for the diets of *S. guttatus* particularly, during the fry to fingerling nursing stage, which is a crucial stage. Protein level and diet for fish in the fingerling stage is a practical choice from the beginning of this period. Therefore, this study aimed to determine the protein required of rabbit fish at an early stage. In this study, the effect of the different dietary proteins were examined, and determined the influence of the protein levels on growth and survival rate, lower feed cost, and suitable industrial diets of the species during the fry to fingerling stage.

2 Materials and Methods

2.1 Materials and animals

Twelve thousand fries were used in this study, before starting the study these fries were acclimatized under laboratory conditions. Once these fries were acclimatized, they were assigned to three tanks, and 1,000 individuals were selected for each tank, with 4 protein levels i.e. 30 percent as control, while 35, 40, and 45% CP were used as a trial, each treatment replicated three times. These fries were supplied from the brood stock station. They had an average weight of 0.04 g $fish^{-1}$ and an average body length of 18.25 mm $fish^{-1}$ at 30 AHD (after hatchery days) and nurtured the fish in each composite tank with a volume of 1 m^3 (water volume = 800 L or 80% of water), the experiment used commercial feed sources with different crude protein contents as mentioned above and balanced for nutritional values the same in trials.

2.2 Experimental set-up

The initial water quality in tanks was uniform and suitable for rearing fish, the water quality is as follows: temperature 28-29°C, salinity 29 – 30%, pH 7.8-8.5; dissolved oxygen (DO) > 5 mg L^{-1}; and stocking density 1,000 fish per tank. The nursing period for fries was 30 to 60 days, and in this period all the fries become fingerlings for delivery to the cultural community. The experiment was arranged on three trials with three replicates, and each practice used industrial diets with three distinct levels of protein trials 1, 2, 3, and 4 as 30; 35; 40; and 45%, respectively. The amount of feed consumed in this experiment was 10% during the first ten days and 9% during the next 10 days. After reaching 8% body weight, the fish were fed 3 times per day.

2.3 Water management and variables in tanks

Fluctuation in water factors such as pH, water temperature, dissolved oxygen (DO), total NH_4/NH_3, and salinity were checked twice a day during the nursery stage. For this, a machine of 9 indicators was used (Machine Automatic Stainless Steel Aluminum Cooper Lampshade contact supplier by sensors).

2.4 Variables and methods

The growth performance of fish was determined at the time of initial weight and stocking (30 days) and weighed at the end (60 days). For this, 30 fish were collected from each tank to determine the weight using a hand-held electronic scale APTP453 (Japan). The length was determined using a graduated ruler and the survival rate at the end of the experiment.

2.5 Variable calculations and accounted

Growth performance indices in terms of percent survival rate, daily weight gain (DG, g day^{-1}), and length (DLG, g day^{-1}) were directly measured, while weight gain (g), length gain (mm), and coefficient of variation (%) were determined by following the equation of weight and length gain given by Rahman et al. (2009) and Yousif et al. (2005).

Weight gain (g) = (final body weight – initial body weight)

Length growth (mm) = (final body length – initial body length)

Daily weight gain (g day^{-1}) = (final body weight – initial body weight)/30 days;

Daily length growth (mm day^{-1}) = (final body length - initial body length)/30 days;

CV (%) = (δ/M) *100 (δ, standard deviation; M, the average value of the weight).

Survival rate (%) = [(Initial number of fish) – (final number of fish)]/initial number *100

2.6 Statistical analysis

The mean value (M) ± standard error (SE, m) was calculated. Data were analyzed using a one-way analysis of variance by ANOVA, T-test through Microsoft Office Excel™ 2010, and SPSS ver. 16.0 software was applied to determine significant differences among dietary CP levels; finally, Tukey's HSD ranking test was used when significant differences for the T-test ($p<0.05$).

3 Results

3.1 Environmental variables

Aquatic animals are thermogenic, and their body temperature changes according to the fluctuations in the living environment, they can adjust their body temperature according to the living environment. Therefore, environmental water factors directly affect fish growth and survival. The results of changes in water environmental factors during the nursery of *Siganus guttatus* are presented in table 1. Results of the study are showing less fluctuation in water environmental indicators during the nursery period of the fish and all the studied parameters are relatively stable and suitable for the development of *S. guttatus* under general and marine conditions.

3.2 Growth rate at different protein levels

The results presented in table 2 showed the daily fish gain after 30 days of nursing, using the commercial diets with different protein levels. Among the carried out trials highest average growth in fingerling weight was reported in trial 3 (1.69 g $fish^{-1}$), this was followed by the fish in trial 4 (1.58 g $fish^{-1}$), trial 2 (1.52 ± 0.11) and trial 1 (1.50^{a} ± 0.12) but these three are not significantly different. Similar trends were observed in daily weight gain and it was reported highest in trial 3 (0.055g/fingerling/day) while the least daily weight gain was reported in trial 1 (0.048g/fingerling/day).

The average growths concerning the length of fish from 30 to 60 days old have been presented in table 3. Results of the study revealed that fish length increased with the increased protein percentage from 40 and 45 % CP. Further results of the study suggested that the length of fish ranged from 47.12, 46.92, 50.97, and 48.89 mm $fish^{-1}$ for the trials 30, 35, 40, and 45% CP

Table 1 Effect of used protein diet on various water indicators during the nursery stage of fingerling

Variables	Time	Trials % CP (M ± m), n = 30			
		30	35	40	45
pH	Morning	7.80 ± 0.01	7.85 ± 0.02	7.90 ± 0.12	7.89 ± 0.12
	Afternoon	8.29 ± 0.05	8.31 ± 0.07	8.20 ± 0.11	8.24 ± 0.10
Temperature (^{0}C)	Morning	28.19± 0.53	28.09 ± 0.73	27.66 ± 0.63	28.26 ± 0.75
	Afternoon	32.45± 0.52	32.55 ± 0.62	32.39 ± 0.51	32.67 ± 0.55
DO (mg L^{-1})	Morning	4.40 ± 0.03	4.50 ± 0.04	4.41 ± 0.08	4.33 ± 0.06
	Afternoon	5.35 ± 0,09	5.45 ± 0,10	5.39 ± 0,05	5.44 ± 0.03
Salinity (‰)	Morning	29.12± 0.41	29.02 ± 0.31	29.33 ± 0.27	29.00 ± 0.22
NH_4/NH_3 (mg L^{-1})	Morning	0.57 ± 0.02	0.67 ± 0.03	0.72 ± 0.08	0.81 ± 0.05

Mean ± SE value followed by the different letters in the same horizontal raw are significantly different at $p> 0.05$; the above-given data are the mean value (M) of thirty replicates

Table 2 Effect of different protein diets on weight gain

Variables	Trials % CP (M ± m), n = 30			
	30	35	40	45
Initial weight (g $fish^{-1}$)	0.04^{a} ± 0.045	0.04^{a} ± 0.045	0.04^{a} ± 0.050	0.04^{a} ± 0.050
Final weight (g $fish^{-1}$)	1.50^{a} ± 0.12	1.52^{a} ± 0.11	1.69^{b} ± 0.14	1.58^{c} ± 0.18
Weight gain (g $fish^{-1}$)	1.45^{a} ± 0.02	1.48^{a} ± 0.02	1.65^{b} ± 0.06	1.55^{c} ± 0.05
DG (g day^{-1})	0.048^{a} ± 0.05	0.049^{a} ± 0.05	0.055^{b} ± 0.07	0.051^{c} ± 0.07

Mean ± SE value followed by the different letters in the same horizontal raw are significantly different at $p> 0.05$; the above-given data are the mean value (M) of thirty replicates

Table 3 Effect of various CP levels on studied Length variables

Variables	Trials % CP (M ± m), n = 30			
	30	35	40	45
Initial length (mm fish^{-1})	18.25^{a} ± 0.29	18.25^{a} ± 0.22	18.25^{a} ± 0.15	18.24^{a} ± 0.05
Final length (mm fish^{-1})	47.12^{a} ± 0.50	46.92^{a} ± 0.60	50.97^{b} ± 0.35	48.89^{c} ± 0.43
Length (mm fish^{-1})	29.87^{a} ± 0.58^{a}	29.67^{a} ± 0.98	34.72^{b} ± 0.60	30.12^{c} ± 0.47
DLG (mm day^{-1})	0.99^{a} ± 0.09	0.98^{a} ± 0.03	1.15^{b} ± 0.02	1.00^{c} ± 0.02

Mean ± SE value followed by the different letters in the same horizontal raw are significantly different at p> 0.05; the above-given data are the mean value (M) of thirty replicates

Table 4 Effect of various CP diets on the survival rate and herd variation coefficient (CV_w)

Variables	Trials % CP (M ± m), n = 30			
	30	35	40	45
Survival rate (%)	67.53^{a} ± 2.80	68.53^{a} ± 1.80	72.33^{b} ± 3.13	67.27^{c} ± 4.76
CV (%)	5.41^{a} ± 1.25	5.31^{a} ± 1.25	5.10^{b} ± 0.88	7.42^{c} ± 2.64

Mean ± SE value followed by the different letters in the same horizontal raw are significantly different at p> 0.05; the above-given data are the mean value (M) of thirty replicates

respectively. The study also indicates that the body length growth increased by 0.99, 0.98, 1.15, and 1.00 mm per fingerling per day. The results of this study are in agreement with the findings of Andam et al. (2016) who reported a 23.90 mm increase in the *S. guttatus* during the fingerling period.

Body length growths (BLG) of the 4 trials were 0.99; 0.98; 1.15, and 1.00 mm day^{-1} for 30, 35, 40 and 45% CP, respectively. The average increasing lengths of the body per day was around 12 – 15% and also have significant differences between trials 2, 3, and 4.

3.3 Survival rate and coefficient of variation

The survival rate and flock division coefficient variation (CV) were important results for the rearing studies. The results of the rearing *S. guttatus* fingerling stage are shown in table 4. There were no significant differences were reported in the survival rate of the fingerlings in the diets with different protein content, and diets with 30, 35, 40 and 45% CP having survival rates of 67.53, 68.55, 72.33, and 67.27%, respectively, which were significantly different ($p > 0.05$) for trials 2, 3, and 4.

4 Discussions

4.1 Water variables

The average pH in the morning and afternoon ranged from 7.85 to 8.31 and are in agreement with the findings of previous studies that suggested the water pH range 6.5-9.0 is suitable for the growth of tropical fish and pH lower or higher than this affects the growth and reproduction of fish. Water temperature also plays an important role in aquaculture and the temperature range from 27.66 to 32.55 °C, is suitable for the species that live in warm water ranges from 25 to 32 °C as mentioned by Carumbana and Luchavez (1979) and Boyd (1998). Further, dissolved oxygen (DO) ranged from 4.33-5.45 mg L^{-1}, salinity ranged from 28 to 29%, and total ammonia (NH_4/NH_3) ranged from 0.67 to 0.81 mg L^{-1} are suitable water characteristics for fish culture (Rahman et al., 2009; Andam et al. 2016). In general, the water environmental factors recorded in this study are within the threshold of good growth and development of fish, and in this study levels of 30, 35, 40, and 45% of dietary protein percentages have not affected the water environmental quality for fish culture, therefore, water control was evaluated every day by leaning of tank bottom, as mentioned the water control by Parazo (1990) and Salem et al. (2021).

4.2 Daily gain and lengths

In a study on mullet (*Liza subviridis*) from the fry to fingerling stage (density 1,000 fish m^{-3}) fed with protein levels from 35 to 45%, the growth performance of fish between the daily weight growth (DG) was significantly different ($p < 0.05$) and reported by Viet et al. (2010) for different protein diets were not significant. Various previous studies on different fish species have shown that fish growth is reduced when fishes are fed on diets that have too high a protein content. In addition, increasing the weight and length is also an important factor to evaluate the development in the fingerling stage. The growth rate of fingerlings at 60 days as shown above indicated the supplement has more protein content up to 40% beneficiation for the nursery of fries to fingerlings. Studies by Tu et al. (2014) also used a protein diet at 35% CP, while research by El-Dakar et al. (2011); Andam et al. (2016); Thiet et

al. (2017) used much higher protein levels (49.9 and 55.5%) at the fingerling stage. Parazo (1990) and Manh et al. (2015) found that while using a diet containing 40% CP and 3832 kcal/kg energy for *S. guttutus* a better growth rate was achieved. The results of this study showed that a protein content of 40% CP had the best growth rate. Another study by Hieu et al. (2018) found these proteins contents levels in diets also showed the highest value of growth rate. The survival rate in this study was higher than that reported by Andam et al. (2016) when using a diet with a protein level of 30 – 40%, which was mentioned as the best growth of the species at 40% CP in the diet. However, the species in the brackish water of Tam Giang lagoon had different characteristics, there was no successful result by artificial reproduction yet and there were still more biological mysteries.

The length of fish during of nursing period from 30 to 60 days is an important fingerling criterion. In this study, best length development was achieved with a dietary level of 40% CP duration 30 days of the nursery, and these results are in agreement with the findings of Izquierdo and Fernandez – Palacios (2019) and Salem et al. (2021) also indicated the length of fish at this stage will be influenced by growth when enough protein content is available, so the discovering a type of protein for this species as soybean meal, is good because at this stage these fingerlings preferred more plant protein than animals.

4.3 Survival rates

Survival results of the current study compared with Hieu et al. (2018) those who fed orange fin loach (*Botia modesta*) with different protein contents ranged from 25 to 55% and it did not affect the survival rate and it was reported between 96.70 to 99.30%. In a further study, Khanh et al. (2020) reported a 100% survival rate for (*T. blochii)* and Thiet et al. (2017) reported a 97.70% survival when reared with different protein contents. A study on the protein levels at 40 and 45% CP also showed a high growth efficiency and optimal survival rate in mullet (*L. subviridis*) at the fingerling stage (Viet et al. 2020). When trying to determine the best growth rate in another marine species based on the fed protein level, studies mentioned by Xu et al. (2019) found that the 35 and 40% level of protein was the most effective. Meanwhile, the coefficient of variation for *T. blochii* using industrial feed (44% CP) reached 0.24% was higher than the treatment combined between industrial feed and trash fish was 0.19%, and the treatment only used trash fish was 0.15% ($p < 0.05$) (Khanh et al. 2020). A commercial feed with 40% crude protein content is suitable for the nursing rabbitfish's fingerling stage, helping the fish to optimal growth and a survival rate of 72.33%, which corresponded to a coefficient variation of 5.10%. In addition to the study of protein levels in commercial feeds, further studies are needed on the respective energy levels to evaluate the effect of the role of energy and protein levels in the feed on the growth of fish.

Conclusion

Nursery of rabbitfish from fry to fingerlings, we can use commercial feed with crude protein of 40% for the best result of growth and survival rate. Although this species was considered a difficult alternative to live food and feedstuff for larvae, fry, and fingerling stages, even many practices were not successfully work in artificial reproduction.

Ethical certification and consent to participate

Ethical certification and consent to participate Certificate Reference Number: HUVNO019 Date 10th March 2022 The Animal Ethics Committee (AEC), I hereby make an ethical approval concerning the commitment of the Principle Researcher and supervisors for the project titled: "Effects of protein levels in commercial feed on growth and performance of Rabbitfishsh *(Siganus guttatus)* from 30 days of fry to fingerling stage"

Consent to publication

The Committee agreed to permit researchers to publish the article in any journal.

Competing interests

Not applicable

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Author contributions

Tran Vinh Phuong, Nguyen Anh Tuan, Nguyen Duy Quynh Tram, Nguyen Duy Thuan, and Ngo Thi Huong Giang collected data and maintained the experiments, Nguyen Quang Linh is leading the research groups and ideas and all conditions, major writing and revised and funding support, Tran Nguyen Ngoc collected data and contributed to experiments.

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Morphological and Morphometric Analysis of *Trypanosoma lewisi* and *Toxoplasma gondii* in Malang City, Indonesia Rats

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ABSTRACT

Rats are reported to be the intermediate hosts and reservoirs of several zoonotic protozoal diseases. *Trypanosoma lewisi* is commonly reported in rats' blood and is considered non-pathogenic protozoa in humans. However, some countries documented several cases in humans with *T. lewisi* infection. Another zoonotic protozoon that develops in rats and can be transmissible to humans is *Toxoplasma gondii*. We intended to present the morphology and morphometry of *T. lewisi* and *T.gondii* in wild rats collected around Malang City to explore the potential risk of transmission nearby. The rats were collected using single live traps followed by identification, sexing, age approximation, and body morphometry. All specimens were euthanized according to the standard procedure followed by blood and peritoneal fluid collection. The fluid smear preparation and Giemsa staining were performed to detect the presence of *T. lewisi* and *T. gondii.* Morphologic and morphometric analyses were conducted using ImageJ software. Among the collected 50 collected rats, 23 were identified as *Rattus norvegicus* (46%), 22 as *Rattus rattus* (44 %), and 5 as *Mus musculus* (1%). In the case of protozoans infection, ten individuals were infected with *T. Lewisi* (20%) from the blood smear check, whereas peritoneal fluid smear examination revealed an infection of *T. gondii* in a specimen (2%). Results of the study proved

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trypanosomiasis and toxoplasmosis in wild rats in Malang City. Though the clinical significance to human and public health impact is questionable, further research and surveillance of rodent-borne parasitic pathogens will provide more information for pre-emptive action.

1 Introduction

Rodents are small mammals, with predominant habitats ranging from natural to urban areas. Rodent species, such as *Rattus sp.* (black rat) and *Mus musculus* (house mouse), are commonly found in Indonesia. Rats are found near human habitation, and they play a crucial role as a reservoir or definitive host of many zoonotic diseases (Gratz 1994). Urban rats potentially bring pathogenic parasites to humans and spread through uptrend contact with a human because of urbanization (Sumangali et al. 2007). The rodent population in an urban area may increase the exposure and susceptibility to rat-borne diseases (Neves Souza et al. 2021). Recently, an increased incidence of cases closely related to zoonotic parasites has been recorded in several countries (WHO 2019).

Trypanosoma lewisi is a hemo-protozoan unicellular parasite and is known as non-pathogenic in rats. According to Sarataphan et al. (2007), *T. lewisi* has zoonotic potential, evidently reported in Thailand. In 2005, an infant was infected by *T. lewisi* and presented general symptoms of fever, cough, and anorexia. Another protozoal disease is Toxoplasmosis, caused by *Toxoplasma gondii*, a tissue, and intracellular parasite. This parasite is categorized as a heterogenous parasite, infecting warm-blooded animals, including domestic and wild animals, livestock, and humans (Weiss and Kim 2007). Further, *T.gondii* has veterinary and medical importance due to congenital diseases in animals and humans. *T. lewisi* and *T. gondii* infecting rats have different morphometry depending on their host and geographic distribution. This study intended to determine the morphologic and morphometric analysis, geographical distribution, and disease proportion of *T. lewisi* and *T. gondii* in wild rats in Malang. Malang has a high population and distribution of syntrophic animals, indicating a high potential for zoonotic diseases transmitted by rats. People living close to rodents are potentially exposed to zoonotic protozoa infection. This study also aimed to explore the possibility of zoonotic protozoan risk associated with rats in the study field area.

2 Materials and methods

2.1 Animals Ethic

Animal Care Committee Universitas Brawijaya adequately approved the protocol of animal ethics. The approval numbers are 090-KEP-UB-2021.

2.2 Research and study area

The location of the study area is in Malang, East Java, Indonesia. Rats were caught from human residences in urban areas and markets. Rats were obtained from the six districts of Malang i.e. Koljen, Lowokwaru, Sukun, Kedungkandang, Blimbing, and Wagir.

2.3 Data collection, and sample collection

Rat collection was carried out from August to October 2021 using live traps. All specimens were euthanized according to the standard procedure followed by blood and peritoneal fluid collection (AVMA: 2020 edition). Morphometric measurements of rats were performed with unit length in millimeters (mm) and weight in grams. Rats' species were determined based on specific characteristics. The standard identification parameters of rats included the total length of body and tail (Total Length), length of tail, hindfoot length, ear length, weight measurement, and added head length measurement (Yuliadi and Muhidin 2016).

2.4 Parasites identification

Blood and peritoneal fluid were smeared on the glass slides and stained using Giemsa staining solution. The prepared smear was observed under 100x microscopes Olympus CX-23 (Olympus Corporation, Japan) with oil emersion and captured by OptiLab Advanced Plus camera (PT Miconos, Indonesia). Spotted parasites were measured using *ImageJ*. According to Desquesnes et al. (2002), *T. lewisi* has konetoplast in the sub-terminal region and a long slight posterior end. The nucleus of this hemo-protozoan was in the anterior region and equipped with free flagella. Montoya et al. (2015) guideline was used for the *T. gondii* morphology study and found that its tachyzoite is crescentic or oval shape and has 5 to 7 length and 2 to 3 µm width. The anterior has a cone-shaped conoid structure with a round posterior end. Dense granules are spread throughout the cytoplasm. The single nucleus is located toward the central area of the cell or the hind end (Vismara et al. 2021).

2.5 Geographical Information System on Parasite

Geographical Information Systems (GIS) maps were created using the QGIS 3.4.5 Madeira. The maps were made by adding the coordinates of the sampling points on the layers of geographic and administrative shapefiles. Shapefiles of the administrative boundaries and detailed bodies of water were derived from the Digital Elevation Model National (DEMNAS) Indonesia. The elevation shapefile was derived from the 50 m Digital Elevation Model database.

2.6 Statistical analysis

Descriptive analyses were used to describe the morphology and morphometry of *T. lewisi* and *T. gondii*. In addition, proportions of protozoan infection were using IBM SPSS (Statistic Package for Social Sciences) 27 version (IBM Corp., Armonk, New York). The Chi-square tests were performed to compare *Trypanosoma* and *Toxoplasma* infection proportions per genus, age, and gender. Risk analyses were conducted using the Odds Ratio test (OR) (Allam et al. 2019).

3 Results

3.1 Identification of Rats species

Total 50 rats were caught and processed for subsequent tests. The results suggested that 10 (20%) rats were positive for blood protozoa and 1 (2%) for tissue protozoa among all collected specimens. Based on quantitative identification of rats (Table 1), all *Rattus rattus* have 39-210 grams body weight, 200-380 mm total length, 70-190 mm tail length, 25-35 mm hindfoot length, 30-55 mm head length, and 10-23 mm ear length. Further, *R. norvegicus* have a more extensive body and have 63-528 grams body weight, 300-560 mm total size, 125-240 mm tail length, 30-45 mm hind foot length, 40-70 mm head length, and 10-25 mm ear length. The specimens of *Mus musculus* have 19-30 grams body weight, 180-220 mm total length, 55-125 mm tail length, 25-35 mm hind foot length, 30-40 mm head length, and 15-20 mm ear length. These results are in agreement with the data reported by Yuliadi and Muhidin (2016), who suggested that *R. rattus* is a medium-sized rat with a total length of 170-370 mm and have a nipple formula 2 + 3. Meanwhile, the *R. norvegicus* is a giant rat with a length of 190-550 mm with a nipple formula. 3 + 3, whereas *M. musculus* is small-sized with a total length of no more than 180 mm and nipple formula 3 + 2.

3.2 Morphology and Morphometry of *T. lewisi*

Microscopic observation revealed that *T. lewisi* showed a leaf-like shape, a long slight posterior end with an oval-shaped kinetoplast located sub-terminal, and a nucleus situated in the anterior part. They are equipped with the free flagellum and undulating membrane (Figure 1). Based on the results of the morphometric examination of *T. lewisi* in specimens, the body length of *Trypanosoma* ranges from 13.36-37.19µm, with an average of 29.26 µm. The body widths were about 1.11 -2.38 µm, with an average of 1,783µm. The visible nuclei were measured 1.41-3.19 µm on average 2.16 µm, and the nucleus widths were around 1.12 – 1.56 µm with an average of 1.27 µm. Further, the length of the kinetoplast raged from 0.32 – 1.19 µm, with an average of 1.08µm. The distances from the nucleus to the kinetoplast were about 4.55 – 8.61 µm with an average of 6.79 µm. Moreover, the free flagellum length was 6.88 – 9.02 µm with 8.11 µmon average (Table 2).

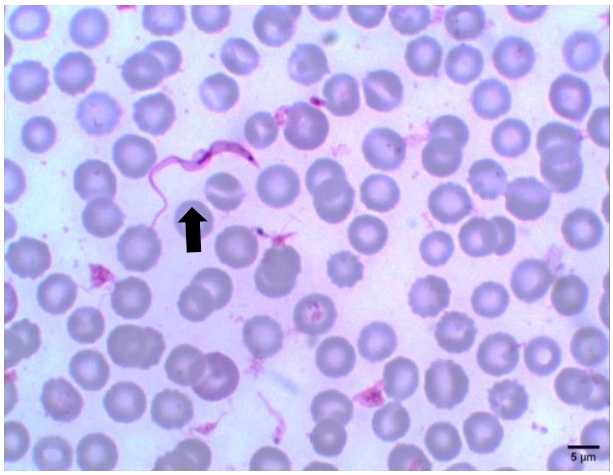

Figure 1 *Trypanosoma lewisi* (arrow) in rat blood, (Giemsa stained; Magnification 100x objective), Scale bar: 5 µm

Table 1 Morphometric recording of rats captured in Malang city

Species of Rats	Morphometric Measure						Nipple Formula
	body weight (grams)	total length (mm)	tail length (mm)	hindfoot length (mm)	head length (mm)	ear length (mm)	
Rattus rattus	39-210	200-380	70-190	25-35	30-55	10-23	2 + 3
Rattus norvegicus	63-528	300-560	125-240	30-45	40-70	10-25	3 + 3
Mus musculus	19-30	180-220	55-125	25-35	30-40	15-20	3 + 2

Table 2 The morphometric measure of trypanosomes in wild rats

Morphometric	*T. lewisi* (µm) In this study	*T. lewisi* (µm) (Sarataphan et al., 2007)	*T. evansi* (Sarataphan et.al, 2007)
Length	13.36-37.19	31.8 ± 6.4	21.0 ± 0.6
Widths	1.11 -2.38	2.7 ± 3.4	1.5 ± 0.3
Distances from the nucleus to kinetoplast	4.55 – 8.61	7.8 ± 1.3	6.1 ± 0.1
Flagellum	6.88 – 9.02	7.8 ± 4.5	4.2 ± 1.1

Table 3 Proportion of Parasite in rats according to the sex of rats

Parasite	Male (n= 27) Positive (%)	Female (n= 14) Positive (%)	T.P (%)
Trypanosoma lewisi	6 (12%)	4 (8%)	10 (20%)
Toxoplasma gondii	1 (2%)	0	1 (2%)
O.P.	7 (14%)	4 (8%)	11 (22%)

O.P: overall proportion; T.P: total Proportion, P%: proportion

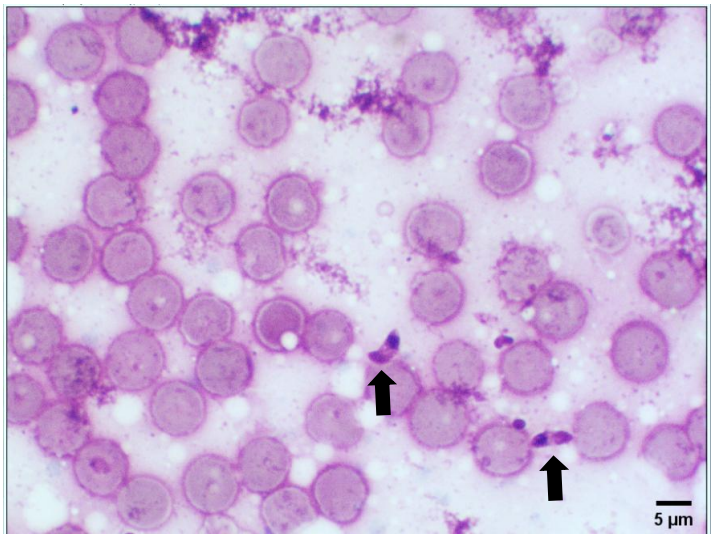

Figure 2 Tachyzoites in image smear of peritoneal fluid, here Note: semi-circular shaped of tachyzoites stadium (arrows), compared with host red blood cells; Giemsa stain. Scale bar: 5 µm

3.3 Morphology and Morphometry of *T. gondii*

The tachyzoite was oval to crescentic shapes and the nucleus was located in the posterior part. The morphometry of *T. gondii* length from posterior to anterior end ranged from 5.67 to 6.83 µm with an average of 6.226 µm. The width ranged from 1.48 to 2.70 µm with an average of 2.091 µm. The visible nuclei were measured 1.44-2.46 µm in length with 1.86 µm in middle, and the nucleus width was around 1.13 – 1.57 µm, with an average of 1.326 µm (Figure 2).

3.4 The proportion of *T. lewisi* and *T. gondii* infection in Rats

Based on the blood smear examination of 50 rats from August to October 2021, the proportion of *T. lewisi* infection was recorded at 20%, while it was recorded at only 2% in the case of *T. gondii*. The results of blood smear examination on rat samples showed that six male rats were positive, and three female rats were infected with *T. lewisi*. In addition, a positive specimen infected with *T. gondii* in the tachyzoite stage based on a peritoneal fluid smear is a male (Table 3). The data obtained were tested using the Chi-square test resulting in the Asymp value at 0.963 (> 0.05). There was no significant correlation between the proportion of infection and rat sex. Furthermore, the Odds Ratio (OR) test was implemented and presented a 0.964 OR score (Cl 95%). This result indicates that gender increases the infection risk at 0.964 times the proportion of the incidence of protozoa infection. Meanwhile, based on the Relative Risk (RR) test results it was found that male rats had a 0.994 times chance (Cl 95%) to be not infected. In contrast, female rats had a 1.030 chance of not being infected with *T. lewisi* and *T. gondii.*

Table 4 Proportion of Parasite in rats according to the genus of rats

Parasite	Genus Rattus (n= 45) Positive (%)	Genus Mus (n= 5) No Positive (%)	T.P (%)
Trypanosoma lewisi	9 (18%)	1 (2%)	10 (20%)
Toxoplasma gondii	1 (2%)	0	1 (2%)
O.P.	10 (20%)	1 (2%)	11 (22%)

O.P: overall proportion; T.P: total Proportion, P%: proportion.

Table 5 The proportion of Parasite in rats according to the age of rats

Parasite	Adults (n= 38) Positive (%)	Juvenile (n= 12) Positive (%)	T.P (%)
Trypanosoma lewisi	8 (16%)	2 (4%)	10 (20%)
Toxoplasma gondii	1 (2%)	0	1 (2%)
O.P.	9 (18%)	2 (4%)	11 (22%)

O.P: overall proportion; T.P: total Proportion, P%: proportion.

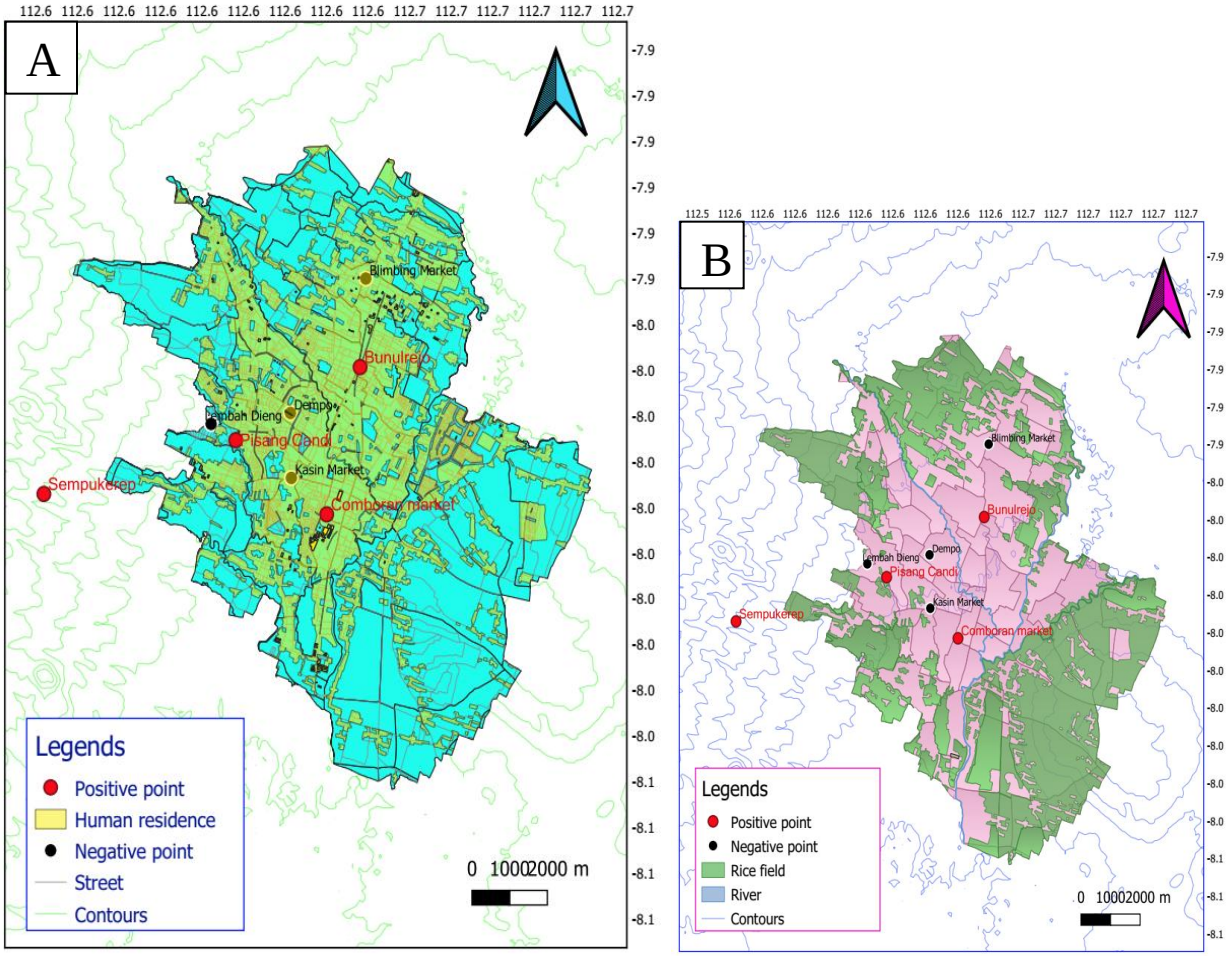

Figure 3 The map shows the distribution of Protozoa in wild rats in Malang East Java; (A) Modern map with human residence and street layers, and (B) Natural map with rice field and river layers.

The data obtained were tested by chi-square and the Asymp value was reported as 0.546 (> 0.05), concluding there was no significant relationship or correlation between the genus of rat and the proportion of infection. Furthermore, the Odds Ratio (OR) test was implemented and the OR value was 2.43 (Cl 95%). This result means that the genus increases the risk by 0.96 times the proportion of the incidence of protozoan infection. Meanwhile, based on the Relative Risk (RR) test results, it was found that genus *Rattus* had a 0.85 times chance (Cl 95%) were not infected, and genus *Mus* had a 1.91 times chance of not being infected with the protozoal disease (Table 4).

Based on the age of the caught wild rats, the study results obtained eight adult rats infected with protozoal diseases, and among these two were positive (Table 5). The data of this study was accomplished by the Fisher's Exact Test. The Exact Sig value was 0.23 because the Exact Sig value was > 0.05, there was no significant correlation between the age of the rat and the proportion of cases of protozoa infection. Furthermore, the Odds Ratio (OR) test was carried out to determine the relationship between protozoal and rat age, and the obtained result was 0.31 (Cl 95%). Meanwhile, based on the results of the Relative Risk (RR) test, it was found that adult rats (adult) had 0.77 times (Cl 95%) chance of not being infected by *T. lewisi* or *T. gondii*. Moreover, young (juvenile) rats had 2.34 times (Cl 95%) chance of not being infected by hemo-protozoan or tissue protozoa.

3.5 Geographical Distribution of Parasites

Based on the results of blood tests, it was found that ten rats were positive for *T. lewisi*, and a specimen was positive for *T. gondii*. Among the infected rats, five rats were reported from the Sempukerep, one rat from Pisang Candi Sukun District, three from the Comboran market Klojen District, and one from Bunulrejo Belimbing District Malang. Moreover, specimen positive *T. gondii* was recorded in Comboran Market Klojen District. Based on these results, a map of the distribution of the blood protozoan *T. lewisi* and *T. Gondii* were illustrated in Figure 3. Through regional distribution, it was found that 5(50%) specimens were positive for *T. lewisi* in Wagir District. In addition, there were 3 (30%) rats infected in the Klojen district. Furthermore, in Sukun and Blimbing Districts only 1 infected rat was found infected from each district (10% from each district).

4 Discussion and conclusion

Blood protozoa, *T. lewisi* is a trypanosome found in rodents and *Xenopsylla cheopis* fleas have a role as vectors. The study chose the dry season months from August to October for sampling. In this season, *Xenopsylla cheopis* often suck blood from animal hosts for egg development, thus potentially spreading *T. lewisi* infection (Hadi and Soviana 2018).

The morphology seen from the blood smear method is the same as Desquesnes et al. (2002). In contrast to *T. evansi* and *T. cruzi*, some differences have been observed in *T. lewisi*, it has a subterminal kinetoplast, a sharp posterior end, a centrally located nucleus, a regular wave shape undulating membrane, and free flagella (Teixeira et al. 2011; Maharani 2016). While in the case of *T. evansi* and *T. cruzi*, the location of the nucleus is in the central part and the undulating membrane has an irregular shape (Truc et al. 2013).

Along with this, in the morphometry description also, *T. lewisi* has some differences as compared to *T. evansi* and *T. cruzi*. The morphometry of *T. lewisi* described in this study is similar to Rabieel et al. (2018) description. *T. lewisi* has a larger size as compared to *T. evansi* and *T. cruzi*, it has an average length of 31 - 44 µm and a width of 1.9 – 3.2 µm while in the case of *T. evansi* length is 15 – 34 µm, 3.15 µm wide, nucleus length 8.21 µm, nucleus width 3.10 µm, kinetoplast length 5.16 µm, undulating membrane length 12.77 µm, and the distance from the nucleus to kinetoplast is 14.8 µm, and similar size was reported for the *T. cruzi* (Levine, 1985; Sarataphan et al. 2007).

T. gondii infection in rats is another important parasite of public health and the veterinary field. Rats potentially transmitted diseases directly or indirectly to humans through contaminated food or water or via an intermediate host (Paramasvaran et al. 2009). The intraperitoneal fluid smear revealed the stadium of tachyzoite *T. gondii*, a parasite of zoonotic importance, with a proportion number of 2%. This finding aligns with studies in Malaysia, which reported a low proportion (6.7%) of *T. gondii* infection in rats (Tijjani et al. 2020). The parasite in rats could be linked with the presence of the stray cat in Malang and poor sanitary conditions in the sampling area. The climatic and environmental conditions in the study area (humidity and tropical situation) influence the persistence and spread of *T. gondii* oocyst. Rats may not transmit toxoplasmosis to a human directly. However, they can be intermediate hosts or serve as a source of infection in cats in this study area. This becomes a source of infection to humans via infective oocysts and consuming poorly cooked meat tissue cysts (Vujanic et al. 2011).

In terms of the proportion of disease incidence, according to Alias et al. (2014), male rats have a greater chance of being infected with blood or tissue protozoa because male rats can explore a larger area than female rats. This allows the broader transmission of protozoan infection. This is in the agreement with this study, where 6 male and 4 female rats were found positive for protozoa. Further, an analysis of the proportion of disease based on the age of the rats was also carried out. The obtained results suggested that among the caught rats, eight adult rats, and two young (juvenile) rats were recorded positive for protozoa. This is in line with the research of Novita (2019) who reported lesser infection in the young rats (juvenile) and

this might be due to the lighter body weight as compared to the adult rats, these researchers reported a correlation between parasite densities with rat body weight. Young rats have a lighter body weight due to the number or density of parasites being more diminutive than adult rats. Therefore, young rats have a lower chance of being infected with blood or tissue protozoan.

Making the map is intended to determine the magnitude of the risk factors for protozoal infection. Based on the mapping, it was found that residential areas, markets, and urban areas are highly prone to infection with protozoa diseases because there are very abundant food sources for rats in these areas. The geographical map presented in figure 3 (A and B) is of Malang. The risk and potential of *T. lewisi* and *T. gondii* spreading were reported in several areas i.e. Sempukerep, Pisang Candi, Bunulrejo, and Comboran markets. The layers shown in map A showed modern layers such as human residences and streets. While map B showed natural layers such as rivers and rice fields. Rats generally live near rivers, water sources, human residences, or urban areas. Hence rats potentially spread the protozoan to animals or humans living near this area. In figure 3, the location of the positive rat sampling is symbolized in the form of a red dot (dot), based on the coordinates of the sampling point, while the black dot represents the negative sampling area. The rapid developments of human residence and agriculture have caused a gradual increase in food products and provided decent live places in urban areas for populations of rats (Khaghani 2007).

In most cases, rats are a significant reservoir for diverse parasites with potential zoonotic diseases (Seifollahi et al. 2016). The data showed that 20% of the exanimate rats were found positive. Further, it was reported that rats caught in the sampling area were infected with two protozoa species, and both have public health concerns. This pushes up a potential risk of disease carried by rodents to humans in Malang. However, research for a better understanding of the dangerous exposure places is needed. The awareness promotion on control and prevention of the rodents population is highly required in most communities that live in close contact with rats. Though the clinical significance to human and public health impact is questionable, further study and surveillance of rodent-borne parasitic pathogens will provide more information for pre-emptive action. In a nutshell, public health is significant in Malang East Java, which can cause infection of zoonotic protozoal in humans, such as *Trypanosomes* and *Toxoplasmosis*. Therefore, it is crucial to take into consideration the role of rats in spreading infectious or parasite diseases in Malang for better control.

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Pre-Harvest Sprouting Tolerance in 36 Bread Wheat Genotypes

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KEYWORDS

Triticum aestivum L.

Germination percentage

PHS selection

Pericarp rupture

Nebulizer

ABSTRACT

Pre-harvest sprouting (PHS), promoted by rainfall during crop maturity, is a high problem in many wheat-producing regions of the world. Considering its importance in Brazil, 36 national and international varieties and advanced lines of wheat were evaluated for their tolerance to PHS. For this purpose, two experiments were conducted over three years. Seed pericarp rupture was used as an indicator of the beginning of germination. The data were analyzed using analysis of variance, the Scott-Knott test, and the Lin and Binns method. The wide range of germination percentage values allowed the genotypes to be classified as tolerant (in experiment 1 - ND 674 and Grandin*2/RL 4137 and experiment 2 - Frontana and Grandin) and moderately tolerant (Alsen, CD 114, and Milan/3/Attila//Fang 69/CIMMYT 3 in Experiment 1; Avante, BRS 177, IAC 5-Maringá, Onix, OR 1, RL 4137, and Rubi in Experiment 2). Because tolerance to PHS is under genetic control and can be improved through breeding programs, the challenge for wheat breeders is to combine increased PHS tolerance with other requirements to meet market demands.

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1 Introduction

Rainfall at harvest time is one of the main factors affecting grain yield and end-use quality of wheat in many regions of the world. Pre-harvest sprouting (PHS) is a polygenic trait governed by genetic and environmental factors (Singh et al. 2021). In a genome-wide association study conducted on 298 bread wheat's, Rabieyan et al. (2022) found that the RRBLUP model is a useful tool for genomic selection.

Screening for PHS tolerance, facilitated by marker-assisted selection, is not a feasible practice in most wheat-breeding programs worldwide. Such screening must be based on easily visible grain characteristics stimulated by PHS, such as grain discoloration, swelling, wrinkling, splitting, and rootlet emergence, as listed by Thomason et al. (2019).

The falling number (FN) test has been considered a superior method for selecting PHS-tolerant genotypes in several studies (Barnard et al. 2005; Nörnberg et al. 2015a; Guarienti et al. 2017; Delwiche et al. 2018). However, the cost of equipment, the requirement of trained personnel, and a great number of samples in PHS selection limit its use as a screening tool. Alternative indirect methods, such as visual scoring (MacMaster and Derera 1976; Franco et al. 2009; Humphreys and Noll 2002) and/or calculation of the percentage of sprouted grains (Bassoi et al. 2006; Zeeshan et al. 2018), have been used in many breeding programs to evaluate PHS tolerance. Recently, Okuyama et al. (2020) found a high negative association between sprouting percentage and FN ($r = -0.9082^{**}$) and between visual germination score and FN ($r = -0.8956^{**}$); they concluded that in the absence of the FN test, calculating the germination percentage and/or grain germination score provided a valid option for PHS selection.

Studies have identified several PHS-tolerant genotypes, including Frontana, RL 4137, and BRS 177 (Andreoli et al. 2006); Frontana, Fundacep Cristalino, Fundacep Raízes, BRS Guamirim, TBIO Mestre, and TBIO Alvorada (Nörnberg et al. 2015b); Frontana, CD 1440, Quartzo, Jadeíte 11, LG Prisma, LG Oro, ORS Vintecinco, TBIO Iguaçu, TBIO Sinuelo, and TBIO Pioneiro (Guarienti et al. 2017); Frontana, CD 1440, and ORS Vintecinco (Rigatti et al. 2019); hard white winter wheat 'KS Venada' (Zhang et al. 2020); and the red-grained cultivar 'AAC Tenacious' (Dhariwal et al. 2021).

Given that the level of PHS tolerance of genotypes is not known in detail to plan the best combinations in a breeding program, the present study was undertaken to classify 36 progenitors to be used in crosses to increase tolerance to PHS.

2 Materials and Methods

Two experiments were conducted over two sowing dates in three years at the Paraná Rural Development Institute, IAPAR-EMATER, Londrina, Brazil. The plot size was 3 rows, 30 cm apart and 2 m long, with a sowing rate of 350 seeds m^{-2}. The following genotypes were evaluated in Experiment 1: Alsen, BRS 208, BRS 220, BRS 210, BRS Guabijú, CD 104, CD 108, CD 114, Chirya 7, Grandin*2/RL 4137, IPR 144, IPR 85, Milan/3/Attila//Fang 69/CIMMYT 3, ND 674, BRS Pardela, and SW 89-5124*2/Fasan. Experiment 2 consisted of the following genotypes: Alcover, Avante, BR 18-Terena, BRS 177, CD 105, CD 116, CEP 24, Frontana, Grandin, IAC 5-Maringá, IAPAR 17-Caeté, Ônix, OR 1, RL 4137, Rubi, and Supera.

At maturity, approximately 100 spikes per genotype were harvested by hand (Hanft and Wych 1982) and kept at room temperature of 20-25ºC. Styrofoam blocks, with pre-drilled holes of 5 cm in the line and 10 cm between the lines, were used to insert 20 spikes per genotype, with two replications. The spikes were kept under artificial rainfall (nebulizer) according to the procedure of Okuyama et al. (2018). The nebulizer was turned on/off in half-hour cycles to produce approximately 280 mm mist per day. After 48 h of nebulization, the spikes were dried in the sun and hand-threshed.

The percentage of grain germination (sprouting) was double-checked each time in a 50 grains sample. A magnifying glass (10×) was utilized to observe pericarp rupture, which was regarded as the beginning of the germination process (Nyachiro et al. 2002; Bassoi and Flintham 2005). The germination percentage data were transformed to arcsine values before analysis according to Snedecor and Cochran (1982). The analysis of variance (ANOVA), Scott-Knott test ($P \leq 0.05$), and the Lin and Binns (1988) methods were performed using Genes (Cruz 2006a; Cruz 2006b) and SAS software was used for statistical analysis (SAS 2001).

3 Results and Discussion

Artificial rainfall (nebulizer) was highly effective in obtaining genotypes with a wide range of germination percentages. Analysis using the Scott-Knott test and Lin and Binns method permitted clear categorization of the genotypes in both experiments. In Experiment 1, combined ANOVA for germination percentage showed that the effects of genotype and year were highly significant ($P \leq 0.01$), the effect of the block (year) was significant at $P \leq 0.05$, and the effect of the interaction genotype × year was not significant ($P > 0.05$) (Table 1). In Experiment 2, the effects of year, genotype, block (year), and the interaction year × genotype were all significant ($P \leq 0.01$) (Table 1).

The range of germinated grains in Experiment 1 varied from 13.83 to 78.83%, whereas it ranged from 8.83 to 85.33% in Experiment 2. Such a large variation in the evaluation of this characteristic allowed the classification of genotypes according to their degree of tolerance to PHS. In the absence of the availability of the FN test in many wheat-breeding programs, a viable option is to use a

Table 1 Variance analysis of the grain germination percentage in wheat genotypes

Source	DF	Mean square Germination (%) Experiment 1	Experiment 2
Year	2	2.6473 **	0.5355 **
Block (year)	3	0.0879 *	0.0985 **
Genotype	15	0.4566 **	0.4414 **
Year x Genotype	30	0.0462 ns	0.0627 **
Error	45	0.0294	0.0189
R-Square		0.9125	0.9206
Coefficient of variation		19.7345	19.0772
Root MSE		0.1715	0.1375
Mean		56.67	44.43

*, ** Significant at P <0.05 and P <0.01, respectively; ns: not significant (P > 0.05).

Table 2 Percentage of germinated grains in genotypes exposed to artificial rain (nebulizer) in Experiment 1

Genotypes	Germinated grains (%) Year 1	Year 2	Year 3	Mean
Alsen	11.50^{b}	68.50^{a}	16.50^{c}	32.17^{b}
BRS 208	56.00^{a}	90.50^{a}	45.00^{b}	63.83^{a}
BRS 210	47.50^{a}	90.00^{a}	80.00^{a}	72.50^{a}
BRS 220	35.00^{a}	96.00^{a}	91.00^{a}	74.00^{a}
BRS Guabijú	53.50^{a}	95.50^{a}	72.00^{a}	73.67^{a}
CD 104	41.50^{a}	89.50^{a}	84.00^{a}	71.67^{a}
CD 108	35.50^{a}	96.50^{a}	91.50^{a}	74.50^{a}
CD 114	7.50^{b}	66.00^{a}	13.00^{c}	28.83^{b}
Chirya 7	55.00^{a}	97.00^{a}	50.50^{b}	67.50^{a}
Grandin*2/RL 4137	14.50^{b}	30.00^{b}	8.50^{c}	17.67^{c}
IPR 144	37.50^{a}	93.50^{a}	78.00^{a}	69.67^{a}
IPR 85	12.00^{b}	85.50^{a}	73.00^{a}	56.83^{a}
Milan/3/Attilla//Fang 69/CIMMYT 3	22.00^{b}	69.50^{a}	29.00^{c}	40.17^{b}
ND 674	3.00^{b}	23.00^{b}	15.50^{c}	13.83^{c}
BRS Pardela	58.00^{a}	95.00^{a}	60.00^{a}	71.00^{a}
SW89-5124*2/Fasan	56.50^{a}	98.00^{a}	82.00^{a}	78.83^{a}
Mean	34.16	80.25	55.59	56.67

*Means followed vertically by the same letters represent statistical homogeneity by the Scott-Knott test (P ≤ 0.05).

germination test (Franco et al. 2009; Gavazza et al. 2012) or sprouting scores to exclude the most susceptible lines (MacMaster and Derera 1976; Humphreys and Noll 2002; Franco et al. 2009). The effectiveness of the percentage of germinated grains was recently confirmed by Okuyama et al. (2020), who reported its high negative correlation with FN (r = −0.91).

Considering the lack of significance in the year × genotype interaction (P > 0.05) for germination percentage in Experiment 1, it is fair to assume that all genotypes showed stable behavior over the years. The mean germination percentage of the genotypes was compared by the Scott-Knott test (P ≤ 0.05). The genotypes ND 674 and Grandin*2/RL4137 showed the lowest percentage of grain

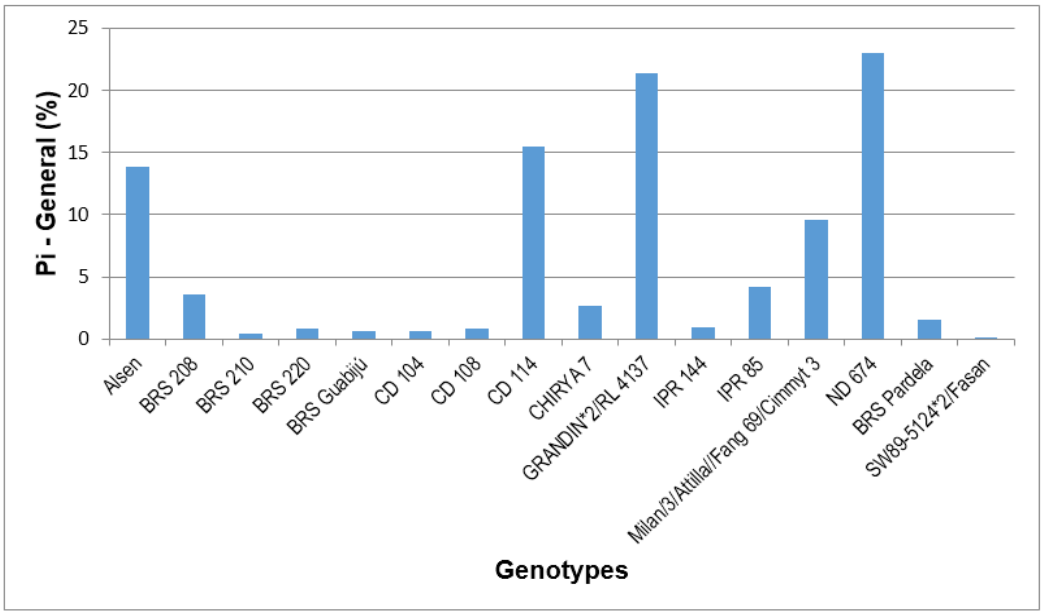

Figure 1 Pi values (estimate of adaptability and stability) of genotypes tested in Experiment 1. The Lowest Pi values correspond to genotypes with the highest percentage of germinated grains (Lin and Binns 1988).

germination and thus can be considered the most tolerant to PHS. The genotypes CD 114, Alsen, and Milan/3/Attila//Fang 69/ CIMMYT 3 were classified as moderately tolerant, whereas IPR 85, BRS 208, Chirya 7, IPR 144, BRS Pardela, CD 104, BRS 220, BRS Guabiju, BRS 220, CD 108, and SW89-5124*2/Fasan were classified as susceptible (Table 2).

The adaptability and stability parameter (Pi) of cultivars was also estimated in Experiment 1 using the Lin and Binns (1988) model, where lower values of Pi represent better model performance. In other words, the genotype with the highest percentage of sprouted grains was the most susceptible to PHS. In addition, the genotypes ND 674 and Grandin*2/RL 4137 exhibited the highest tolerance to PHS, as indicated by the lower percentage of germinated grains. In another group, the genotypes CD 114, Alsen, and Milan/3/Attila//Fang69/CIMMYT3were considered moderately tolerant, and IPR 85, BRS 208, and Chirya 7 were moderately susceptible. The genotypes with the lowest Pi values were IPR 144, BRS Pardela, CD 104, BRS 210, BRS Guabijú, BRS 220, CD 108, and SW 89-5124*2/Fasan; these genotypes resulted in the highest values of sprouted grains and were, therefore, most susceptible to PHS (Figure 1).

In Experiment 2, three years of evaluation by the Scott-Knott test ($P \leq 0.05$) showed that Frontana and Grandin were the most tolerant to PHS, whereas BRS 177, Rubi, and IAC 5-Maringá were moderately tolerant. The cultivars OR 1, Onix, Avante, CD 105, CEP 24, CD 116, and IAPAR 17-Caeté were moderately susceptible, and Supera, Alcover, and BR 18-Terena were classified as susceptible (Table 3).

The Lin and Binns (1988) model in Experiment 2 showed that the Frontana and Grandin genotypes had the highest Pi values, thereby representing the highest tolerance to PHS. In this group, genotypes RL 4137, BRS 177, Rubi, IAC 5-Maringá, OR 1, Onix, and Avantewere considered moderately tolerant. Other genotypes, such as CD 105, CEP 24, CD 116, and IAPAR 17-Caeté, were classified as moderately susceptible, whereas Supera, Alcover, and BR 18-Terena, with the lowest Pi values, were susceptible to PHS (Figure 2).

Considering both experiments together, the most tolerant genotypes to PHS were ND 674 and Grandin*2/RL 4137 (Experiment 1), with 13.8 and 17.7% of germinated grains, and Grandin and Frontana (Experiment 2), with 8.8 and 19.8% of germinated grains, respectively. Comparing the PHS data from these experiments with an earlier study by Okuyama et al. (2020), it can be surmised that all genotypes with seed germination above 46% would have FN values of less than 200s, between 32 and 46% would associate with FN values in the range of 200 to 250s, and between 19 and 32% would have FN values in the range of 250 to 300 s. The most tolerant genotypes, representing seed germination below 19%, would all be associated with high FN values over 300s. Based on this comparison, we can infer that even after 48 h under artificial rain conditions, the genotypes ND 674, Grandin*2/RL 4137, and Grandin had FN values higher than 300 s and Frontanahad FN values between 250 and 300s.

The genealogy and characteristics of PHS-tolerant genotypes (ND 674 and Grandin*2/RL 4137) and moderately tolerant genotypes (CD 114, Alsen, and Milan/3/Attila//Fang 69/CIMMYT 3) in

Table 3 Percentage of germinated grains of genotypes exposed to artificial rain (nebulizer) in Experiment 2

Genotypes	Germinated grains (%)			
	Year 1	Year 2	Year 3	Mean
Alcover	83.00^{Aa*}	95.00^{Aa}	58.50^{Ba}	78.83
Avante	38.50^{Ac}	49.50^{Ab}	36.00^{Ab}	41.33
BR 18-Terena	87.00^{Aa}	94.50^{Aa}	74.50^{Aa}	85.33
BRS 177	5.00^{Bc}	18.50^{Bc}	53.50^{Aa}	25.67
CD 105	18.50^{Cc}	83.00^{Aa}	54.50^{Ba}	52.00
CD 116	73.00^{Aa}	83.00^{Aa}	33.50^{Bb}	63.17
CE P24	47.50^{Ab}	54.50^{Ab}	66.50^{Aa}	56.17
Frontana	29.00^{Ac}	23.50^{Ac}	7.00^{Ac}	19.83
Grandin	3.00^{Ac}	20.50^{Ac}	3.00^{Ac}	8.83
IAC 5-Maringá	14.50^{Ac}	30.50^{Ac}	43.50^{Aa}	29.50
IAPAR 17-Caeté	33.50^{Cc}	93.00^{Aa}	68.50^{Ba}	65.00
Ônix	19.50^{Ac}	42.00^{Ab}	34.50^{Ab}	32.00
OR 1	12.00^{Bc}	58.50^{Ab}	25.00^{Bb}	31.83
RL 4137	9.50^{Bc}	42.00^{Ab}	20.50^{Bb}	24.00
Rubi	25.50^{Ac}	27.50^{Ac}	25.00^{Ab}	26.00
Supera	68.00^{Aa}	82.00^{Aa}	64.00^{Aa}	71.33
Mean	35.44	56.09	41.75	44.43

*Means followed by the same uppercase letters horizontally or lowercase letters vertically indicate a statistically homogeneous group by the Scott-Knott test ($P \leq 0.05$).

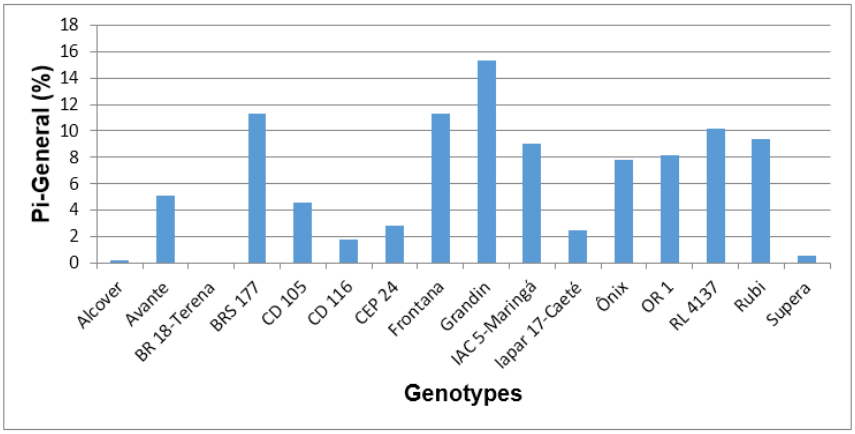

Figure 2 Pi values (estimate of adaptability and genotype stability) of genotypes tested in Experiment 2. The Lowest Pi values were those with the highest percentage of germinated grains (Lin and Binns 1988).

Experiment 1 are presented in Table 4. RL 4137, which is considered a source of PHS resistance (DePauw et al. 2009; Rasul et al. 2012; Liton et al. 2021), probably derives its resistance from multiple sources in its pedigree, including Frontana, Thatcher, Kenya farmer, and RL 2265 (Exchange/McMurachy).

The genotypes Frontana and Grandin were also classified as tolerant to PHS in Experiment 2, whereas RL 4137, Avante, BRS 177, IAC 5-Maringá, Ônix, and Rubi were classified as moderately tolerant. The genealogies and characteristics of the genotypes are presented in Table 5.

Table 4 Genealogy and characteristics of PHS superior genotypes in Experiment 1

Genotype	PHS in Experiment 1	Genealogy	Characteristics
ND 674	Tolerant	Grandin (PI 531005)*2/Glupro (Frohberg et al. 2006)	High and stable bread quality characteristics (Andrade et al. 2001; Singh et al. 2006; Mergoum et al. 2008)
Grandin*2/RL 4137	Tolerant	Grandin*2/RL 4137 (Frontana//RL2265/2*Redman/3/Thatcher *6/ Kenya Farmer) (Martynov and Dobrotvorskaya 2016)	The parent line RL 4137, in the cross, is considered a source of PHS resistance (DePauw et al. 2009; Rasul et al. 2012; Liton et al. 2021)
CD 114	Moderately tolerant	PF 89232 / OC 938 (Souza and Caierão 2014)	Classified as moderately susceptible to PHS (Reunião 2007)
Alsen	Moderately tolerant	ND674//ND2710 (PI 633976)/ND688 (Frohberg et al. 2006).	Resistance to *Fusarium* Head Blight and leaf rust caused by *Puccinia triticina* (Oelke and Kolmer 2005)
Milan/3/Attila//Fang 69/CIMMYT 3	Moderately tolerant	CIMMYT advanced line	Resistance to several isolates of Pyriculariaoryzae (Marangoni et al. 2013)

Table 5 Genealogy and characteristics of PHS superior genotypes in Experiment 2

Genotype	PHS in Experiment 2	Genealogy	Characteristics
Frontana	Tolerant	Fronteira/Mentana (Souza and Caierão 2014)	Tolerant to PHS (Andreoli et al. 2006; Czarnecki 1986; Souza 2001; Nörnberg et al. 2015a), source of resistance against leaf and stripe rust (Chaves et al. 2020) and Fusarium Head Blight (Zhu et al. 2019)
Grandin	Tolerant	Len/Butte*2/ND507/3/ND593 (Dagou and Richard 2016)	High grinding requirements and cooking quality (Mergoum et al. 2006), adult plant resistance to leaf rust disease based on Lr13 and Lr34 genes (Liu and Kolmer 1997) as well as used in multiple crosses (Mergoum et al. 2005; Dagou and Richard 2016; Zhao et al. 2018; Thambugala et al. 2021)
RL 4137	Moderately tolerant	Frontana//RL2265/2*Redman/3/Thatcher *6/ Kenya Farmer (Martynov and Dobrotvorskaya 2016)	Consistently high levels of PHS resistance across years and environments (DePauw et al. 2009; Martynov and Dobrotvorskaya 2016)
Avante	Moderately tolerant	PF89232/2*OR1 (Souza and Caierão 2014)	Moderately resistant to PHS and a moderate level of resistance to foliar blights (Reunião 2007)
BRS 177	Moderately tolerant	PF83899/PF813//F27141 (Caierão et al. 2014)	Superior performance to PHS (Andreoli et al. 2006), moderate level of resistance to foliar leaf blights, and soil-borne mosaic virus (Reunião 2007)
IAC 5-Maringá	Moderately tolerant	Frontana/Kenya 58//Ponta Grossa 1 (Souza and Caierão 2014)	Adapted to the warmer regions of Brazil (Souza and Caierão 2014)
Ônix	Moderately tolerant	CEP 24/Rubi "S" (Souza and Caierão 2014)	Classified as resistant/moderately tolerant for PHS (Reunião 2007). Widely adopted in Brazil and Argentina (Souza and Caierão 2014)
Rubi	Moderately tolerant	Embrapa 27/Klein H3450 C3131 (Souza and Caierão 2014)	Moderate level of tolerance to PHS (Reunião 2007) and source of resistance against Soil Borne Mosaic Virus in Brazil

Some genotypes such as ‘AC Majestic', 'AC Domain', and 'Red RL 4137' (Rasul et al. 2012), have the potential to be used as a parent to incorporate PHS resistance in a breeding program. However, in the absence of locally adapted or high-yielding parents with tolerance to PHS, another strategy suggested by Andreoli et al. (2006) emphasizes on the pre-breeding effort to transfer major genes from Frontana or obtained lines, such as BRS 177 or RL 4137, into modern breeding lines. Our results confirm the findings of these authors that the genotypes Frontana, RL 4137, and BRS 177 are good options for combining PHS resistance with locally or regionally adapted modern breeding lines.

Additional studies (data not shown) have demonstrated an increased level of PHS tolerance in newer lines derived from ND 674, Grandin*2/RL 4137, Grandin, and Frontana. Beyond PHS, Nörnberg et al. (2015b) reported that TBIO Mestre and TBIO Alvorada combination with Fundacep Cristalino led to the

identification of many superior genotypes exhibiting PHS tolerance and high grain yield.

According to Singh et al. (2021), back-cross breeding can be effectively applied for the introgression of identified major quantitative trait loci (QTLs) for PHS tolerance. The importance of identifying QTLs with the potential to enhance PHS resistance in spring wheat has been highlighted by Liton et al. (2021). In their study, a combination of RL 4137 carrying three QTLs on chromosomes 4A, 6B, and 6D and 'Roblin' carrying a new QTL on 1D increased resistance to PHS in the Roblin/RL 4137 population.

Despite advances in the molecular marker-assisted selection, many breeding programs may not be able to use these tools to develop PHS-tolerant genotypes. On the other hand, the identification and transfer of PHS-related traits from genotypes such as Frontana, Grandin, ND 674, and RL 4137 into locally adapted and high-yielding germplasms are successful. Therefore, we believe that the utilization of tolerant and moderately tolerant germplasms identified from this study and previous studies is key to global wheat-breeding programs affected by PHS. Besides improving sprouting tolerance, these progenitors will help enhance end-use quality, disease resistance, and other traits required by the market.

Conclusions

This study confirms that grain germination percentage under controlled spike wetting allows the classification of genotypes for PHS tolerance. Genotypes such as Frontana, Grandin, ND 674, and Grandin*2/RL 4137 were classified as tolerant to PHS, while Alsen, Avante, BRS 177, CD 114, IAC 5-Maringá, OR 1, Onix, RL 4137, Rubi, and Milan/3/Attila//Fang 69/ CIMMYT 3 were classified as moderately tolerant. Tolerance to PHS can be further enhanced by combining tolerant and moderately tolerant genotypes and/or incorporating the tolerance characteristic of these genotypes into other agronomically desirable germplasms. PHS tolerance should be further combined with high grain yield, disease resistance, good end-use quality, and other traits of interest in a region.

Conflict of interest

The authors declare that there is no conflict of interest.

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Effect of different growing media on selected growth performance parameters of *Raphanus pugioniformis* and *Raphanus raphanistrum*

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ABSTRACT

Raphanus raphanistrum and *R. pugioniformis* (*Brassicaceae*) are wild radishes, native to the Eastern Mediterranean region. This study aimed to evaluate the effect of growing soil media (perlite, sand, and terra rossa) on the growth performance of two *Raphanus* species. For this, seeds of the selected species were germinated and seedlings were transferred to plastic cylinders, filled with growing soil media. At harvest, various growth parameters including shoot length, shoot fresh weight, shoot dry weight, root length, root fresh weight, and root dry weight were determined. Root and shoot fresh and dry weight, before and after oven dry for 24 h at 70 °C was measured. Results of the study revealed statistically significant differences (P value ≤ 0.05) among the various studied growth parameters for the selected *Raphanus* species and are affected by different growing media including types of soil and growing time (days after potting from 33 to 78). After 33 days of potting, the average shoot length for *R. pugioniformis* was found 6.6, 8.0, and 8.6 cm in terra rossa, sand, and perlite growing media respectively. On the other hand, the fresh (0.8, 1.6, and 2.5g) and dry (0.25, 0.48, and 0.72g) shoot weight for *R. pugioniformis* was reported in terra rossa, sand, and perlite soil media respectively. From the results of the study, it can be concluded that among the tested growing media, perlite growing medium is the best medium for the growth of both studied *Raphanus* species. This study demonstrated that the three studied growing media affected all the growth performance parameters of both *Raphanus pugioniformis* and *Raphanus raphanistrum* differently.

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1 Introduction

Weedy radish belongs to the family *Brassicaceae* genus *Raphanus* and is a native population in the Mediterranean region (Conner et al. 2011; Al-Shehbaz 2012; Conner et al. 2014; Klinger et al. 1991). *Raphanus sativus* comprise four major types including two fruit crops (Oilseed and edible pod Rat-tail) and two root crops (European radish and Asian daikon) but the relationships among these species are not well known (Ziffer-Berger et al. 2020). According to the Campbell et al., (2006) cDNA sequence of *Raphanus* taxa revealed monophyly of native and weedy *R. raphanistrum.*

Under many ecological and evolutionary studies, weedy radish has been widely used as a genomic resource for both origins and adaptation studies of agricultural weeds (Irwin et al. 2003; Moghe et al. 2014). More populations of *R. raphanistrum* are found in sandy soils along the Mediterranean coastal plain in Palestine, while the *R. pugioniformis*, covers heterogeneous habitats along a gradient of environmental conditions (Ziffer-Berger et al. 2015). It is assumed that these two species differ in the structure of their population genetic, i.e., short-distance dispersal of *R. pugioniformis* contributed to inter-populations phenotypic differentiation, whereas long-distance dispersal reduces the possibility of ecotypic differentiation in *R. raphanistrum*. The various dispersal strategies of wild radishes and their consequence on their broad-scale geographical and small-scale spatial distribution, allow assessing the impact of environmental conditions on the phenotypic variation of ecologically important life history traits in annual plants, at the inter-species as well as intra-species levels (Waitz et al. 2021).

Different studies evaluated the effect of different growing media on the growth performance of different plants (Abbadi 2017a; Abbadi 2017b; Abbadi et al. 2017; Abbadi et al. 2019). Other investigators also confirmed the importance of the selection of an appropriate growth medium for better growth at different stages (Špulák and Hacurová 2021; Madhavi et al. 2021). A growing media consist of three components i.e. solid (33-50%), water and gases (50-70%), 12% oxygen, and dissolved nutrients. This combination was found good for vigorous growth and a stronger root system (Gil et al. 2012). Špulák and Hacurová (2021) investigated the influence of growing medium composition on pine and birch seedling response during the period of simulated spring drought. Results of this study showed that the birch responded more intensively to the fertilizer concentration than to the clinoptilolite admixture and was more vulnerable to drought damage. Similarly, Abid et al. (2017) studied the growth response of *Dracaena reflexa* to various growing substrates and reported that the combination of silt and farm yard manure is useful growing media for *D. reflexa* as compared to others. Sardoei and Shahdadneghad (2015) studied the effect of seven different growing media on the growth and development of zinnia (*Zinnia elegans*) under different climatic conditions. Similarly, Popescu and Popescu (2015) studied the effects of different potting growing media on photosynthetic capacity, leaf area, and flowering potential on two ornamental plants (*Petunia grandiflora* and *Nicotiana alata* Link & Otto and confirmed the effects of growing media on the studied parameters. Younis et al. (2015) also studied the influence of various growing substrates on the growth and flowering of the potted miniature rose cultivar "Baby Boomer" where four different growing substrates with variable composition and combinations were compared using garden soil as a control. Results of this study indicated that leaf compost exhibited overall better performance as compared to other media for plant height, number of leaves per plant, number and length of branches per plant, and the number and diameter of flowers. Similarly, Madhavi et al. (2021) studied the influence of different growing media on the growth and development of strawberry plants. The results of this study demonstrated that bio plus compost with synthetic nutrients act as a better source for the growth and production of strawberries in the greenhouse.

Therefore, this study aimed to evaluate the effect of different growing media (sand, perlite, and terra rossa) on selected growth performance parameters (shoot length, shoot fresh weight, shoot dry weight, root length, root fresh weight, and root dry weight) of two *Raphanus sps* namely *R. pugioniformis* and *R. raphanistrum.*

2 Materials and Methods

2.1 Experimental design

Ten seeds of each one of 10 representing populations (a total of 100 seeds) were collected and surface sterilized by 6% (v/v) hypochlorite solution for 1 min, followed by the many time washing with sterile distilled water. These surface sterilized seeds of both selected species were inoculated on the moistened Whatman filter paper No. 1 set in 9cm Petri dishes and placed in sufficient daylight. After 2 to 3 days of germination, seedlings were transferred to germination trays (when rootlets are about 2-3 cm long) having potting soil mixture as substrate. When they develop 4-5 true leaves, the seedlings were transplanted to the final cylindrical pots (20cm diameter x 150 cm height) with different potting soil types (perlite, sand, and terra rosa) separately. The experiment was conducted in a greenhouse in the nursery of Thinnaba cooperative in Tulkarm in November 2019 during the natural growing season. During the experimental period, the average day and night temperatures ranged from 20–22°C and 18–20°C with midday peak temperatures between 23 and 28° C in July, and relative humidity was reported between 40 -80%. Plants received sufficient water and nutrient by irrigating them with a Hoagland solution (0.01x) nutrient solution as per plant requirement. The pots of different soil treatments were arranged in

a completely randomized design. At the flowering stage (50% flowering), plants were harvested and roots were washed for soil particle separation and morphological observations.

2.2 Root and shoot measurements

Root structure was evaluated two times, first at the potting and second at the flowering stage. For this, five plants of each population were harvested, roots were washed and observation related to the root morphology and leaves length was recorded. At harvest, the plants were washed with water, and the vegetative growth length was measured with a measuring scale. Plant biomass (shoot and root) was also determined as root and shoot fresh and dry weight was measured by weighing them before and after oven drying for 24 h at 70 °C, respectively.

2.3 Statistical analysis

All statistical analyses were performed using SAS (SAS Institute Inc., Cary, USA, Release 8.02, 2001). Mean comparisons were carried out using the GLM procedure, treating main factors separately using one-way analysis of variance (ANOVA). The Bonferroni procedure was employed with multiple t-tests to maintain an experiment-wise of 5%. Differences were considered significant if P values were lower than 0.05.

3 Results

3.1 Effect of growing media on shoot length

In this study, the shoot length of *R. pugioniformis* and *R. raphanistrum* species was measured in three different growing media (perlite, sandy, and terra rossa soil). The shoot length (in cm) was measured with varying day's intervals (33 to 78 days) after potting. As presented in Figure 1A, pots containing perlite as growing media have a shoot length range between 8.6 - 21cm, and 11.7 - 32.6cm after 33 to 78 days of potting for *R. pugioniformis* and *R. raphanistrum*, respectively. Similarly, the shoot length of *R. pugioniformis* and *R. raphanistrum* was reported between 8 - 15cm, and 9.2 - 12.8cm for sandy soil as growing media (Figure 1B) and 8 - 15cm, and 7.3 - 29.4 cm for terra rossa soil as growing media (Figure 1C) after 33 to 78 days of potting respectively. This implies that shoot length increases as days after potting increase for both *Raphanus* species. Statistical analysis showed that shoot length for *R. raphanistrum* is significantly higher than that for *R. pugioniformis* after 33, 48, 63, and 78 days of potting in perlite and terra rossa soil growing media, while in the case of sandy soil shoot length was reported significantly higher for *R. raphanistrum* after 33 and 48 days of potting and the reverse was obtained at 63 and 78 days.

Further, figure 1D and 1E summarize the effect of growing media on the shoot length for both *Raphanus* species. Results presented in figure 1D show the *R. pugioniformis* shoot length in selected three-growing media. After 33 days of potting, the average shoot length was reported 6.6, 8.0, and 8.6cm in terra rossa, sand, and perlite growing media, respectively. Similar trends were observed after 48 and 63 days of potting. Further, it was reported that amongst the selected three growing media, the highest *R. pugioniformis* shoot length was reported in the plant grown in perlite growing media and it was followed by the sandy and terra rossa growing media. Further, results presented in figure 1E show the *R. raphanistrum* shoot length in selected three growing media. After 33 days of potting, the shoot length was reported as 7.3, 9.2, and 11.7cm using terra rossa, sand, and perlite growing media, respectively, and significant differences were reported in these three-growing media. A similar trend was observed after 48 days of potting for *R. raphanistrum* where shoot length was significantly different and increased in the order of terra rossa, sand, and perlite respectively. On the other hand, after 63, and 78 days of potting, the trend was found different and the highest shoot length was reported in perlite, followed by terra rossa and sand-growing media (Figure 1E).

3.2 Effect of growing media on shoot fresh weight

The effect of different growing media (perlite, sandy, and terra rossa soil) on *R. pugioniformis* and *R. raphanistrum* shoot fresh weight was studied and found significant differences with varying days after potting (33 to 78 days) and type of growing media (Figure 2). As presented in Figure 2A using perlite as growing media, the range of shoot fresh weight was found to be from 2.5 - 16.7g and 5.7 - 16g as 33 to 78 days after potting for *R. pugioniformis* and *R. raphanistrum*, respectively. While in the case of sandy soil potting medium, the range of shoot fresh weight was found 1.6 - 5g and 2.5 - 7.4g for *R. pugioniformis* and *R. raphanistrum* after 33 to 78 days after potting respectively (Figure 2B). When terra rossa soil was used as growing media, the range of shoot fresh weight was found from 0.8 - 25.4g and 1.2 - 16.2g for *R. pugioniformis* and *R. raphanistrum*, after 33 to 78 days of potting respectively (Figure 2C). These results imply that shoot fresh weight increases as days after potting increase for both *Raphanus* species. Statistical analysis of data suggested that in perlite growing medium, after 33 and 48 days of potting shoot fresh weight for *R. raphanistrum* is significantly higher than that for *R.pugioniformis* while the reverse was observed for 63, and 78 days of potting. While in the case of sandy soil, significantly higher fresh shoot weight was reported for *R. raphanistrum* than that for *R. pugioniformis* for four periods of days after potting studied (33, 48, 63, and 78 days). Statistical analysis of terra rossa soil growing media showed that the shoot fresh weight for *R. raphanistrum* is significantly higher than the *R. pugioniformis* for 3 periods of days after potting studied (33, 48, 63) while a reverse trend was observed after 78 days after potting.

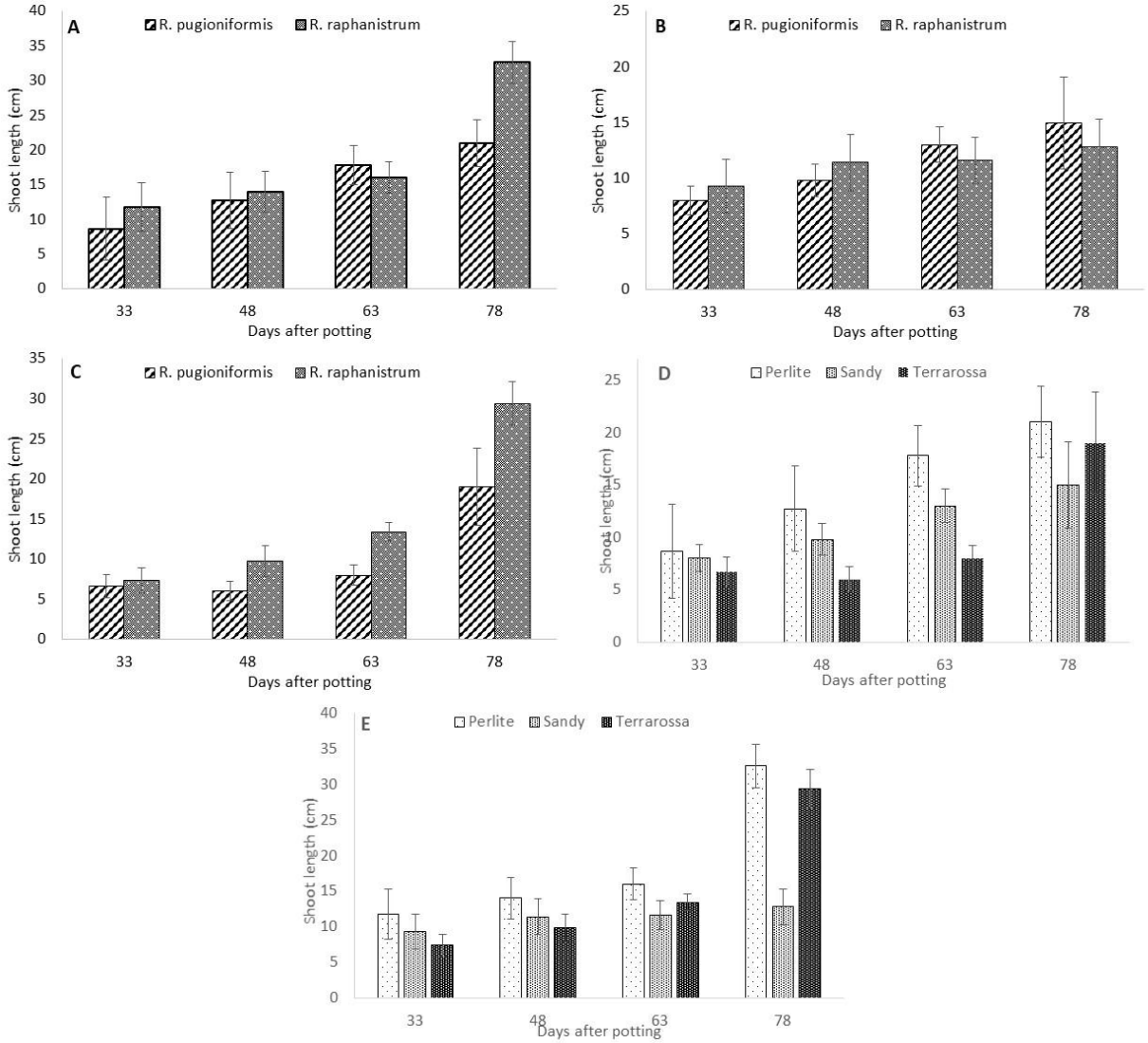

Figure 1 Effect of three growing media (perlite, sandy, and terra rossa soil) on shoot length (cm) of *R. pugioniformis* and *R. raphanistrum*; A: perlite growing media, B: sandy soil growing media, C: terra rossa growing media, D: shoot length of *R. pugioniformis* in all three growing media, and E*:* shoot length of *R. raphanistrum* in all three growing media

Figures 2D and 2E summarize the effect of growing media on the shoot fresh weight for both *Raphanus* species. Results presented in figure 2D show the *R. pugioniformis* shoot fresh weight for the used three-growing media. After 33 days of potting, the shoot fresh weight was found 0.8, 1.6, and 2.5g using terra rossa, sand, and perlite growing media, respectively. The same trends were observed after 48 days of potting while different trends were observed after 63 and 78 days of potting (Figure 2D). After 63 days, shoot fresh weight was not significantly different in terra rossa and sand as growing media which is significantly lower than shoot fresh weight when the growing media is perlite. On the other hand, after 78 days of potting the trend was found to be different where the shoot fresh weight was highest when terra rossa is the growing media followed by perlite, and the least was found for sand as the growing media (Figure 2D).

Figure 2E shows the shoot fresh weight for *R. raphanistrum* using the three-growing media. After 33 days of potting, the shoot fresh weight was found 1.2, 2.5, and 5.7g using terra rossa, sand, and perlite growing media, respectively. The same trend was observed for shoot fresh weight after 48 days of potting for *R. raphanistrum*. On the other hand, after 63 days of potting, the trend was found different where the shoot fresh weight was recorded highest when perlite is growing medium (Figure 2E) and after 78 days of potting, the highest shoot fresh weight was for both terra rossa, and perlite growing media, and these two are significantly higher than that of the sandy growing media.

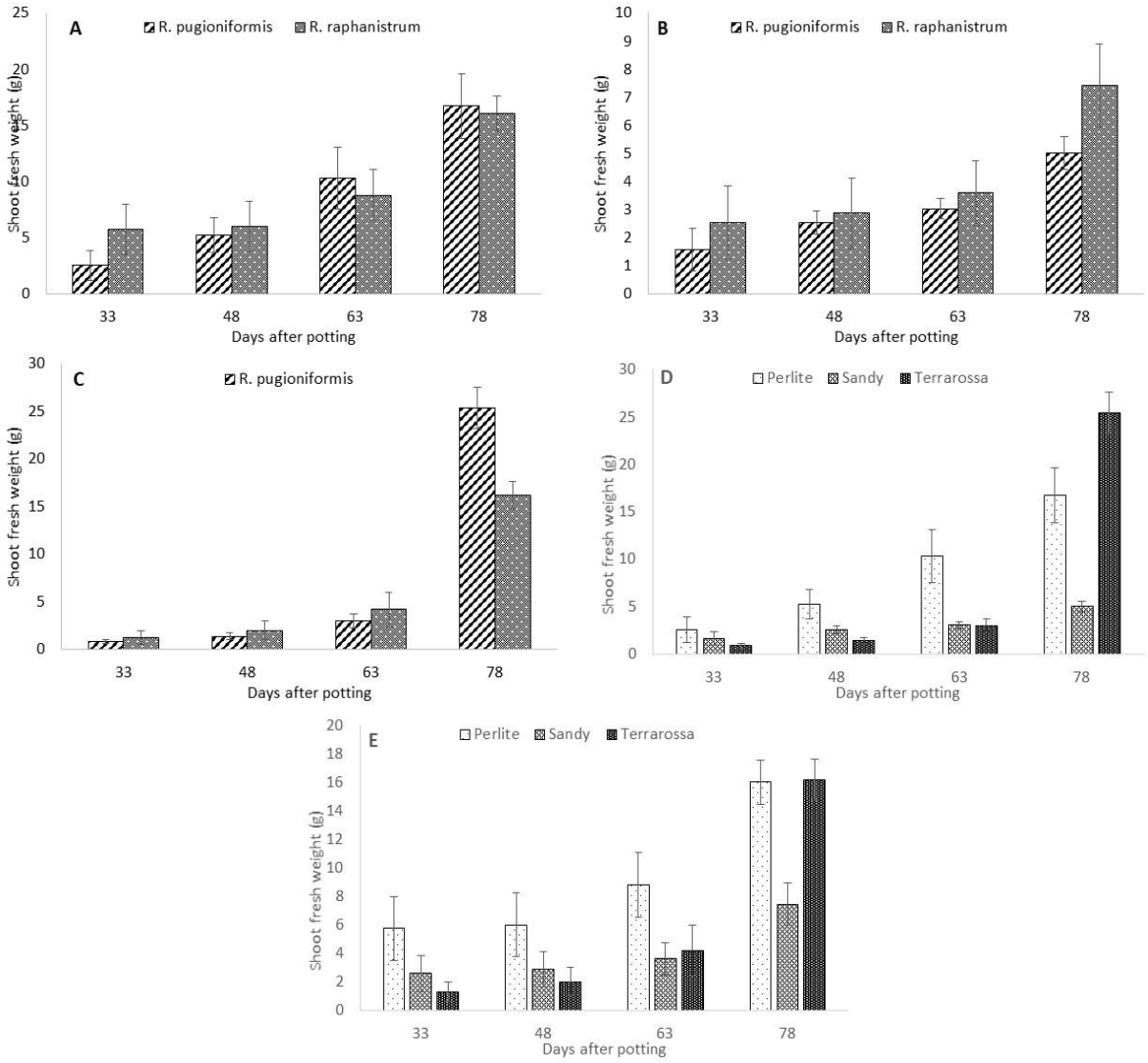

Figure 2 Effect of different growing media on shoot fresh weight (g) of *R. pugioniformis* and *R. raphanistrum*, A: perlite growing media, B: sandy soil growing media, C: terra rossa growing media, D: shoot fresh weight of *R. pugioniformis* in all three growing media, and E*:* shoot fresh weight of *R. raphanistrum* in all three growing media

3.3 Effect of growing media on shoot dry weight

The effect of different growing media on the shoot dry weight of *R. pugioniformis* and *R. raphanistrum* species was studied after 33 to 78 days of potting (Figure 3). As it is obvious from Figure 3A using perlite as growing media, the range of shoot dry weight was found from 0.72 - 4.7 g, and 1.6 - 4.0 g for *R. pugioniformis* and *R. raphanistrum* respectively as the days after potting increases from 33 to 78. When sandy soil was used as growing media, the range of shoot dry weight was recorded as 0.48 - 1.6g and 0.85 - 2.5g as the days after potting increased from 33 to 78 for *R. pugioniformis* and *R. raphanistrum*, respectively (Figure 3B). Regarding the shoot dry weight of *R. pugioniformis* and *R. raphanistrum* species using terra rossa soil as growing media, results showed that the range of shoot dry weight was found from 0.25 - 4.5g and 0.29 - 3.6g for *R. pugioniformis* and *R. raphanistrum* respectively as the days after potting increases from 33 to 78 (Figure 3C). Results of the study also revealed that the shoot dry weight of *R. raphanistrum* is significantly higher than that of *R. pugioniformis* when the growing media is perlite and terra rossa at 33, 48, and 63 days after potting while the reverse was observed for 78 days of potting. While in the case of sandy soil, statistical analysis of data showed that shoot dry weight for *R. raphanistrum* is significantly higher than that for *R. pugioniformis* for 4 periods of days after potting studied (33, 48, 63, and 78 days).

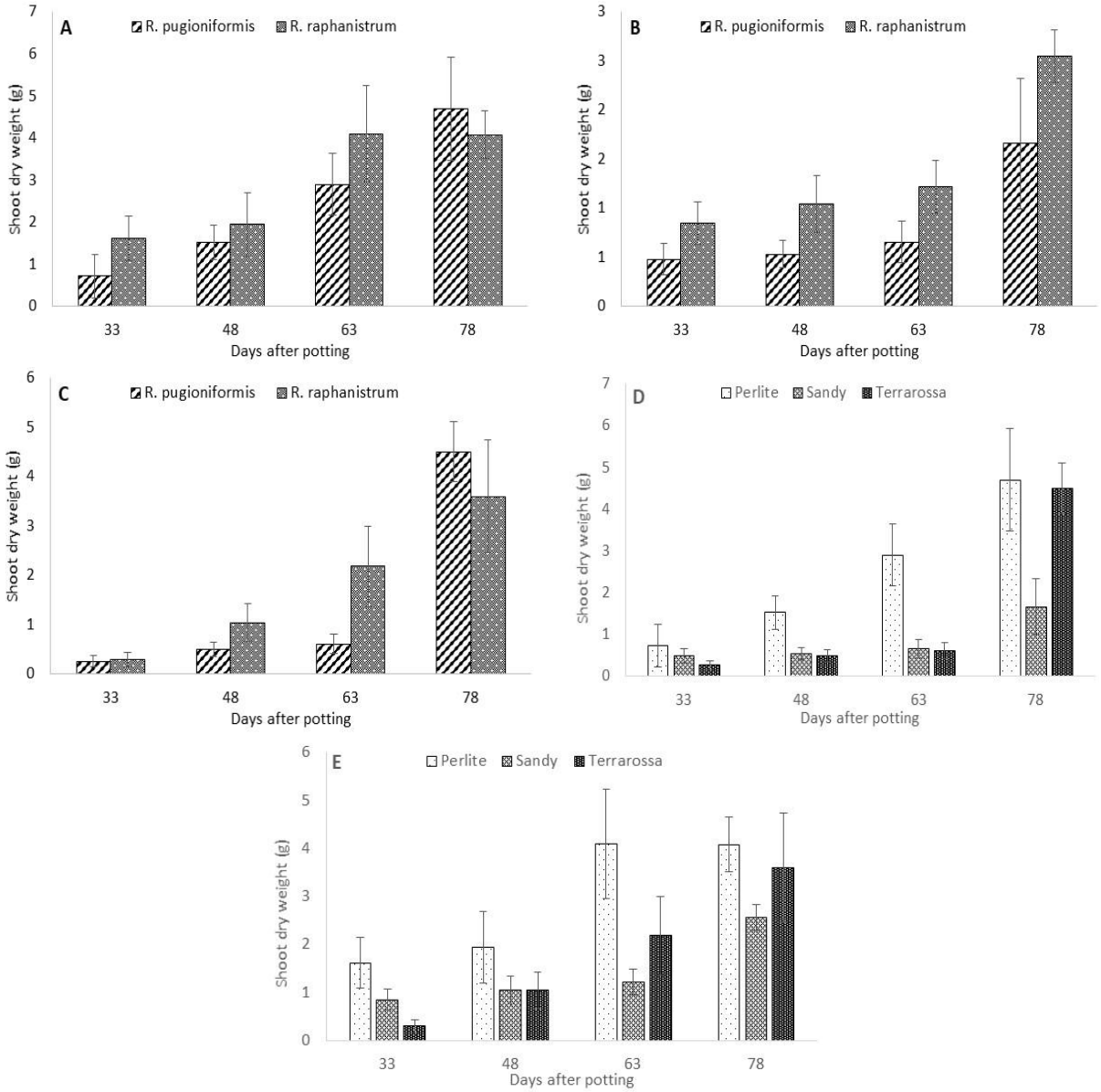

Figure 3 Effect of different growing media on shoot dry weight (g) of *R. pugioniformis* and *R. raphanistrum*, A: perlite growing media, B: sandy soil growing media, C: terra rossa growing media, D: shoot dry weight of *R. pugioniformis* in all three growing media, and E*:* shoot dry weight of *R. raphanistrum* in all three growing media

Further, figure 3D shows the shoot dry weight for *R. pugioniformis* using the three-growing media. After 33 days of potting, the shoot dry weight of *R. pugioniformis* was reported 0.25, 0.48, and 0.72g using terra rossa, sand, and perlite growing media, respectively. Different trends were observed for shoot dry weight after 48, 63, and 78 days (Figure 3D). Figure 3E shows the shoot dry weight for *R. raphanistrum* using the three-growing media. After 33 days of potting, the shoot dry weight was recorded as 0.29, 0.85, and 1.61g using terra rossa, sand, and perlite growing media, respectively. While the different trend was observed for shoot dry weight after 48, 63, and 78 days (Figure 3E).

As it is obvious from figure 3D, Perlite was the best-growing media for *R. pugioniformis* for the 4 different potting periods while terra rossa and sand-growing media have a similar effect for 33, 48, and 63 days. Terra rossa has a higher effect on shoot dry weight compared to sand at 78 days (Figure 3D). Regarding *R. raphanistrum*, Perlite was the best-growing media for the 4 different potting periods while terra rossa and sand growing media have a similar effect at 48 days (Figure 3E). Terra rossa has a higher effect on shoot dry weight compared to sand at 63 and 78 days, while the reverse was observed at 33 days after potting (Figure 3E).

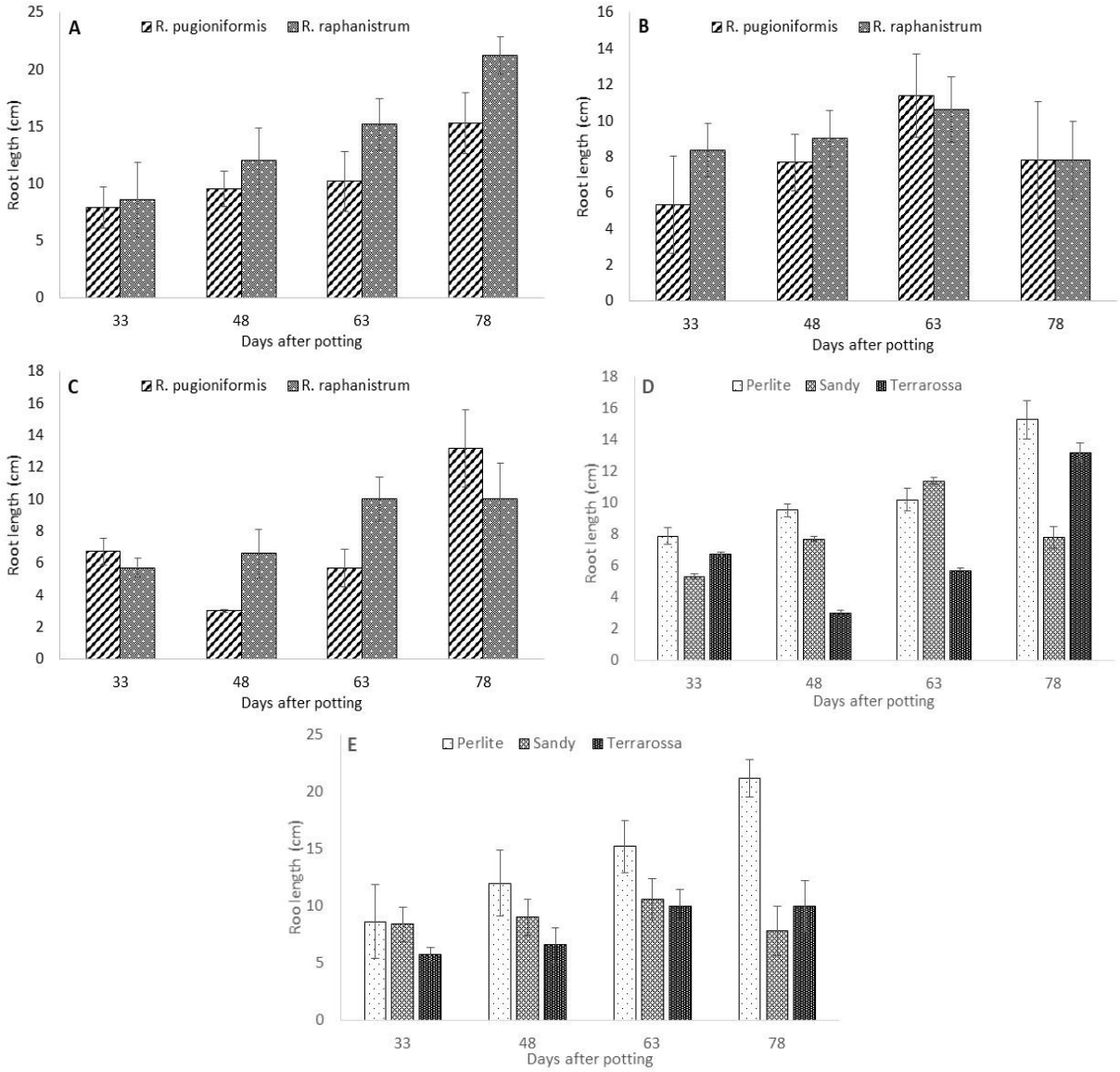

Figure 4 Effect of different growing media on root length (cm) of *R. pugioniformis* and *R. raphanistrum;* A: perlite growing media, B: sandy soil growing media, C: terra rossa growing media, D: root length of *R. pugioniformis* in all three growing media, and E*:* root length of *R. raphanistrum* in all three growing media

3.4 Effect of growing media on root length

The effect of selected growing media on *R. pugioniformis* and *R. raphanistrum* root length was studied after potting from day 33 to 78 days (Figure 4). The range of root length was recorded from 7.9 - 15.3 cm and 8.62 - 21.2 cm for *R. pugioniformis* and *R. raphanistrum* has grown under a pot containing perlite growing media after 33 to 78 days of potting. Using sandy soil as a growing media, the range of root length was recorded from 5.32 - 11.4cm, and 7.8 - 10.6cm for *R. pugioniformis* and *R. raphanistrum*, respectively (figure 4B). While in the case of terra rossa soil, root length was reported 5.72 - 10.0cm, and 3.02 - 13.2cm for 33 to 78 days after potting for *R. pugioniformis* and *R. raphanistrum*, respectively (Figure 4C). This implies that root length increases as days after potting increase for both *Raphanus* species. Statistical analysis showed that root length for *R. raphanistrum* is higher than that for *R. pugioniformis* when the growing media is perlite and sandy soil for the 4 periods of days after potting studied (33, 48, 63, and 78 days), with a significant difference at 63 and 78 days. While in the case of terra rossa soil, statistical analysis of obtained data showed that root length for *R. raphanistrum* is higher than that for *R. pugioniformis* for 48 and 63 days after potting, while the reverse trend was observed after 33 and 78 days after potting but this difference was not significantly different.

Further, amongst the tested growing medium, perlite was found the best growing media in terms of root length for *R. pugioniformis* for the 3 different potting periods (33, 48, and 78 days) with significant differences (figure 4D), while sand growing media have significantly higher effect at 63 days compared to perlite and terra rossa growing media. Regarding *R. raphanistrum*, here also perlite gave the highest root length for 3 potting periods (48, 63, and 78 days) while perlite and sandy growing media have a similar effect at 33 days (Figure 4E).

3.5 Effect of growing media on root fresh weight

The effect of different growing media on the fresh root weight of *R. piriformis* and *R. raphanistrum* species was studied, and it varies with the study time from day 33 to 78 (Figure 5). As shown in figure 5A using perlite as growing media, the range of root fresh weight was recorded from 0.25 - 0.71 g, and 0.82 - 1.10 g for *R. pugioniformis* and *R. raphanistrum*, respectively for various time intervals i.e. 33 to 78 days after potting. This implies that root

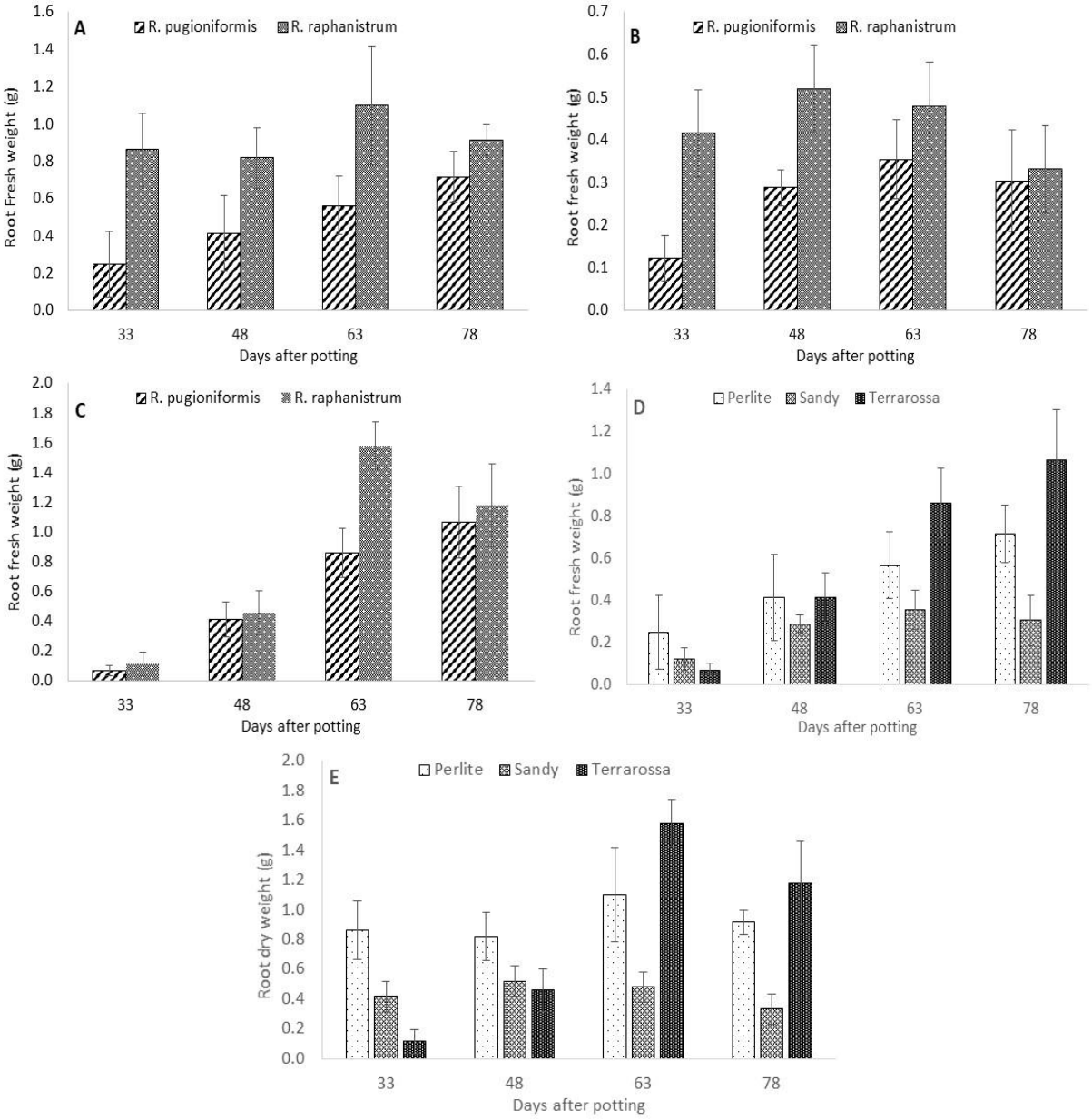

Figure 5 Effect of different growing media on root fresh weight of *R. pugioniformis* and *R. raphanistrum;* A: perlite growing media, B: sandy soil growing media, C: terra rossa growing media, D: root fresh weight of *R. pugioniformis* in all three growing media, and E*:* root fresh weight of *R. raphanistrum* in all three growing media

fresh weight increases as days after potting increased and it was reported highest for *R. raphanistrum* followed by the *R. pugioniformis* grown in perlite grown media and differences in these two are significantly different for all four observation periods (33, 48, 63, and 78 days). Using sandy soil as growing media, the range of root fresh weight was found 0.12 - 0.35g and 0.33 - 0.52g for *R. pugioniformis* and *R. raphanistrum*, respectively and statistical analysis of the obtained data suggested that the root fresh weight for *R. raphanistrum* is higher than that for *R. pugioniformis* for 4 periods of days after potting but only for 33 and 48 days a significant difference was reported (figure 5B). Regarding the root fresh weight of *R. pugioniformis* and *R. raphanistrum* species using terra rossa soil as growing media (Figure 5C), results showed that the range of root fresh weight was found to be from 0.07 - 1.06g and from 0.12 to 1.58g for the days after potting from 33 to 78 for *R. pugioniformis* and *R. raphanistrum*, respectively. Statistical analysis showed that root fresh weight for *R. raphanistrum* is higher than that for *R. pugioniformis* for the 4 periods of days after potting studied, but it showing statistically significant differences only at 63 days.

Among the tested growing media, perlite was reported as the best-growing media in terms of root fresh weight for *R. pugioniformis* at 33 days after potting, while terra rossa growing media have significantly higher root fresh weight at 63 and 78 days compared to perlite and sandy growing media, and perlite and terra rossa gave comparable root fresh weight at 48 days (Figure 5D). Regarding *R. raphanistrum*, perlite gave the highest root fresh weight for two potting periods (33, and 48 days) with significant differences, while terra rossa growing media gave the highest root fresh weight for 63 and 78 days (Figure 5E).

3.6 Effect of growing media on root dry weight

Like other growth parameters, the effect of different growing media on the root dry weight of *R. piriformis* and *R. raphanistrum* species was also reported (Figure 6). Using perlite as growing media, the range of root dry weight was found from 0.1 - 0.21 g and 0.12 - 0.39 g as the days after potting increased from 33 to 78 for *R. pugioniformis* and *R. raphanistrum*, respectively. Similarly, in the case of sandy soil, the root dry weight was recorded from 0.04 to 0.19g and 0.12 - 0.52g for *R. pugioniformis* and *R. raphanistrum*, respectively (Figure 6B), and this was reported from 0.0**3** - 0.24g and 0.03 - 0.88g for *R. pugioniformis* and *R. raphanistrum* grown in terra rossa soil respectively (Figure 6C). These results suggested that root dry weight was higher for *R. raphanistrum* and it was followed by the *R. pugioniformis* for all studied growing media. In the case of perlite, a significant difference was reported between the two *Raphanus* sps., and three growing periods i.e. 33, 63, and 78 days after potting while the reverse was observed at 48 days without significant difference. Like perlite, in the case of sandy soil, the highest root dry weight

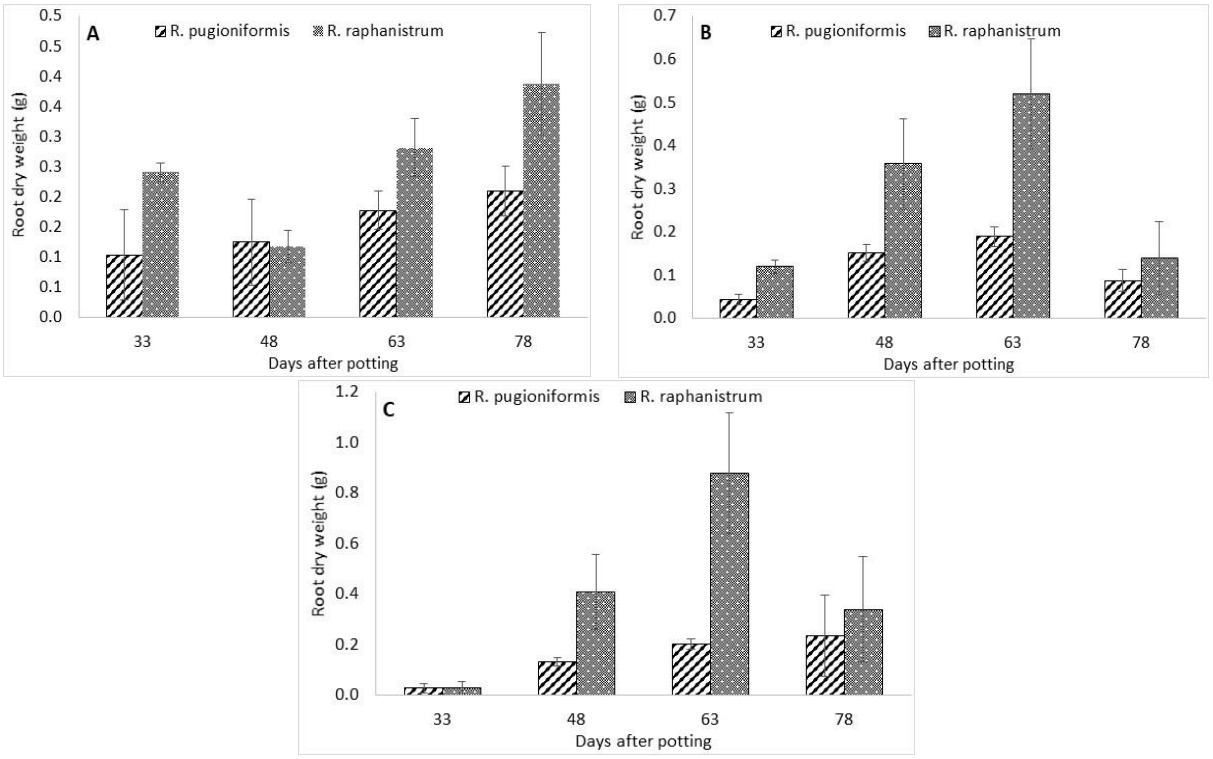

Figure 6 Effect of different growing media on root dry weight (g) of *Raphanus pugioniformis*and*Raphanus raphanistrum;* A: perlite growing media, B: sandy soil growing media, C: terra rossa growing media

was reported for *R. raphanistrum* and it shows a statistically significant difference for 33, 48, and 63 days after potting while the reverse was observed at 78 days. Among the plant grown in terra rossa soil also, *R. raphanistrum* is showing higher root dry mass and it was significantly different from the *R. pugioniformis* for the 3 growing periods (48, 63, and 78 days) while the reverse was observed at 33 days. The individual effects of the soil growing media on root dry weight of *Raphanus* sps., and its association with four-time intervals was not studied.

4 Discussion

The present study determines the efficiency of three potting media (perlite, terra rossa, and sandy soils) on the various growth parameters (shoot and root) of two *Raphanus* species. Terra rossa (Italian for "red soil") is a well-drained, reddish, clayey to silty soil with neutral pH conditions and is typical of the Mediterranean region. Compared to most clay-rich soils, terra rossa has good drainage characteristics (Popescu and Popescu 2015). On the other hand, perlite is a non-organic additive used to aerate the media. Growth parameters like shoot length, shoot fresh and dry weights, root fresh and dry weight, and root length was measured after different periods of potting from 5 to 11 weeks (33, 48, 63, and 78 days). Results of the study revealed that all the studied growth parameters were reported highest for perlite growing media for both *R. pugioniformis* and *R. raphanistrum* and significant differences were reported after 33, 48, and 63 days of potting. In the case of individual *Raphanus* sps., *R. raphanistrum* showed superiority over the *R. pugioniformis* in all studied plant growth parameters. Plant biomass (root and shoot) is frequently used for the calculation of net primary productivity and growth rates of plants and also plays an important role in determining their adaptive responses to stress situations (Cornelissen et al. 2003; Moghe et al. 2014; Umar et al 2019; Gyewali et al. 2020), and considered as an important parameter in growth analysis (Niklas and Enquist 2002). Nutrient absorption by plants from the soil depends on the interactions between plant roots and soil, and it is highly influenced by the nutrient quantity, availability, mobility in soil, and the uptake kinetics of the root system (Lambers et al. 2006). Further, nutrient uptake by plants is also affected by root morphological characteristics (Fageria and Moreira 2011), and the root morphology and root length have been shown higher influence on nutrient uptake efficiency (Jia et al. 2008). The root length is an important root property that affects the contact between soil nutrients and root surface, root distribution in different soils, and transport of nutrients (Barber 1984).

Conclusion

Wild radish (*Raphanus*) is an invasive weed that hurts different land use types and more research has not been done yet on the weed. Therefore, this study was conducted to understand differences in growth patterns in two allopatric and closely related *Raphanus* species i.e. *R. raphanistrum* and *R. pugioniformis* of the East-Mediterranean (Med.) under three different soil types. Results of the study can be concluded that soil type has a significant effect on the selected Raphanus sps., and among the studied three soil types, perlite is the best-growing media in terms of root length and root fresh weight for *R. pugioniformis* and *R. raphanistrum*. Further, the growth parameters of the two *Raphanus sps.*, are different and these are depending on the used soil type, which indicates that *R. raphanistrum* and *R. pugioniformis* have different adaptive strategies in different soil types.

Conflicts of interest and financial disclosures

Authors declare no conflict of interest.

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Assessing the competitive ability of the invader *Senna obtusifolia* with coexisting natives species under different water stress regimes

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Senna obtusifolia

Competitive ability

Invasive species

Sensitivity index

Greenhouse

ABSTRACT

Invasive species tend to pose a threat to ecosystem biodiversity, functioning, and ecosystem service provision. This study was conducted in Burkina Faso to assess the competitiveness of an invasive species *Senna obtusifolia* that is a less palatable legume plant in West African Sahelian rangelands. To address the research hypothesis that the recurrent drought in the Sahel results in *S. obtusifolia* being more competitive in the land invasion, we conducted an interspecific competition involving *S. obtusifolia* and 3 herbaceous species (*Andropogon gayanus*, *Chamaecrista mimosoides*, and *Pennisetum pedicellatum*) in a greenhouse experiment under four water stress regimes using a replacement series design. The height and biomass of each species were measured throughout four months experiment. In the severe water regime, *S. obtusifolia* was the most sensitive to water deficit while the 3 other species were found to be resistant. In addition, in all water regimes, the aggressivity index revealed that *S. obtusifolia* was less competitive than the grass species *A. gayanus* and *P. pedicellatum*. Further, the study discovered that drought in the Sahel made *S. obtusifolia* more vulnerable than the other species. Hence the invasion of Sahelian rangelands by *S. obtusifolia* could be favored by overgrazing that reduces fodder species' dominance and competitiveness. Good management of sahelian rangelands by controlling grazing could help to reduce *S. obtusifolia* invasion and provide more fodder for livestock.

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1 Introduction

Invasive plant species represent a major threat to biodiversity and the ecosystem (Vilà et al. 2011; Shackleton et al. 2019; Bogale and Tolossa 2021). They directly affect native plant communities by causing biodiversity loss, ecosystem change, and reduction in ecosystem services (Wardle et al. 2011; Barros et al. 2020). Previous studies showed that the success of these species depends on several abiotic and biotic factors (Martin and Coetzee 2014; Leal et al. 2022) as well as on biological traits related to their ability to spread such as copious seed production, high germination, survival rates, efficient dispersal and good competitive ability (Wickert et al. 2017; Yu et al. 2018; El-Barougy et al. 2020).

In general, abiotic factors such as water, light, and nutrients for which plants compete in the process of growth and development (Wang et al. 2012; Gao et al. 2021) may also influence the intensity of competition (Yu et al. 2018) when these resources are present in limited quantity between nearby individuals with a common requirement (Bastiani et al. 2016; Zhou et al. 2018). With ongoing climate change impacts, water availability could decrease in severe drought conditions and this may also facilitate the competitive ability and success of invasive species (Diez et al. 2012; Paudel et al. 2018; Barros et al. 2020). For example, in West African Sahel rangeland, the arid climate characterized by severe drought (Issaharou-Matchi et al. 2016) has favoured the proliferation of invasive plant species due to their high physiological performance (Oliveira et al. 2014; Duell et al. 2021). In this situation, the species with functional traits that are consistent with high resource acquisition may overcome the congener and become invasive (Funk et al. 2016; Broadbent et al. 2018; Kumar and Garkoti 2021). These species often become a disturbance or even harm to their environment. During the last decades, many species have been listed in the national biodiversity report of Burkina Faso as invasive species and their proliferation contributes to the degradation of ecosystems (SP/CONEDD 2010, 2014). This list includes *S. obtusifolia* Linn, a herbaceous legume species belonging to the Fabaceae family commonly known as sicklepod. This species, with a 1.5–2.5 m height size (Holm et al. 1997), represent a serious threat to fields and rangelands in many tropical and subtropical regions (Palmer and Pullen 2001; Sosnoskie et al. 2021). *S. obtusifolia* has copious seed production and can germinate and grow under a wide range of environmental conditions (Retzinger 1984; Chaves Neto et al. 2020). Under favorable conditions of its development, *S. obtusifolia* can displace coexisting native species and form dense monospecific stands. This situation can lead to significant ecological changes (Mackey et al. 1997).

It is widespread in the Sahelian zone of Burkina Faso (Kiema et al. 2008, 2012; Kadeba et al. 2015) where rainfall (around 300 to 600 mm annually) is irregular and limited to only three or four months followed by a long drought period (Kiema et al. 2014).

In fact, *S. obtusifolia* has become an invader of rangelands and can completely dominate grass species, eradicate rangeland growth, and exclude livestock leading to unproductive monoculture in semi-arid rangelands (Palmer and Pullen 2001; Gebreyesus 2017). It is also known as “bush pasture” due to the damage it causes to rangelands (Chaves et al. 2002). Moreover, owing to the presence of toxic substances in its green leaves (Peres et al. 2010; Augustine et al. 2020) livestock do not graze this species in its green stage (Kiema et al. 2012). Therefore, the invasion of *S. obtusifolia* constitutes a threat to rangelands as well as to livestock which is the main source of household income in rural areas and contributes 18% of Burkina Faso’s Gross Domestic Product (MRA 2010).

Despite its high proliferation in Burkina Faso, *S.obtusifolia* has been the subject of very few studies. These studies are mainly focused on its nutritional and medicinal potential (Nacambo et al. 2021, Zare et al. 2022) and its impact on fodder species availability (Ouedraogo et al. 2021). No studies on the invasive performance of the species at the national level have been carried out yet.

To assess the competitive ability of *S. obtusifolia* and its sensitivity to water deficit in a controlled environment, three palatable species including *Chamaecrista mimosoides* L. (an annual legume), *Andropogon gayanus* Kunth (perennial grass) and *Pennisetum pedicellatum* Trin (annual grass) were used each in a mixture with *S. obtusifolia* in a greenhouse experiment under water deficit conditions. *Chamaecrista mimosoides* L. is a prostrate leguminous herb in open grasslands along roads and paths (Bargali and Bargali 2016; Li et al. 2022). It is an erect annual herb up to a meter long, sometimes woody at the base with many slender, pubescent branches. It is not very common and grows widely on grassy hills slopes in the wild form. It is spread over the entire climatic range of Burkina Faso and is considered as highly palatable for livestock (Schmidt-Groh et al. 2019). *A. gayanus* kunth and *P. pedicellatum* Trin particularly have become abundant in some areas. They are regarded among the important abundant grasses in Burkina Faso because they have high reproductive output and high dispersal ability (Grice et al. 2013; dos Santos et al. 2022; Ojo et al. 2022). Moreover, *A. Gayanus* is a C_4 perennial tufted or bunch grass that grows up to 3 m high (Baruch 1994) and possesses several positive characteristics: It exhibits higher photosynthetic efficiency and nutrient uptake (Setterfield et al. 2010; Zhang et al. 2022)owing to its ease of establishment, fast growth rate, high productivity, resilience and long growing season (Adams and Setterfield 2013). Also, *P. pedicellatum*, a fodder species widely spread in the Sudan and Sahel, is a herbaceous annual C_4 grass that grows upright up to 40-150cm in height depending on the environmental conditions where it grows. It produces high

biomass that greatly improves ground cover which in turn reduces soil erosion by controlling runoff and soil loss (Issaharou-Matchi et al. 2016; Beyene 2021). These characteristics may suggest that these species may be good competitors in a low-pressure grazing system. These plants (grasses and legumes) commonly co-occur in many habitats to improve forage yield and quality (Eisenhauer and Scheu 2008). They also represent important variations in photosynthetic and growth rate physiology, morphology, and canopy architecture that may contribute to the difference in competitive ability (Roush and Radosevich 1985; Petruzzelli's et al. 2021). Furthermore, the competitive ability between legumes and non-legumes (grasses) for resources has already been tested by several authors (DeBoer et al. 2020; Grüner et al. 2020; Khatiwada et al. 2020). To achieve the purpose of the study, single container experiments were conducted to determine (i) the effect of water deficit on above-ground biomass and height of each species; (ii) the effect (in terms of biomass loss) and the nature (intra-interspecific competition) of the competition among the focus species underwater different regimes; (iii) the intensity of the competition under different water regimes.

2 Materials and Methods

2.1 Study area

Seeds of the two annual legumes (*S. obtusifolia* and *C. mimosoides*) and two grass species *A.gayanus* (perennial grass) and *P.pedicellatum* (annual grass) were collected from the rangelands of the Sahelian zone of Burkina Faso, particularly in the Séno province whose capital town is Dori (figure 1). The province, with an estimated population of 385,900 inhabitants (INSD 2019), is cohabited by several ethnic groups including Fulani, Fulcé, Mossi, Gourmantché, Bella, Rimaïbé, etc. (Kiema et al. 2012). Agriculture and husbandry are the main socio-economic activities of the study area (Ayantunde et al. 2020). The province falls within the Sahelian zone of Burkina Faso which is characterized by average rainfall between 400 and 600 mm/year and average annual temperature ranges between 22°C to 37°C. Vegetation in this zone is dominated by desert grasslands and scrub (Kiema et al. 2014). The woody layer is dominated by *Combretum glutinosum* Perr. ex DC., *Balanites aegyptiaea* (L.) Del., *Acacia tortilis* subsp. *Acacia tortilis* (Forssk.) Hayn,

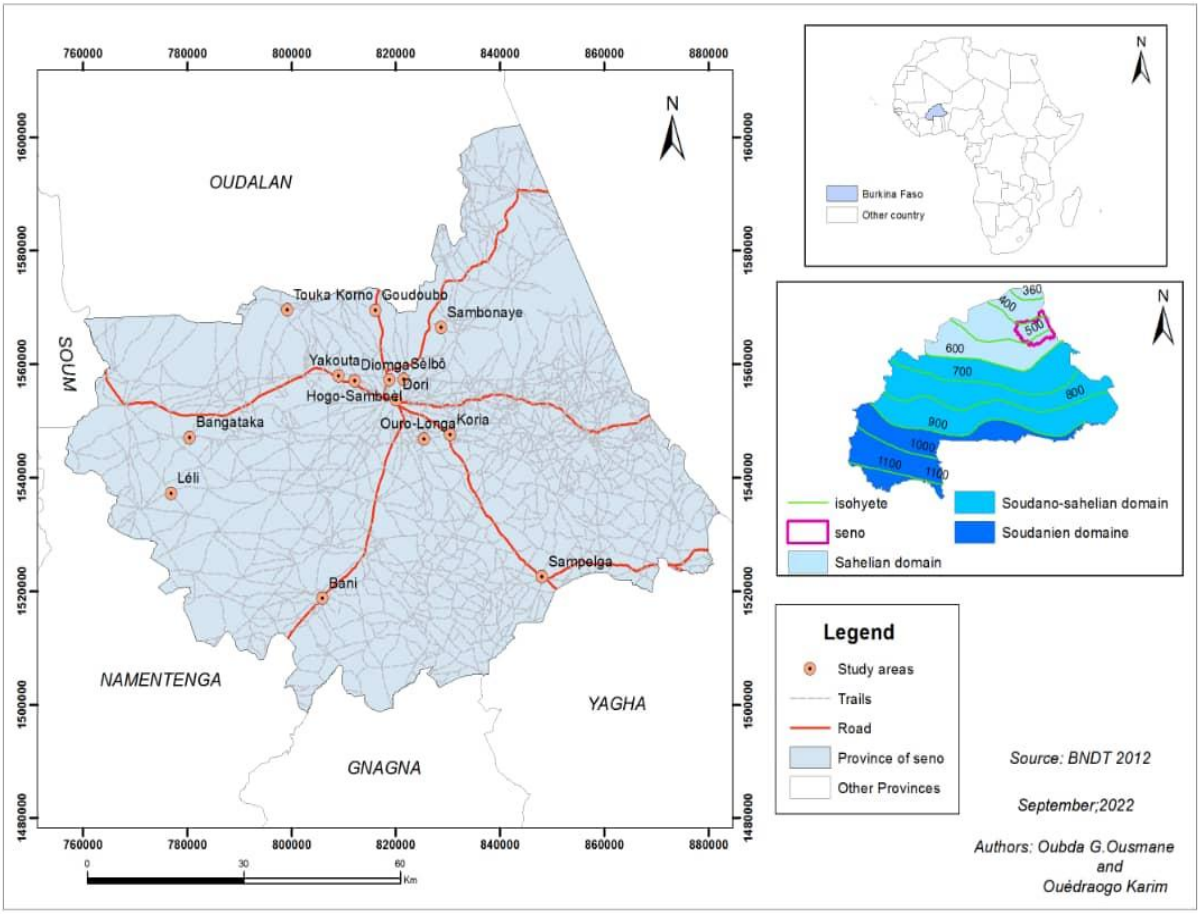

Figure 1 Geographical situation of the study area

Faidherbia albida (Delile) A. Chev., (Fontes and Guinko 1995). The vegetation of the herbaceous layer remains mainly dominated by annual grasses and characteristic pasture grasses such as *Pennisetum pedicellatum* Trin., *Loudetia togoensis* (Pilg.) Hubb., *Triumfetta pentandra* A. Rich, *Achyranthes aspera* L., *Andropogon gayanus*, *Andropogon pseudapricus* Stapf, *Aristida mutabilis* Trin, *Senna obtusifolia* (L.) H.S. Irwin & Barneby, *Tephrosia pedicellata* Bak, *Acanthospermum hispidum* DC, *Schoenefeldia gracilis* Kunth; *Zornia glochidiata* C.Rchb. ex DC. (Kiema et al. 2014; Nacoulma et al. 2019). The pods containing seeds were harvested at maturity in mid-November directly from standing individuals to avoid infestation with soil and put into envelopes. The harvested seeds were stored in the laboratory at ambient temperature (25 to 30°C) for seven months from November 2018 to June 2019.

2.2 Experimental conditions

The experiment was conducted under greenhouse conditions at the National Forest Seed Center (12°24'-31°78'N and 01°28'-59°33'W) in Ouagadougou Burkina Faso. The average minimum and maximum temperatures within the greenhouse are 28.13°C and 36.3°C respectively while those in the open environment are 27.32°C and 34.76°C. The experiment (from laboratory to greenhouse) started on 22 June 2019 corresponding to the beginning of the rainy season in Burkina Faso and was completed on 07 September 2019 when the leaves of *S. obtusifolia* were almost yellowing. First of all, a substrate was prepared and it is composed of soil, sand, and manure in a ratio of 3:2:1. This was prepared by mixing 3-wheel barrows of soil, 2-wheel barrows of sand, and 1 wheelbarrow of manure. The complete substrate mixture was sterilized and from this 30 kg of the substrate was filled in a container with a 50 cm diameter and 25cm depth. The bottom of each container was carefully perforated to allow the excess water to drain after watering. To ensure that planting density would be enough to result in competition, laboratory studies were conducted before sowing. These studies consisted in lifting the tegumentary dormancy of the seeds by pre-treating them with acid and water. The first pre-treatment consisted of soaking the seeds of *P. pedicellatum* and *A. gayanus* in tap water for 24 hours. The second pre-treatment was immersion of seeds in 96% concentrated sulfuric acid (H_2SO_4), in this, *S. obtusifolia* seeds were treated for 5 minutes and *Chamaecrista mimosoides* seeds for 3 minutes. The acid-pretreated seeds were then washed with water for 15 minutes.

The germination rate was about 90% for all species. After two weeks of sowing in the greenhouse, an adjustment was carried out to obtain seedlings with similar sizes which 3; 6; 9, and 12 individuals in monoculture and 12 individuals per container in the appropriate proportions in a mixture (figure 2).

2.3 Experimental design

Among methods to investigate the competitive relationships between plants, the experiments in replacement series have been widely used since its introduction by De Wit (1960) where one species gradually replaces another in a mixture at constant overall density (in this study constant overall density was 12). This method allows the understanding of the competitive process between plants. In particular, its application includes an aspect of inter-and intraspecific interactions between wild plants (Solbrig et al. 1988; Savić et al. 2021). In order to assess the competitive ability of *S. obtusifolia*, this method was used to assess how one species growing in a mixture with another species can affect the performance of this one. The focus was to determine how *S. obtusifolia* responds in terms of biomass production when it competes with *A. gayanus*, *C. mimosoides*, and *P. pedicellatum*. Also, the variation of water regimes in the experiment aimed at understanding how differences in water availability may change the competitive relationship between *S. obtusifolia* and the target species. Each of the species in this experiment was grown in monoculture with 3:0; 6:0; 9.0 and 12:0 densities on one side. On the other side, *S. obtusifolia* was grown in a mixture with the other species in the proportion of 9:3; 6:6; 3:9 as shown in figure 2. A total of 25 containers/pots were used in the experiment, among these, 16 containers are in monoculture, and 9 containers are in mixed species planting. Each container was replicated five times giving a total of 80 containers in monoculture and 45 containers in mixed culture i.e. 125 pots per block. Further, the total plant density was twelve plants per container (60 plants m^{-2}). The appropriate combined proportion of *S. obtusifolia* would reflect the level of invasion of *S. obtusifolia* in the field.

For the water regime, a split-plot approach was used in a randomized complete block design to avoid the microclimatic

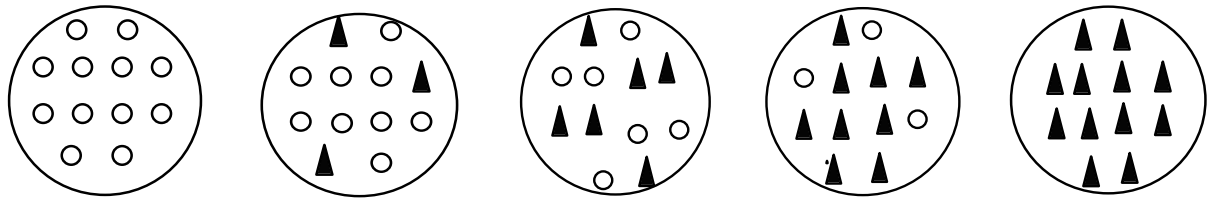

Figure 2 Schematic diagram of the replacement series (O circles represent *S.obtusifolia* and ▲triangular represent one of the 3 other species)

effect. Four (04) blocks corresponding to four watering regimes were applied. The watering regime consisted of watering plants in each container every two days with a determined amount of water from the germination to the mature phenology stage. This quantity of water or volume of water (VW) varied progressively from one block to another according to the predefined level of stress. The applicable volume of water was determined by calculating the field capacity which is the mass of water remaining in the soil after a rapid drainage of 1 to 2 days. It is expressed as a percentage of the dry weight of the soil. A sample of dry soil placed in a container was first weighed to determine the dry weight (DWs) and then soaked in water to saturation. After 48 hours, the water infiltrated through the perforated bottom of the container. The soil was weighed again to determine its wet weight (WWs). Then the field capacity (FC) was determined in grams of water per gram of dry soil using the following formula (Veihmeyer and Hendrickson 1931):

$$FC = \frac{WWs - DWs}{DWs}$$

Where FC is the field capacity, WWs is the wet weight expressed in kg and DWs is the volume of dry soil which is expressed in kg.

The volume of water (VWs) for each regime is determined from the following formula VWs=VS*FC *X% with VWs being the volume of water. VS represents the volume of dry soil, FC is the field capacity, and X the portion corresponding to the level of water stress applied. This portion varies progressively from 25%; 50%; 75% and 100%. Thus 100% FC is the proportion of water irrigation in the control regime and the other three are considered stressed regimes depending on the amount of water irrigation. All blocks were under a plastic-covered greenhouse preventing rainwater infiltration. The water stress application at the end of germination consisted of watering all the plants regularly every two days in the water regimes until the end of the experiment.

2.4 Measured parameters

To characterize the effects of water regime and competition on the plants, the height and above-ground biomass of each plant species were measured. Generally, plant biomass and height have been used to provide information on the size and aggressiveness of a species which may determine its competitive ability (Radosevich 1987; Reeves et al. 2021). The height (cm) for each species was measured using a tape measure. Because of the difficulty of accurately separating the roots of individual plants in each pot for analysis and in particular for perennial grasses, only above-ground biomass was examined (Meekins and McCarthy 1999; Bolinder et al. 2002). When plants were in a vegetative state (50 days after planting), above-ground plant materials were cut at soil level and above-ground dry biomass of the plants was determined after drying in an oven at 65°C for 72 h using a precision balance (0.01g). These parameters provide a better understanding of the mechanism adopted by each species under conditions of water deficit combined with competition and thus help to evaluate species adaptation potential (Kagambèga et al. 2019).

2.4.1 Sensitivity index

To characterize and compare the degrees of tolerance of the different species to water deficit, the stress sensitivity index (SSI) was calculated at the end of the experiment following the different regimes. In this study, the stress sensitivity index developed by Fischer and Maurer (1978) was used because it is successfully applied in many studies (Nana et al. 2009; Kagambèga et al. 2019) and was determined from the height of the plant species using the formula below:

$$SSI(\%) = 100 \times \frac{H1 - H2}{H1}$$

Where H_1 is the value of the plant's height in the control plants and H_2 is the value of the plant's height in the stressed plants. Thus stress sensitivity index based on plant height (SSI-H) was determined for each species following the different regimes and the species with the higher sensitivity value is the most sensitive.

2.4.2 Competition Index

Weigelt and Jolliffe (2003)have grouped the competition index into three categories to quantify the effect and outcome of the competition. In this study, three indices were calculated notably the Relative Yield (RY), Relative Yield Total (RYT) to quantify the effect of competition, and the Agressivity (A) to quantify the intensity of the competition. All of these indices were calculated using the weight of the average above-ground dry biomass per species in each container.

2.4.3 Relative competitive ability

Relative values are used to compensate for absolute differences in biomass between different species and to allow interspecies comparisons to be made (Fowler 1982). The Relative Yield (RY) value indicates the type of competition experienced by the species. If RY is greater than 1.0, the interspecific competition is less than the intraspecific competition. If RY is less than 1.0, the interspecific competition is more than the intraspecific competition. When RY is equal to 1.0, it indicates that the degree of competition of the species in mixture and monoculture is the same. The RY values were obtained according to the following equation:

$RYA = \frac{MA}{PA}$ and $RYB = \frac{MB}{PB}$ (De Wit and Van den Bergh 1965)

PA & PB = mean yield of species `A' and `B' in monoculture; Ma & Mb = mean yield of species `A' and `B' in the mixture.

Where, RY expresses the relationship between the mean yield of species A (Ma) when grown in a mixture containing species B with the mean yield of species A (Ma) when grown in monoculture and conversely for species B. In this study, A represents *S.obtusifolia* and B was represented by each of the 3 other target species.

Furthermore, the method of graphical analysis of the relative yield can be used (Radosevich 1987; Galon et al. 2022). This model can also involve the construction of a diagram based on the relative yield (RY) and also the relative total yield (RYT) for the studied plant proportions (0; 3; 6; 9 and 12). Equal competition between species would be represented by lines with constant slopes across all proportions resulting in an intersection point of the two curves at the 50:50 proportions.

Expected RY (straight line) for a species occurs when plants of a given species grow equally well in mixture and monoculture. That means there is no effect of one species over the other or the ability of the species to interfere with one another is equivalent. Comparisons of the actual RY of each species with their expected RY (diagonal dashed line in replacement diagrams) indicate competition if the actual RY curve of one species is concave and that of the second convex. When the RY of A is a convex curve, it indicates that the growth of A in the mixture was positively affected whereas the growth of B with a concave curve was negatively affected. However, niche differentiation occurs if the actual RY curves of both species are convex and there is mutual antagonism when the actual RY curves of both species are concave.

Relative Yield Total (RYT) is a measure of resource complementarities (Kumar et al. 2017). The RYT data were represented by the ½ sum of the relative yield of A and B (McGilchrist and Trenbath 1971). RYT equal to 1.0 (straight line) means that there was competition for the same resources. However, a value greater than 1.0 (convex line) means that there was no competition either because the supply of resources exceeds the demand or because the demands for the resources are different for each species. Finally, a value of RYT less than 1 (concave line) indicates the occurrence of antagonism that harmed the growth of both species (Bastiani et al. 2016).

2.4.4 Intensity of competition

Aggressivity (A) is an index that has been proposed to measure the strength of competition (Willey and Rao 1980). RY can be used to determine the agressivity index (McGilchrist and Trenbath 1971; Roush and Radosevich 1985). It is obtained according to the following formula.

$$A_A = ½ (RY_A - RY_B)$$

$$A_B = ½ (RY_B - RY_A)$$

Where RY_A is the relative yield of species A and RY_B is the relative yield of species B. An aggressivity value of zero indicates that component plants are equally competitive with the sign of the dominant species being positive and that of the dominated species being negative (Connolly et al. 2001). When two species are grown together in a pot, the more aggressive species will have the higher A value. The plant with the higher aggressivity value is assumed to be the stronger competitor (Meekins and McCarthy 1999).

2.5 Statistical Analysis

To assess the differences between water regimes at each species level, data obtained from the measured parameters (height and biomass) were subjected to a one-way ANOVA analysis of variance. A two-factor analysis of variance (ANOVA) was used to assess differences in performance (SSI) between species in response to the water regime. The interactive effects were evaluated with the two-way ANOVA procedure. Means were separated and ranked using the Tukey HSD (Honestly Significant Difference) test at the 5% threshold. Index data were analyzed by performing a three-way GLM ANOVA procedure. The effects of species, proportions, and water regime on plant above-ground biomass were examined using relative yield, and aggressivity index as the dependent variables. Species combination and water regime represent the fixed factors. RY and RYT from each mixed culture were compared to the value of 1.0 using t-tests (P=0.05). The aggressivity value from each mixed culture was compared to the zero value using t-tests (P=0.05). All analyses were performed using software version 4.1.0 (RCore Team 2021).

3 Results

3.1 Effect of water deficit condition on height and above-ground biomass

Water deficiency significantly reduced the vegetative growth of the four herbaceous species. Regarding the morphological parameters, height and biomass values were negatively influenced by water regimes i.e. control (100% FC); sufficient water supply (75% FC); moderate water stress (50% FC), and severe water stress (25% FC)for all species (Figures 3 and 4). Height was higher in the control block containers and progressively reduced until severely water-stressed blocks. A similar trend was observed for above-ground biomass (Figures 3 and 4) indicating a positive correlation (r=0. 69) between these two parameters.

Concerning the plant height, the sensitivity stress index (SSI-H), increased progressively from the water regime of 100% FC to the most stressed one 25% FC (Figure 5). Significant variability in stress sensitivity stress index (SSI-H) was observed for each species under different water regimes (Figure 5). The stress sensitivity index varied significantly with the water stress regime and

with the combination of species (Table1). However, the sensitivity stress index did not vary significantly between species in the regime 75% FC and 50% FC. In opposite, in the regime 25% FC there is a highly significant variation of the SSI-H (dl = 3; F =26.72 and p < 7.81 e-14) (Figure 5).With its highest sensitive index, *S. obtusifolia* was the most sensitive species to water deficit compared respectively to the other three species, i.e. *C. mimosoides*, *P. pedicellatum*, and *A. gayanus* (Figure 6).

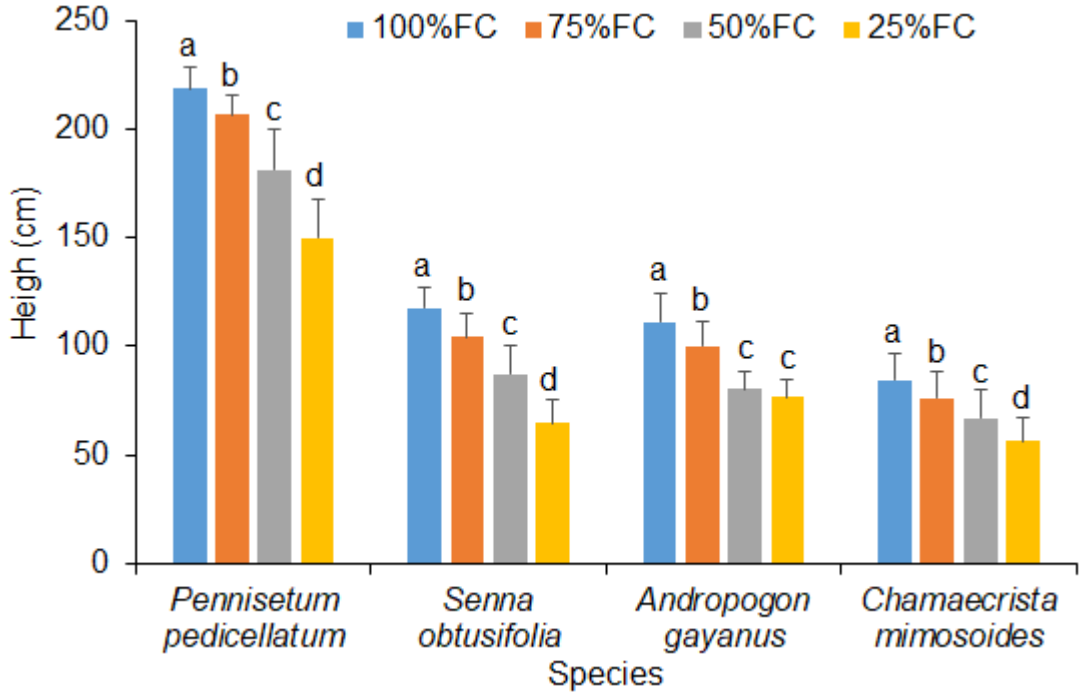

Figure 3 Variation in species height under different water regimes (Values without common letter differ significantly at P ≤ 0.05 as per Tukey HSD test); error bars represent standard deviation

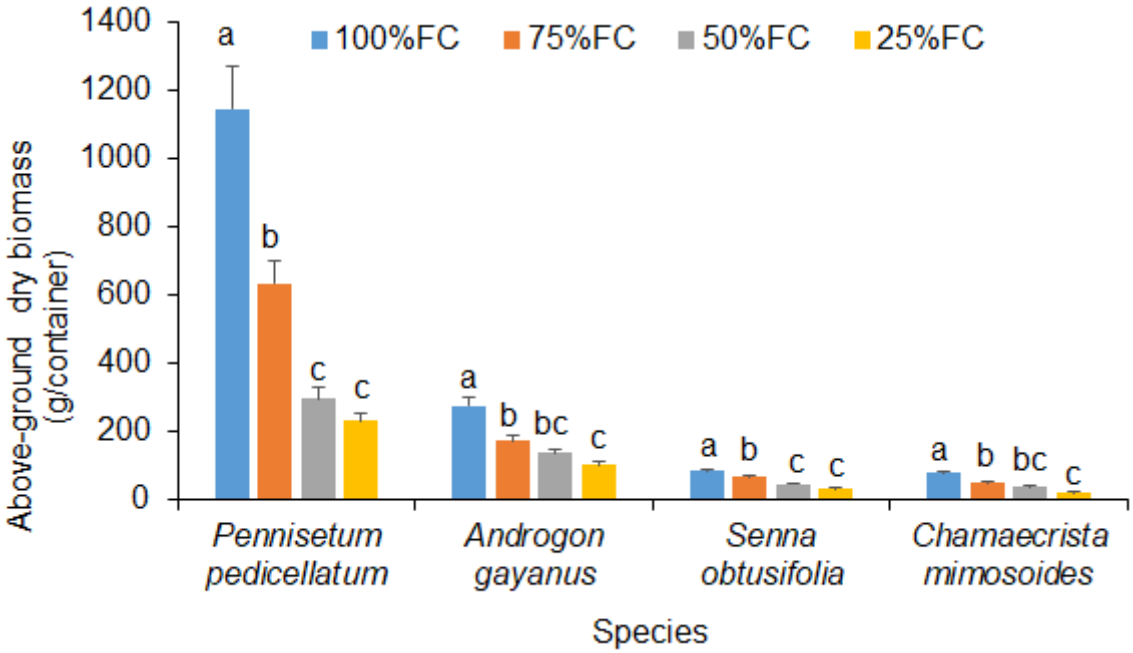

Figure 4 Variation in species biomass under different water regimes (Values without common letter differ significantly at P ≤ 0.05 as per Tukey HSD test); error bars represent standard deviation

Table1 Effect of species combination and water regime on the stress sensitivity index (SSI-H)

Source of variation	SSI-H		
	df	F value	Pr(>F)
Species	3	23.244	0.006*
Water regime	3	689.092	<0.002*
Species x water regime	9	7.907	0.007*

*means significantly different

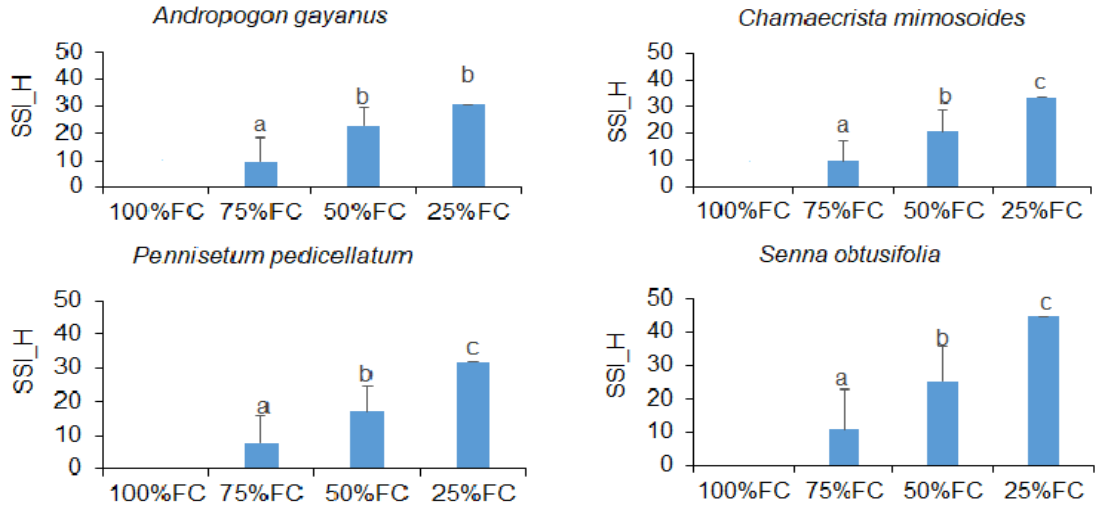

Figure 5 Variations in species sensitivity index to different water stress regimes (Values without common letter differ significantly at P ≤ 0.05 as per Tukey HSD test); error bars represent standard deviation; SSI-H: stress sensitivity index based on plant height

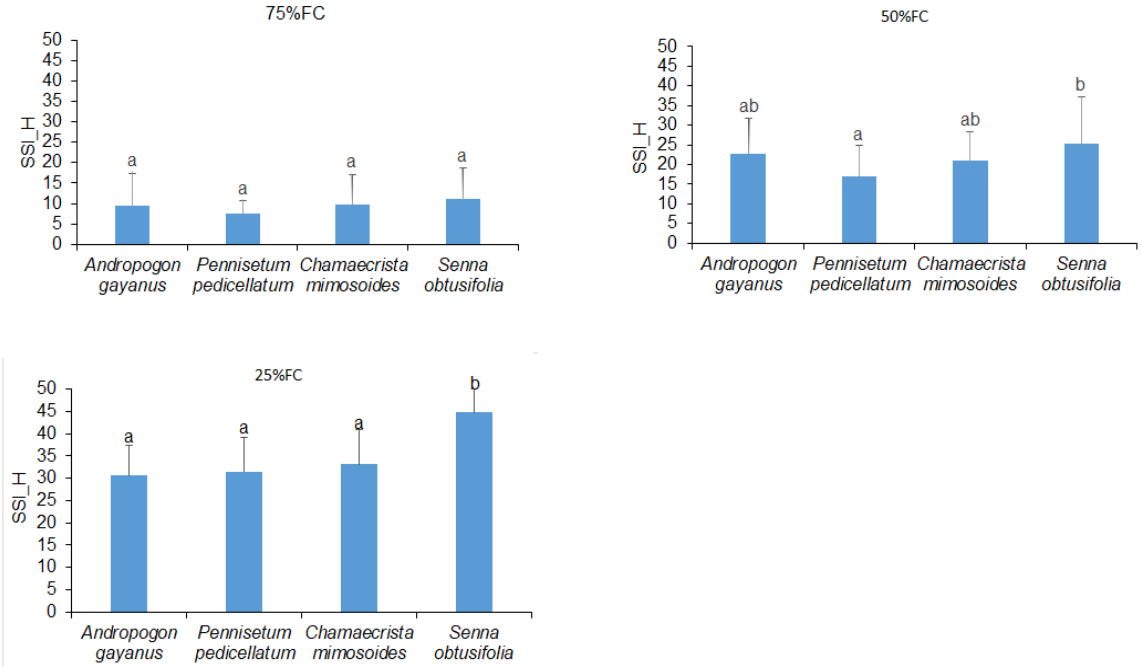

Figure 6 Sensitivity of the four species in severe water stress; (Values without common letter differ significantly at P ≤ 0.05 as per Tukey HSD test); error bars represent standard deviation; SSI-H: stress sensitivity index based on plant height

Table 2 Dry above-ground biomass (g) of plant species in monoculture and mixed culture under different water regimes

	Monoculture		Mixed culture			
Water regime	Species	Mean	Species	Mean	F	P
Control (100%FC)	*S. obtusifolia*	90.98 ± 35.97	SA	59.97 ± 2.10	-2.77	0.037*
			SC	86.97 ± 28.23	-0.36	0.984ns
			S P	60.17 ± 29.41	-2.75	0.039*
	A. gayanus	385.57 ± 108.85	AS	376.21 ± 103.88	0.06	0.81ns
	C. mimosoides	93.77 ± 36.71	CS	53.97 ± 28.77	10.92	0.002***
	P. pedicellatum	1490.83 ±688.03	PS	1476.42 ± 68.55	0.003	0.950ns
	S. obtusifolia	74.54 ± 32.25	SA	43.06 ± 21.21	-3.28	0.001***
			SC	68.55 ± 28.98	-0.62	0.924ns
			SP	41.66 ± 20.78	-3.42	0.001*
Sufficient water supply (75%FC)	*A. gayanus*	237.94 ± 93.96	AS	226.39 ± 81.47	0.13	0.72ns
	C. mimosoides	64.71 ± 22.74	CS	32.73 ± 14.91	20.75	0.001***
	P. pedicellatum	830.75 ± 377.76	PS	818.33 ± 382.69	0.008	0.93ns
	S. obtusifolia	47.82 ± 23.34	SA	27.83 ± 15.09	-3.29	0.001***
			SC	43.94 ± 13.72	-0.64	0.91ns
			SP	24.96 ± 12.03	-3.77	0.002***
Moderate water stress (50% FC)	*A. gayanus*	187.05 ± 66.30	AS	177.85 ± 54.96	0.17	0.68ns
	C. mimosoides	47.97 ± 15.21	CS	25.01 ± 13.43	19.19	0.001***
	P. pedicellatum	342.63 ± 130.87	PS	330.98 ± 128.90	0.06	0.80ns
	S. obtusifolia	38.86 ± 19.92	SA	17.21 ± 5.60	-4.90	0.001***
			SC	22.25 ± 6.03	-3.75	0.002***
			SP	14.66 ± 11.02	-5.47	0.001***
Severe water stress (25%FC)	*A. gayanus*	147.10 ± 43.18	AS	131.00 ± 23.38	1.61	0.21
	C. mimosoides	25.27 ± 3.33	CS	13.66 ± 3.18	95.14	0.001*
	P. pedicellatum	290.05 ± 115.70	PS	286.11 ± 115.81	0.009	0.92ns

The values used are averages and (*) indicate significance at the 5% threshold according to the Tukey HSD test; *** Highly significant; ns not significant; ± standard error Here SA: *Senna obtusifolia* in mixture with *Andropogon gayanus*; SC: *Senna obtusifolia* in mixture with *Chamaecrista mimosoides*; SP: *Senna obtusifolia* in mixture with *Pennisetum pedicelatum*; AS: *Andropogon gayanus* in mixture with *Senna obtusifolia*; CS: *Chamaecrista mimosoides* in mixture with *Senna obtusifolia*; PS: *Pennisetum pedicellatum* in mixture with *Senna obtusifolia*

3.2 Effect of competition under the water regime

3.2.1 Biomass production

The above-ground dry biomass of *S. obtusifolia* was negatively influenced in combination with *A. gayanus* and *P. pedicellatum* under the four water regimes. In contrast, the above-ground biomass of *S. obtusifolia* was not influenced in combination with *C. mimosoides*. Indeed, a significant difference (P<0.05) was found between *A. gayanus* and *P. pedicellatum* under the four water regimes (table 2). In contrast to this, no significant difference (P> 0.05) was reported between the biomass of *S. obtusifolia* in monoculture from its production in mixed culture with *C. mimosoides* under a water regime of 100% FC; 75% FC, and 50% FC. While in the case of 25% FC water regime a significant difference (P<0.05) was found between the biomass of *S. obtusifolia* in monoculture from its production in mixed culture with *C. mimosoides*. Biomass of *A. Gayanus* and *P. pedicellatum* in monoculture was not significantly different (P> 0.05) from their biomass production in mixed culture with *S. obtusifolia* in all regimes (table 2). Further, the biomass of *C. Mimosoides* in monoculture under severe water stress (regimes of 25% FC) was significantly different from its biomass in mixed culture with *S. obtusifolia*.

3.2.2 Relative Yield and Relative Yield Total

Relative Yield (RY) and Relative Yield Total (RYT) based on the shape of the curves differed significantly among regimes, proportions, and species ($P<0.05$). Under all water regimes regarding *S.obtusifolia* and *C. mimosoides*, RY of *S.obtusifolia* over-yielded the expected (diagonal dashed line) and had always convex curves except for proportions 6:6; 9:3, and 12:0 under

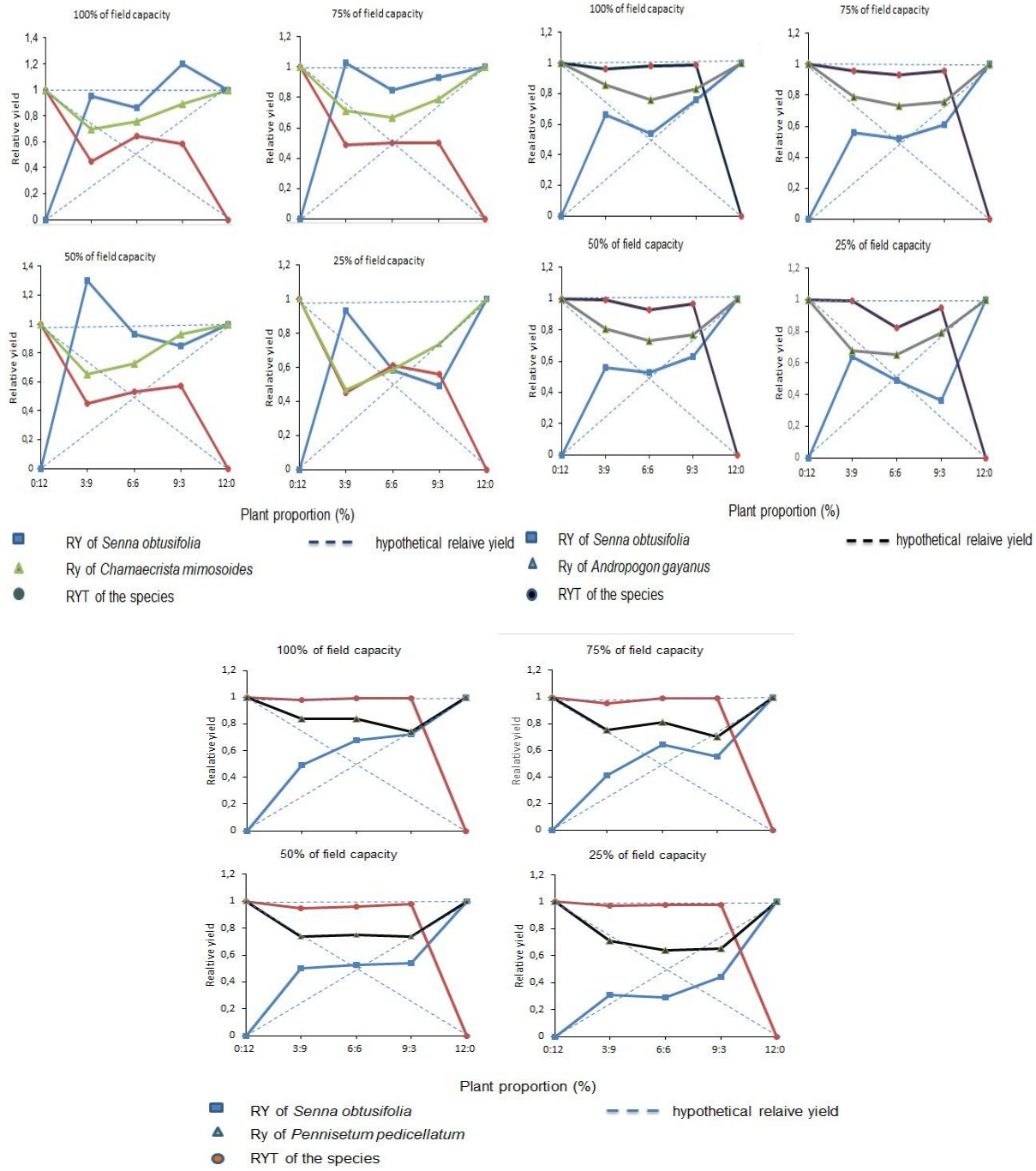

Figure 7 Replacement series diagrams illustrating mean ± sd relative yield of *S. obtusifolia* (□), *C. mimosoides*(Δ), *A. gayanus* (Δ), *P. pedicellatum* (Δ) and relative yield total (●) as a function of species proportion. Dashed lines represent the expected values in the absence of competition and solid lines represent the observed values when the species competed in different plant proportions. Proportions 100%, 75%, 50%and 25% represented different water regimes.

water deficit (25%FC) where it under-yielded the expected and had a concave curve (figure 6). Whereas, the curve of *C. mimosoides* was concave for 0:12 to 6:6 proportion and became convex until proportion 12:0.

RY of *S. obtusifolia* and *A. gayanus*, when grown together were significantly different from expected values in each mixture proportion under all water regimes. *S. obtusifolia* over-yielded the expected and had a convex curve for 0:12 to 6:6 proportions and later on these curves became progressively concave for the proportion 12:0 whereas *A.gayanus* over-yielded the expected in all proportions under all regimes (figure 6).

RY of *S. obtusifolia* and *P. pedicellatum* were significantly different from expected values in each mixture proportion under all water regimes. RY of *S. obtusifolia* was significantly higher than expected and had convex curves from proportions 0:12 to 6:6. These curves became progressively concave after proportion 6:6, whereas *P.pedicellatum* over-yielded the expected in all proportions under all regimes. Mixtures were under-yield i.e. RYT and RY of the four species in the mixture at all regimes were each other less than 1.0 as represented by the dashed line in figure 7.

3.2.3 Intensity of competition

The aggressivity index (A) differed significantly between species and proportions of species combination in pairs but not for the regime (Table 3). *S. obtusifolia* had a positive aggressivity value while *C. mimosoides* had negative aggressivity across all proportions at almost all water regimes. In contrast, when *S.obtusifolia* was grown in a mixture with *A. gayanus* and *P. pedicellatum*, the aggressivity value of *S.obtusifolia* was negative whereas the aggressivity of *A.gayanus and P.pedicellatum* were positive (table 3). Water regime, species combination, and interactions of species significantly affected the aggressivity index (A). However, the Relative yield total (RYT) was not affected by the interaction between the water regime and species combination (table 4).

Table 3 Effect of different water regimes on the Aggressivity of selected species

Water regimes	Proportions	Mean ± sd *S. obtusifolia* X *C. mimosoides*	Mean ± sd *S. obtusifolia* X *A. gayanus*	Mean ± sd *S. obtusifolia* X *P. pedicellatum*
Control	75 :25	0.25 ± 0.06*	-0.09 ± 0.02*	-0.12 ± 0.04*
	50 :50	0.1 ± 0.09	-0.21 ± 0.03*	-0.15 ± 0.03*
	25 :75	0.31 ± 0.11*	-0.16 ± 0.09*	-0.25 ± 0.04*
Stand mean		0.22 ± 0.08	-0.15 ± 0.05	-0.17 ± 0.04
Sufficient water supply	75 :25	0.22 ± 0.04*	-0.17 ± 0.04*	-0.19 ± 0.3
	50 :50	0.17 ± 0.09*	-0.21 ± 0.05*	-0.18 ± 0.3*
	25 :75	0.25 ± 0.10*	-0.2 ± 0.04*	-0.29± 0.3
Stand mean		0.21 ± 0.07	-0.19 ± 0.04	-0.22 ± 0.3
Moderate water stress	75 :25	0.2 ± 0.06*	-0.18 ± 0.03*	-0.2 ± 0.04
	50 :50	0.19 ± 0.25	-0.2 ± 0.05*	-0.21 ± 0.03*
	25 :75	0.36 ± 0.17*	-0.2 ± 0.03*	-0.23 ± 0.03
Stand mean		0.25 ± 0.09	-0.19 ± 0.04	-0.13 ± 0.03
Severe water stress	75 :25	0.01 ± 0.01	-0.31 ± 0.01*	-0.26 ± 0.03*
	50 :50	-0.01 ± 0.07	-0.16 ± 0.09*	-0.34 ± 0.04*
	25 :75	0.18 ± 0.06*	-0.16 ± 0.05*	-0.34 ± 0.05*
Stand mean		0.18 ± 0.09	-0.21 ± 0.05	-0.31 ± 0.04

Aggressivity value (mean± sd) of *C. mimosoides*, *A. gayanus*, *P. pedicellatum* when grown with *S. obtusifolia* at different combinations under different water regimes control (100% FC); sufficient water supply (75% FC); moderate water stress (50%FC) and severe water stress (25% FC); here the proportion is the ratio of the percentage of *S. obtusifolia* plants to the target species in a container; A: t-test being considered significant (*) when differing ($p \leq 0.05$) from 0.

Table 4 The effects of species combination and water regime on the Aggressivity (A) and Relative yield total (RYT)

Sources of variation		A		RYT	
	DF	F-value	P-value	F-value	P-value
Species	3	30.36	0.001***	15.26	0.001***
Proportion	2	8.23	0.001***	28.71	0.001* **
Regime	3	1.40	0.25ns	3.16	0.031 *
Species-proportion	4	9.05	0.001***	1.47	0.212ns
Species-regime	6	2.44	0.03*	2.03	0.061ns
Proportion-regime	6	1.29	0.03*	1.50	0.182ns
Species-proportion-regime	12	1.55	0.11ns	1.19	0.291ns

The values used are averages and (*) indicate significance at the 5% threshold according to the Tukey HSD test examined with three-way of Anova.. (***) Highly significant; (ns) not significant.

4 Discussion

4.1 Effect of water stress on plant's height and above-ground biomass

In this study, all four species' height and above-ground biomass were negatively affected by water stress. This could be explained by the juvenile state of the plants (16-day-old plants) during the induction of water deficiency and duration of stress. Indeed, the response of a plant to water deficit is complex and depends on the stage of development of the plant, the intensity of the deficit, the duration of the stress, the genotype of the plant, and the state the plant was in when the stress occurred (Sawadogo et al. 2006; Aziadekey et al. 2014; Peng et al. 2022). In addition, when the plants are subjected to long-term severe water stress during the vegetative growth stage, the stress may be great enough to cause substantial yield losses (Wijewardana et al. 2019).

Plants subjected to soil moisture deficit conditions experienced a decrease in the performance of their parameters (height and biomass) compared to plants of the control regime. This reduction in growth following a water deficiency is considered a regulatory mechanism allowing the adaptation of plants to water restrictions by a reduction in the transpiration surface (Chaves et al. 2002; Yang et al. 2021; Wu et al. 2022).

Furthermore, the sensitivity index (SSI-H) varies significantly for each species under different water regimes. The differences observed between species showing their degree of sensitivity to water stress would be due to the intrinsic characteristics of each species as reflected in the sensitivity index. Grasses are empirically considered "tolerant" to drought (Baruch 1994; Pardo and VanBuren 2021). Hence *A. gayanus* was one of these grasses relatively more tolerant and less affected by drought due to a slowing down of the growth of the aerial organs in favour of root development which preferentially glues the layers of the substrate with residual humidity (Buldgen 1997). In the severe stress regime, *S. obtusifolia* was the most sensitive species to water deficit (having the highest sensitive index) compared respectively to the other species i.e. *C. mimosoides*, *P. pedicellatum*, and *A. Gayanus*.

However, a plant is tolerant when it can maintain its metabolic activity under low water potentials (Kagambèga et al. 2019). The species with the lowest sensitivity index to water stress, in decreasing order, were *A. Gayanus*, *P. pedicellatum*, and *C. Mimosoides* suggest that these species are the most tolerant to water deficits. Previous studies (Bargali and Bargali 2016; de Moura et al. 2020) have reported that *A. Gayanus* and *P. pedicellatum* are drought-tolerant species. In addition, it is argued that *C.mimosoides* can withstand low water deficit (Bargali and Bargali 2016). On the contrary, *S. obtusifolia*, characterized by the highest sensitivity index, is the least tolerant to water deficit in soil. This shows that *S.obtusifolia* prefers well-drained soils (DAF 2016). This situation could suggest that *S.obtusifolia* used less water under water deficit than the other species. Similarly, Moreshet et al. (1996) reported that *S.obtusifolia*tends to use less water than *Arachis hypogea* when grown together in a mixture with Arachis hypogea (peanut) under water deficit.

4.2 Effect of competition

4.2.1 Biomass production

The biomass of *S. obtusifolia* is significantly reduced when grown in a mixture with *A.gayanus* or *P.pedicellatum*as compared to its biomass in monoculture under all regimes except C. *mimosoides.* In contrast to this, the biomass of *A. gayanus* and *P. pedicellatum* are not significantly influenced when comparing their performance in mixtures versus monocultures. According to Hartvigsen (2000), the growth of some species is restrained when grown in mixtures. Furthermore, the combination of *S. obtusifolia* and *C. mimosoides* revealed that in the three least stressed regimes, the biomass of *S. obtusifolia* was not influenced while the biomass of *C. Mimosoides*

decreased as compared to its biomass in monoculture. However, the biomass of *S. obtusifolia* decreased when grown in a mixture with *C. mimosoides* under the severe water regime. Our results are in line with the evidence showing that invasive plant species often produced more biomass than competing plant species under high water conditions while the opposite pattern has been found under drought as the invaders became less competitive than competing species (Kelso et al. 2020).

4.2.2 Relative competitive ability

Interaction among *S. obtusifolia* and other species in the mixture was negative showing a frequent occurrence of interspecific competition. In general, equal competition between species would be represented by lines with constant slopes across all ratios resulting in an intersection point of the two curves at the 50:50 ratio. In this study, the curve representing the relative yield of *S.obtusifolia* and the curves of the relative yield of each species in a mixture with *S. Obtusifolia* usually do not intersect at the 6: 6 proportion. Moreover, the curve of *S. obtusifolia* is generally concave and yields less than the expected quantities while curves of *A. gayanus* and *P. pedicellatum* are convex and yield higher than the expected quantities across all regimes. These trends suggest that *S. obtusifolia* was subordinate whereas *A. gayanus* and *P. pedicellatum* were the dominant species. In contrast to *A. gayanus* and *P. pedicellatum, C. mimosoides* was negatively affected by the presence of *S. obtusifolia* under all regimes. The RY curve of *C. mimosoides* was concave and produced yields less than expected while the RY curve of *S. obtusifolia* produced a yield higher than expected.

However, in *S. obtusifolia* combinations with the other three species (*A. gayanus, C. mimosoides,* and *P. pedicellatum*), the relative yields total values were less than expected and indicated that there was intense competition between their populations. The relative yields total may be lower than expected (dashed line) if one species or both are more affected than expected when there is crowding for the same space (Baghdadi et al. 2016). When the total value of relative yield is lower than expected, it may be a case of mutual antagonism (Dekker et al. 1983). This suggests that one species may produce a toxin that reduces the growth of the other species (allelopathy) or one of the species in combination may lose its ability to grow due to the presence of its congener.

Similar studies on legume and non-legume mixtures had attributed high values of relative yields total through the use of different nitrogen sources in addition to differences in root and above-ground characteristics (Zand and Beckie 2002; Ball et al. 2020). Contrary to their study, the results of this study showed a lower value of relative yield totals through the use of water stress as the main factor in addition to above-ground biomass. Our results are in agreement with Martin and Field (1984) who found relative yields total values less than expected by using grass-legume mixtures in shallow boxes in addition to differences in root and above-ground biomass. This situation suggested that the value of relative yields total in grass-legume competition depends on the nature of the species, resources, and the measured parameters.

4.2.3 Intensity of competition

S. obtusifolia was the subordinate species and *A. Gayanus* and *P. pedicellatum* were dominant except *C. mimosoides*. The aggressivity of *S.obtusifolia* was negative and lower while the aggressivity of *A. gayanus* and *P. pedicellatum* were positive and greater when grown in all proportions under all water regimes. The highest aggressiveness value of *A. gayanus* and *P. pedicellatum* may be due to their efficient C_4 photosynthetic pathway and high nitrogen and water use efficiency. This result supports the view of Zhang et al. (2011), and Sheppard (2019) who reported that the species with the greater competitive ability is usually termed as the dominant species or superior competitor and has a greater ability to acquire resources and to occupy the superior ecological niche. *S. obtusifolia* was expected to be the best competitor in its proliferation in the Sahel rangelands. In addition, previous studies show that this species is the most troublesome or best competitor when in a mixture with soybean (*Glycine max*), tobacco (*Nicotiana tabacum* L.), peanut (*Arachis hypogaea* L.), cotton (*Gossypium hirsutum*) and vegetables (Buchanan et al. 1980; Monks and Oliver 1988; Webster and Macdonald 2001) due to its many competitive attributes such as fast-growing, deep-rooting, rapid biomass accumulating and forms a close canopy cover to recycle nutrients from the subsoil and suppress the growth of noxious weeds (Hauser et al. 1975; Holt 1995). However, the experimentation in the greenhouse under water stress revealed that this species is a poor competitor suggesting that its invasion could be favoured by other factors. These results are in agreement with Chambers et al. (2014), and Waddell et al. (2020) who report that generally invasion is facilitated by land uses or management activities that disturb native vegetation increase resource availability, and promote the establishment and spread of the species. Moreover, the degree of this disturbance may be partly due to overgrazing (Hobbs and Huenneke 1992; He et al. 2022)

Conclusion

From the results of the study, we can deduce that the predictive competitive ability of *S. obtusifolia* is not due to its biology but due to the presence of other factors. Specifically, the current invasion of *S. obtusifolia* could be favored by overgrazing in the rangeland which reduces fodder species' dominance and competitiveness. Further, good and sustainable management of sahelian rangelands by controlling grazing would help to reduce *S. obtusifolia* invasion and provide more fodder for livestock.

Author contributions

OO and AZ conceived and designed the experiments. OO, KO, PCB, and AZ performed the experiments. KO, PCB, and AZ analyzed the data. AZ wrote the paper. All authors reviewed and approved the final manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Effects of hormone and fertilizers on early flower induction of *Dendrobium anosmum* hybrid seedlings under *ex vitro* condition

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Hybrid.

Ex vivo

ABSTRACT

Early flowering of new orchids is important to save time for selecting valuable flowers and artificial induction of flowering is a critical consideration in the orchid production industry. In this study, a new *Dendrobium anosmum* hybrid was generated by cross-breeding between *D. anosmum* 'Chau Nhu' and *D. anosmum* 'Di Linh'. The ancestors and hybrid seedlings from *in-vitro* culture were trained in the net house and their growth and flowering were evaluated under *ex vivo* conditions with specific fertilizers and hormones. The results suggest that the hybrid plants grew better than their parents in terms of stem height, stem diameter, and leaf number. Growth hormones were applied to stimulate early flowering in matured hybrids and it was discovered that 'Keiki pro', a commercial hormone product, produced the best results, with a flowering rate of 66.67% after two applications. Hybrid flowers varied in width from 36.36% (3.0-6.0 cm) to 63.64 % (more than 6.0 cm) from ancestral width in medium-sized and large-sized flowers, respectively. Also, the hybrid flower colours was mostly a combination of pink/violet (75C) and purple/pink (68A), which is different from their parents. Importantly, the dorsal sepal, petal colours, and shape of hybrid flowers varied significantly among individual hybrids, between hybrids and their progenitors. Some mutations in the lips and columns of the novel hybrid flowers were also visualized. Hence, the *D. anosmum* hybrid seedlings

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successfully induced flowers after a year of culture under optimal hormones and fertilizers conditions. The results can serve as a critical reference for the early flowering of the orchid seedlings.

1 Introduction

Dendrobium is the third-largest genus in the Orchidaceae with an upper range of 1400 species, including a large number of hybrid (Da Silva et al. 2015; Da Silva and Ng 2017), and is also the most important global floricultural, ornamental, and medicinal plants (Leitch et al. 2009; Takamiya et al. 2011; Da Silva and Ng 2017). This genus is mainly distributed across Southeast Asia, including the Philippines, Indonesia, Vietnam, Papua New Guinea, Sri Lanka, Malaysia, and Thailand (Teoh 2005; Xiaohua et al. 2009). *Dendrobium* species have fascinated people since early times because of their sheer beauty with the myriad of shapes and vibrant colours (Da Silva et al. 2014). In 1986, the first species of the *Dendrobium* genus, introduced into Hawaii from the Philippines, was known as *D. anosmum* and became a favorite flower among gardeners (Kamemoto et al. 1999). In Vietnam, there are more than 100 indigenous *Dendrobium* species (Tran 1998), but their natural populations have contracted due to their low rate of regeneration and seed germination, global climate change, human population increase, and over-exploitation for illegal trade (Maharjan et al. 2020). Therefore, the conservation of *Dendrobium* has recently emerged as a global environmental strategy (Kishor et al. 2006).

Hybridization is a method of recombining the genotypes of parents to create hybrid plants with novel characteristics, which is an exciting dimension in the floriculture industry (Kishor et al. 2006). In particular, cross-breeding is an important hybridization method to generate new hybrid orchids, which serve as the primary material for selecting desired flowers with different shapes, free-blooming habits, superior quality, and a blend of colourss. In addition, many *Dendrobium* hybrids produce flowers several times a year, and their flowers are in high demand for the cut-flower and pot plant markets (Da Silva et al. 2015). Some research groups have attempted to cross-pollinate between different orchid varieties to generate novel hybrids. Zheng et al. (2009) reported that the success rate of *D. nobile* cross-pollination was 58.62%, and two kinds of hybrid plants (No.5029 and No. 5041) were developed for mass production. Further, Galdiano et al. (2012) successfully applied cryopreservation protocols for *D.* Swarts hybrid 'Dong Yai' seed germination and seedling development. Hartati and Cahyono (2014) successfully cross-pollinated a female (♀) *D.liniale* and male (♂) *D. bigibbum*, ♀ *D.bigibbum* and ♂ *D.liniale*, ♀ *D. mirbelianum* and ♂ *D.liniale*, ♀ *D. liniale* and ♂ *D. mirbelianum*, and achieved 100% seed germination. Recently, at the Laboratory of Cells, Institute of Biotechnology, Hue University, Vietnam, hybrid seedlings, resulting from a cross between ♀ *D.anosmum* 'Chau Nhu' and ♂ *D.anosmum* 'Di Linh', was successfully transplanted to the orchid net house (unpublished works).

However, hybrid orchid seedlings are often slow to flower in *ex vitro* conditions, usually taking more than one year after transplanting to the greenhouse (Kishor et al. 2006; Sim et al. 2007; Hartati et al. 2021). Kishor et al. (2006) highlighted those hybrid seedlings of *Vanda coerulea* ×*Ascocentrum auranticum* first flowered after two years under *ex vitro* conditions. Sim et al. (2007) showed that many commercial orchid hybrid species took from two to four years for their juvenile periods. Therefore, it takes a long time for researchers and gardeners to characterize novel hybrid species. To reduce juvenile periods, some research groups attempted to stimulate orchid flowering in the early plant growth regulators. Wang et al. (2009) showed the successful *in-vitro* flowering of *D.nobile* Lindl. seedlings on MS (Murashige and Skoog) medium supplemented with PBZ (paclobutrazol) and TDZ (thidiazuron) at different concentrations. The *in vitro* floral bud induction rate was 33.3% to 34.8% after 4 months of culture on ½ strength MS medium supplemented with 0.5 mgL^{-1}PBZ or on MS containing 0.1 mgL^{-1}TDZ. In addition, Guan and Shi (2009) demonstrated that floral buds of *D. denndanum* seedlings were induced at a rate of 10% by culturing on MS medium supplemented with 2.0 mgL^{-1} BA (6-benzylaminopurine). Cen et al. (2010) induced flowering in non-root plantlets of *D. officinate* by culturing on MS medium supplemented with 1.0 mgL^{-1} BA + 0.2 mgL^{-1}NAA (Naphthalene acetic acid), 3.0% sucrose and 0.1% activated carbon (AC). Zhao et al.(2012) reported 96% flower bud initiation in *D. strongylanthum* seedlings cultured on MS medium supplemented by 2 mgL^{-1}BA + 0.2 mgL^{-1}NAA and 3.0% sucrose after 70 days of culture. However, these studies focused on laboratory scale rather than a real application in orchid production. There is a huge market demand for the characterization of new orchid flowers (pot or cutting) in the *exvitro* condition instead of *in vitro* conditions. Unfortunately, studies of *ex vitro* growth and flowering are poorly reported. *D. tosaense* is the first *Dendrobium* orchid successfully acclimated and flowering from *in-vitro* seedlings after 18 months (Lu et al. 2014). Recently, *Anacamptis sancta*, a tuberous orchid was successful in symbiotic germination and flowering under *ex vitro* conditions after 30 months (Deniz et al. 2022). In Vietnam, this research field is currently under-investigatigation, especially in *D. anosmum* hybrid seedlings. Therefore, this study aimed to evaluate the effect of fertilizer and plant growth regulators on *D. anosmum* hybrid flowering induction and to characterize hybrid flower morphology.

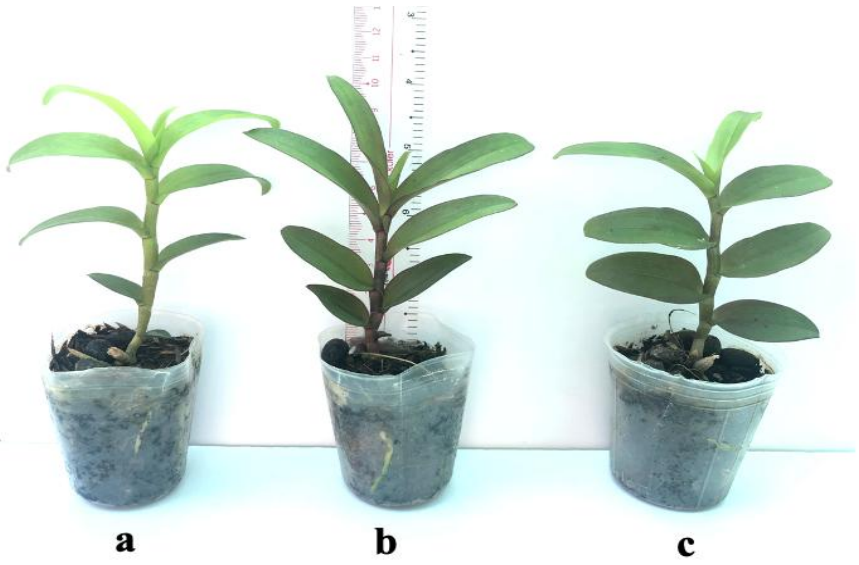

Figure 1 *Dendrobium anosmum* seedlings a) 'Chau Nhu', b) 'Di Linh', and c) hybrid

2 Materials and methods

2.1 Plant materials and cultivation

Three cultivars of *D. anosmum* orchid seedlings were transplanted to *ex vitro* condition at approximately four months. Plants chosen for this study were *D. anosmum* 'Chau Nhu', *D. anosmum* 'Di Linh', and hybrid seedlings of ♀ *D. anosmum* 'Chau Nhu'× ♂*D. anosmum* 'Di Linh' and having 10 cm in height and 8 true leaves (Figure 1). These seedlings were provided by the Laboratory of Cells, Institute of Biotechnology, Hue University, Vietnam. Seedlings were grown under the orchid net house (Institute of Biotechnology, Hue University, Vietnam) without an air conditioning system. The net house was set up with an irrigation system to produce mist which reduces the temperature during hot weather, so the range of temperature in the house was usually 20^{o}C to 35^{o}C. Irrigation was applied to all cultivars following the instruction of the Vietnams National Standard of technical requirements for spray irrigation method (TCVN 9170-2012).

2.2 Methods

2.2.1 Application of fertilizer in optimizing hybrid seedling growth and development

The fertilizer experiments were based on Diem et al. (2021) study of *D. anosmum* 'Di Linh' seedlings cultivation. This experiment was designed to compare the growth indicators of hybrid seedlings with their parent. All the treatments were sprayed once a week with foliar fertilizer which was prepared by dissolving 1 gram of N (nitrogen): P (phosphorus): K (Potassium) 30:10:10 (Grow More; Gardena, CA, USA) and 2 grams of Vitamin B1 (Thailand T-REX) in 1 litter of water. A slow-release fertilizer Hyponex MagamP (NPKMg 6: 40: 6: 15) (Hyponex Japan, Co. Ltd, Osaka, Japan) was applied 5 grams on the surface of every pot, which contained the seedlings after acclimation a month (Diem et al. 2021). Experimental treatments were arranged randomly in a Randomized Complete Block Design (RCBD), each treatment had 3 replicates, and each replicate had 50 plants. Plant growth parameters, including the number of new leaves, shoot height, stem diameter, leaf length, and width were measured at intervals of 10 days for 120 days. Shoot height (in cm) was measured using a measuring tape from the base to the tip of the apical leaf. The leaf length and width (cm) were measured at the longest and widest points with a measuring tape, respectively. The number of new leaves was counted during plant growth, and the stem diameter was measured at the widest point (cm).

2.2.2 Effect of plant growth regulator application on *D. anosmum* hybrid seedling flower induction

This experiment was carried out to identify the effect of plant growth regulators on the diverse flowering of hybrid seedlings. After 4 months of growth in *ex vitro* conditions, the flower induction with PGR was carried out as outlined in Table 1.

Firstly, the apical leaf was slightly injured to exposure meristem and the designed dose of PGR (plant growth hormone) was spread on it as mentioned in table 1. Repeat treatments were carried out on the second and third days. Treated hybrid plants were moved to the shaded area without water for 24 hours to avoid removing the PGR. The treatment had three replicates, and five plants were used for each. After three weeks, data was collected and counting the rate of floral bud, shoot bulb, top shoot disease, inflorescence number, and flower number.

Table 1 The Plant Growth Regulator treatment on the precocious flower of *D. anosmum* hybrid seedlings

Plant Growth Regulator (PGR)	Concentrations	Volume using	Time repeat	References
Keiki pro orchid growth hormone	Proprietary	20 µl	1, 2, or 3 times*	Commercial product from Kelley's Korner Orchid Supplies
TDZ and PBZ	0.1 mgL^{-1} TDZ + 0.5 mgL^{-1} PBZ	20 µl	1, 2 or 3 times*	Wang et al. 2009
BA and NAA	2 mgL^{-1} BA + 0.2 mgL^{-1} NAA	20 µl	1, 2, or 3 times*	Zhao et al. 2012
Control	Without PGR			

* Note: Each time repeat is one day later

Table 2 Growth indicators of three D. anosmum seedlings

Times	Beginning					After four months				
Species	Stem height (cm)	Stem diameter (cm)	Leaf number	Leaf length (cm)	Leaf width (cm)	Stem height (cm)	zStem diameter (cm)	Leaf number	Leaf length (cm)	Leaf width (cm)
Chaunhu	10.11[az]	0.40[a]	8.00[a]	4.40[c]	1.51[b]	18.57[b]	0.57[c]	11.85[b]	5.99[c]	2.00[b]
Dilinh	10.15[a]	0.40[a]	8.00[a]	5.44[a]	1.52[b]	18.59[b]	0.50[b]	12.05[b]	7.47[a]	2.55[a]
Hybrid	10.03[a]	0.40[a]	8.00[a]	5.02[b]	1.57[a]	19.56[a]	0.60[a]	12.45[a]	6.54[b]	2.53[a]
LSD<0.05	ns	ns	ns	<0.05	<0.05	<0.05	<0.001	<0.05	<0.001	<0.05

[z]Means labeled with the same letter in a column were not significantly different at the P <0.05 (Tukey's HSD)

2.2.3 Estimation of morphological characters

The morphological characteristic of hybrid plant flowers was based on De (2020) and colours was assessed following the Royal Horticultural Society (RHS) colours chart. When the blooming of floral buds reached around 80%, the characteristics of flowers were assessed to identify flower variation of hybrid plants including a) flower width (cm) in front view (tip distance of two lateral petals), predominant flower colours, flower fragrance, flower longevity on plants (days); b) dorsal sepal outside and inside colours, outside and inside ornamentation, shape, apex, and curvature; c) lateral sepal outside and inside colours, outside and inside ornamentation, shape, apex, and curvature; d) lip outside and inside colours, outside and inside ornamentation, shape, apex, and curvature; e) column colours and shape (Vu et al., 2022).

2.3 Statistical analysis

Statistical analysis was performed by Microsoft Excel 2016, and by one-way analysis of variance (ANOVA) followed by Turkey's test, using the SPSS statistic 20.0 software (SPSS Inc., Chicago, IL, USA). The difference in the table represents significant differences as $p< 0.05$.

3 Results and Discussion

3.1 Effect of fertilizer on optimizing hybrid seedling growth and development

A combination of foliar fertilizer NPK 30:10:10 and vitamin B1 with a slow-dissolving fertilizer NPKMg (6:40:6:15) showed a positive effect on the growth characteristics of three *D. anosmum* seedlings after four months of experiment (Table 2).

The shoot height of each seedling cultivar dramatically increased from 10 cm to approximately 19 cm The hybrid height was estimated at 19.56 cm which was higher than that of 'Chau Nhu' (18.57 cm) and 'Di Linh' (18.59 cm). Further, the stem diameter in hybrid seedlings was 0.60 cm, which was higher than their parents. In addition, there were more leaves in hybrid seedlings (12.45 leaves) compared to their parent (11.85 leaves in the mother and 12.05 leaves in the father). Interestingly, the leaf length and leaf width of hybrid plants were intermediate, between those of their progenitors (Table 2).

Supplying adequate types and kinds of fertilizer was critical for the vegetative growth stage of all cultivars. The use of fertilizer in this study was consistent with the findings of previous studies (De 2020; Diem et al. 2021; Khuraijam et al. 2017). These authors highlighted that an ample supply of NPK 30:10:10 fertilizer during the vegetative stage of *Dendrobium* species was required not only to facilitate robust shoot and leaf production but also for subsequent orchid flowering. In addition, a slow-release fertilizer NPKMg (6:40:6:15) was also investigated and had been identified as the best for orchid root growth (Kawakami et al. 2008; Sendo et al. 2010). Vitamin B1 helps in increasing plant root system development, chlorophyll production, light photosynthesis, and extreme hot and cold water resistance (Fitzpatrick and Chapman 2020; Herastuti and Siwi Hardiastuti 2020). Both hybrid and parent seedlings of *D. anosmum* in the present study showed improved growth compared to the study on *D.* 'Sunya Sunshine' by Zhang et al. (2019). One year

after transplanting, *D.*"Sunya Sunshine" was approximately just 16 cm tall with six leaves (Zhang et al. 2019), whereas, *D. anosmum* reached approximately 19 cm and had 12 leaves.

Therefore, the use of fertilizers in the recent study optimized the growth of hybrid seedlings. Moreover, the growth parameters of hybrid seedlings, including plant height, stem diameter, and leaf numbers, were significantly greater than those in their progenitor. These outcomes determined the success of the hybridization experiment

3.2 Effect of plant growth regulator on *D. anosmum* hybrid seedling flower induction

The application of PGR significantly influenced the floral bud initiation of the *D. anosmum* hybrid (♀ *D. anosmum* "Chau Nhu" × ♂*D. anosmum* "Di Linh") (Figure 2). Over 40% of hybrid plants were induced floral buds by Keiki pro with the highest induction rate obtained 66.67%, after two treatments. Moreover, the hybrid plants treated with the mixture of TDZ and PBZ showed 53.33% of induced plants had floral buds after three treatments, which was remarkably higher than a single treatment (33.33%) or two treatments (40.00%) (Figure 2). The combination of BA and NAA was less effective on hybrid plant flower induction than Keiki pro, or the mixture of TDZ and PBZ, with the rate of hybrid plant floral bud induction was 20.00% (treatment one time), 33.33% (treatment two times), and 13.33% (treatment three times) (Figure 2).

PGR played a key role in the specific flowering time, especially after the dormancy of hybrid plants. The results revealed a significant finding of this study that precocious flowering can be initiated in hybrid plants one year after transplanting, notwithstanding different effect levels among the treatments. Remarkably, the PGR commercial product was more effective than the two other PGR treatments. This may be due to the particular combination of phytohormones, which may have more PRG factors that activate dormant orchid node growth (Cen et al. 2010). Furthermore, the mixture of PBZ and TDZ induced floral buds of the hybrid plants better than that of BA and NAA, implying that PBZ and TDZ could enhance meristematic activity and influence inflorescence initiation (Huang et al. 2021). In addition, the percentage of hybrids treated with PBZ and TDZ combination achieved significantly higher floral bud initiation and it was higher than those in *in-vitro* culture. For instance, around 34% of *D. nobile* Lindl seedlings induced floral buds when seedlings were treated with 0.5mgL^{-1} PBZ or 0.1 mgL^{-1} TDZ; while it was only 29% in Friederick's *D.orchid* when treated seedlings with 0.05 mg L^{-1} PBZ under *in vitro* condition, respectively (Te-Chato et al. 2009; Wang et al. 2009). The explanation was that PGR had different effects on different kinds of *Dendrobium.*

Besides floral bud induction, PGR also affected the shoot bud stimulation. The rate of shoot bud induction in plants was approximately 20% in all experiments, except for those involving three treatments, which induced 13.33% of plants to form shoot buds (Figure 2). There was no shoot induction in the untreated hybrid plants. TDZ, BA, and IAA are the three main PGRs used in shoot stimulation for the orchid *in vitro* propagation (Naing et al. 2011; Sjahril et al. 2016; Regmi et al. 2017; Luan et al. 2019), so these could also initiate shoot bud formation in orchid plants under *ex vitro* conditions. Moreover, disease at the top of the

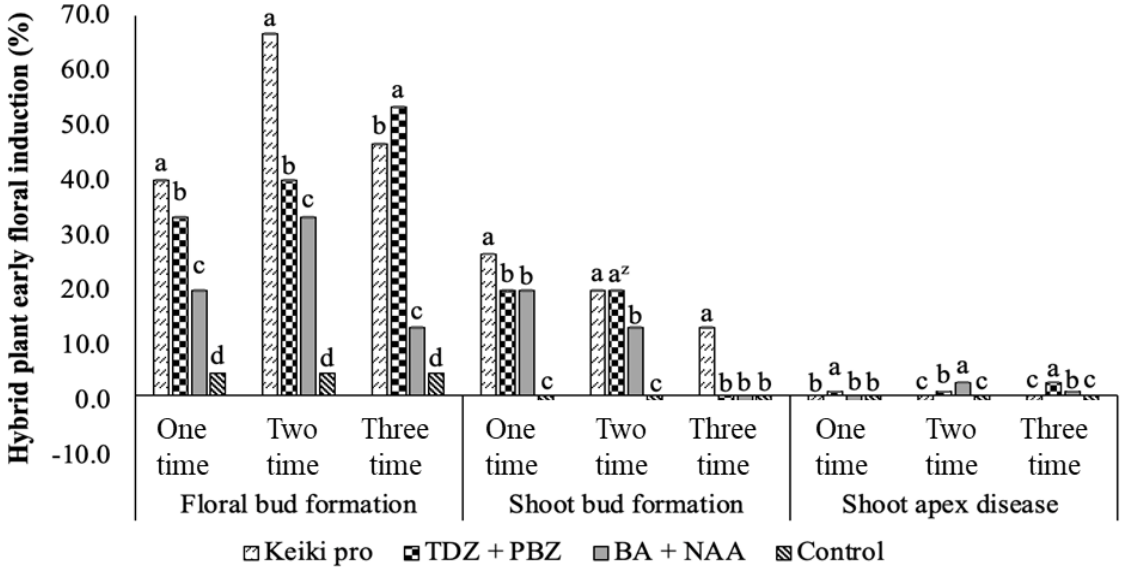

Figure 2 The percentage of hybrid plants induced to form floral buds and shoot buds and the percentage with a disease of the shoot apex when treated with three PGR preparations; ᶻMeans labeled with the same letter were not significantly different at the P < 0.05 level (Tukey's HSD)

Table 3 The Influence of PGR on hybrid plant inflorescence and flower number

Type	Inflorescence number			Flower number		
PGR	One time	Two time	Three times	One time	Two time	[z]Three times
Keiki pro	1.0[az]	1.6[a]	1.0[b]	2.0[a]	3.6[a]	1.6[b]
TDZ + PBZ	1.0[a]	1.2[b]	1.4[a]	1.6[b]	2.0[b]	2.6[a]
BA + NAA	1.0[a]	1.0[b]	1.2[b]	1.6[b]	1.6[c]	1.6[b]
Control	1.0[a]	1.0[b]	1.0[b]	1.5[b]	1.5[c]	1.5[b]
LSD<0.05	Ns	<0.05	<0.05	<0.05	<0.05	<0.05

[z]Means labeled with the same letter within a column were not significantly different at the P < 0.05 level (Tukey's HSD)

Figure 3 Flower characteristics of a) *D.anosmum*'Chau Nhu', b) *D.anosmum*'Di Linh',c) hybrid flowers, d) hybrid flower treated twice with Keiki pro, e) hybrid flower of medium size and f) large hybrid flower

shoot occurred when hybrid plants were treated with PGR. However, this phenomenon did not cause concern as it was less than 4% (Figure 2).

PGR had a critical influence on the inflorescence and flower numbers of hybrid plants (Table 3). In general, hybrid plants had approximately one inflorescence each when treated once with PGR. However, the inflorescence number significantly increased in the plants treated twice with Keiki pro 100000 ppm (1.6 inflorescences/plant) or three times with the mixture of TDZ and PBZ (1.4 inflorescences/plant). Consequently, the highest flower number was 3.6 flowers/plant in plants treated twice with Keiki pro (Table 3).

The number was approximately 2.6 flowers/plant in hybrid plants treated three times with TDZ and PBZ and around 1.6 flowers/plants in all other treatments. These results were in contrast to Mishra et al. (2018), who studied the effect of PGR (GA3, BA, Kinetin, and IAA) on early flower induction in *Phalaenopsis* hybrids and reported that the application of different PGR did not affect the number of spikes/inflorescences per plant. Conversely, Zhang et al. (2019) determined that applying TDZ 30mgL^{-1} as a spray to *D.* "Sunya Sunshine" for early flower stimulation increased the number of inflorescences per plant. These divergent findings could be a result of using different *Dendrobium* in each study.

3.3 Hybrid flower characteristics

Four weeks after floral bud induction, most of the buds were flowering. Hybrid flower characteristics showed dramatic variation compared to those in their parents (Table 4).

Table 4 Comparative analysis of parental and hybrid flower characteristics

Type	Characteristic	Cultivars			[z]Percentage (%)	Type of assessment
		D. anosmum 'Chau Nhu'	*D. anosmum* 'Di Linh'	Hybrid		
Flower	Flower width (cm)	Large (> 7.0 cm)	Large (>7.0 cm)	Medium (3.0-7.0 cm)	36.36	MS
		-	-	Large (>7.0 cm)	63.64	
	Flower predominant colours	Light violet (69C), purple/pink (68A)	Early white (N155B), purple/pink (68A)	Pink violet (75C), Purple pink (68A)	100.00	
	Flower fragrance	Heady	Sweet	Slightly + sweet	58.33	
				Heady	41.67	
	Flower longevity on plants (days)	20	20	20	100.00	
Dorsal sepal	Outside dorsal sepal colours	Light violet (69C),	Early white (N155B), purple/pink (73A)	Early white (N155B), Pink violet (75C)	100.00	VS
	Inside dorsal sepal colours	Light violet (69C),	Early white (N155B), purple/pink (73A)	Early white (N155B), Pink violet (75C)	91.67	
		-	-	Yellow-green (149D), Pink/violet (75C)	8.33	
	Outside dorsal sepal ornamentation	Absent	Straight stripe	Straight stripe	100.00	
	Inside dorsal sepal ornamentation	Absent	Straight stripe	Straight stripe	100.00	
	Dorsal sepal shape	Lanceolate	Lanceolate	Lanceolate	83.33	
		-	-	Oblong	16.67	
	Dorsal sepal apex	Acute	Obtuse	Acute	100.00	
	Dorsal sepal curvature	Deflexed	Deflexed	Deflexed	100.00	
Lateral sepal	Outside lateral sepal colours	Light violet (69C),	Early white (N155B), purple/pink (73A)	Early white (N155B), Pink violet (75C)	100.00	VS
	Inside lateral sepal colours	Light violet (69C),	Early white (N155B), purple/pink (73A)	Early white (N155B), Pink violet (75C)	100.00	
	Outside lateral sepal ornamentation	Absent	Absent	Straight stripe	100	
	Inside lateral sepal ornamentation	Absent	Straight stripe	Absent	83.33	
				Stripe/ Netted	16.67	
	Lateral sepal shape	Lanceolate	Lanceolate	Lanceolate	100.00	
	Lateral sepal apex	Acute	Acute	Acute	100.00	
	Lateral sepal curvature	Deflexed	Deflexed	Deflexed	100.00	

Type	Characteristic	Cultivars			[z]Percentage (%)	Type of assessment
		D. anosmum 'Chau Nhu'	*D. anosmum* 'Di Linh'	Hybrid		
Petal	Outside petal colours	Light violet (69C),	Early white (N155B), purple/pink (73A)	White (N999D)	16.67	VS
		-	-	Early white (N155B), Pink/violet (75C)	83.33	
	Inside petal colours	Light violet (69C)	Early white (N155B), purple/pink (73A)	White (N999D)	16.67	
		-	-	Early white (N155B), pink/violet (75C)	16.67	
		-	-	Early white (N155B), pink/violet (75D)	66.66	
	Outside petal ornamentation	Absent	Absent	Absent	100.00	
	Inside petalornamentation	Absent	Straight stripe	Absent	83.33	
		-	-	Stripe/ Netted	16.67	
	Petal shape	Oblong	Oblong	Oblong	16.67	
		-	-	Ovate	66.66	
		-	-	Elliptic	16.67	
	Petal apex	Obtuse	Acute	Acute	40.00	
				Obtuse	60.00	
	Petal curvature	Deflexed	Deflexed	Deflexed	100.00	
Lip	Lip outside colours	Base: purple/pink (68A); Apex: white (155A)	Base: purple/pink (68A); Apex: pink violet (75C)	Base: Purple/pink (68A); Apex: white (155A)	100.00	VS
	Lip inside colours	Base: purple/pink (68A); Apex: white (155A)	Base: purple/pink (68A); Apex: pink/violet (75C)	Base: Purple/pink (68A); Apex: white (155A)	100.00	
	Lip shape	Ovate	Heart	Heart	28.94	
		-	-	Obovate	66.66	
		-	-	Mutation	4.40	
	Lip apex	Acute	Acute	Obtuse	50.00	
		-	-	Acute	33.33	
		-	-	Retuse	16.67	
	Lip curvature	Straight	Straight	Straight	100.00	
	Lip margin	Fimbriate	Fimbriate	Fimbriate	100.00	
Column	Column colours	Purple/pink (68A)	Purple/pink (68A)	Purple/pink (68A)	100.00	VS
	Column shape	Round	Round	Round	95.60	
		-	-	Mutation	4.40	

[z]Percentage of hybrid flower varieties; MS: measurement of individual plant number or parts of plants; VS: visual assessment by observations of individual plants or parts of plants

In general, hybrid flowers have one dorsal sepal, two lateral sepals, two petals, one lip, and a column, similar to their ancestors, however, some other traits were significantly varied (Figure 3a, 3b, 3c, and 3d). Taking large (> 6 cm) parental flower width as 100%, hybrid flower width was 36.36% in a medium-sized (3.0-6.0 cm) and 63.64% in large-sized flowers (Table 4, Figure 3e, 3f). The segregation of this trait may be due to early flower induction, and the vegetative growth of some hybrid plants was not as pronounced as that of others. The cross-breeding of the mother flower, which was light violet (69C) and purple/pink (68A), and the father flower, which was early white (N155B) and purple/pink (68A) generated hybrids with the dominant colours was a combination of pink/violet (75C) and purple/pink (68A) (Figure 3a, 3b, and 3c). The fragrance of novel flowers was a combination of their originator fragrance with slightly sweet and heady aromas (Table 4), whereas flower longevity was the same as their parent's (20 days).

The dorsal sepal colours of hybrid flowers was significantly different from those of their progenitors. The dorsal sepal colourss of 91.67% of plants were early white (N155B) and pink/violet (75C) while those of 8.33 % were yellow-green (149D) and pink/violet (75C) colourss compared to those of their mother (light violet -69C) and father (early white - N155B) and purple/pink (73A) (Figure 3a, 3b, and 4a). Outside and inside dorsal sepal ornamentation of the hybrid flowers involved a straight stripe that was similar to their father (Table 4). The dorsal sepal apex of the hybrids flowers was 83.33% similar to their parents, which showed a lanceolate shape, and 6.67% was oblong shaped, which differed from their ancestors (Figure 3a, 3b, 3e and 3f).

The lateral sepal colours of hybrid flowers was the same as their dorsal sepal colours and differed only slightly from those of their ancestors. Excitingly, the most variable change was in the petal shape of hybrid flowers. The petal shape was not only oblong (16.67%) as seen in their parents but also ovate (66.66%) and ellipse (16.67%) (Figure 4b). Outside and inside lip colour were inherited from their mother plants, but lip shape and apex traits were more varied than in the parents (Table 4). Hybrid lips were heart-shaped (28.94%), obovate-shaped (66.66%) or mutated (4.40%) (Figure 4c, 4d, and 4e), however, the mother and father flower lip shapes were ovate and heart-shaped, respectively (Figure 3a and 3b). Hybrid flower lip apex was obtuse (50%), acute (33.33%), or retuse (16.67%) as compared to only acute in their ancestors (Table 4). Mutation of hybrid flowers also occurred in the column shape of 4.40% of plants (Figures 4f and 4g).

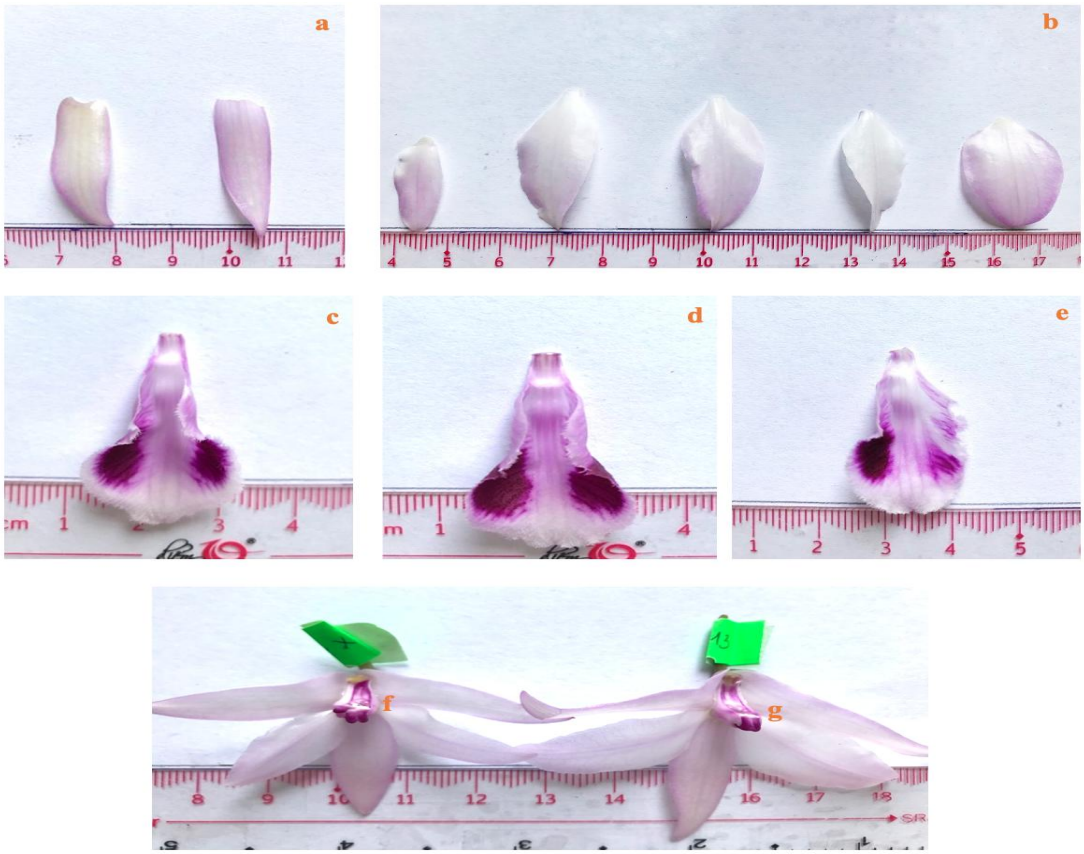

Figure 4 Hybrid flower characteristic. a) dorsal sepal colours and shape assessment, b) petal colours and shape assessment, c) lip heart shape, d) lip obovate shape, e) lip mutation, f) column mutation and g) normal column

The current study reports for the first time that the characteristics of hybrid varieties of ♀ *D. anosmum* 'Chau Nhu' X ♂*D. anosmum* 'Di Linh', despite previous attempts to investigate the commercially important, flowering hybrid orchid family (Mishra et al. 2018; Zhang et al. 2019). The results of this study revealed variations in the traits of *D. anosmum* hybrid flowers, especially the segregation of dorsal sepal and petal traits. In addition, mutations occurred in lip and column traits of hybrid flowers. These followed Mendel's theory of inheritance that cross-pollination uncovers variations inherited by hybrid plants from their ancestor (Bateson and Mendel 2013; Ellis et al. 2011). Therefore, the outcomes of this study would be a valuable resource for further study on the genetic variation of *D. anosmum* hybrid flowers.

Conclusions

The Fertilizer was optimized to maximize the vegetative growth of *D. ansomum* hybrid plants. The application of PGR at a specific time point induces the highest level of precocious flowering in hybrid plants around one year after transplanting to *ex-vitro* conditions. The study identifies various traits of hybrid flowers, adding to *D. anosmum* orchid historical research. Further research is needed to study the genetic variation of these hybrid flowers and the genetic events behind the phenotypic changes.

Conflicts of interest and financial disclosures

The authors declare no competing interests.

Author contribution

T.O.N, T.D.N. and T.K.C.N. designed the experiment. T.D.N., T.O.N., T.T.H and H.T.N carried out the experiments and data analyses. T.D.N. and T.O.N. prepared all of the figures, and all authors contributed to data interpretation. T.D.N. wrote the first draft of the manuscript, T.T.H and T.K.C.N edited the draft. All authors reviewed the manuscript.

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Aspirin regulates oxidative stress and physio-biochemical attributes in *Brassica juncea* under cadmium toxicity

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Toxicity

ABSTRACT

The current study aimed to evaluate the effects of aspirin (Asp) on growth, physio-biochemical variables, and oxidative stress in *Brassica juncea* subjected to cadmium toxicity. Cadmium (Cd) toxicity decreased the root and shoot development by 67.53 % and 64.4 % respectively, over the control. However, treatment with Asp showed improved root and shoot growth in Cd treated seedlings. This study demonstrates elevation in total soluble sugar (TSS), proline, and glycine betaine levels and suppressed total protein concentrations in Cd treated seedlings over control. On the treatment of Asp to Cd exposed plants, an enhanced level of the above said variables was reported. The activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and ascorbate (ASC) increased in plants with Cd stress over control, followed by enhanced elevation of the same on supplementation of Asp. Supplementation of Asp reduces the accumulation of malondialdehyde (MDA) and H_2O_2, confirming the plant metals' stress protection properties of Asp. Thus studies confirm aspirin's involvement in protecting plant growth and development against cadmium toxicity.

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1 Introduction

Plants being immobile are exposed to a wide range of abiotic and biotic stress. Among abiotic stress, metal-induced toxicity is the most prevailing cause of extensive water and soil pollution (Ahmed et al. 2016). Few metals are important for the regular functioning of plants, but, many more are deleterious and impede normal plant growth and development (Ahmed et al. 2012). Cadmium is an environmental pollutant and biologically toxic metal (Godt et al. 2006). Cadmium tends to build up in the soil of anthropogenic and natural activities (Vitória et al. 2001). Elevated levels of heavy metals in the environment, particularly soil result in the generation of reactive oxygen species (ROS) (Liu et al. 2010), this impacts the germination of seeds, growth, and development of plants (Jonak et al. 2004) and disrupts the transport of electrons in the chloroplast (Cui and Wang 2006). Heavy metals also impede photosystems I, and II and interfere with the transfer of K^{2+}, $Ca^{2+,}$ and abscisic acid in the guard cells of the plants. Further, these metals also disturb the Ca^{2+}, $Zn^{2+,}$ and Fe^{2+} in proteins which results in the release of free radicals (Minglin et al. 2005). Metals cause the oxidation of biomolecules causing oxidative stress and thus cell damage (Romero et al. 2002). Oxidative stress results in lipid peroxidation, leading to cell membrane disruption (Nouairi et al. 2006). Plants prevent the harmful effect of free radicals by enhancing the generation of antioxidant enzymes during heavy metal stress. Plants have inbuilt enzymatic and nonenzymatic systems like catalase, ascorbate peroxidase, and superoxide dismutase to scavenge free radicals generated from oxidative stress due to phytotoxicity (Sharath Chandra and Sukumaran 2020).

Exposure to cadmium toxicity changes the enzyme activity of superoxide dismutase, catalase, and ascorbate peroxidase, which are essential to preserving normal cellular hydrogen peroxide levels to shield the cell from oxidative stress-induced cellular and tissue damage. Antioxidant enzymes like catalase, SOD, and peroxidase are elevated during stress (El-Beltagi et al. 2010). Catalase and peroxisomes are found in the cytosol and peroxisomes of plants respectively. Cadmium-associated deprivation of glutathione causes intracellular hydrogen peroxide accumulation which results in cell death (Schutzendubel and Polle, 2002). Similarly, the accumulation of proline during heavy metal stress facilitates protecting the biomolecules from denaturation, thus increasing the plant tolerance to abiotic stress (Lesko and Simon-Sarkadi 2002).

Salicylic acid (SA) is not only a plant growth regulator but also an important non-enzymatic oxidant, playing a significant part in numerous physiological mechanisms in plants (Fariduddin et al. 2003). Acetylsalicylic acid or aspirin (Asp) is one among the many derivatives of salicylic acid. It elicits plants' defense mechanisms against diseases and protects plants from viral, bacterial, and fungal infections. Aspirin mimics the role of plant growth hormone and is involved in the promotion of plant growth. Thus, aspirin functions similarly to salicylic acid as a plant hormone (Pallag et al. 2014). *Brassica juncea* (Indian mustard) is extensively used as a model plant for phytoremediation, because of its higher biomass and capacity to accumulate high concentrations of heavy metals, like cadmium up to 400 μg/g DW in shoots (Haag-Kerwer et al. 1999). *B. juncea* possesses ten times more biomass generation capacity as compared to other heavy metal accumulators. It also shows a fast growth rate and collects other toxic heavy metals available in the soil. Thus *B. juncea* is selected as a suitable plant system for phytoremediation studies (Salt et al. 1998). The main purpose of the present study is to understand the role of aspirin (Asp) in tolerating cadmium-induced toxicity in *B. juncea* by studying growth and development, physio-biochemical variations, and oxidative stress.

2 Materials and Methods

2.1 Seed collection and Experimental setup

Certified and viable seeds of *B. juncea* were surface sterilized for 10 min with a 5 % sodium hypochlorite (NaOCl) solution. Further, priming of seeds was performed with 0.5mM of aspirin (Asp), for 10 h. Aspirin exposed and non-exposed seeds were grown in Petri dishes covered with Whatman filter paper which is set aside in a growth chamber with a photoperiod of 24 hrs and incubated for 8 days. Eight days old germinated seedlings were further transferred to trays containing perlite: sand: peat (1:1:1 v/v/v) added with 200 µM of cadmium solution (cadmium chloride). The control plants were subjected to only distilled water. Each exposure is the mean of three replications and each replicate contains five plants. The samples were obtained for experimentation 12 days after treatment. Determination of cadmium and aspirin concentrations was made based on earlier reports (Senaratna et al. 2000; Shanmmugaraj et al. 2013).

2.2 Plant growth analysis and biomass accumulation

The amount of germinated seed was counted and the ratio of germination was calculated using the formula

$$\text{Germination (\%)} = \frac{\text{Total number of seeds germinated}}{\text{Total number of seeds inoculated}} \text{ x } 100$$

The length of the root and shoot were measured manually using a scale. Dry weight (W) of the root, shoot, and leaves of the mustard plant was dried for 60 h at 65ºC in the oven and then evaluated.

2.3 Estimation of Glycine betaine and proline levels

Glycine betaine levels were evaluated based on Grieve and Grattan (1983) method. The results were determined at 365 nm by spectrophotometer. The dosage for glycine betaine was taken at

50-200 mgml^{-1} which was dissolved in 1N H_2SO_4. Levels of proline were evaluated by Bates et al. (1973) method. Absorbance was analyzed at 520 nm in a spectrophotometer, with toluene as blank.

2.4 Estimation of total soluble sugars (TSS) and total protein

Total soluble sugars (TSS) were determined by Dev (1999) method. The absorbance was determined at 485nm using a spectrophotometer. Total protein content was assessed by Lowry et al. (1951) method. The absorbance was recorded at 595 nm by spectrophotometer with BSA as control.

2.5 Determination of Lipid peroxidation (MDA), Hydrogen peroxide (H_2O_2), and Ascorbate

Lipid peroxidation (accumulation of malondialdehyde "MDA") was determined by the method of Heath and Packer (1968). Optical density was recorded at 600 nm with 20 % TCA (trichloroacetic acid) and 1% TBA (thiobarbituric acid) as blank. Hydrogen peroxide level was determined by the procedure of Velikova et al. (2000). Hydrogen peroxide concentration was expressed as µM g^{-1} FW. Ascorbate was measured by the method of Foyer et al. (1983). The absorbance was recorded at 265 nm and expressed as µM g^{-1} FW.

2.6 Antioxidant enzyme assays

Plant material (2g) was homogenized at pH 7.5 in 100 mM Tris HCl in the presence of 10 mM magnesium chloride, 5 m Mdithiothreitol, 1 mM EDTA, 1.5% polyvinyl pyrolidone, 5 mM magnesium acetate and 1 mM phenylmethanesulfonyl. The sample was filtered, and the homogenate was centrifuged for 15 min at 10,000 rpm. Subsequently, after the centrifugation, the supernatant was used as a source of enzyme. For determination of APX activity, tissues were homogenized separately with 2 mM Ascorbate.

Superoxide dismutase (SOD) activity estimation was performed according to Kono (1978), which resulted in the photo-reduction of nitroblue tetrazolium (NBT). The readings were taken at 540 nm in a spectrophotometer. SOD unit indicates the enzyme quantity that impedes 50 % photo-reduction of nitroblue tetrazolium. SOD levels were expressed as U g^{-1} FW.

Catalase activity was determined by the procedure of Aebi (1984). The absorbance was read at 240 nm in a spectrophotometer and reported as mmol g^{-1} FW. APX activity was assessed by the method of Nakano and Asada (1981). The absorbance was measured at 265 nm, and the activity was reported as mmol min^{-1} g^{-1} FW.

2.7 Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) and Duncan`s multiple range test (DMRT) was employed to determine the significant difference between the samples. The values indicate the mean ± SE (n=3). $P \leq 0.05$ significantly differs.

3 Results

3.1 Effect of Aspirin on germination and growth under cadmium stress

The observations regarding the influence of cadmium and aspirin on the germination and growth of *B. juncea* are shown in Table 1. The toxicity of cadmium reduces the percentage of germination by 75.86% in comparison to the control. But the application of aspirin to cadmium exposed plants exhibited only a 3.44 % decrease in germination over the control. Further, cadmium stress led to a decrease in the length of both root and shoot (Table 1). Root length declined by 67.53 % with cadmium application, however, plants exposed to cadmium in presence of aspirin demonstrated enhanced root length and were almost similar to the control. Shoot length was reduced by 64.4 % under cadmium influence against control (Table 1). Supplementation of aspirin improved the shoot length by 71 % when compared to cadmium-applied plants alone. Dry weight (DW) was reduced by 57 % in cadmium-treated plants over the control. Aspirin co-application with cadmium exhibited enhanced dry weight by 56 % over the plants exposed to only cadmium.

3.2 Aspirin betters Glycine betaine and proline levels under cadmium toxicity

Cadmium toxicity increased glycine betaine content by 24.23 % in treated plants over the control (Table 2). However, aspirin co-

Table 1 Effect of Cd (200 µM) and Aspirin (0.5 mM) individually and in combination on various growth parameters of *Brassica juncea*

Treatment	Germination	Root length (cm)	Shoot length (cm)	Dry weight (mg)
Control	87 ± 0.57^{d}	6.53 ± 0.21^{d}	13.12 ± 0.44^{d}	63.52 ± 0.25^{d}
Aspirin	85 ± 0.71^{d}	6.66 ± 0.43^{d}	13.85 ± 0.52^{d}	61.65 ± 0.14^{d}
Cadmium	21 ± 0.14^{b}	2.12 ± 0.29^{b}	4.67 ± 0.06^{b}	27.53 ± 0.19^{b}
Cd+Asp	84 ± 0.32^{a}	6.48 ± 0.41^{a}	13.55 ± 0.26^{a}	63.37 ± 0.07^{a}

Date values are the means of three replicates ± SE. All the values represent a significant difference ($P \leq 0.05$). Similar superscript denotes no significant difference between the means of the treatment in the columns.

Table 2 Effect of individual and combined application of Cd (200 µM) and Aspirin (0.5 mM) on Protein, Total soluble sugar (TSS), proline, and glycine betaine level in *Brassica juncea*

Treatment	Protein mg g^{-1} FW	TSS µgg^{-1} FW	Proline µgg^{-1} FW	Glycine betaine µmolg^{-1}FW
Control	7.65 ± 0.24[d]	4.66 ± 0.18[d]	9.62 ± 0.04[d]	3.72 ± 0.12[d]
Aspirin	7.81 ± 0.43[d]	4.95 ± 0.16[d]	14.19 ± 0.25[c]	3.79 ± 0.55[d]
Cadmium	3.77 ± 0.51[b]	6.22 ± 0.37[b]	17.33 ± 0.51[b]	4.91 ± 0.02[b]
Cd+Asp	6.53 ± 0.15[a]	8.16 ± 0.12[a]	26.48 ± 0.28[a]	5.53 ± 0.19[a]

Date values are the means of three replicates ± SE. All the values represent a significant difference (P ≤0.05). Similar superscript denotes no significant difference between the means of the treatment in the columns

Table 3 Effect of an individual or combined application of Cd (200 µM) and aspirin (0.5 mM) on various enzymes and oxidative stress markers

Treatments	Antioxidant enzymes			Non-enzymatic oxidants	Oxidative stress markers	
	SOD (U g-1 FW)	CAT (mmolg-1 FW)	APX (mmolmin-1 g-1 FW)	ASC (µmg-1FW)	MDA (µMg-1FW)	H_2O_2 (nmg-1 FW)
Control	45 ± 0.12[d]	0.58 ± 0.06[d]	2.54 ± 0.17[d]	253 ± 1.2[d]	2.19 ± 0.04[d]	336 ± 1.6[d]
Aspirin	52 ± 0.55[c]	1.02 ± 0.13[d]	2.42 ± 0.23[d]	295 ± 1.7[c]	2.21 ± 0.16[d]	323 ± 2.6[d]
Cadmium	69 ± 1.2[b]	1.72 ± 0.55[b]	4.17 ± 0.44[b]	342 ± 0.9[b]	7.05 ± 0.32[b]	697 ± 3.4[b]
Cd+Asp	76 ± 0.73[a]	2.11 ± 0.09[a]	6.09 ± 0.36[a]	422 ± 1.5[a]	4.16 ± 0.07[a]	431 ± 1.5[a]

Date values are the means of three replicates ± SE. All the values represent a significant difference (P ≤0.05). Similar superscript denotes no significant difference between the means of the treatment in the columns

application to cadmium exposed plants demonstrated a further increase of 12 % over cadmium treated plants alone. Proline levels were also increased under cadmium stress by 45 % when compared to the control (Table 2). Application of aspirin along with cadmium exhibited further accumulation of proline levels by 35 % over cadmium alone treated plants.

3.3 Aspirin increases total soluble sugar and total protein under cadmium toxicity

Plants exposed to cadmium stress demonstrated an elevation of 25 % in total soluble sugar as compared to the control (Table 2). However, the co-application of aspirin to Cd-induced stressed plants further elevated the total soluble sugars by 24 % over cadmium exposed plants. While in the case of total protein levels, it diminished by 50 % in cadmium stress-induced plants as compared to the control (Table 2). Supplementation of aspirin to cadmium stressed plants exhibited a 53 % increase in protein levels over cadmium alone treated plants.

3.4 Aspirin preserves MDA and hydrogen peroxide content under cadmium toxicity

Accumulation of malondialdehyde (MDA) was 70 % under cadmium toxicity as compared to the control. Aspirin co-application with cadmium demonstrated a reduction in MDA accumulation by 41 % in comparison to cadmium treated plants (Table 3). Hydrogen peroxide levels in plants increased by 52 % under cadmium stress over the control (Table 3). However, the addition of aspirin to cadmium exposed plants lowered the accumulation of hydrogen peroxide by 38 % in comparison to only cadmium treated plants. Plants treated with only aspirin displayed no significant changes in MDA and hydrogen peroxide levels.

3.5 Impact of aspirin and cadmium on antioxidant enzyme activity

The observations of antioxidant enzymes and non-enzymatic responses on individual or combined application of cadmium and aspirin treated *B.juncea* plants are presented in table 3. Further, superoxide dismutase activity increased by 35 % in cadmium treated plants; further elevated by 41 % upon co-application of aspirin to cadmium exposed plants. Catalase and APX elevated by 66 % and 39 % respectively in cadmium stresses plants in comparison to the control (Table 3). However, aspirin supplementation to cadmium treated plants further elevated by 73 % and 59 % over cadmium stressed plants alone. The Ascorbate (ASC) levels also increased by 26 % in cadmium stressed plants over control; further, the levels of ascorbate enhanced by 40 % on co-application of aspirin to only cadmium treated plants.

4 Discussion

Abiotic stress due to heavy metal pollution is a globally frequently seen phenomenon. At higher concentrations, heavy metals can aggregate in plants that cause toxic effects leading to undesirable

variations in morphological, biochemical, and physiological mechanisms (Chandra et al. 2017; Mahadimane and Chandra 2020;). In this study, cadmium treated plants demonstrated significant variation in the germination of seeds, biomass content, root length and shoot length over the control plants. The seeds treated with cadmium exhibited underdeveloped growth and limited root formation. Inhibition of plant growth and decrease in biomass content is the primary phenomenon that takes place in response to abiotic stress due to heavy metal toxicity (Shekhawat et al. 2010; Rashmi et al. 2019). The reduction in the root length and shoot length observed may be associated with the buildup of cadmium in the plant roots that diminishes the uptake of minerals and water, which will eventually influence the plant's biochemical mechanisms and physiology. Elevated concentration of cadmium in the soil leads to root tip damage, a decrease in the transportation of water to multiple tissues that in due course results in declined transpiration rate and inhibition of photosynthesis by disturbing the enzymes catalyzing the Calvin cycle, finally leading to stunted growth (Baudhha and Singh 2011; Shanmmugaraj et al. 2013; Ranjitha and Sharath Chandra 2020). During metal induced stress third of the root growth decreased and it matched with the underdeveloped shoot system. The observations were in correlation with Baudhh and Singh (2011) and Shanmmugaraj et al. (2013).

Cadmium toxicity elevates proline levels in the current study (Table 2) and results are in correlation with the findings of Sirhindi et al. (2016) who also described the elevation of proline levels in *T. aestivum* under heavy metal toxicity. Proline buildup under heavy metal toxicity has been identified as a potential marker of abiotic stress tolerance (Ashraf and Foolad 2007). Proline helps in rebuilding chlorophyll, activates the citric acid cycle, and amounts to the energy source (Ramon et al. 2003). Proline also plays a major role in osmotic regulation and stabilized biomolecules. Proline possesses the ability to scavenge free radicals and protect cells and tissues from oxidative damage (Ahmad et al. 2015). Glycine betaine (GB) also increases with cadmium toxicity (Table 3) and is reported as an important solute under heavy metal stress (Munns 2005). Similarly, glycine betaine also plays multiple roles in maintaining membrane integrity, stabilizing the PS II complex, osmotic regulation, preserving RUBISCO activity, and detoxification of reactive oxygen species (Ashraf and Foolad 2007). Glycine betaine is also involved in protecting the protein's structural integrity from stress due to heavy metals (Sakamoto and Murata 2002). Under metal toxicity, glycine betaine and proline have been shown to regulate gene expression by activation of transcription and replication (Rajendrakumar et al. 1997). Aspirin, a derivative of natural plant growth regulator salicylic acid, was found to be playing a potentially similar biological role to that of salicylic acid in the present study. Proline with free radical scavenging capacity might be triggered by aspirin to defend the cell from the oxidative burst. Glycine betaine content elevates aspirin exposure and the results correlate with reports on plant growth regulators by Gao et al (2004). External application of aspirin enhances the GB levels due to the up-regulation of betaine aldehyde dehydrogenase (BADH) expression (Gao et al. 2004).

Soluble proteins are known to reduce with an increase in heavy metal toxicity (Perva et al. 2020). The reduction in protein levels in response to cadmium toxicity and similar heavy metal stress increases protease activity, which causes protein degradation (Palma et al. 2002). Cadmium caused protein content decline, which may be due to the production of free radicals and binding of heavy metals to protein –SH groups that denature protein structure and further diminish the activity of –SH-containing enzymes (Seregin and Kozhevnikova 2006). In the present study, aspirin increases the total protein levels which corroborates with reports on similar plant growth regulators. Several reports suggest that many proteins synthesized during abiotic stress may be due to growth regulators like salicylic acid, and jasmonic acid (Thaler 1999). Plant stress regulators have been known to increase the expression of various proteins during abiotic stress. The elevation in total soluble sugar levels under cadmium toxicity may be attributed to over resistance of photosynthetic organelle (Prokopiev 1978) and depleted transport of starch to cells of the mesophyll. Increased accumulation of toxic heavy metals disrupts the metabolism of carbon because of the undesirable interaction of the ribulose-bisphosphate carboxylase enzyme (Stiborova et al. 1987). Elevated sugar may also be associated with the degradation of starch. Sugar build-up results in the plants absorbing extra water from the neighboring environment (Hajar et al. 1996)

Hydrogen peroxide is a lethal reactive oxygen species (ROS) and increases with cadmium stress, and the findings (Table 3) correlate with Hao et al. (2006). Heavy metal toxicity is also known to facilitate the accumulation of hydrogen peroxide in wheat leaves (Gajewska et al 2006). Malondialdehyde (MDA) is a result of lipid peroxidation and is an indicator of oxidative stress (Chandra and Sukumaran 2020). Heavy metal stress has been reported to increase hydrogen peroxide and lipoxygenase activity, which causes lipid peroxidation. Increased MDA content is also reported in eggplant during heavy metal stress (Pandey and Rajeev 2010). Additionally, several heavy metals are reported to cause an elevation in MDA content in *Bruguiera gymnorrhiza* thus considered an important biomarker in the identification of abiotic stress (Zhang et al. 2007). In the present study co-application of aspirin curtails the generation of hydrogen peroxide, which can preserve the impact on membrane lipids. Reduction in MDA levels during cadmium toxicity with supplementation of growth regulator was also reported in *Kandelia obovata* (Chen et al. 2014). Aspirin might play a similar role to other plant regulators in scavenging free radicals thus avoiding the accumulation of hydrogen peroxide, avoid lipid peroxidation, and accumulation of MDA.

Ascorbate, which is found in most flora and fauna, is an important non-enzymatic antioxidant synthesized in the mitochondria. Ascorbate levels were increased in cadmium induced toxicity in the present study. The oxidative outburst caused by cadmium is negated by the ascorbate cycle thus defending cellular damage (Singh et al. 2006), however, supplementation of aspirin further increased the concentration of ascorbate, confirming the antioxidant system response in plants for the growth regulator.

The elevation in antioxidant enzyme activities can be seen in Table 3 and agrees with the study of Awasthi and Sinha (2013) conducted on *Luffa cylindrical* during heavy metal toxicity. Superoxide dismutase (SOD) which is considered the primary defense enzyme system in living beings increases under metal toxicity in eggplant (Pandey and Rajeev 2010). Heavy metal stress is found to increase ascorbate peroxidase activity in several plant models as wheat (Gajewska et al. 2006) and rice (Maheshwari and Dubey, 2009). Increased catalase and ascorbate peroxidase activity has been reported in *Wolfia arrhiza* under abiotic stress due to heavy metals (Piotrowska et al. 2009). Aspirin increases the activity of antioxidant enzymes in the present study (Table 3), which coincides with the findings of Chen et al. (2014).

B. juncea is shown to increase the expression of the catalase 3 gene (CAT3) under cadmium toxicity (Minglin et al. 2005). Gene expression of SOD, CAT, and APX is also been reported to increase in Chickpea under saline conditions (Rasool et al. 2013).

Conclusion

Elevated levels of cadmium in the soil are harmful to plant growth, development, and productivity. Biochemical and physiological stress induced by cadmium toxicity is irreversible and leads to adverse effects on root and shoot growth, biochemical variables, and oxidative stress in the present study. However, the co-application of aspirin alleviates the toxic effects of cadmium through the regulation of osmotic and biochemical mechanisms. Application of aspirin in soil known to be affected with cadmium content may be a sustainable way to protect the plants and thus enhance their productivity.

Conflict of interest

None declared

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Genetic improvement for drought tolerance in rice using mutation induction

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ABSTRACT

Thirty-three percent of the world's farmland is subject to drought, making it the most difficult abiotic stress on rice production. Ten different M_4-rice mutants were tested, along with three check varieties (Giza 179, Sakha 107, and IET1444 - International check variety for drought stress), to see how well they fared in drought conditions. These genotypes were tested in well-watered (WW: irrigation every 4 days), water-stressed (WS1: irrigation every 8 days), and severe water-stressed (WS2: irrigation every 12 days) conditions across generations M_5 to M_8. Drought stress was measured regarding its effect on agronomic traits and drought tolerance indices. Of the ten tested mutants, seven high-tillering mutants had higher yields under normal and stress conditions than the check varieties did in the field. The STI, MP, YI, and GMP indices show that, compared to IET444 (DT check variety), the mutant EN25 performed best under drought stress, followed by the mutant EN27. According to the data analysis of SCoT markers, only 34 of the 46 primers used amplified 377 bands (alleles) across 53 different markers. There was a wide range of genetic similarities among mutants, parents, and the check varieties, and it ranged from 17% to 78%. These seven mutants shared 13 common bands with the most drought-tolerant check variety (IET444) using SCoT markers, which indicates that these mutants carried some drought-tolerant genes. Hence, these mutants hold great potential for use in drought-stressed rice breeding programs.

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1 Introduction

Rice, *Oryza sativa* L is the staple food source of more than half of the world's population (Rasheed et al. 2020). Rice is the second largest source of calories in the human diet after wheat, accounting for 20% of the total dietary energy supply worldwide (Babaei et al. 2011). Furthermore, it is grown on about 164 million hectares around the world, with a total production of 756.7 million tonnes. Many restrictions exist in Egypt due to limited water resources; rice is grown on approximately 0.66 million hectares each year, yielding 5.5 million tonnes (FAO 2020). Producing promising drought-tolerant rice cultivars with high yielding is one of the main targets for rice breeders. Drought stress is a severely limiting factor to rice production and quality, which decreased the agronomic traits and rice yields by 53-92% (Lafitte et al. 2007). Drought stress affects more than one-third of the world's total cultivated area. Drought resistance is a plant's ability to produce its maximum economic yield when water is scarce (Moussa 2011; Rollins et al. 2013). It is a complex trait that is determined by the action and interaction of various morphological, biochemical, and physiological responses. Breeding for drought-tolerant rice varieties is a thought-provoking task because of its complex nature and multigenic control.

Breeding rice varieties that are resistant to drought stress provides an economically viable and long-term solution for increasing rice productivity (Pandey and Shukla 2015). Mutation breeding has proven to be an effective method for introducing new traits that may lead to crop improvement and can be used in conjunction with plant breeding (Babaei et al. 2010). Mutation induction techniques can be utilized for crop improvement through increasing genetic diversity, which enables plant breeders to select according to the desired breeding objectives (Abdul Haris et al. 2013). Mutation breeding involves developing new varieties characterized by abiotic stress tolerance, early maturity, and high productivity using physical and chemical mutagens (Oladosua et al. 2016). Gamma rays have been successful in inducing genetic variability in rice. The mutant variety database contains more than 3,364 mutants, mainly consisting of cereal species (47.13%) with 851 mutants in rice crops, among them 248 tolerant to abiotic stress (FAO 2020). Molecular markers are effective tools for assessing genetic variation and elucidating genetic relationships within and between species (Chakravarthi and Naravaneni 2006), increasing the efficacy of selection in breeding programs. Start codon targeted polymorphism (SCoT) is a novel marker system for gene differential expression developed based on the short conserved regions flanking the ATG start codon in the plant genome. These markers can be used to find new genes (Collard and Mackill 2009). So, this study was conducted to evaluate rice mutants for tolerance to drought and salinity stress as well as yield and yield-related traits using gamma rays and molecular techniques.

2 Material and Methods

Ten rice mutants in the M_4 generation, namely EN7, EN14, EN17, EN24, EN25, EN26, EN27, EN28, En32, and EN46, were selected from populations of 2 local cultivars, Giza 178 (Gz178) and Sakha 101 (Sk101) that irradiated with varying doses of gamma rays (0, 200, 250 and 300 Gy). These mutants, the parent of most mutants (Giza 178) and three drought tolerance check varieties (Giza 179; Sakha 107 and IET1444) were used to investigate genetic diversity for drought tolerance. The visual selection was based on drought tolerance, early maturity, and high grain yield.

2.1 Evaluation of tolerance to drought stress

Ten rice mutants, a Gz178 cultivar (parent), and three tolerant check cultivars were evaluated for drought tolerance in the El-Sharkyia Governorate location for four years from M_5 to M_8 generations (2017-2020) under three irrigation intervals: as well-watered (WW) (irrigation every 4-days), water-stressed 1 (WS1) (irrigation every 8-days) and severe water-stressed 2 (WS2) (irrigation every 12-days) conditions in loam soil. The grains of rice genotypes were planted as individual plants in separate rows in a split-plot design with three replications. Plant height (PH), Number of grains per panicle (NGP), Number of panicles per square meter (NPM), and Grain yield per square meter by gram (GYM) were recorded.

2.2 Drought tolerance indices

Drought tolerance indices were calculated by using the following formulas (Afify et al. 2022).

Stress susceptibility index (SSI) = [1- (Ys / Yp)] / [1-($\overline{Ys}$/($\overline{Yp}$)] (Fischer and Maurer 1978). Tolerance index (TOL) = Yp – Ys (Rosielle and Hamblin 1981).

Mean productivity (MP) = (Yp + Ys) / 2 (Rosielle and Hamblin 1981).

Geometric mean productivity (GMP) = $\sqrt{Ys \times Yp}$ (Fernandez 1992).

Stress tolerance index (STI) = (Ys×Yp) / $\overline{Yp}^2$ (Fernandez 1992).

Yield index (YI) = Ys/ $\overline{Ys}$ (Gavuzzi et al. 1997).

Yield stability index YSI= Ys / Yp (Bouslama and Schapaugh, 1984).

Sensitivity drought index SDI= (Yp-Ys) /Yp (Farshadfar and Javadinia 2011).

Relative drought index RDI= (Ys/Yp) / ($\overline{Ys}$/($\overline{Yp}$) (Fischer and Maurer 1978).

Where Ys and Yp represent yield in stress and non-stress conditions respectively. Also, Ys^- and Yp^- are the mean yield of all genotypes in stress and non-stress conditions respectively. Si is the stress intensity and is calculated as $Si= 1- (YS^- / Yp^-)$.

2.3 SCoT analysis

According to Dellaporta et al. (1983), DNA was extracted using a modified CTAB method. Forty-six SCoT markers were used for PCR amplification for the selected rice genotypes (Table 1). PCR reactions were conducted at a final volume of 12.5 µl, containing 6.25 µl PCR master mix (KAPA2G Fast Ready Mix PCR Kit), 1 µl genomic DNA, 1 µl for each primer and 3.25 µl dH2O. The PCR reactions were performed in a thermal cycler (TECHNE TC-412) programmed as follows: 94°C/3 min for pre-denaturation followed by 35 cycles 94°C/1 min, annealing temperature 50°C/1 min, 72°C/2 min), and 72°C/5 min for final extension then held at 4°C. The PCR products were separated by electrophoresis in 1.2% agarose gel at 80 V for 50 min in 1x TAE buffer, stained with ethidium bromide, and visualized on a UV transilluminator.

2.4 Statistical analysis

Analysis of variance for agronomic and yield traits of M_7 and M_8 generations were subjected to the combined analysis of split-plot design with three replications over two years using the statistical software MSTAT-C. Furthermore, the mean comparisons among the treatments were carried out by Duncan's Multiple Range Test (DMRT).

3 Results and Discussion

3.1 Analysis of variance

Analysis of variance for yield and yield-related traits of 14 rice genotypes in the combined analysis under normal and water stress conditions over two years (M_7 and M_8) is presented in Table 2. The results revealed that mean squares due to the watering stress (WS), genotypes (G), and G x WS interaction were significant for all studied traits in two years except the NGP, suggesting that water stress had a significant effect among the mutants and check varieties. The significance of interaction variance indicates that the

Table 1 Description of SCoT markers used in the study

primer	Sequence (5′-3′)	primer	Sequence (5′-3′)
SCoT1	CAACAATGGCTACCACCA	SCoT24	CACCATGGCTACCACCAT
SCoT2	CAACAATGGCTACCACCC	SCoT25	ACCATGGCTACCACCGGG
SCoT3	CAACAATGGCTACCACCG	SCoT26	ACCATGGCTACCACCGTC
SCoT4	CAACAATGGCTACCACCT	SCoT27	ACCATGGCTACCACCGTG
SCoT5	CAACAATGGCTACCACGA	SCoT28	CCATGGCTACCACCGCCA
SCoT6	CAACAATGGCTACCACGC	SCoT29	CCATGGCTACCACCGGCC
SCoT7	CAACAATGGCTACCACGG	SCoT30	CCATGGCTACCACCGGCG
SCoT8	CAACAATGGCTACCACGT	SCoT31	CCATGGCTACCACCGCCT
SCoT9	CAACAATGGCTACCAGCA	SCoT32	CCATGGCTACCACCGCAC
SCoT10	CAACAATGGCTACCAGCC	SCoT33	CCATGGCTACCACCGCAG
SCoT11	AAGCAATGGCTACCACCA	SCoT34	ACCATGGCTACCACCGCA
SCoT12	ACGACATGGCGACCAACG	SCoT35	CATGGCTACCACCGGCCC
SCoT13	ACGACATGGCGACCATCG	SCoT36	GCAACAATGGCTACCACC
SCoT14	ACGACATGGCGACCACGC	SCoT37	CAACAATGGCTACCAGCG
SCoT15	ACGACATGGCGACCGCGA	SCoT38	AAGCAATGGCTACCACCG
SCoT16	ACCATGGCTACCACCGAC	SCoT39	ACGACATGGCGACCAGCG
SCoT17	ACCATGGCTACCACCGAG	SCoT40	ACGACATGGCGACCACGT
SCoT18	ACCATGGCTACCACCGCC	SCoT41	ACGACATGGCGACCGCGG
SCoT19	ACCATGGCTACCACCGGC	SCoT42	ACCATGGCTACCACCGAT
SCoT20	ACCATGGCTACCACCGCG	SCoT43	ACCATGGCTACCACCGGT
SCoT21	ACGACATGGCGACCCACA	SCoT44	GCAACAATGGCTACCACG
SCoT22	AACCATGGCTACCACCAC	SCoT45	CATGGCTACCACCGGCCG
SCoT23	CACCATGGCTACCACCAG	SCoT46	CCATGGCTACCACCGGCA

Table 2 Mean squares for the studied traits of 14 rice genotypes in combined analysis under normal and water stress conditions over two years

S.V	d.f	PH	NPM	NGP	GYM
Years (Y)	1	2.28	16.20	3.02	0.00
Replications (Y)	4	0.19	967.30**	283.5**	0.003
Water Stress (WS)	2	2352.7**	41741.0**	1398.4**	0.883**
Y x WS	2	2.65	20.95	3.33	0.000
Error	8	2.82	128.31	8.14	0.001
Genotypes (G)	13	145.7 **	72050.1**	3301.1**	0.775**
Y x G	13	1.17	0.83	0.44	0.000
GxWS	26	9.009 **	552.29**	24.84	0.012*
Y x GxWS	26	0.48	0.41	0.34	0.000
Error	156	3.10	311.41	117.9	0.007

*, **indicate significant at p≤0.05 and p≤0.01, respectively; PH: Plant height (cm); NTM: Number of panicles per M^2; NFGP: Number of grains per panicle; GYP: Grain yield per M^2(g)

Table 3 Performance of plant height trait among 14 rice genotypes under drought stress over two years

Genotypes	WW	WS1	Change (%)	WS2	Change (%)	Mean
EN7	101.7^{ab}	91.73^{j-m}	9.80	86.73^{pq}	14.72	93.40^{d}
EN14	100.0^{bc}	95.33^{e-h}	4.67	90.00^{l-o}	10.00	95.11^{bc}
EN17	100.9^{a-c}	95.60^{e-g}	5.25	91.27^{k-n}	9.54	95.93^{b}
EN24	100.0^{bc}	95.70^{e-g}	4.30	91.03^{k-n}	8.97	95.59^{b}
EN25	99.43^{b-d}	96.60^{ef}	2.85	91.27^{k-n}	8.21	95.77^{b}
EN26	101.2^{a-c}	93.70^{g-j}	7.41	88.03^{op}	13.01	94.31^{cd}
EN27	99.27^{cd}	94.60^{f-i}	4.70	88.27^{op}	11.08	94.04^{cd}
EN28	103.0^{a}	97.50^{de}	5.34	91.17^{kn}	11.49	97.22^{a}
EN32	100.9^{a-c}	94.73^{f-i}	6.11	89.73^{m-o}	11.07	95.12^{bc}
EN46	92.50^{i-k}	88.50^{op}	4.32	83.17^{s}	10.09	88.06^{g}
Gz178	96.83^{ef}	92.17^{j-l}	4.81	85.17^{q-s}	12.04	91.39^{e}
Gz179	96.57^{ef}	91.07^{k-n}	5.70	84.07^{rs}	12.94	90.57^{ef}
IET1444	93.97^{g-j}	90.97^{k-n}	3.19	85.63^{qr}	8.88	90.19^{ef}
Sk107	93.13^{h-k}	89.30^{no}	4.11	85.80^{qr}	7.87	89.41^{f}
Mean	98.54^{a}±0.57	93.39^{b}±0.458	5.18	87.95^{c}±0.47	10.71	

WW: Well-watered (every 4-days); WS1: Water- stressed (every 8-days);WS2: Water stressed (every12 days); (mean for each treatment ±SE); LSD = 2.011.

rank of mutant differs from well-watering to water stress environment for all studied traits.

3.2 Evaluation of tolerance to drought stress

3.2.1 Plant height (cm)

The study's findings revealed that EN28 and EN17 mutants had the highest average plant height values (97.22 and 95.93 cm, respectively) under drought conditions (Table 3). On the other hand, EN46 and SK107 have the lowest values for PH over two years (88.06 and 89.41 cm, respectively). Drought stress caused a significant reduction in PH traits, ranging from 2.85% to 14.72%, as observed in EN25 and EN7, respectively. The lowest PH reduction (2.85% and 3.19%) was seen for EN25 and IET 1444, respectively, in the WS1 condition. Furthermore, in the WS2 condition, SK107 and EN25 were reduced by 7.87% and 8.21%, respectively. However, EN7 showed a significant reduction (14.72%).

Drought stress decreases metabolic activity because of a shortage of water, which leads to a decrease in turgor pressure, which impacts plant cell division and elongation processes and lowers PH. Furthermore, the decrease in PH can be attributed to a decrease in

gibberellins, which are required for stem elongation and are associated with water stress (Yang et al. 2001). Similar findings were recorded by Yeo (1999), who discovered that a lack of water reduced the PH of rice. Furthermore, Terra et al. (2013) found a significant reduction in PH in upland rice genotypes that were submitted to water deficiency. Also reduction in the PH of rice genotypes under water stress has been reported in numerous studies by Davatgar et al. (2009); Hussain et al. (2018) and Hussain et al. (2021).

3.2.2 Grains yield per square meter

The results presented in Table 4 revealed that drought stress caused a significant reduction in grain yield per square meter in two generations; this reduction ranged from 7.33 to 15.94%. Also, the grain yield/m^2 ranged from 0.933 kg to 1.689 kg per square meter in Gz178 and EN25, respectively, under drought conditions. Although tolerant drought check variety (Gz179) gave the lowest reduction in grain yield (0% and 9.09%) under WS1 and WS2 conditions, with grain yield (1.07 kg per square meter) under WS2 conditions, respectively. The drought-tolerant rice mutants (EN25 and EN27) exhibited the highest mean for grain yield (1.69 and 1.39 kg/m2) under irrigation conditions (WS2 every 12 days). On the other hand, the EN28 mutant was more influenced under WS1 and WS2 conditions since it had the highest reduction percentage in grain yield (26.45%).

The primary important trait for improving drought tolerance is grain yield under stress. Drought stress inhibits rice growth by influencing various traits such as seedling biomass, stomatal conductance, starch metabolism, plant water relations, and photosynthesis. The photosynthesis process is essential to maintain crop growth and development. Furthermore, chlorophyll content is one of the major chloroplast components for photosynthesis, and it has a positive relationship with photosynthetic rate. As a result, decreased chlorophyll content due to water stress may produce reactive oxygen species (ROS), which can lead to chlorophyll destruction (Ahmadikhah and Marufinia 2016; Sarkarung et al. 1997; Quampah et al. 2011). Furthermore, Pantuwan et al. (2000) revealed that depending on the timing, length, and intensity of the plant water stress, it was found that under drought conditions, the grain production of some rice cultivars could drop by up to 81%.

3.2.3 Number of panicles per square meter

The results presented in Table 5 showed that drought stress caused a significant reduction in the number of panicles per square meter in two generations, ranging from 5.69% to 10.97%. Also, the number of panicles per square meter ranged from 309.2 in EN28 to 531.5 in EN25. Minimum reduction in (NPM) was shown in EN28 (1.47%) by WS1 and 3.03% by WS2, while maximum reduction (17.60%) was reported in EN46.

Ahmadikhah and Marufinia (2016) found that severe water deficit compared to normal irrigation significantly reduced the tiller number in rice. Under water stress conditions, effective tiller production may be reduced due to a limited supply of assimilates, less water uptake to prepare sufficient food, and inhibition of meristematic tissue cell division (Zubaer et al. 2007). Furthermore, the decrease in the number of tillers could be due to decreased photosynthesis and plant growth (Quampah et al. 2011).

Table 4 Performance of grain yield trait among 14 rice genotypes under drought stress over two years

Genotypes	WW	WS1	Change (%)	WS2	Change (%)	Mean
EN7	1.433^{c-e}	1.233^{f}	13.96	1.200^{fg}	16.26	1.289^{d}
EN14	1.133^{f-i}	1.067^{h-k}	5.83	1.000^{j-l}	11.74	1.067^{fg}
EN17	1.233^{f}	1.167^{f-h}	5.35	1.067^{h-k}	13.46	1.156^{e}
EN24	1.400^{de}	1.333^{e}	4.79	1.233^{f}	11.93	1.322^{cd}
EN25	1.800^{a}	1.733^{a}	3.72	1.533^{bc}	14.83	1.689^{a}
EN26	1.167^{f-h}	1.067^{h-k}	8.57	0.9667^{kl}	17.16	1.067^{fg}
EN27	1.567^{b}	1.400^{de}	10.66	1.200^{fg}	23.42	1.389^{b}
EN28	1.133^{f-i}	0.9333^{lm}	17.63	0.8333^{m}	26.45	0.967^{hi}
EN32	1.167^{f-h}	1.133^{f-i}	2.91	1.033^{i-l}	11.48	1.111^{ef}
EN46	1.100^{g-j}	1.000^{j-l}	9.09	0.9333^{lm}	15.15	1.011^{gh}
Gz178	1.033^{i-l}	0.933^{lm}	9.65	0.8333^{m}	19.33	0.933^{i}
Gz179	1.100^{g-j}	1.100^{g-j}	0.00	1.000^{j-l}	9.09	1.067^{fg}
IET1444	1.467^{b-d}	1.400^{de}	4.57	1.200^{fg}	18.20	1.356^{bc}
Sk107	1.133^{f-i}	1.067^{h-k}	5.83	0.9667^{kl}	14.68	1.056^{fg}
Mean	1.267^{a}±0.036	1.183^{b}±0.034	7.33	1.071^{c}±0.029	15.94	

WW: Well -watered (every 4-days); WS1: Water- stressed (every 8-days); WS2: Water stressed (every12-days);(mean for each treatment ± SE); LSD = 0.09542

Table 5 Performance of the number of panicles per square meter among 14 rice genotypes under drought stress over two years

Genotypes	WW	WS1	Change (%)	WS2	Change (%)	Mean
EN7	448.7^{de}	414.7^{g}	7.58	393.6^{h}	12.28	419.0^{d}
EN14	351.4^{k-m}	324.9^{n-r}	7.54	325.2 $^{n-r}$	7.46	333.8^{h}
EN17	377.2^{h-j}	352.2^{k-m}	6.63	332.5^{m-q}	11.85	353.9^{ef}
EN24	465.4^{d}	441.0^{ef}	5.24	424.5^{fg}	8.79	443.6^{c}
EN25	567.3^{a}	536.4^{b}	5.45	490.8^{c}	13.48	531.5^{a}
EN26	362.3^{i-l}	341.2^{l-n}	5.82	325.3^{n-r}	10.21	342.9^{f-h}
EN27	382.3^{hi}	350.8^{k-m}	8.24	321.5^{n-r}	15.90	351.6^{ef}
EN28	313.9^{p-r}	309.3^{qr}	1.47	304.4^{r}	3.03	309.2^{i}
EN32	354.8^{j-m}	337.5^{m-p}	4.88	314.2^{p-r}	11.44	335.5^{h}
EN46	382.9^{hi}	335.0^{m-p}	12.51	315.5 $^{o-r}$	17.60	344.4^{f-h}
Gz178	353.3^{k-m}	339.7^{l-n}	3.85	319.5^{n-r}	9.57	337.5^{gh}
Gz179	374.0^{h-k}	363.0^{i-l}	2.94	339.1^{l-o}	9.33	358.7^{e}
IET1444	489.60^{c}	469.10^{d}	4.19	435.8^{e-g}	10.99	464.8^{b}
Sk107	367.5^{i-k}	355.2^{j-m}	3.35	324.9^{n-r}	11.59	349.2^{e-g}
Mean	399.3^{a}±10.7	376.4^{b}±10.0	5.69	354.8^{c}±8.7	10.97	

WW: Well-watered (every 4-days); WS1: Water- stressed (every 8-days); WS2: Water stressed (every12-days); (mean for each treatment ± SE); LSD = 20.13

Table 6 Performance of the number of grains per panicle among 14 rice genotypes under drought stress over two years

Genotypes	WW	WS1	Change (%)	WS2	Change (%)	Mean
EN7	142.5^{e-j}	133.0^{h-l}	6.67	130.3^{i-l}	8.56	135.2^{fg}
EN14	166.9^{bc}	156.1^{c-e}	6.47	150.6^{e-g}	9.77	157.8^{b}
EN17	156.5^{c-e}	147.5^{e-h}	5.75	143.8^{e-i}	8.12	149.3^{cd}
EN24	147.0^{e-h}	141.9^{e-k}	3.47	140.3^{f-l}	4.56	143.1^{de}
EN25	154.6^{c-f}	151.4^{d-g}	2.07	150.2^{e-g}	2.85	152.1^{bc}
EN26	154.3^{c-f}	151.3^{d-g}	1.94	150.3^{e-g}	2.59	152.0^{bc}
EN27	185.5 ^{a}	180.6^{a}	2.64	175.6 ^{ab}	5.34	180.6 ^{a}
EN28	144.8^{e-i}	140.4^{f-k}	3.04	140.1^{f-l}	3.25	141.8^{d-f}
EN32	165.8^{b-d}	157.1^{c-e}	5.25	156.9^{c-e}	5.37	159.9^{b}
EN46	133.4^{h-l}	131.2^{i-l}	1.65	127.0^{kl}	4.80	130.6^{g}
Gz178	156.6^{c-e}	150.3^{e-g}	4.02	150.4^{e-g}	3.96	152.4^{bc}
Gz179	143.1^{e-i}	137.1^{g-l}	4.19	137.1^{g-l}	4.19	139.1^{ef}
IET1444	156.6^{c-e}	150.3^{e-g}	4.02	150.4^{e-g}	3.96	152.4^{bc}
Sk107	130.6^{i-l}	127.8^{j-l}	2.14	125.4^{l}	3.98	127.9^{g}
Mean	152.7^{a}±2.5	146.9^{b}±2.4	3.81	144.9^{c}±2.3	5.09	

WW: Well-watered (every 4-days); WS1: Water- stressed (every 8-days); WS2: Water stressed (every12 days); mean for each treatment ±SE; LSD = 12.39

3.2.4 Number of grains per panicle

The results showed that drought stress caused a significant reduction in the number of grains per panicle in two studied generations, ranging from 3.81% to 5.09%, as presented in Table 6. Also, NGP ranged from 127.9 in SK107 to 180.6 in EN27. The lowest reduction of NGP was seen for EN46 (1.65%) in WS1 and were 2.59% and 2.85% in (EN26 and EN25) mutants, respectively in WS2 treatment. However, the highest reduction was reported at 9.77% in EN14.

Under water stress conditions, grain size, number, and ultimately weight were reduced due to decreased water content in the plant, which limits reproductive development and grain growth (Pantuwan et al. 2000). Additionally, decreased NGP under water stress levels as a result of inhibition of assimilating to grains translocation (Zubaer et al. 2007). Cha-um et al. (2010) reported a similar result in rice, reporting differential responses of two tolerant rice genotypes to moisture deficit for fertile grains. These tolerant genotypes were not significantly reduced in NGP, resulting in higher productivity than the two sensitive varieties.

3.3 Drought tolerance indices

Drought tolerance indices were developed to select drought-tolerant genotypes based on grain yield potential in well-irrigated and stress conditions (Bennani et al. 2017). From the obtained

Table 7 Drought tolerance indices of 14 rice genotypes over two years

Genotypes	Yp	Ys	STI	MP	GMP	TOL	SSI	YI	YSI	SDI	RDI
Water Stress 1											
EN7	5.73	4.93	1.09	5.33	5.31	0.80	1.87	1.04	0.86	0.14	0.93
EN14	4.53	4.27	0.74	4.40	4.40	0.26	0.77	0.90	0.94	0.06	1.02
EN17	4.93	4.67	0.89	4.80	4.80	0.26	0.71	0.99	0.95	0.05	1.02
EN24	5.60	5.33	1.15	5.47	5.46	0.27	0.65	1.13	0.95	0.05	1.03
EN25	7.20	6.93	1.92	7.07	7.06	0.27	0.50	1.47	0.96	0.04	1.04
EN26	4.67	4.27	0.77	4.47	4.47	0.40	1.15	0.90	0.91	0.09	0.99
EN27	6.27	5.60	1.35	5.94	5.93	0.67	1.43	1.19	0.89	0.11	0.97
EN28	4.53	3.73	0.65	4.13	4.11	0.80	2.37	0.79	0.82	0.18	0.89
EN32	4.67	4.53	0.81	4.60	4.60	0.14	0.40	0.96	0.97	0.03	1.05
EN46	4.40	4.00	0.68	4.20	4.20	0.40	1.22	0.85	0.91	0.09	0.98
Gz178	4.13	3.73	0.59	3.93	3.92	0.40	1.30	0.79	0.90	0.10	0.98
Gz179	4.40	4.17	0.71	4.29	4.28	0.23	0.70	0.88	0.95	0.05	1.02
IET1444	5.87	5.60	1.26	5.74	5.73	0.27	0.62	1.19	0.95	0.05	1.03
SK107	4.53	4.27	0.74	4.40	4.40	0.26	0.77	0.90	0.94	0.06	1.02
Mean	5.10	4.72	0.95	4.91	4.91	0.39	1.03	1.00	0.92	0.08	1.00
Water Stress 2											
EN7	5.73	4.80	1.06	5.27	5.24	0.93	1.02	1.12	0.84	0.16	1.00
EN14	4.53	4.00	0.70	4.27	4.26	0.53	0.74	0.93	0.88	0.12	1.05
EN17	4.93	4.27	0.81	4.60	4.59	0.66	0.84	1.00	0.87	0.13	1.03
EN24	5.60	4.93	1.06	5.27	5.25	0.67	0.75	1.15	0.88	0.12	1.05
EN25	7.20	6.13	1.70	6.67	6.64	1.07	0.94	1.43	0.85	0.15	1.01
EN26	4.67	3.87	0.69	4.27	4.25	0.80	1.08	0.90	0.83	0.17	0.99
EN27	6.27	4.80	1.16	5.54	5.49	1.47	1.48	1.12	0.77	0.23	0.91
EN28	4.53	3.33	0.58	3.93	3.88	1.20	1.67	0.78	0.74	0.26	0.87
EN32	4.67	4.13	0.74	4.40	4.39	0.54	0.73	0.96	0.88	0.12	1.05
EN46	4.40	3.73	0.63	4.07	4.05	0.67	0.96	0.87	0.85	0.15	1.01
Gz178	4.13	3.33	0.53	3.73	3.71	0.80	1.22	0.78	0.81	0.19	0.96
Gz179	4.40	4.00	0.68	4.20	4.20	0.40	0.57	0.93	0.91	0.09	1.08
IET1444	5.87	4.80	1.08	5.34	5.31	1.07	1.15	1.12	0.82	0.18	0.97
SK107	4.53	3.87	0.67	4.20	4.19	0.66	0.92	0.90	0.85	0.15	1.02
Mean	5.10	4.29	0.86	4.69	4.68	0.82	1.00	1.00	0.84	0.16	1.00

Ys and Yp represent yield (ton/acre) in stress and non-stress conditions, respectively. Also, WS1: Water- stressed (every 8-days); WS2: Severe Water stressed (every 12-days); SSI: Stress susceptibility index; TOL: Tolerance index; MP: Mean productivity; GMP: Geometric mean productivity; STI: Stress tolerance index; YI: Yield index; YSI: Yield stability index; SDI: Sensitivity drought index and RDI: Relative drought index.

mutants in this study, EN24, EN25, EN26, EN27, EN32, and IET1444 (cultivar) had the largest STI, YP, and YS indicating they might be the best promising tolerant. On the other hand, Gz178, EN28, and EN46 were the most susceptible genotypes because they showed the smallest STI by WS1 and WS2 treatments.

These results are in agreement with Moghaddam and HadiZadeh (2002). They found that STI was a more helpful index for identifying genotypes that produce high yields under favorable and water-stress conditions. Further, they recommended that a high value of STI implies higher tolerance to abiotic stress. Similar findings are also documented by Farshadfar et al. (2013); Abdelghany et al. (2016); Eid and Sabry (2019) and El-Hosary et al. (2019).

Rosielle and Hambin (1981) also reported that MP refers to the average yield of genotypes between water stress and well-irrigated. In this study, the genotypes with high values of MP were EN25 (7.07), EN27 (5.94), IET1444 (5.74), and EN24 (5.47); these genotypes were considered tolerant to drought. On the other side, Gz178 cultivar (3.93), EN28 (4.13), and EN46 (4.20) mutants had lower values as presented in table 7. Also, Genotypes EN25, EN27, IET1444, and EN24 exhibited the highest values for GMP indices, therefor these genotypes are drought tolerant, however, cultivar Gz178 (3.93), mutants EN28 (4.11) and EN46 (4.20) were the most susceptible genotypes. Besides EN25, EN27, IET1444, and EN24 were drought-tolerant genotypes based on STI, MP, and GMP indices. While the cultivars Gz178, EN28, and EN46 were found the most sensitive genotypes. Therefore, STI, MP, and GMP are considered more efficient indices in the high selection yielding drought-tolerant genotypes under well-irrigated and water-stress conditions. The same outcomes were reported by Mursalova et al. (2015); Ali and El-Sadek (2016), and Eid and Sabry (2019).

The highest Tol values were related to mutants EN7 and EN28, which recorded values of 0.80 in WS1, while EN27 had the highest Tol value (1.47 in WS2). The high amount of Tol is a sign of susceptibility to stress (Parchin et al. 2013; Eid and Sabry 2019). On the other side, EN32 (0.14), EN24 (0.27), Gz179 (0.23), IET1444 (0.27), EN25 (0.27), EN14 (0.26), EN17 (0.26) and SK107 (0.26) has the lowest values recorded in WS1, and these mutants were considered as tolerant genotypes which, showed a lower value of TOL (stress tolerance). Similar findings were documented by various previous researchers (Raman et al. 2012; Pantuwan et al. 2002; Ouk et al. 2006; Sio-Se Mardeh et al. 2006).

The mutants which showed stress susceptibility index (SSI) values <1 could be considered drought tolerant as compared with those of stress susceptibility index > 1. As shown in Table 7, SSI ranged from 0.40 for EN32 to 2.37 for EN28. The lowest values of 0.40, 0.50, 0.57, 62, and 0.65 were reported for the genotypes EN32, EN25, Gz179 IET1444, and EN24, respectively. So, these genotypes were considered to be more tolerant to drought. These current mutants had the same trend as SDI. These results are in agreement with Kumar et al. (2012). Whereas EN28, and EN7, with high SSI values of 2.37 and 1.87, respectively, can be considered susceptible to drought and only suitable for normal irrigation conditions. Similar results were recorded by Abdi et al. (2013); Raman et al. (2012); Eid and Sabry (2019) and Afiah et al. (2019).

A genotype is deemed suited for drought circumstances if it has a high Yield index (YI) value. The genotype which has>1 value is considered tolerant, while the genotype having <1 value is denoted as a susceptible one. EN25, EN27, IET1444, and EN24 exhibited the highest YI values of 1.47, 1.19, 1.19, and 1.13, respectively, indicating tolerant genotypes as in the case of STI cross-testing of genotypes suitable for drought conditions. Similarly, lower values of YI were noted in the genotypes that exhibited susceptibility to drought, while all other genotypes were intermediate. The highest YSI values were recorded for EN32 (0.97), EN25 (0.96), EN24 (0.95), EN17 (0.95), Gz179 (0.95), and IET1444 (0.95). These current genotypes had the same trend as RDI. These findings are in harmony with Karimizadeh and Mohammadi (2011).

3.4 SCoT markers for drought tolerance

Forty-six SCoT markers were used to screen the status of drought tolerance genes in 10 mutants and the check varieties. Out of 46 markers, thirteen were found to be linked with drought tolerance genes (Table 8). The results of genotypic screening of the 14 rice genotypes for the presence or absence of rice drought tolerance genes linked to SCoT markers are shown in Table 8. The electrophoretic pattern for each SCoT marker linked to these studied genes is shown in Figure 1.

The genetic frequencies of the 13 major rice drought tolerance genes ranged from 64.3% to 78.6%, according to the data. The drought tolerance genes linked to SCoT markers are SCoT8, 1.674 bp, SCoT40, 3.615 bp, and SCoT44, 0.878 bp, and these are distributed in all tolerant genotypes with the check variety for drought tolerance IET1444 which shared the same bands. However, the sensitive genotypes did not have these SCoT markers. Whereas, SCoT11 (1.703 bp and 1.925 bp), SCoT40 (1.179 bp), and SCoT44 (0.709 bp) showed 71.4 % of gene frequency in all genotypes. Also, drought tolerance genes which are linked to SCoT1 (2.555 bp), SCoT6 (0.53 bp), SCoT12 (2.64 bp), SCoT23 (1.222 bp), SCoT29 (1.572 bp) and SCoT40 (0.946 bp) showed the lowest gene frequency across all genotypes. According to the data in Table 8, cultivars IET1444, Gz179 and mutants EN24 and EN28 are high tolerance to drought stress and possess thirteen genes that are linked to all selected SCoT markers in table 8. Although the mutant EN28 is drought tolerant, unfortunately, it has a relatively low yield. While in case of EN7

Table 8 Genotypic screening of 10 rice mutants and check cultivars for drought tolerance genes linked with SCoT markers

Primers	MS	Gz179	Gz178	EN32	EN28	EN27	EN26	EN25	EN24	EN17	EN14	EN46	EN7	SK107	IET	Gene Frequency
SCoT1	2.555	1	1	1	1	1	1	1	1	0	0	0	0	0	1	64.3
SCoT6	0.53	1	1	1	1	1	0	0	1	1	1	0	0	0	1	64.3
SCoT8	1.674	1	1	1	1	1	1	1	1	1	1	0	0	0	1	78.6
SCoT11	1.925	1	1	1	1	0	1	1	1	1	1	0	0	0	1	71.4
SCoT11	1.703	1	1	1	1	0	1	1	1	1	1	0	0	0	1	71.4
SCoT12	2.64	1	1	0	1	0	1	1	1	1	1	0	0	0	1	64.3
SCoT23	1.222	1	0	0	1	1	1	1	1	1	1	0	0	0	1	64.3
SCoT29	1.572	1	1	1	1	0	1	1	1	1	0	0	0	0	1	64.3
SCoT40	3.516	1	1	1	1	1	1	1	1	0	1	0	0	1	1	78.6
SCoT40	1.179	1	0	1	1	1	0	1	1	1	1	0	0	1	1	71.4
SCoT40	0.946	1	0	1	1	1	0	1	1	1	1	0	0	0	1	64.3
SCoT44	0.878	1	1	1	1	1	1	1	1	1	1	0	0	0	1	78.6
SCoT44	0.709	1	0	1	1	1	1	1	1	1	1	0	0	0	1	71.4
Total of tolerance genes		13	9	11	13	9	10	12	13	11	11	0	0	2	13	

The rice drought tolerance gene scored as the presence (1) and absence (0) of band linked to allele of SCoT markers; MS: Molecular size

and EN46 (highly susceptible) do not possess these genes as well as SK107 cultivar, which belongs to the same genus (Japonica) does not have most of the drought tolerance genes except for SCoT40 (1.179 bp and 3.516 bp) alleles. Therefore, the tolerance of this cultivar can be attributed to the SCoT40 gene.

Our results were similar to Patidar et al. (2022) and Xiong et al. (2011). They reported that the SCoT marker technique corresponds to functional genes and their correlating characters in rice. SCoT is a targeted marker with multilocus nature; besides, SCoT can generate more information correlated with biological traits and help in case of high genetic polymorphism. Evaluation of SCoT markers in diversity analysis and diagnostic finger printing has already been established in *Vigna unguiculata* (Igwe et al. 2017). Moreover, Gorji et al. (2011) presented that SCoT markers were more informative and compelling, followed by ISSRs and AFLP markers in fingerprinting of potato varieties. However, the simplicity and reproducibility of SCoT have been successfully applied to the assessment of genetic diversity and taxonomic study of Citrus (Han et al. 2011); rice (Collard and Mackill 2009); and barley (Aboulila and Mansour, 2017; Dora et al. 2017).

Out of 46 SCoT primers, 34 primers revealed a total of 377 PCR bands, while the remaining primers did not amplify bands among the studied genotypes. The total numbers of polymorphic bands were 373 (98.9%), while the remaining four were monomorphic (1%). The total number of polymorphic amplified bands by each primer ranged from 4 (primer SCoT 24) to 19 (primer SCoT 12) (Table 9), with 100% polymorphism for all primers except SCoT 44 and SCoT 2 (75 and 81.81, respectively). Also, four monomorphic bands were only amplified by primers SCoT 2 (2) and SCoT 8 (2), where the studied rice genotypes contained two sensitive ones for drought. The mediocre number of amplicons/primers was 11 (10.9 and 0.1 polymorphic and monomorphic bands, respectively). As well as, targeting regions in plant genes confers great importance on unique bands amplified with SCoT primers, especially in elite genotypes. The present investigation revealed fifty-three positive specific distinctive markers for high-yielding drought-tolerant rice genotypes, which suggests a role of such unique sequences in yield and drought tolerance (Table 9 and Figure 1). The amplification of unique SCoT bands in drought-tolerant genotypes was also recorded by Shaban et al. (2022). In harmony with our results, Emam et al. (2022) amplified unique SCoT bands with drought tolerance. Therefore, SCoT can be applied to differentiate between different drought stress tolerances according to markers associated with new alleles for this trait in given selected genotypes.

The UPGMA-based dendrogram (Figure 2) showed the genetic relationships among the rice genotypes for SCoT analysis. The dendrogram of the fourteen rice genotypes using the UPGMA procedure clustered these genotypes into three major groups by their

Table 9 The polymorphism for 14 rice genotypes using 34 SCoT markers

Primers	MB	PB	UB	TB	P (%)
SCoT1	0	11	0	11	100
SCoT2	2	9	0	11	81.81
SCoT5	0	16	1	16	100
SCoT6	0	10	2	10	100
SCoT7	0	12	1	12	100
SCoT8	0	13	2	13	100
SCoT9	0	6	1	6	100
SCoT10	0	7	1	7	100
SCoT11	0	15	2	15	100
SCoT12	0	19	2	19	100
SCoT13	0	9	2	9	100
SCoT14	0	8	5	8	100
SCoT16	0	9	1	9	100
SCoT19	0	12	3	12	100
SCoT20	0	13	4	13	100
SCoT21	0	10	0	10	100
SCoT22	0	9	0	9	100
SCoT23	0	13	1	13	100
SCoT24	0	4	0	4	100
SCoT26	0	12	5	12	100
SCoT28	0	13	3	13	100
SCoT29	0	13	2	13	100
SCoT31	0	11	1	11	100
SCoT32	0	9	2	9	100
SCoT33	0	11	2	11	100
SCoT34	0	14	3	14	100
SCoT35	0	14	2	14	100
SCoT36	0	9	2	9	100
SCoT37	0	7	1	7	100
SCoT39	0	13	1	13	100
SCoT40	0	13	0	13	100
SCoT42	0	12	1	12	100
SCoT44	2	6	0	8	75
SCoT46	0	11	0	11	100
Total	4	373	53	377	-----
Average	0.1	10.9	1.6	11.0	98.7

MB: Monomorphic bands, PB: Polymorphic bands, UB: Unique bands, TB: Total bands. P%: Polymorphism

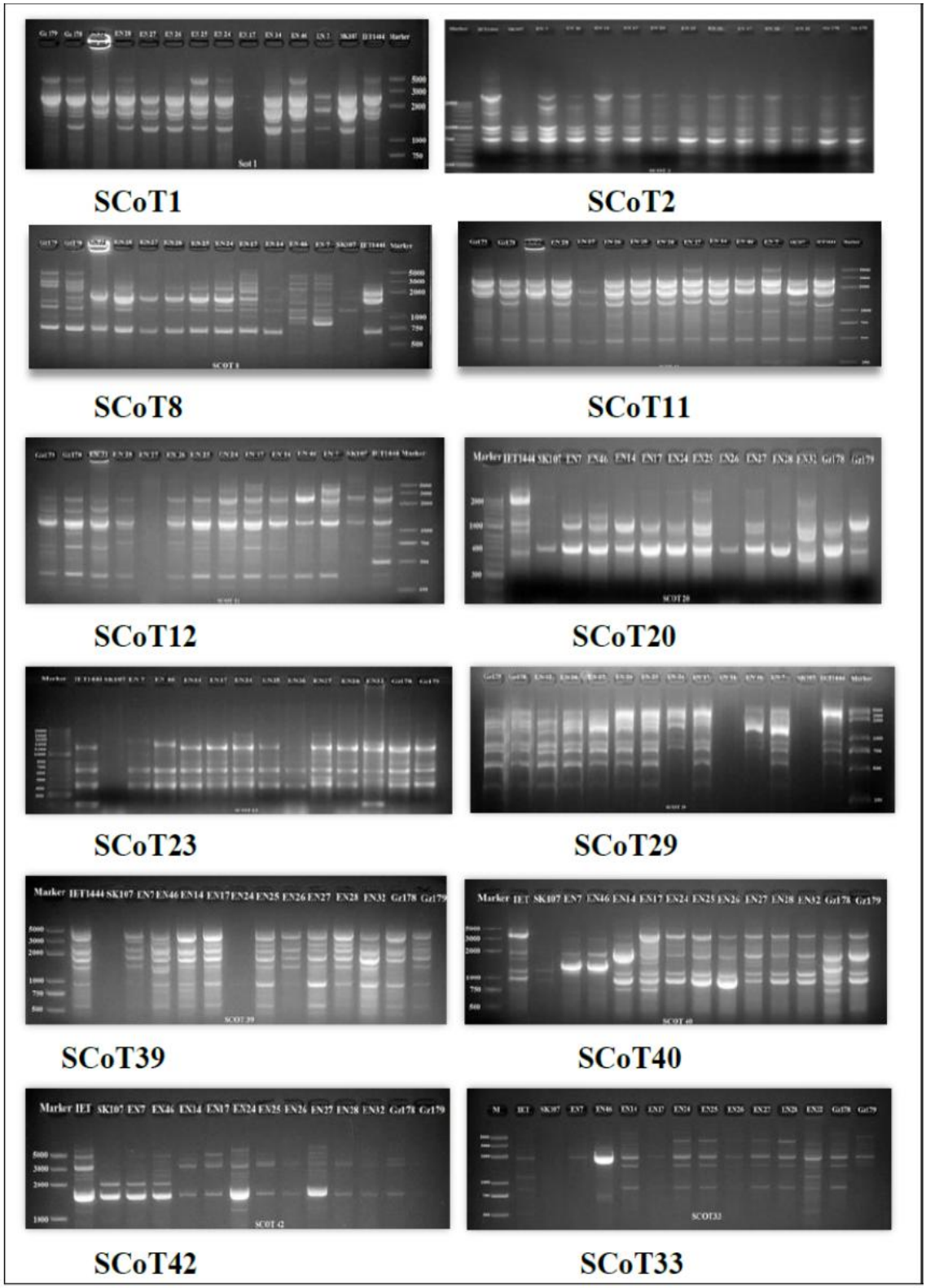

Figure1 SCoT profiles of 10 mutants of rice were produced using gamma radiation in M_8 generation and the check varieties using SCoT 1, SCoT 2, SCoT8, SCoT11, SCoT 12, SCoT 20, SCoT 23, SCoT29, SCoT33, SCoT 39, SCoT 40 and SCoT 42 primers

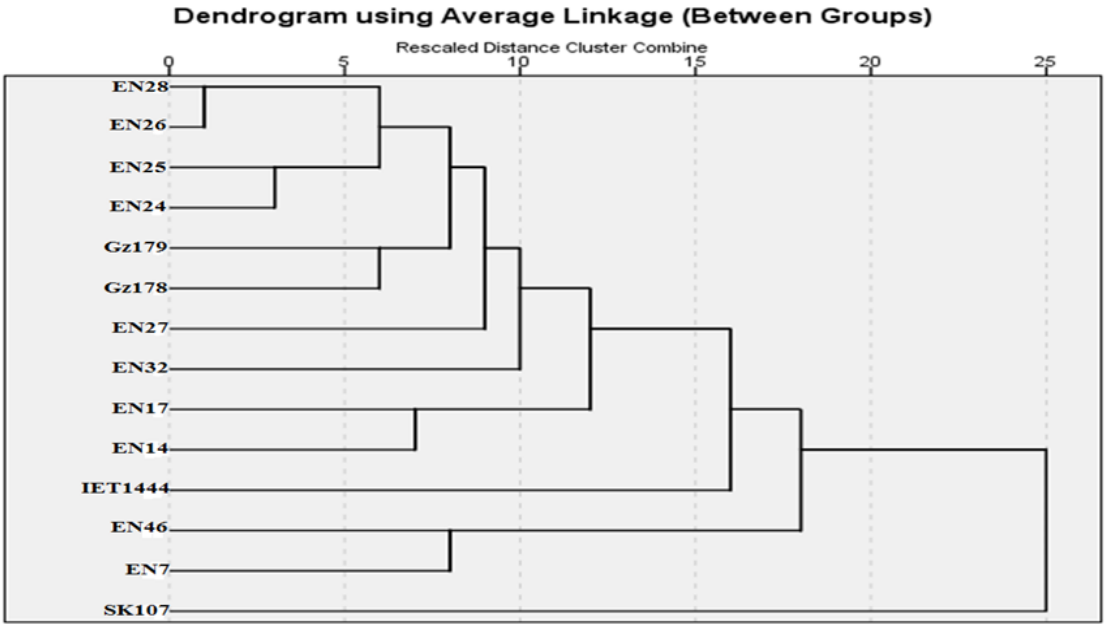

Figure 2 Dendrogram for Ten selected M_8 rice mutants constructed from SCoT data using (UPGMA) that computed according to Dice coefficients

Table 10 The similarity indices for all selected mutants of rice from M_8 and check varieties with SCoT primers

Genotypes	Gz179	Gz178	EN32	EN28	EN27	EN26	EN25	EN24	EN17	EN14	EN46	EN7	SK107
Gz178	68%												
EN32	59%	64%											
EN28	68%	71%	68%										
EN27	57%	58%	59%	70%									
EN26	61%	58%	57%	78%	61%								
EN25	62%	59%	54%	72%	59%	72%							
EN24	62%	61%	53%	70%	60%	58%	72%						
EN17	60%	56%	49%	59%	49%	53%	68%	62%					
EN14	56%	57%	49%	56%	46%	49%	55%	60%	64%				
EN46	41%	38%	35%	41%	32%	38%	45%	41%	50%	42%			
EN7	46%	36%	32%	44%	35%	45%	51%	46%	51%	41%	63%		
SK107	23%	17%	20%	27%	16%	27%	28%	29%	27%	32%	42%	39%	
IET	48%	49%	46%	47%	36%	40%	48%	48%	49%	55%	43%	42%	28%

reaction to drought tolerance response and the type of these mutants. Interestingly, the eleven drought-tolerant rice genotypes, i.e. EN28, EN26, EN25, EN24, Gz 179, Gz 178, EN27, EN32, EN17, EN14, and IET1444 belong to Indica- Japonica types were far from the other genotypes and were clustered together in one group (cluster I). A moderate drought-tolerant variety, Sk107 (Japonica type), was closer to the drought-tolerant genotypes (cluster II). The drought-sensitive rice genotypes EN46 and EN7 were very close and grouped in the same cluster (cluster III), representing the Japonica type. The cluster I genotypes showed a genetic similarity percentage ranging from 36% to 78% in the similarity indices (Table 10). On the other hand, cluster II included the Sk107 variety, which was isolated in a single group (a moderate japonica drought-tolerant variety). Drought-sensitive rice mutants EN46 and

EN7 (japonica) were separated into the same group (cluster III) with a genetic similarity percentage of 63%. The highest similarity value of 78% was recorded between the two mutants EN26 and EN28, followed by 72% among EN25 and EN24, EN26, and EN28; these values indicate that every two mutants with high similarity were closely related. While the lowest value recorded was 16% among genotypes SK107 and EN27; this indicates that these two genotypes were genetically distant types of genotypes. These results confirm the capability of SCoT as an excellent marker to establish the genetic relationships between various cultivars and obtain new specific clustering (Xiong et al. 2011; Etminan et al. 2016).

Conclusions

Mutation breeding is a beneficial method to create new rice genotypes that are resistant to the effects of drought. Ten mutants were evaluated under three irrigation intervals (irrigation every 4, 8, and 12 days) for yield and yield-related traits. Moreover, these mutants were evaluated using different drought-tolerant indices. According to the previous methods, the obtained results exhibited seven high-tillering drought-tolerant mutants with high yields compared with check varieties under irrigation conditions. The STI, MP, YI, and GMP indices present that the mutant EN25 showed the highest ability under drought stress followed by EN27 in comparison with IET444 (DT check variety). These mutants will save more than 30% of irrigation water with maintaining high productivity and can be used as sources of tolerance genes in breeding programs. Furthermore, using DNA markers linked to the tolerance genes is a powerful tool in identifying and screening these specific genes between rice genotypes. Moreover, SCoT markers in this study successfully evaluated the genetic relationships among the 14 rice genotypes. All PCR primers generated a high level of polymorphism 100%, except SCoT2 and SCoT44. The 14 genotypes were clustered into three clusters using the UPGMA dendrogram based on their tolerance to drought stress. SCoT showed that these seven mutants share 13 of the same bands with IET444 (check variety). The results of the present study will be useful for releasing new drought-tolerant rice cultivars.

Conflict of interest

The authors declare no conflict of interest

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The future of Mango farmers post COVID-19 pandemic outbreak: The Household Livelihood Resilience Approach

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ABSTRACT

The future and welfare of mango farmers are important and if mango farmers can apply agribusiness behavior, namely perseverance, resilience, hard work, saving, carefulness, discipline, and respecting time, they stand a better chance of survival during a pandemic. How mango farmers' livelihood will be going forward is, yet unknown since many aspects such as economic, physical, financial, and human factors of their way of earning a living are affected hugely by Covid-19. This study was conducted to determine the socio-economic nature of mango farmers in Vhembe district Limpopo province, determine the livelihoods of mango farmers after the Covid-19 pandemic, and identify challenges that mango farmers encountered during the Covid-19 pandemic. Descriptive statistics and household were employed to help analyze the results from the collected data. In the study area, 77% of the farmers were males and pensioners since they were above the age of 60. Currently, mango farmers are highly impacted by the outbreak of Covid-19 in terms of production and marketing their products, thus these changes in their livelihood and their survival in farming are in the line since 54% of the farmers currently have access to loans, which makes it easy for them to cover for their loss of income. The government should ease up other restrictions on farmers to enable them to farm and issue necessary support to those farmers who have lost the least they had due to Covid-19. Concerning access to information about Covid-19, the results of the study posit the significance of the relationship between finance and human capital, which the study recommended that the government should subsidize the farmers.

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1 Introduction

It is advisable to employ the notion of resilience to sustainably manage resources for both ecosystem function and farmers' development and well-being (Berkes et al. 2002). However, Tanner et al. (2015) proposed that the lens of resilience requires greater attention to mango farmers' livelihoods if it is to address the limits of adaptation strategies and the development needs of the planet's poorest and most vulnerable farmers. Most rural area-based mango farmers are oriented to product quality and market demand, and oriented towards the addition of value, able to control and utilize nature, responsive to innovation this value allows mango farmers to survive and have a better future (Gatto and Busato 2020)

Agriculture in rural areas continues to be the main economic activity that sustains the livelihoods of most farmers 'households. Most mango farmers depend on mango farming as their source of income. The covid-19 pandemic forced the country to go on lockdown, where mango farmers marketing channels had to close down. During the pandemic, farmers' households and indeed the district's food security seems threatened (Yamba et al. 2017). The capacity of all farmers to sustain and improve their livelihood opportunities and well-being despite any form of disturbances is a livelihood-resilient approach. This approach expands beyond the technical approaches to minimizing harm and loss by bringing issues of farmers' lives. Importantly livelihood approach acknowledges that farmers' characteristics are different and their perceptions of different situations affect their ability to adapt to changes (Quandt 2018; Rahman et al. 2019).

As illustrated by Lebel (2017), there is a need to expand resilience work to include more analysis of the processes and perceptions that affect people's ability to adapt to the impacts of various changes to their livelihood. Integrating livelihood approaches with resilience thinking can reinforce the understanding of the dynamics of rural household livelihood and of how rural households pursue and improve their livelihood to cope with changes and perturbations (Alexander 2013; Speranza et al. 2014). Livelihood resilience is a coping strategy used by farmers during stressful times. These coping strategies can be spontaneous but often involve planning and preparation shocks. Coping strategies can be specific responses or activities used to adjust to changing conditions of production, both short and long-term (Quandt 2018).

1.1 Household livelihood resilience

The Household Livelihoods Resilience Approach (HLRA) is a method of understanding the lives of people experiencing poverty and disadvantage. A participatory approach is based on the belief that people experiencing poverty have abilities and assets that can be used to help them manage and improve their lives (Altendorf 2017). Some attempts to measure livelihood resilience only provide a theoretical framework instead of practical methods. Most of the efforts to measure resilience only rely on the objective measure of resilience, whereas many interventions to build livelihood resilience focus on the community scale. Many efforts to measure livelihood resilience ignore human agency, the importance of power relationships, and access to assets (Quandt 2018).

A livelihood is sustainable and can cope with and recover from stress and shocks, maintain or enhance its capabilities and assets, and provide sustainable livelihood opportunities for the next generation and which contributes net benefits to other livelihoods at the local and global levels and in the short and long term (Clark and Carney 2008). Tanner et al. (2015) promote the livelihood resilience approach because it highlights human agency and capacity to prepare and cope with different shocks. This is important because it shows that people can take an active role in building resilience. Measuring livelihood resilience through the five livelihood capital assets can highlight how people actively build and accumulate capital to better prepare for shocks.

1.2 Impacts of Covid-19 on mango production, food security, and livelihood of farmers

Coronavirus is a contagious virus that spread continuously, coronavirus disease 2019 (Covid-19) is continuing to spread around the world; it is a hard time for many economic sectors, including agriculture (Andersen et al. 2020). The virus has posed serious challenges to the sustainable functioning of agricultural food markets. As of 1 May 2020, more than 3.2 million cases were confirmed with over 231,000 deaths (FAO 2020a). The WHO is still reporting a continuous rise in the number of cases, with the pandemic now spreading to virtually all countries of the world (Jámbor et al. 2020). The impact of Covid-19 on perishables vegetables, fruits, milk, eggs, and poultry producers has been even more severe than on the producer of cereals, pulses, and oil seeds. Most current assessments generally foresee a contraction in both supply and demand for agricultural products and point to possible disruptions in trade and logistics (Galán Saúco 2004; Scoone 2020).

To avoid a total disaster that is food insecurity and economic instability in the rural sector during the Covid-19 crisis all-available mechanisms enable the drawdown of additional financial resources mustered. It should be possible to identify which previous experiences may be relevant and applicable this time around in the Covid-19 crisis; then target the populations most likely to benefit, and support them in scaling up as necessary once the interventions have been determined and put in place (Scoone 2020). The 2008 financial crisis was primarily a crisis of prices and speculation, linked to the global financial situation including the vast market in sub-prime mortgages in the US. As it happens, global food stocks in 2020 are in good shape (AFD 2020).

1.3 Previous literature on Livelihood Resilience Approaches (LRA)

There are a growing number of approaches to thinking about resilience. Bahadur et al. (2010) review 16 approaches among these and identify many common features. Since the publication of these articles, multiple organizations have also defined their resilience frameworks. Several of these frameworks also come with long lists of resilience characteristics or features. Depending on this, resilience can be defined as a process, an outcome, an overarching objective, or a dimension of sustainability.

Today, livelihoods approaches are most useful as an analytical or heuristic tool (Clark and Carney 2008). They provide a way to order information and understand not only the nature of poverty but also the links between different aspects of people's livelihoods. In this way, they help users to understand complex and changing situations (Ellis and Freeman 2007). They broaden the policy dialogue and assist in identifying the relevance of programs as well as where key constraints and opportunities lie. Furthermore, livelihood approaches are still essential within social and economic research on poverty and food security, both as embedded in research strategies and as a research tool (Speranza et al. 2014).

2 Materials and Methods

Limpopo Province is comprised of five districts. These are Capricorn district, Mopani district, Sekhukhune District, Vhembe district, and Waterberg district. However, the current study was conducted in the Vhembe district of Limpopo province, South Africa. The district comprised four local municipalities namely Makhado, Musina, Collins Chabane, and Thulamela. The area of the district is 25 597 km² with a population of approximately 1.2 million (Stats SA, 2018). Data for the study was collected from the Vhembe district in the Limpopo Province, where the population of registered mango farmers is approximately 400. To determine the sample size of the study, Raosoft sample size calculator was used to determine the sample size from the list of smallholder sunflower farmers in the study area. With the help of the above-mentioned calculator, and a sample size of 111 was selected for this study.

2.1 Household Livelihood Resilience Approach

The household livelihood resilience approach was used to analyze the impact of the Covid-19 pandemic on mango farmers after the Covid-19 pandemic outbreak. According to Thulstrup (2015), this method is based on participatory research applied anthropology and rapid rural appraisal. According to this approach, livelihood should be considered in terms of people's access to capital assets such as financial, physical, natural, human, and social assets (Quandt 2018). HLRA process is applied in steps as follows:

Step 1: Mango farmer's indicators

The HRLA indicators of livelihood resilience are developed using a literature review of the capital assets. This approach to determining indicators of resilience is similar to the stakeholder assessment method proposed by Campbell et al. (2001). The indicators are organized around the five-livelihood capital, financial, physical, natural, human, and social assets as has been done by previous research (Erenstein et al. 2010). While some of these indicators will be contextual-based, it does not mean that the context and responses will be the same for all households within a community. As explained by Twigg (2007) researchers and development practitioners need to move away from thinking of community as 'homogenous', and instead recognize internal variability and differences as shown in table 1.

Step 2: Composite asset index

A composite asset index is created for each household, to create the index the results of each indicator will be converted so that the answer choices for questions will be on a scale of 0 to 1. It is assumed that higher scores should indicate higher levels of livelihood assets and greater livelihood resilience (Quandt 2018). The importance of converting the results of each indicator question into a scale of 0 to 1 is that it allows indicators to be averaged together and generally makes it easier to analyze as shown in figure 1.

2.2 Econometric Model

The probit model is a popular specification for an ordinal or a binary response model that employs a probit link function (Thulstrup 2015). The Binary Probit model was used to analyze the impact of access to general information about the Covid-19 outbreak. General information includes; how to produce mangoes under Covid-19 regulations, how to market mangoes and the labour requirements in the new normal with Covid-19. Access to information can help farmers deal with the Covid-19 outbreak. In the study area, some farmers had access to information while other farmers had no access. Therefore we assume that Y* can be specified as follows:

$$Y^*i = B0 + B1X1 + B2X2 + \ldots\ldots\ldots + BkXk + Ui$$

And that: $Yi = 1$ if $Y^* > 0$;

$Yi = 0$ Otherwise

Where X1, X2… Xk represents the explanatory variables, B represents a vector of unknown parameters and U represents the disturbance term (Nagler 1994).

Table 1 livelihood resilience indicators

Assets	Quantitative indicator
Financial capital	Income (Rand per annum) Access to loan (Yes or No) Household belongings (No of belongings) Size of farmland (No of hector) Ownership of farm equipment (Own, Rent, Borrow, or lease) Non-Agriculture salaried job (Yes or No)
Human capital	Family labour availability (Yes or No) Education level (Level or respondent) Health problems affecting practice (Yes or No) General health of family labour (Scale from Poor to good) Availability of healthy facilities (Yes or No)
Social capital	Access to social information about health (Yes or No) Participation in other Agriculture activities (Yes or No) Strength of relationship with the neighbour farm (Scale from poor to good) Participation in farmers' groups (Yes or No)Access to extension services (Yes or No)
Physical capital	Road conditions (Scale from bad to good) Availability of storage facilities (Yes or No) Access to market (Yes or No) Ownership of farming equipment (No of equipment) Distance to the market (No of KM)
Natural capital	Size of farmland (No of Hectors) Own farm (Yes or No) Diverse farming activities (Yes or No) Diversity of farm crops (Yes or No) Availability of natural resources (Yes or No)

Source: Author computation 2021

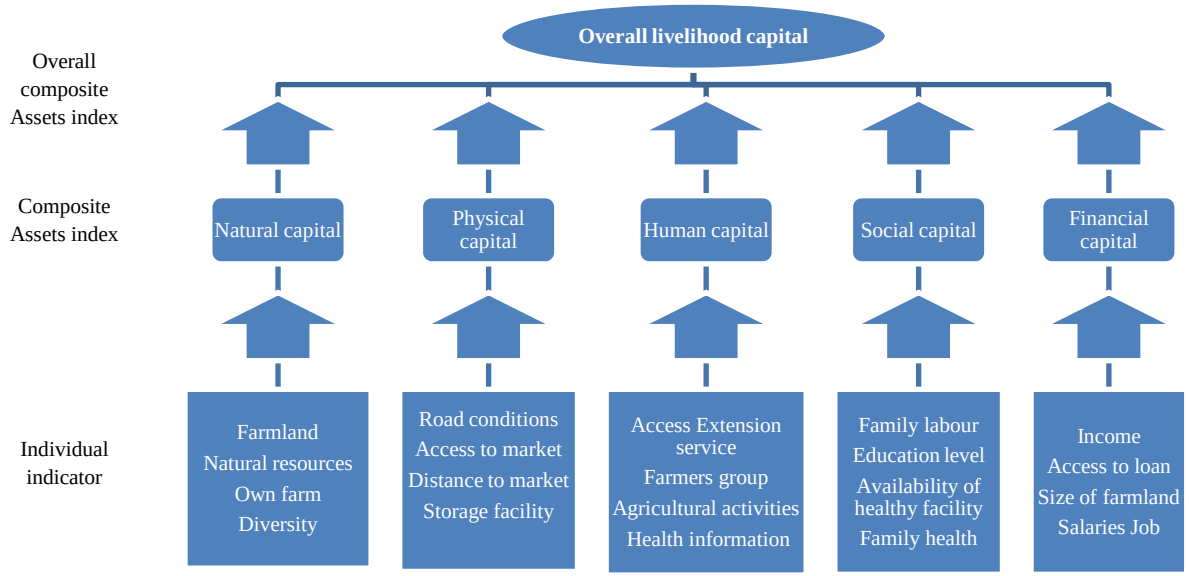

Figure 1 Schematic representation of data types and linkages for sustainable livelihood framework (*Source*: Author computation 2021)

3 Results and Discussion

Results presented in table 2 showed different socio-economic characteristics of mango farmers, the results of the study revealed that about 40% of farm workers had comorbidities while 60% had no comorbidities, this implies that 40% or farm workers are in danger of being attacked by Covid-19 and can be vital to them and it will alter their livelihood. Andersen et al. (2020) stated that Covid-19 is continuing to spread around the world, causing hard times for many economies and sectors, including the future of agriculture and since people with comorbidities are prone to this virus.

Table 2 Socio-economic characters of respondents

Parameters				N=111
Gender			Male	Female
			77 %	23 %
Age	21-40 years	41-60 years	61-80 years	81-100 years
	9%	29%	52%	10%
Employment status	Employed	Unemployed	Pension	Other
	31%	9%	60%	0%
Education level	No schooling	Primary	Matriculated	Tertiary
	9%	9%	64%	18%
Access to loan			Access Granted	No access
			54%	46%
Access to extension service			Have access	No access
			100%	0%
Access to COVID-19 Information			Have access	No access
			87%	13%

Source: Authors computation 2021.

3.1 Access to credit

With Covid-19 affecting different parts of the economy, many of them will require loans to revive their production for the future and be able to produce again, the farmer who had access to short-term and long-term loans from commercial banks made up 54% of the study and 46% of farmers had no access to loans. This can be due to a lack of collateral or any form of property in their name and a lack of information on how to acquire loans and this poses danger to the continuity or revival of their farming projects post Covid-19 and maintaining their livelihood. Lack of access to loans can also be due to high-interest rates, according to Schmidhuber et al. (2020), since the outbreak of Covid-19, interest rates have changed around the world and risen by about 3.5% for low and middle-income countries

3.2 Access to information regarding Covid-19

Importantly most of the farmers had information about the impact of Covid-19 and ways of dealing with it. Farmers with general information regarding Covid-19 constituted 87% of the study and those who lacked info were 13% of the sample population in the study area. Lack of Covid-19 information access can be due to the inability to access the internet and digital media (DFID 2020). The WHO is still reporting a continuous rise in the number of cases, with the pandemic now spreading to virtually all countries of the world. After the first shock, many media platforms have started to post articles on the different effects of the virus related to agriculture. This enabled many farmers to have access to information regarding the Covid-19 outbreak and this will help them in the future to deal with the virus.

The results of the study revealed that males as compared to female farmers dominated the study conducted, 77% of the participants were males and 23% were females as indicated in table 2. Respondents indicated that mango production is dominated by males because it is labour intensive in table 2. Females do land a helping hand but mostly the ownership is dominated by males, According to Campbell et al. (2001), across sub-Saharan Africa, men have been found to produce between 4 to 25% more crops per hectare as compared to women while in case of mangoes this number is higher and men produced above 40% higher than women.

The second category is of farmers who are of the age between 41 to 60 years constitute 29% of the sample population in the study area. This indicates that most of the people consider mango farming after retirement but they start preparing early so that they can gain more experience. The other group is farmers of age between 21, 40, and 81 to 100 who make 9% and 10% respectively. The rest of the respondents between the age of 21 and 40 showed less participation this indicated that they are still engaged in other activities such as schooling and other economic activities and most of them do not see farming as a means of survival that can provide them with enough income to maintain their livelihood.

During the analysis of the results, the value of 1 was assigned to the most desirable response while 0 was to the least desirable response. For the question that had two answers, yes or no, 1 was assigned to the most desirable answer which is yes and 0 was allocated to the least desirable answer which is no. Questions with multiple answer choices were assigned values within the range of 0

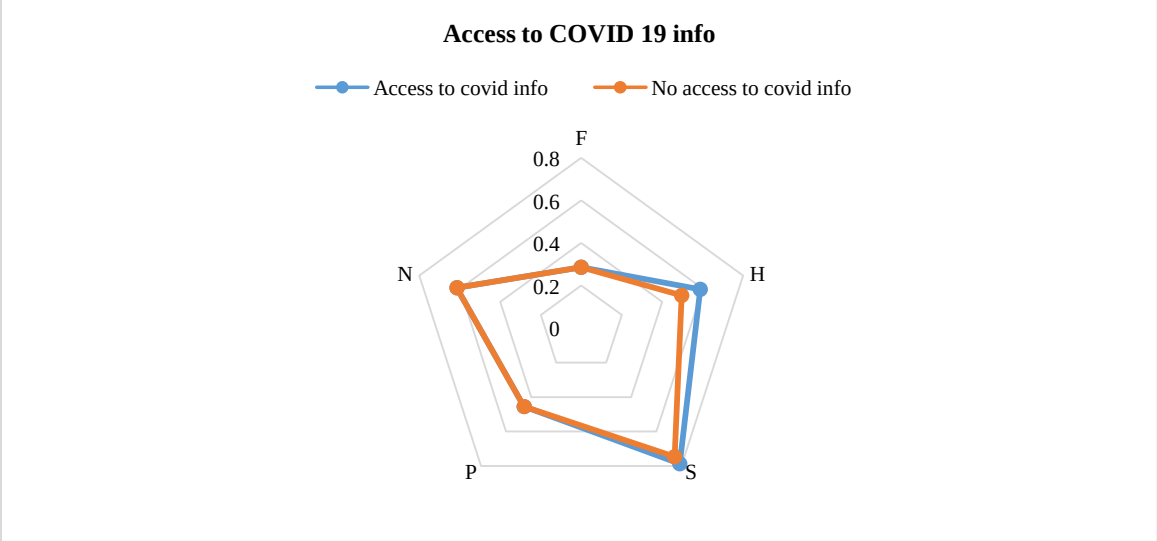

Figure 2 Spider diagram comparing the five livelihood capitals between, the farmers with access to Covid-19 information and farmers without access to Covid-19 information. How farmers with access to information can be resilient to Covid-19 outbreak and survive to continue with livelihood.

to 1. Quandt (2018), assumes that higher scores should indicate higher levels of livelihood assets and greater livelihood resilience. The interrelationship between the five individual composite indexes have been shown in figure 2.

3.2.1 Financial capital

Results presented in table 3 revealed that the average cost of production is 0.10, which indicates that due to the COVID-19 outbreak the cost of production has increased and this affects the livelihood of mango producers. The cost has increased due to certain factors such as the increase in transport. According to Rawal et al. (2020), the sudden imposition of the lockdown caused a massive disruption in the entire agricultural marketing system. No preparations were made to ensure the continuation of agricultural marketing or to ensure the safety of food supply chains before the lockdown was announced. There was a shortage of inputs and farmers had to pay a higher price for the inputs at the beginning of the Covid-19 pandemic. Mango farmers had to pay more to get the input for their products such as spray and fertilizers. Marketing costs since farmers need to deal with locked-down regulations before reaching the preferred market. The entire agricultural sub-sector future of farming for profit rather than subsistence is threatened by any type of transport restriction, or by limits to the scope for selling produce (URD 2020).

The change in livelihood caused by the lockdown has resulted in a considerable additional economic burden on farmers because of higher costs, increased debt burden, inability to sell products at reasonable prices, and crop losses. A large number of farmers, in particular, producers of pulses, oilseeds, vegetables, and fruits, have been forced to sell their produce at low prices to local traders because of disruptions in the functioning of the markets (Folke et al. 2002).

The income average is 0.41, which is below 0.50 and is close to the least desired income, which can help to influence the future livelihood of mango farmers. According to Rawal et al. (2020), the sales of fruits and vegetables have contracted very significantly because of the decline in demand. Mango is harvested in different parts of the country between April and July (DAFF 2019). Producers of all these fruits have incurred massive losses because of a collapse of both export and domestic demand due to lockdown regulations.

The financial sector plays a fundamental role in enabling successful country responses to the food supply and production by farmers and maintaining their livelihood. Other countries such as China are implementing lower percent interest rates for loans to Agri-SMEs in providence through the Agricultural Bank of China to help farmers to gain their lost income (FAO 2020b).

Most mango farmers have lost income due to Covid-19 and some will have to apply for loans in the future so that they can regain their normal livelihood before Covid-19 in their production. Farmers' export average is (0.00) which shows that farmers are also not exporting their products and this influence their income and it plays a huge role when it comes to the survival of outbreaks such as Covid-19. According to Smith (2012), the surge in food prices bans on exports, and loss of revenue because of economic contraction have severe problems for food security. During the pandemic, governments across the world impose lockdowns and shut down their borders. Therefore, the fear that food markets have logistical constraints and shortages in labour puts pressure on the prices.

Table 3 Livelihoods of mango farmers after the covid-19 pandemic outbreak (HLRA)

Indicators	Averages
Financial	
Income (Rand per annum)	0.41
Access to loan (Yes or No)	0.54
Cost of production	0.10
exporting	0.00
Non-Agriculture salaried job (Yes or No)	0.35
Human	
Family labour availability (Yes or No)	0.12
Education level (Level or respondent)	0.63
Farmers with commodities	0.24
Access to info about COVID	0.95
Availability of healthy facilities (Yes or No)	1.00
Social	
Access to irrigation schemes	0.94
Challenges when producing	1.00
Access to info about COVID 19	0.95
Access to general support from NGOs	0.05
Access to extension services (Yes or No)	1.00
Physical	
Market channel available	1.00
Availability of storage facilities (Yes or No)	0.28
Access to market (Yes or No)	1.00
Ownership of farming equipment (No of equipment)	0.00
Access to market information	1.00
Natural	
Size of farmland (No of Hectors)	0.56
Own farm (Yes or No)	1.00
Diverse farming activities (Yes or No)	0.35
Variety produced the most	0.58
Variety that sells the most	0.58

3.2.2 Physical capital

Due to storage of facilities, mango farmers fail to keep on supplying their market, and as such when the Covid-19 outbreak mango farmers suffered a setback when it comes to supplying the market. Mango storage has an average of 0.28, which shows that mango farmers lack storage facilities. A mature crop, if not stored properly, is spoilt because of pests and fungus. The storage capacity at the farm level is limited and often not good enough for prolonged storage. This is particularly a problem for poor and middle peasants and producers of perishable crops. While perishable crops such as potatoes and tomatoes need cold storage, even grain is at risk of spoilt (Rewal et al. 2020).

Since the Covid-19 outbreak, countries are taking advantage of digitally enabled innovation and developing digital ecosystems in the agricultural and rural sectors to provide market information (UNESCAP 2019). Participants had access to the market even after the Covid-19 outbreak. Access to market information and access to the market both have an average of 1.00 respectively. This factor will help farmers to maintain their livelihood and since they will still have access to the market in the future this is mainly because most farmers have various markets available to them.

3.2.3 Human capital

In any year, the need for employment creation in farming peaks in May and June, when the labour demand in agriculture is lowest in most parts of the country (FAO 2020b). The availability of labour average is 0.12 which shows that there is a lack of labour available; this is because most mango farmers use family labour as their workers on the farm. Agriculture is a sector that typically hires a large number of part-time or seasonal workers, and the virus causes a scarcity of farm labour force. As the virus limits the free flow of labour, farmers are also worried that they will not be able to hire enough workers, particularly for planting and harvesting (Jambor et al. 2020).

To create the overall weighted index all indicators were averaged since they are given equal weight. The average was used to show the influence of access to information about Covid-19 on farmers' future livelihoods (Table 4). Each indicator was given equal weight to aid interpretation and reduces ambiguity, as done by Erenstein et al. (2010).

3.2.4 Natural capital

Participants that have enough natural capital (0.614) help them to maintain their livelihood. All participants have their land which they are using for production; they do not have to go for loans to pay the rent for the land. This also helps them to keep their farming projects afloat. The physical capital value is 0.456, due to owning the land farmers are well equipped to build storage facilities in the future on the land so that they can store their mangoes, and support natural capital. If mangoes are well stored in proper facilities, farmers will have sufficient mangoes to supply the market in the future and this will have less impact on their future livelihood.

3.2.5 Social capital

Socially (0.788) farmers are getting all the necessary support to be resilient against the Covid-19 outbreak. All the participants indicated that they have full access to extension services offered by the Department of Agriculture. Farmers also have their support groups where they support each other in terms of difficult situations such as the Covid-19 outbreak. Concerning information about Covid-19, most of the participants are covered because the government made the information available to the public, this includes symptoms and prevention measures.

The decrease in social average from 0.788 to 0.747 showed how important it is for farmers to know more about Covid-19. This is because farmers will be able to produce more at less cost and apply necessary measures such as the use of storage facilities to store the mangoes. Socially mango farmers can also use the help available from NGOs to support their livelihood.

Access to Covid-19 information has a huge impact on human capital causing the change in average from 0.588 to 0.497 when farmers do not have access to Covid-19 information. Many farm workers are individuals with comorbidities and they are prone to the Covid-19 virus, so they need to remain in a safe place. This will affect the farm activities such as harvesting and fertilizing negatively due to the lack of labour availability.

3.3 Mango farmers' access to Covid-19 information

Table 5 summarizes the results of the binary probit regression coefficients of indicators affecting the mango farmer's access to general information about the Covid-19 outbreak. The binary probit model is a type of regression where the dependent variable can only take two values (Albert and Chib 1993). As such, it was used to analyze the data obtained from 111 mango farmers who were interviewed using a structured questionnaire, of the 111 farmers sampled, six (6) farmers had no access to general information on COVID-19, and 105 had access to general information about Covid-19.

A positive sign on an explanatory variable's coefficient indicates that the higher the values of the variable increase and decrease vice versa (Nagler 1994). In the table given above income coefficient is

Table 4 Overall weighted average index

Capitals	Averages with access to information about COVID-19	Averages without access to information about COVID-19
Financial	0.286	0.286
Human	0.588	0.598
Social	0.788	0.747
physical	0.456	0.456
Natural	0.614	0.614

Source: Authors computation 2021

Table 5 Binaryprobit results

Variable	Coefficient	Standard error	ratios	P value
Income (Rand per annum)	-2.79**	1.8531	0.061	0.131
Cost of production	0.999	1.9780	2.716	0.614
Family labour availability (Yes or No)	1.060**	0.9351	2.887	0.257
Education level (Level or respondent)	0.699	1.3441	2.011	0.603
Challenges when producing	3.237	3.3855	25.421	0.339
Access to irrigation schemes	-0.387	0.9165	0.679	0.673
Size of farmland (No of Hectors)	-4.477*	2.3299	0.011	0.055
Variety produced the most	0.838	2.6250	2.311	0.755
log likelihood	22.6			
Observation	Yes=6 (5.4%); No=105 (94.6%)			

**Significance at 10%; *significant at 5%; Source: Authors computation 2021.

-2.79 which is significant at 10%, which implies that when the income increases it enables farmers to get enough information about Covid-19. Income as financial capital indicator implies that a farmer's financial asset can help farmers in the future get information that will enable them to be resilient to the Covid-19 outbreak and this is because financially a farmer will be able to follow Covid-19 protocols and access far-distanced market as such their livelihood will be maintained.

Family labour availability value is 1.060 which is significant at 5%, indicating that if farmers have access to information they will know who to hire since they are well aware of who is at risk and who is safe regarding the Covid-19 virus. This implies that human capital has a huge influence on the future of mango farmers' livelihood because if farmers hire the right labour or have safe labour available they will be able to produce and sell their mangoes in the future.

Conclusion and recommendations

Mango farmers with access to Covid-19 information are more resilient and are more to survive the outbreak and continue to produce their mangoes. Farmers with less information are facing a lot of changes in their livelihood; they are bound to make adapting measures so that they produce again and earn income. Farmers encounter a lot of challenges when producing and marketing their products. Since the outbreak of Covid-19, production and marking has changed and the challenges have increased in numbers as compared to when the situation is normal.

The overall conclusion is that Covid-19 has changed the mango farmer's household livelihood and as such their production and marketing, this is due to the challenges that farmers encounter when producing and marketing their products. Covid-19 has also exposed farmers to a new way of doing things than their normal way.

The study recommends that farmers get work-shopped on how to deal with the pandemic for them to keep on producing. The workshop can offer well need needed education on what strategies to employ when farmers are facing a pandemic such as Covid-19 since it is new to them. Farmers lack support from the NGOs and banks to be resilient enough against the pandemic since the Covid-19 outbreak most farmers have lost income and spent more when producing. The study recommends that farmers should join hands and start their funding programs so that they can be able to help each other when there is a financial need. When mango farmers are a team they can also reduce, the money spent when they are taking their products to the market because they can share a load price.

Most of the mango farmers do not have a store room where they can store their products and their inputs as chemicals used for spraying their mangoes. Since most farmers own, the land that they are producing the study recommends that they build storage rooms for their products and input to avoid having to source every time to avoid price fluctuations. The study also recommends the sharing of storage among farmers who already have storage to avoid the loss of mangoes.

Based on the results of the study it can be recommended that youth needs to engage in mango production and farmers should hire them as workers this will help reduce the dominance of pensioners in the industry and allow for the survival of the farmers. Farmers' lack of access to the market is due to the restriction imposed by the government to curb the spread of Covid-19 and as such, farmers cannot export their products anymore. The study recommends that the government ease up the regulations on farmers and markers where farmers can sell their products so that they can be able to access the market. The study also recommends that farmers should be work-shopped on the importance of exporting their products

because they can earn more than what they are earning by targeting markets around them.

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