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PHARMACEUTICAL POTENTIAL OF LABORATORY GROWN CULTURES OF BLUE-GREEN ALGAE: A COMPREHENSIVE REVIEW AND FUTURE POSSIBILITIES

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Development

ABSTRACT

COVID-19 pandemic has taught the world researchers the urgent need for new sources and novel pharmaceuticals not only for existing diseases but also for both seasonal epidemics and future pandemics. Pharmaceutical drug discoveries for the past fifty years depended deeply on the procedure of empirical transmission of a huge number of pure bioactive compounds to provide new leads. The screening of extracts or isolating compounds is a common way to discover novel biologically active molecules. Most of the valuable Blue-Green algal metabolites are concentrated in their biomass. For existence in nature, Blue-Green algae (BGA) secrete and contain various organic substances like proteins, fatty acids, vitamins, pigments, primary and secondary metabolites, and these compounds are explored for potential biological activities such as antibacterial, antifungal, antiviral (including the anti-SARS-CoV-2 virus that causes COVID-19), anticancer, antioxidant, antidiabetic, protease inhibitory activity, anti-inflammatory activity, etc. Due to their diverse application, pharmaceutical companies have shown commercial interest in the Blue-green algal group for the discovery and development of novel molecules to combat deadly diseases for the benefit of society and mankind. The current review paper highlights and discusses the diverse pharmaceutical potential of laboratory-grown cultures of BGA along with comprehensive and current knowledge on bioactive compounds discovered by researchers globally.

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1 Introduction

In the extremely competitive environment of current pharmaceutical research and development of new molecules, natural products offer a unique element of molecular diversity and biological functionality, which is essential for drug discovery (Bernardini et al., 2018; Chatterjee et al., 2019; Atanasov et al., 2021). The study of secondary metabolites that organisms such as microbes including BGA and plants have evolved, largely for their survival, has historically proved of immense benefit in drug discovery and development (Petersen et al., 2020). They are providing a rich source of structurally novel bioactive molecules such as lipopeptides, amino acids, fatty acids, etc., many of which have become life-saving drugs (Singh et al., 2021a). In recent decades, pharmaceutical inventions are focused on natural sources (microbial sources such as bacterial, fungi, algal including Blue-green algae) which can deal with recent diseases. Medicinal chemistry is the backbone of lead generation in early drug invention where small molecule hits from high throughput screen (HTS), which leads to limited optimization and identification of lead compounds (Jimenez-Lopez et al., 2021). Despite these efforts, some new chemical entities have reached the market, and researchers throughout the world now are giving more attention to exploring these groups of microorganisms (including microbes from extreme environments i.e., Arctic, Antarctic) for extractions of novel compounds with diverse pharmaceutical applications.

1.1 The need for novel Pharmaceutically important compounds

The less accessibility and high cost of new generation antibiotics necessitate looking for the substances from alternative medicines with claimed antimicrobial activity. Today, most of the diseases caused by pathogens can be cured with the help of available antibiotics, but the discovery of any new antibiotic generally follows up with a course of resistance mechanism building up against it among the target organisms (Dimri et al., 2018). This phenomenon is known as 'antibiotic resistance is developing among microbial species at an appreciable rate, is a formidable complication of prudent and overuse of available antibiotics, and is imposing a serious health threat to human welfare. World Health Organization (WHO) in February 2017 published a report of antimicrobial-resistant bacteria for which new pharmaceutical compounds are urgently needed (WHO, 2017). Keeping in view the urgent requirement, the current review highlights various important aspects about the pharmaceutical potential of laboratory-grown cultures of BGA.

1.2 Blue-Green Algae

Blue-green algae are a group of extraordinary, diverse, gram-negative, oxygenic, photosynthetic prokaryotic, microscopic oldest organisms that originated 3.5 billion years ago (Kaushik et al., 2009). Blue-green algae are found all over the world, shows remarkable ecological diversity of habitats such as Freshwater (Khatoon et al., 2018; Chittapun et al., 2020), Terrestrial (Radzi et al., 2019; Riba et al., 2020), Marine (Basu et al., 2019; Uma et al., 2020), Hot spring (Tang et al., 2018; Cheng et al., 2020), etc. These BGA are also widely distributed in the polar region such as the Arctic, Antarctic, Southern Ocean, and Himalayas (Singh & Elster, 2007; Rego et al., 2019; Zaki et al., 2020). It has been estimated that about 2000 strains of freshwater and marine BGA are distributed all over the world. The capability to grow in adverse conditions and their autotrophic nature makes them an eligible candidate to grow in low nutrient-deficient lakes, ponds, and oceans which pose a serious threat to water and result in eutrophication. This may cause unpleasant tastes and odors of water through the secretion of volatile compounds. Random screening of blue-green algae will continue to play an important role in the drug discovery process for the foreseeable future. Several studies have been conducted for the isolation and identification of Blue-Green Algae from water, soil, sediments, algal mats, etc. (Figure 1-8) using advanced morphological, physiological, and molecular characterization techniques (Bellinger & Sigee, 2015; Hokmollahi et al., 2016; Radkova et al., 2020). These BGA have successfully grown on a laboratory scale using selective media i.e. BG-11, BG-13, Chu 10 (Chu, 1942; Rippka et al., 1979; Kaushik et al., 2010), Allen and Arnon Medium (Allen & Arnon, 1955), Fogg's Medium (Fogg's. 1965), Modified Bristol's Medium (Bold, 1949) and Pringsheim's Medium (Pringsheim, 1946). Blue-green algae do not require carbon or energy sources in their growth medium. Thus, they require only a basic inorganic medium, which has several logical advantages when performing the mass culture and purification of active compounds. Flask cultivation and mass cultivation for instance open pond system, hybrid system, closed photobioreactors are very well-known culturing methods used for generating biomass maintaining proper light, temperature, water, CO_2 supply, pH, nutrient supply, and proper mixing (Kaiwan-arporn et al., 2012; Troschl et al., 2017; Al-Saman et al., 2020; Jo et al., 2020). Lyophilization (freeze-drying), air drying, and sun-drying are some known popular techniques to convert biomass into powder (Smetana et al., 2017). Aqueous extraction i.e. cold and hot water extraction, organic extraction i.e. polar solvent extraction, semi-polar solvent extraction, non-polar solvent extraction, mix solvents extraction and sequential extractions, soxhlet extraction have been used extensively to isolate medicinal value active ingredients (Fatima et al., 2017; Vanlalveni et al., 2018; Yücer et al., 2018; Saurav et al., 2019). Generally, all blue-green algae vegetative cells contain carboxysomes, pseudocrystalline aggregates of the key enzymes of CO_2 fixation via the reductive pentose phosphate pathway and glycogen is a general carbohydrate reserve material of cyanobacteria. Other cellular inclusions include Poly-β-hydroxybutyrate (PHB) granules, cyanophycean granules, polyphosphate granules, carboxysomes or polyhedral bodies, and gas

vesicles (Stanier, 1988). They show notable ecological diversity. Because of extensive eutrophication of lakes, ponds and some parts of oceans BGA often forms blooms, which lead to water hygienic problems (Chorus et al., 2000; Duy et al., 2000). They may cause unpleasant tastes and odors through the excretion of volatile compounds (Jones & Korth, 1995; Liu et al., 2006).

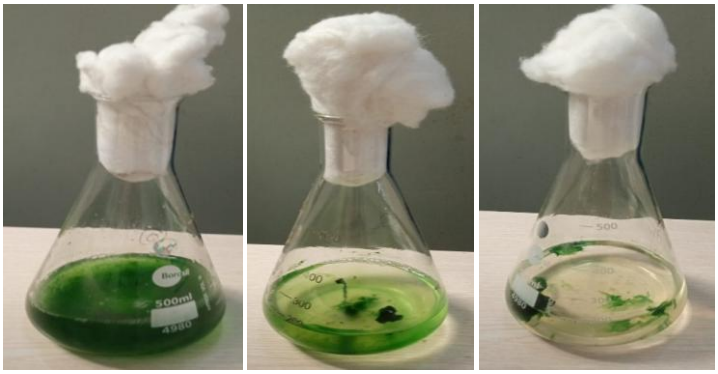


Figure 1 Laboratory Grown Culture of BGA (Medium: BG-11): A. *Calothrix* sp., B. *Spirulina* sp. C. *Oscillatoria* sp.

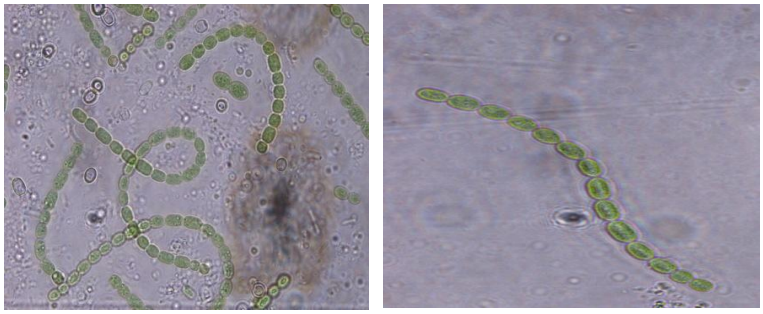


Figure 2 Microscopic images of *Anabaena* species (Chauhan & Jindal, 2020)

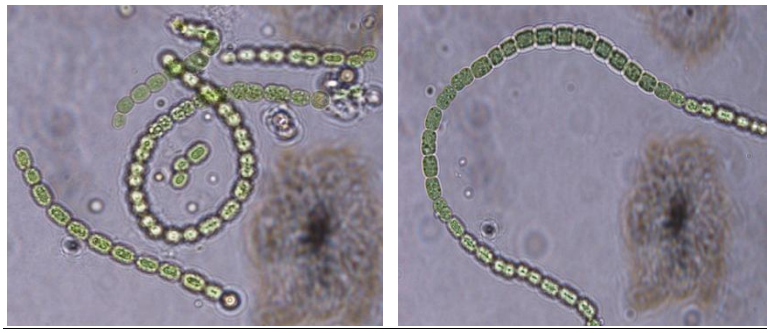


Figure 3 Microscopic images of *Nostoc* species (Chauhan & Jindal, 2020)

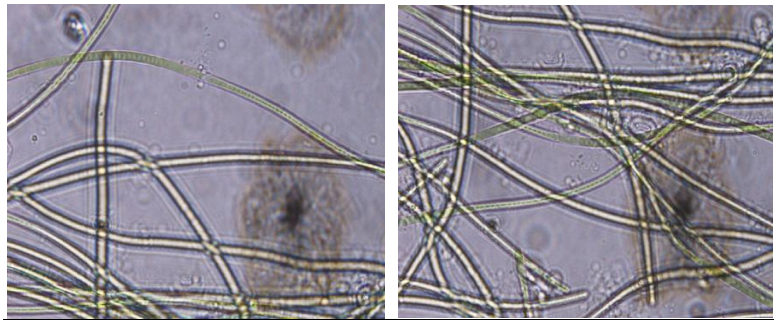


Figure 4 Microscopic images of *Calothrix* species (Chauhan & Jindal, 2020)

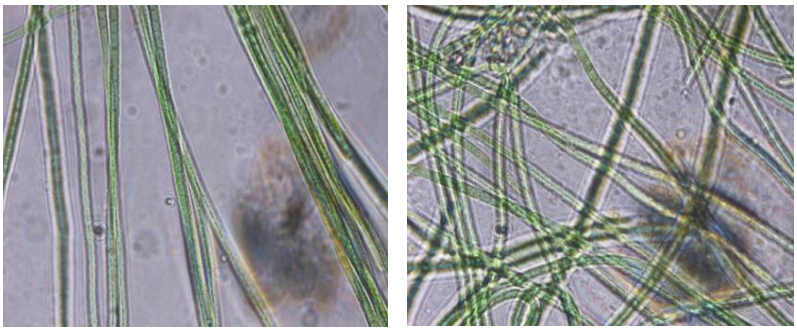


Figure 5 Microscopic images of *Oscillatoria* species (Chauhan & Jindal, 2020)

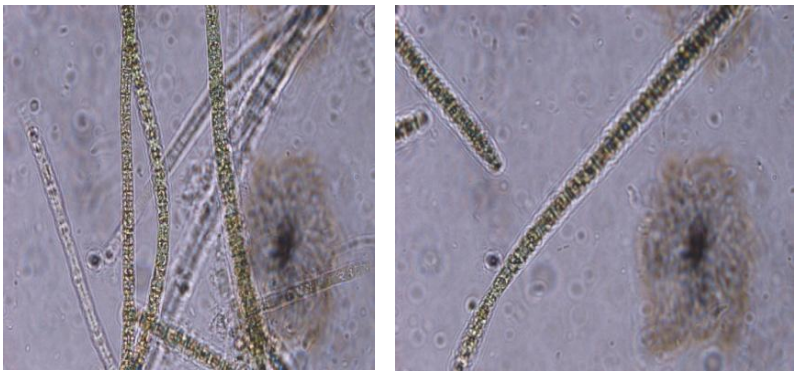


Figure 6 Microscopic images of *spirulina species* (Chauhan & Jindal, 2020)

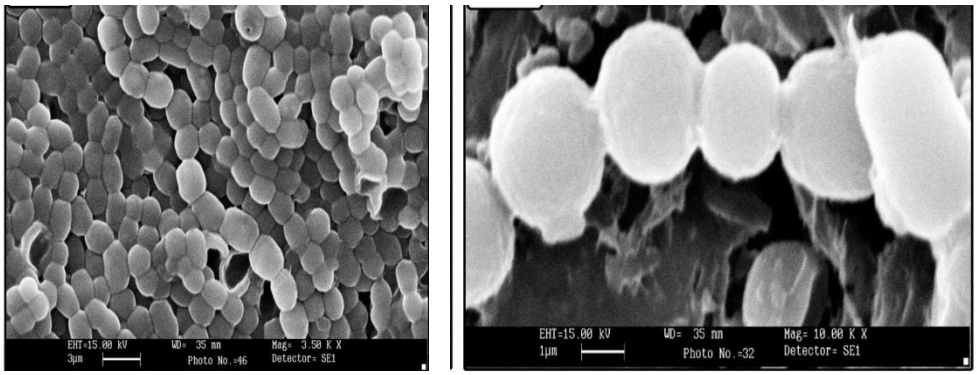


Figure 7 Scanning Electron Micrograph (SEM) image of *Anabaena* sp. (A) and *Nostoc* sp. (B) as per Kaushik & Chauhan (2008a)

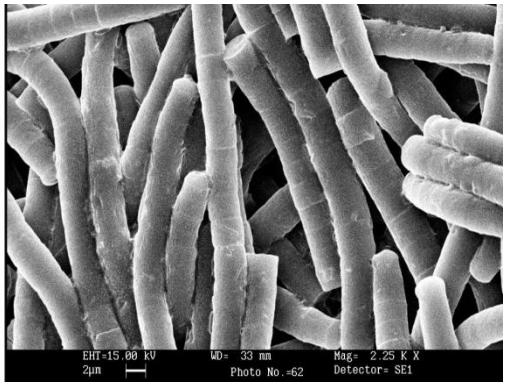


Figure 8 Scanning Electron Micrograph (SEM) image of *Spirulina platensis* (Kaushik & Chauhan, 2008b)

2 Pharmaceutical Potential of BGA

Blue-green algae are the rich source of structurally novel and biologically active metabolites with diverse antibacterial, antifungal, antiviral (including the anti-SARS-CoV-2 virus that causes COVID-19), anticancer, antioxidant, antidiabetic, protease inhibitory activity, anti-inflammatory activity, immunomodulatory activity, larvicide, and protease inhibitory activity, etc. (Figure 9) (Nainangu et al., 2020; Jafari et al., 2021). The first time before 1500 BC, medicinal and nutritional properties have been investigated for Nostoc algal species to treat gout, fistula, and cancer (Cardellina et al., 1979a; Shishido et al., 2020).

These photosynthetic microorganisms can yield proteins, carbohydrates, and lipids as a result of photosynthesis thus referred to as important biological resources having a wide range of biotechnological applications in the modern world due to their ability to grow rapidly even in harsh environmental conditions (Padmini et al., 2021). A search of these organisms for medicinal purposes has revealed important chemical prototypes for the finding of new agents, stimulating the use of refined physical techniques and new syntheses of molecules with the pharmaceutical application for human welfare. Phytochemical's constituents described from extracts of Blue-Green algae have been described by researchers (Figure 10) and are very well documented (Vasudevan et al., 2020; Nainangu et al., 2020; Gabr et al., 2020; Vasudevan et al., 2020)

2.1 Pharmaceutically important compounds isolated from BGA

Secondary metabolites refer to those compounds that are not used by the organisms for their primary metabolisms. Secondary metabolites influence other organisms in the vicinity and are thought to be of phylogenetic importance (Carpine & Sieber, 2021). Secondary metabolites include several types of compounds that may act as hormones, antibiotics, allelochemicals, toxins, and biotoxins that are found in surface supplies of fresh water (Carmichael, 1992). The ability of such compounds to kill bacteria and fungi have been well documented (Bonjouklian et al., 1988). The properties of secondary metabolites in nature are not completely understood (Metting & Pyne, 1986; Inderjit & Dakshini, 1994; Vasudevan et al., 2020).

The blue-green algae bear the characteristics to secrete vitamins, amino acids, fatty acids, carbohydrates, and various primary and secondary metabolites like amines, histamines, histidine, tannins, terpenoids, bromophenol, and polysaccharides (Figure 11). Few of these compounds are proven to be biologically active (Metting & Pyne, 1986; Padmini et al., 2021). The recent examples are cyanovirin-N secreted by *Nostoc* ellipososporum and anti-HIV glycolipids secreted by Isochyrosis and bromophenol are secreted by *Calothrix* sp. (Jaspars & Lawton 1998; Safari et al., 2020).

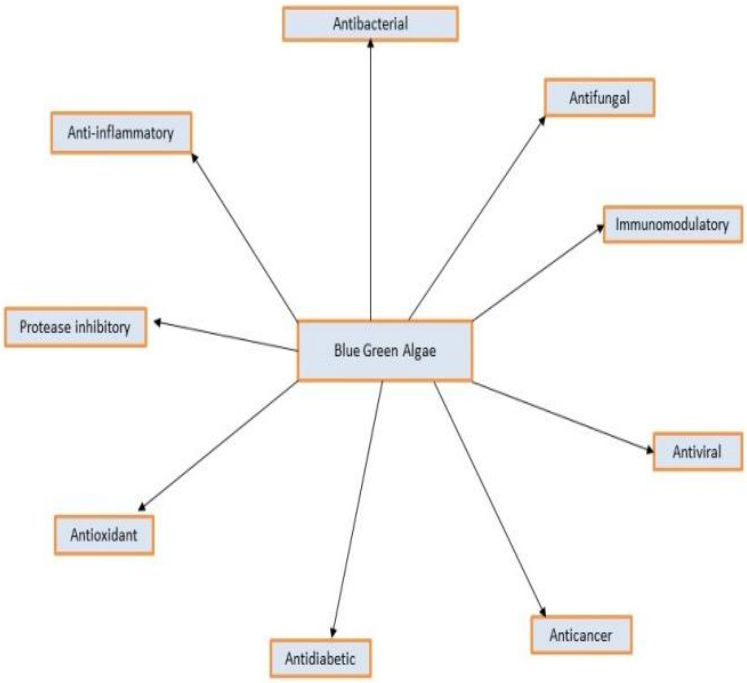


Figure 9 Pharmaceutical Potential of Blue-Green Algae

Figure 10 Major Phytochemicals constituents described from extracts of Blue-Green algae

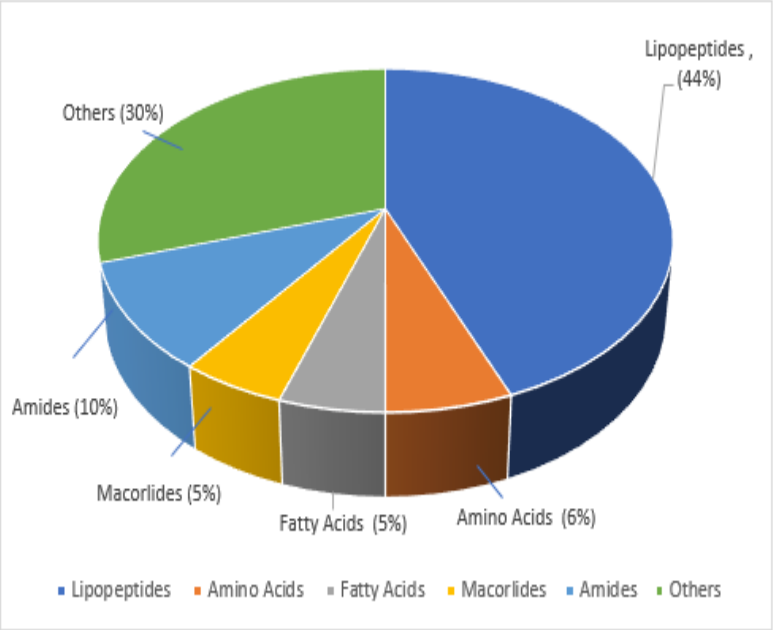


Figure 11 Pharmaceutically Important Compounds isolated from BGA

2.1.1 Antibacterial Potential of BGA

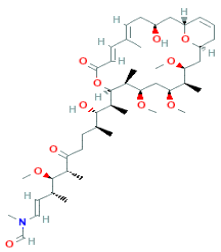
BGA are known for the secretions of antibacterial compounds with potential antibacterial activity against both Gram-positive and Gram-negative bacteria. Several strains such as *Anabaena*, *Lyngbya*, *Calothrix*, *Spirulina*, *Nostoc*, *Hapalosiphone*, *Phormidium*, and *Oscillatoria* have been identified by researchers from different habitats which can produce a wide variety of antibacterial molecules having therapeutic potentials (Chauhan et al., 2022). These organisms are even being altered genetically using biotechnological interventions for the production of various active compounds having antibacterial activity such as Bacteriocin Ambigol A, Parsiguine, Hapalindole, Hormothamnin A. *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus sanguinis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *L. monocytogenes*, *Salmonella typhimurium*, *Enterobacter aerogenes*, *Klebsiella pneumonia*, *Methicillin-resistant B. anthracis* are the examples of some gram-positive and Gram-negative bacteria which have studied for the inhibitory action of BGA (Luesch et al., 2001; Muller et al., 2006; Mo et al., 2009; Sturdy et al., 2010). The first partly identified antimicrobial compound isolated from algae were obtained from unicellular green algae particularly, *Chlorella* which contained a substance termed as '*chlorellin*' that exhibited inhibitory activity against both Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* (Pratt et al., 1944). *Chlorellin* is composed of peroxides of unsaturated fatty acids (Spoehr & Milner, 1949). Kaushik & Chauhan (2008a) had reported the antibacterial activity of several species of cyanobacteria such as Anabaena, *Lyngbya*, *Calothrix*, *Spirulina*, *Nostoc*, *Hapalosiphone*, *Phormidium*, and *Oscillatoria*, etc. against both Gram-positive (*Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, etc.) and Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, etc.). In a similar study, various species of *Anabaena* were evaluated for their antimicrobial activity and active antibacterial extracts were further screened for the presence of various chemical constituents through HPTLC techniques (Kaushik et al., 2009; Chauhan et al., 2010). Extracts of *Nostoc commune* and *Lyngbya majuscula* were studied for potent antimicrobial activity against clinically significant microorganisms (Kaushik & Chauhan, 2008a; Kaushik & Chauhan 2008b; Kaushik et al., 2009; Verma et al., 2016). HPTLC analysis were also performed to identify novel pharmaceutical compounds responsible for the activity. In a recent study, El-Sheekh et al. (2021) have evaluated the antibacterial activity of *Oscillatoria* sp. and *Spirulina* mediated silver and gold nanoparticles. Two new antibacterial molecules namely Arachidonoyl dopamine and fluocinolone recently discovered from methanolic extracts of *Arthrospira platensis*, a BGA isolated from a hypersaline lake in Rajasthan, India (Singh et al., 2021b). In another study, Antibacterial efficacy extracts of *Oxynema thaianum* have been assessed against multi-drug-resistant bacteria such as *E. coli* and *K. pneumoniae* (Padmini et al., 2021). Antibacterial compounds discovered from various species of BGA have been listed in Table 1 and Figure 12.

Table 1 Antibacterial compounds reported from BGA

BGA Sps.	Antibacterial Compounds	Detail of Chemical compounds	References
Scytonema pseudo hofmanni	Scytophycins C	MF: $C_{45}H_{75}NO_{11}$ MW: 806.1g/mol IUPAC Name: N-[(E,3R,4R,5R,9S,10S,11S)-10-hydroxy-11-[(1S,3S,4R,5S,7R,8S,9R,12E,14E,17S,19R)-17-hydroxy-3,5,7-trimethoxy-4,8,14-trimethyl-11-oxo-10,23-dioxabicyclo[17.3.1]tricosa-12,14,20-trien-9-yl]-4-methoxy-3,5,9-trimethyl-6-oxododec-1-enyl]-N-methylformamide	Ishibashi et al., 1986
Scytonema ocellatum, Tolypothrix conglutinate	Tolytoxin	MF: $C_{46}H_{75}NO_{13}$ MW: 850.1g/mol IUPAC Name: N-[(E,3R,4R,5R,9S,10S,11S)-10-hydroxy-11-[(1S,3S,4S,5S,7R,8S,9R,12E,14E,16S,17R,19R)-16-hydroxy-3,5,7,17-tetramethoxy-8,14-dimethyl-11-oxospiro[10,23-dioxabicyclo[17.3.1]tricosa-12,14,20-triene-4,2'-oxirane]-9-yl]-4-methoxy-3,5,9-trimethyl-6-oxododec-1-enyl]-N-methylformamide	Moore, 1982
Tolypothrix nodosa	Tolyporphin J	MF: $C_{24}H_{22}N_4O_4$ MW: 430.5g/mol IUPAC Name: 3,13-dihydroxy-3,7,13,18-tetramethyl-22,24-dihydroporphyrin-2,12-dione	Prinsep et al., 1992
Fischerella ambigua	Ambigol A	MF: $C_{18}H_8Cl_6O_3$ MW: 485g/mol IUPAC Name: 3,5-dichloro-2(3,5-dichloro-2-hydroxyphenyl)-6-(2,4-dichlorophenoxy)phenol	Falch et al., 1995
Nostoc muscorum	Muscoride A	MF: $C_{28}H_{40}N_4O_5$ MW: 512.6g/mol IUPAC Name: 3-methylbut-2-enyl 5-methyl-2-[5-methyl-2-[1-[3-methyl-2-(2-methylbut-3-en-2-ylamino)butanoyl]pyrrolidin-2-yl]-1,3-oxazol-4-yl]-1,3-oxazole-4-carboxylate	Nagatsu et al., 1995
Fischerella ambigua	Tjipanazole D	Compound CID: 10087661 MF: $C_{18}H_{10}Cl_2N_2$ MW: 325.2g/mol IUPAC Name: 3,8-dichloro-11,12-dihydroindolo[2,3-a] carbazole	Falch et al., 1995
Microcystis aeruginosa	Kawaguchipeptin A	MF: $C_{68}H_{92}N_{16}O_{18}$ MW: 1421.6g/mol IUPAC Name: 2-[(3S,9S,12S,15S,17S,25R,28S,31S,34S,40S,43R,46S,48S,56S)-28,31,40-tris(2-amino-2-oxoethyl)-9-[(1R)-1-hydroxyethyl]-12-(hydroxymethyl)-17,48-bis(3-methylbut-2-enyl)-43-(2-methylpropyl)-2,8,11,14,27,30,33,36,39,42,45-undecaoxo-1,7,10,13,24,26,29,32,35,38,41,44,55-tridecazaoctacyclo[44.10.0.03,7.015,26.017,25.018,23.048,56.049,54]hexapentaconta-18,20,22,49,51,53-hexaen-34-yl]acetic acid	Ishida et al., 1997
Nostoc spongiaeforme var. tenue	Tenuecyclamide A	MF: $C_{19}H_{20}N_6O_4S_2$ MW: 460.5g/mol IUPAC Name: (4S)-4,7,11,18-tetramethyl-6-oxa-13,20-dithia-3,10,17,22,23,24-hexazatetracyclo[17.2.1.15,8.112,15]tetracosa-1(21),5(24),7,12(23),14,19(22)-hexaene-2,9,16-trione	Banker & Carmeli, 1998
Nostoc commune	1,8-dihydroxy-4-methyl anthraquinone	MF: $C_{15}H_{10}O_4$ MW: 254.24g/mol IUPAC Name: 4,5-dihydroxy-1-methylanthracene-9,10-dione	Jaki et al., 2000
Calothrix sp.	Calothrixin A	MF: $C_{19}H_{10}N_2O_3$ MW: 314.3g/mol IUPAC Name: 20-oxido-10-aza-20-azoniapentacyclo[11.8.0.03,11.04,9.014,19]henicosa-1(13),3(11),4,6,8,14,16,18,20-nonaene-2,12-dione	Doan et al.,2000

BGA Sps.	Antibacterial Compounds	Detail of Chemical compounds	References
Nostoc commune	Comnastin A	MF: $C_{27}H_{40}O_4$ MW: 428.6g/mol IUPAC Name: 3-[[(3R,3aR,5aS,6R,7R,9aS,9bR)-3-(hydroxymethyl)-3,3a,6,7,9a-pentamethyl-2,4,5,5a,7,8,9,9b-octahydro-1H-cyclopenta[a]naphthalen-6-yl]methyl]-4-hydroxybenzoic acid	Jaki et al., Jaki et al., 2000
Nostoc sp.	Nostocyclyne A	MF: $C_{23}H_{34}O_2$ MW: 342.5g/mol IUPAC Name: 15-propylbicyclo[14.2.2]icosa-1(19),16(20),17-trien-2-yne-17,20-diol	Ploutno & Carmeli, 2000
Nostoc spongiaeforme	Nostocine A	MF: $C_5H_5N_5O$ MW: 151.13g/mol IUPAC Name: 7-methyl-2H-pyrazolo[4,3-e][1,2,4]triazin-3-one	Hirata et al., 2003
Oscillatoria redekei	a-dimorphecolic acid	MF: $C_{18}H_{32}O_3$ MW: 296.4g/mol IUPAC Name: (9S,10E,12Z)-9-hydroxyoctadeca-10,12-dienoic acid	Mundt et al., 2003
Scytonema hofmanni PCC 7110	Scyptolin A	MF: $C_{45}H_{69}ClN_8O_{14}$ MW: 981.5g/mol IUPAC Name: (2S,3R)-2-[[(2S)-2-(butanoylamino)propanoyl]amino]-N-[(2S,5S,8S,11R,12S,15S,18R,21R)-5-[(3-chloro-4-hydroxyphenyl)methyl]-21-hydroxy-2-[(1R)-1-hydroxyethyl]-4,11-dimethyl-15-(2-methylpropyl)-3,6,9,13,16,22-hexaoxo-8-propan-2-yl-10-oxa-1,4,7,14,17-pentazabicyclo[16.3.1]docosan-12-yl]-3-hydroxybutanamide	MacMillan & Molinski, 2005; Matern et al., 2001
Lyngbya sp.	Pahayokolide A	MF: $C_{72}H_{105}N_{13}O_{20}$ MW: 1472.7g/mol IUPAC Name: [1-[(6R,10R,13S,19R,22S,25E,28S,31Z,34S,37S)-6-(3-amino-3-oxopropyl)-34-benzyl-25,31-di(ethylidene)-9-hydroxy-22-[(1R)-1-hydroxyethyl]-28-(hydroxymethyl)-2,5,8,12,18,21,24,27,30,33,36-undecaoxo-19-(2-phenylethyl)-1,4,7,11,17,20,23,26,29,32,35-undecazatricyclo[35.3.0.013,17]tetracontan-10-yl]-4,5-dihydroxy-7-methyloctan-2-yl] (2S)-2-[acetyl(methyl)amino]-4-methylpentanoate	Berry Berry et al., 2004
Microcoleous lacustris	Abietane	MF: $C_{20}H_{36}$ MW: 276.5g/mol IUPAC Name: (2S,4aS,4bR,8aS,10aS)-4b,8,8-trimethyl-2-propan-2-yl-1,2,3,4,4a,5,6,7,8a,9,10,10a-dodecahydrophenanthrene	Thajuddin & Subramanian, 2005
Fischerella sp.	Hapalindole T	MF: $C_{21}H_{23}ClN_2OS$ MW: 386.9g/mol IUPAC Name: (2S,6S,7R,8R,10R)-8-chloro-7-ethenyl-7,11,11-trimethyl-3-thia-5,17-diazapentacyclo[10.6.1.02,6.02,10.016,19]nonadeca-1(18),12(19),13,15-tetraen-4-one	Asthana et al., 2009
Fischerella ambigua	Ambigol B	MF: $C_{18}H_8Cl_6O_3$ MW: 485g/mol IUPAC Name: 3,5-dichloro-2,6-bis(2,4-dichlorophenoxy)phenol	Raveh & Carmeli, 2007
Nostoc sp.	Carbamidocyclophane A	MF: $C_{38}H_{54}Cl_4N_2O_8$ MW: 808.6g/mol IUPAC Name: [(2R,3S,13R,14S)-13-carbamoyloxy-8,19-bis(4,4-dichlorobutyl)-10,21,24,26-tetrahydroxy-3,14-dimethyl-2-tricyclo[18.2.2.29,12]hexacosa-1(22),9,11,20,23,25-hexaenyl] carbamate	Bui et al., 2007
Nostoc sp.	Nostocarboline Hydroiodide; Nostacarboline Iodide	MF: $C_{12}H_{10}ClIN_2$ MW: 344.58g/mol IUPAC Name: 6-chloro-2-methyl-9H-pyrido[3,4-b]indol-2-ium;iodide	Becher et al., 2007
Fischerella ambigua	Ambiguine A isonitrile	MF: $C_{26}H_{31}ClN_2$ MW: 407g/mol IUPAC Name: (2S,3R,4R,5R,7S)-5-chloro-4-ethenyl-3-isocyano-4,8,8-trimethyl-15-(2-methylbut-3-en-2-yl)-14-azatetracyclo[7.6.1.02,7.013,16]hexadeca-1(15),9(16),10,12-tetraene	Mo et al., 2009

BGA Sps.	Antibacterial Compounds	Detail of Chemical compounds	References
Fischerella ambigua	Ambiguine A isonitrile	MF: $C_{26}H_{31}ClN_2$ MW: 407g/mol IUPAC Name: (2S,3R,4R,5R,7S)-5-chloro-4-ethenyl-3-isocyano-4,8,8-trimethyl-15-(2-methylbut-3-en-2-yl)-14-azatetracyclo[7.6.1.02,7.013,16]hexadeca-1(15),9(16),10,12-tetraene	Mo et al., 2009
Leptolyngbya crosbyana	Crossbyanol A	MF: $C_{30}H_{15}Br_7O_6$ MW: 1030.8g/mol IUPAC Name: 3-bromo-4-[2-bromo-4-(3-bromo-4-hydroxyphenoxy)-6-(2,4-dibromophenoxy)phenoxy]-2-(2,4-dibromophenoxy)phenol	Choi et al., 2010
Nostoc sp. MGL001	9-Ethyliminomethyl-12-(morpholin-4- ylmethoxy) 5, 8, 13, 16-tetraaza-hexacene-2, 3 dicarboxy-licacid (EMTAHDCA)		Niveshika et al., 2016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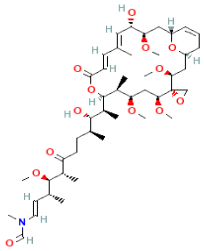
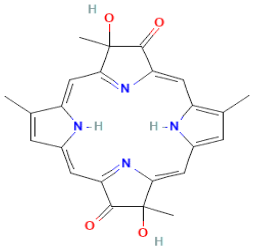

Tolytoxin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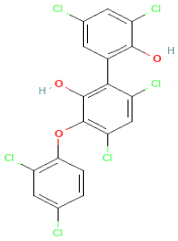
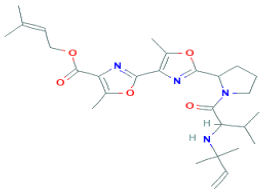
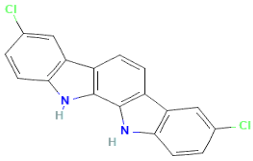
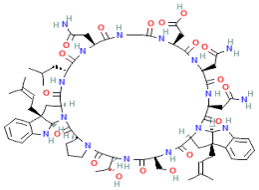
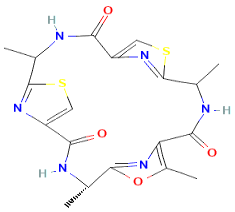

Ambigol A


Muscoride A


Tjipanazole D


Kawaguchipeptin A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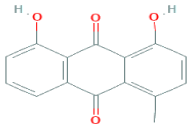
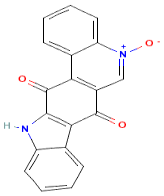
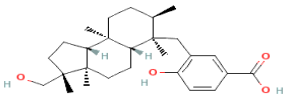
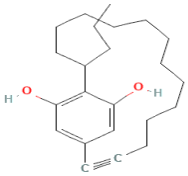
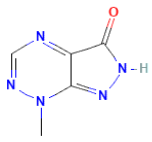
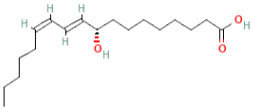

1,8-dihydroxy-4-methyl anthraquinone

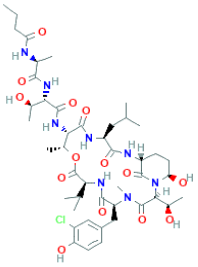

Calothrixin A

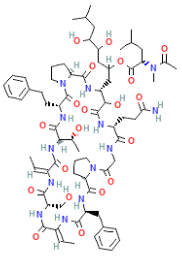

Comnastin A

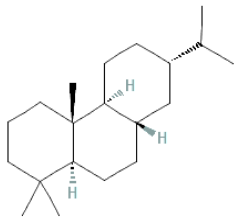

Nostocyclyne A


Nostocine A

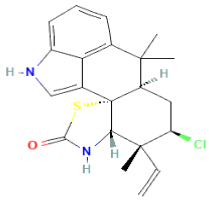

a-dimorphecolic acid


Scyptolin A

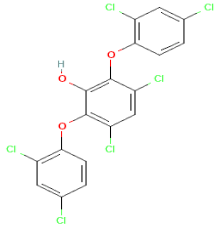

Pahayokolide A



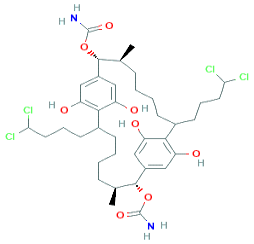
Abietane

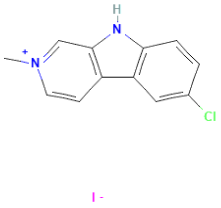


Hapalindole T

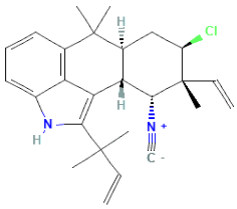


Ambigol B

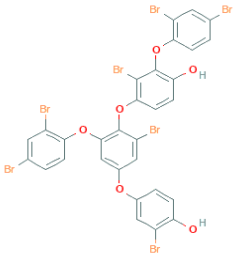




Nostocarboline Hydroiodide; Nostacarboline Iodide



Ambiguine A isonitrile



Crossbyanol A

9-Ethyliminomethyl-12-(morpholin-4- ylmethoxy) 5, 8, 13, 16-tetraaza-hexacene-2, 3 dicarboxy-licacid (EMTAHDCA)

Figure Not available

Figure 12 Chemical structures of the active ingredients isolated from various BGA and having antibacterial potential

2.1.2 Antimycobacterial potential of BGA

BGA extracts and compounds have been tested against various species of Mycobacteria. Rao et al. (2007) reported the antimycobacterial activity of different spp. of BGA *viz., Hapalosiphon sp*., *Anabaena sp. Lyngbya sp., Westeillopsis prolifica, Spirulina sp. Anabaena variabiles, Anabaena cylindrica, Oscillatoria sp.* and*Scytonema sp*. against *Mycobacterium tuberculosis ATCC 27294, M. tuberculosis MDR, M.avium, M. intracellulare,* and *M. aurum.* Other BGA species which exhibit antimycobacterial potential are *Tychonema* sp., *Fischerella ambigua* are *Lyngbyama juscule* (Muller et al., 2006; Mo et al., 2009; Sturdy et al., 2010; Luesch et al., 2001). Antimycobacterial compounds produced by these BGA strains are summarized in Table 2.

2.1.3 Antifungal potential of BGA

Antifungal properties of BGA strains have been documented globally. Fungal and yeast strains such as *Candida friedricki, Fusarium oxysporum, Aspergillus fumigatus, Alternaria alternate, A. niger, C. albicans, A. parasiticus, A. flavus, A. westerdijikia, A. ochraceus* ITAL 14, *A. carbonarius* ITAL 204, *A. steynii* IBT LKN 23096, *Penicillium verrucosum* BFE 500, *F. verticillioides* ITEM 10027, *F. proliferatum* MPVP 328 have been tested with BGA extracts and compounds (Marrez & Sultan, 2016; Vanlalveni et al., 2018; Saurav et al., 2019). Antifungal compounds reported from BGA strains have been listed in Table 3 and Figure 13**.**

2.1.4 Antioxidant potential of BGA

Blue-green algae produce large amounts of antioxidants to protect themselves from harmful stress conditions so that the cells can be protected from the effect of the reactive oxygen species (ROS) produced during stress. Hydrogen peroxide and oxygen free radicals are the two harmful reactive oxygen species formed in the cells during oxidative stress and can damage the cells (Vasudevan et al., 2020). Antioxidant properties of various extracts have been evaluated using different assay methods such as DPPH radical-scavenging Assay, $ABTS^{+}$ (2, 2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid); Nitric oxide radical scavenging Assay; Total Antioxidant capacity determination kit, and β-carotene bleaching assay (Table 5). BGA produces several antioxidants that can scavenge these free radicals. These antioxidants have been explored as a novel source of dietary supplements because the cyanobacteria are rich in phenolics, vitamins, and carotenoids, the most common being carotenoids, which can be used for alleviating oxidative stress and limiting health problems (Nainangu et al., 2020; Gabr et al., 2020). ROS are also formed in animals and humans during oxidative stress and cause damage to the biomolecules such as lipids, proteins, and DNA. Oxidative stress is associated with several diseases such as cancer, neurodegeneration, retinopathy, aging, and other diseases. Algae produce several substances that have antioxidant effects, and these substances can be used as dietary supplements by humans (Guerreiro et al., 2020; Safari et al., 2020; Li et al., 2020). Antioxidant compounds recently purified from Blue-Green Algae are summarized in Table 4.

Table 2 Antimycobacterial compounds reported from BGA

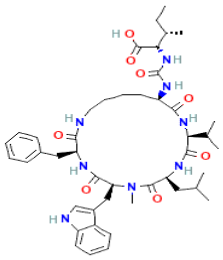
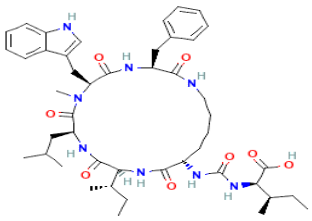
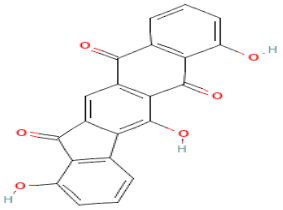
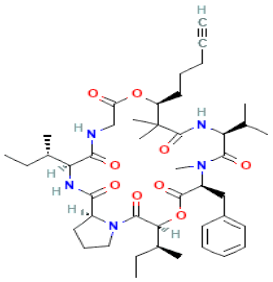
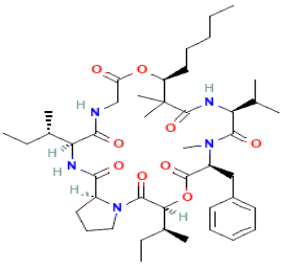
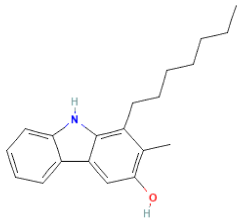
BGA Spp.	Antimycobacterial Compounds	Chemical Structure	References
Tychonema sp.	Brunsvicamide A MF: $C_{45}H_{64}N_8O_8$ MW: 845g/mol IUPAC Name: (2S)-2-[[(3S,6S,9S,12S,15S)-3-benzyl-6-(1H-indol-3-ylmethyl)-7-methyl-9-(2-methylpropyl)-2,5,8,11,14-pentaoxo-12-propan-2-yl-1,4,7,10,13-pentazacyclononadec-15-yl]carbamoylamino]-3-methylpentanoic acid		Muller et al., 2006
Tychonema sp.	Brunsvicamide B MF: $C_{46}H_{66}N_8O_8$ MW: 859.1g/mol IUPAC Name: (2S)-2-[[(3S,6S,9S,12S,15S)-3-benzyl-12-butan-2-yl-6-(1H-indol-3-ylmethyl)-7-methyl-9-(2-methylpropyl)-2,5,8,11,14-pentaoxo-1,4,7,10,13-pentazacyclononadec-15-yl]carbamoylamino]-3-methylpentanoic acid		Muller et al., 2006
Fischerella ambigua	Eucapsitrione MF: $C_{21}H_{10}O_6$ MW: 358.3g/mol IUPAC Name: 2,8,19-trihydroxypentacyclo[11.8.0.03,11.04,9.015,20]henicosa-1(13),2,4(9),5,7,11,15(20),16,18-nonaene-10,14,21-trione		Sturdy et al., 2010
Lyngbya majuscula	Pitipeptolide A MF: $C_{44}H_{65}N_5O_9$ MW: 808g/mol IUPAC Name: (3S,6S,9S,13S,19S,22S)-6-benzyl-3,19-bis[(2S)-butan-2-yl]-7,12,12-trimethyl-13-pent-4-ynyl-9-propan-2-yl-4,14-dioxa-1,7,10,17,20-pentazabicyclo[20.3.0]pentacosane-2,5,8,11,15,18,21-heptone		Luesch et al., 2001
Lyngbya majuscula	Pitipeptolides C MF: $C_{44}H_{69}N_5O_9$ MW: 812g/mol IUPAC Name: (3S,6S,9S,13S,19S,22S)-6-benzyl-3,19-bis[(2S)-butan-2-yl]-7,12,12-trimethyl-13-pentyl-9-propan-2-yl-4,14-dioxa-1,7,10,17,20-pentazabicyclo[20.3.0]pentacosane-2,5,8,11,15,18,21-heptone		Mo et al., 2009

Table 3 Antifungal compounds reported from BGA

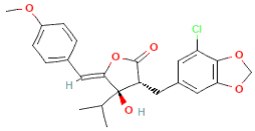
BGA Spp.	Antifungal Compounds	Detail of Chemical compounds	References
Hyella caespitosa	Carazostatin	MF: $C_{20}H_{25}NO$ MW: 295.4g/mol IUPAC Name: 1-heptyl-2-methyl-9H-carbazol-3-ol	Cardellina et al., 1979a
Scytonema hofmanni	Cyanobacterin	MF: $C_{23}H_{23}ClO_6$ MW: 430.9g/mol IUPAC Name: (5Z)-3-[(7-chloro-1,3-benzodioxol-5-yl)methyl]-4-hydroxy-5-[(4-methoxyphenyl)methylidene]-4-propan-2-yloxolan-2-one	Mason et al., 1982
Tolypothrix tenuis	Toyocamycin	MF: $C_{12}H_{13}N_5O_4$ MW: 291.26g/mol IUPAC Name: 4-amino-7-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]pyrrolo[2,3-d]pyrimidine-5-carbonitrile	Moore, 1982
Tolypothrix tenuis	Tubercidin	MF: $C_{11}H_{14}N_4O_4$ MW: 266.25g/mol IUPAC Name: (2R,3R,4S,5R)-2-(4-aminopyrrolo[2,3-d]pyrimidin-7-yl)-5-(hydroxymethyl)oxolane-3,4-diol	Moore, 1982; Banker & Carmeli, 1998
Hapalosiphon fontinalis	Anhydrohaloxindole A; Anhydrohapaloxindole A	MF: $C_{21}H_{21}ClN_2O$ MW: 352.9g/mol IUPAC Name: (3R,4R,5R,7R)-5-chloro-4-ethenyl-3-isocyano-4,8,8-trimethyl-14-azatetracyclo[7.6.1.02,7.013,16]hexadeca-1,9(16),10,12-tetraen-15-one	Moore et al., 1987
Hapalosiphon fontinalis	Fontonamide	MF: $C_{20}H_{22}ClNO_2$ MW: 343.8g/mol IUPAC Name: N-[(6R,7R,10aR)-6-chloro-7-ethenyl-7,10,10-trimethyl-9-oxo-6,10a-dihydro-5H-anthracen-1-yl]formamide	Moore et al., 1987
Nostoc sp.	Nostocyclamide	MF: $C_{20}H_{22}N_6O_4S_2$ MW: 474.6g/mol IUPAC Name: (4S,18R)-4,7-dimethyl-18-propan-2-yl-6-oxa-13,20-dithia-3,10,17,22,23,24-hexazatetracyclo[17.2.1.15,8.112,15]tetracosa-1(21),5(24),7,12(23),14,19(22)-hexaene-2,9,16-trione	Moore et al., 1988
Hormothamnion enteromorphoides	Hormothamnin A	MF: $C_{60}H_{97}N_{11}O_{14}$ MW: 1196.5g/mol IUPAC Name: (3Z)-28-benzyl-19,22-di(butan-2-yl)-3-ethylidene-36-hydroxy-6,31-bis(2-hydroxyethyl)-16,25-bis(2-methylpropyl)-10-pentyl-1,4,7,11,14,17,20,23,26,29,32-undecazabicyclo[32.3.0]heptatriacontane-2,5,8,12,15,18,21,24,27,30,33-undecone	Gerwick et al., 1989
Calothrix fusca	Calophycin	MF: $C_{19}H_{18}N_2O_3$ MW: 322.4g/mol IUPAC Name: (3R)-2-[(4-hydroxyphenyl)methyl]-1,3,4,9-tetrahydropyrido[3,4-b]indole-3-carboxylic acid	Moon et al., 1992
Dichothrix baueriana	Bauerine B	MF: $C_{12}H_8Cl_2N_2$ MW: 251.11g/mol IUPAC Name: 7,8-dichloro-9-methylpyrido[3,4-b]indole	Larsen et al., 1994
Nostoc sp. ATCC 53789)	Cryptophycin 1	MF: $C_{35}H_{43}ClN_2O_8$ MW: 655.2g/mol IUPAC Name: (3S,6R,10R,13E,16S)-10-[(3-chloro-4-methoxyphenyl)methyl]-6-methyl-3-(2-methylpropyl)-16-[(1S)-1-[(2R,3R)-3-phenyloxiran-2-yl]ethyl]-1,4-dioxa-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetrone	Trimurtulu et al., 1994
Hapalosiphon welwitschii, *Westiella intricate*	Welwitindolinone A isonitrile	MF: $C_{21}H_{21}ClN_2O$ MW: 352.9g/mol IUPAC Name: (3S,3'S,4'R,6'R)-4'-chloro-3'-ethenyl-2'-isocyano-3',7',7'-trimethylspiro[1H-indole-3,8'-bicyclo[4.2.0]oct-1-ene]-2-one	Stratmann et al., 1994

BGA Spp.	Antifungal Compounds	Detail of Chemical compounds	References
Nostoc commune	Nostofungicidine	MF: $C_{48}H_{76}N_{10}O_{18}$ MW: 1081.2g/mol IUPAC Name: 2-[16-(2-amino-1-hydroxy-2-oxoethyl)-27-hydroxy-19-[hydroxy-(4-hydroxyphenyl)methyl]-3,22-bis(hydroxymethyl)-10-(3-hydroxypentadecyl)-2,5,8,12,15,18,21,24-octaoxo-1,4,7,11,14,17,20,23-octazabicyclo[23.3.0]octacosan-13-yl]-2-hydroxyacetamide	Kajiyama et al., 1998
Fischerella muscicola	Fischerellin B	MF: $C_{20}H_{29}NO$ MW: 299.4g/mol IUPAC Name: (3R,5S)-3-methyl-5-[(E)-pentadec-5-en-7,9-diynyl]pyrrolidin-2-one	Srivastava et al., 1999
Lyngbya majuscula	Tanikolide	MF: $C_{17}H_{32}O_3$ MW: 284.4g/mol IUPAC Name: (6R)-6-(hydroxymethyl)-6-undecyloxan-2-one	Singh et al., 1999
Scytonema pseudo hofmanni	Scytophycin A	MF: $C_{45}H_{75}NO_{12}$ MW: 822.1g/mol IUPAC Name: N-[(E,3R,4R,5S,9S,10S,11S)-6,10-dihydroxy-11-[(1S,3S,4S,5S,7R,8S,9R,12E,14E,17S,19R)-17-hydroxy-3,5,7-trimethoxy-8,14-dimethyl-11-oxospiro[10,23-dioxabicyclo[17.3.1]tricosa-12,14,20-triene-4,2'-oxirane]-9-yl]-4-methoxy-3,5,9-trimethyldodec-1-enyl]-N-methylformamide	Matern et al., 2001
Tolypothrix byssodea	Tolybyssidin A	MF: $C_{71}H_{116}N_{16}O_{17}$ MW: 1465.8g/mol IUPAC Name: [(1R)-1-[(3S,6S,9S,12S,15S,18Z,21S,24S,27S,30S,33R,36S,39S)-21-benzyl-24,27-bis[(2S)-butan-2-yl]-36-[3-(diaminomethylideneamino)propyl]-18-ethylidene-9,30-bis[(1R)-1-hydroxyethyl]-33-(2-methylpropyl)-2,5,8,11,14,17,20,23,26,29,32,35,38-tridecaoxo-3,6,15-tri(propan-2-yl)-1,4,7,10,13,16,19,22,25,28,31,34,37-tridecazabicyclo[37.3.0]dotetracontan-12-yl]ethyl] acetate	Jaki et al., 2001
Tolypothrix byssodea	Tolybyssidin B	MF: $C_{72}H_{114}N_{16}O_{16}S$ MW: 1491.8g/mol IUPAC Name: 2-[3-[(2S,5S,8S,11S,14S,17S,20S,23S,26S,29S,32S,35S,38E)-32-benzyl-17-[(2S)-butan-2-yl]-38-ethylidene-14,20-bis[(1R)-1-hydroxyethyl]-5-[(4-hydroxyphenyl)methyl]-8-(2-methylsulfanylethyl)-3,6,9,12,15,18,21,24,27,30,33,36,39-tridecaoxo-11,23,26,29,35-penta(propan-2-yl)-1,4,7,10,13,16,19,22,25,28,31,34,37-tridecazacyclononatriacont-2-yl]propyl]guanidine	Jaki et al., 2001
Lyngbya confervoides	Lobocyclamine B	MF: $C_{65}H_{115}N_{13}O_{20}$ MW: 1398.7g/mol IUPAC Name: 3-[(3S,6R,9S,12R,15R,18S,21R,24S,28R,31S,34R,37S,39R)-9-[(2S)-butan-2-yl]-6-[(1R)-1,2-dihydroxyethyl]-28-heptyl-39-hydroxy-3,31-bis[(1R)-1-hydroxyethyl]-15,21-bis[(1S)-1-hydroxy-2-methylpropyl]-10,18-dimethyl-34-(2-methylpropyl)-2,5,8,11,14,17,20,23,26,30,33,36-dodecaoxo-24-propan-2-yl-1,4,7,10,13,16,19,22,25,29,32,35-dodecazabicyclo[35.3.0]tetracontan-12-yl]propanamide	MacMillan et al., 2002
Nostoc commune	Nostodione A	MF: $C_{18}H_{11}NO_3$ MW: 289.3g/mol IUPAC Name: (3E)-3-[(4-hydroxyphenyl)methylidene]-4H-cyclopenta[b]indole-1,2-dione	Bhadury & Wright, 2004
Hassallia sp.	Hassallidin A	MF: $C_{62}H_{99}N_{11}O_{24}$ MW: 1382.5g/mol IUPAC Name: N-[(2S,3R)-1-[[(3S,6S,12S,15Z,18S,21S,24S,25R)-3,12-bis(3-amino-3-oxopropyl)-15-ethylidene-21-[(1R)-1-hydroxyethyl]-18-[(4-hydroxyphenyl)methyl]-7,25-dimethyl-2,5,8,11,14,17,20,23-octaoxo-6-[(1R)-1-[(2R,3S,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxyethyl]-1-oxa-4,7,10,13,16,19,22-heptazacyclopentacos-24-yl]amino]-3-hydroxy-1-oxobutan-2-yl]-2,3-dihydroxytetradecanamide	Neuhof et al., 2005

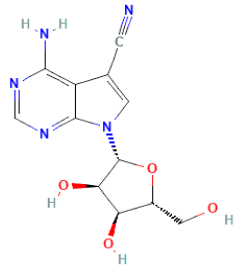
BGA Spp.	Antifungal Compounds	Detail of Chemical compounds	References
Fischerella ambigua	2,4-dichlorobenzoic acid	MF: $C_7H_4Cl_2O_2$ MW: 191.01g/mol IUPAC Name: 2,4-dichlorobenzoic acid	Wright et al., 2005
Geitlerinema sp.	Swinholide A	MF: $C_{78}H_{132}O_{20}$ MW: 1389.9g/mol IUPAC Name: (1R,3S,5E,7E,11S,12S,13R,15S,16S,17S,19S,23R,25R,27Z,29E,33S,34S,35R,37S,38S,39S,41S)-3,13,15,25,35,37-hexahydroxy-11-[(2S,3S,4R)-3-hydroxy-6-[(2S,4R,6S)-4-methoxy-6-methyloxan-2-yl]-4-methylhexan-2-yl]-33-[(2S,3S,4S)-3-hydroxy-6-[(2S,4R,6S)-4-methoxy-6-methyloxan-2-yl]-4-methylhexan-2-yl]-17,39-dimethoxy-6,12,16,28,34,38-hexamethyl-10,32,45,46-tetraoxatricyclo[39.3.1.119,23]hexatetraconta-5,7,21,27,29,43-hexaene-9,31-dione	Andrianasolo et al., 2005
Fischerella ambigua	Tjipanazole B	MF: $C_{23}H_{18}Cl_2N_2O_4$ MW: 457.3g/mol IUPAC Name: (2R,3R,4S,5R)-2-(3,8-dichloro-11H-indolo[2,3-a]carbazol-12-yl)oxane-3,4,5-triol	Wright et al., 2005
Nadularia harveyana	Norharmane	MF: $C_{11}H_8N_2$ MW: 168.19g/mol IUPAC Name: 9H-pyrido[3,4-b]indole	Volk & Furkert, 2006
Synechocystis sp.	AK-3	MF: $C_9H_{16}N_4OS$ MW: 228.32g/mol IUPAC Name: 2-(dimethylamino)-N-(5-propyl-1,3,4-thiadiazol-2-yl)acetamide	Yoon et al., 2006
Lyngbya majuscula	Hectochlorin	MF: $C_{27}H_{34}Cl_2N_2O_9S_2$ MW: 665.6g/mol IUPAC Name: [(5S,12S,13S,16S)-12-(4,4-dichloropentyl)-16-(2-hydroxypropan-2-yl)-4,4,13-trimethyl-2,10,14-trioxo-3,11,15-trioxa-7,18-dithia-20,21-diazatricyclo[15.2.1.16,9]henicosa-1(19),6(21),8,17(20)-tetraen-5-yl] acetate	Gademann & Portmann, 2008
Calothrix elenkinii	Benzoic Acid	MF: $C_7H_6O_2$ MW: 122.12g/mol IUPAC Name: benzoic acid	Natarajan et al., 2012



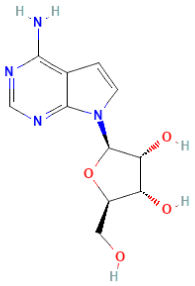
Carazostatin



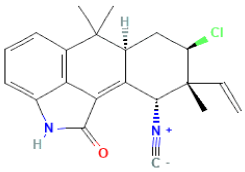
Cyanobacterin



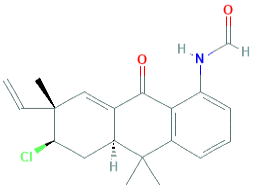
Toyocamycin



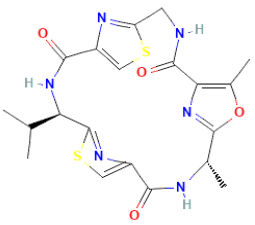
Tubercidin



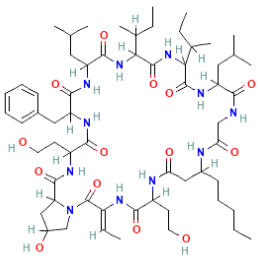
Anhydrohaloxindole A; Anhydrohapaloxindole A



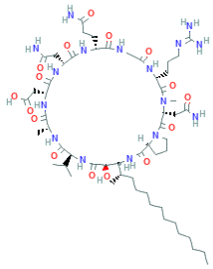
Fontonamide



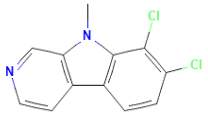
Nostocyclamide



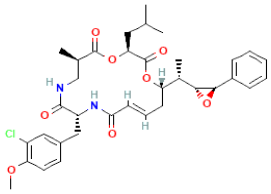
Hormothamnin A



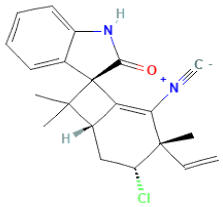
Calophycin



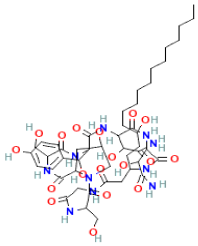
Bauerine B



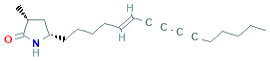
Cryptophycin 1



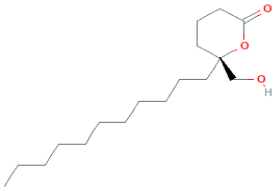
Welwitindolinone A isonitrile



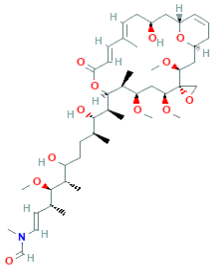
Nostofungicidine



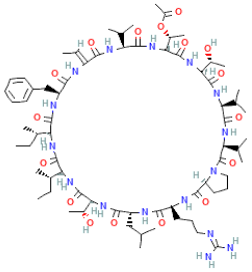
Fischerellin B



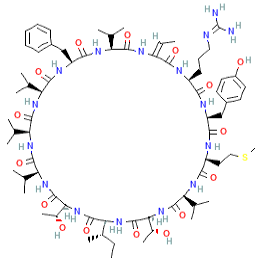
Tanikolide



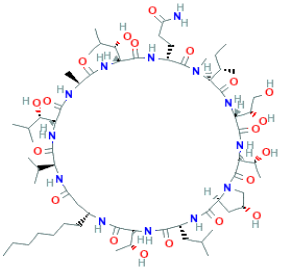
Scytophycin A



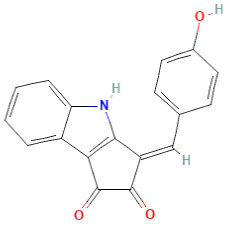
Tolybyssidin A



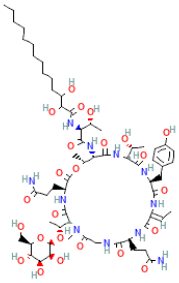
Tolybyssidin B



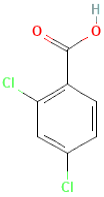
Lobocyclamine B



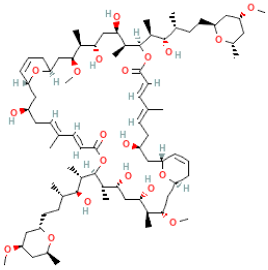
Nostodione A



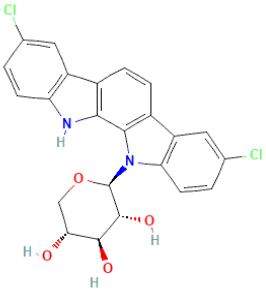
Hassallidin A



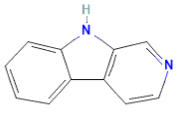
2,4-dichlorobenzoic acid



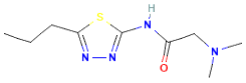
Swinholide A



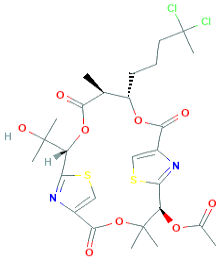
Tjipanazole B



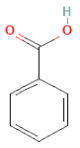
Norharmane



AK-3



Hectochlorin



Benzoic Acid

Figure 13 Chemical structures of the active ingredients isolated from various BGA and having antifungal propeties

Table 4 Antioxidants compounds recently purified from Blue-Green Algae

Antioxidants compounds	Source	References
Pyrogallol, E-Vanillic, Hespirdin	*Spirulina platensis*	Gabr et al., 2020
Benzeneacetanomide and Norvaline, n-propargyloxycarbonyl	*Microcystis aeruginosa*	Vasudevan et al., 2020
BHA, Beta tocopherol, Phytosterols	*Spirulina maxima*	Gamal et al., 2020
Caffeic acid, syringic acid, ferulic acid, p-coumaric acid, chlorogenic acid, kaempferol, quercetin and apigenin γ-linolenic acid, α-linolenic acid	*Spirulina platensis*	Bellahcen et al., 2020
Benzoic acid, 4-(1-azepinyl)azo-, ethyl ester (b) 1,6-methanonaphthalen-1(2H)-ol, octahydro-4,8a,9,9-tetramethyl, (c) Dibutyl phthalate, (d) 1,2-benzene dicarboxylic acid, (e) Hexadecanoic acid, 2-pentadecyl-1,3-dioxan-5-yl ester	*Oscillatoria sp.* SSCM01	Nainangu et al., 2020

Table 5 Types of BGA Extracts, Assay methods and their antioxidant potential

Blue-Green Algae	Type of Extract	Assay methods	Maximum Activity	References
Spirulina platensis	Ethanolic and aqueous extract	DPPH radical-scavenging Assay	96.33%	Gabr et al., 2020
Microcystis aeruginosa	Methanol Extract	Scavenging ability on 1, 1-diphenyl-2-picrylhydrazyl radicals (DPPH) Hydroxyl radical scavenging assay	54% 49%	Vasudevan et al., 2020
Spirulina maxima	-	DPPH radical-scavenging Assay	25.73 %	Gamal et al., 2020
Spirulina platensis	Ethanolic, aqueous, and lipidic extracts	Diphenyl-1-picrylhydrazyl (DPPH) Azino-bis (ethylbenzthiazoline-6-sulfonicacid (ABTS)	(IC50=449 µg/mL ± 83) (IC50=740 µg/mL ± 12)	Bellahcen et al., 2020
Oscillatoria sp. SSCM01	MeOH: CHCl3 fraction	DPPH radical scavenging assay	48%	Nainangu et al., 2020
Spirulina platensis	crude extracts	DPPH radical-scavenging activity	45.75%	Safari et al., 2020
Oscillatoria acuminate	Methanolic extract	DPPH (2, 2- diphenyl-1-picrylhydrazyl) $ABTS^{+}$ (2, 2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)	6.58 % 34.60 %	Gheda & Ismail (2020).
Dolichospermum flos-aquae HSSASE2		DPPH radical scavenging assay Nitric oxide radical scavenging assay Anti-lipid peroxidation assay	(467.7 µg/ml) (IC50 = 28.7 ± 0.1 µg/ml) (IC50 11.9 ± 0.2 µg/ml)	Senousy et al., 2020
Anabaena sp., *Stigonaema* sp. and *Oscillatoria* sp.	Methanol Extract	Total Antioxidant capacity determination kit	0.346 (mM/L); 0.36 (mM/L) and 0.37 (mM/L)	Seddek et al., 2019
Aphanizomenon gracile (LMECYA 009), *Aphanizomenon flos-aquae* (LMECYA 088), *Nostoc* (LMECYA 291), *Plankto thrixmougeotii* (LEGE 06224)	Methanolic and ethanolic	DPPH scavenging method, β-carotene bleaching assay	10.7% 828.94 AAC	Guerreiro et al., 2019

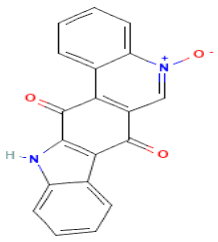
2.1.5 Anti-cancer potential of BGA

BGA extracts and compounds are known to exhibit anticancer properties (Shishido et al., 2020; Gara-Ali et al., 2021). Research by Jaspers & Lawton (1998) has focused on various biologically active compounds from BGA. The *curian A*, a novel lipid compound isolated from *Lyngbya majuscula*, is a potent inhibitor of microtubule assembly with very low IC_{50} values against L1210 leukemia cells and CD-46 Burkitt lymphoa cells, at par with those for colchicines. Cryptophycin 1 and 8, another anticancer compound was first isolated from *Nostoc sp*. by researchers at Merck. The oral supplement of *Spirulina fusiformis* is known for regression of subjects with homogenous leukolakia (Mathew et al., 1995). The extracts of *Spirulina* and *Dunaliella* inhibited the chemically induced carcinogenesis in model hamster buccal pouches (Schwartz et al., 1988). Studies have also shown that sulphated polysachharide, calcium spirulans appears to inhibit tumor invasion of melanoma cells and basement membrane (Mishima et al., 1998). *Aphanizomenon flosaquae* extract containing a high concentration of phycocyanin inhibited the *in vitro* growth of tumour cells, indicating the sensitivity of cell lines to the phycocyanin. A filamentous cyanobacterium *Phormidium tenue* contains several diacylglycerols that inhibit chemically induce tumors on mice (Tokuda et al., 1996). Similarly, cryprophycin 1 isolated from *Nostoc* sp. (ATCC 53789) is the most potent suppressor of microtubule dynamics *i.e*. it blocks all cell cycles in G2/M phase. Curacin A is isolated from *Lyngbya majuscule*. This compound is found to be a potent inhibitor of microtubule assembly. There is a need for immediate attention for more novel anticancer drugs so that carcinogenic cells are capable of resisting some drugs, like vinca alkaloids and taxanes. These drugs failed to treat cancer in a chemotherapeutic way. Cancer is known to be the major cause of mortality worldwide. Recently some new types of cancer e.g. gliobastoma are increased rapidly. Anticancer compounds reported from BGA are summarized in Table 6 Figure 14.

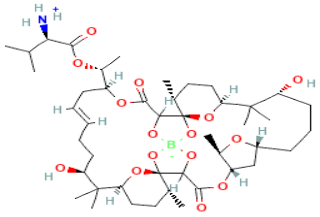
Table 6 Anticancer compounds reported from BGA

BGA Spp.	Anticancer Compounds	Chemical Structure	References
Calothrix sp.	Calothrixin A	MF: $C_{19}H_{10}N_2O_3$ MW: 314.3g/mol IUPAC Name: 20-oxido-10-aza-20-azoniapentacyclo[11.8.0.03,11.04,9.014,19]henicosa-1(13),3(11),4,6,8,14,16,18,20-nonaene-2,12-dione	Cardellina et al., 1979b
Nostoc sp.	Boromycin	MF: $C_{45}H_{74}BNO_{15}$ MW: 879.9g/mol IUPAC Name: [(2R)-1-[(1R)-1-[(1R,5S,7E,11S,13S,16R,17R,24S,25R,27R,31R,33S,36R)-11,31-dihydroxy-12,12,16,25,32,32,36-heptamethyl-3,22-dioxo-4,18,20,23,26,37,38,40,41-nonaoxa-19-boranuidaheptacyclo[17.17.1.11,33.12,19.113,17.124,27.017,21]hentetracont-7-en-5-yl]ethoxy]-3-methyl-1-oxobutan-2-yl]azanium	Banker & Carmeli, 1998; Gupta, 2012
Scytonema varium	Scytovirin	MF: $C_{66}H_{91}N_{19}O_{24}S$ MW: 1566.6g/mol IUPAC Name: (4S)-4-[[(2S)-4-amino-2-[[(2S)-2-[[(2R)-2-[[(2S)-2-[[(2S,3R)-2-[[(2S)-1-[2-[[(2S)-2-[(2-aminoacetyl)amino]-3-hydroxypropanoyl]amino]acetyl]pyrrolidine-2-carbonyl]amino]-3-hydroxybutanoyl]amino]-3-(4-hydroxyphenyl)propanoyl]amino]-3-sulfanylpropanoyl]amino]-3-(1H-indol-3-yl)propanoyl]amino]-4-oxobutanoyl]amino]-5-[[(2S)-1-[[(2S)-4-amino-1-[[(2S)-4-amino-1-[(2S)-2-(carboxymethylcarbamoyl)pyrrolidin-1-yl]-1,4-dioxobutan-2-yl]amino]-1,4-dioxobutan-2-yl]amino]-1-oxopropan-2-yl]amino]-5-oxopentanoic acid	Shi et al., 1999
Symploca genus	Largazole	MF: $C_{29}H_{42}N_4O_5S_3$ MW: 622.9g/mol IUPAC Name: S-[(E)-4-[(5R,8S,11S)-5-methyl-6,9,13-trioxo-8-propan-2-yl-10-oxa-3,17-dithia-7,14,19,20-tetrazatricyclo[14.2.1.12,5]icosa-1(18),2(20),16(19)-trien-11-yl]but-3-enyl] octanethioate	Luesch et al., 2001
Nostoc sp.	Apratoxin A	MF: $C_{45}H_{69}N_5O_8S$ MW: 840.1g/mol IUPAC Name: (2S,3S,5S,7S,10S,16S,19S,22S,25E,27S)-16-[(2S)-butan-2-yl]-7-tert-butyl-3-hydroxy-22-[(4-methoxyphenyl)methyl]-2,5,17,19,20,25-hexamethyl-8-oxa-29-thia-14,17,20,23,30-pentazatricyclo[25.2.1.010,14]triaconta-1(30),25-diene-9,15,18,21,24-pentone	Grinberg et al., 2002

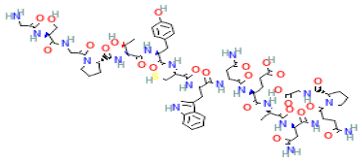
BGA Spp.	Anticancer Compounds	Chemical Structure	References
Dolabella auricularia	Dolastatin 15	MF: $C_{45}H_{68}N_6O_9$ MW: 837.1g/mol IUPAC Name: [(2S)-1-[(2S)-2-benzyl-3-methoxy-5-oxo-2H-pyrrol-1-yl]-3-methyl-1-oxobutan-2-yl] (2S)-1-[(2S)-1-[(2S)-2-[[(2S)-2-[[(2S)-2-(dimethylamino)-3-methylbutanoyl]amino]-3-methylbutanoyl]-methylamino]-3-methylbutanoyl]pyrrolidine-2-carbonyl]pyrrolidine-2-carboxylate	Stevenson et al., 2002
Cyanobacteria	Astaxanthin	-	Chen et al., 2003
Nostoc sp.	Cryptophycin	MF: $C_{35}H_{43}ClN_2O_8$ MW: 655.2g/mol IUPAC Name: (3S,6R,10R,13E,16S)-10-[(3-chloro-4-methoxyphenyl)methyl]-6-methyl-3-(2-methylpropyl)-16-[(1S)-1-[(2R,3R)-3-phenyloxiran-2-yl]ethyl]-1,4-dioxa-8,11-diazacyclohexadec-13-ene-2,5,9,12-tetrone	Back & Liang 2005; Medina et al., 2008
L. majusculata	Curacin A	MF: $C_{23}H_{35}NOS$ MW: 373.6g/mol IUPAC Name: (4R)-4-[(1Z,5E,7E,11R)-11-methoxy-8-methyltetradeca-1,5,7,13-tetraenyl]-2-[(1R,2S)-2-methylcyclopropyl]-4,5-dihydro-1,3-thiazole Isomeric SMILES: C[C@H]1C[C@H]1C2=N[C@@H](CS2)/C=C\CC/C=C/C=C(\C)/CC[C@H](CC=C)OC	Xiong et al., 2006
Lyngbya sp.	Dragonamide C	MF: $C_{33}H_{57}N_5O_6$ MW: 619.8g/mol IUPAC Name: (E)-N-[(2S)-1-[[(2S)-1-[[(2S)-1-[[(2S)-1-amino-3-methyl-1-oxobutan-2-yl]-methylamino]-3-methyl-1-oxobutan-2-yl]-methylamino]-3-methyl-1-oxobutan-2-yl]-methylamino]-3-methyl-1-oxobutan-2-yl]-3-methoxy-N-methyloct-2-en-7-ynamide	Gunasekera et al., 2008
Lyngbya sp.	Dragonamide D	MF: $C_{32}H_{55}N_5O_6$ MW: 605.8g/mol IUPAC Name: N-[(2S)-1-[[(2S)-1-[[(2S)-1-[[(2S)-1-amino-3-methyl-1-oxobutan-2-yl]-methylamino]-3-methyl-1-oxobutan-2-yl]-methylamino]-3-methyl-1-oxobutan-2-yl]-methylamino]-3-methyl-1-oxobutan-2-yl]-N-methyl-3-oxooct-7-ynamide	Gunasekera et al., 2008



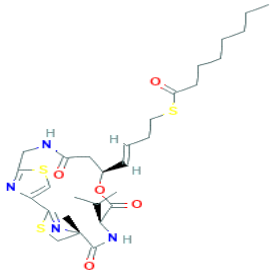
Calothrixin A



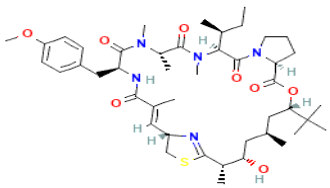
Boromycin



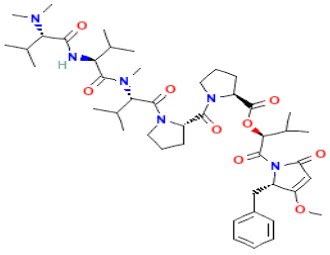
Scytovirin



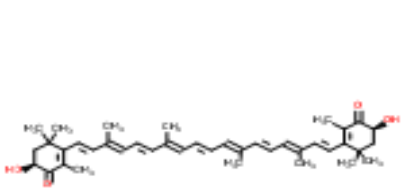
Largazole



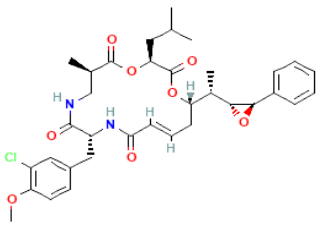
Apratoxin A



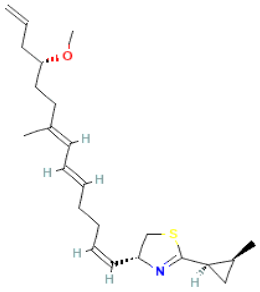
Dolastatin 15



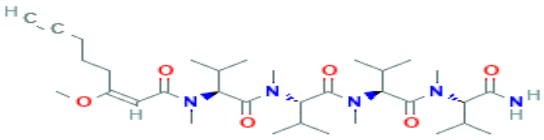
Astaxanthin



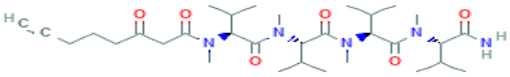
Cryptophycin



Curacin A



Dragonamide C



Dragonamide D

Figure 14 Chemical structures of the active ingredients isolated from various BGA and having anticancerous properties

2.1.6 Antiviral Potential including SARS-CoV-2

The globe is so much affected by the dreadful diseases caused by infection of viruses such as HIV-acquired immune deficiency syndrome. There is also another viral deadly disease that is dengue which may have many consequences. Despite two former major outbreaks of coronavirus infections i.e. the SARS and MERS, the world is still underprepared to effectively manage the current COVID-19 pandemic outbreak. The researchers were in search of a novel and potent drug which will be able to resist those deadly and dreadful viral infections throughout the world. Scientists have now invented novel, potent, and safe anti-viral agents that are very useful in this urgent situation. Recently there is a new scientific treatment or therapy which is named highly active antiretroviral therapy in short HAART. This is triple therapy which is very fruitful and capable in the treatment of HIV infections which is very helpful and makes control and resistance power in carcinogenic treatment. This therapy can create strong viral resistance. But this therapy cannot stop the viral agent which is causing such kinds of issues. BGA species are also known to produce substances that have been proved to be anti-HIV, therefore can be exploited in therapy against AIDS (Schaeffer & Krylov, 2000; Carpine & Sieber, 2021)

Gustafson et al. (1989) used a tetrazolum-based micro-culture to screen extracts of cultured marine cyanobacteria, *Lyngbyalager heimii*, and *Phormedium tenue*, for the inhibition of HIV-1. This led to the discovery of sulfonic acid containing glycolipids as a new class of HIV-1- inhibitory compounds. Other cyanobacteria, *Phormedium cebennse*, *Oscillatoria raciborskii*, *Scytonem aburmanicum*, *Calothrix elenkinii*, and *Anabaena variabilis*, gave extracts that inhibited HIV-1 and gave positive tests for the presence of sulfolipids.

Compounds and extracts with anti-HIV activity are also active against other retroviruses such as Herpes simplex virus (HSV) and respiratory syncytial virus, but the amount of antiviral activity varies with the compound and the virus. Most of the research has focused on sulphated homopolysaccharides and heteropolysaccharides, sulfoglycolipids, carrageenans, fucoidan, sesquiterpene hydroquinones, and other classes of compounds with an anti-HIV activity that has been isolated from algae have received less attention. Hayashi et al. (1996) isolated calcium spirulan, a sulfated polysaccharide obtained from a marine blue-green alga, *Spirulina platensis* which inhibited the Herpes simplex virus. Subsequently, Ayehunie et al. (1998) determined that an aqueous extract of *S. platensis*, at a concentration that was non-toxic to human cells, inhibited syncytium formation and HIV-1

replication in human T-cell lines, peripheral blood mononuclear cells, and Langerhans cells. The antiviral effects of polysaccharides from marine algae towards mumps virus and influenza B virus were reported by Gerber et al. (1958). Subsequently, polysaccharides fractions from extracts of red algae were found to inhibit the herpes simplex virus (HSV). Similarly, Boyd et al. (1997) isolated Cyanovirin-N from an aqueous cellular extract of cyanobacterium *Nostoc ellipsosporum* which has been proved to be antiviral. Lau et al., (1993) reported that the lipophilic and hydrophilic extracts of over 900 strains of cultured blue-green algae *in vitro* for their ability to inhibit the reverse transcriptases of avian myeloblastosis virus (Table 7)

Various compounds have been isolated from a variety of blue-green algaeBGA-derived polysaccharides that have been reported for the inhibition of SARS-CoV-2 (Sami et al., 2021). Few organizations are actively involved in developing algae-based edible vaccines for SARS-CoV-2 (Jafari et al., 2021) (Table 8)

2.1.7 Antidiabetic Potential of BGA

Blue-green algae are known to exhibit potential antidiabetic properties. In a study conducted by Priatni et al. (2016) methanol extract of marine cyanobacterial strains such as *Oscillatoria limnetica*, *Coelastrella* sp., *Oscillatoria* sp., *Chroococcus* sp., *Leptolyngbya* sp., *Pseudanabaena* sp., *Lyngbya* sp., *Aphanothece* sp., *Phormidium*sp, and *Synechococcus* sp. have potential antidiabetic potential. The metabolites of *Pseudanabaena* sp. showed the highest α-glucosidase inhibition. In another study, Egyptian Scientists evaluated extracts of *Fischerella* sp. BS1-EG for antidiabetic

Table 7 Antiviral Compounds reported from BGA

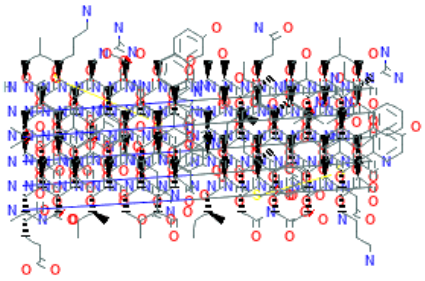
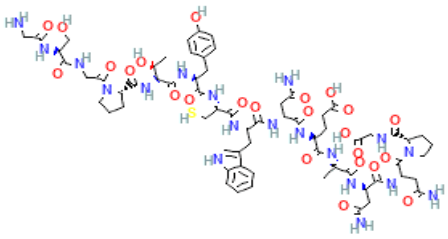
BGA Sp.	Anti Viral Compound	Chemical Structure	References
Nostoc ellipsosporum	Cyanovirin-N		Boyd et al., 1997;; Bewley, 2001
Microcystis aeruginosa	Microvirin		Kehr et al., 2006
Scytonema varium	Scytovirin MF: $C_{66}H_{91}N_{19}O_{24}S$ MW: 1566.6g/mol IUPAC Name: (4S)-4-[[(2S)-4-amino-2-[[(2S)-2-[[(2R)-2-[[(2S)-2-[[(2S,3R)-2-[[(2S)-1-[2-[[(2S)-2-[(2-aminoacetyl)amino]-3-hydroxypropanoyl]amino]acetyl]pyrrolidine-2-carbonyl]amino]-3-hydroxybutanoyl]amino]-3-(4-hydroxyphenyl)propanoyl]amino]-3-sulfanylpropanoyl]amino]-3-(1H-indol-3-yl)propanoyl]amino]-4-oxobutanoyl]amino]-5-[[(2S)-1-[[(2S)-4-amino-1-[[(2S)-4-amino-1-[(2S)-2-(carboxymethylcarbamoyl)pyrrolidin-1-yl]-1,4-dioxobutan-2-yl]amino]-1,4-dioxobutan-2-yl]amino]-1-oxopropan-2-yl]amino]-5-oxopentanoic acid		Bokesch et al., 2003
M. viridis NIES-02	Microcystis Viridis Lectin	NA	Yamaguchi et al., 1999
Arthrospira platensis	Calcium Spirulan	NA	Hayashi et al., 1996

Table 8 Anti-HIV activity of compounds from BGA

BGA strains	Compounds	References
Anabaena variabilis, Calothrix elenkinii, Lyngbya lagerheimii, Phormidium tenue, Phormedium cebennse, Oscillatoria raciborskii, Scytonema burmanicum	Extracts containing sulfolipids (sulfoquinovosyl diacylglycerols)	Gustafson et al., 1989
Spirulina platensis	activity in polysaccharide fraction	Ayehunie et al., 1998
Spirulina platensis (Marine)	Calcium spirulan, Ca-SP (sulfated polysaccharide fraction) Dextran sulfate	Hayashi et al., 1996
Nostoc ellipsosporum	Cyanovirin- N (11-k Da antiviral protein	Gustafson et al., 1996
Nostoc ellipsosporum	Cyanovirin- N (11-k Da antiviral protein	Boyd et al., 1996
900 strains	Lipophilic and Hydrophilic extracts	Lau et al., 1993
Scytonema spp.	Sulfoglycolipids	Reshef et al., 1997
Oscillatoria spp.	Acylated diglycolipids	Reshef et al., 1997
Oscillatoria raoi(TAU IL-76-12), *Syctonema spp.* (TAU SL-30-1-4), *Oscillatoria trichoides*(TAU IL 104-3-2), *Phormidium tenue* (TAU IL-144-1), *Oscillatoria limnetica, Lammermann* (TAU NG-4-1-2)	Sulfolipids, Sulfoglycolipids, hydrolysis products, synthetic derivative	Loya et al., 1998

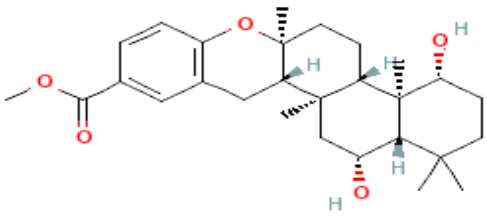


Figure 15 Chemical structure of Anti-inflammatory compounds Tolypodiol isolated from *Tolypothrix nodosa*

potential and have reported certain bioactive compounds responsible for the activity (Ahmed et al., 2018). In a recent study, Sridhar et al. (2021) have evaluated phycocyanin of *S. platensis* for its antidiabetic potential by assessing α-amylase and β-glucosidase enzyme inhibition using spectroscopy techniques. In this *in vitro* test, significant Antidiabetic activity (88%) was observed at a concentration of 250 µg/ml. In some studies, lesser antidiabetic properties have been reported, like Ghosh et al. (2016) evaluated in vitro antidiabetic properties of molecules from *Lyngbya*, *Microcoleus*, and *Synechocystis* sp. by α-amylase inhibition method and stated the lesser enzyme inhibition effect. Similarly, Xu et al. (2012) described the lowest α-amylase enzyme inhibition activity of *Phlorotannins* pigments extracted from Eckloniakurome. However, Hwang et al. (2014) reported 65 - 80% inhibitory activity at the concentration of 250 µg/ml while this was reported 51 - 67% at the dose of 200 µg/ml dose of *S. platensis* phycocyanin. Lesser enzyme inhibition even in higher concentrations was reported in a study conducted by Priatni et al. (2016).

2.1.8 Anti-inflammatory Activity of BGA

Blue-Green Algae contain a significant amount of carotenoids *i.e.* β-carotene, lycopene, lutein having antioxidant properties. By the quenching action on the reactive oxygen species, these carotenoids also have anti-inflammatory activity. This anti-inflammatory activity might be due to the presence of phycocyanin, a photo harvesting pigment. Further, the anti-inflammatory effect seemed to be the result of leucotriene formation inhibition by phycocyanin, an inflammatory metabolite of arachidonic acid (Romay et al., 1999). *Apbanizomenon flosaquae* decrease the level of arachidonic acid. Further, *A. flosaquae* and *Spirulina* contain significant amount of omega-3-alpha linolenic acid which inhibits the formation of inflammatory prostaglandins and arachidonate metabolites (Figure 15).

3 Conclusion and Future possibilities

BGA are groups of extraordinary, diverse, gram-negative, oxygenic, photosynthetic prokaryotic microscopic organisms.

Blue-green algae are found all over the world, showing remarkable ecological diversity of habitats such as freshwater, terrestrial, marine, hot spring, etc. These BGA are also widely distributed in the polar region such as the Arctic, Antarctic, Southern Ocean, and the Himalayas. Several studies have been conducted for the isolation and identification of Blue-Green algae from the water, soil, sediments, algal mats, etc. using advanced morphological, physiological, and molecular characterization techniques. Various selective media are known for their Cultivation. It has been now proven that BGA offers a great opportunity as these are considered to be one of the potential organisms useful to mankind in many ways. They exhibit diverse biological activities (Antibacterial, Antifungal, Anticancer, Antiviral Antidiabetic, and many more). Various bioactive molecules have been reported by researchers globally. In pharmaceutical companies especially in the new drug discovery research division, for the last many year's research is going on at various levels starting from extraction, purification, and identification of new compounds or drugs from various species of BGA. The major challenge in front of the current world is to fight effectively against the new emerging diseases and microbes specifically WHO priorities list of multiple antibiotic-resistant bacteria, microbial infections including SARS-CoV-2 virus and Cancer, etc., and to discover new pharmaceutical compounds for mankind and society. At the same time, there is an urgent need to think from basic to applied research to commercialize several value-added products. The use of nanomaterials to enhance biological activity could be one of the ways. Inventions of these drugs using nanotechnology can lead to the development of novel pharmaceuticals. Based on the cultures of cells, activities of enzymes, and receptors binding with ligands, various new technologies are invented to develop novel things of miniaturized screens. As a result, there is a conformational analysis, i.e., an analysis of the spatial arrangement of the component atom within a molecule that can be rotated about one or more single bonds. The known ligands result in the development of new compounds of structure-based drug design. Hence, the pharmaceutical potential of blue-green algae deserves more scientific attention and interdisciplinary research, and BGA strains from still unexplored and extreme habitats such as the Antarctic, Arctic, and the Himalayas can serve as good candidates in this regard.

Authors' contributions

All authors contributed significantly to the conception and design of the study, the interpretation of data, and the drafting and revision of the manuscript. All authors read and approved the final manuscript**.**

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Conflict of Interest

The authors hereby declare no conflict of interest.

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NEURODEGENERATIVE DISEASES: IMPACT OF PESTICIDES

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Environmental toxicants

Pesticides

Word Health Organization

Neurotransmitters

ABSTRACT

Pesticides are widely used to fulfill the higher yield requirement for humans in agricultural practices and the repellents to kill the unwanted insects but excess uses of these pesticides combat various diseases and are also responsible for environmental pollution. Total 234 pesticides are registered in India out of these, four pesticides are WHO class 1a pesticide, 15 are WHO class 1b pesticides, and 76 are WHO class 2nd mentioned pesticides together constituting 40% registered pesticides. Excess use of pesticides can cause fatigue, headache, respiratory problems, and neurodegenerative diseases in human beings. Neurodegenerative disease is the result of a process called neuron degeneration in which the structure and functions of the neurons are progressively degenerate. Alzheimer's, Parkinson's, Amyotrophic lateral sclerosis impose a burden on most of society. In the present study, we are emphasizing the mode of action of the various pesticides that influenced neurodegenerative diseases that is necessary to check the effect of neurotoxicants.

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1 Introduction

Pesticides are defined as substances intended to kill the pests, insects and other biological organisms (National pesticide information center). The major classes of pesticides are insecticides, herbicides, fungicides, and nematicides (Aktar et al., 2009), which are used to control insects, herbs, fungus, and nematodes respectively. More than 500 non-biodegradable formulations of pesticides are used in agricultural practices that enhance environmental toxicity and synthetic pesticides are more dangerous to the environment and result in serious environmental and human health concerns (Harlow et al., 2020).

1.1 Production and Contamination of Pesticides in India

In 1952, India started the production of pesticides after china and rank, twelfth globally. India is the second-largest manufacturer of pesticides in Asia (Aktar et al., 2009). India used 76% of insecticides used which is the highest in the world at 44%. According to the "All India Coordinated Research Project Pesticide Residues (1999) (AICRPPR) report that food commodities were affected by pesticides. This report finds that 43.4% of a sample of milk exceeds DDT MRLs. In Uttar Pradesh and Kerala-like, more than 40% of samples of fruits and vegetables exceeded the MRLs (Maximum residue limits) (Bhushan et al., 2013). Pesticides affect people who spray the pesticides like agricultural workers as it is hazardous more at the time of manufacture and formulation (Aktar et al., 2009; Javaid et al., 2016).

1.2 Ways of Pesticides Exposure in Human

Air, soil, water, and food are different ways through which; humans get exposed to pesticides and are sensitive to the various adverse effects of pesticides (National Research Council U.S., 1993). When the pesticides are applied directly to the target pest, the whole site gets affected, including the crop plants, soil organisms, and humans. The pesticides use differently for exposure; in the air during spraying and vapors are come to contact with the human by their respiratory system (Boyd et al., 2012). Through soil, the route is hand-to-mouth behavior and dermal contact, while the children are exposed when the children are surrounding the spraying area. Another route of exposure is water and food, and fruits, vegetables, meat, dairy are the exposure sources (Pesticides, 2008). Pesticides are easy and inexpensive substances to control the weeds, pests, and insects to protect agricultural fields (WHO 1990; Aktar et al., 2009). Many repellents, sprayers, coils, are used to control the insects in the house and gardens. In this repellent, the companies used pesticides and aerosols that stay in the air and affect the human being with breathing and causes diseases (Rahman et al., 2001).

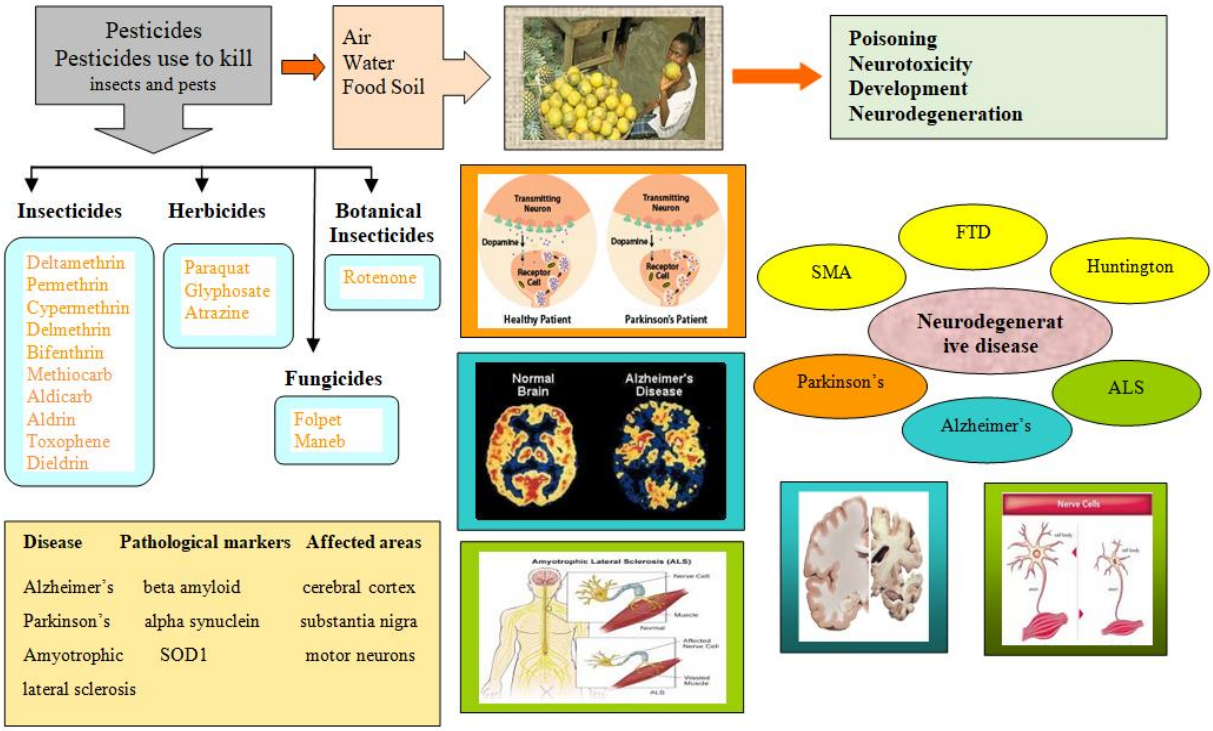


Figure 1 Representative diagram of pesticides exposure ways and the related diseases

Table 1 Showing the list of various classes of pesticides that is commonly used in commercial as well as agricultural practices and have ability to cause neurodegenerative diseases

	Path of action	Neurodegenerative diseases
Carbamate	Inhibit Acetylcholinestrase	Alzheimer's disease (Fukuto, 1990)
Organophosphate	Dopamine Acetylcholinestrase	Parkinson's disease and Alzheimer's diseases (Yadav et al., 2016)
Pyrethroid	Dopamine	Parkinson's disease (Elwan et al., 2006)
Botanical insecticides	Dopamine	Parkinson's disease (Freire & Koifman, 2012)

2 Pesticides Mode of Action

In various modes of action, pesticides can come into the contact with humans and cause neurodegenerative diseases due to the over expression and mutation in the gene that directs the production of enzymes like aspartoacylase (ASPA) this allows the buildup of N-acetyl aspartic acid (NAA) in the brain. This causes the damage of the myelin sheet that provides the protective coating to the nerve fiber in the brain that ensures that nerve impulses are properly transmitted in the brain (Palop & Mucke, 2010). Loss of glutamate transporter EAAT2 (excitatory amino acid transporter 2) protein in the motor cortex and spinal cord, glutamate-mediated excitotoxicity of nitric oxide synthesis containing neurons results in the Paraquat induced neurotoxicity (Uversky, 2004). Pesticides may also be one of the reasons for the degeneration of the neurons as earlier studies show that the pesticides like Rotenone, Carbamates are the known pesticides that are causing neurodegenerative diseases like Huntington's and Alzheimer's respectively (Harms et al., 2021). As longevity increases, neurodegenerative diseases become more problematic, specifically when the treatments for these diseases are unavailable or ineffective.

2.1 Alzheimer's Disease

The mechanism of carbamates poisoning involves the inactivation of acetylcholinesterase which causes the loss of acetylcholine neurotransmitters. Carbamate causes the inhibition of acetylcholinesterase (AChE) enzyme important for transmission in nerve impulses (Alhewairini et al., 2016). Exposure of organophosphates affects the nervous system by the accumulation of acetylcholine which results in the inhibition of acetylcholinesterase therefore the use of organophosphates increases the risk to develop Alzheimer's disease in the exposed population (Yadav et al., 2016). Acetylcholine was first discovered in the 1920s as the first known neurotransmitter and the role of Acetylcholine found in the brain plays a role in learning and memory (Alexander et al., 2014). When acetylcholinesterase enzyme inhibited the cholinergic toxicity which, resulted in the continuous stimulation of cholinergic receptors throughout the central and peripheral nervous systems (The United States Environment Protection Agency, 2007). Alzheimer's is virtually based on targeting of amyloid precursor protein (APP), Presenilin 1 or Presenilin 2, and Tau/PTL - 1 gene (Palop & Mucke, 2010).

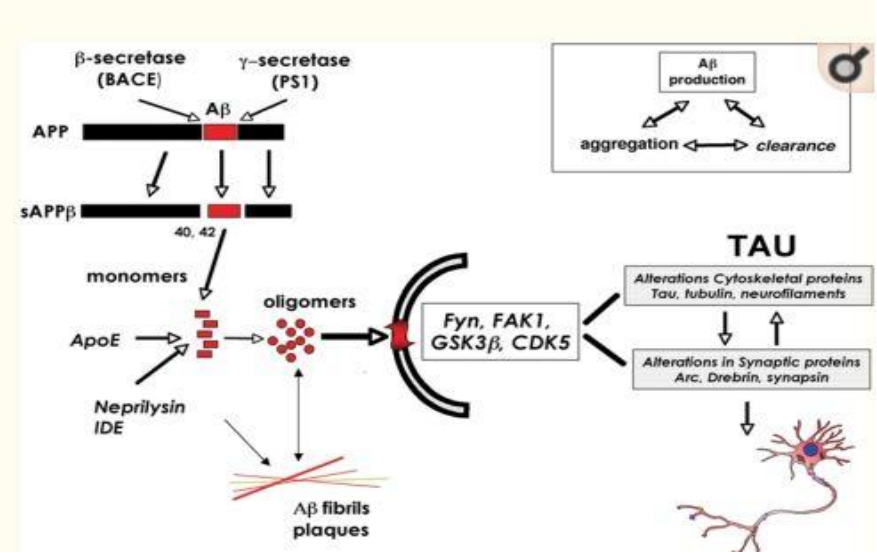


Figure 2 Mechanism of formation of beta amyloid plaques causes tau protein aggregation (Crews & Masliah, 2010)

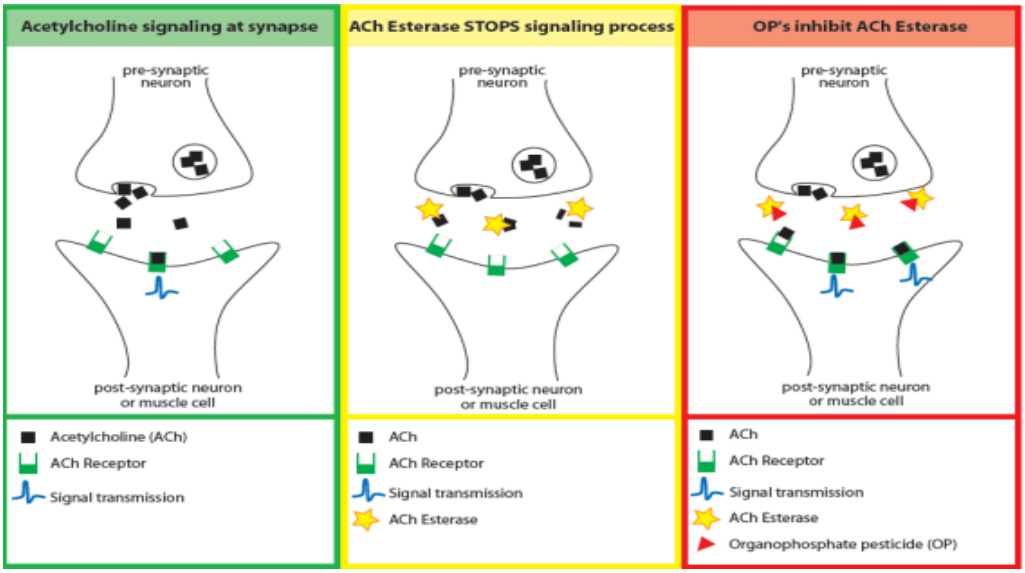
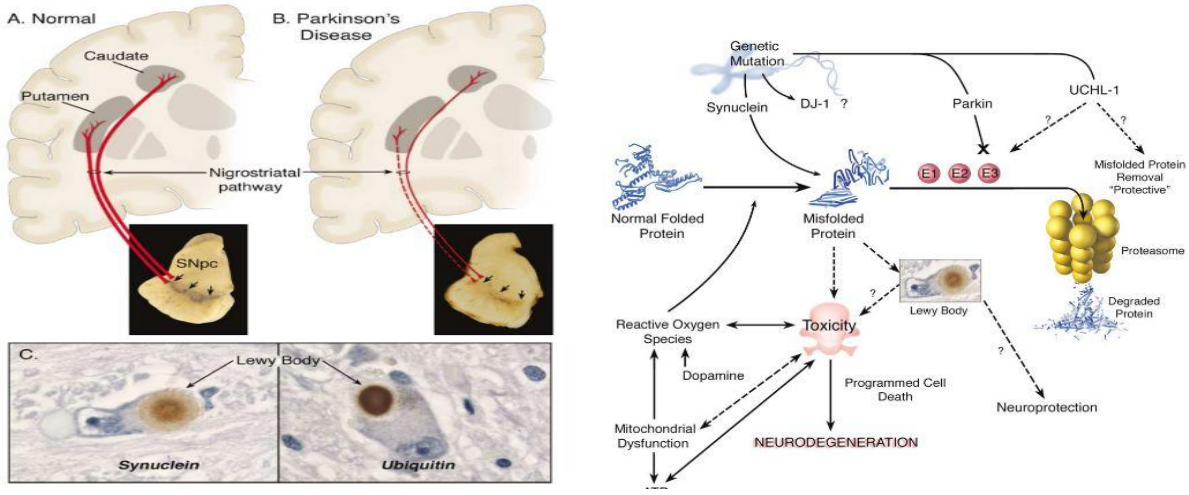


Figure 3 Mode of action of pesticide in the development of Alzheimer's disease (Organophosphate pesticides and child health, 2007)

2.2 Parkinson's Disease

The pyrethroids are also responsible to inhibit the dopaminergic neurons within the basal ganglia region of substantia nigra pars compacta that causes Parkinson's disease (Zhou et al., 2013). Dopaminergic neurons present during this region of the brain correlate with the motor cortex area that is liable for all the voluntary and involuntary movement within the body. Pyrethroids are utilized in many commercially available products that also are wont to control mosquitoes, professionals apply pyrethroids as an ultra-low volume (ULV) spray it releases very tiny aerosol droplets. These droplets stay within the air and kill the adult mosquitoes on contact. Parkinson's is that the second most common neurodegenerative disease including the selective neuronal degeneration by the nigrostriatal pathway through the protein accumulation of alpha-synuclein within the Lewy body inclusions (Cooper & Raamsdonk, 2018). First described by James Parkinson in 1817 it's a disorder of the central nervous system that affects nerve cells within the part of the brain controlling muscle movement and impairs motor skills and speech (Nass et al., 2001). The symptoms of Parkinson's disease are tremor, rigidity, Bradykinesia (absence of movement), abnormalities, fatigue, soft speech, and learning disabilities. PARKIN, PINK 1, DJ-1, LRRK2 are the genes linked to Parkinson's disease which have an ortholog within the *Caenorhabditis elegans* model organism (Wolozin et al., 2011). *C.elegans* is used to predict chemical activity and identify mechanisms that can able to influence toxicological outcome (Harlow et al., 2020).



[a] [b]

Figure 4 Parkinson's disease due to genetic mutation caused dopaminergic neurodegeneration (Dauer & Przedborski, 2003)

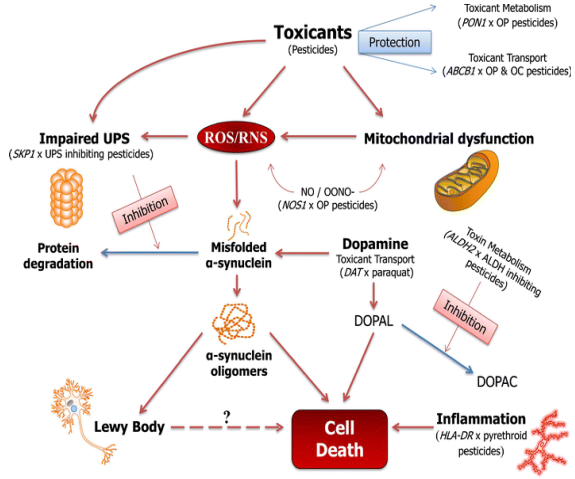


Figure 5 Mode of action of Parkinson's disease due to the effect of pesticides (Ritz et al., 2016)

2.3 Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) may be a disease during which has the degeneration of upper and lower motor neurons (originating within the brainstem). A serious breakthrough of this disease includes the mutation within the Cu/Zn superoxide dismutase (SOD1) gene with cytoplasmic inclusions in the proximal axon and cell body (Cluskey & Ramsden, 2001). An individual who suffers from (ALS) has a 30-95% loss of the glutamate transporter EAAT2 (excitatory amino acid transporter 2) protein within the part of the motor cortex and the spinal cord region (Lin et al., 1998). ALS patients have muscular atrophy, weakness, and therefore the degeneration of motor neurons within the brainstem and spinal cord (Cluskey & Ramsden, 2001).

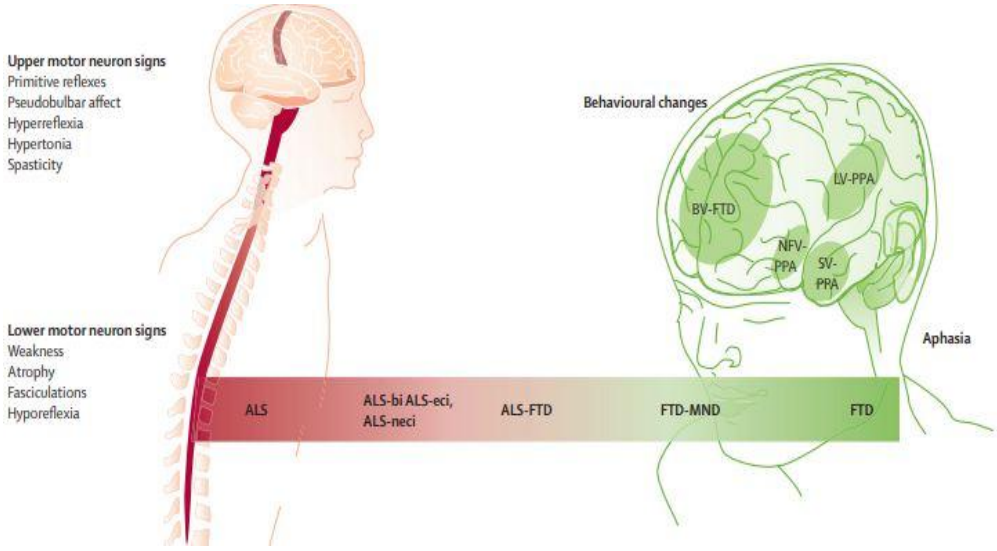


Figure 6 Representation of the upper and lower neurons identifiable for ALS (Michael et al., 2017)

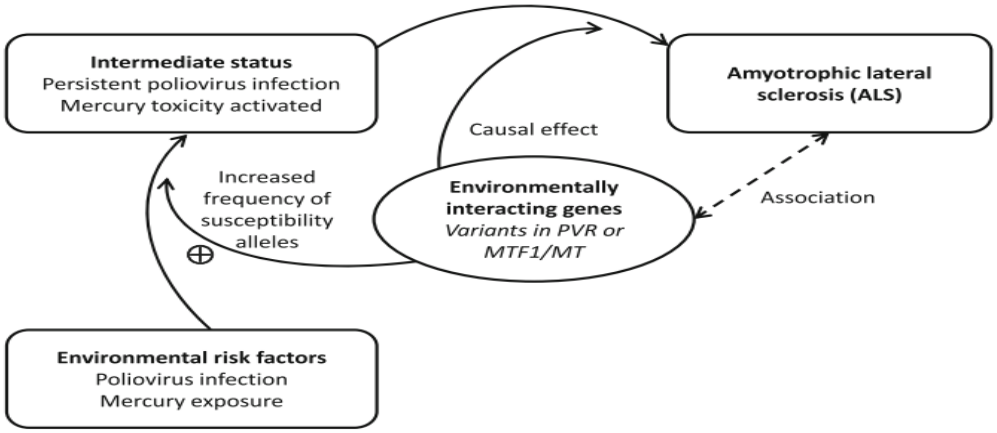


Figure 7 Mode of action of Amyotrophic lateral sclerosis due to the effect of pesticides (Yu & Pamphlett, 2017)

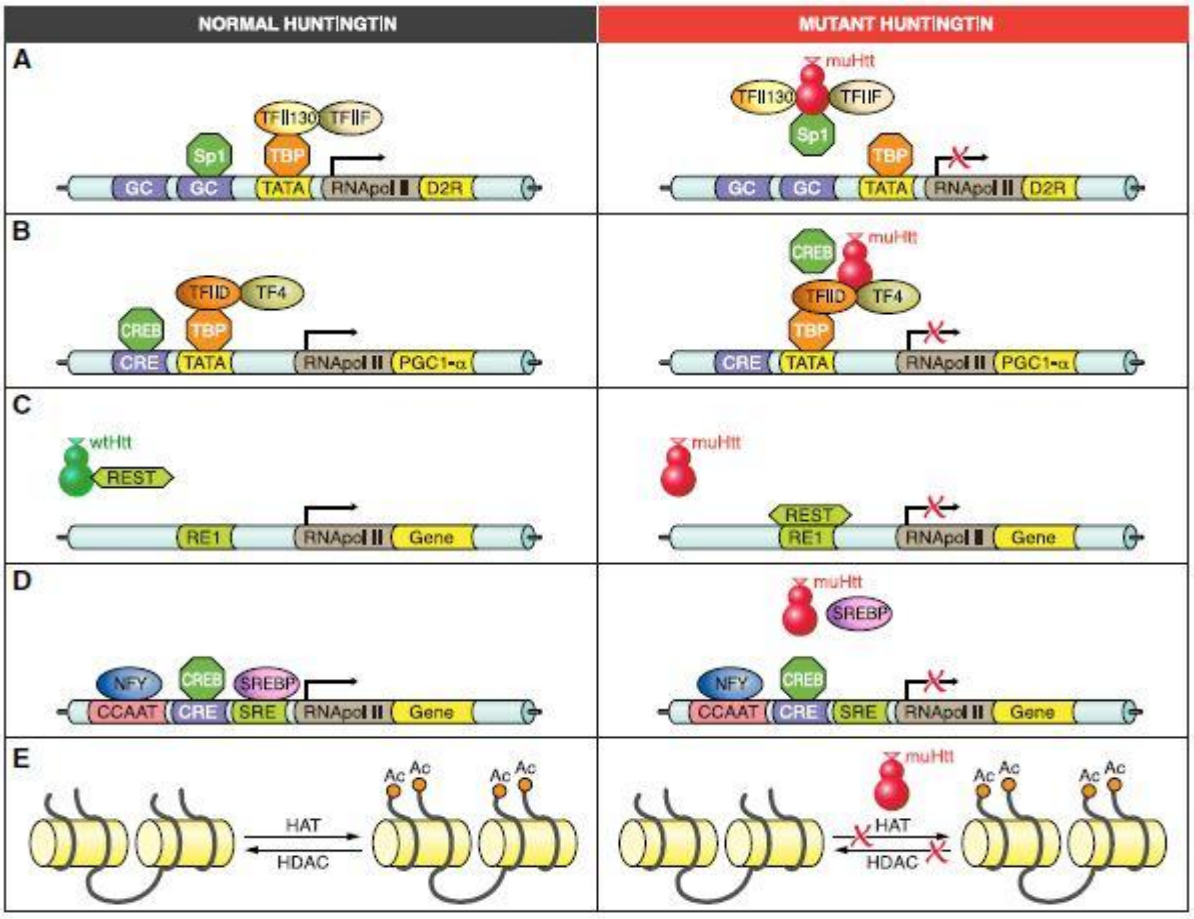


Figure 8 Molecular representation of normal and Huntington's disease pathway (Zuccato et al., 2010)

2.4 Huntington's Disease

Huntington's disease is additionally a neurodegenerative disease characterized by the degeneration of striatal GABAergic projection neurons but this do not affecting the striatal interneuron. It has been first introduced in 1872 and it's identified its mutated genes in 1993 (Zuccato et al., 2010). It's a dominant inherited neurodegenerative disorder caused due to the unstable expansion of a CAG repeat within the coding region of the IT-15 gene which encodes for a protein called Huntingtin, and the mutation in this elongation of glutamine on the NH2 terminus of the protein (HD Collaborative Research Group, 1993).

Conclusion

Through the literature survey, was reported that there is a large number of pesticides such as Rotenone, Paraquat, Deltamethrin, Permethrin, and nicotine which belongs to herbicides, pyrethroids, and Botanical insecticides respectively. These pesticides classes can cause neuronal cell death and abnormalities in the motor cerebral cortex in the cerebellum and also in the basal forebrain region that denotes the neurodegenerative diseases i.e. Alzheimer's and Parkinson's. There have many diseases that are responsible to cause neurodegenerative diseases due to exposure of pesticides. Many researchers are currently working in the present area, to find out the pesticides having neurotoxic potential. The present study includes an overview of the work going on in the present area to explore the mechanism of neurotoxicity after exposure to pesticides. Therefore further work is required to check the activity of the various other pesticides that are used in agriculture as well as in household practices to control the pests.

Author's Contribution

NS is the first author who carried out the experiments, statistically analyzed and interpreted the data, and wrote the manuscript. PG was oversight responsibility for the research activity planning and acted as the corresponding author.

Conflict of Interest

The authors state that they have no conflict of interest.

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ROLE OF SUBSTANCE P IN PANCREATITIS AND ASSOCIATED DISEASES

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Substance P

Inflammation

NK1R

Pancreatic cancer

Pancreatitis

ABSTRACT

Substance P (SP) is a neuropeptide that has its place in the tachykinin family and helps in the transmission of neurogenic signals. SP is also a neuromodulator that plays a crucial part in pain during inflammatory processes. It is produced by the capsaicin-sensitive unmyelinated C fibers sensory neurons by the central and peripheral nervous systems. Substance P is known as a critical primary responder to most of the extreme stimuli, i.e., specifically those with the ability to destabilize the biological integrity. Hence, SP can be considered as an instantaneous system for defense, stress, healing, etc. SP is known to perform a vital role in neurogenic inflammation and the pathophysiology of acute pancreatitis. Out of these, neurogenic inflammation is responsible for acute interstitial pancreatitis as a result of oedema. SP binds itself to the G-protein coupled neurokinin-1 receptor and causes plasma leakage, cell proliferation, and invasion resulting in pancreatic cancer. SP along with comparable neuropeptides seems to be crucial targets with the capability of satisfying several unfulfilled medical requisites. This review article mainly focuses on compiling the available evidence to show that SP could be a novel therapeutic target for pancreatic diseases, and more exploration into the SP signaling pathways is the call of the hour.

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1 Introduction

Peptides are an essential category of molecules that play vital roles as transmitters as well as modulators in our nervous system. Out of these peptides, Substance P (SP) is considered to be highly characterized in terms of its distribution and function. SP is a well-known 11 amino acid (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH2) containing neuropeptide with a total positive charge in physiological pH (Qureshi, 2007). SP is synthesized by neuronal and non-neuronal cells along with immune cells. SP is proven to exert its biological activities via G protein-coupled neurokinin or tachykinin receptors (NKRs or TACRs), namely NK1R, NK2R, and NK3R. It is also known to engage in an essential role in asthma, arthritis in addition to several other inflammatory processes (Keeble & Brain, 2004).

SP is released from primary sensory nerve filaments which are a part of the tachykinin family and have been associated with a multitude of physiologic activities (di Mola & di Sebastiano, 2008; Hökfelt et al., 2001). SP is extensively expressed in both central as well as peripheral nervous systems along with peripheral tissues like B and T cells in an autocrine or paracrine manner (Esteban et al., 2006). NK1R, a vital receptor of SP, is also expressed excessively in several standards and neoplastic cell types (Guha et al., 2005; Muñoz et al., 2010; Muñoz & Rosso, 2010). SP regulates loads of biological functions and has also been concerned with neurogenic inflammatory reactions, pain, and depression. Nerves, enclosing SP, are known to innervate primary lymphoid organs, the thymus, and the bone marrow, as well as secondary lymphoid organs including the lymph nodes, spleen, tonsils, etc. (Marriott & Bost, 2002). This advocates the fact that SP may perhaps act as a facilitator between nervous and immune systems. Besides their synthesis from neurons, SP coupled with its receptor NK1R is well known to be expressed in several other immune cell types.

The release of SP primarily influences the triggering of the inflammatory reaction cascade in the marked tissue. SP is capable of enhancing the release of the pro-inflammatory facilitators towards the peripheral tissue. For instance, SP can intensify the synthesis and secretion of prostaglandin E2 (Lotz et al., 1987), the secretary process of lysosomal enzymes (Johnson & Erdös, 1973), along with the secretion of interleukin 1 coupled with the neutrophil chemoattractant interleukin 6 (Lotz et al., 1988). In the course of inflammation, entering macrophages express NK-1 receptors (Marriott & Bost, 2002) proposing that SP inside the peripheral tissues can prolong the inflammatory cascade.

It is well established that SP is also present in the pancreas and it may act identical to a neurotransmitter for the sensory afferent nerves in the pancreas. Receptors dedicated to SP have also been discovered on acinar cells present in the pancreas of guinea pigs. Furthermore, the neuropeptide exhibits as a secretagogue, accelerating the process of secretion of amylase from acinar cells through a mechanism involving the G protein, the phospholipase along with inositol phosphate and calcium-mediated reactions in the species (Song et al., 1988; Sjödin et al., 1991; Sjödin et al., 1994). Pancreatic acinar cells of the rat do not exhibit receptors for SP and the concerned neuropeptide does not promote the release of enzymes from the rat's acinar cells (Song et al., 1988).

The expressions of both NK-1 receptors besides substance P levels present in acinar cells are amplified during the development of pancreatitis induced by the cerulean (Bhatia et al., 1998). Cerulein stimulated pancreatitis depicted by pancreatic plasma leakage, raised myeloperoxidase (MPO) levels in both pancreas and lungs was also considerably diminished in NK1 knockout mice, showing that SP significantly plays a part in acute pancreatitis s(AP) and AP allied lung injury (Bhatia et al., 1998; Grady et al., 2000). Additionally, an NK1 receptor inhibitor (CP-96345) has been demonstrated to defend against AP and accompanying lung injury stimulated by injecting cerulein in rats and mice (Grady et al., 2000; Lau et al., 2005). The employment of both NK1 in cooperation with neural endopeptidase knockout mice established that SP plays a crucial role in regulating the toxicity in the case of the necrotizing classical pancreatitis model (Maa et al., 2000).

2 Substance P Mediated Physiological Mechanisms in the Pancreas

As mentioned above, SP is known to interact with three specific G protein-coupled neurokinin or tachykinin receptors, namely the NK1-R plus NK2-R, as well as NK3-R, with varying affinities (Helke et al., 1990). In the case of the mouse pancreas, the presence of the NK1 receptor is already identified with the help of immunohistochemistry in acinar cells. However, it is undetermined whether the other cells localized in the pancreas, like neurons or endothelial cells, are capable of expressing neurokinin receptors or not.

The functional aspect of SP in the case of the pancreas is not entirely understood to date. This is primarily entitled to the complications involved in the neurohormonal communications that are known to affect the secretory process of the exocrine pancreatic region. Secretin stimulated secretion concerned with the exocrine pancreas is constrained using SP in rats (Zhou et al., 2018). Likewise, SP obstructs; equally, the basal and secretin promoted fluid release in isolated pancreatic duct cells of rats (Ashton et al., 1990). It is also well known that intravenous SP increases the amylase secretion induced by venous infusion of cerulein in the sedated intact rat (Katoh et al., 1984) but then

prevents the secretion of pancreatic protein triggered by cerulean (Konturek et al., 1981). Venous administration of SP induces countless systemic consequences that may modify the pancreatic secretion containing hypotension, variations in case of visceral blood flow (**v** Euler & Gaddum, 1931; Konturek et al., 1981), hyperglucagonemia, as well as hyperglycemia (Horiuchi et al., 1993; Edwards & Bloom, 1994; Schönfeld & Müller, 1994). Therefore, SP intravenous administration perhaps does not imitate the biological effect of this locally operating neuropeptide in numerous ways.

Research has reported that SP antagonizes cholecystokinin (CCK) analogcerule in stimulated amylase secretion and pancreatic discharge of juice caused by the administration of secretin in the case of rat pancreas. However, the above effects can be partly reversed by blocking either the NK1-R or the NK2-R. SP stimulated blockage of exocrine secretion contains a specific neural mechanism. The stimulus concerned with sensory nerves caused by capsaicin additionally results in a dose-related constraint of the exocrine secretory cascade, which is somewhat blocked by antagonists of the concerned NK1 receptor. These results further suggest that the peptidergic sensory nerves are essential in the secretory regulation of the exocrine pancreatic region (Figure 1) as suggested by Kirkwood et al. (1999).

3 Neurogenic Mechanism of Inflammation

Several tissues such as the skin, eye, joints along with systems such as the digestive, respiratory, etc. have shown the presence of neurogenic inflammation. The activity of SP is primarily vasoconstrictive. Contrariwise, there are descriptions that SP can perform vasodilation in part by triggering the secretion of histamine from the mast cells (Owman, 2019) via straight action, which is not reliant on the secretion of histamine. Inhibitors concerned with the NK1 receptor might also eradicate the vasodilation caused by SP (Couture & Cuello, 1984; Xu et al., 1991). Calcitonin gene correlated peptide is an effective vasodilator that, in low concentrations, can perform vasodilation through direct action (Yan et al., 2019). In the case of visceral tissues, neurogenic inflammation cannot be considered merely as a pathophysiological mechanism resulting in various diseases because it also takes part in the tissue response mechanism to injury. Neurogenic inflammation appears as an adaptive reaction, advocating rapid escalations in tissue substances by priming cells for neighboring defense and increasing fluid transportation to separate the bacteria and toxins. Still, this course of action may turn maladaptive, as noticed in the case of asthma, migraine as well as arthritis (Holzer & Barthó, 1996).

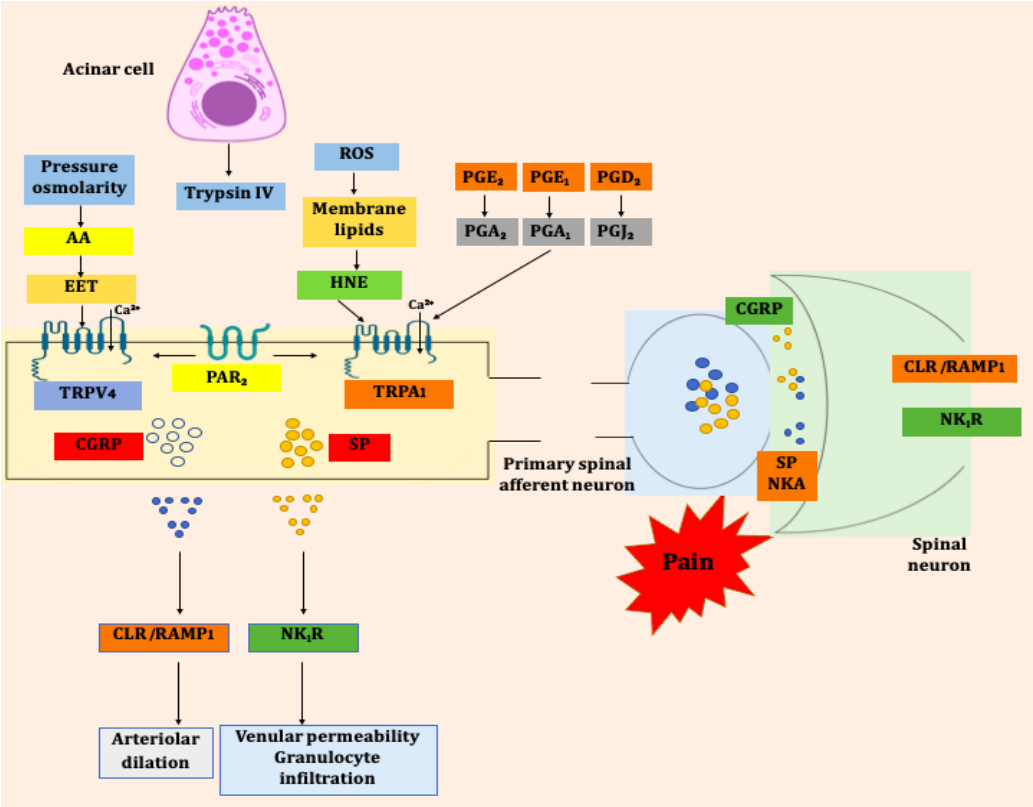

Figure 1 Illustration of SP molecular mechanism in pancreas

Table 1 Available Clinical Trials of SP against Pancreatic Diseases

Drug Name	Condition/Disease	Therapeutic Mechanism	Clinical Trial Phases	Time period	Status
Rolapitant	Prevention of chemotherapy-induced nausea and vomiting (CINV).	Rolapitantorvarubibelongs to a class of anti-cancer agents that is aselective NK1 receptor antagonist. Given in combination with other anti-vomiting drugs in emetogenic cancer chemotherapy.	Phase 3	2011-2016	completed
[18F]-Labeled Substance P Antagonist Receptor Quantifier	Pancreatic Cancer	Non-randomized interventional trial to study proportion of pancreatic tumors detected by [18F]-labeled substance P antagonist receptor quantifier positron emission tomography.	Phase 1	2007-2012	completed
Palonosetron and Fosaprepitant	Cholelithiasis-Postoperative nausea and vomiting	Cholelithiasis-PONV resultant pancreatitis. ComparisonbetweenSP antagonists Palonosetron and FosaprepitantintheProfilaxis of PONVin women.	Phase 4	2018-2019	Recruiting
Aprepitant	Pancreatitis	Reduce the risk of pancreatitis by blocking SP/NK1R mechanism in patients undergoing Post-Endoscopic Retrograde Cholangiopancreatography (ERCP).	N.A	2008-2015	Completed
Substance P biomarker	Acute pancreatitis	Clinical Evaluation of Novel Biological Markers for the Prediction of Severe Acute Pancreatitis.	N.A	2008-2012	Completed
Paricalcitol	Pancreatic Cancer	Man-made Vitamin D that blocks the signal in cancer tumour cells that leads to growth and spreading of tumour	Phase 2	2020	Recruiting

The process of injecting SP to mice triggers leakage of plasma from the postcapillary venules located in the pancreas and this consequence can be hindered by inhibitors of the NK1 receptor (Figini et al., 1997). Additionally, it has been established that the genetic elimination of the NK1 receptor from mice decreases the seriousness of the experimental mice model of pancreatitis (Bhatia et al., 1998). These outcomes show that SP is one of the essential facilitators of pancreatic inflammatory reactions.

The aforementioned support indicates a vital role of SP as one of the pro-inflammatory mediators throughout pancreatitis; however, its function as a mediator concerned with pain besides neurogenic inflammation is quite unspecified. As discussed above, SP is capable of stimulating the activation of several pro-inflammatory cytokines due to its function as a pro-inflammatory factor. These released cytokines are capable of producing hyperalgesia (Watkins & Maier, 2000). Hence, this might be one of the several mechanisms causing pancreatic pain. The manifestation of SP, as well as IL-8 in the case of pancreatitis, are interconnected (Di Sebastiano et al., 2000). In addition, the upregulation of receptor NK1 located in the pancreatic region is associated with aspects of pain in pancreatitis (Shrikhande et al., 2001).

4 Role of SP in Pancreatitis

Pancreatitis is a state of disorder in which the primary pathology is related to the inflammatory condition of the pancreatic tissue. The classification of the condition generally depends on the duration or course of progression of the disease. Acute pancreatitis (AP) can be described as a self-limited, mild state, usually associated with higher serum levels of the pancreatic digestive enzymes (Singer et al., 1985). Chronic pancreatitis (CP) frequently represents the gradual damage to the pancreatic tissue by a recurrent outburst of silent or symptomatic AP (Vera-Portocarrero & Westlund, 2005).

The mechanisms followed by SP to intensify the rigorousness associated with pancreatitis are not evident. Studies conducted denote the probable role of SP in inflammatory reactions and the fact that the neuropeptide primarily acts on the endothelial cells to escalate vascular penetrability and stimulates the formation of edema (Bhatia et al., 1998).

4.1 Acute Pancreatitis

The CCK analog, cerulein stimulated, NK1 receptor facilitated, plasma leakage in AP is probably associated with the intrapancreatic secretion of SP. Pancreatic levels of SP escalate

after cerulein administration (Bhatia et al., 1998). As already mentioned, the pathway involved in the cerulein stimulatory secretion of SP in the pancreatic tissue is not well understood. In several other tissues, several toxic chemicals along with mechanical or thermal stimuli trigger the subgroups of afferent neurons that include SP, which is subsequently released either centrally or peripherally, leading to stimulation and internalization of the NK1 receptor (Bowden et al., 1994; Mantyh et al., 1995). It appears that the sensory nerves are stimulated during AP progression resulting in the secretion of intrapancreatic SP and activation of the above-mentioned NK1 receptor (Grady et al., 2000). Transient receptor potential vanilloid type 1 (TRPV1) conduits are situated on the primary sensory neurons, which, when activated, instigate the neuronal secretion of accumulated SP. It was confirmed in vivo that the capsazepine, a TRPV1 inhibitor, considerably diminished inflammatory reactions and pancreatic tissue injury in caerulein-induced AP (Nathan et al., 2001). Contrary-wise, the activation of TRPV1 via capsaicin initiated the release of SP and amplified caerulein stimulated AP (Hutter et al., 2005). The consequences caused by SP can be stopped by employing enzymatic degradation with the help of neutral endopeptidase (NEP). Genetic deletion of these NEP intensifies pancreatic tissue damage and accompanying lung injury and correspondingly enhanced mortality rate in a choline-deficient and ethionine supplemented regime diet (CDE) provoked AP (Grady et al., 2000). In addition to the pro-inflammatory influences of SP in AP, it facilitates nociception in typical animal models representing AP.

4.2 Chronic Pancreatitis

Further, there are no sufficient efforts to evaluate the probable role of SP in chronic pancreatitis. SP was acknowledged as a facilitator of pain responses in the case of CP. Levels of NK1R mRNA in CP patients were considerably associated with the intensity, incidence, and interval of pain (Shrikhande et al., 2001). Comparable relationships were also witnessed between NK2R mRNA levels, coupled with pain (Michalski et al., 2007). A noticeable upsurge of SP comprised nerve endings which was witnessed from the swollen or inflammatory pancreatic tissue contrasted to healthy subjects (Büchler et al., 1992). Contrarywise this, intrapancreatic PPTA mRNA levels of expression were not enhanced in those patients, advocating that the site of SP synthesis is external to the pancreas (Di Sebastiano et al., 2000).

5 Role of SP in Pancreatic Cancer

In the preceding few years, the expression and release of peptides by the tumors have been a significant and expanding field of interest (Munoz & Covenas, 2013). SP is an 11 aa long peptide that is extensively scattered all over the body. When SP binds to its highly specific receptor, NK1, it kicks off multiple biologic activities, such as the proliferation of tumor cells, angiogenesis (Guha et al., 2005), along with migration, which is crucial for the process of tumor cell infiltration and metastasis (Esteban et al., 2006; Muñoz et al., 2010). The mentioned biological functions are reversible using the NK1 receptor antagonists. These experimental findings insinuate that SP/NK1 receptor signaling may carry out an indispensable role in the process of cancer advancement and metastasis since SP could be a mitogen in tumor cells that express the NK1 receptor (Muñoz et al., 2010; Muñoz & Rosso, 2010).

Also, SP, along with NK1 receptors, have been pinpointed in the neoplastic cells and peritumoral blood vessels (Hennig et al., 1995; Munoz et al., 2011; Muñoz et al., 2010). Furthermore, it has lately been described that the discharge of malignant tumor cells to the brain to develop cerebral metastases can be an SP stimulated process (Lewis et al., 2013). More precisely, it has been also testified that SP/NK1 receptor signalling is concerned with pancreatic cancer by provoking pancreatic cancer propagation, neo-angiogenesis, in addition to the movement of malignant pancreatic cells. Conflicting this, the NK1 receptor inhibits pancreatic tumor cell proliferation as well as angiogenesis. (Friess et al., 2003; Guha et al., 2005; Muñoz et al., 2006; Muñoz & Rosso, 2010; Li et al., 2013).

The enriched expression levels of the NK1 receptor were not reported to be correlated to the tumor grade, although they were linked with an advanced tumor phase coupled with a feeble prognosis. In comparison to healthy individuals the immunoreactivity and mRNA expression levels of the NK1 receptor, are higher in pancreatic cancer test samples (Friess et. al., 2003). Furthermore, utilizing the western blot assessment, the NK1 receptor was noticed to be amplified 26-fold in the case of samples with pancreatic cancer when compared to normal healthy controls. Research suggests that the NK1 receptor mRNA was seen in 5 pancreatic cancer cell lines where the highest concentration via a quantitative RT-PCR was seen in CAPAN-1 and the lowest being ASPC-1 cell lines. It also suggested that SP along with its analog agonists stimulate pancreatic cancer progression based on the concentration of the respective NK1 receptor levels.

Moreover, this effect can be obstructed by a specific NK1 receptor inhibitor in a concentration subjected fashion (Friess et al., 2003; Muñoz et al., 2006). It is well-known that the possibility of progressing pancreatic cancer is exceedingly high in individuals with CP and probably is not dependent on country, gender, and the nature of pancreatitis (Lowenfels et al., 1993). It is also established that the process of up-regulation involved with mRNA expression levels of the NK1 receptor in CP has a convincing association with the pain disorder that these patients undergo (Shrikhande et al., 2001). Accordingly, excess expression of the engaged NK1 receptor may be involved in CP correlated with cancer. Truncated NK1 receptor is known to be overexpressed in colonic epithelial

cells obtained from individuals with colitis-allied cancer; however, the entire length is not distressed (Gillespie et al., 2011).

6 Pathological Role of SP in Inflammatory Diseases

SP causes pain through a mechanism called nociception, in which the nocireceptor reacts to a potentially damaging stimulus by signalling the brain and the spinal cord alongside its pro-inflammatory mechanisms (Nichols et al., 1999). SP has known to be involved in numerous physiological responses and diseases. SP increases the sensitivity to pain due to its indirect inflammatory effects while some other literature also suggests that SP has an in-vitro pain-relieving effect that gives a possible explanation of the failure of NK-1R antagonists in clinical trials.

Interaction of SP with NK-1R triggers neurogenic inflammation augmenting the inflammatory process in several parts of the body (Gherardini et al., 2020). Neurogenic inflammation is characterized by vasodilation and increased vascular permeability to immune cells besides leucocyte extravasation and direct action upon resident infiltrating cells to augment their immune functions. There is a dynamic cross-talk between the nervous system and the immune system, a plausible answer for why the expression of SP and its receptor can be regulated by the immune cells (Green et al., 2019).

6.1 Rheumatoid Arthritis (RA)

The literature suggests that Substance P is found in higher concentrations in the synovial joints of patients suffering from RA as a result of which they stimulate the synovial cells, increasing the release of prostaglandins and collagenase from them (Keeble & Brain, 2004). The number of the receptor of SP is different in the rheumatoid tissues pointing out their contribution to RA. Blocking the NK-1R is thought to be an efficient treatment strategy against RA (Menkes et al., 1993).

6.2 Skin Disorders

SP causes the accumulation of the mast cells, the WBC involved in allergic and inflammatory responses leading to eczema(Zhan et al., 2017). Higher blood concentrations of SP have been associated with psoriasis progression due to its role in inflammation and cell growth. SP has also been known to cause pruritus via the release of histamine from the skin mast cells (Amatya et al., 2010). Fibromyalgia patients have shown higher levels of SP in their spinal cord fluid, partly due to chronic stress, a key factor linked with fibromyalgia (Karlsson et al., 2019; Tsilioni et al., 2019).

6.3 Inflammatory CNS Disorders

Despite being highly protected by the blood-brain-barrier, neuroinflammation leads to infiltration of macrophages, T-cells, dendritic cells, and other immune responders due to the disruption of blood-brain-barrier by the pro-inflammatory substances like IL-12 secreted by the activated glial cells leading to devastating conditions like meningitis and encephalitis (Burmeister et al., 2017). Neurocysticercosis, a parasitic infection of the Central Nervous System caused by *Taenia solium* shows the presence of SP positive cells adjacent to the degenerating worms (Prodjinotho et al., 2020). In other CNS disorders like Parkinson's and Alzheimer's, a decreased level of SP has been reported in the animal models followed by a severe depletion in the NK1R expression neurons in MSA and Parkinson's disease (Przedborski, 2017; Severini et al., 2016). In some cases, the SP-NK1R interactive is required to reduce the disease burden such as murine gamma herpesvirus 68 and *Salmonella* infections driving the cell-mediated immunity for disease clearance showing the neuroprotective effects of this neuropeptide (Johnson et al., 2017; Rytel et al., 2020). However, the lack of SP-NK1R intection either due to the pharmacological response of NK1R antagonists or SP deficient mice contributes significantly to the bacteria-induced neuroinflammation and the consequent CNS damage.

6.4 Asthma

SP plays a major role in causing the typical changes in the asthmatic airways like bronchoconstriction, vasodilation, and plasma protein leakage, etc. by the activation of the postganglionic cholinergic nerves and mast cells. The expression of the NK-1R is upregulated in the asthmatic lung compared to normal making asthmatics hyper-responsive to SP. Steroid therapy may be used to down-regulate the expression of the NK-1R gene thereby curbing the SP-NK1R interactions (Kytikova et al., 2019).

Blocking the SP in the lungs increases the apoptosis of the lung cancer cells, a possible treatment option in non-small cell lung carcinoma patients. Targeting SP can also protect mice against TNF-alpha-mediated apoptotic liver damage. SP is also a key player in a non-apoptotic form of programmed cell death which is independent of caspase activation (Mashaghi et al., 2016).

6.5 Gastrointestinal Tract Diseases

Repeated acidification of the esophagus occurs in Gastro-oesophageal reflux disease (GERD) due to the relaxation of the lower oesophageal sphincter causing SP release from the extrinsic afferent nerve endings activating local inhibitory pathways to the lower oesophageal sphincter via the neurokinin-1 receptors (Takeda et al., 2020). SP also plays a crucial role in regulating acute-pancreatitis and pancreatitis-associated lung injury since they are potent pancreatic circulatory stimulants (Suvas, 2017; Tamizhselvi et al., 2011).

6.6 Inflammatory Bowel Disease (IBD)

The ulcerative colitis patients show elevated levels of SP in the rectum and colon while patients with Crohn's disease exhibit decreased mucosal levels of SP in the rectum (Patel et al., 2020). There is a 1000 fold up-regulation of SP binding sites in the lymphoid follicles and submucosal vasculature in patients suffering from IBD. Colonic inflammation triggers SP-induced inflammatory and proliferative effects leading to diseased conditions (Rychlik et al., 2020).

6.7 Depression

SP and NK-1R are present in abundance in those areas of the brain that are involved in emotion and behavioral stress response and hence patients with these mental health problems show significantly high levels of SP in the cerebrospinal fluid (Richter et al., 2018; Won et al., 2017).

6.8 Sickle Cell Anaemia

SP may be considered as a pain sensitization marker for sickle cell disease patients. SP promotes the production of cytokines that encourage adherence of the sickle red blood cells to the vessel wall, raising the risk of sickle cell crisis (Brandow et al., 2016).

7 Conclusions

Substance P, a member of the tachykinin family of neuropeptides is a critical molecule associated with inflammatory processes in a variety of tissues. As one of the very first messengers activated upon a stimulus, it plays an important role in pancreatic tissue injury/infection. Various other pathological conditions such as rheumatoid arthritis, skin disorders, inflammatory CNS disorders, asthma, gastrointestinal tract diseases, inflammatory bowel disease, and depression along with sickle cell anemia also see a very significant contribution of SP in their pathogenesis. In each case, SP plays a role in both – pain and progression of inflammation. However, resolving the inflammatory response is a dynamic series of events to prevent neutrophil sequestration, granulocyte clearance, and disruption in the homeostasis of the pro-and anti-inflammatory mediators. Therefore, the evolving role of SP in inflammation with better experimental evidence may help in the development of novel SP inhibitors in the future.

8 Future Perspectives

Since SP is an amplifier of inflammatory pathways, inhibition of SP could lead to a better prognosis of concerned tissues. A general curbing of SP activity, however, could affect surrounding cell types. On the other hand, specific blocking of SP activity in pancreatitis proves to be a promising pharmacological target. The NK-1R inhibitors against pancreatic conditions are not been well explored *in vivo* studies so far. Hence, they are not yet been clinically approved in any human pancreatic conditions so far. Further research in the area, thus shows the potential of better resolution of pancreatitis.

Abbreviations

AP- Acute pancreatitis; CCK-Cholecystokinin receptors; CDE-Choline-deficient and ethionine supplemented regime diet; CP-Chronic pancreatitis; GERD- Gastro-oesophageal reflux disease; IBD - Inflammatory Bowel Disease; IL-8- Interleukin 8; IL-12-Interleukin 12; MPO-Myeloperoxidase; NEP- Neutral endopeptidase; NF-κB-nuclear factor kappa B; NK1R-Neurokinin 1 Receptor; PPTA-Preprotachykinin A; SP-Substance P; TACR-Tachykinin receptor; TNF α-Tumor Necrosis Factor α; TRPV1-Transient receptor potential vanilloid type 1; RA- Rheumatoid Arthritis; WBC- White blood cells.

Conflicts Of Interest

The authors declare that there are no potential conflicts of interest.

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QUASISPECIES FEATURE IN SARS-CoV-2

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ABSTRACT

Since the identification of the SARS-CoV-2, genus Beta- Coronavirus, in January 2020, the virus quickly spread in less than 3 months to all continents with a susceptible human population of about a 7.9billion, and still in active circulation. In the process, it has accumulated mutations leading to genetic diversity. Regular emergence of variants of concern/significance in different ecology shows genetic heterogeneity in the base population of SARS-CoV-2 that is continuously expanding with the passage of the virus in the vast susceptible human population. Natural selection of mutant occurs frequently in a positive sense (+) single-stranded (ss) RNA virus upon replication in the host. The Pressure of sub-optimal levels of virus-neutralizing antibodies and also innate immunity influence the process of genetic/ antigenic selection. The fittest of the mutants, that could be more than one, propagate and emerge as variants. The existence of different lineages, clades, and strains, as well as genetic heterogeneity of plaque purified virus population, justifies SARS-CoV-2 as 'Quasispecies' that refers to swarms of mutant sequences generated during replication of the viral genome, and all mutant sequences may not lead to virion. Viruses having a quasispecies nature may end up with progressive antigenic changes leading to antigenic plurality that is driven by ecology, and this phenomenon challenges vaccination-based control programs.

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1 Introduction

'Quasispecies' is described as mutant clouds comprising of a population of a virus comprising of a large number of genomic variants (Domingo et al, 2012; Domingo & Perales, 2019). Inside infected cells, a parent virion RNA molecule seldom produces daughter RNA of identical nucleotide sequence, and this occurs as part of the biology of mRNA sense viruses. This variation in the nucleotide sequence in form of substitution/deletion is consequent to error(s) during copying of template/parent RNA molecule by viral RNA-dependent-RNA-polymerase. The significance of a mutant genome sequence, beyond phylogenetic and evolutionary analyses, lies with the existence of matching mutant virions. Mutant virus populations with non-identical genome continuously generate due to error-prone viral RNA genome replication, and the spectrum of mutants in a virus population, either plaque purified or not, vary with the progress in virus replication and subsequent selection process that is influenced by ecology comprising of host, environment/ landscape and the virus itself (Domingo & Perales, 2019). Viruses restrict themselves by either eliminating the host or immunizing the host following primary infection. Such natural restriction in controlling the virus population is usually breached by the emergence of variants that vary in major immunogenic domains. This has been the reason for the second wave of Covid-19 in India and elsewhere when many variant virus populations with antigenic dissimilarity have been identified.

2 Human CoVs

Human respiratory sickness caused by coronavirus was identified in 1962 (Habas et al., 2020). There are seven species of hCoV, which include α-CoVs of HCoV-NL63 & 229E,β-CoVs of HCoV-OC43, HKU1, SARS-CoV-1 and 2, and MERS-CoV. HCoV-229E, -OC43, -NL63, and HKU1 are distributed globally. Even though HCoVs have been identified to create epidemics every 2–3 years with a significant risk of reinfection, there is a scarcity of data on the epidemiology and clinical symptoms of these four HCoVs around the world (Phelan et al., 2020; Ye et al., 2020).

3 The Coronavirus disease- 2019 (Covid-19)

Consequent to the steady transboundary transmission of an emerging Coronavirus, SARS-CoV-2, since December 2019 from Wuhan, PRC, causing highly transmissible respiratory disease, named as Covid-2019, in humans across the world (first Covid-19 case in India was detected on 30 January 2020), the World Health Organization (WHO of the UN) declared' Covid-19 pandemic on March 12, 2020 (Li et al., 2020a; Pattnaik &Yadav, 2020; Platto et al., 2021). The real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) targeting multiple virus genes (E, N, RdRpetc) is the gold standard method for detection of SARS-CoV-2 with variable sensitivity that primarily depends on virus load in the clinical sample, and duration and severity of clinical disease (Corman et al., 2020; Wang et al., 2020a; Li et al., 2020b). During sampling from nostrils and throat or elsewhere, the stage of infection in the individual is largely unknown. Spot antigen test is also being used as a point-of-care (POC) test, and negative samples are subjected to rRT-PCR for the nucleic acid test (NAT). Antibody (IgM and IgG) tests are also in use for retrospective diagnosis. NAT positivity is taken as an active infection. There has been NAT positivity with no clinical sickness; also, NAT negativity with respiratory sickness. Radiological examination of the chest by computerized tomography (CT) of patients with suspected SARS-CoV-2 infection is highly sensitive in arriving at a clinical decision based on the degree of lung pathology (Inui et al., 2020; Yang et al., 2020). The Dutch Radiological Society developed the 'COVID-19 Reporting and Data System' (CO-RADS) that combines chest CT results, clinical data, and NAT results (Prokop et al., 2020; Zayed et al., 2021). Pulmonary pathology is not exclusive to Covid-19, and immuno-histochemistry is required to diagnose SARS-CoV-2 infection.

There is no specific treatment for Covid-19, however chemotherapeutic compounds like Remdesvir/ Veklury (ATP analog), Flavipiravir (pyrazine), aminoquinoline, etc., have been used selectively (Majumder & Minko, 2021). In treatment, though ACE2 enzyme inhibitors and receptor blockers (ARBs) have been used in cardiovascular diseases; but increase in expression of ACE2 receptors on cells following treatment with inhibitor/blocker has been observed (Albini et al., 2020). The use of live attenuated vaccines of Polio, MMR, and BCG (bacillus Calmette-Guerin) could boost non-specific immunity (NSI)/ innate immunity and help in reducing the severity of Covid-19 (Majumder & Minko, 2021).

4 The virus

The SARS-CoV-2 is a *Sarbecovirus* in the Genus beta-Coronavirus (β-CoV). Like any other CoV, the genome of SARS-CoV-2 is (+) ssRNA (mRNA sense) of < 30 kb with 5' - cap, 3'- poly(A) tail (Pattnaik & Yadav, 2020). The organization of the viral RNA genome is 5′ cap-L-UTRs-polymerase-Spike-Env-Membrane-Nucleocapsid-3′UTR-poly(A) (Lu et al., 2020). There are 14 open reading frames (ORFs) in the viral RNA genome, that encode 4 structural proteins of the spike (S), envelope (E), membrane/matrix (M), and nucleocapsid (N), and 16 non-structural proteins (NSPs; from proteolytic processing of polyproteins pp1A and 1ab). The structural genes are interspaced with 9 accessory genes; ORFs 3a, 3b, 6, 7a, 7b, 8, 9b, 9c, and 10, and the accessory proteins are essential for virus replication, pathogenesis, and virus- morphogenesis processes (Thomas, 2021). Six of the NSPs, viz., 3,9,10,12,15, and 16 are essential in virus replication (Krichel et al., 2020). The S glycoprotein is a transmembrane that binds through residues in

the Receptor Binding Domain (RBD; in S1 domain) to ACE2 expressed on cells of most human organs. The host TMPRSS2 facilitates membrane fusion with the involvement of the S2 domain of the S glycoprotein. There is a similarity as well as distinction in amino acid composition in RBD between SARS-CoV-1 and -2 (Wan et al. 2020), as both the virus use ACE2 receptor (Hofmann et al., 2020; Wang et al., 2020b). Six different amino acid residues in the RBD are critical in the attachment of the virus to the ACE2 receptor, viz., Leu^{455}, Phe^{486}, Gln^{493}, Ser^{494}, Asn^{501}, and Tyr^{505} in SARS-CoV-2, and in contrast Tyr^{442}, Leu^{472}, Asn^{479}, Asp^{480}, Thr^{487} and Tyr^{4911} in SARS-CoV-1 (Mohamadian et al., 2020). Both the SARS-1 and -2 viruses are antigenically distinct and different. Highly antigenic epitopes in RBD had A348V, V367F, and A419S (Singh et al., 2020). There was attenuation of SARS-CoV-2 upon deletion of 30 amino acid residues in the S1-S2 junction of S glycoprotein (Lau et al., 2021). Deletion of 382 nucleotides in the accessory protein ORF8 (Δ382 variant of SARS-CoV-2) is associated with mild disease (Young et al., 2020). The N protein attaches to the viral RNA during the morphogenesis of new virion particles and also contributes to immune evasion by the virus (Mu et al., 2020). The M protein is conserved and in association with N protein and accessory proteins, 3a and 7a facilitate the budding of new virion particles (Roy et al., 2020; Ysrafil, 2020). The E protein facilitates virion maturation and releases from infected cells (Naqvi et al. 2020). Globally, NSP1/NSP2 and ORF7a/3a are the most mutable genes of SARS-CoV-2 (Roy et al., 2020). Both the NSPs and ORF7a and ORF3a are involved in virus replication (Bianchi et al., 2021; Thomas, 2021).

5 Variations in the genome

The S gene is variable in the nucleotide sequence, and the virion surface S glycoprotein determines host susceptibility. Mutations and evolution of the Spike gene have been elaborated (Winger & Caspari, 2021). The most dominant transition and trans-version in the S gene across the globe were C→U and G→U (Roy et al., 2020). The S protein substitution D^{614}→G was the first major event in the mutation and evolution of SARS-CoV-2 in the COVID-19 pandemic (Korber et al., 2020; Plante et al., 2020; Yurkovetskiy et al. 2020). This substitution is located in a B- cell epitope in the S1 domain that classified the SARS-CoV-2 sequences in 2 subtypes, viz. SARS-CoV-2a and -2b, having amino acid residues D614 and G614 respectively. Lineages of SARS-CoV-2b have spread the world over. Both the subtypes differ in immunogenicity, and subtype 2b is reported to be less immunogenic compared to subtype 2a, the parent virus. This evolution possibly made the host hypo-responsive so that the virus could persist in the human population (Kim et al., 2020). Further G614 increased the stability of the virion and has dominated the global Covid-19 scenario. Mutants with mutations around the ACE2 binding site of the virus are in circulation (http://cov-glue.cvr.gla.ac.uk; https://www.gisaid.org). Naturally occurring mutations in the S gene at positions E484, F490, Q493, and S494 reduced binding of the virus to the Mabs C121 and C144, and mutations at R346, N439, N440, K444, V445, and G446 lead to reduced affinity to the Mab C135 (Robbiani et al., 2020; Baum et al., 2020). This observation shows the presence of virus populations differing in epitope profile. The selection of neutralization escape mutants in vivo will depend upon the concentration and affinity of the neutralizing antibodies (Weisblum et al., 2020).

Several clades and Spike variants have evolved during the pandemic in different countries., viz., Alpha (B.1.1.7; U.K.), Beta (B.1.351; South Africa), Gamma (P.1; Brazil and Japan), Delta (B.1.617.2; India), Delta Plus (K^{417} →N), Epsilon (B.1.429 and B.1.427), Kappa (B.1.617.1; India), and Eta (B.1.525), etc. An S variant with substitution N^{501}→Y and deletion of histidine and valine codons at positions 69 and 70, were identified in the UK (Tang et al., 2020). The worldwide prevalence of clade B.1.617 was followed by the generation of its variant strains of Kappa (κ), Delta (δ), and Delta plus (Figure 2).

Several non-synonymous mutations were detected in the transmembrane and C-terminus domains of the E protein (Hassan et al., 2020). Recurrent non-synonymous mutations are linked to adaptation in humans (vanDorp et al., 2020). Several sites of variation in ORFs 1a, 1b, S, 3a, M, 8, and N indicate elective mutations in the virus (Wang et al., 2020c).

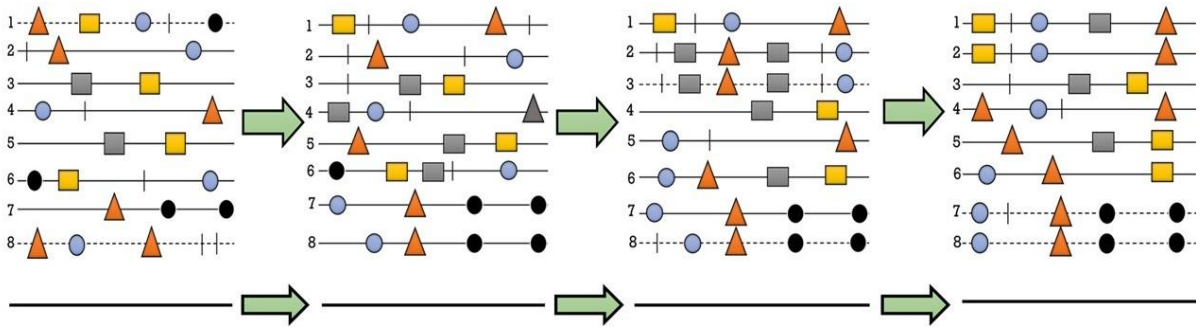

Figure 1 Representation of the evolution (change in composition) of a viral quasispecies (modified from the source: Domingo et al., 2012)

Conclusion and Future Prospects

There is a continuous evolution of SARS-CoV-2 leading to SNP variants and many lineages. SARS-CoV-2 has undergone strong selection pressure over a short period since December 2019. Forces of selection (fitness is the criterion) by the host immune system during replication of the virus and its transmission between hosts play an important role (Roy et al., 2020). The virus also undergoes genetic evolution due to mutations accruing over time and space, producing variants differing from the parent strain(s). The mutation is independent of the fitness of the parent and mutated genome; evolution rate includes time factor. A comprehensive investigation of thousands of SARS-CoV-2 genome sequences identified > 1000 mutations. Virus strain having less sensitivity to neutralizing antibodies in the immunosuppressed patients was identified. The asymmetric antigenic relationship observed between strains (Yadav et al., 2021) is of epidemiological significance. The phenomenon of quasispecies in foot and mouth disease virus (FMDV; Aphthovirus genus) that has (+) ssRNA (mRNA sense) of about 8.5 kb with 5' -cap, 3'- poly(A) tail has been described, where even virion population in a purified plaque is genetically heterogeneous (Figure 1). CoVs have a higher mutation rate (0.44 – 2.77 × 10-2 per site per year) compared to the Foot-and-mouth disease virus (6 ×10-3 per site per year), though both have (+) ss RNA genome. Plaque purification of SARS-CoV-2 in Vero E6 cells revealed genetic variants in small plaques that could not be detected in the original clinical material. This shows that the variants appeared upon replication of the virus. These

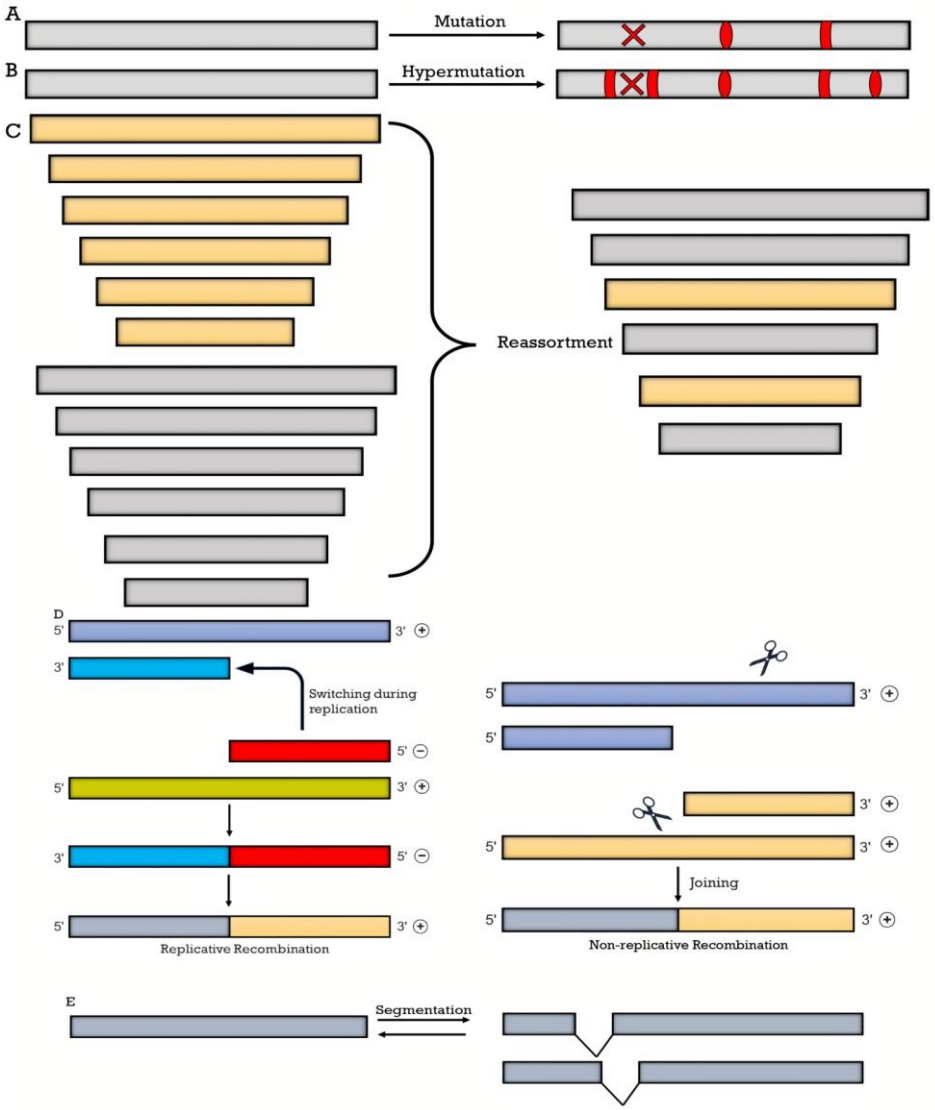

Figure 2 Representation of several types of genetic modifications that can alter the composition of viral quasispecies (modified from the source: Domingo et al., 2012)

variants were either deletion mutants (15-30 nucleotides deleted) or were having point mutations in the S1-S2 interface. Analysis of 55,189 SARS-CoV-2 sequences identified 2175 sequences having non-synonymous mutations in the Spike gene. The frequency of variation varied from 5.48 in coding regions to 6.96 in the noncoding regions (Cao et al., 2021). These observations support quasispecies in SARS-CoV-2. Despite the ongoing global mass vaccination program, the emergence of new a variant virus threatens control of Covid-19. The proposed future directions are (i) random and regular screening of symptomatic patients for SARS-CoV-2 infection is needed, (ii) regular sequencing and analysis of SARS-CoV-2 isolates should be conducted, (iii) mass awareness about the variants and their effect on human health is to be carried out, (iv) more stress should be given to the vaccination of the entire population and other personal hygienic measures.

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Conflict of interest

None

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BIPLOT ANALYSIS FOR IDENTIFICATION OF SUPERIOR GENOTYPES IN A RECOMBINANT INBRED POPULATION OF WHEAT UNDER RAINFED CONDITIONS

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ABSTRACT

Physiological traits of wheat genotypes and their trait relation to drought conditions are important to identify the genotype in target environments. Thus, genotype selection should be based on multiple physiological traits in variable environments within the target region. This study was conducted at Punjab Agricultural University during *rabi* crop seasons 2012-13 and 2013-14 to study the recombinant inbred lines (RILs) of wheat genotypes derived from traditional landraces and modern cultivars (C518/2*PBW343) based on various morpho-physiological traits. A total of 175 RILs were selected for this study based on various tolerance indices. The genotype by trait (GT) biplot analysis was applied to data from seven high-yielding RILs grown under irrigated (E_1) and rainfed environments (E_2). The GGE biplot explained 100% of the total variation for chlorophyll content, grain filling period, peduncle length, water-soluble carbohydrates, grain number, grain yield, and 95.1% for canopy temperature, 94.9% for thousand-grain weight. GT-biplots indicated that the relationships among the studied traits were not consistent across environments, but they facilitated visual genotype comparisons and selection in each environment. RIL 84 and RIL108 were close to the average environment (ideal genotype) for all traits studied except chlorophyll content. A well-performing genotype with great environmental stability is called an "ideal genotype. Among all entries, these genotypes performed well. Therefore, among the traits studied, grain filling period, peduncle length, canopy temperature, water soluble carbohydrates, and 1000 grain weight contributed to grain yield under a stress environment. Furthermore, it may be used as a donor material in breeding programs and QTLs mapping.

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1 Introduction

Globally, wheat is the most cultivated crop and is the second most staple cereal crop in India. As per the Ministry of Agriculture, Government of India, 68% of the total net sown area (142.2 mha) in India was documented as drought-prone (Yaduvanshi et al., 2015). Various researchers have found wheat susceptibility to drought stress, which can result in yield losses of up to 90% depending on the development stage, genotype, and intensity and length of the dry period (Wang et al., 2019; Gupta et al., 2020; Tian et al., 2020). Drought and water scarcity threaten the agricultural productivity of many developing countries including India to feed their increasing demand and ever-growing population. Drought stress not only affects the plant behavior but also severely affects the metabolism of the plant, physiology, and biochemical responses in plants. These responses include leaf rolling, destruction of the cell membrane, leakage of electrolyte, dysfunction of photosystem II, inactivation of enzyme synthesis which leads to stomatal closure, repression of cell growth and photosynthesis, and activation of photorespiration (Gururani et al., 2015; Alyammahi & Gururani, 2020; Kappachery et al., 2021). Therefore, physiological and biochemical approaches have great importance in elucidating the complex phenomenon of drought tolerance in plants and understanding the underlying mechanism.

The enhancement of crop productivity under drought conditions requires genotypes with improved physiological traits like better stomatal conductance, higher relative water content, less transpiration utilizing minimum water, cooler canopy, better stem reserve mobilization, and yield stability (Srivastava et al., 2017; Chowdhury et al., 2021) The primary goal of many breeding programs is to identify superior genotypes through considering multiple physiological traits under drought conditions (Yan & Rajcan, 2002). Physiological aspects of drought tolerance have been shown in a recombinant inbred population of wheat. Association of stomatal density and low canopy temperature with drought stress was found in high-yielding cultivars. Furthermore, a correlation has been shown in various traits like peduncle length and water-soluble carbohydrates as well as 1000 grain weight under drought-stressed conditions (Srivastava et al., 2016). It is also reported a trait correlation in chlorophyll fluorescence and chlorophyll content with drought tolerance index which reflects the reproductivity of mapping population of wheat (C306 x HUW206) under rainfed stress conditions (Kumar et al., 2012).

Biplot analysis of a genotype by traits (GT) can help to understand and identify the crop as an integrated system with interconnected components, i.e., multiple breeding objectives. Simultaneously, it helps to unleash the trait profiles (strength and weakness) of the genotypes, which are important for identifying those that are superior in desired traits and hence could be candidates for use as parents in a breeding program (Yan & Kang, 2002). It also discloses information on the key traits of cultivars besides it assisting in the detection of less significant traits and recognizing those that are suitable for indirect selection of a target characteristic. Furthermore, GGE biplot analysis may be better and more accurate to the graph AMMI1 mega-environment for being more efficient in explaining the sum of squares of GE and G + GE, due to confirmed by its greater predictive accuracy (Bozovic et al., 2020).

Under drought stress conditions, the relationships between yield and physiological traits become the choice of selection and breeding strategies for the breeder. There is also a strong negative relationship or volatile interaction between breeding targets, which makes breeding very difficult and complicated (Jha et al., 2020; Ranjith & Rao, 2021). Therefore, more - attention must be paid to undesirable associations among breeding objectives when performing independent choice, because only selection based on desirable traits or culling for undesired levels of one trait may lead to the loss of useful materials or even render the selection useless (Ranjith & Rao, 2021). A biplot is a statistical tool to evaluate genotype-by-trait cultivars based on many characteristics and to classify those that are superior in desirable characteristics and consistent yield production in variable environments in the target area. Moreover, it could be used as donor parents in a breeding program or directly released for commercial production. The GT biplot analysis allows a graphical representation of the genetic correlations among traits and their degree and nature of association among the traits (Yan & Frégeau-Reid, 2008; Bozovic et al., 2020). However, there are pitfalls in interpreting a GT biplot when the biplot does not fully approximate the data. The relationships among physiological traits in bread wheat are frequently influenced by unpredictable conditions in the Mediterranean rain-fed areas. Therefore, the selection of genotypes within the target area should be ground on different traits in an erratic environment. However, little is known about the trait relations and trait profiles of RILs derived from the cross of (C518/2*PBW343) wheat genotypes in rainfed conditions. Both the parents PBW343 (modern cultivar) and C518 (traditional landraces) have contrast characters offering several morpho-physiological traits (Table.1). PBW343 is a modern cultivar and known for high-yielding variety but input responsive in drought conditions. Although C518 is a long-height old cultivar and can mobilize stalk reserve in stressful conditions because of larger peduncles and low input (Bala et al., 2010). The present study was undertaken to assess the performance of promising RILs under two different water regimes and to identify superior genotypes better adapted to rainfed conditions by examining different physiological traits.

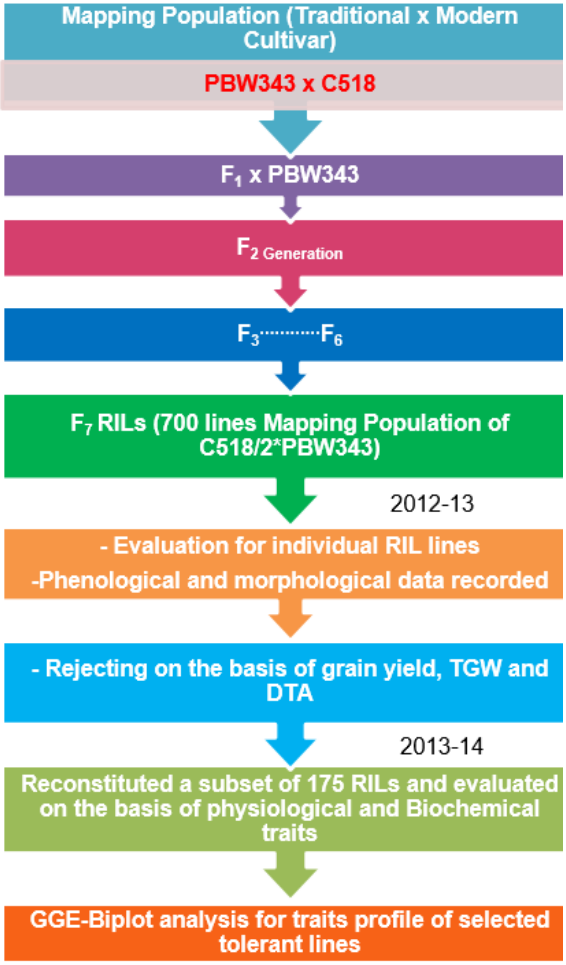
Table 1 Characteristic traits of parental lines

S. No.	Traits	Parents		RILs				
		PBW343	C518	32	47	80	84	108
1	Plant Height	Medium (95-100 cm)	Tall (125-130 cm)	Medium-tall (104-108 cm)	Tall (114-118 cm)	Small (84-88 cm)	Medium-tall (104-108 cm)	Medium-tall (106-110 cm)
2	Peduncle Length	Medium	Long	Medium	Long	small	Medium	Medium
3	Stomatal Conductance	High	Low	Moderate high	Moderate Low	High	Moderate high	Moderate high
4	Grain Filling Period	Medium	Longer	Longer	Medium	Medium	Longer	Longer
5	Chlorophyll Content	High	Low	Medium	Low	Medium	High	High
6	Peduncle and leaves colour	Green	Green turn golden color	Green yellow	Green yellow	Green	Green turn golden color	Green turn golden color
7	Agronomic condition	Input responsive	Low input	Medium input	Low input	Input responsive	Low input	Low input

2 Materials and Methods

2.1 Experimental design and data collection

The paired experiments (rain-fed and irrigated conditions) were conducted at Punjab Agricultural University, Ludhiana, India, during the cropping seasons 2012-13 and 2013-14. The experimental trial was conducted in a randomized block design with two replications, with a plot size of 1 m per row and a row-to-row spacing of 20 cm. Recombinant inbred lines (RIL) population (700 lines including parents) were procured from Wheat Section, Punjab Agricultural University, Ludhiana. In the first-year experiment, a set of 700 RILs including parents (PBW 343 and C518) were evaluated for morpho-physiological traits viz., canopy temperature, chlorophyll content, water-soluble carbohydrates, stem research mobilization, grain filling period, and from this a small subset of 175 RILs reconstituted for further evaluation based on yield percent reduction. Later the subsequent year, a total of 175 RILs derived from drought tolerance and moderately susceptible wheat cultivars (C518 and PBW343 respectively) along with parents were evaluated in this study (Figure 1). Thereafter, based on the tolerance index, seven promising genotypes viz., RIL 32, RIL 47, RIL 80, RIL 84, RIL 108, PBW343, and C518 were selected for biplot analysis. Normal recommended agronomic practices were followed for growing wheat crops. Meteorological data weekly were recorded from the agrometeorological station, Punjab Agricultural University, Ludhiana, presented in Figure 2. The traits recorded for each genotype under both rain-fed and irrigated conditions during cropping seasons were: peduncle length (PL), chlorophyll content index (CCI), grain filling period (GFP), water-soluble carbohydrates (WSC), Number of grains per spike (GN) and grain yield (GY) as described earlier (Kumar, 2015).



mapping population

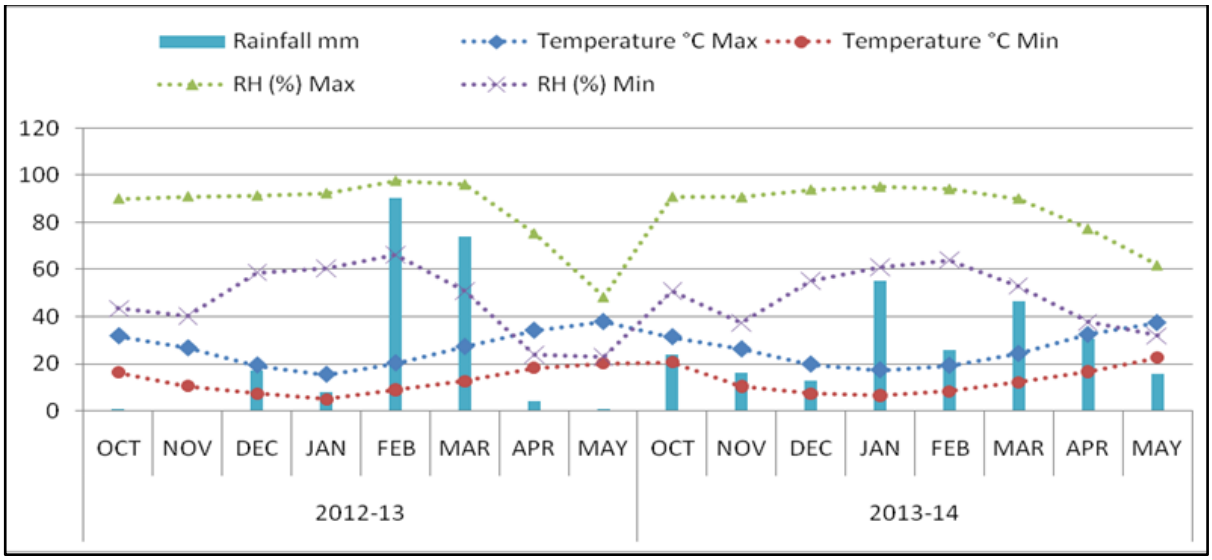


Figure 2 Agrometerological data during crop season 2012-13 and 2013-14

2.2 Statistical Analysis

The GGE biplot software (version 2.2.210) was employed to display the genotype-by-trait relations two-way data in a biplot for each environment. A vector is created from the biplot origin to the relevant trait markers in the GT biplot to show the relationship between the characteristics. The correlation coefficient of any two traits is approximated by the cosine of the angle between their vectors. Acute angles show positive correlations, obtuse angles suggest negative correlations, and right angles indicate no association. A short vector may mean that the trait is not linked to any other trait (Yan, 2001).

3 Results and Discussion

3.1 Interrelationship between genotypes and environments (irrigated E_1 and rainfed E_2)

Environment vectors are the lines that link the biplot origin to the markers. The correlation coefficient between two conditions is proportional to the angle between their vectors (Yan, 2002). Broad obtuse angle, or strong negative associations among the environment, indicated a strong genotype-by-environment relationship (Katuuramu et al., 2020). Concerning genotype discrimination, the distance between two environments tests their dissimilarity. According to GGE analysis, ideal cultivars are those that should have large PC1 scores (high mean value) and small (absolute) PC2 scores (high stability). The GGE biplot explained 100 % of the total variation for chlorophyll content, grain filling period, peduncle length, water soluble carbohydrates, grain number, grain yield, and 95.1 % for canopy temperature, 94.9 % for 1000 grain weight (Figure 3). This relatively high percentage variation reflects the accuracy of interrelationships among the measured traits. Most angles between their vectors are acute. The genotype's vector length indicates the extent of its influence (positive or negative) on a specific phenotype (Yan & Tinker, 2005). Thus, the majority of RILs were highly correlated with parent C518 except for chlorophyll content and grain yield (Figure 3a-h). PBW343 showed high chlorophyll in E_1 and E_2 environments as their vector lay between E_1 and E_2 environments. The interspace between a genotype and the biplot origin is a special indicator of the genotype's difference from an ordinary genotype (Yan & Frégeau-Reid, 2008). Genotypes having a long distance from the biplot origin exhibits disproportionately high levels of one or more characteristics. These genotypes are not always superior, but they do act as parents for specific traits. Thus, RIL 80 had the longest distance from biplot origin followed by RIL 84 and RIL108. However, RIL 84 had a negative interaction with E_2 and RIL 80 had a positive interaction with E_1. Genotypes in the biplot's centre show no interaction, implying that their mean value is stable across two environments E_1 and E_2. Therefore, RIL 47, RIL 32, RIL 108, and C518 are located in this category, and it can be considered as stable performance (Figure 3a). In the case of grain filling period (Figure 3b), C518 showed the maximum grain filling period under both E_1 and E_2 but positive interaction with E_2 as the length of the vector is more and their angle is less than 90° and hence specific adaptability with E_2. However, PBW343 had a negative interaction with E_2. Moreover, among the tested RIL, the RIL108, RIL 47, and RIL 80 were found an inverse relationship with C518 as their vector showed an obtuse angle, i.e., it had a minimum grain filling period than C518. While RIL 32 and RIL 84 had a close relationship with C518 by their acute angle, i.e., it showed at par the mean value of C518. For peduncle length (Figure 3c), C518 had maximum peduncle length over E_1 and E_2. However, PBW showed an inverse relationship with C518 as their

obtuse angle, i.e., minimum peduncle length in comparison to C518. Likewise, RIL 80 and RIL 30 were also found an inverse relationship with C518 but a close relationship with PBW343 by their vectors showed an acute angle. Moreover, RIL 108, RIL 84, and RIL 47 showed a close relationship with C518 as their vectors showed acute angle, but RIL 47 showed nearest to biplot origin, i.e., their mean value is stable under both E_1 and E_2 environments.

For canopy temperature at anthesis and post-anthesis (Figure 3d), PBW343 showed a close relationship with C518 under E_1 and E_2 as their vectors showed acute angle but PBW343 and RIL 80 showed nearest to biplot origin as it has a stable mean value under E_1 and E_2 environments. Likewise, RIL 108 showed an acute angle between them and had a close relationship with E_1 and E_2 environments, i.e., they have higher temperatures in E_1 and E_2. However, RIL 84 and RIL 47 showed an inverse relationship with E_1 and E_2 as their vectors showed an obtuse angle between them, i.e., they have minimum temperature under both E_1 and E_2 environments. Similarly, for mobilized water-soluble carbohydrates (Figure 3e), C518 had maximum mobilized water-soluble carbohydrates under both E_1 and E_2 environments as their vectors lied between E1 and E_2 and had closed to environment vector. Whereas PBW343 and RIL 108 showed a negative relationship with C518 as their vector showed an obtuse angle (>90°) i.e., they have minimum water-soluble carbohydrates in contrast to C518. However, RIL80, RIL 47, RIL 32, and RIL 84 had a close relationship between them as their vectors showed an acute angle, but RIL 80 showed closer to biplot origin, i.e., they have stable values at par to C518 under E_1 and E_2 environments.

Like in the previous, C518 showed stable performance, i.e., maximum 1000 grain weight (grain weight in normal and defoliation) under both E_1 and E_2 environments as their vector lied between them and closed to biplot origin. Whereas PBW343 showed an inverse relationship with C518 as well as E_1 and E_2 environments as their vectors showed an obtuse angle between them. RIL 108 and RIL 84 had a closer relationship with E_1 and E2, but RIL 47 and RIL 84 showed stable performance under both environments as their position was closed to the biplot origin (Figure 3f). Biplot analysis for the number of grains per spike showed an inverse relationship between C518 and PBW343 (Figure 3g). However, RIL 80 showed a maximum number of grains per spike as their vector lied approximately middle position between E_1 and E_2 environments followed by C518 which showed a close relationship between them while PBW343 had an inverse relationship with E_2. RIL 32, RIL 108, and RIL 84 have a close relationship with PBW343 as their vectors are shown by an acute angle. Similarly, for grain yield, C518 and PBW343 showed an inverse relationship between them as their vectors showed an obtuse angle (>90°) but the performance of C518 showed higher in comparison to PBW343 as their vector lines on a positive axis. However, RIL 32, RIL 47, RIL 108, and RIL 80 showed maximum grain yield under both E_1 and E_2 environments as their vectors closed to the environmental vector, but RIL 32 and RIL 47 had stable performance under both E_1 and E_2 as they showed closet position form biplotorigin (Figure 3h). Similar studies have also reported the effectiveness of GGE plot in identifying superior genotypes under different environments (Bányai et. al., 2020; Bishwas et. al., 2021)

3.2 Which-won-where pattern of biplot analysis

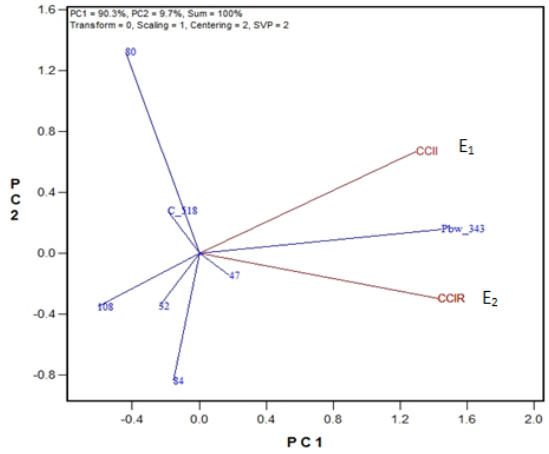
Yan et al. (2000) reported the depiction of the "which-won-where" pattern of biplot analysis is essential for investigating genotype interactions in diverse contexts. To identify the stable genotypes, polygon view analysis of bioplot is the best tool to visualize the relationship between genotypes and environments (Yan & Kang, 2002). The farthest genotypes are joined to create a polygon to form the Which-Won-Where graph. Following that, perpendicular lines are drawn from the biplot's origin to each of the polygon's sides, dividing the biplot into multiple sectors, each with a single genotype at the polygon's vertex. These lines are known as lines of equality (Yan, 2001). In one or more conditions, the genotypes at the polygon's vertices are the best or the worst. Within the environments, the genotype near the polygon's vertex has the highest performance (Yan & Tinker, 2005). Which-won-where biplots for chlorophyll content, canopy temperature, grain filling period, water-soluble carbohydrates, peduncle length, 1000 grain weight, number of grains per spike, and grain yield over two environments E_1 and E_2 are presented in Figure 3i-p respectively. Polygon for chlorophyll content showed vertex genotype PBW343, RIL 80, RIL 108, and RIL 84 (Figure 3i). While PBW343 showed higher chlorophyll content under both E_1 and E_2 environments followed by RIL 47. The vertex RIL 80, RIL 108, and RIL 84 showed minimum chlorophyll content as they showed no environment in their sector. In sector 2, C518 showed stable mean values under E_1 and E_2 environments as their position showed close to biplot origin. Similarly, canopy temperature at anthesis and post-anthesis showed vertex genotype C518, RIL 108, RIL 32, RIL 84, and RIL 47. C518 showed higher canopy temperature at anthesis in E_1 and E_2 followed by PBW343. RIL 108 at the vertex in the sector showed higher canopy temperature at post-anthesis in E_1 (irrigated) and E_2 (rainfed). However, RIL 80 showed a stable mean value i.e., low canopy temperature at anthesis and post-anthesis in E_1 and E_2 environments (Figure 3l). Polygon view analysis for the grain filling period showed vertex genotypes C518 RIL 47, PBW343, RIL 80, and RIL 84. C518 showed the maximum grain filling periods as they were farthest from biplot origin under E_1 and E_2 followed by RIL 32. However, PBW343, RIL 47, RIL 80, and RIL 84 showed the minimum grain filling period in comparison to C518 as there were no environments in their sector (Figure 3j). Similarly, for mobilized water-soluble

carbohydrates (Figure 3m), polygon showed vertex genotypes C518, RIL 84, RIL 47 PBW343 and RIL 32. Parent C518 showed maximum water-soluble carbohydrates under both E_1 and E_2 as their position showed farthest to biplot origin in E_1 and E_2 environments followed by PBW343 but not stable in mean value as their sector does not fall in E_1 and E_2. Moreover, RIL 47, RIL 32, and RIL 84 showed less water-soluble carbohydrates in comparison to C518, but they showed stable values in E_1 and E_2 as their position showed close to biplot origin on PC2. For peduncle length, the which-won-where pattern of biplot analysis showed vertex genotypes C518, RIL 84, RIL 80, PBW343, and RIL 108. Parent C518 showed maximum peduncle length under E_1 and E_2 followed by RIL 108 and RIL 84 as their sectors fall in a certain environment. However, genotypes PBW343 and 80 showed minimum peduncle length in comparison to C518 as they showed an inverse relationship (Figure 3k). Polygon view for 1000 grain weight showed vertex genotypes RIL 32, RIL 80, PBW343, and RIL 108. RIL 82 showed the maximum 1000 grain weight (grain weight in normal condition) under E_1 followed by C518 (Figure 3n). While RIL 108 showed a maximum 1000 grain weight (grain weight defoliation) under E2 followed by RIL 84 i.e., at par value of C518. However, PBW343 showed a minimum 1000 grain weight (under normal and defoliation) under E_1 and E_2 as their sectors showed inverse form under certain environments. Similarly, for grain number (Figure 3o), vertex genotypes RIL 80, RIL 47, PBW343, RIL 32, and C518. While RIL 80 showed the maximum grain number under E_1 and E_2 as their position showed farthest to biplot origin in a certain environment followed by C518 and RIL 47. Moreover, PBW343 showed unstable in mean value under E_1 and E_2 environment as their position showed farthest to biplot origin on PC2. Likewise, in previous polygon view for grain yield showed vertex genotypes RIL 108, RIL 84, C518, PBW343, and RIL 80. RIL 108 showed maximum grain yield under E_1 and E_2 environment followed by RIL 32 and RIL 47 as their sector fell in E_2 environment and showed stable value with high performance as their position closed to biplot origin. However, parents C518 and PBW343 showed an inverse relationship as they fell in different sectors and showed the opposite in certain environments (Figure 3p).

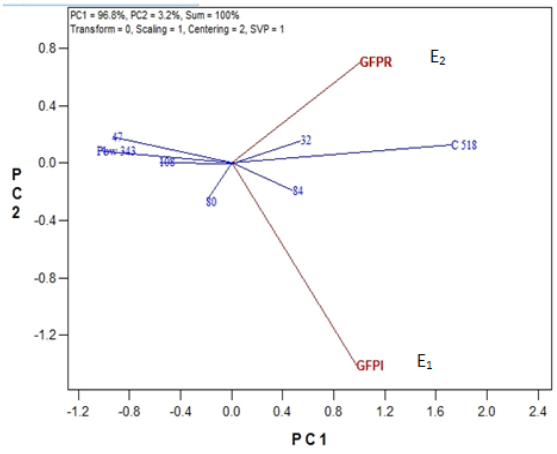
3.3 Mean performance and stability of genotypes

The selected RILs and their parents based on their mean values of different traits and stability performance were shown in Figure 3q-x. The passing line through the biplot is called the average environment coordinate (AEC), which is determined in all environments by the average PC1 and PC2 values. The AEC abscissa is the single arrowhead line. The AEC abscissa runs across the biplot root and the average environmental marker, pointing upwards the higher average values. The perpendicular lines to the AEC passing through the biplot origin are referred to as the AEC ordinate. These ordinates are represented by a pair of arrowed lines in Figure 3q–x. The longer a cultivar's projection is, the less stable it is. In addition, the estimates of their markers of the AEC abscissa are used to estimate the average significance of genotypes for various traits (Kaya et. al., 2006; Mwiinga et al., 2020). However, the stable genotypes have a shorter vector from the AEC, and yielded genotypes have the most distance on the right side of the biplot from the confluence point of AEC and double arrow lines. Accordingly, the chlorophyll content of PBW343 showed a maximum value with stability under E_1 and E_2 environments as their position showed farthest to biplot origin on AEC followed by RIL 47, RIL 84, C518, RIL 32, RIL 80, and RIL 108. However, C518 and RIL 32 showed more stability than RIL 80 and RIL 84 as their shorter length of projection (Figure 3q). Similarly, for canopy temperature at anthesis and grain filling (Figure 3t), C518 showed higher canopy temperature at anthesis under E_1 and E_2 environment followed by RIL 108 PBW343 and RIL 80. While RIL 80 and PBW343 showed more stability than other entries as their project line, close AEC ordinate and RIL 108 showed higher canopy temperature in E_1 (CT at grain filling under irrigated) and E_2 (CT at grain filling under rainfed) environments as their greater projection in E_1 and E_2. Moreover, RIL 84 showed the lowest canopy temperature while RIL 32 and RIL 47 exhibited the lowest stability under certain environments. C518 showed the maximum grain filling period followed by RIL 32 and RIL 84. While PBW343 showed the minimum grain filling periods and their mean values at par with RIL47 and RIL 108 (Figure 3r). Similarly, C518 showed higher mobilization of water-soluble carbohydrates under E_1 and E_2 environments followed by RIL 84 and RIL 80. RIL 84, RIL 80, RIL 32, and RIL 47 showed higher mobilization of water-soluble carbohydrates in the E_2 environment. Moreover, PBW343 showed relatively unstable under E_1 and E_2 environments (Figure 3u). For peduncle length, C518 showed maximum length with more stability under E_1 and E_2 environments followed by RIL 108, RIL 84and RIL 47 but relatively less stable in mean values. PBW showed minimum peduncle length with stability and their mean values at par with RIL 80 (Figure 3s).

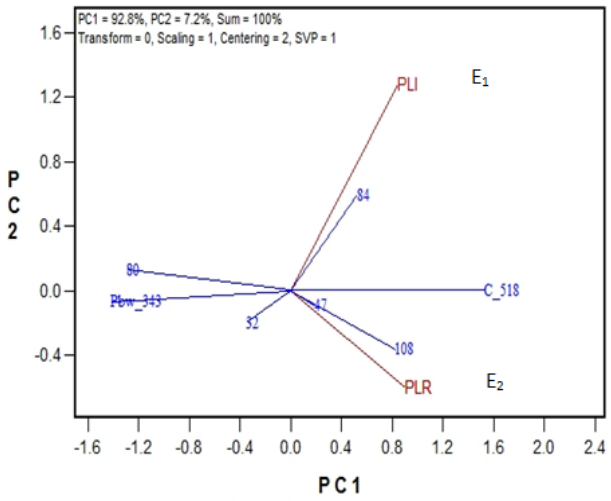
RIL 32 showed a maximum 1000 grain weight (grain weight in normal and defoliation) under E_1 followed by C518, RIL 108, and RIL 84. While C518 showed stability under both environments for grain weight (Figure 3v). Similarly, for grain number, RIL 80 showed the maximum grain number with stability under E_1 and E_2 environment followed by C518 and RIL 47. PBW343 showed higher instability in grain number under E_1 and E_2 environments followed by RIL 47 (Figure 3w). Mean values of grain yield showed maximum yield with more stability for RIL 108 and RIL 84 under E_1 and E_2 environments followed by RIL 80, RIL 32, and RIL 47. While C518 and PBW343 showed minimum yield relative to other tolerance RILs as their positive lied inverse to certain environments (Figure 3x).



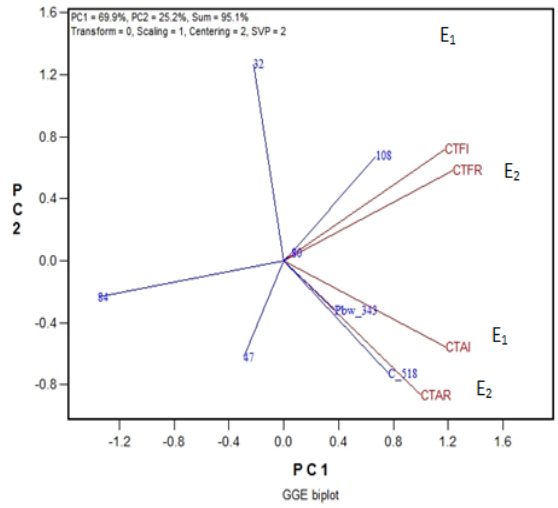
(a) Chlorophyll content index (CCI)



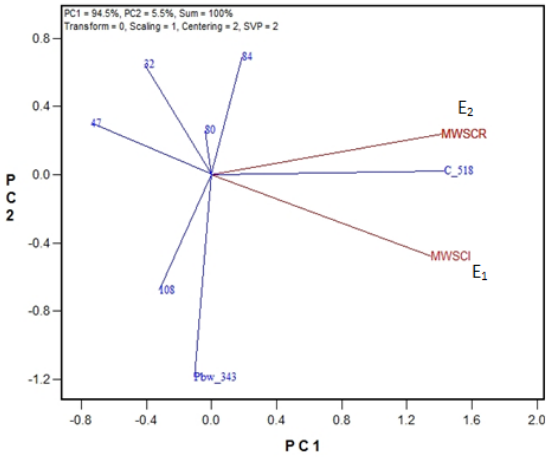
(b) Grain filling period (GFP)



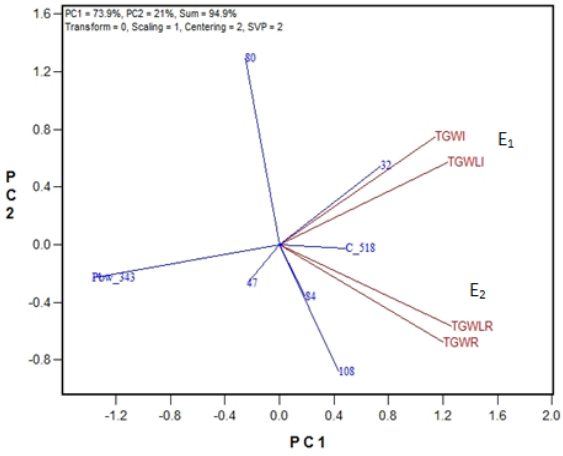
(c) Peduncle length (PL)



(d) Canopy temperature at anthesis and grain filling

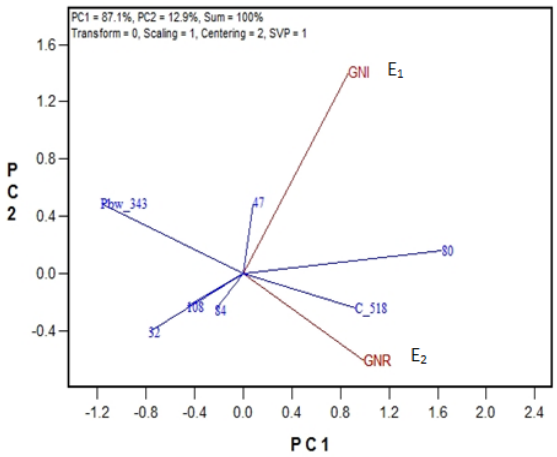


(e) Mobilized water soluble carbohydrates (MWSC)

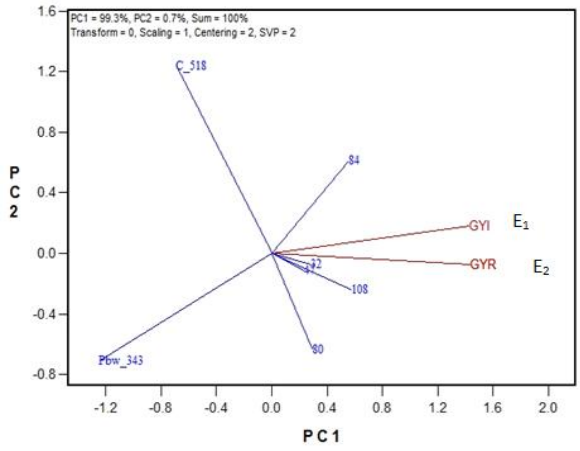


(f) 1000 grain weight under normal and defoliation (TGW & TGWL)

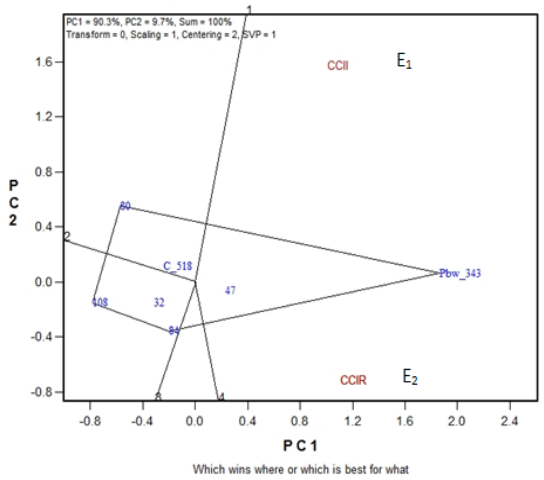
Contd…Figure 3



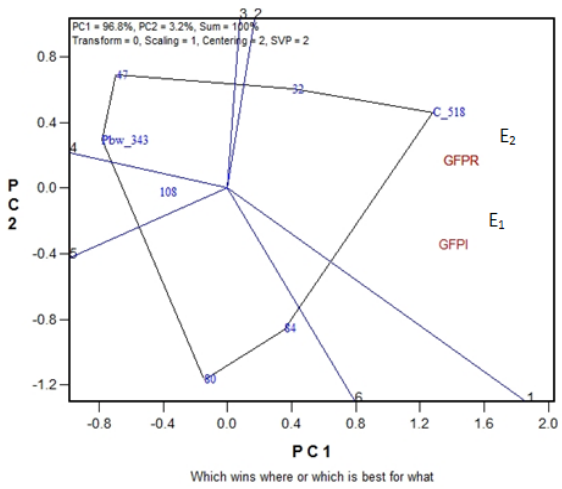
(g) Grain number (GN)



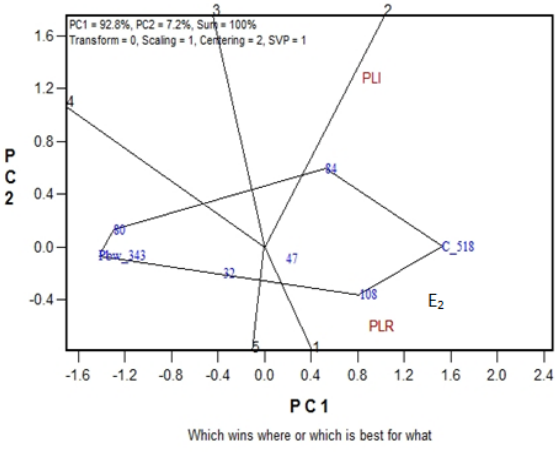
(h) Grain yield (GY)



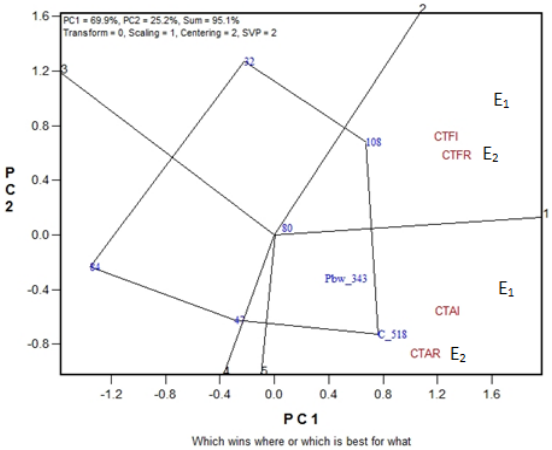
(i) Chlorophyll content index (CCI)



(j) Grain filling period (GFP)

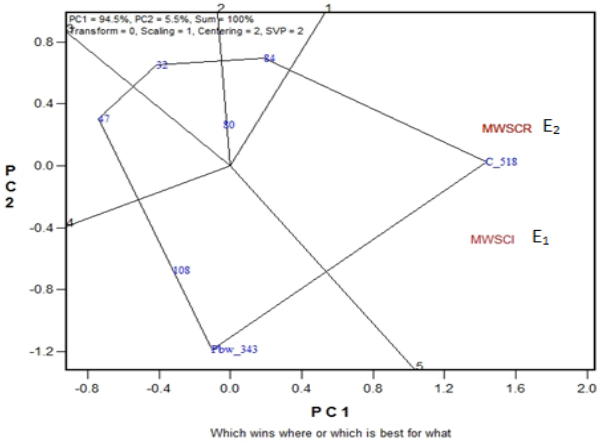


(k) Peduncle length (PL)

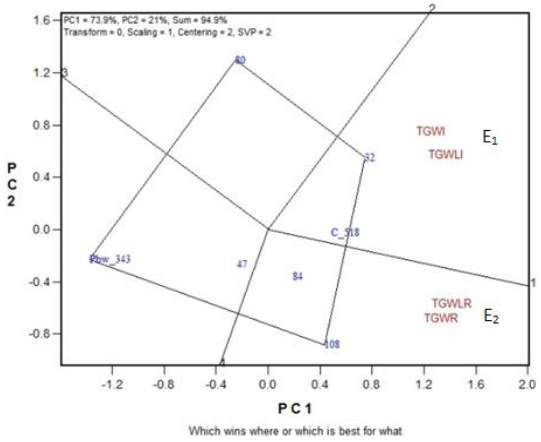


(l) Canopy temperature at anthesis and grain filling

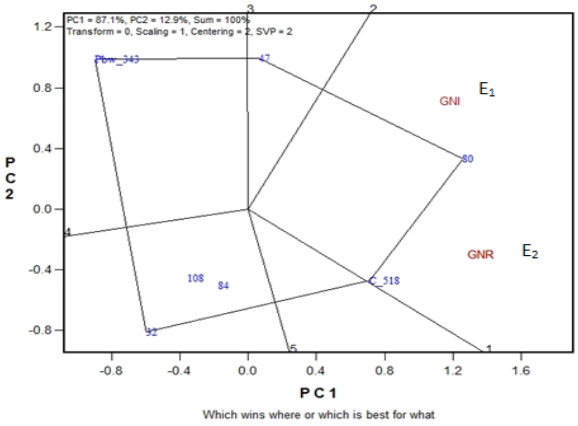
Contd…Figure 3



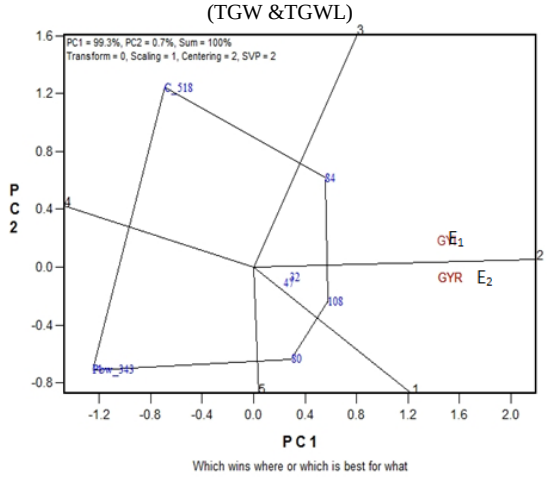
(m) Mobilized water soluble carbohydrates (MWSC)



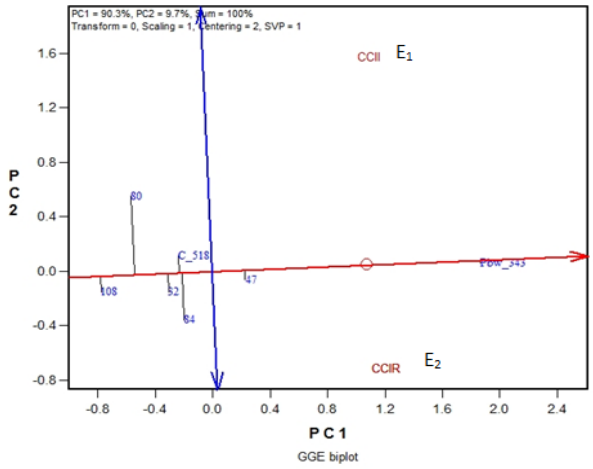
(n) 1000 grain weight under normal and defoliation (TGW &TGWL)



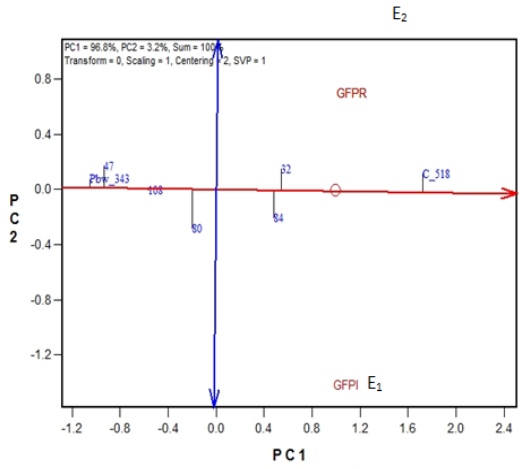
(o) Grain number (GN)



(p) Grain yield (GY)

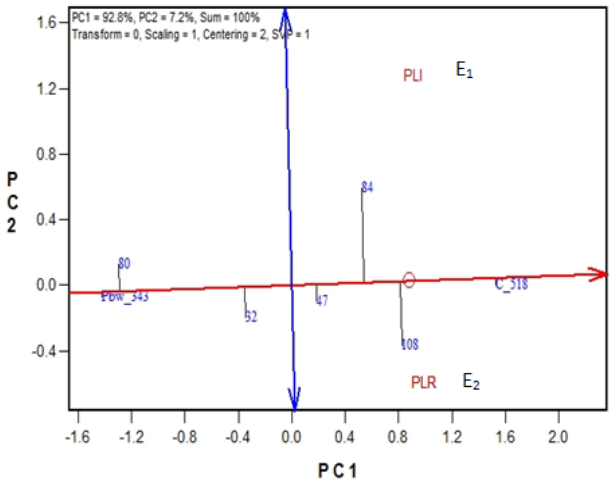


(q) Chlorophyll content index (CCI)

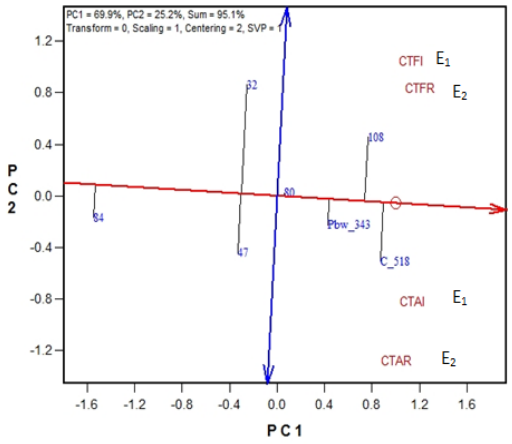


(r) Grain filling period (GFP)

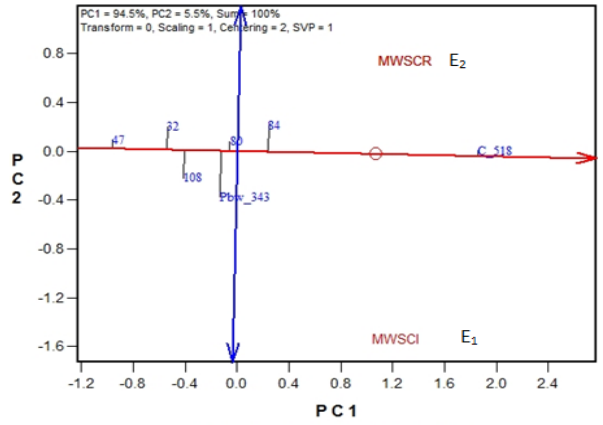
Contd…Figure 3



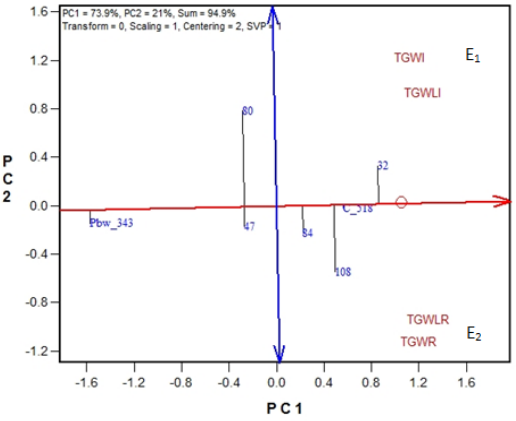
(s) Peduncle length (PL)



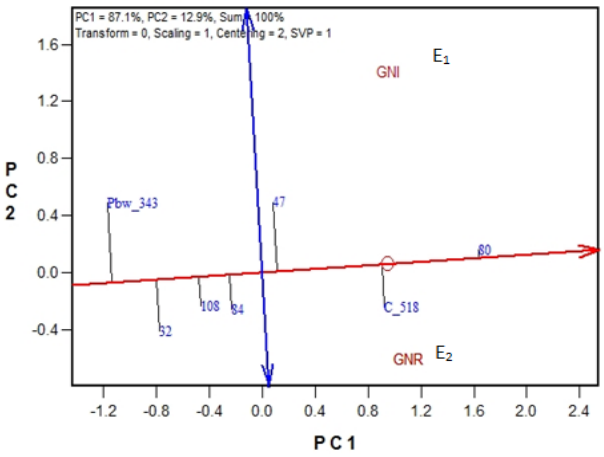
(t) Canopy temperature at anthesis and grain filling



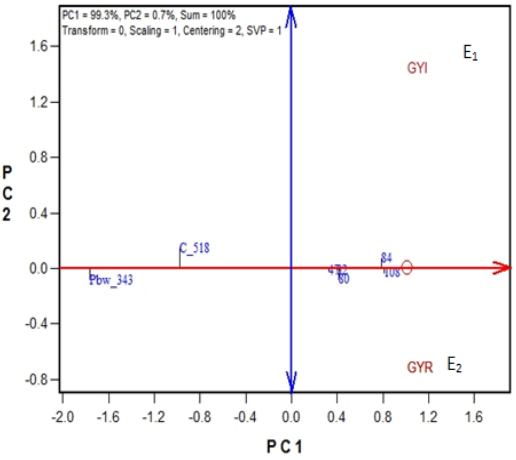
(u) Mobilized water soluble carbohydrates (MWSC)



(v) 1000 grain weight under normal and defoliation (TGW &TGWL)



(w) Grain number (GN)



(x) Grain yield (GY)

Figure 3 GGE biplots analysis for chlorophyll content index, grain filling period, peduncle length, canopy temperature at anthesis and grain filling, mobilized water soluble carbohydrates, 1000 grain weight under normal and defoliation, grains number and grain yield; a–h Relation among the genotypes and environments; i–p Which-won-where analysis of the genotypes; q-x Mean versus stability of the genotypes

The graphical representation of the GT biplot showed that the genotype performances concerning different Agro-physiological traits in two different environments. The relationship between traits and genotypes varied by the environment, indicating genotype-by-traits-by-environment interaction. Further association between genotypes and traits differed under rainfed as well as irrigated environments. So far, these interactions complex the understanding of genotype-by-traits analysis in a breeding program. GT biplot graphical representation helps in finding stable and ideal genotypes associated with important traits. To find the most stable high-yielding genotypes and their association with physiological traits, we applied GGE biplot analysis in a mapping population of wheat and results showed clear interrelationships among traits, genotypes, and environments other than commonly used methods such as path coefficient analysis and Pearson's correlation analysis. Similarly, the GT biplots were found to be a more accurate method for graphically depict of genotype-by-trait data and disclosing trait interrelationships (Peterson et. al., 2005; Egesi et. al., 2007; Fernández-Aparicio et. al., 2009).

Conclusion

The above studies stated that RIL 84 and RIL 108 were projected close to the average environment (ideal genotype) for all traits studied except chlorophyll content. Vector projected close to the average environment is considered as ideal genotypes as it has higher stability across the environments. Therefore, among the traits studied, grain filling period, peduncle length, canopy temperature, water-soluble carbohydrates, and 1000 grain weight contributed to grain yield under stress environment. These identified RILs may serve as a donor in further breeding programmes for the development of drought-tolerant cultivars.

Conflict of Interest

The authors state that they have no conflict of interest.

Author Contributions

The experiment conducted, Ashutosh Srivastava; Formal analysis, Ashutosh Srivastava; Investigation, Ashutosh Srivastava and Puja Srivastava; Resources, Mayank Gururani; Supervision & Validation, Puja Srivastava and RS Sarlach; Writing – original draft, Ashutosh Srivastava; Writing – review & editing, Mayank Gururani.

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SEROTYPES, TOXINS AND ANTIBIOTIC RESISTANCE OF *Escherichia coli* (E.COLI) STRAINS ISOLATED FROM DIARRHEIC RABBITS IN PHU VANG, THUA THIEN HUE

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KEYWORDS

Antibiotic susceptibility

Diarrhea

E. coli

Resistant

Rabbits

ABSTRACT

This study was conducted to determine the prevalence of *E. coli* in rabbits, their biochemical and serological characteristics, common virulence genes, and antibiotic resistance. The diarrhea rabbit feces were collected from households and rabbit farms in Phu Vang - Thua Thien Hue with a total of 250 samples for testing. The results showed that rabbits age from 31 to 45 days old had the highest incidence of diarrhea disease caused by *E.coli* (92.0%) and the lowest infection rate was observed in rabbits over 60 days old with an infection rate of 30%. Further, the isolated *E.coli* strains tested biochemical characteristics showed 100% motile, positive for indole and methyl red, fermenting glucose and lactose. Simultaneously these strains were detected belong to 7 serotypes O103, O157, O158, O169, O44, O125, O153 and susceptible to cefuroxime (95.45%), akamicin (86.37%), streptomycin (81.82%), amoxicillin (81.82%), tetracycline (68.18%), colistin (68.18%), ampicillin (63.63%), gentamycin (59.10%) and levofloxacin (50.0%), whilst resistant to doxycycline (100%), sulfamethoxazole-bactrim (95.46%), and neomycin (86.37%). By using PCR assay for detection of virulence genes of the isolated *E. coli* strains, there were 7 strains carried virulence genes, of which 4/7 *E. coli* strains carried *eaeA* and *tsh* genes (57.14%), 2/7 strains carried *stx2* gene (28.57%); 1/7 *E. coli* strains carried *stx1* gene (14.28%) and the F4, F5 and F6 genes were not found in all serotypes in this study.

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1 Introduction

Rabbit farming in recent years has increased for various purposes including medical and educational laboratory animals. Rabbit meat is efficiently converted from vegetable protein into high-quality animal protein. However, the large-scale production of rabbit meat is severely affected by the high mortality of rabbits, which hinders mass production (Okerman, 1994). Gastrointestinal syndrome in young rabbits often has a clinical manifestation of diarrhea and leads to secondary infections, which is the cause of reduced resistance and increased mortality in rabbits, and serious economic impacts for the rabbit industry (Yang et al., 2017). *E.coli* is a very common bacteria in the gastrointestinal tract and it does not directly disease in rabbits, but stress or exposure to other pathogens can lead to its growth in the gut which results in death (Milon., 1996). Enteropathogenic *E. coli* (EPEC) is the main causative agent of acute intestinal disease in rabbits, characterized by intestinal lesions (Licois, 2004). Highly virulent *E.coli* strains causing diarrhea in rabbits were identified as belonging to 12 different O serotypes (Pisoni et al., 2004). Intestinal infections in rabbits caused by serotypes of O are known as diarrheal *E. coli* strains, so their pathogenicity is related to several virulence properties (Xia, 2010). In addition, the majority of *E.coli* isolates were resistant to different antibiotics (Wang, 2021). Currently, there is a lack of information on the incidence of diarrheagenic *E. coli* in rabbits in Vietnam. Therefore, in this study, we collected fecal samples from rabbit farms to identify the prevalence of *E. coli* in rabbits, biochemically, serologically, common virulence genes, and analyze their drug resistance characteristics to provide information to determine reasonable use of antibiotics.

2 Materials and Methods

2.1 Collection of samples

A total of 250 fecal samples were collected from 1 to 60 and over 60 days old New Zealand white rabbits with diarrhea from households and farms in Phu Vang–Thua Thien Hue, Vietnam. Collected samples were directly transferred to the Laboratory of immunology and vaccine, Institute of Biotechnology, Hue University for *E.coli* isolation and identification without any delay.

2.2 Isolation and biochemical identification of *E. coli*

The nutrient broth was used to enrich the collected samples and incubated for 20-24 hours at 37°C. Then inoculated separately from each sample onto Mac Conkey agar and further incubated for 24 hours at 37ºC. Suspect colonies were selected for gram staining; morphological and biochemical characteristics of the isolated bacterial strains were determined according to Cruickshank et al. (1975).

2.3 Serological identification of isolated *E. coli* strains

The serotypes of isolated *E.coli* strains were determined by slide agglutination assay for detection of O antigen using *E.coli* antisera (Edwards & Ewing, 1972).

2.4 Antibiotic Sensitivity Test

The antibiotic susceptibility of isolated *E. coli* strains were determined by using the disk diffusion method as suggested by Bauer et al. (1966). Antibiotics used in this study including ampicillin (AM-10 µg), amoxicillin(AX-10 µg), colistin (Co-10 µg), gentamycin (GE- 10 µg), akamicin (AK-30 µg), streptomycin (SM-10 µg), tetracycline (TE-30 µg), doxycycline (DX-30 µg), sulfamethoxazole-bactrim (BT- 23.75 µg), cefuroxime (CU-30 µg), levofloxacin (LV-5 µg) and neomycine (NM-30 µg). The diameter of the zone of inhibition of *E. coli* bacteria by antibiotics on the diffusion disk was determined according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2015).

2.5 Detection of common virulence genes of isolated *E. coli*

Genomic DNA of *E.coli* bacteria was extracted using the QIAamp DNA Mini Kit following the manufacturer's instructions. PCR conditions and primer sequences are shown in Table 1. The volume of the reaction was carried out in 25µl including (DNA template, primers of each, dNTP, PCR buffer, and Taq DNA polymerase). PCR products were separated by electrophoresis in 1.0% agarose gel with TAE buffer at 80V.

2.6 Statistical analysis

The data were analyzed using the Minitab statistical package version 14.0. The results were compared by X^2 test (Chi-square test) and analysis of variance (ANOVA), p-value < 0.05 was considered statistically significant.

3 Results

3.1 Prevalence of *E. coli* in rabbit samples

The prevalence of *E. Coli* isolated from rabbits with diarrhea in Thua Thien Hue is presented in Table 2. The results showed that an overall infection rate of 55.2%. Rabbits with the age group of 31-45 days old had the highest incidence of diarrhea caused by *E.coli* (92.0%), followed by rabbits of age between 16-30 days old, 46-60 days old, and 1-15 days old with an infection rate of 74.0 %, 48.0 %, and 32.0%, respectively. The lowest infection rate was observed in rabbits over 60 days old with an infection rate of 30%.

3.2 Results of isolation and biochemical identification of *E. coli*

The morphological characters of *E. coli* showed that all isolates were Gram-negative, rod-shaped, non-sporulated bacteria and arranged

Table 1 Conditions of PCR for virulence gene amplification

Target Gene	Oligonucleotide sequences (5´- 3´)	Size Fragment (bp)	Annealing Temperature (ºC)	Reference
Fimbrial (F4)	GAA TCT GTC CGA GAA TAT CA GGT ACA GGT CTT AAT GG	505	53	Boerlin et al., 2005
Fimbrial (F5)	AAT ACT TGT TCA GGG AGA AA AAC TTT GTG GTT AAC TTC CT	230	50	
Fimbrial (F6)	GTAACTCCACCGTTT GTATC AAGTTACTGCCAGTCTATGC	409	53	
Attaching and effacing *(eaeA)*	ATG CTT AGT GCT GGT TTA GG GCC TTC ATC ATT TCG CTT TC	248	51	Bisi-Johnson et al., 2011
Temperature sensitive hemagglutinin *(tsh)*	AGT CCA GCG TGA TAG TGG AGT CCA GCG TGA TAG TGG	620	54	Delicato et al., 2003
Shiga-Like Toxin *(stx1)*	ACACTGGATGATCTCAGTGG CTGAATCCCCCTCCATTATG	641	58	Dipineto et al., 2006
Shiga-Like Toxin *(stx2)*	CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCAGCACTTTG	779	58	

Table 2 Prevalence of *E. coli* isolated from diarrhea rabbits

Days old	Number of examined samples	Number of positive samples	Percentage (%)
1-15	50	16	32.00
16-30	50	37	74.00
31-45	50	46	92.00
46-60	50	24	48.00
>60	50	15	30.00
Total	250	138	55.20

singly or in pairs (Table 3). Bacterial colonies appeared as smooth, shiny, donut-shaped, and are surrounded by dark pink area, strong lactose fermenting colonies on MacConkey's agar. The typical biochemical reactions of isolated *E.coli* strains are lactose and glucose-fermenting colonies and positive tests for indole, methyl red, and motility; tests were negative for citrate administration, urea hydrolysis, voges-proskauer, and no H_2S generation (Table 3).

3.3 Determination of serotypes of isolated strains of *E. coli*

The serotypes of isolated *E. coli* strains were determined by the slide agglutination test (Table 4). The results showed that from 45 isolated *E. coli* strains were classified into 7 serotypes including O103 (20.00%), O158 (20.00 %), O125 (15.55%), O153 (13.34%), O169 (13.34%), O44 (8.89%), O157 (6.66%) and Untypable (2.22%).

Table 3 Biochemical identification of isolated *E. coli*

Biochemical test	Reaction
Glucose	+
Lactose	+
Motility	+
Indole	+
Methyl red	+
Vogus-proskauer	-
Citrate utilization	-
Urea utilization	-
Hydrogen sulphide	-

Table 4 Serotypes of isolated *E.coli* strains from diarrhea rabbits

Serotypes	Number	Percentage (%)
O103	9	20.00
O158	9	20.00
O125	7	15.55
O153	6	13.34
O169	6	13.34
O44	4	8.89
O157	3	6.66
Untypable	1	2.22
Total	45	100

Table 5 Susceptibility of isolated *E. coli* to 12 commonly used antibiotics

Antibiotics	Number	Susceptible		Intermediate		Resistance	
		No	Percentage (%)	No	Percentage (%)	No	Percentage (%)
Ampicillin	22	14	63.63	6	27.28	2	9.09
Amoxicillin	22	18	81.82	3	13.63	1	4.55
Colistin	22	15	68.18	4	18.18	3	13.64
Gentamycin	22	13	59.10	0	0	9	40.9
Akamicin	22	19	86.37	2	9.09	1	4.54
Streptomycin	22	18	81.82	1	4.54	3	13.64
Tetracycline	22	15	68.18	2	9.09	5	22.73
Doxycycline	22	0	0	0	0	22	100
Sulfamethoxazole -bactrim	22	1	4.54	0	0	21	95.46
Cefuroxime	22	21	95.45	0	0	1	4.55
Levofloxacin	22	11	50.0	5	22.72	6	27.28
Neomycine	22	2	9.09	1	4.54	19	86.37

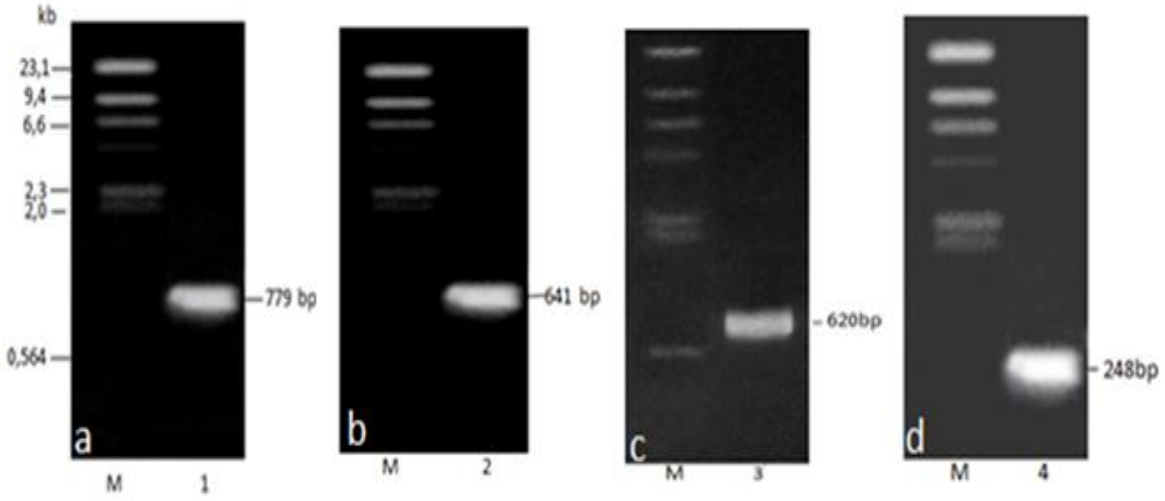

Figure 1 Detection of virulence genes of isolated *E. coli* strains by PCR on Agarose gel (a): *stx2* gene, lane 1 with 779 bp; (b): *Stx* 1 gene, lane 2 with 614 bp;(c): *tsh* gene, lane 3 with 620bp; (d): *eaeA* gene, lane 4 with 248 bp;M; λ DNA/HindIII Markers.

3.4 Determination of antibiotic susceptibility of isolated *E. coli* strains

The antibiotic susceptibility of isolated *E. coli* strains was conducted with 12 antibiotics that are often used at rabbit farms in Vietnam. The results of the study showed that isolated *E. coli* have a higher sensitivity to cefuroxime (95.45%), akamicin (86.37%), streptomycin (81.82%), amoxicillin (81.82%), tetracycline (68.18%), colistin (68.18%), ampicillin (63.63%), gentamycin (59.10%) and levofloxacin (50.0%) while isolated *E.coli* were resistance to doxycycline (100%), sulfamethoxazole-bactrim (95.46%), neomycine (86.37%) (Table 5).

3.5 Detection of common virulence genes of isolation *E.coli* by PCR

A total of 44 isolated *E.coli* strains, only 7 different serotypes for each strain were tested by PCR to detect the presence of virulence genes including *eaeA*, *tsh*, *stx1*, *stx2*, F4, F5, and F6. The results showed that the tested *E.coli* strains had the presence of one of the virulence genes (*eaeA*, *tsh*, *stx1* and *stx2*) accounting for 100% (7/7). Whereas, the virulence genes F4, F5, and F6 were not found in all serotypes in this report (Table 6, Table 7, and Figure 1).

Table 6 The common virulence genes of isolated *E. coli* strains

Virulence genes	*E.coli* isolates	Percentage (%)
eaeA	4/7	57.14
Tsh	4/7	57.14
stx1	1/7	14.28
stx2	2/7	28.57
F4, 5, 6	0/7	0.00

Table 7 Virulence genes in serotypes of isolated *E.coli* strains

Serotypes	Virulence genes				
	eaeA	*Tsh*	*stx1*	*stx2*	*F4,5,6*
O103	-	-	-	+	-
O157	-	-	-	-	-
O158	+	+	-	-	-
O169	-	+	-	+	-
O44	+	-	+	-	-
O125	+	+	-	-	-
O153	+	+	-	-	-

4 Discussion and conclusions

E.coli is one of the common bacteria in the gut microbiota of rabbits, when the body is exposed to adverse conditions, they will become pathogens causing infections inside and outside of the digestive tract (Okerman, 1994). Rabbit farms are severely affected economically by the high mortality rate in rabbits caused by Enteropathogenic *E. coli* (Stakenborg et al., 2006). Diarrhea caused by *E.coli* leads to a high mortality rate in a rabbit farm with a rate of 75% (Hong et al., 2017). In this study, *E. coli* were isolated from the diarrheic rabbits with an overall incidence rate of 55.2% (Table 1). This number is similar to the findings of Alton et al. (2013), Entssar et al. (2000), and Sawsan (2012), those who have isolated *E. coli* from diarrheic rabbits with percentages of 53.7%, 61%, and 64%, respectively. In contrast, lower rates of *E. coli* from diarrheic rabbits were recorded by Sharada et al. (2010), Hasan et al. (2011), Literak et al. (2013), and Radwan et al.(2014) with 44.61%, 36.20%, 35.74%, and 41.5%, correspondently. In the current study, the highest incidence of diarrhea caused by *E.coli* was reported at the age of 31 to 45 days old, accounting for 92% (Table 2). The reason may be that at this stage, rabbits are often weaned, so their ability to be affected by adverse factors such as housing, food, and care conditions are high which lead to rabbits at a high disease rate (Dung, 2011, Bekheet, 1983; Shahin et al., 2011).

The classification of serotypes isolated from 44 *E. coli* strains are O103, O158, O125, O153, O169, O44, and O157 (Table 4). These serotypes are similar to O125 and O158 which were reported by Aisha & Yousief (1999). Further, the serotypes O44 and O158 are associated with the diarrhea rabbits (Shahin et al., 2011) while serotype O125 is associated with the rabbit's enteritis (Alshimaa, 2007). Besides, the presence of different serotypes such as O111 and O114 were also reported by Scaletsky et al. (1984), serotypes O119, O55, and O128 by Morsy et al. (2002), and serotypes O109, O15, and O8 by Walaa & Lamyaa (2016). Some reports indicated that the common serotypes among the *E. coli* strain associated with rabbit diarrhea are O103, O49, O26, O128, O92, and O44 in which serotype O44 is present most frequently (Blanco et al., 1997; Marches et al., 2000; Morsy et al., 2002).

Regarding antibiotic sensitivity of isolated *E.coli* serotypes, out of *25 E.coli* O serotypes identified, only 22 different serotypes were used for antibiotic susceptibility testing (Table 5). Results of antibiotic sensitivity showed variable sensitive to cefuroxime (95.5%), akamicin (86.37%), streptomycin (81.82%), amoxicillin (81.82%), tetracycline (68.18%), colistin (68.18%), ampicillin (63.63%), gentamycin (59.10%), and levofloxacin (50.0%). High resistance was reported against the doxycycline (100%), sulfamethoxazole (95.46%), and neomycine (86.37%). Similarly, *E. coli* strains isolated from diarrhea rabbits were resistant to sulfamethoxazole and susceptible to gentamycin (Moharam et al. 1993; Abd-El Rahman et al. 2005; Rhouma et al., 2020). In this study, all isolated *E.coli* showed high resistance to doxycycline, sulfamethoxazole, and neomycine. This is consistent with the fact that these antibiotics were widely used for the prevention and treatment of diseases in rabbits. Besides, they were very susceptible to cefuroxime, akamicin, streptomycin, amoxicillin, tetracycline, and different antibiotics. This suggests that these antibiotics are less frequently used in rabbit farms.

Out of 44 serotypes, only 7 isolates of *E.coli* were identified with different serotypes to detect the presence of common virulence genes by PCR (Table 6, 7). The results showed that the presence of the *eaeA* gene in serotypes O157, O158, O125, and O153 with 57.14% (4/7). Further, 57.14% (4/7) were positive to *tsh* gene present in serotypes O158, O169, O125, and O153; while 28.57% (2/7) were positive to *stx2* gene present in serotypes O103, O169; and 14.28% (1/7) were positive to *stx1* gene present in serotype O44. Camarda et al. (2003) reported that the *eaeA* gene accounted for 28.57% of the isolated *E.coli* strains tested by PCR, while Alexis & James (2003) reported that, 25% of the *eaeA* gene was present in a total of 28 samples tested, Rhouma et al. (2020) suggested that out of isolated 40 *E.coli* strains, 17 strains carry the *eaeA* gene. Similarly, Pohl et al. (1993) and Blanco et al. (2006) showed that the *eaeA* gene accounts for a high proportion in serotypes isolated from diarrhea rabbits. Hassan & Al-Azeem (2009) reported that 31% of the isolated strains of *E. coli* carried the *eaeA* gene and possessed an *stx1* gene. Mohamed et al. (2019) suggested that the serotypes of *E. coli* O148 and O44 isolated from

diarrheal rabbits carried the *eaeA*, *stx1*gene. Regarding, the *tsh* gene, Ashraf et al. (2014) detected in serogroups of *E. coli* O55, O125, and O146 possessing *tsh*, *eaeA* genes while virulence gene *stx2* was detected in serotypes O55 and O125. In this report, the presence of the serotypes carries virulence genes F4, F5 and F6 were not found in all serotypes in this study.

Conclusion

In this study, *E.coli* strains from diarrhea rabbits were isolated from households and farms in Thua Thien Hue, Vietnam. Isolated strains of *E.coli* were identified as belonging to seven serotypes including O103, O158, O125, O153, O169, O44, O157, and Untypable. The classified serotypes carry different virulence genes that cause diarrhea in rabbits. In addition, isolated strains of *E.coli* showed high resistance to some antibiotics commonly used in rabbit farms in Vietnam.

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Conflict of Interest

The authors declare that they have no conflict of interest**.**

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EFFECTIVENESS OF SOLAR THERMOELECTRIC COOLER FOR FISH PRESERVATION: EXPERIMENTAL STUDY ON QUALITY CHARACTERISTICS OF *Pangasius bocourti* FISH FILLETS DURING STORAGE

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Sensory attributes

ABSTRACT

Preservation of fish products is a big issue where inconsistent electricity supply. In the current study, a solar thermoelectric cooler (STC) was fabricated by exploiting the solar energy and its cooling potential for fish preservation was evaluated. The STC consists of a photovoltaic (PV) panel, battery, PV charge controller, thermoelectric cooling system, and cooler box. The temperature of the STC decreased to 7.4ºC within 90 minutes and then reached 5±0.2ºC in 150 min. The cooling capacity and coefficient of performance of the STC were 23.8 W and 0.44, respectively, at an input electric current of 3.5 A. The *Pangasius bocourti* fish fillets were stored in the STC for 10 days and tested its quality at 2 days intervals. On day 10, thiobarbituric acid, peroxide, pH, water binding ability, total plate count values were 1.65mg MDA/kg, 5.04 mEqO_2/kg, 7.16, 26.18%, and 4.26 log CFU/g, respectively. A significant reduction in hardness, springiness, and chewiness values was observed, whereas no cohesiveness changes. The color values L^* and a^* decreased significantly, whereas b^* and ΔE increased. The sensory attributes were found in the range of 5.2-6.0 on the 10^{th} day. As the quality parameters showing an acceptable level, STC could be an alternate green option for fish preservation.

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1 Introduction

Fish is a rich resource of protein, omega-3 fatty acids, vitamins, and minerals and plays a significant role in human nutrition. Supplementation of fish, a minimum of two times a week in the human diet, is recommended and considered to be healthy (Khalili Tilami & Sampels, 2018). Among farmed fish species, basa *(Pangasius bocourti*) is one of the most consumed fish worldwide due to its excellent taste, color, deliciousness, great acceptability, affordable price, and commercial appearance in the form of fillet without skin or thorns (Guimarães et al., 2016). *Pangasius* primarily existed in fresh and brackish waters across the south and southeast Asian countries. Despite the above, such fishes are highly perishable due to microbial attack, lipid oxidation, and enzymatic activities leading to the deterioration of their quality (Nielsen & Jessen, 2007).

As fish spoilage is temperature-dependent, storing at a low temperature is the best option. Refrigeration can slow down the chemical and microbiological activities and associated deterioration during storage (Secci & Parisi, 2016). The studies have been reported that the fish stored at a chilled temperature ranging from 0 to 5ºC, has a storage life of 7-16 days, this depending on the species (Mohan et al., 2008; Viji et al., 2015; Remya et al., 2018). The shelf life of fish can also be increased up to 3 months during frozen storage at -20ºC (Nielsen & Jessen, 2007; Sriket & La-ongnual, 2018). However, preservation of perishable foods by cooling is generally a big issue where there is an inconsistent electricity supply. Moreover, in the tropical climate, maintaining the quality of perishable foods such as meat, fish, drink, and milk is a great challenge (Dai et al., 2003).

Solar energy, an abundant supply of natural energy resources, has a great potential to encounter the growing energy demand. The solar radiation is directly converted into electrical energy through photovoltaic (PV) cells. In the solar cooling system, solar energy is converted into electrical energy by the PV system and used for running refrigerators. Solar cooling emerges to be an attractive proposition as the cooling demand increased (Dai et al., 2003). The thermoelectric device is used as a cooler by converting electrical energy into a temperature difference. This system has many advantages, including compact size, portable, reliable, low production cost, less maintenance, no harm to the environment, and possible outdoor use combined with solar-PV cells (Chen et al., 2014).

The thermoelectric cooling system is working on the principles of the Peltier effect, briefly, when an electric current is made to flow in a closed circuit made of two dissimilar metals, thermal energy is evolved at one metal junction and discharged at the other junction depending on the direction of the current flow (Guo et al., 2020; Aboelmaaref et al., 2020). The thermoelectric cooling system has many advantages; in fact, it does not require liquid refrigerants, consumes less electric power, is easy to develop as portable and lightweight devices for outdoor use, is highly suitable in small and temporary places, no irreversible harm to the environment, noiseless, compact in size, lightweight, portable, high reliability, low-cost production, less maintenance, long lifetime, viable for outdoor use combined with solar photovoltaic cells, and attractive to use as mini-refrigerator for preserving foods and drugs in small places (Chen et al., 2014; Mirmanto et al., 2019; Aboelmaaref et al., 2020).

Various studies have been reported on the cooling performance of the solar thermoelectric refrigerator. Dai et al. (2003) reported that the solar-powered thermoelectric refrigerator could maintain the temperature in the range of 5-10°C and has a coefficient of performance (COP) of 0.3 under test conditions. Abdul-Wahab et al. (2009) demonstrated that the refrigeration temperature decreased from 27°C to 5°C within 45 min, and the COP of the device was about 0.16. Chen et al. (2014) demonstrated that the thermoelectric chiller driven by solar cells was cooled water from 18.5°C to 13°C. In contrast, COP of the device varied between 0.55 and 1.05, while solar insolation altered between 450 W/m^2 and 1000 W/m^2. Ohara et al. (2015) designed a portable thermoelectric refrigerator for vaccine storage. The system achieved a minimum temperature of 3.4ºC and diminished power consumption by 50% when it reached twice the temperature difference.

The retail fish sellers used ices to preserve fish, and the retailers in the rural and remote areas were not easy to obtain the ices. Moreover, these ices occasionally do not fulfill the food grades and become significant sources of contamination. The solar thermoelectric cooler could be helpful in those areas where inconsistent conventional electricity supply. Besides, research on the preservation of fish products in thermoelectric coolers is not found in the literature. It has mainly prompted the formulation of the present study. The objectives of this study were (i) to evaluate the cooling performance of the fabricated solar thermoelectric cooler (ii) to assess the chemical, texture, color, microbial load, and sensory attributes of basa fish fillets during storage.

2 Materials and Methods

2.1 Sample preparation

The study was conducted in the Department of Agricultural Engineering, Visva-Bharati University, Santiniketan, West Bengal, India in the hot climate conditions during March-May 2019. Freshly harvested live basa fish (*P. bocourti*) were procured from a local fish farm at Bolpur and immediately brought to the Department of Agricultural Engineering, Visva-Bharati University, Santinietan, West Bengal. The fish were stunned by applying a

blow on the skull with a hammer before decapitation. After stunning, the slaughtering and bleeding of the fish were carried out immediately by cutting the gill arches and main blood vessels from the heart with a sharp knife (Leary et al., 2016). Then manually filleted the fish, after being scaled, beheaded, and gutted. The fillets were packed in low-density polyethene pouches about 100 g per pouch, prepared 12 pockets, and sealed using a zip lock. The fillet pockets were stored in the solar thermoelectric cooler for 10 days. The samples were withdrawn from the cooler at a time interval of 2 days to evaluate changes in the quality in terms of texture, color, thiobarbituric acid, peroxide value, pH, water binding ability, microbial load, and sensory attributes.

2.2 Fabrication of the solar thermoelectric cooler

The solar thermoelectric cooler (STC) consists of a cooler box, solar-PV module, storage battery, energy control unit, thermoelectric module, heat sinks, and cooling fans. The cooler box is a roto-moulded plastic container made up of food-grade double tough high-density polyethene (HDPE), each 6 mm thick, and a polyurethane insulation foam layer of 50 mm thick filled in the double tough wall construction. The internal dimension of the cooler box is 0.36 m × 0.28 m × 0.25 m with a volume of 0.0252 m^3. A stretch holds down loops having a constant pressure mechanism used for closing the lid. Thermal conductivities of HDPE and polyurethane foam insulation materials are 0.44 and 0.028 W/mK, respectively. The insulation is used to inhibit the backflow of heat and prevent any loss in the cooler's performance affected by external heat. The estimated refrigeration of the STC was 41.52 W (Eq. 1). The cooler box was purchased from Aristoplast Products Pvt. Ltd, Mumbai, India.

A polycrystalline type solar-PV module (power rating: 100 W_p; efficiency: 13.2%; size: 1032 × 672 mm) used in this study. The PV module's power rating is based on the power requirement of the STC (see Eq. 8). The electrical power generated by the PV module is utilized to drive the STC. The PV module was supplied by Vinova Energy Systems Private Limited, Tamil Nadu, India. The specifications of the PV module are presented in Table 1. A tubular type lead-acid battery (storage capacity: 80 Ah) is used to store the PV power and exploit the store electricity when there is no sunshine. Based on the thermoelectric cooler, the battery capacity was chosen. Silvex Exports Private Limited, Mumbai, India, supplied the storage battery. A solar charge controller regulates the supply of electric power from the PV module to the storage battery.

The thermoelectric cooling system consists of a thermoelectric module, heat sinks, and fans. The thermoelectric module (TEM) is a solid-state heat pump that builds hot and cold sides when electric current flows across the module. The specifications of the TEM are presented in Table 1. The TEM converts the direct solar current (DC) to alternate current (AC) power. Two heat sinks, one (size: 10×10×3 cm) at the hot side and the other (size: 4×4×2.6 cm) at the cold side of the TEM, were fixed. Besides, two low power consumption fans (3 W), one at the backside of the hot side heat sink and the other at the cold side heat sink, were fixed as shown in figure 1. This complete thermoelectric assembly was fixed onto the cooler box wall, as shown in figure 2. The heat sink at the hot side amplifies the heat transfer rate from the hot side of the TEM; thus, it will dispel the heat outside of the cooler box; it also protects TEM from overheating and maintains an ambient environment. The heat sink at the cold side of the TEM is used to cool the cooler cabinet. The cooling fans are used to reject extra heat from the hot side of the TEM to the ambient environment, better ventilation, and maintain the TEM's effectiveness. The schematic diagram of an experimental solar thermoelectric cooler is shown in figure 2.

Table 1 Specifications of the solar PV module and thermoelectric module

Solar PV module		Thermoelectric module	
PV module type	VE12100	TEM type	TEC1-12705S
Maximum rated power (W_p)	100 W_p	I_{max}	5.3 A
Maximum power voltage (V_{mp})	18.58 V	U_{max}	15.4 V
Maximum power current (I_{mp})	5.98 A	$Q_{max.}$@ΔT=0	57 W
Open circuit voltage (V_{oc})	22.58 V	ΔT_{max}	67 ºC
Short circuit current (I_{sc})	5.96 A	T_h	40 ºC
Module efficiency (η_m)	13.2 %	N-P junction	127 couples
Output tolerance	5 %	Maximum temperature	138ºC
Maximum series fuse rating	10 A	Dimension	40×40×3.8 mm
Operating temperature	- 40 to 85 ºC	Thermal conductance (K)	0.411 W/m^2K
Module dimension	1032×672 mm	Seebeck coefficient (α)	0.1882 V/K
Weight	8 kg	Electrical resistance (R)	1.989 Ω
		Weight	29 g

PV module at standard test condition: solar irradiance 1.0 kW/m^2; module temperature 25°C; wind speed 1.0 m/s.

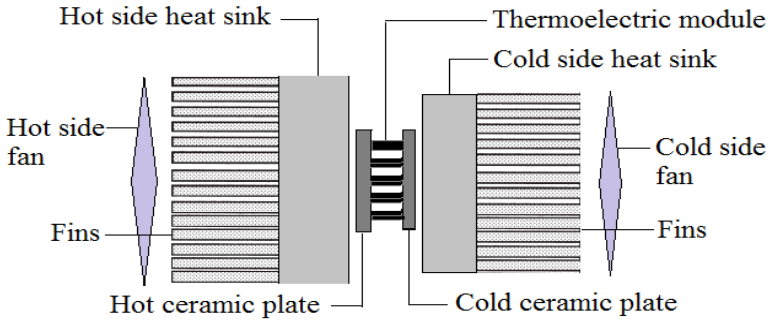

Figure 1 Schematic arrangement of components of thermoelectric cooling system

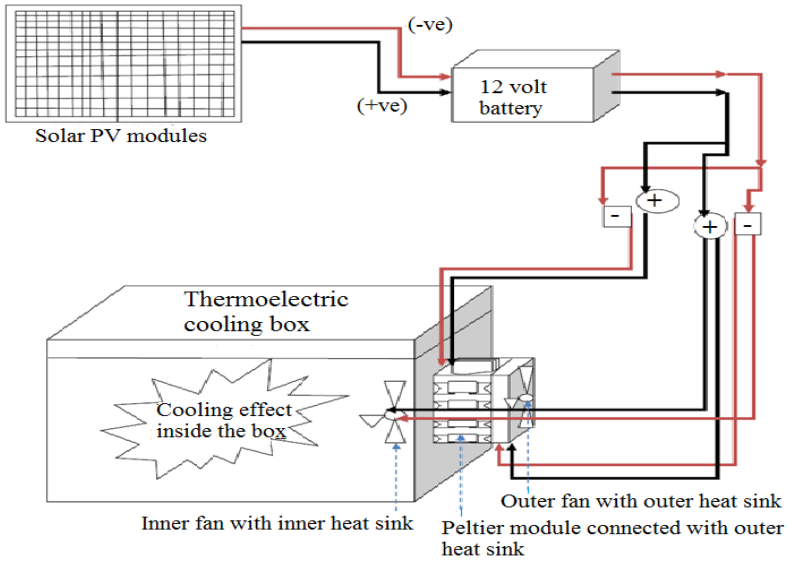

Figure 2 Schematic diagram of the experimental solar thermoelectric cooler

2.3 Testing of STC

The solar-PV panel is fixed with the required angle (40º) to receive maximum solar radiation incidence on the panel. It was connected with the battery terminals through a solar charge controller using respective cables. The positive side of the TEM turns the cold side due to absorption of heat, which transferred to the other side, which became the hot side. The cold side of the TEM was fixed inside of the cooler box, and the hot side fitted outside (Figure 2). The temperature of the cold and hot sides of the TEM was measured using T-type thermocouples, which connected to the cold and hot sides, other ends with the digital temperature indicator. The voltage and electric current was measured using a digital energy meter. The PV power was utilized to drive the STC in the daytime (from 8:00 to 17:00); after, battery power was exploited from 17:00 to 23:30.

2.4 Estimation of heat gain of cooled space

The TEM can pump the total heat gain of the cooled space (refrigeration load) of the STC. The total heat energy (Q_T) is the sum of the heat flow entering the cooler box, conduction heat loss inside the TEM, product heat, and heat given off by the fan.

$$Q_T = Q_w + Q_m + Q_p + Q_f \quad (1)$$

The heat loss through the wall of the cooler box can be estimated using the following formula (Gökçek & Şahin, 2017):

$$Q_w = A_c\, U\, (T_0 - T_i) \quad (2)$$

$$U = \frac{1}{\frac{1}{h_{in}} + \frac{X_p}{K_p} + \frac{X_i}{K_i} + \frac{1}{h_{out}}}$$

The conduction heat loss inside the TEM can be estimated as (Abdul-Wahab et al., 2009; Bjørk et al., 2014):

$$Q_m = \frac{K_m A_m (T_h - T_c)}{X_m} \quad (3)$$

The product heat refers to the heat given off by the stored product. In this study, 2.0 kg fish fillets were kept in the STC as product material at 5±0.2ºC. The specific heat capacity of the fish fillets is about 3.85 kJ/kg K. The product heat in the cooler can be determined as the method described by Gökçek & Şahin (2017):

$$Q_p = \frac{M_p C_p (T_{pi} - T_{pf})}{3.6\,\Delta t} \quad (4)$$

The heat given off by the fan on the cold side of the TEM was calculated using its rated electrical power consumption (Abdul-Wahab et al., 2009; Gökçek & Şahin, 2017):

$$Q_f = N_f F_r \quad (5)$$

2.5 Evaluation of cooling performance

The cooling capacity of the TEM or amount of heat absorbed at the cold side of the TEM can be estimated as follows (Dai et al., 2003; Chen et al., 2014):

$$Q_c = \alpha I T_C - 0.5 I^2 R - K\Delta T \quad (6)$$

The electrical energy consumption of the TEM (P_t) can be estimated as follows (Dai et al., 2003; Chen et al., 2014):

$$P_t = I^2 R + \alpha I \Delta T \quad (7)$$

The total power consumption of the STC and its coefficient-of-performance (COP) can be estimated as follows (Gökçek & Şahin, 2017):

$$P_{stc} = P_t + P_f \quad (8)$$

$$COP = \frac{Q_c}{P_{stc}} \quad (9)$$

The heat energy in the cooled space of the STC (Q_T) is absorbed in the cold side of the TEM (Q_c) during the cooling process. Therefore, we can use either Q_T or Q_c to calculate the COP of a thermoelectric cooler (Gökçek & Şahin, 2017; Aboelmaaref et al., 2020).

2.6 Chemical analysis

Fish fillets were minced using a mixer grinder for chemical analysis. Thiobarbituric acid (TBA) is a measure of oxidative stability of the fish muscle. A spectrophotometer (Model: LT2203, Labtrinics, India) was used to estimate the TBA at 538 nm, expressed as mg of malonaldehyde per kg sample (Tarladgis et al., 1960). TBA value is a measure of oxidative stability of the fish muscle. The standard iodometric method (AOCS, 1992) was used to determine the peroxide content, expressed as milliequivalents oxygen per kg sample. The fish sample's pH value was estimated using a digital pH meter after homogenization in distilled water (1:5 w/v). Gravimetrically, the water-binding ability of the fish sample was calculated by taking the weight difference of the initial and centrifuged sample, expressed in percent (Hassan et al., 2016).

2.7 Texture profile analysis

A texture analyzer (TA-HDi, Stable Micro Systems, U.K.) fitted with a cylindrical platen probe of 50 mm diameter was used to measure texture profile (Sun et al., 2018). The fish fillet samples size of 3.0×2.0×1.5 cm were placed on the heavy-duty platform fixture and compressed perpendicular to the muscle fibre posture using the probe at a test speed of 2 mm/s through the two-cycle sequence, trigger force of 0.15 N, and using 50 kg load cell. The texture profile parameters are computed as described by the manufacturer. Hardness was measured as resistance at a maximum compressive force of the first bite to deform the sample, expressed in Newton. Cohesiveness is the ratio of the second compression's positive force to the first compression, dimensionless. Springiness is measured as the distance recovered by the sample between the first and second compressions expressed in mm. The chewiness was calculated by the multiplication of hardness × cohesiveness × springiness, expressed in Newton. Each experiment was replicated three times.

2.8 Color analysis

The color analysis was done using a Minolta colorimeter (Minolta Co. Ltd., Japan) under the hunter lab system. The color measurements of L^*(lightness), a^*(redness), and b^*(yellowness) were recorded at three different locations on the fish fillets surface and determined the mean value. The reference color values (L_b^*, a_b^*, b_b^*) before storage and color values of stored samples (L_s^*, a_s^*, b_s^*) were used in the determination of change in color (ΔE), as defined by Kandasamy & Mukherjee (2019):

$$\Delta E = \sqrt{(L_b^* - L_s^*)^2 + (a_b^* - a_s^*)^2 + (b_b^* - b_s^*)^2} \quad (10)$$

2.9 Microbial analysis

Total plate count (TPC) was estimated using the plate count agar method (APHA, 1992). Ten grams of minced fish fillet sample was homogenized with 100 ml standard saline solution to obtain a 1/10 dilution. One mL from this dilution was transferred into a test tube containing a 9 mL saline solution to get the subsequent dilution. One mL was transferred aseptically to sterile Petri-plates from each dilution, added 15 mL melted agar medium (45ºC), mixed, and solidified. The inoculated plates were incubated for five days at 7ºC for total psychrotrophic counts. The colony counter was used to count the colonies, an average number of colonies was multiplied by the dilution factor to obtain complete counts as a colony-forming unit (CFU) per gram of sample. This count converted to the total plate count of log CFU/g of the sample. Triplicate measurements were done in each experiment.

2.10 Sensory evolution

Sensory evaluation of fish fillets was carried out with slight modifications in the method given by Kulawik et al. (2016). The fish fillets were cooked with standard local culinary practice. At the evaluation time, the samples were warmed using a microwave oven for 1 min, cut across the center to make equal size, and arranged treatment wise and then served warm to panel lists for sensory evaluation. The panel lists consist of 10 semi-trained male and female staff members and research scholars of the Institute. The panel members should have neither vegetarians nor allergic to fish and seafood. The members were requested not to consume meals, drinks, mints, etc., for at least one h before the session. Portable water was provided to rinse their mouth between the samples. The panel lists asked to evaluate the samples for flavor, juiciness, texture, taste, and overall acceptability according to their perception. An 8-point descriptive scale rating (where 8 = like extremely and 1 = dislike extremely) was used to evaluate the sensory attributes. On each sensory parameter, determined the average value and standard deviation.

2.11 Statistical analysis

Average values of each experiment with standard deviations reported. One-way analysis of variance used to analyze the data adopted a completely randomized design. To analyze the experimental data, we used SPSS software (SPSS Inc., Chicago, USA).

3 Results

3.1 Performance of solar thermoelectric cooler

The temperature variation of the TEM's cold and hot side with the loaded item is shown in Figure 3(a). The cold side temperature (T_c) of TEM decreased to 7.4ºC from 30ºC within 90 min, and then gradually reduced, reached to 5±0.2ºC in 150 min. The hot side temperature (T_h) increased to 40.2°C. The temperature difference (ΔT) depends on the hot and cold side's temperature, raised over the experimental time. Besides, the temperature of fish fillets dropped to 6.5°C from an initial temperature of 21.5°C. It also absorbed that the temperatures continued till the end of the experiment. On the other hand, no significant change in outdoor temperature was observed.

The effect of electric current input on cooling capacity and COP of the STC are shown in Figure 3(b). The results showed that increasing electric current input results in decreasing the COP of the STC. The raising electric power consumption of the TEM while increasing the electric current input, and therefore reducing the COP of the STC (see Eq. 10). On the other hand, the cooling capacity increased while increasing the electric current input. The increased flow of electrons within the TEM decreases the cold side temperature, thus increasing the cooling capacity of the TEM. By increasing electric current intake from 0.9 A to 3.5 A, cooling capacity increased from 12.9 W to 23.8 W, but the COP decreased from 0.98 to 0.44. The maximum cooling capacity of 23.8 W and the COP of 0.44 was observed at the electric current input of 3.5 A.

The STC has driven continuously for a period of about 5-6 h after the sunset. The sunset times varied from 18:09 to 18:38 during the study period. The store electricity in the battery is exploited to drive the cooler during this time. A temperature change in the STC was noticed in this study while using the battery power and observed that the temperature at the cold side maintained about 5ºC and gradually increased. The current flow rate also decreased gradually.

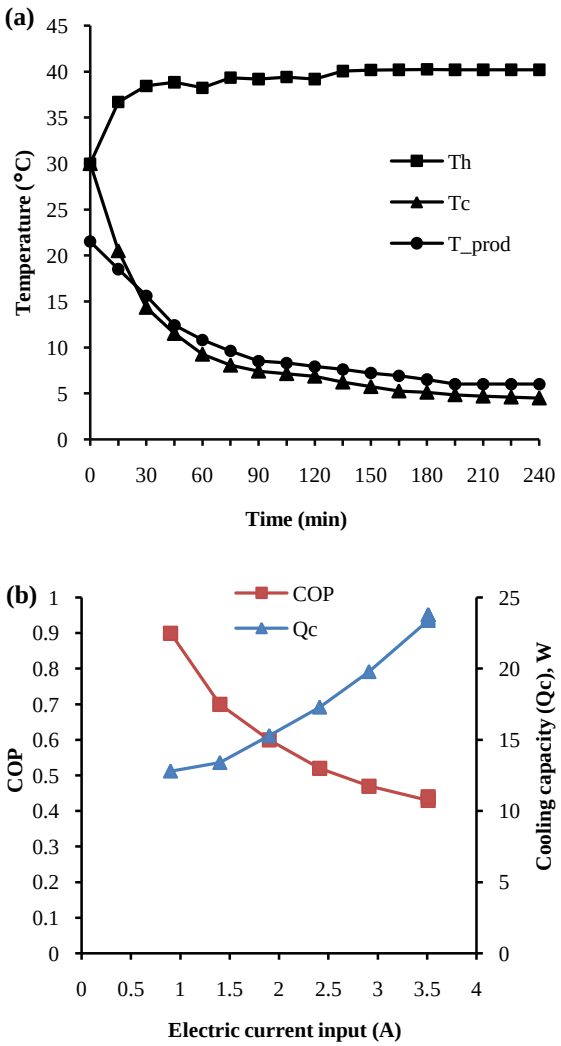

Figure 3 Performance of the solar thermoelectric cooler
(a) temperature variations of product, hot and cold sides of the thermoelectric module (b) variations of COP and Q_c against electric current input

3.2 Thiobarbituric acid value

The changes in thiobarbituric acid (TBA) value of basa fish fillets during storage in the STC at 5±0.2ºC are presented in Table 2. It is clear from the table that the TBA value increased gradually and reached 1.65 mg MDA/kg sample on the 10th day from an initial value of 0.21 mg MDA/kg sample. The increase in TBA value during storage may be due to the denaturation of the fish muscle cells that liberates the kind of catalysts that accelerate the lipid oxidation (Sriket & La-ongnual, 2018). The limit of TBA value's acceptability is in the range of 1.0-2.0 mg MDA/kg sample; an undesirable odor and taste will develop beyond this limit (Mohan et al., 2008; Viji et al., 2015). In the present study, the TBA value showed about 1.65 mg MDA/kg sample on the 10th day, which is within the general range of 1.0-2.0 mg MDA/kg sample.

3.3 Peroxide value

The changes in peroxide value of basa fish fillets during storage in STC at 5±0.2ºC are reported in Table 2. The peroxide value of fish fillets significantly increased from an initial value of 1.26 to 5.04 $mEqO_2$/kg sample during 10 days storage period. In the current study, the peroxide value of fish fillets showed less than 5.5 $mEqO_2$/kg fat in STC during the storage period of 10 days, and no off-flavor or odor was registered.

3.4 pH value

The pH value of the basa fish fillets increased significantly during the storage period of 10 days in STC at 5±0.2ºC (Table 2). The initial pH value was found to be 6.56, whereas, after 10 days, the value was 7.16.

Table 2 Quality parameters of *P. bocourti* fish fillets during storage in the solar thermoelectric cooler at 5±0.2°C for 10 days

Quality categories	Parameters	Days of storage					
		0	2	4	6	8	10
Biochemical quality	TBA	0.21 ± 0.06^{a}	0.47 ± 0.05^{a}	0.73 ± 0.03^{a}	0.95 ± 0.08^{a}	1.19 ± 0.08^{a}	1.65 ± 0.09^{a}
	Peroxide	1.27 ± 0.08^{b}	2.54 ± 0.13^{b}	3.48 ± 0.10^{b}	3.94 ± 0.13^{b}	4.58 ± 0.12^{b}	5.04 ± 0.21^{b}
	pH	6.56 ± 0.18^{c}	6.67 ± 0.15^{c}	6.79 ± 0.18^{c}	6.92 ± 0.25^{c}	7.08 ± 0.24^{c}	7.16 ± 0.24^{c}
	WBA	15.02 ± 0.11^{d}	18.16 ± 0.18^{d}	20.53 ± 0.30^{d}	22.75 ± 0.16^{d}	24.38 ± 0.13^{d}	26.18 ± 0.21^{d}
Texture profile	Hardness (N)	28.71 ± 0.23^{a}	26.72 ± 0.51^{a}	25.53 ± 0.39^{a}	24.29 ± 0.51^{a}	22.59 ± 0.53^{a}	20.36 ± 0.55^{a}
	Cohesiveness	0.27 ± 0.03^{a}	0.28 ± 0.09^{b}	0.27 ± 0.04^{c}	0.27 ± 0.05^{d}	0.26 ± 0.06^{e}	0.27 ± 0.08^{f}
	Springiness (mm)	0.94 ± 0.09^{c}	0.87 ± 0.09^{c}	0.84 ± 0.08^{c}	0.82 ± 0.06^{c}	0.81 ± 0.06^{c}	0.79 ± 0.06^{c}
	Chewiness (N)	7.31 ± 0.87^{d}	6.34 ± 1.40^{d}	5.99 ± 1.64^{d}	5.45 ± 1.55^{d}	4.76 ± 0.72^{d}	4.45 ± 1.72^{d}
Color values	L^{*}	63.74 ± 0.47^{a}	61.93 ± 0.12^{a}	60.73 ± 0.66^{a}	59.44 ± 0.79^{a}	58.65 ± 0.30^{a}	57.83 ± 0.56^{a}
	a^{*}	4.86 ± 0.23^{b}	4.16 ± 0.20^{b}	3.63 ± 0.29^{b}	3.50 ± 0.35^{b}	3.19 ± 0.25^{b}	3.07 ± 0.19^{b}
	b^{*}	15.27 ± 0.20^{c}	16.17 ± 0.11^{c}	17.07 ± 0.24^{c}	17.83 ± 0.18^{c}	18.25 ± 0.21^{c}	18.76 ± 0.20^{c}
	ΔE	0.0 + 0.0	2.14 ± 0.10^{d}	3.69 ± 0.09^{d}	5.21 ± 0.05^{d}	6.13 ± 0.11^{d}	7.06 ± 0.14^{d}
Microbial load	TPC	2.13 ± 0.07^{e}	2.61 ± 0.16^{e}	3.12 ± 0.25^{e}	3.38 ± 0.11^{e}	3.85 ± 0.23^{e}	4.26 ± 0.14^{e}
Sensory attributes	Flavour	6.96 ± 0.11^{a}	6.66 ± 0.03^{a}	6.54 ± 0.11^{a}	6.38 ± 0.18^{a}	6.15 ± 0.08^{a}	5.94 ± 0.14^{a}
	Juiciness	6.53 ± 0.13^{b}	6.46 ± 0.07^{b}	6.18 ± 0.16^{b}	6.05 ± 0.14^{b}	5.74 ± 0.12^{b}	5.20 ± 0.08^{b}
	Texture	6.63 ± 0.07^{c}	6.51 ± 0.17^{c}	6.37 ± 0.13^{c}	6.13 ± 0.15^{c}	5.63 ± 0.15^{c}	5.32 ± 0.20^{c}
	Taste	6.86 ± 0.04^{d}	6.71 ± 0.09^{d}	6.53 ± 0.22^{d}	6.37 ± 0.12^{d}	6.21 ± 0.13^{d}	5.98 ± 0.16^{d}
	Overall acceptability	6.61 ± 0.09^{e}	6.53 ± 0.13^{e}	6.22 ± 0.12^{e}	6.08 ± 0.18^{e}	5.84 ± 0.21^{e}	5.61 ± 0.13^{e}

Data are presented as mean ± standard deviation (n = 3). Mean value with same superscript in each row showed significant variation at 1%. TBA = Thiobarbituric acid (mg MDA/kg), Peroxide ($mEqO_2$/kg), WBA = Water binding ability (%), L^{*} = Lightness, a^{*} = Redness, b^{*} = Yellowness, ΔE = Change in colour, and TPC = Total plate count (log CFU/g).

3.5 Water binding ability

In the present investigation, the water binding ability (WBA) increased significantly during the storage period. At the beginning of the experiment, the WBA of the fish fillets was 15.02%, whereas it increased up to 26.18% on the 10th day (Table 2).

3.6 Texture profile analysis

Changes in the texture profile of *pangasius* fish fillets during storage under STC at 5±0.2°C are presented in Table 2. A significant reduction in hardness value was observed throughout the storage period, which gradually reduced from an initial value of 28.71 N to 20.36 N. The reduction in hardness may be due to the weakening of the muscle fibrils aggregation over the storage time. The cohesiveness value of 0.27 was recorded, which indicates the fish sample had a sufficient elasticity. It is an indication of the goodness of the internal bonding of muscle fibrils during storage. A decreasing trend in springiness was observed over the storage period. The springiness value decreased to 0.79 N from an initial value of 0.94 N. The decreasing trend in springiness may be due to diminishing muscle fibrils aggregation's strength over the storage time. In chewiness value, a decreasing trend was noticed during the storage period, which decreased to 4.45 N from an average initial value of 7.31 N. The chewiness value could decrease while decreasing the hardness value directly proportional to hardness (chewiness = hardness × cohesiveness × springiness).

3.7 Color change

Changes in color characteristics of basa fish fillets during storage in STC at 5±0.2°C are presented in table 2. The results showed that the L^*(lightness) and a^*(redness) values decreased significantly, whereas b^*(yellowness) and color change (ΔE) values increased over the storage period. The L^* value dropped from an initial value of 63.74 to 57.83. The a^* value also decreased from an initial value of 4.86 to 3.07. The b* value was observed to be 15.27 on the initial day however increased to 18.76 on the 10th day. On day 10, the ΔE value was 7.06.

3.8 Microbial load

In the present study, the psychrotrophic bacteria counts in p*angasius* fish fillets were determined by plate count agar (PCA) test, and results are reported in Table 2. The work shows that the total psychrotrophic count increased significantly over the storage period. The total psychrotrophic count in the *pangasius* fillets was 2.13 log CFU/g at the beginning of a trial, which indicates the initial quality of fish fillets used in this study was good. On the other hand, it increased to 4.26 log CFU/g on the 10th day. The total psychrotrophic count in the study was in good agreement with the general limit of 7 log CFU/g for raw fish products (Mohan et al., 2008).

3.9 Sensory characteristics

The sensory scores of cooked *Pangasius* fillets during storage is presented in Table 2. It is clear from the table that the sensory values decreased significantly over the storage period. The sensory scores for flavor, juiciness, texture, taste, and overall acceptability were recorded between 6.0 and 7.0 on the 6th day. On the other hand, their values were in the range of 5.2-6.0 on the 10th day. However, the sensory scores on juiciness were slightly less than other scores on other parameters during storage. The present study samples were acceptable for up to 10 days since the sensory score of 4.0 is an account of a lower limit of the overall acceptability.

4 Discussion

The degree of lipid oxidation can be assessed by the TBA index, which measures malonaldehyde (MDA) content (Mohan et al., 2008). The MDA is a principal component formed during the oxidation of polyunsaturated fatty acids. The higher TBA value indicates fat degradation compounds such as free fatty acids and peroxide. Lipid oxidation, corresponding to the oxidative deterioration of polyunsaturated fatty acids in fish muscle, leads to off-flavor production, thereby shortening the shelf life of fish (Secci & Parisi, 2016). The results were confirmed with the findings of Viji et al. (2015), who demonstrated the TBA value of 1.0 mg MDA/kg sample for gutted sutchi catfish on the 20th day at 1-2°C. Hassan et al. (2016) reported that the TBA value of *P. hypophthalmus* mince was 1.33 mg MDA/kg sample on the 6th day at 3-5°C. On the other hand, Sriket & La-ongnual (2018) showed the TBA value of 2.0 and 4.0 mg MDA/kg sample on 2nd and 4th week, respectively for basa fish fillets at -20°C. This fluctuation in TBA may be due to temperature; the rate of lipid oxidation is directly associated with the weather (Mohan et al., 2008).

Peroxide is a measure of the concentration of hydroperoxides, products of lipid oxidation, which leads to degradation of fish meat quality during storage. The literature showed that meat and oil's peroxide value to maintain their quality without rancidity flavor is in the range of 10-20 $mEqO_2$/kg fat (Kong & Singh, 2011). The increase in peroxide value could be attributed to the oxidation of unsaturated fatty acids, thus increasing free oxygen availability. A similar increasing trend in peroxide value was reported by Mohan et al. (2008) for catfish steaks during chilled storage (0-2ºC) for 10 days, Ehsani & Jasour (2012) for rainbow trout fillets during refrigerated storage (4ºC) for 12 days, and Viji et al. (2015) for gutted sutchi catfish during ice storage at 1-2°C.

pH is an indicator of the spoilage pattern of fish during storage. Storage time tends to increase the pH value, which can be associated with the production of ammonia, volatile alkali, and trimethylamine due to internal enzymatic activity (Chamanara et

al., 2012) and due to lactic acid build-up because of the disintegration of glycogen during the post-mortem process (Remya et al., 2018). pH also influences the degradation of myofibrillar proteins, which stimulates the gel-forming ability of minced fish. The myofibrillar proteins are unstable and rapidly lose ATPase activity with excessive pH changes (Hassan et al., 2016).

The Water binding ability refers to the bound moisture available in the muscle. It is an indicator of the water holding capacity of minces that has a direct correlation between myofibrillar protein content and gel strength of the product (Smith, 1987). The present results were confirmed with the findings of Binsi et al. (2015) and Hassan et al. (2016), who reported an increasing trend in drip loss of vacuum-packed freshwater catfish during chill storage at 4±2°C and *P. hypophthalmus* mince during storage in the refrigerator (3-5°C), respectively.

Food texture is referred to as the degree of hardness or softness, measured in terms of force (kgf) required to deform or penetrate or cut out the food material. The compressive force depends on the hardness or softness of the food tissues and is directly proportional to the hardness (Kandasamy & Mukherjee, 2019). The texture profile describes the muscle tissue properties such as hardness, cohesiveness, springiness, gumminess, and chewiness (Sun et al., 2018). The connective tissues in fish muscle ruptured and turned softer due to degradation of the myofibrillar protein (Viji et al., 2015). The protein degradation may be attributed to the activity of the endogenous enzymes and the reaction between protein and water molecules (Sun et al., 2018). Similarly, Liu et al. (2013) observed that the hardness of grass carp fillets decreased sharply within the first 3 days, both at -3°C and 0°C. In this study no significant change in cohesiveness throughout the storage period.

Cohesiveness and springiness are texture properties used for describing the elastic behavior, i.e., the ability of muscles to deform when compressed and return their original form after compression is removed and their resistance to consequent deformation (Hassoun & Karoui, 2016). A cohesiveness value of 0.0 indicates plasticity characteristics, and a value of 1.0 indicates total elasticity (Viji et al., 2015; Manju et al., 2007). The present results were in good agreement with findings of the published literature, showed no significant change in the cohesiveness of whiting fillets at 4°C during 15 days (Hassoun & Karoui, 2016) and mandarin fish (*Siniperca chuatsi*) fillets at 4°C over 4 days (Sun et al., 2018).

Springiness deals with the elastic behavior of the fish muscle. The degree of softness of fish fillets over the storage period could be the reason for decreasing in the chewiness value. A similar decreasing trend in springiness and chewiness values were reported by Manju et al. (2007) for Pearl spot fish under air and vacuum during 17 days storage at 0-2°C, Gao et al. (2014) for pompano fillets at 4±1ºC over 15 days, Viji et al. (2015) for sutchi catfish at 1-2ºC over 22 days, Hassoun & Karoui (2016) for whiting fillets during 15 days storage at 4°C and Sun et al. (2018) for mandarin fish during 5 days storage at 4ºC.

The color is an important physical property that describes the freshness of fish meat products and enhances consumer acceptability and market value (Gao et al., 2014); thus, color stability during storage is an essential factor in fish preservation. The color of fish muscle is mainly influenced by the concentration of pigment and muscle structure characteristics (Gao et al., 2014), and they reported that a decreasing trend in lightness (L*) and whiteness values whereas an increasing trend in blueness (b*) value for pompano fish fillets during storage at 4±1°C for 15 days. Lipid oxidation, pigment degradation, reactions between aldehydes, ketones, and amino acids over the storage time lead to discoloration of the fish muscle (Sriket & La-ongnual, 2018). Reduction in redness due to heme oxidation leads to browning of the bloodline, increasing yellowness (Kulawik et al., 2016). We also confirmed our results with the findings of Sriket & La-ongnual (2018). They showed a significant reduction in $L^*$and a^* values and an increase in b^* value for basa (*P. bocourti*) fillet during frozen storage at -20°C for 20 weeks. Greeshma et al. (2019) also reported a similar decreasing trend in L^*, a^*, and b^* values for vacuum packaged *P. hypophthalmus* fillets during chilled storage at 2 ± 1°C for 18 days.

The physiological condition, temperature, and microbial contamination are the primary sources for the deterioration of fish during storage. Low temperature is the most decisive impact in controlling the growth of endogenous enzymes and bacteria. The development of psychrotrophic microorganisms is effective retards below 5ºC (Erkmen & Bozoglu, 2016). An increasing trend in psychrotrophic counts was reported by Remya et al. (2018) for barracuda *Sphyraena jello* steaks during chilled storage at 0-2ºC.

Sensory characteristics play a significant role in describing the overall acceptability of fish products for human consumption. The lowest limit of the acceptable sensory score for human consumption is 4.0 (Manju et al., 2007). The sensory attributes indicate the solar thermoelectric cooler has a great potential in increasing the shelf life of basa fish fillets up to 10 days. Our results were in good agreement with the findings of Manju et al. (2007), who reported the sensory scores of pearl spot fish packed in different packs were declined to 3.4-3.8 on the 10th day from an initial value of 8.6 at 0-2°C. Mohan et al. (2008) reported similar findings for air-packed catfish steaks and Binsi et al. (2015) for vacuum-packed catfish.

Conclusion

In this work, STC was fabricated and evaluated its coefficient of performance. The STC's cooling impact on quality parameters of

the basa fish fillets includes TBA, peroxide, pH, WBA, TPC, texture profile, color, and sensory attributes, were evaluated. The result indicates that the STC temperature decreased to 7.4ºC from 30ºC within 90 min, and then gradually reduced, reached to 5±0.2ºC in 150 min. The maximum voltage of 12 V was observed when the input electric current of 3.5 A. Therefore, it was assumed as a design point to estimate the cooling capacity and COP of the STC. The cooling capacity and COP were found to be 23.8 W and 0.44, respectively. The quality parameters, TBA, peroxide, pH, WBA, and TPC, were increased significantly during the storage period of 10 days. The texture profile properties, hardness, springiness, and chewiness values decreased, whereas no significant change in cohesiveness. The color L^* and a^* values were increased significantly, whereas b^* and decreased color change (ΔE) values. However, such changes were within the acceptable level concerning the published works. The sensory scores were found in the range of 5.2-6.0 on the 10^{th} day. Since the sensory score of 4.0 is an account of the lower limit of the overall acceptability, the stored fish fillets are acceptable for 10 days. It can conclude that the STC could be an alternate green option for the preservation of fish and other perishable products where inconsistent electricity supply.

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Conflict of interest

The authors declare that they have no conflict of interest.

Nomenclature

Q_w - amount of heat transfer through walls of cooler box (W); A_c - area of the cooler walls (m^2); U - overall heat transfer coefficient (W/m^2K); T_0 - average outside air temperature (°C); T_i - inside temperature of the cooling chamber (°C); K_p - thermal conductivity of HDPE wall (W/mK); K_i - thermal conductivity polyurethane insulation foam (W/mK); X_p - thickness of HDPE wall (m); X_i - thickness of insulating material (m); Q_m - conduction heat loss inside the TEM (W); K_m - thermal conductivity of Bismuth telluride, Bi_2Te_3 (W/mK); A_m - cross-sectional area of the TEM (m^2); X_m - thickness of length of the TEM (m); Q_p - heat energy of the product (W); M_p - mass of the fish fillets (kg); C_p - specific heat capacity of the fish product (kJ/kg K); T_{pi} - initial temperature of the fish product (ºC); T_{pf} - final temperature of fish product (ºC); Q_f - heat energy of the fan (W); N_f - number of fans; F_r - rating of fan (W); Q_c - cooling capacity or amount of heat pumped by the TEM (W); α - Seebeck coefficient of the TEM (V/K); R - electrical resistance of the TEM (Ω); K - thermal conductance of TEM (W/m^2K); T_c - temperature at the cold side of the TEM (°C); T_h - temperature at the hot side of the TEM (°C); ΔT - temperature difference between hot and cold side of the TEM (°C); I - electrical current (A); V - voltage (V); P_f - power consumption of the fans (W)

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COMBINED USE OF HYALURONIC ACID WITH NANO-BIOACTIVE GLASS ENHANCED BIOCEMENT BASED SILICATE STIMULATED BONE REGENERATIVE CAPACITY IN TIBIAL BONE DEFECTS OF RABBITS: IN-VIVO STUDY

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Bioactive glass

Calcium silicate cement

Hyaluronic acid

Tibial bone defect

ABSTRACT

An ideal biomaterial for bone regeneration is a longstanding quest nowadays. This study aimed to evaluate the osteogenic potentiality of nano-bioactive glass enhanced biocement based silicate with or without hyaluronic acid seeded in rabbits' tibial bone defects**.** For this, 24 male rabbits with two 5 mm defects (1 defect per tibia) were divided into three equal groups. Among the predefined three groups, for the rabbits of group 1(control) bone defects were left untreated while for the members of group 2 defects received nano-bioactive glass enhanced biocement based silicate cement, and group 3 defects received nano-bioactive glass cement mixed with hyaluronic acid. Animals of each group were divided equally for euthanization after 3 and 6 weeks. Bone specimens were processed and examined histologically with histomorphometrically analysis of new bone area percentage. The bone defects in group 3 showed significantly improved osseous healing histologically as compared to the group 1&2. The morphometric analysis also revealed a significant increase in the new bone area percentage in group 3 as compared to the group 1 and 2 ($P < 0.05$). The results of the present study can be concluded that bone defects could be treated with nano-bioactive glass and hyaluronic acid cement. Although, nano-bioactive glass alone was capable of bone regeneration the combination of both had significant regenerative capacity.

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1 Introduction

Bone defects represent a serious pathological condition that can cause severe complications and affect vital components of bone. Bone fractures' healing and the union is an obstacle due to precarious blood supply that may complicate the treatment (Rodriguez-Merchan & Forriol, 2004; Ibrahim, 2006;). The demand for an ideal biosynthetic material with the same structure and function as natural bone used for replacement and repair of bone tissue loss has increased significantly due to the complications of autografts, allografts, and xenografts (Wei et al., 2020). Despite the increasing number of these materials, there is no ideal bone graft substitute (Nandi et al., 2010; Wu & Chang, 2013). Bone tissue engineering (BTE) is an advanced biomedical technique that is considered an effective approach for bone regeneration and reconstruction of lost bone tissue (Collins et al., 2021). Currently, the paradigm of BTE depends on bone substitute materials that can promote the human body's regenerative capacity in the repair process by stimulating the expression of osteogenic genes. In this regard, the scaffold should be designed as bone tissue "regeneration" rather than mere "replacement"(Salinas et al., 2013). Synthetic materials used for bone regeneration have great potential in clinical applications, this includes metal materials, inorganic non-metallic materials, organic materials, and composites. Bioactive glass (BG) has been applied extensively for bone repair and regeneration as they have shown excellent bone bioactivity and in vivo-bone forming ability (Kaur et al., 2014). The nanoscale of BG showed improvements in its bioactivity, this can be explained by the higher surface area of nanoscale BG that permits rapid release of ions and higher protein adsorption. Previous researches have proven that bone and teeth tissues mineralization was accelerated when these tissues were in contact with nanoscale particles in comparison with micron scaled particles (MačKović et al., 2012). Biocement based silicate was developed more than 20 years ago. The main advantage of silicate-based cement is the fact that silicate plays an essential role in mineralization and gene activation in bone regeneration process. It was reported that silicate can be combined with Ca^{2+} ions, which have shown its superiority in pre-osseous and osseous tissue repair *in vitro* and *vivo* (Zhao et al., 2005; Xu et al., 2008). Calcium silicate has been shown to facilitate cell attachment and integration with opposing hard tissues as well as their active role in bone regeneration. Many researchers reported that biomaterials containing $CaO–SiO_2$ enhance minerals deposition in addition to bonding capability to living bone and soft tissues through the development of a biologic hydroxyapatite layer on their surface (Wu & Chang, 2013). However, the degradation of pure tricalcium silicate cement is too slow to match the rate of new bone formation, which limits its application in bone regeneration (Liu et al., 2015). Numerous studies reported the efficacy of combining silicates with other materials to design bioactive biomaterials with better properties for bone tissue regeneration (Abdulghani & Mitchell, 2019). Hyaluronic acid (HA) acts as an important natural polymer that improves and modifies the biological properties of a synthetic scaffold. HA was found to be capable of binding to extracellular matrix molecules and cell surface receptors. Subsequently, it helped in regulating cellular behavior via control of the tissues' macro- and micro-environments (Chen & Abatangelo, 1999). It has been proven that HA has a great role in angiogenesis, wound healing, and tissue regeneration. HA-based scaffolds represented a source of osteoinductive elements that can subsequently promote the osteogenic effects of implanted scaffolds (Amini et al., 2012). HA was able to accelerate the degradability rate of scaffolds when added to other biomaterials such as chitosan to coincide with the new bone formation rate (Filho et al., 2021). Several previous reports on the use of nano-bioactive glass, calcium silicate cement and hyaluronic acid in bone regeneration were found. Yet, none incorporated them together as a biocomposite mixture. Therefore, this study aimed to introduce a novel composite scaffold with extrudable nanostructured bioactive glass and calcium silicate based biocement pastes using hyaluronic acid as a solvent, which may serve as an alternative for bone tissue regeneration.

2 Materials and Methods

2.1 Materials

Tetraethyl orthosilicate (TEOS), triethyl phosphate ethanol (TEP), nitric acid (65%) used as a catalyst, calcium nitrate tetrahydrate ($Ca(NO_3)_2.4H_2O$), ammonia (NHOH), and silver nitrate ($AgNO_3$) were used to prepare the silver bioactive glass and calcium silicate cement by sol-gel method. Nano-BG particles were prepared by adding TEOS, TEP, calcium nitrate, and silver nitrate in sequence under constant stirring. Forty-five minutes were given for each reagent to react completely. The solution gels were aged for 8 days at room temperature. The gel was subsequently dried at 70 °C for 72 h to reach the monolith. The dry gel was fired at 500 °C for 8 h. The glass system reached was $60SiO_2$:$35CaO$:$4P_2O_5$:1 Ag_2O_3 (Kozon et al., 2016). The novel biocement was prepared by mixing 80% of calcium silicate cement with 20% of silver bioactive glass (Lee et al., 2018). Either high molecular weight hyaluronic acid (1750 kDa) (Sigma-Aldrich Merck KGaA, Darmstadt, Germany), or distilled water was used to prepare the cement paste which was subsequently moulded into the critical-sized bone defect (Ahmadzadeh-Asl et al., 2011).

2.2 Nanocomposite characterization

Transmission electron microscopy (TEM) was used to analyze the particles size and morphology of nanopowders. The prepared nanocomposites were subjected to TEM analysis by applying JEOL-JEM 2100F to monitor the particle size with an accelerating voltage of 200 kV.

2.3 Ethical statement

The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) - Cairo University with approval number (CU/III/F/46/19). All surgical procedures were performed under general anesthesia, and all efforts were done to minimize suffering.

2.4 Experimental animals

This experiment was conducted on 12 weeks old 24 healthy male New Zealand white rabbits weighing about 2.5 to 3.5 kg. Animals were purchased and housed in the animal house Faculty of Medicine, Cairo University. The rabbits were randomly allocated into three groups. Each group consisted of 8 rabbits. Animals were kept in separate cages and maintained under controlled temperature at 25˚C± 2˚C with a 12 h light/dark cycle. They were fed pellets containing several nutritional ingredients including wheat, hay grass, oats, vitamin D, and calcium. The animals were given fresh tap water available ad libitum with good ventilation conditions throughout the experiment.

2.5 Bone defect preparation

The surgical procedure was performed under general anesthesia upon intramuscular injection of a combination of 5mg/kg 2% Xyaline (Xyla-Ject®, PhoenixTM, Pharm. Inc., St. Joseph, USA), and 40mg/kg Ketamine Chlorhydrate (Ketamine, AmounPharm. Company, Egypt)**.** A single bone defect 5 mm in diameter was created in each tibia using a round surgical bur coupled to a low-speed handpiece used under constant copious irrigation with physiological saline solution to prevent the overheating of the periphery of the bone (Doostmohammadi et al., 2019). The bone defect was drilled until the medullary canal was reached. The defects of group 1 (control group) were left untreated (filled with a blood clot), while group 2 defects were filled with nanoBG enhanced biocement based silicate mixed with distilled water. Group 3 defects were packed with nanoBG enhanced biocement based silicate mixed with HA. Postoperatively, the periosteum, muscle, and fascia were repositioned properly over the defects and sutured with resorbable #2.0 catgut, and the skin was sutured with interrupted #3.0 silk sutures. Systemic antibiotic Amikacin® 1.5 mg/ kg (Amoun pharmaceutical company, Egypt) was administrated as an intramuscular injection per 12 hours for 1 week (Dang et al., 2019)**.** Analgesic 10 mg/kg Cataflam (Novartis, Egypt) was administrated to relieve postoperative pain. Topical antibiotic spray Bivatracin (Egyptian Company for Advanced Pharma, Egypt) was applied to avoid local infection. Half of the animals in each group were euthanized with an intra-peritoneal overdose of Ketamine/Xylazine mixture3 weeks postoperatively while the other half was euthanized after 6 weeks (Zhao et al., 2016)**.** Both tibiae were dissected to be free from any soft tissues; the bone specimens including the defect of each group were cut by a disc under constant irrigation to include the entire defect sites.

2.6 Histological and histomorphometry examination

Bone specimens were fixed in 10% calcium formol solution for 48 hours and demineralized in 10% EDTA (El-Gomhouria Co., Egypt) solution for 4-5 weeks. The specimens were subsequently dehydrated in ascending grades of alcohol, cleared by xylol, and then embedded in paraffin blocks. Serial 5-6 µm paraffin cross sections were prepared and stained with haematoxylin and eosin (H&E) stain. Histomorphometric analysis of the newly formed bone area percentage was obtained using Leica Qwen 500 image analyzer Computer system (Leica Imaging System Ltd., Cambridge, the U.K. in Research unit in faculty of Oral and Dental Medicine, Cairo University). The image analyzer consisted of a colored video camera, colored monitor, hard disc of IBM personal computer connected to the microscope and controlled by Leica Qwen 500 software.

2.7 Statistical analysis

The data obtained from the histomorphometric analysis were statistically described in the terms of mean and standard deviation (SD) values. A two-way ANOVA test was used to compare different observation times within the same group. Followed by Tukey's post hoc test to compare multiple 2-group comparisons. The significance level was set at $p < 0.05$. Statistical analysis was performed with IBM SPSS 18.0 (Statistical Package for Scientific Studies, SPSS, Inc., Chicago, IL, USA) version 22 for windows.

3 Results

3.1 TEM analysis of nanoBG biocement

TEM analysis of novel nanoBG silicate based biocement showed the heterogeneous shape of the nanoparticles in form of crystalline dark and amorphous transparent nanoparticles. The average particle size of nanoparticles of the clumped distributions was between 9.46 and 18.36 nm (figure 1A). While the TEM images of novel biocement mixed with HA showed a uniform distribution with large hydrated cloudy clusters encapsulating many nanoparticles of different morphology. The average nanoparticles size ranged from 12.09 to 15.31 nm in diameter (figure 1B).

3.2 Histological (H & E stain) results

Three weeks postoperatively, group 1 showed an almost open bone defect with some granulation tissue in the middle of the defect and few newly formed bone trabeculae at the bony edges enclosing large bone marrow cavities in-between (figure 2 A & B). Thin and interconnected neobone trabeculae were formed around the graft material in group 2 with wide bone marrow cavities in-between

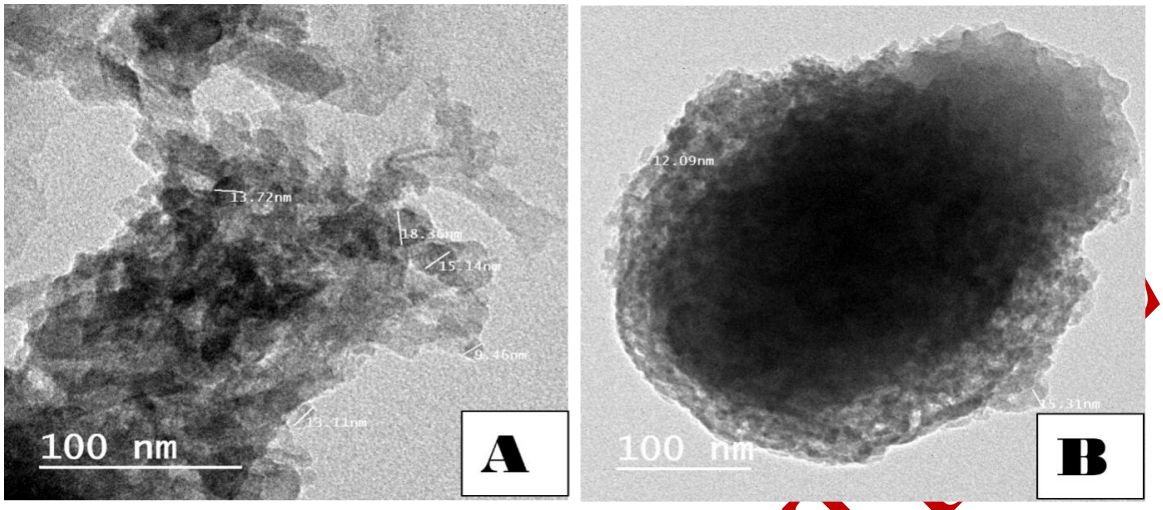

Figure1 TEM image of sliver nanoBG/calcium silicate cement nanopowder (A) TEM image of sliver nanoBG/calcium silicate cement nanopowder mixed with HA (B) (x100 nm)

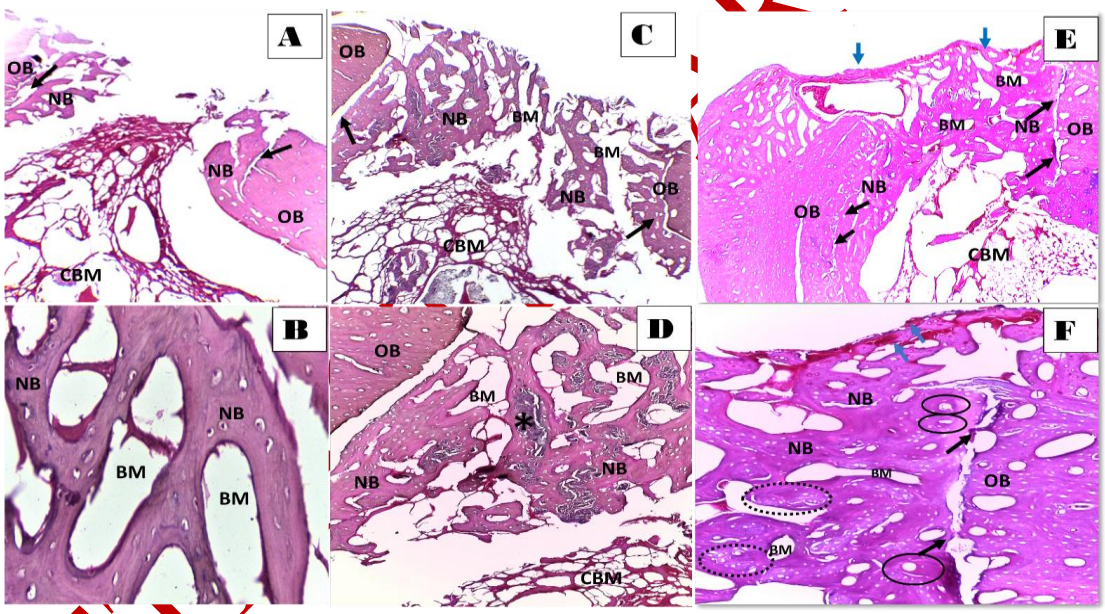

Figure 2 Photomicrographs of H& E-stained sections of bone defects (3 weeks). Group 1 or control group (A &B), Group 2 (C&D), Group 3 (E&F). Here OB: old bone, NB: new bone, BM: bone marrow, CBM: central bone marrow, black arrows: interface between old pre-existing and new bone, blue arrows: periosteum, black asterisk: graft material remnants, black circles: primary osteons, dashed black circle: woven bone (A, C&E x40, B x400, D&F x100)

(figure 2 C & D). Group 3 revealed newly formed bone trabeculae filling the defect with thick trabeculation and appearance of primary osteons having wide have rsian canals as well as scattered areas of woven bone. The bone defect showed highly vascularized periosteum coverage. The interface between newly formed bone and old pre-existing bone was about to be sealed with a scalloped border (figure 2 E & F). Six weeks postoperatively, group 1 defects revealed newly formed interconnecting bone trabeculae filling almost all the defects as compared to the same group at 3 weeks postoperatively. Dispersed areas of woven bone with different degrees of basophilia were detected (figure 3 A & B). Group 2 showed bone defect almost filled with newly formed lamellar bone with thick trabeculation enclosing small bone marrow spaces. The indistinguishable interface was observed between old bone and newly formed bone with a significant change in the orientation of the lamellae between old and neobone (figure 3 C & D). Group 3 demonstrated a completely restored defect with densely packed compact bone tissue that could not be distinguished from the old bone with a completely sealed

interface. Dense compact bone compromised lamellae assumed in concentric arrangement around a have rsian canal, forming a typical osteon (figure 3 E & F).

3.3 Histomorphometry

The histomorphometry analysis of the newly formed bone area percentage between groups during both time intervals showed the highest newly formed bone area percentage in group 3 which revealed a statistically significant increase in the mean of bone area percentage relative to groups 1 and 2. Moreover, all groups showed statistically significant higher newly formed bone area percentage mean value at 6 weeks as compared to 3 weeks ($p<0.05$). Tukey's post hoc test revealed a significant difference between each 2 observation times in all groups (figure 4, table 1 and 2).

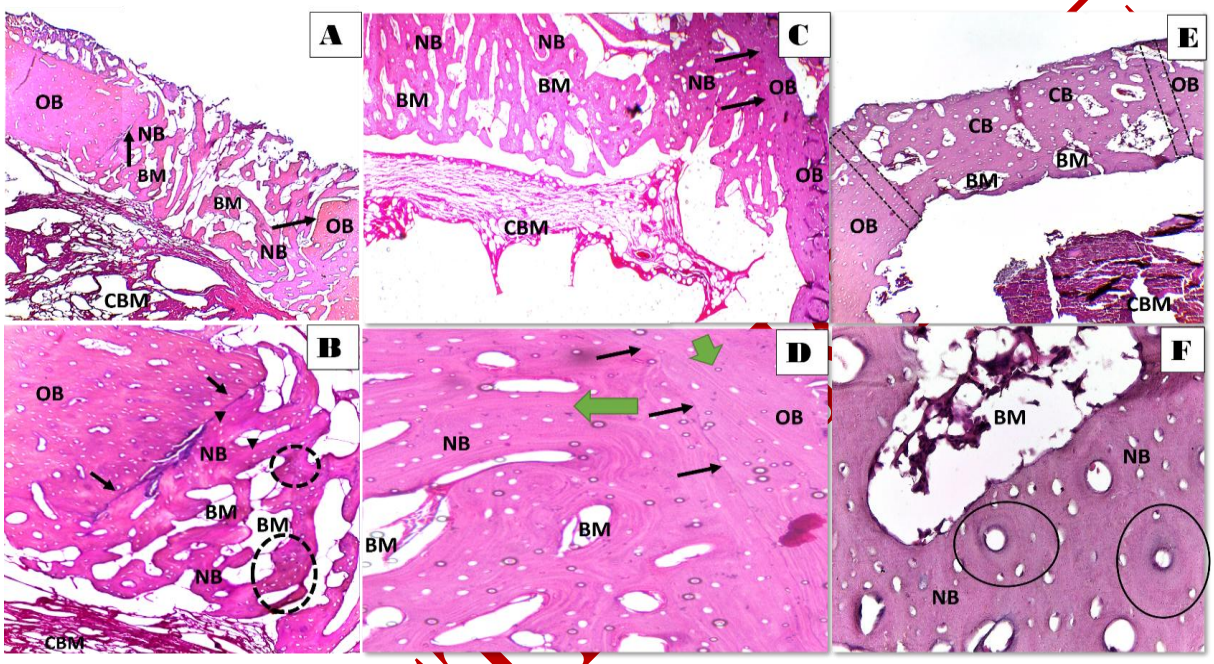

Figure 3 Photomicrographs of H& E-stained sections of bone defects (6 weeks).Group 1 or control group (A &B), Group 2(C&D), Group 3(E&F), OB: old bone, NB: new bone, BM: bone marrow, CBM: central bone marrow, CB: compact bone, black arrows and dashed rectangle: interface between old pre-existing and new bone, green arrows: different orientation of bone lamellae, black circles: typical osteons, dashed black circle: woven bone (A, C&E x40, B x100,D&F x400).

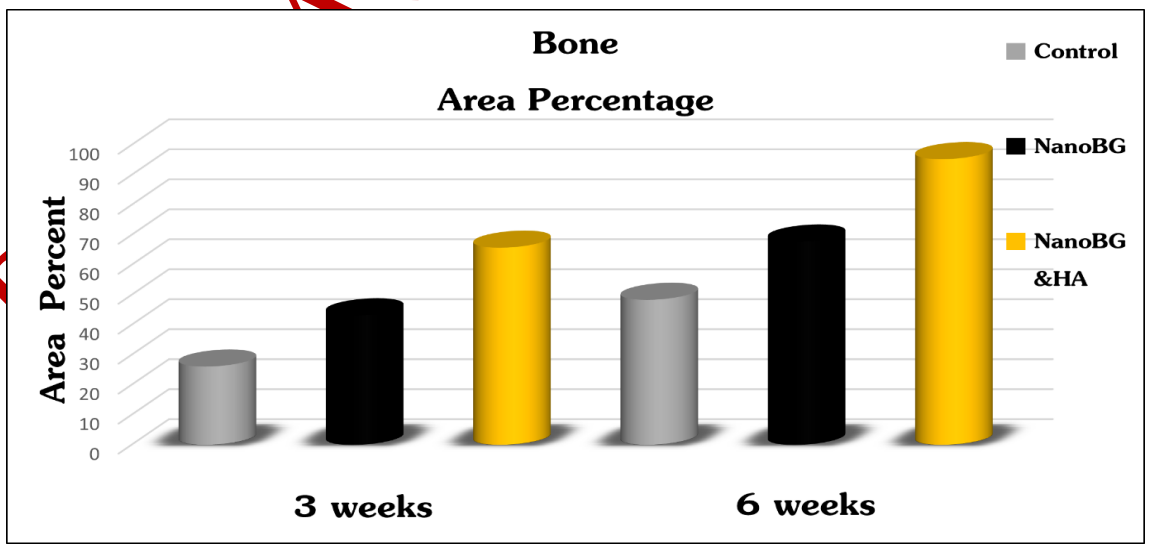

Figure 4 Column chart showing newly formed bone area percentage mean value of with 95% confidence interval error bars in all groups for 3 and 6 weeks postoperatively

Table 1 Newly formed bone area percentage between groups.

Duration	Group	Mean	Standard deviation	Std. Error Mean
3 weeks	Group 1	26.13^{D}	3.35	1.18
	Group 2	43.221^{C}	2.589	0.915
	Group 3	65.79^{B}	4.65	1.64
6 weeks	Group 1	48.40^{C}	4.17	1.47
	Group 2	67.84^{B}	4.31	1.52
	Group 3	95.317^{A}	2.039	0.721

ANOVA test: Significant means with different superscript letters are significantly different.

Table 2 Newly formed bone area percentage for each group at different observation times

Group	Duration	Mean	Standard deviation	Std. Error Mean	T-Value	Adjusted P-Value
Group 1	3 weeks	26.13	3.35	1.18		
	6 weeks	48.40	4.17	1.47	12.22	0.000*
Group 2	3 weeks	43.221	2.589	0.915		
	6 weeks	67.84	4.31	1.52	13.51	0.000*
Group 3	3 weeks	65.79	4.65	1.64		
	6 weeks	95.317	2.039	0.721	16.2	0.000*

Tukey's post hoc test: Pairwise comparison of newly formed bone area % between different time intervals within the same group.(*Significance level P≤0.05)

4 Discussion

Before evaluating biomaterials in humans, a perfect bone substitute ought to be tried in vitro and in vivo, to be certain beyond any doubt that it works viably and securely. Therefore, establishing an appropriate animal model is an essential step when assessing the mechanical property and biocompatibility of bone tissue biomaterials (Schlegel et al., 2006). Silica based BG has been exclusively applied for bone repair and regeneration as they showed excellent bone bioactivity and in vivo bone forming ability (Al-Harbi et al., 2021). Numerous works of literature indicate that HA acts primarily to promote healing at fracture sites by stimulating callus formation. Furthermore, HA of a specific molecular weight when used in vitro was reported to significantly increase alkaline phosphatase activity and stimulate osteoblastic cell proliferation and differentiation (Zhai et al., 2020).

The histological results of the current study in the nanoBG group at both time intervals showed better bone regeneration than the control group. They showed interconnected bone trabeculae filling almost all the defect perimeters which appeared thicker with smaller bone marrow cavities 6 weeks postoperatively. Moreover, the newly formed bone area percentage was significantly higher in the nanoBG group than in the control group. BG showed unique properties in bone tissue regeneration by the formation of the carbonated hydroxyapatite layer (HCA) when exposed to biological fluid. This layer is responsible for the strong bonding between bioactive glasses and human bone (Jones et al., 2007). In coincidence with our findings, Abiraman et al., (2002) concluded that after 6 weeks of BG implantation in tibial bone defects in rabbits the periosteal and the endosteal regions were completely closed. As well as Pinto et al., (2013) reported that tibial bone defects implanted with biosilicate ceramics showed highly organized newly formed bone filling the whole defect after 45 days postoperatively. Another study demonstrated that the quantitative woven bone volume was significantly higher in the BG group than in the control group after 20 days of implanting BG in tibial bone defects of rats (Granito et al., 2011).

NanoBG with HA group showed superior histological results than the other 2 groups throughout the whole experiment with the highest newly formed bone area percentage in form of dense uniform and organized compact after 6 weeks. Superior bone regenerative results seen in the nanoBG and HA group could be assumed to the characteristic role of HA in cell adhesion, chemotaxis, differentiation, and proliferation signaled through several macromolecules and especially during wound healing and tissue regeneration (Huang et al., 2003; Prestwich, 2011). Similarly, Shamma et al. (2017) confirmed that the addition of HA into bone graft around dental implants placed in sockets of extracted mandibular third premolar of dogs after 6 weeks showed filled mature well-formed bone with obvious complete osseointegration with the native bone.

On contrary, El behairy et al. (2019) revealed that HA implanted in combination with biphasic calcium phosphate cement in femoral bone defects of rats didn't give superior bone regeneration in comparison with the cement alone 4 and 10 weeks postoperatively. They explained their findings by assuming that the low molecular weight (less than 1000 kDa) of the HA used in their study was the reason. The HA ability to enhance the osteogenic and osteoinductive properties of bone graft materials was dependent on its dose and molecular weight. It was found that HA of higher molecular weight (more than 1000 kDa) promoted mesenchymal stem cells (MSCs) proliferation and differentiation (Huang et al., 2003). This may confirm the osseous regenerative potentiality of HA used in the current study which had a high molecular weight (1750 kDa). However, Kuo et al. (2021) revealed that hybrids of HA with different molecular weights combined with bone grafting material enhanced bone regenerative capacity in rabbit femoral bone defects, suggesting that 50:50 (high molecular weight: low molecular weight) of HA showed ideal degradability and significant new bone formation.

Parallel to our results, Elkarargy, (2013) demonstrated that combining HA to synthetic bone graft increased the newly formed bone area percentage upon implantation in sockets of extracted lower lateral incisors in rabbits when compared with bone graft alone and empty control group after 4 weeks and 8 weeks postoperatively. Moreover, Shirakata et al., (2021) concluded that adding HA either alone or combined with collagen matrix in 5 mm intrabody defects on the walls of mandibular premolars in dogs enhanced the periodontal wound regeneration. Also, Filho et al., (2021) confirmed that the addition of 1% HA to the chitosan-gelatin scaffold in rodents' intrabuccal bone defects revealed improved gingival healing after 21 days with a better degradability rate of scaffolds in comparison to chitosan-gelatin alone.

Conclusions

From the results of the current study, it can be concluded that the combined use of HA and nanoBG enhanced silicate biocement for osteogenic regeneration of bone defects is a potential alternative treatment for accelerated healing than using these biomaterials alone. This conclusion is a breakthrough in the field of bone graft materials since BG overcomes the limitations associated with other synthetic and natural bone grafts and makes it a promising bone substitute material in critical bone defects in clinical applications.

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Conflict of interest

The authors declare that they have no conflict of interest.

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PHYTOCHEMICAL SCREENING OF DIFFERENT ROOT EXTRACTS OF *Ageratum conyzoides* AND THEIR POTENTIAL BIOACTIVE PROPERTIES

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Antimicrobial activity

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Medicinal plants

ABSTRACT

The present study was conducted to determine different classes of secondary metabolites of *Ageratum conyzoides* root extracts and to evaluate their antioxidant, antibacterial, and antifungal potentialities using various pathogenic fungal and different gram-positive/gram-negative bacterial strains. The roots powder was subjected to ultrasonic-assisted extraction with *n*-hexane, acetone, ethanol, and EtOH:H_2O(1:1). The screening of phytochemicals indicated the existence of terpenoids, alkaloids, coumarins, sterols, flavonoids, and glycosides in the root extract of *A. conyzoides*. However, the absence of saponins, tannins, anthocyanidins, anthraquinones, and phlobatannins was observed. The results indicated a reasonable antibacterial (against gram-negative and gram-positive bacteria), and antifungal potential. The antibacterial activity of the ethanolic extract was highest against all four strains of bacteria and was also comparable to the standard medicines used. However, antifungal activity was highest in EtOH:H_2O (1:1) extract. Moderate antioxidant properties were also demonstrated, favoring the importance of the roots of this plant from a medicinal point of view.

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1 Introduction

Ageratum conyzoides is a weed commonly present in cultivated lands,e.g., wastelands, grasslands, pastures, and even in forests areas (Batish et al., 2006; Harjanti et al., 2019). This plant is commonly found in subtropical and tropical regions of the world. It has spread to Southeast Asia, West Africa, South America, Southeastern North America, Central America, the Caribbean, and Florida (USA) (Okunade 2002; Bamidele et al., 2010). In Pakistan, it is widely distributed in Islamabad, Rawalpindi, and Changa Manga Forest near Lahore (Zafar et al., 2006). The roots and leaves of *A.conyzoides* are of great medicinal value. They are used for the treatment of an array of disorders such as burns and wounds. Due to excellent antioxidant, antibacterial, and antifungal properties, medicinal plants have been used for several bacterial infections, dyspnea, arthrosis, headaches, analgesic, anti-inflammatory, pneumonia, anti-asthmatic, anti-spasmodic, and haemostatic effects, stomach ailments, gynaecological diseases, and other skin disorders (Sachin et al., 2009; Kamboj et al., 2010; Al-Kaabi et al., 2021). In a murine ascites Dalton's lymphoma *in-vivo*, the aqueous extract of roots of *A. conyzoides* declined glutathione in the liver and the lymphoma cells of the tumor-bearing mice. In the opinion of the authors, this could be one step in producing the anti-tumor effect (Rosangkima & Prasad, 2004).

There is a growing scientific concern in finding plants having high medicinal potential. Due to their chemical structure, plant extracts have been shown to exhibit improved capability to substitute these mediators of their multi-step processes for the efficient treatment of painful and inflammatory processes (Wang et al., 2013; Khalid et al., 2017a,b,c; Bilal et al., 2018; Shafiq et al., 2021). Nowadays, diarrhea is a very common disease in several countries, and it is the leading cause of morbidity and mortality, especially among the lower-age population (Wendel et al., 2008; Muhammad et al., 2012). Medicinal plants have a central role in the advancements of modern technologies on anti-diarrheal activities of naturally extracted compounds (Panda et al., 2012; Rawat et al., 2017). *S. aureus*, also known as golden staph causes pimples, scalded skin, pneumonia, and toxic shock syndrome. *Pseudomonas aeruginosa* is a casual microorganism of the urinary tract, pulmonary tract, and blood infections, while *Klebsiella pneumonia* causes pneumonia, wound infection, diarrhea, urinary tract infections, and upper respiratory tract infection. The research for efficient and safer agents for biomedical purposes has continued to be a hot area among the scientific community (Ishaq et al., 2019; Khalid et al., 2019; Bilal et al., 2020; Munir et al., 2021). Therefore, the WHO has encouraged scientific work on the evaluation of traditional medicinal plants for the treatment of common health-causing issues worldwide (Uddin et al., 2009; Al-Kaabi et al., 2021). This study was designed to determine the phytochemicals present in the root extract of *A.conyzoides*. Moreover, the evaluation of their antioxidant, antibacterial and antifungal potential was a key purpose of broadening the role of *A. conyzoides* chemicals in the modern biomedical industry.

2 Materials and Methods

2.1 Collection of raw materials

The root samples of *A.conyzoides* were obtained from I-9/4 Islamabad, Punjab-Pakistan. The plant species (specimen number–84902) was authenticated by the Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan. The samples were preserved in the herbarium of the Department of Plant Sciences. The plant specimen was cleaned with tap water and air-dried before preservation.

2.2 Development of plant extract

Powdered (1.5 kg) roots were successively extracted with the increased polarity of solvents such as *n*-hexane, dimethyl ketone (Me_2CO), ethanol (EtOH), and EtOH:H_2O (1:1). At each stage, a sufficient amount of solvent was added, and the resulting slurry was subjected to mechanical stirring for at least 24 h. The slurry was also irradiated with ultrasonic waves for efficient extraction. The extract was decanted, and the solvent was driven off under the vacuum. Plant extracts with 1 mg/ml concentration were used for the determination of bioactivities.

2.3 Screening of phytochemicals

The phytochemicals screening of the above-mentioned plant extracts was performed by following the previously reported protocols of Trease & Evans (1989) and Cannell, (1998). A brief description of methodologies has been given in sub-sections below to determine the secondary metabolites such as alkaloids, tannins, saponins, flavonoids, terpenoids, sterols, glycosides, coumarins, anthraquinones, anthocyanidins, and phlobatannins (Okamoto et al., 2006; Tiwari et al., 2011; Ganesh et al., 2011; Mulugeta et al., 2021)

2.3.1 Alkaloids (Dragendroff's test)

For the determination of alkaloids, plant extracts (0.2 g) were added with 2% sulfuric acid and incubated for 2 minutes. The mixtures were filtered using filter paper, and a few drops of Dragendroff's reagent were added. The formation of orange-red precipitates indicated the existence of alkaloids in root extracts.

2.3.2 Tannins ($FeCl_3$ test)

The plant extracts (0.5g) were added to a test tube containing 10 mL of H_2O. The samples were boiled and filtered. Few drops of $FeCl_3$ (0.1%) were added. The appearance of blue-black or brownish-green coloration confirmed the existence of tannins in the root extracts of the plant.

2.3.3 Saponins (Frothing test)

The plant extracts (0.5g) were dissolved in boiling water. The mixtures were gently shaken for mixing and allowed to cool for a few minutes. Persistent froth appeared indicating the existence of saponins in the extracts.

2.3.4 Flavonoids (Alkali test)

The plant extracts (0.2g) were dispersed in dilute sodium hydroxide and added with the dilute HCl. The yellowish color of the solution was disappeared indicating the existence of flavonoids in the plant extracts.

2.3.5 Terpenoids (Salkowski test)

The roots extracts(0.5g) were added with $CHCl_3$(2 mL) along with concentrate H_2SO_4 (3 mL). The appearance of the reddish-brown color of the interface indicated the existence of terpenoids in plant extracts.

2.3.6 Sterols (Lieberman-Burchard reaction)

The concentrate H_2SO_4 (1 mL) was added to 1 mL of each extract of *A. conyzoides* roots using a test tube. The appearance of a red coloration indicated the existence of sterols in plant extracts.

2.3.7 Glycosides (Keller-Killiani test)

The root extracts (0.5g) were diluted by the addition of H_2O (5 mL), followed by the addition of glacial HOAc (2 mL). The mixture was added with one drop of $FeCl_3$ solution and 1 mL of concentrate H_2SO_4. The interfacial brown ring formation showed the existence of deoxy-sugar properties of glycosides.

2.3.8 Coumarins (NaOH test)

The root extracts (0.5g) were taken in test tubes, wrapped with filter paper, and soaked with NaOH (1M). Test tubes were kept in a water bath for few minutes. The test tubes were examined under UV light for yellow fluorescence which showed the existence of coumarins.

2.3.9 Anthraquinones

Plant extracts (0.5 g) were placed in 10% of HCl and boiled for few minutes in the water bath. After gentle filtration, the mixtures were allowed to cool. The mixture was equilibrated with $CHCl_3$ along with a few drops of 10% solution of NH_3. The mixture was heated, and the formation of rose-pink color confirmed the presence of anthraquinones.

2.3.10 Anthocyanidins

The root extracts (0.5g) were added with HCl (1 mL). The presence of red coloration indicated the existence of anthocyanidins.

2.3.11 Phlobatannins

The root extracts (0.5g) were mixed in dH_2O and filtered. The filtered solution was boiled with 2% of HCl. The formation of red precipitates confirmed the existence of phlobatannins.

2.4 Determination of antibacterial activity

Antibacterial activities of different root extracts were analyzed against four different bacteria (*Bacillus subtilis*–ATCC 6059, *Streptococcus aureus*–ATCC 6538, *Klebsiella pneumonia*–ATCC 4352, and *Pseudomonas aeruginosa*–ATCC 7221) using the previously described protocol (Mehreen et al., 2016). For this purpose, samples were prepared by mixing crude extracts (1 mg) in 1 mL of dimethyl sulfoxide (DMSO) as a negative control, whereas two different antibiotics were used as a positive control e.g., Chloramphenicol (positive control-1) and Streptomycetes (positive control-2). The nutrient agar media was prepared by the standard protocol. Seeded agar plates were prepared by pouring 75 mL of media into the petri dish. When the agar was solidified, the sterile cork borer (5 mm) was used for the formation of four wells per plate. The samples (100 µL) were poured along with the positive as well as a negative control in the respective wells of the petri dish with the help of a micropipette. The plates were placed at 37 ºC for incubation. After 24 hrs, the zone of inhibition was calculated with the help of a scale.

2.5 Determination of antifungal activity

Antifungal activities of crude root extracts were analyzed using the agar-tube-dilution process (Washington & Sutter, 1980). Two toxic fungal strains (*Fusarium moniliforme* and *Helminthosporium sativum*) were used for the evaluation of anti-fungal potentialities. The strains were obtained from the Microbiology Department of Quaid-i-Azam University, Pakistan. Both fungal cultures were maintained on Sabouraud dextrose agar (SDA) media at kept at refrigerator temperature (4 ºC). The samples were prepared from the initial stock of 1 mg of crude extracts in 1 mL of DMSO. Another tube from each specimen was prepared without plant extract which acted as a negative control. While standard fungicide Chloramphenicol (1 mg/ml) was kept as a positive control. The test tubes were placed at 28 °C for seven days. Inhibition of fungal growth was measured by the length of fungal growth, and the growth inhibition was analyzed according to positive and negative controls. Percent inhibition of fungal growth for each sample was determined by the following formula:

$$\text{Percent inhibition of fungal growth} = \frac{100 - \text{Linear growth in sample test tube (mm)}}{\text{Linear growth in control test tube (mm)}} \times 100$$

2.6 Determination of antioxidant activity

For the determination of antioxidant potentialities, different concentrations of the test sample and standard (ascorbic acids) were prepared e.g., 100, 50, 25, 12.5, 6.25, 3.15, and 1.56 µg/mL, respectively. A set of three clean and dry test tubes were taken and 2.7 mL of DPPH solution was added to each test tube. Ascorbic acid solution (0.3 mL) in EtOH was added in another three test tubes including 2.7 mL of DPPH solution. Twenty-one clean and dry test tubes were taken in which 2.7 mL of DPPH reagent and 0.3 mL of different concentrations of test sample (100, 50, 25, 12.5, 6.25, 3.15, and 1.56 µg/mL) concentrations were added and mixed thoroughly. Seven test tubes were taken in which 0.3 mL of various concentrations of test samples (100, 50, 25, 12.5, 6.25, 3.15, and 1.56 µg/mL) and 2.7 mL EtOH solvent were added and run in UV as blank. After 30 min incubation period at 37 ˚C in dark, the absorbance of the standard solution and the resulting mixtures was determined at 517 nm with UV/vis spectrophotometer (model UV-1700 (E) 23 OCE, Schimadzu, Japan).

3 Results and Discussion

3.1 Screening of phytochemicals

Phytochemical screening of four different extracts i.e., *n*-hexane, acetone, ethanol, and EtOH:H_2O (1:1) of *A. conyzoides* was carried out to determine the detailed information regarding phytochemical constituents of *A. conyzoides* roots, which may provide an incentive for proper evaluation of this plant for its medicinal potential. Table 1 summarizes the results obtained after the screening of secondary metabolites whose presence was indicated by the above-mentioned phytochemical tests. According to the results obtained phlobatannins, anthraquinones, anthocyanidins, saponins, and tannins were absent in all extracts. However, flavonoids, glycosides, sterols, terpenoids, and alkaloids were found in all plant extracts except for flavonoids which were absent in *n*-hexane extract and cardiac glycosides in EtOH:H_2O (1:1) extract. Coumarins were also absent in *n*-hexane and EtOH:H_2O (1:1) extracts but present in acetone and ethanol extracts. Therefore, it is concluded that phytoconstituents isolated from *A. conyzoides* roots possess a wide range of biological activities. However, their effects on the cardiovascular system, diuresis, anti-viral, spasmolytic, anti-inflammatory, and other biomedical properties are yet to be studied. Phytochemical constituents and their biological activities showed the medicinal importance of this plant (Kamboj et al., 2011; Al-Kaabi et al., 2021). Parveen et al., (2014) studied the efficiency of *Artemisia absinthium* and *A. conyzoides* in-vitro on *Rhipicephalus microplus* using the AIT test. Five different concentrations of plant extract from 1.25–20% were used in the evaluation process. The results revealed 66.7% and 40% of mortality at 20% concentration of *A.absinthium* and *A.conyzoides*, respectively. *A.conyzoides*reduced 90% of egg hatching; however, *A. absinthium* indicated 100% inhibition. The study revealed that *A.absinthium* presents higher acaricidal characteristics. Chah et al., (2006) reported excellent antibacterial and wound healing properties of *A. absinthium*.

3.2 Antibacterial activities

Antibacterial activity of different root extracts was determined against four different bacteria [Gram-positive e.g., *B. subtilis*(ATCC 6059), *S. aureus*(ATCC 6538), and Gram-negative e.g., *K. pneumonia*(ATCC 4352) and *P. aeruginosa* (ATCC 7221)] using agar-well-diffusion method. Bioassays of four different extracts of roots of *A.conyzoides* was carried out to compare the relative activities of these extracts. In the current study, the results

Table 1 Screening of phytochemicals of the different root extracts of *A. conyzoides*.

S. No.	Metabolites	Extracts			
		n-hexane	Me_2CO	EtOH	EtOH:H_2O(1:1)
1	Alkaloids	+	+	+	+
2	Tannins	-	-	-	-
3	Saponins	-	-	-	-
4	Sterols	+	+	+	+
5	Flavonoids	-	+	+	+
6	Terpenoids	+	+	+	+
7	Glycosides	+	+	+	-
8	Coumarins	-	+	+	-
9	Anthocyanidins	-	-	-	-
10	Anthraquinones	-	-	-	-
11	Phlobatannins	-	-	-	-

signified that all the crude extracts have exhibited strong in-vitro antibacterial activities against the different bacteria that was employed in the experiments in various degrees. The bioassays findings have been represented in Figure 1. These results showed that the antibacterial activity of EtOH extract was highest against all four strains of bacteria and was also comparable to the standard medicines used. The activity of EtOH:H_2O extract was found relatively less than EtOH extract but greater than *n*-hexane and acetone extracts, whereas the activity of *n*-hexane extract was the least. From the obtained results it can be concluded that slightly more polar compounds present in EtOH extract possess superior antibiotic character in nature in comparison to non-polar components of *n*-hexane extract and highly polar components of EtOH:H_2O extract. The graphical representation of these results has been shown in Figure 1.

3.3 Antifungal activities

Antifungal activities of crude plant extracts were determined using the agar-tube-dilution method. Two toxic fungal strains (*F.moniliforme* and *H.sativum*) were used to analyze the antifungal activities of these *A. conyzoides* extracts. *A. conyzoides* very strongly suppressed the growth of the fungal strains used, therefore this plant is anticipated for the isolation of the compounds with excellent antifungal characteristics. Results obtained after antifungal activities have been shown in Figure 2. In the present study, the obtained results illustrated that all the crude extracts have demonstrated strong in-vitro antifungal activities against two toxic fungal strains that were used in the experiments in various degrees. Results of antifungal activity indicated the highest activity of EtOH:H_2O extract against both fungal strains. Whereas activity of acetone and ethanol extracts were also appropriate and comparable to standard drugs against *F. moniliforme*. These results indicated that polar compounds extracted by polar solvents were more antifungal. Therefore, the extract of highest polarity i.e., EtOH:H_2O extract showed the highest activity and the extract of lowest polarity i.e., *n*-hexane extract, showed the lowest activity. Results of antifungal activities have been shown in Figure 2.

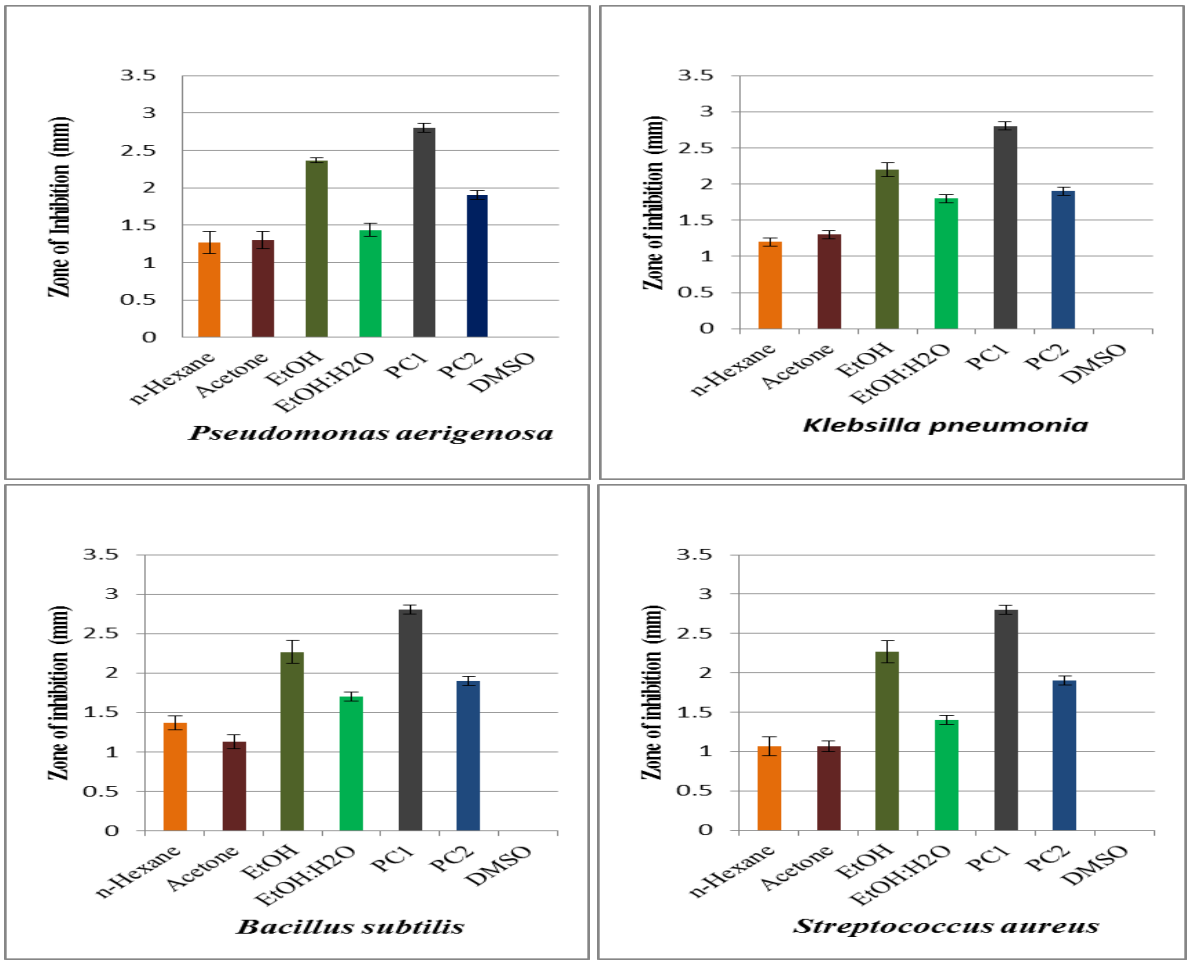

Figure 1 Graphical representation of antibacterial activities of four different extracts of *A. conyzoides* against different bacterial strains.

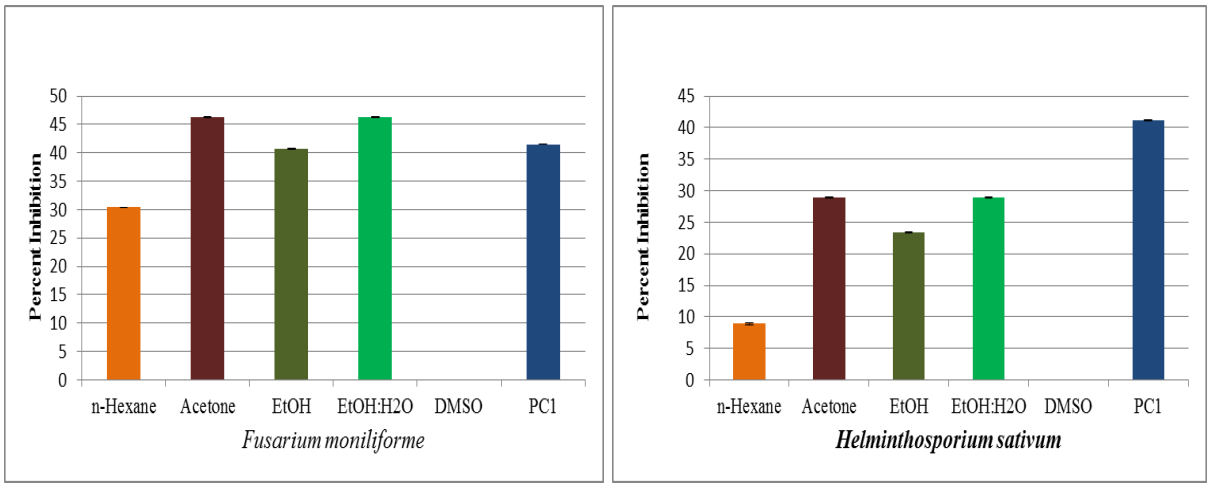

Figure 2 Graphical representation of the antifungal activity of four extracts of *A. conyzoides* against *Helminthosporium sativum* and *Fusarium moniliforme*.

Table 2 DPPH Radical Scavenging Activity of Me_2CO extract of roots of *A. conyzoides.*

S.N.	DPPH	AA	100 µg	50 µg	25 µg	12.5 µg	6.25 µg	3.12 µg
1	0.723	0.024	0.378	0.488	0.545	0.594	0.624	0.63
2	0.736	0.024	0.381	0.485	0.561	0.609	0.631	0.648
3	0.735	0.023	0.388	0.495	0.567	0.601	0.634	0.65
4	2.194	0.071	1.147	1.468	1.673	1.804	1.889	1.928
Mean	0.731	0.024	0.382	0.489	0.558	0.601	0.630	0.643
Test sample + Solvent	-	-	0.028	0.013	0.006	0.002	0.001	0.000
Final absorbance	0.731	0.024	0.354	0.476	0.552	0.599	0.629	0.643
Inhibition (%)	-	-96.72	-51.57	-34.88	-24.48	-18.058	-13.95	-12.04

3.4 Antioxidant activities

The DPPH free radical scavenging activities were presented as inhibition percentage and results are exhibited in Table 2. The obtained results varied from 12.04 to 96.72 % for the acetone extract. The antioxidant activity was considerably observed in the Me_2CO extract as compared to other extracts. The IC_{50} values of standard ascorbic acid and Me_2CO extract were calculated with the help of EZ fit ™ enzyme kinetic software (statistical software) (Table 2). From the IC_{50} value, it could be observed that Me_2CO extract exhibits moderate antioxidant activity. Acetone extracts showed moderate hydroxyl free radical scavenging activity. This study leads to the conclusion that the polar constituents of roots of this plant exhibited higher antioxidant activity as compared with non-polar constituents.

Conclusion

The phytochemical screening exhibited the presence of various useful compounds in the root extract of *A. conyzoides* including terpenoids, alkaloids, coumarins, sterols, flavonoids, and glycosides. Moreover, the results indicated reasonable antibacterial (against both gram-positive and gram-negative bacteria), antifungal, and antioxidant potentialities, thus favoring the importance of the roots of this plant from a medicinal point of view. Keeping in view the biological activities of the phytochemicals identified in the roots of this plant, it can be stated that this plant could be a potential candidate for modern medicinal formulations.

Conflict of interest

The authors declared that they have no conflict of interest.

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A WEB-BASED CROSS-SECTIONAL STUDY AMONG INDIANS REVEALS A WILLINGNESS SHIFT REGARDING COVID-19 VACCINE UPTAKE AFTER THE SECOND WAVE

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ABSTRACT

This study aimed to determine the shift in willingness regarding the COVID-19 vaccine after the second wave. The study attempts to understand the willingness towards COVID-19 vaccination by assessing the public's knowledge, concerns, and attitude regarding the vaccine. Between May 16 to May 28, 2021, the individuals of the general population were invited to fill the online questionnaire. Total 711 participants had given their informed consent and completed the questionnaire on their background and vaccination behavior-related variables such as knowledge, practices, and their concerns regarding the vaccine. Before the launching of the vaccine, people were less likely to get vaccinated (63.6%); however, once the second wave hit India, attitudes towards vaccines shifted dramatically, and the figure increased to 84.4%. A significant proportion of the population is now willing to take the vaccine. There are several socio-demographic differences regarding knowledge and concerns related to vaccines, especially in age and gender groups. The success of a COVID-19 vaccination program is determined not just by the vaccine's efficacy, but also by its uptake. To ensure optimum vaccination uptake, there is an immediate need for the most effective policy and communication.

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1 Introduction

WHO declared COVID-19 as a global pandemic on March 11, 2020 (World health organization, archived: WHO timeline-COVID-19, 2021) and India reported its first case of COVID-19 in Kerala on January 27, 2020 (Ministry of home affairs, India, https://www.mygov.in/covid-19 accessed on 02.06.2021). Since then, India has been hit by the two waves of COVID-19 and the second surge has been more devastating than the first one. Till the date of manuscript writing of this survey report, the total tally of COVID-19 infected people reached 2,98,27,268 and total causality 3,85,220 (Ministry of home affairs, India, https://www.mygov.in/covid-19 accessed on 02.06.2021). Grappled with the second wave, medical professionals predicted that the third wave will arrive sooner (India could see a third wave in 6-8 weeks if Covid-appropriate behavior is not followed: AIIMS chief Randeep Guleria, 2021). Since there was no effective therapy available at the time, the government urged individuals to take precautions such as hand washing, wearing a facemask, and social distancing however, the latter has taken its toll on the mental health and social well-being of the people (Sulprizio et al., 2021).

These preventive measures were having minimal success in limiting the spread of the deadly COVID-19 disease, and vaccination was the only ray of hope for the population. The fact that vaccination is the only weapon to fight against this pandemic and avoid the accompanying problems has been highlighted in earlier research (Sallam, 2021). There have been 13 distinct vaccinations provided worldwide (across four platforms), with India having three primary vaccines, namely Covaxin, Covisheild, and Sputnik V, which have shown encouraging effects against coronavirus infection (World health organization, Coronavirus disease: Vaccine, 2021). With such a large population, the mammoth task of vaccinating the entire country is another challenge for the Indian government and relevant agencies. However, after the approval of COVID-19 vaccines, the government was trying to vaccinate the people based on risk priority. To maintain continued success, it is necessary to maintain a high level of vaccine coverage. Still, the biggest hurdle before the vaccination drive was the hesitancy of people reported in earlier studies (Dubé et al., 2013).

According to the SAGE working group, vaccine hesitancy signifies the delay in acceptance and refusal of vaccine despite its availability (MacDonald et al., 2015). It was also included in the list of threats to global health in 2019 (Vaccine Hesitancy, 2015). This Vaccination hesitancy poses a significant barrier to achieving herd immunity against the COVID-19 pandemic. To overcome this issue, it is critical to have a positive attitude toward the vaccine, as well as knowledge and perceptions about it. The causality in the second wave, as well as the anticipated threat of the third wave, terrified the general public, causing them to change their minds about getting the COVID-19 vaccine.

In India, there have been few studies on public readiness or apprehension to get vaccinated. The goal of this study was to learn more about India's perceptions after the devastating second wave of COVID-19 hit the country. To begin, we assessed participants' knowledge, attitudes, and practices toward the COVID-19 vaccine. Finally, we looked at how perceptions about the COVID-19 vaccine changed over time, as well as how people thought about COVID-19 vaccination uptake.

2 Materials and Methods

A validated questionnaire with some adjustments was used in the web-based cross-sectional survey of the general populace (Kumari et al., 2021a) The internal consistency of the questionnaire was tested using Cronbach's alpha in Excel, and the result was 0.74, which is acceptable. The sample size was calculated using EpiInfo™ 7.2.4.0 software from the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA. The minimal sample size required to represent the adult Indian population as per the 2011 census (Office of the Registrar General & Census Commissioner, India, 2021) with a 95% confidence interval and an acceptable margin of error of 3.7%, given 50% predicted frequency, was found to be 702. All the statistical analysis (Descriptive statistics and Chi-square test) was performed in SPSS 16 (IBM statistics).

This broad web-based cross-sectional survey was conducted via an online platform between May 16, 2021, and May 28, 2021. The questionnaire was anonymous. The Google form questionnaire was sent using a snowball sampling approach. Investigators sent the Google form link by email, Whatsapp chat, Instagram, and Facebook to their personal and social acquaintances. The questionnaire was written in both English and the respondent's native language, keeping in mind that the respondents came from a variety of educational backgrounds. Respondents were told about the study aims, declaration of confidentiality, and voluntary participation before beginning to fill out the questionnaire. In cases when respondents lacked technological skills, investigators conducted the telephone interview on their behalf and recorded their responses in the Google form. Respondents above the age of 18 belonging to different socio-demographic backgrounds such as age, gender, marital status, chronic disease history, socioeconomic status, and residence were included in the study to fulfill maximum diversity.

3 Results

3.1 Participant's characteristics

In total, 711 participants from 26 different states aged 18 and above took part in the survey. Among these, 312 (43.9%) females and 399 (56.1%) males with different educational backgrounds participated in this survey with the majority having an undergraduate or postgraduate degree. The urban population accounts for 77.7% of the total number. Only 31 people (4.4%) said they had a history of chronic disease, while the remainder said they were healthy. There were 166 (23.3%) participants who had already had the vaccine, but there were still a considerable number of people who had not received the vaccine. The healthcare system was represented by 15.6 % of the participants and more than half of participants were unmarried (Figure 1).

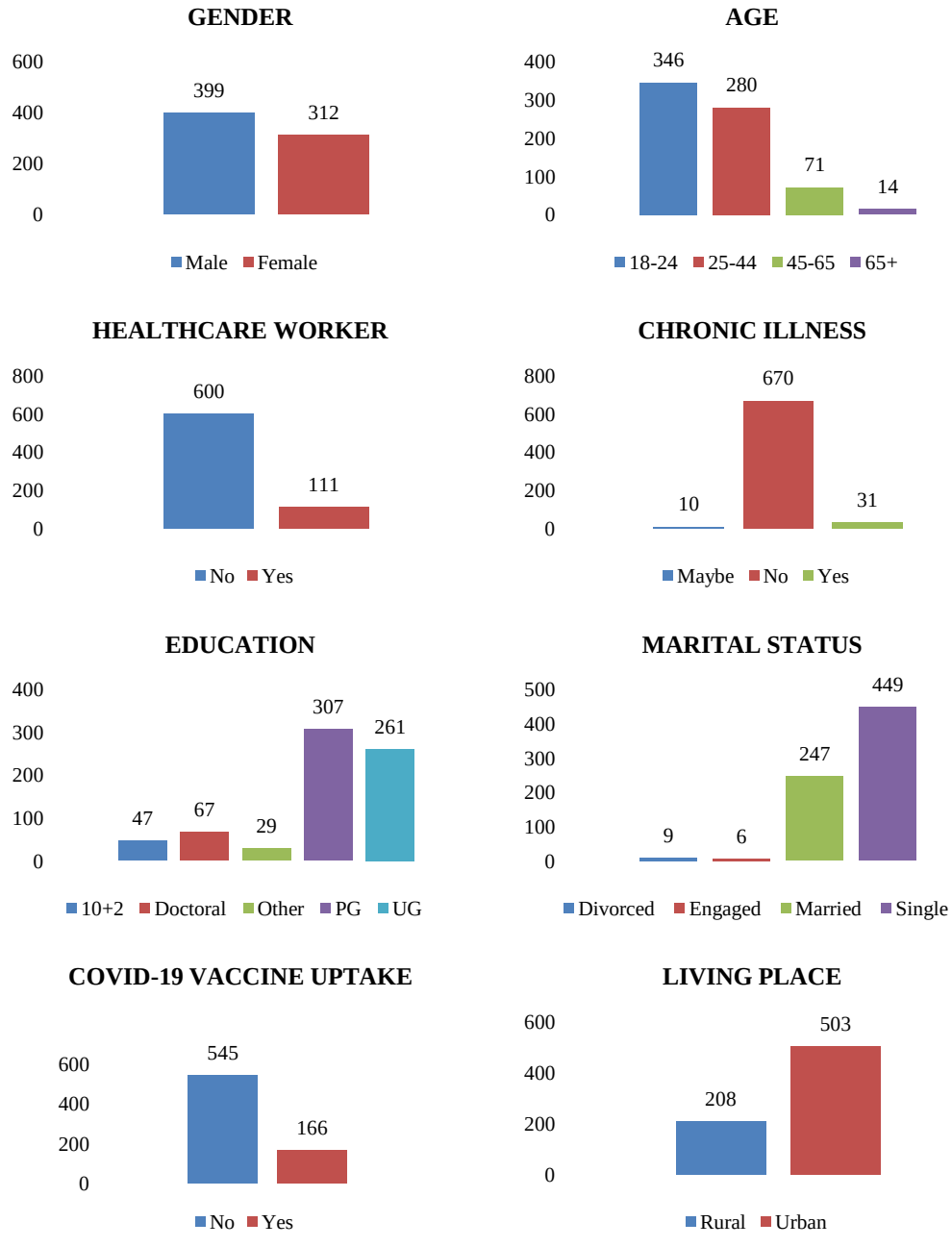

Figure 1 Socio-demographic profile of participants

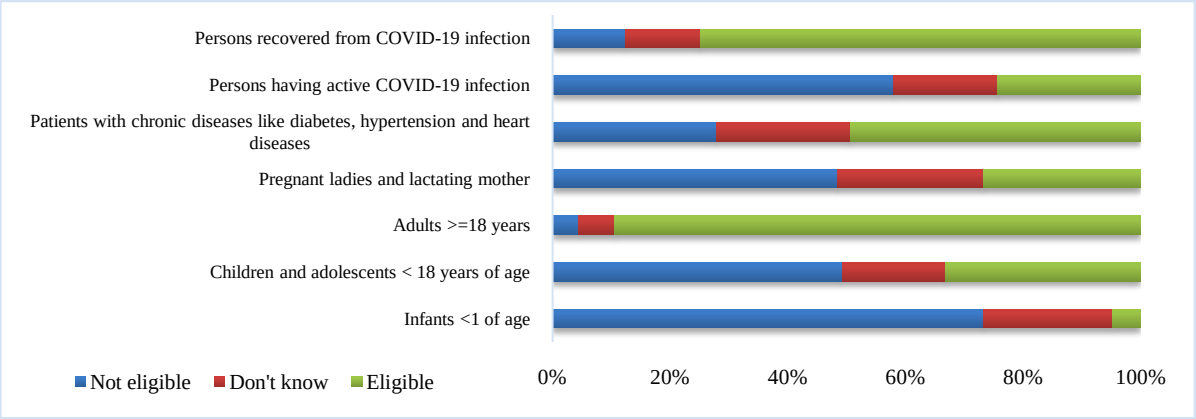

Figure 2 The responses of participants regarding the knowledge associated with COVID-19 vaccination.

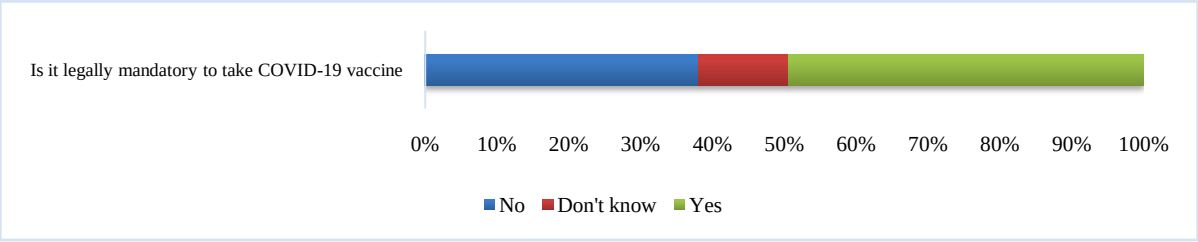

Figure 3 The response of participants regarding the knowledge associated with COVID-19 vaccination.

3.2 Knowledge regarding COVID-19 vaccine

The knowledge regarding the COVID-19 vaccine is quite varied, with the majority of people correctly identifying who should be vaccinated, such as adults (89.4%) and people who have recovered from COVID-19 (74.8%), and who should not be vaccinated, such as infants or children under the age of 18 (49.4%).

However, there is still a lack of information about inoculation for pregnant women and breastfeeding mothers, since the majority of people either don't know (24.9%) or give incorrect answers (26.7%). A similar tendency has been observed in the immunization of children under the age of 18, individuals with chronic illnesses, and people who are infected with COVID-19. The immunization of infants, children, pregnant women and lactating mothers, chronic disease patients, and those with active COVID-19 infection has a significant correlation with where they live ($p<0.05$). People belonging to the urban area were having more knowledge regarding vaccination (Figure 2). The knowledge of pregnant women and breastfeeding mothers about vaccination has been linked to gender ($p<0.01$).

Individuals are perplexed by vaccination's legal requirement, as just 38.11% of people are aware that it is not legally obligated. This is strongly associated with age, location of residence, and education ($p<0.05$), as persons in urban regions with higher age groups and education, have better information than people in rural areas or with lower education (Figure 3).

3.3 Source of information influencing vaccination

During the pandemic, news organizations and social media platforms are the key sources of information about COVID-19 and its prophylaxis. However, it also promotes some falsehoods, making individuals skeptical of the information. Various sources of information, such as news networks (80.9%), government organizations (76.2%), social media platforms (73.3%), discussions among friends and family (85.2%), and healthcare providers (80.7%), have influenced people's decision-making regarding vaccination (Figure 4). The information gained from reputable sources such as government agencies is substantially associated with education ($p<0.05$), as persons with a higher level of education are more likely to believe in a more trustworthy source of information. Because people living in urban regions have more access to diverse social media platforms than people living in rural areas, the influence of non-reliable sources such as social media platforms is strongly associated with residency ($p<0.05$).

Furthermore, additional reputable sources, such as healthcare providers, have shown a significant relationship with age ($p<0.05$). It has been observed that healthcare practitioners have a strong effect on the younger age group (18-24), making it the major obligation of healthcare providers to deliver accurate vaccination information to their patients.

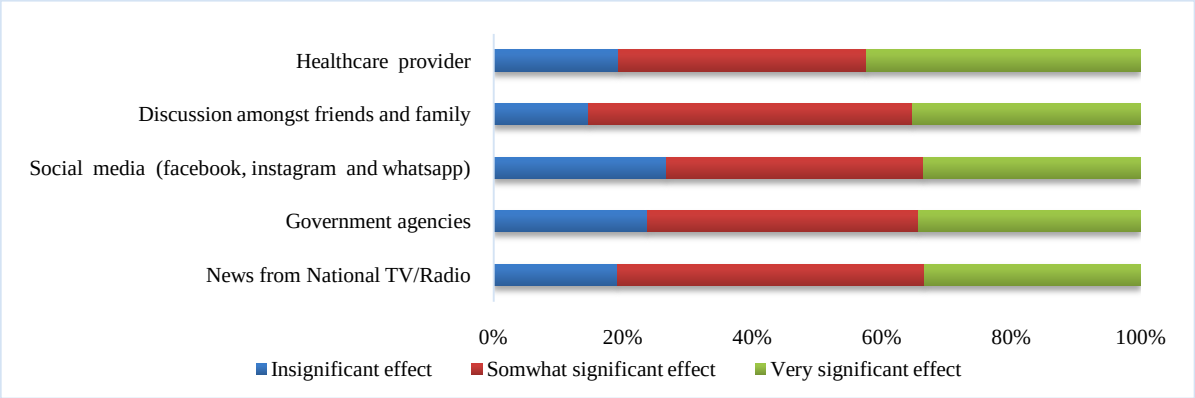

Figure 4 The response of participants regarding the source of information that affect their opinion associated with COVID-19 vaccination.

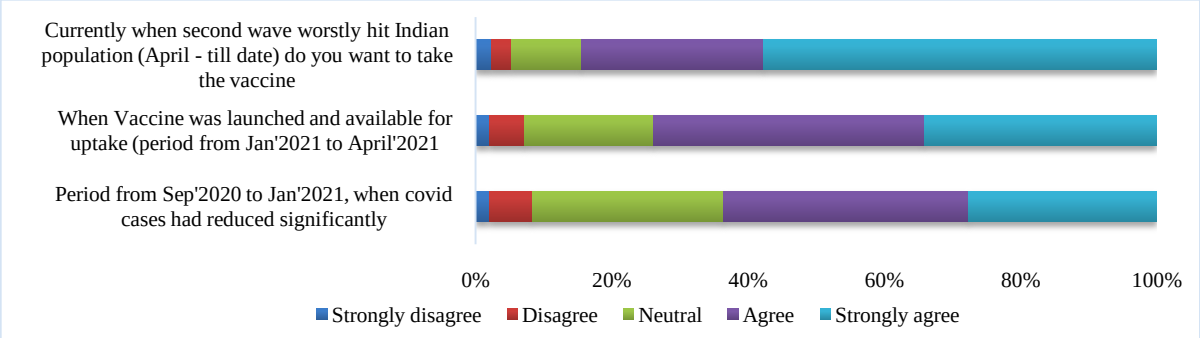

Figure 5 The responses of willingness about COVID-19 vaccine uptake during different period.

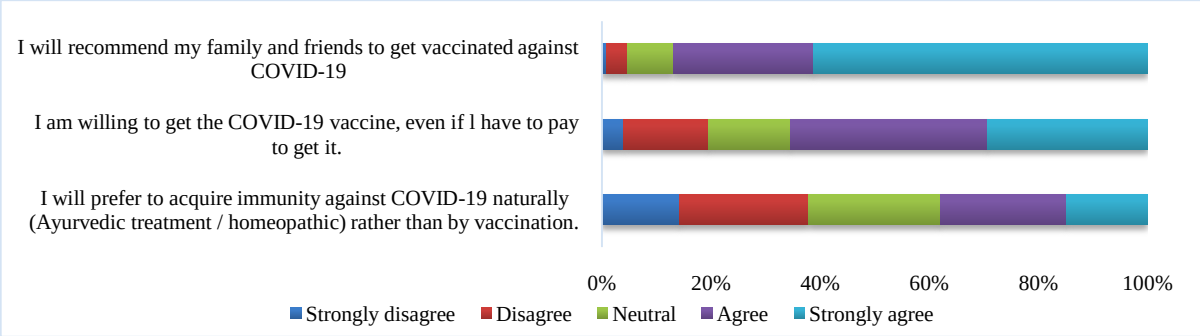

Figure 6 The responses of questions related to different aspect about COVID-19 vaccine.

3.4 Opinions regarding COVID-19 vaccine

From September 2020 to January 2021, Pre-launch of the vaccine, the vaccination was met with a lot of skepticism; just 63.6% of people were willing to take it. The willingness during this period is significantly associated with marital status ($p<0.05$). From January 2021 to April 2021, there was a considerable increase in the number of people wanting to get the vaccine (73.8%). When the second wave of COVID-19 hits India, people's perceptions were radically altered, and 84.4% of the population agreed to take the vaccine (Figure 5).

There is an overall positive attitude towards the vaccination; people were willing to take vaccination even if they had to pay (65.4%) and were ready to recommend it to their family and friend (86.9%) (Figure 6). The willingness to pay for the vaccine is significantly associated with age ($p<0.05$), gender ($p<0.01$), living ($p<0.01$), and marital status ($p<0.01$). However, when it came to naturally acquired immunity, only 38% of people strongly disagreed with the statement, while the rest of the population was quite perplexed; this factor is significantly associated with age ($p<0.01$), living ($p<0.01$), education ($p<0.01$), marital status ($p<0.01$), and socioeconomic status ($p<0.05$).

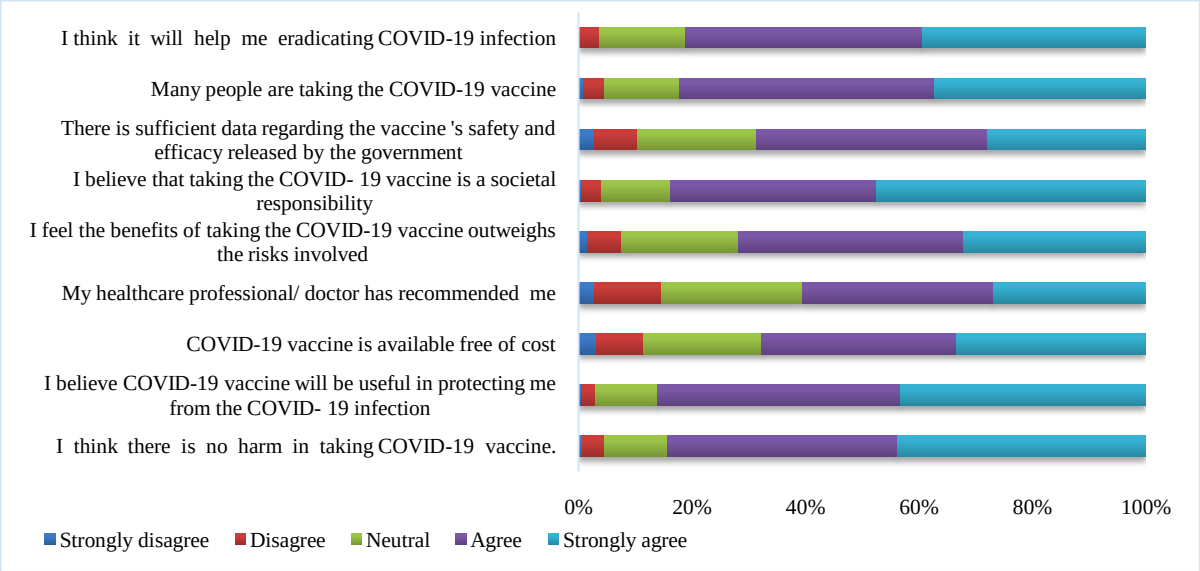

Figure 7 The responses of factors related to decision making about COVID-19 vaccine.

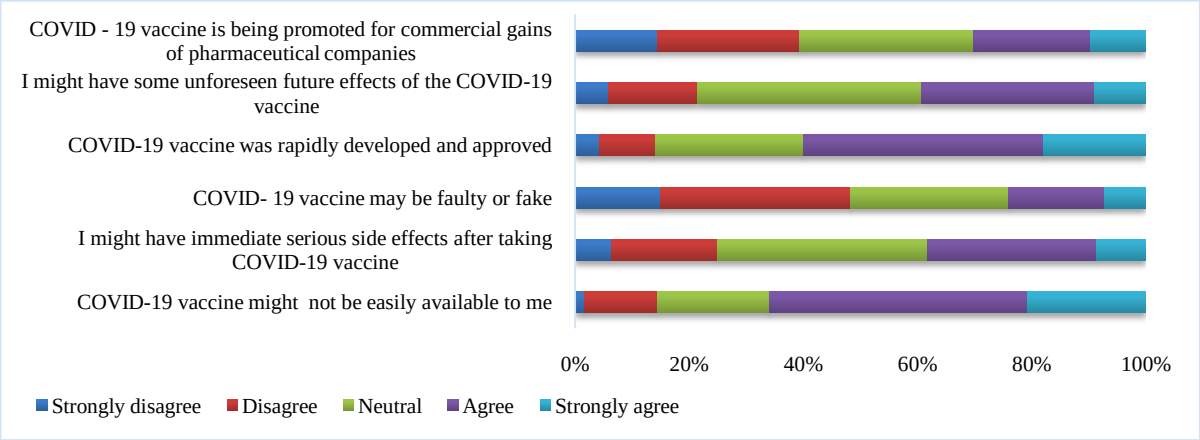

Figure 8 The responses of participants regarding the Concerns associated with COVID-19 vaccination.

3.5 Factors responsible for the decision making of vaccination

Several factors affected the COVID-19 vaccine acceptance among people, such as people believed that there is no harm in taking the vaccine (84.3%), vaccination will help in protecting them from infection (86.1%), there is sufficient data available on the COVID-19 vaccine (68.6%), the vaccine will aid in the eradication of COVID-19 infection (81.2%). Various motivating factors such as no harm in taking vaccine ($p<0.05$), recommendation by the health care professionals ($p<0.05$), its availability for free of cost ($p<0.05$) are significantly associated with age, as the higher the age of the people, the greater the motivation to get vaccinated (Figure 7).

Motivating factors such as the benefits of the vaccine outweigh the risk (71.1%) and people considering it as a societal responsibility (83.8%)are significantly associated with age ($p<0.05$), and living place ($p<0.05$) as better the place of residence, better knowledge regarding the vaccine. People considering it as a societal responsibility is also significantly associated with education ($p<0.01$) as a more educated person will have a better knowledge regarding vaccines (Figure 8).

3.6 Concerns regarding COVID-19 vaccination

Results of the current survey suggested that the participants seemed to have a wide spectrum of opinions. There are several concerns regarding the availability of vaccine (66%), rapid development and approval of vaccine (59.9%), major adverse effects following vaccination (38.2%), undiscovered future vaccine effects (39.4%). The majority of the respondents showed their faith in the COVID-19 vaccine (75.4%), ruling out the possibility that it is flawed or fake.

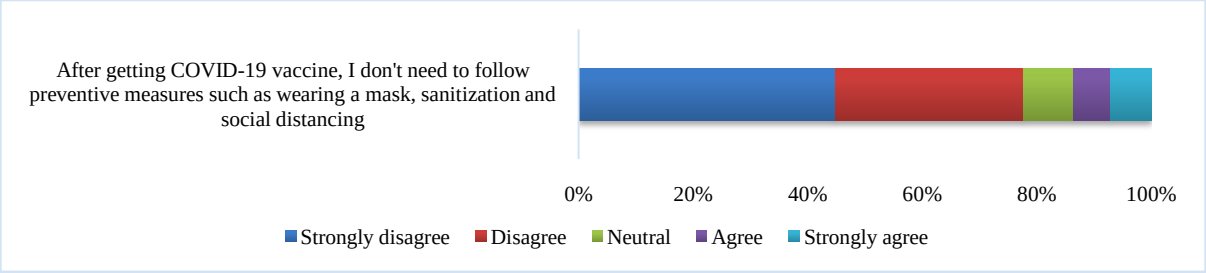

Figure 9 The response of participants related to follow preventive measures even after vaccination.

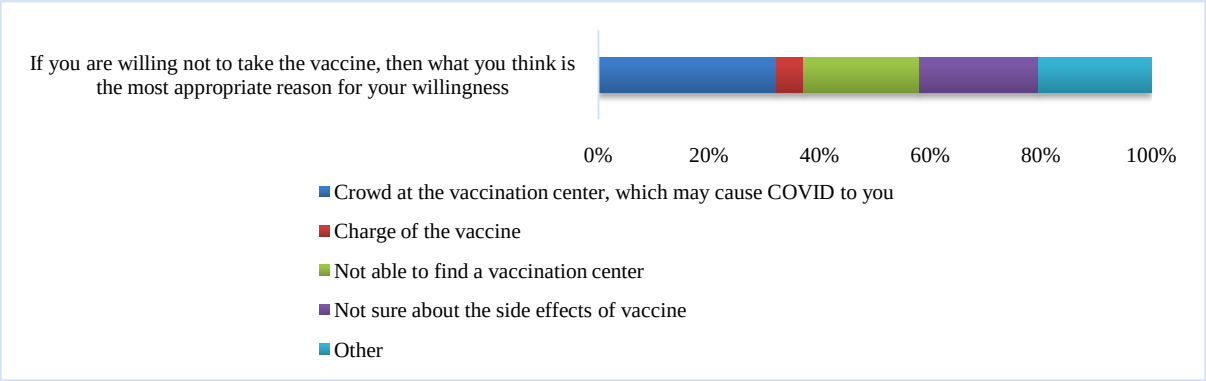

Figure 10 The response of participants about possible reason for reluctancy of COVID-19 Vaccine uptake.

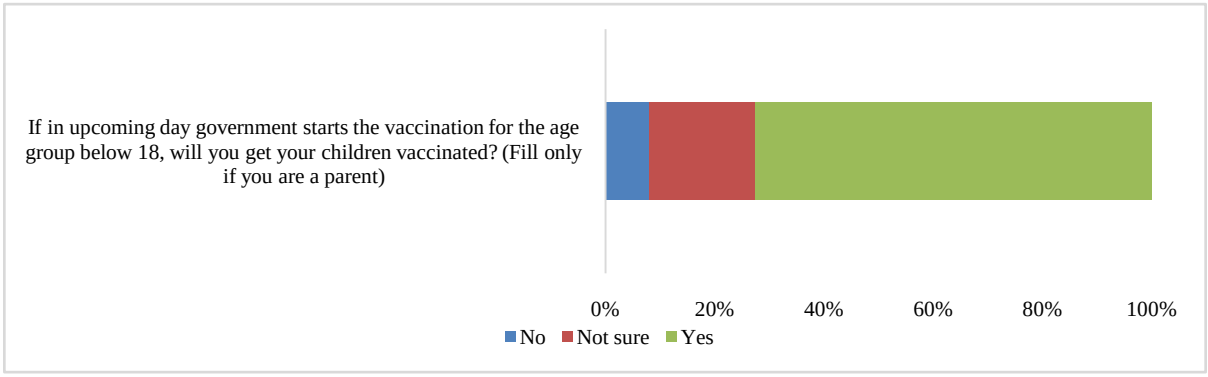

Figure 11 The response of parents about the willingness to get their children vaccinated.

Even after vaccination, the majority of respondents (77.7%) strongly support the idea of implementing all preventive measures, such as sanitization, wearing masks, and social distancing, as recommended by government health organizations (Figure 9).

In response to the question, if you are unwilling to take the vaccine, then what do you think is the most appropriate reason for your unwillingness. Most of the respondents showed their concern about getting infected at the vaccination center (32%). A nearly equal proportion of participants (21%) answered that they would be unable to locate a vaccination center and that they were unsure of the potential negative effects (Figure 10).

The concern regarding the availability of vaccines is significantly associated with age ($p<0.01$), as the lower the age, the more concerns regarding the vaccine. Other factors like the authenticity of the vaccine, the rapid development of a vaccine, unforeseen future effects, a personal gain of pharmaceutical companies, and following preventive measures are significantly associated with residence ($p<0.01$) as more the backward area, lesser the knowledge regarding the vaccine. The authenticity of the vaccine was also associated with education ($p<0.05$), as the more educated the person, the greater the queries regarding the vaccine. Factor like promoting personal gain of pharmaceutical companies is also associated with marital status ($p<0.05$) and socioeconomic status ($p<0.05$) as more the socioeconomic status, more the concerns.

4 Discussion

This web-based cross-sectional survey was done to investigate the paradigm shift in COVID-19 vaccination uptake and try to connect it to people's knowledge, opinions, and concern about COVID-19 immunization during the pandemic's second wave. The total number of participants who participated in this survey is 711 to complete the validated questionnaire based on their knowledge regarding the COVID-19 vaccine.

This study has found some similarities and dissimilarities regarding the willingness of vaccine uptake and consistent with the previous studies, the intent of vaccine uptake is associated with various socio-demographic aspects such as age, gender, place of residence, and education. (Kumari et al., 2021b)

There is a myriad of information sources that have a significant impact on vaccine knowledge. Even though the majority of the population (80.9%) trusts credible sources of information, the information obtained through social media is a subject of concern. It was highlighted in previous studies that those who obtained their information from social media are less likely to get vaccinated (Wilson & Wiysonge, 2020). This necessitates the communication of accurate vaccine information, particularly to persons who live in rural regions and have a lower socioeconomic standing. Results of the current study revealed that healthcare workers played quite a significant role in spreading factual information regarding the vaccine. This result is following a study in France which reveals that 72.4% of the participating health care are willing to get vaccinated, and 79.6% of them are eager to recommend it to their patients (Janssen et al., 2021).

Our result indicated that the majority of our participants are willing to accept the vaccine (84.4%) which indicate that India has a higher vaccine acceptance than the U.S. (56.9%) (Reiter et al., 2021) and France (58.9%) (Schwarzinger et al., 2021) but much lesser than countries like Indonesia (93.3%) (Harapan et al., 2020) and China (91.3%) (Wang et al., 2020).

The people of 45-65 years living in the urban area show more willingness to take vaccines even if they have to pay for them and are willing to recommend them to their family members.In line with the study conducted in China by Zhang et al. (2020), the parents in our study expressed a strong desire (71.9%) to get their kids vaccinated as soon as it became accessible for children below age 18 (Figure 11).

Current knowledge and intention of vaccine uptake are generally higher than reported in the previous studies in India (Kumari et al., 2021b) as well as in Australia (Seale et al., 2021) and UK (Sherman et al., 2021). This is most likely due to a massive rise in corona cases during the second wave, which prompted people to learn more about the COVID-19 vaccine and change their habits. This might be the possible reason for the change in willingness about COVID-19 vaccine uptake.

The study revealed that the willingness to vaccine uptake increased however, the lack of knowledge regarding eligible candidates for the vaccine is a significant hurdle in the vaccination drive. People residing in rural areas having lesser education can be an essential factor for this. There is a high tendency towards acceptance of vaccine uptake in middle and high socioeconomic status populations.

Several facilitators that influence the vaccine uptake include the availability of vaccines at no cost, the opinion of their healthcare workers, the risk and benefits ratio of the vaccine, and its usefulness in eradicating the infection. A large number of participants felt that it is their social responsibility to get vaccinated. The zeal to eradicate this infection is the key driver of this mission and can be associated with vaccine acceptance. Though many factors influence vaccine acceptance, our study showed that there are a few concerns that may prevent people from getting vaccinated, such as vaccine availability and authenticity, serious side effects or unforeseen future effects, and pharmaceutical companies promoting commercial gain. Several additional research, similar to ours, have reported the same role of barriers and facilitators in vaccination campaigns (MacDonald et al., 2015; Graichen, 2021; Seong et al., 2021; Fisk, 2021; Saied et al., 2021).

Conclusion

This is a web-based cross-sectional survey that assessed the change in public intention towards vaccine acceptance during the pre-launching of vaccine to the second wave of COVID-19 periods. As the consequences of the second wave were terrifying, leading to high mortality. This changed the intent of participants about COVID-19 vaccine uptake. Several risks and trust factors are significant predictors of the intention of vaccine uptake. Vaccine apprehension is complicated and context-dependent, altering across time, place, and vaccine, as stated by the SAGE working group. It is critical to keep an eye on factors like complacency, convenience, and confidence as we are approaching the third wave. Aside from that, the Indian government is prioritizing vaccination based on several factors, including age, vaccination of healthcare workers, and immunization of frontline workers. Because other countries have either experienced or are experiencing the third wave, the Indian government should be prepared. It is critical to vaccinate an increasing number of people.

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Disclosure statement

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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ROLE OF COPPER METAL AND BIOLOGICAL MARKERS IN PATIENTS WITH ENTEROBIASIS

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Enterobiasis

Diarrhea

Copper

IgE

TRFC

Kufa

Iraq

ABSTRACT

The primary goal of this study was to determine the effect of copper in patients with *Enterobius vermicularis* infection, as well as its association with IgE and TRFC. The study was carried out with 583 suspected and thirty healthy respondents of the same age who visited the AL-Zahra maternity and pediatrics laboratory, AL-Hakeem hospital, in AL-Najaf province(Iraq) from July 2020 to June 2021. This study aimed to estimate the level of copper, IgE, and TRFC in patients suffering from enterobiasis. The concentration of three biomarkers (copper, IgE, and TRFC) in serum was determined using the ELISA technique as per the manufacturer's instructions while the concentration of copper was assessed by using the colourimetric method. Results of the study revealed that the concentrations of IgE and TRFC significantly increased (P≤0.05) in the blood sample of *E. vermiculris* infected patients, while the serum concentrations of copper decreased significantly (P≤0.05) compared to the control group. The results of the current study suggested that the *E. vermicularis* infection alters serum IgE and TRFC concentrations, which significantly impacts copper levels in the blood.

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1 Introduction

Enterobiasis, also known as oxyuriasis, is a common infection caused by the helminthic parasite *Enterobius vermicularis*, which has a global distribution but is most prevalent in temperate areas (Mohammad & Koshak, 2011; Meletis et al., 2019). *E. vermicularis* is a parasitic helminth that infects about 300 million people globally, that making it the most common human parasitic helminth infestation (Dezsényi et al., 2018). *E. vermicularis* spreads via the fecal-oral route when a patient ingests eggs previously present in the perianal area of infected individuals (Meletis et al., 2019).

Many disorders such as malnutrition, intestinal disorders, and intestinal malabsorption are associated with copper metal deficiency (Stephenson et al., 2000). To achieve optimal growth, better immune response, and intellectual aptitude, intestinal parasite infections must be diagnosed and treated at the early stage (Pegelow et al., 1997; Stephenson et al., 2000). Furthermore, Koltas et al. (1997) suggested a significant effect of parasite infection on the mean copper concentration, which explained that the mean level of serum copper was considerably lower in the E. vermicularis infected group than the control group children. T-helper (Th) cells differentiate into Th1, Th2, Th17, and T-regulatory (Treg) subpopulations due to innate immunological identification, immune stimulation, activation, and their subsequent functional contact with B lymphocytes (Abd-Aljabar & Aljanaby, 2021). The presence of intestinal helminths stimulates the immune system, it responds with a type-2 directed response that attempts to expel the worms (Patsantara et al., 2016). T-helper (Th2) cytokines such as interleukin 4, 5, and 13 are commonly used in the immune response against pinworms (Sulk & Ehrchen, 2019; Rashad & Aljanaby, 2021). During pinworm infection, eosinophils and immunoglobulin E (IgE) levels notably rise in the youngsters (Dawood et al., 2016; Kara & Volkan, 2018). Intestinal parasites use host carbohydrates, lipids, minerals, vitamins, and other food sources to get essential energy for the life cycle. These minerals have an important role in the human body's metabolism and physiology, especially in growing children. Several studies have been reported that mineral elements such as copper and zinc have a vital role in children's regular growth and development (Hesham et al., 2004). The significant reduction in serum copper level in parasite-infected children is probably due to malabsorption and anorexia (Uwah et al., 2012). To understand the relationship between the parasite and human immune response especially on the level of copper, IgE, and TRFC, this study was carried out in patients with enterobiasis.

2 Materials and Methods

In the current study, identification of *E. vermicularis* infections was based on the various visible clinical symptoms such as severe anal itching (especially at night - the most common), irritability, lack of appetite, abdominal pain, urinary tract infections, yellowing of the face, and weight loss. The samples were collected from the 583 possible respondents from July 2020 to June 2021 and identified using a light microscope and wet mount preparation for adult pinworms or eggs detection at the microbiological laboratory, department of biology, college of science, Al-Kufa University's. To detect the presence of *E. vermicularis* eggs, all faeces samples were examined using the saline wet mount technique, which involved placing a drop of saline (0.9 percent) on a glass slide. For this, a small amount of faeces samples were spread over the glass slide, mixed with a saline drop, covered with a coverslip, and observed at 100 X and 400 X, magnifications under a light microscope. Positive samples were defined as those that contained one or more eggs; all positive samples were frozen at -20 °C to preserve them for further analysis (Garcia et al., 2018; Al-labban et al., 2021).

2.1 Serum biomarker detection

From the selected 583 respondents, 60 were found positive for the *E. vermicularis* infection, blood samples of these 60 patients and 30 healthy youngsters who served as controls were collected for the serum collection and further analysis. The ELISA technique (Human reader, Germany) was used to determine the concentration of three biomarkers in serum (copper mg/dl, IgEng/ml, and TRFC ng/ml), as suggested by the manufacturer Company. The presence of the copper is assessed using the colourimetric method (Al-Hadraawy et al., 2016; Azeez el al., 2021) and BTLAB provided these biomarkers units with Cat.No. E0188Hu, and Cat.No.E0259Hu respectively.

2.2 Statistical analysis

The healthy and control groups were compared using Graph-pad prism version 10-computer software (T-test). Statistical significance was defined as a P-value of less than 0.05 (Al-Hadraawy et al., 2016; Al-Hadraawy et al., 2019; Mohy et al., 2019).

3 Results and Discussion

Results of this study are presented in table 1 and suggested a significant increase (P = 0.05) in the concentrations of IgE (ng/ml), and TRFC (ng/ml) in the *E. vermicularis* infected patients as compared to the healthy control while a significant reduction was reported in the concentration of copper (mcg/dl). Further, no significant gender effect was reported in the current study, and both males and females are showing similar values of IgE, TRFC, and copper. Overall, the concentration of IgE, TRFC, and copper was reported 373.39 (ng/ml), 972.93 (ng/ml) and 90.08 (mcg/dl) in the total patients infected by *E. vermicularis* while this value was reported 299.09 (ng/ml), 739.09 (ng/ml) and 128.17 (mcg/dl) respectively in the healthy control.

Table 1 Effect of *E. vermicularis* infection on the selected biological markers

Respondents	Biological Markers		
	Copper (mcg/dL)	IgE (ng/ml)	TRFC (ng/ml)
Male (Patients)	89.75±0.92	372.46±4.68	961.96±12.10
Male (Control)	130.20±2.23	321.24±16.42	749.31±9.50
Female (Patients)	90.42±0.89	374.33±4.42	983.91±10.53
Female (Control)	126.13±1.26	276.95±7.33	728.75±22.09
Total Patients	90.08±0.64	373.39±3.19	972.93±8.09
Total Control	128.17±1.31	299.09±9.75	739.03±11.97

As compared to the control group, the concentration of copper in males and females infected with *E. vermicularis* infection was significantly lower. This could be attributed to intestinal parasites that convert the host's carbohydrates, lipids, minerals, vitamins, and other dietary sources into necessary energy for the life cycle (Hesham et al., 2004). Minerals (copper, zinc, and magnesium) are essential for growth and development in developing children and play an essential role in the human body's metabolism and physiology (Hesham et al., 2004; Ramana, 2012). These findings are consistent with the findings of Culha & Sangün(2007) and Arbabi et al. (2015), those who found a significant reduction in the serum copper levels in children infected with enterobiasis. Similarly, Uwah et al. (2012) reported lower serum zinc, copper, and magnesium level in patients with parasite infection as compared to the control group. While the results of the current study are contradictory to the finding of Sadraei et al. (2007), those who reported significantly higher serum copper levels in patients infected with *E. vermicularis* intestinal infection as compared to the healthy control in Hatay Province.

The results of the study showed that IgE concentration in males and females infected with *E. vermicularis* was significantly higher than the control group; this could be due to allergic and pruritus action caused by enterobiasis infection (Arruda & Santos, 2005; Schroeder et al., 2018). Chronic infection with geohelminths causes polyclonal activation which resulted in high serum IgE levels (Scrivner et al., 2001; Sales et al., 2002). The helminths boost antiparasite IgE production and accelerate polyclonal IgE synthesis, which results in extremely high levels of total IgE in the bloodstream (Cooper et al., 1998). Although the link between helminthic infections and IgE antibody levels is controversial, IgE antibody is an essential component of the immune response against parasites (Allen & Maizels, 1996). IgE appears to play a role in the host's defenses against toxins, as tissue injury appears to be the primary trigger of immune responses in Th2 cells (Kelly & Grayson, 2016; Mukai et al., 2016). Similarly, various previous studies also suggested that patients infected with *E.vermicularis* had higher levels of IgE than those in the control group (Ganguly et al., 1988; Zarebavani et al., 2012; Al-kabee et al., 2014; Solmaz et al., 2018). Khazaal et al. (2020a) explained that immunoglobulin levels increased dramatically, with a mean of 227.07 IU/ml compared to 48.66 IU/ml in the control group. While the results of the current study are contradictory to the findings of Essawy et al. (1989), Delialiglu &Nutman (2004), and Al-Daoody& Al-Bazzaz (2020), those who reported no significant effect of the *E. vermicularis* on the levels of serum total IgE.

Th2 cell' response determines the acquired immunity of the host against the worms, other infections, poisons, and hazardous chemicals (Al-Hasheme et al., 2020). The results demonstrate that the concentration of TRFC in males and females infected with *E. vermicularis* was significantly higher than in the control group. This could be because infection hinders iron absorption from the gastrointestinal tract (Stoltzfus et al., 1997; Olsen et al., 1998; Tomanakan et al., 2020; Khazaal et al., 2020b).

These findings are differing from the results of Kuvibidila et al. (1995), those who found that TRFC levels are unaffected in *Plasmodium falciparum* infection. At the same time, the findings of the current study were consistent with those of Kuvibidila et al. (1995) those who reported lower TRFC levels in patients with acute malaria. Also, the study by Mockenhaupt et al. (1999) found that mean TRFC values were higher in *P. falciparum* infected children as compared to the non-infected children. Similarly, Stoltzfus et al. (1997) and Verhoef et al. (2001) found an increase in sTfR concentrations with parasite density in children with malaria infection. These researchers reported that sTfR concentrations increased with parasite density in children with asymptomatic malaria Infection. According to Hung et al. (2007), parasites do not influence the level of the transferrin receptor in parasite-infected individuals.

Conclusions

Results of the current study can be concluded that *E. vermiculris* infection has a significant effect on the various biomarkers and the children infected with *E. vermiculris* had lower copper levels and higher IgE and TRFC levels in the blood serum.

Conflict of Interest: Nil

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PRELIMINARY STUDY ON POTENTIAL EDIBLE COATINGS DERIVED FROM CARBOXYL METHYLCELLULOSE AND FUNGI CULTURED METABOLITES ON THE SHELF-LIFE EXTENSION OF SWEET-ORANGE (*CITRUS SINENSIS*)

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Synergistic effects

ABSTRACT

This study focused on assessing the potential of formulated edible coatings derived from a metabolite of *Trichoderma viride* and *Penicillium chrysogenum*, combined with carboxyl methylcellulose (CMC) on the postharvest storage quality of orange fruits. The cultured metabolite of fungal bioagents combined with CMC, as well as glycerol (plasticizer), inadequate solution ratio based on wettability, was evaluated for microbiological quality and shelf-life extension of sweet orange. Thereafter, ascorbic acid, total soluble solids, pH, percentage weight loss, among other parameters were assessed for 7 weeks. The results of the study revealed that the pH of CMC + *Trichoderma viride* and CMC + *P. chrysogenum* coatings had 3.8 ± 0.02 and 3.17 ± 0.06 respectively, while it was reported 2.90 ± 0.04 for uncoated treatment. Also, the ascorbic acid and total soluble solids of the edible coated oranges were higher than the control. In addition, the percentage of weight loss was higher in the uncoated control compared to the potential edible coated oranges. Further, the microbial load count of the potential edible-coated oranges was less compared to the un-coated oranges. In conclusion, this formulated potential edible coating could be further improved upon and optimized for use in prolonging the storage of sweet oranges.

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1 Introduction

The foremost fruit crop produced globally is citrus. It is cultivated in most of the nations of the world with well over a hundred million tons (Mahawar et al., 2020). Citrus fruit is globally accepted for its impact on human well-being. It is widely available and possesses enhanced dietary, nutritional, and other value-added bioactive properties along with superior taste (El-Otmani et al., 2011; Ghoshal, 2019). According to Mishra & Patel (2020) consuming enough citrus fruits enhanced immunity and can reduce the disease incidence in human beings. Recently, the COVID-19 outbreak led to an increase in demand for fresh fruit worldwide.

However, yearly a significant percentage of harvested fruits are spoiled by pathogenic organisms (Onah et al., 2019). Post-harvest spoilage pathogens lead to the citrus fruit spoilage globally and make a loss in their economic and aesthetic value (Shi et al., 2018). Sweet oranges are prone to microbial infestations during the postharvest process of storing and transporting the fruit (Zhou et al., 2018). The optimum potential of commercial cultivation and consumption of citrus fruit in terms of value-added therapeutic advantages and economic impacts are being threatened due to the problem of postharvest microbial infestations, and lack of good post-harvest handling facilities, thereby leading to huge losses financially (Terao et al., 2017). It is estimated that food that is lost yearly throughout the world is in the range of over a billion tons (Ishangulyyev et al., 2019). Also, about 35% of fruits and vegetables are part of these losses in a lot of countries.

Despite this challenging scenario, the collection of research funds is mainly focused on productivity enhancement of the foods (up to 95 %), while only 5% of investment is directed at research on food losses (Kitinoja & Alhassan, 2012). The approach that is currently used in combating postharvest diseases of citrus fruits is by applying synthetic fungicides spray (Costa et al., 2019). However, there are various drawbacks associated with synthetic fungicides uses on edible orange fruits due to the cost implications, environmental pollution influence, and it also poses health hazards to mankind if some of these chemical residues remain on the orange fruit peels.

Hence, the trending scenario all over the world is an increase in public demand for a reduction in the use of fungicide, to produce healthy and eco-friendly safe “fruits”, without chemical inputs for combating postharvest infestation of fruits (Padmaperuma et al., 2020). Metabolic activities such as transpiration and respiration occur in fresh fruits after their harvesting; thereby inducing textural changes and loss of water (Jalali et al., 2020; Nourozi & Sayyari 2020). Thus, minimizing this bioactivity of fruits and vegetables postharvest is required through actions like the use of edible coatings thereby creating barrier/permeability to gaseous exchange (Md Nor & Ding, 2020).

Edible coatings are a sustainable alternative that could be used as a postharvest treatment method for the benefit of perishable vegetables and fruits by extending their shelf life (Abhirami et al., 2020; Jafarzadeh et al., 2021). Edible coatings (Carboxymethyl-cellulose and Fungi cultured metabolites) are good alternatives to combat the menace of post-harvest losses of fruits and vegetables, thereby extending their shelf life (Amiri et al., 2021; Iniguez-Moreno et al., 2021). They are natural and eco-friendly options for tackling this huge problem (Tosati et al., 2017). However, the optimum result is not always attainable with just a single source of coating materials. Research reports indicated that edible coating materials that are combined synergistically with other bioactive materials and compounds perform better (Tosati et al., 2018; Yang et al., 2019).

This study is, therefore, focused on assessing the potential of deploying carboxyl methylcellulose in combination with cultured metabolites from two well-known rhizosphere-competent filamentous fungi, as bioactive forming coatings to extend the shelf-life of sweet oranges.

2 Materials and Methods

2.1 Plant materials

Sweet oranges (*Citrus sinensis*), without any diseased or injury symptoms, were freshly collected from an orchard and were quickly transported to the laboratory for processing. The oranges were rinsed under tap water and followed by surface sterilization with 3% hypochlorite solution and 70 % ethanol, and finally rinsed with sterile distilled water.

2.2 Preparation of Carboxyl methylcellulose solution

Carboxyl methylcellulose (CMC) sodium salt powder 1g/L was hydrolyzed in sterile water at 70° C for 2 h, with constant stirring to obtain 1% (W/V) carboxyl methylcellulose solution.

2.3 Culturing of Fungi in submerged liquid fermentation and extraction of metabolites

Freshly prepared mycelia of *T. viride* and *P. chrysogenum* that was characterized in a previous study (Omomowo et al., 2020) were used to obtain the fermented cultured metabolites. The submerged liquid fermentation processing was done by using potato dextrose broth, under shaking incubation conditions at 150 rpm for 7 days at 28°C, to obtain the bioactive cultured metabolites used for bioactive coatings.

2.4 Postharvest treatment and storage conditions of oranges

The oranges were subjected to three treatments, using a combination of an equal volume of cultured metabolites of *T.*

viride and *P. chrysogenum* combined with a 1% (W/V) carboxyl methylcellulose solution. The third treatment was the control experiment that entails dipping the oranges in sterilized distilled water only. The oranges were processed with the bioactive coating solutions as per the Jiang et al. (2005) protocol. Treated oranges were stored at 25℃ with a relative humidity between 70-80 % for the seven weeks.

2.5 Determination of the firmness of the orange fruits

The firmness of the treated fruits was determined using a penetrometer probe (Gong et al., 2020). This was calculated as the maximum force (N) attained for tissue breakage. The result obtained with this probe was delineated in Kg / cm^{-2}.

2.6 Determination of Percentage loss in weight of orange

Loss in the weight of the treated and stored oranges was calculated by weighing orange fruit at the beginning of the experiment and reweighing it at the end of each week. It was calculated and expressed as a percentage, using the following equation.

$$\text{Weight loss (\%)} = (M1 - M2)/\,M1) \times 100$$

Where M_1= Weight of orange fruit before bioactive coatings and storage.

M_2 = Weight of orange fruit after bioactive coatings and storage in grams (Adetunji et al., 2018).

2.7 Determination of pH

After orange fruit firmness analysis, the oranges were cut into small pieces (approximately 10 g equivalent) and homogenized in a grinder, while the supernatant was diluted with 100 ml of distilled water and filtered. The solutions were titrated (phenolphthalein used as an indicator) to the endpoint of pH 8.2 and the acidity result was taken as citric acid percentage equivalence. The pH of the samples was assessed using a pH meter (MonjazebMarvdashti et al., 2020).

2.8 Determination of the Total SSC, and Ascorbic acid content

The total soluble solid content of the oranges was evaluated using a hand refractometer with drops of orange fruit juice that had been previously extracted (Abd Allah et al. 2011).

Also, the ascorbic acid content was measured using the 2,6 – dichlorophenolindophenol dye method (Deepa et al., 2006). Titration of the juice with Trichloroacetic acid (TCA) led to the color changing to pink. The results were delineated as ascorbic acid equivalent (AOAC, 2006).

2.9 Microbiological analysis

The effects of the bioactive forming coatings on the oranges were determined by doing a total microbial plate count for bacteria, yeasts, and mold. For this, 1g of the treated and stored oranges were cut and dissolved in 9 ml of sterile distilled water. 10-fold sample dilutions were carried out and inoculated on PDA medium and glucose yeast extract agars incorporated with antibiotics, and then it was incubated at 28 ℃ and the colony-forming units (CFU) of the incubated plates were counted after 5 days. Also, the total mesophilic and psychrophilic bacteria were determined following the same protocol on plate count agar, but incubation was for 48 hrs at 37 °C. The (CFU) was observed in triplicate and represented as CFU × g^{-1} (Sogvar et al., 2016; Shah et al., 2017).

2.10 Data analysis

The data analysis was subjected to Duncan's test which provided the significance level for the differences of any pair of means.

3 Results

3.1 Fungal metabolites and carboxyl-methylcellulose as bioactive coatings

The potential of the bioactive edible coatings, obtained from the cultured filtrates of the fungal bioagents, when combined with carboxyl methylcellulose was screened for the ability to increase the shelf life of orange by maintaining its post-harvest qualities. The experiment was conducted for 7 weeks. The results obtained indicated that the potential bioactive edible coatings gave better physiological and microbiological results after 7 weeks of postharvest treatment storage compared with the control. For illustration, at the end of the 7 weeks, the ascorbic acid level in the orange fruits coated with CMC + *T. viride* solution was 5.8 ± 0.04 while it was reported 4.7 ± 0.04 for the CMC + *P. chrysogenum* bioactive solution (Table 1). These were better ascorbic acid content results, compared to the control having (4.0 ± 0.04). Also, the results obtained for the total soluble content of the orange fruits due to postharvest treatment with the potential bioactive edible coatings, indicated that after 7 weeks, the total soluble solid of fruits coated with CMC + *P. chrysogenum* bioactive solution had the highest value of 3.22 ± 0.01, followed by the CMC +*T. viride* (3.1 ± 0.02) and control (2.90 ± 0.01). The result showed that potential bioactive edible-coated fruits performed significantly ($p < 0.05$) better than the control after seven weeks of observation (Table 2). In addition, the effects of the potential edible coatings derived from carboxyl methylcellulose with metabolites of *T. viride* and *P. chrysogenum* on the pH of the postharvest treated orange fruits are shown in Table 3. After the 7 weeks of experimentation, the pH of fruits coated with CMC + *T. viride* solution had the

highest pH value of 3.8 ± 0.02, this was followed by CMC + *P. chrysogenum* bioactive solution (3.17 ± 0.06) and the lowest pH value was observed in the control (2.90 ± 0.04). The result showed that potential bioactive edible-coated fruits performed better compared to un-coated control fruits. Furthermore, the resultant effects of potential bioactive edible coatings with carboxyl methylcellulose solution, combined with the cultured metabolites of *T. viride* and *P. chrysogenum* on the percentage weight loss of the orange fruits are illustrated in Table 4. After seven weeks of experimental storage, it was reported that potential bioactive edible-coated fruits performed significantly ($p < 0.05$) better than the uncoated orange fruits, and the highest value of weight loss was reported 53 ± 0.76 percentage for the orange fruits used as uncoated control while this value was reported 44.0 ± 0.4 and 37 ± 0.4 for the CMC + *P. chrysogenum* and CMC +*T. viride* bioactive edible-coated respectively.

Table 1 Effect of potential bioactive edible coatings derived from carboxyl methylcellulose and fungal metabolites on ascorbic acid content of orange fruits

Week(s)	*T. viride* filtrates + 1% (CMC) v/v (CFU/g)	*P. chrysogenum* filtrates + 1% (CMC) v/v (CFU/g)	Control (Sterile distilled water)
1	7.8 ± 0.05^{a}	7.8 ± 0.04^{a}	7.8 ± 0.01^{a}
2	7.5 ± 0.04^{b}	6.3 ± 0.03^{b}	6.0 ± 0.15^{b}
3	7.4 ± 0.04^{b}	6.0 ± 0.02^{c}	5.9 ± 0.04^{b}
4	7.0 ± 0.01^{c}	5.7 ± 0.04^{d}	5.0 ± 0.02^{c}
5	6.5 ± 0.04^{d}	5.3 ± 0.08^{e}	4.7 ± 0.08^{d}
6	6.3 ± 0.06^{d}	5.1 ± 0.04^{e}	4.3 ± 0.08^{e}
7	5.8 ± 0.04^{e}	4.7 ± 0.04^{f}	4.0 ± 0.04^{f}

Values are means ± standard error. Means with similar alphabetical superscripts within a column is non-significant at ($P < 0.05$)

Table 2 Effect of potential bioactive edible coatings derived from carboxyl methylcellulose and fungal metabolites on the total soluble solids content of orange fruits

Week (s)	*T. viride* filtrates + 1% (CMC) v/v (CFU/g)	*P. chrysogenum* filtrates + 1% (CMC) v/v (CFU/g)	Control (Sterile distilled water)
1	5.53 ± 0.01^{a}	5.53 ± 0.04^{a}	5.53 ± 0.01^{a}
2	5.42 ± 0.01^{b}	4.21 ± 0.01^{b}	4.21 ± 0.01^{b}
3	5.32 ± 0.01^{c}	4.01 ± 0.01^{c}	3.78 ± 0.01^{c}
4	4.92 ± 0.01^{d}	3.92 ± 0.01^{d}	3.51 ± 0.01^{d}
5	4.75 ± 0.01^{e}	3.71 ± 0.01^{e}	3.42 ± 0.01^{d}
6	4.62 ± 0.01^{f}	3.41 ± 0.01^{f}	3.11 ± 0.04^{e}
7	3.10 ± 0.02^{g}	3.22 ± 0.01^{g}	2.90 ± 0.01^{f}

Values are means ± standard error. Means with similar alphabetical superscripts within a column is non-significant at ($P < 0.05$).

Table 3 Effect of potential bioactive edible coatings derived from carboxyl methylcellulose and fungal metabolites on pH of orange fruits

Week(s)	*T. viride* filtrates + 1% (CMC) v/v (CFU/g)	*P. chrysogenum* filtrates + 1% (CMC) v/v (CFU/g)	Control (Sterile distilled water)
1	5.8 ± 0.20^{a}	5.8 ± 0.01^{a}	5.8 ± 0.01^{a}
2	5.2 ± 0.10^{b}	4.6 ± 0.10^{b}	4.3 ± 0.10^{b}
3	4.8 ± 0.02^{c}	4.4 ± 0.10^{b}	4.2 ± 0.04^{bc}
4	4.7 ± 0.02^{cd}	4.2 ± 0.10^{b}	4.0 ± 0.10^{bc}
5	4.5 ± 0.02^{d}	3.8 ± 0.04^{c}	3.8 ± 0.10^{c}
6	4.2 ± 0.02^{e}	3.5 ± 0.10^{cd}	3.0 ± 0.11^{d}
7	3.8 ± 0.02^{f}	3.17 ± 0.06^{d}	2.9 ± 0.04^{d}

Values are means ± standard error. Means with similar alphabetical superscripts within a column is non-significant at ($P < 0.05$).

Table 4 Effect of potential bioactive edible coatings derived from carboxyl methylcellulose and fungal metabolites on percentage weight loss of orange fruits

Week(s)	*T. viride* filtrates + 1% (CMC) v/v (CFU/g)	*P. chrysogenum* filtrates + 1% (CMC) v/v (CFU/g)	Control (Sterile distilled water)
1	25 ± 0.40^{e}	23 ± 1.13^{e}	25 ± 0.76^{e}
2	26± 0.40^{e}	28 ± 0.40^{d}	30 ± 0.40^{d}
3	28.67± 0.60^{d}	30 ± 0.40 ^{d}	38 ± 1.13^{c}
4	31 ± 0.40^{cd}	35 ± 0.76^{c}	40 ± 0.76^{c}
5	33 ± 0.40^{bc}	38 ± 0.40^{b}	46 ± 0.40^{b}
6	35 ± 0.40^{ab}	42 ± 0.40 ^{a}	48 ± 0.60^{b}
7	37 ± 0.40 ^{a}	44 ± 0.40 ^{a}	53 ± 0.76 ^{a}

Values are means ± standard error. Means with similar alphabetical superscripts within a column is non-significant at ($P < 0.05$)

Table 5 Effect of potential bioactive edible coatings derived from carboxyl methylcellulose and fungal metabolites on psychrophilic bacteria isolated from oranges

Week(s)	*T. viride* filtrates + 1% (CMC) v/v (CFU/g)	*P. chrysogenum* filtrates + 1% (CMC) v/v (CFU/g)	Control (Sterile distilled water)
1	2.3 ± 0.04^{e}	2.27 ± 0.06^{f}	2.3 ± 0.08^{g}
2	2.5 ± 0.08^{e}	2.7 ± 0.08^{e}	3.9 ± 0.04^{f}
3	2.8 ± 0.04^{d}	3.87 ± 0.06^{d}	5.7 ± 0.08^{e}
4	3.0 ± 0.05^{d}	4.87 ± 0.06^{c}	6.8 ± 0.10^{d}
5	3.4 ± 0.04^{c}	5.2 ± 0.08^{c}	7.8 ± 0.07^{c}
6	4.5 ± 0.30^{b}	5.67 ± 0.09^{b}	12.5 ± 0.20^{b}
7	4.8 ± 0.04^{a}	6.27 ± 0.06^{a}	18.3 ± 0.08^{a}

Values are means ± standard error. Means with similar alphabetical superscripts within a column is non-significant at ($P < 0.05$)

Table 6 Effect of potential bioactive edible coatings derived from carboxyl methylcellulose and fungal metabolites on mesophilic bacteria isolated from oranges

Week(s)	*T. viride* filtrates + 1% (CMC) v/v (CFU/g)	*P. chrysogenum* filtrates + 1% (CMC) v/v (CFU/g)	Control (Sterile distilled water)
1	1.6 ± 0.10^{d}	1.6 ± 0.07^{f}	1.6 ± 0.06^{g}
2	2.3 ± 0.04^{c}	2.33 ± 0.02^{e}	3.4 ± 0.01^{f}
3	2.5 ± 0.04^{c}	3.67 ± 0.06^{d}	4.6 ± 0.04^{e}
4	2.5 ± 0.04^{c}	4.3 ± 0.04^{c}	5.2 ± 0.10 ^{d}
5	3.6 ± 0.08^{b}	8.5 ± 0.04^{b}	5.8 ± 0.01 ^{c}
6	3.8 ± 0.04^{ab}	8.4 ± 0.04^{b}	16.8 ± 0.04^{b}
7	3.93 ± 0.06^{a}	8.9 ± 0.02^{a}	19.3 ± 0.08^{a}

Values are means ± standard error. Means with similar alphabetical superscripts within a column is non-significant at ($P < 0.05$)

3.2 Potentials of bioactive coatings on microbial qualities of orange fruits

The plate count results of the potential bioactive edible coatings revealed that the bioactive film-forming coating gave a decrease in microbial population during ambient storage conditions when compared to the uncoated fruits. The results presented in Table 5 indicated that the potential bioactive edible coatings led to the reduction of colony-forming units of psychrophilic bacteria. Further, the treatment containing CMC + *T. viride* bioactive solution produced the lowest colony forming unit per gram (4.8 ± 0.04 CFU/g) at the end of the 7 weeks storage with followed by CMC + *P. chrysogenum* (6.27 ± 0.06CFU/g), while the highest value (18.3 ± 0.08 CFU/g) was recorded for the uncoated control fruits.

Further, a similar trend of reduction in the number of mesophilic bacteria was observed due to the application of potential bioactive edible coatings on orange fruits (Table 6). Treatment with CMC + *T viride* bioactive edible coatings produced the lowest colony-forming unit (3.9 ± 0.06 CFU/g) at the end of the 7th week while this was followed by CMC + *P. chrysogenum* bioactive edible coating solution (8.9 ± 0.02 CFU/g), while a higher value of (19.3 ± 0.08 CFU/g) was recorded for the uncoated control fruits.

Table 7 Effect of potential bioactive edible coatings derived from carboxyl methylcellulose and fungal metabolites on microbial load reduction of mold and yeast from oranges

Week(s)	*T. viride* filtrates + 1% (CMC) v/v (CFU/g)	*P. chrysogenum* filtrates + 1% (CMC) v/v (CFU/g)	Control (Sterile distilled water)
1	1.2 ± 0.01^{d}	1.2 ± 0.11^{f}	1.2 ± 0.08^{f}
2	1.4 ± 0.06^{cd}	1.6 ± 0.05^{e}	1.8 ± 0.08^{e}
3	1.4 ± 0.11^{cd}	1.9 ± 0.07^{d}	2.7 ± 0.04^{d}
4	1.7 ± 0.08^{c}	2.0 ± 0.01^{d}	3.0 ± 0.04^{d}
5	2.3 ± 0.08^{b}	2.5 ± 0.06^{c}	3.7 ± 0.06^{c}
6	2.4 ± 0.04^{b}	2.8 ± 0.04^{b}	4.3 ± 0.06^{b}
7	2.8 ± 0.01^{a}	3.9 ± 0.08^{a}	5.8 ± 0.12^{a}

Values are means ± standard error. Means with similar alphabetical superscripts within a column is non-significant at ($P < 0.05$)

More so, microbial load reduction of molds and yeasts was also attributed to the treatment of orange fruits using potential bioactive edible coatings as presented in (Table 7). Postharvest orange fruits treatments with CMC + *T. viride* solutions produced the lowest colony-forming unit (2.8 ± 0.01 CFU/g) at the end of the 7 weeks treatment storage with followed by CMC + *P. chrysogenum* bioactive solution (3.9 ± 0.08 CFU/g) and the highest value (5.8 ± 0.12 CFU/g) was recorded for the uncoated control oranges. Generally, these results revealed that there was variation in the effects of the potential bioactive edible coatings on microbial populations of orange fruits.

4 Discussion and conclusion

A preliminary investigative study on the potential of bioactive edible coatings that were derived from the culture filtrates of two fungal bioagents was subjected to submerged liquid fermentation to obtain the metabolites, these metabolites were tested in combination with carboxyl methylcellulose solution to assess the synergistic effects on the postharvest shelf-life extension of sweet oranges during a 7-week experimental setup. The results of the study suggested good prospects in this preliminary screening investigation of the potential bioactive edible coatings. The population of the microbial load was reduced significantly due to the effects of the potential bioactive edible coatings on orange fruits. The plate count analysis indicated a reduction in the microbial counts of mesophilic and psychrophilic bacteria, as well as the molds and yeast count as reflected in their colony-forming units respectively. The CMC + *T. viride* bioactive solution led to a reduction in the population of psychrophilic bacteria with a (4.8 ± 0.04 CFU/g), while (CMC + *P. chrysogenum*) bioactive solution gave microbial load value of (6.27 ± 0.06 CFU/g). The highest microbial load value of (18.3 ± 0.08 CFU/g) was recorded for the uncoated control fruits. Also, a similar trend was observed for the result of mesophilic bacteria population count, as well as the population of molds and yeast due to the potential bioactive edible coated orange fruits as compared with the uncoated control fruits. For example, the potential bioactive edible coated (CMC + *P. chrysogenum*) orange fruits, had a mesophilic bacteria count of 8.9 ± 0.02 CFU/g, and the highest mesophilic bacteria count was 19.3 ± 0.08 CFU/g which was recorded for the uncoated control fruits. These results imply that the potential bioactive edible coatings have antimicrobial properties that led to a reduction in the microbial count of the coated oranges when compared to the uncoated control of orange fruits. Earlier studies that are in agreement with our findings reported the efficacy of using edible coatings in improving the microbiological and physiological status of fruits and vegetables, thereby extending their storage life (Nayak et al., 2019; Nair et al., 2020; Pirozzi et al., 2021). According to the previous studies(Arnon et al. 2015; Chen et al. 2016; Munhuweyi et al. 2020; Zhao et al. 2020; Atta et al. 2021), the postharvest storage quality of fruits using carboxyl methylcellulose indicated an enhancement in the storage quality of the fruit attributable to the following mechanisms; the creation of hydrogen bond inside the CMC coating and forming of an extra layer of cuticle on the outer surface of the fruit. Also, the CMC coatings inhibited the proliferation of microbial life on the fruits, it reduces moisture loss and the rate of transpiration. More so, in a study on Agege sweet orange, Adetunji et al. (2018) utilize the synergistic influence of chitosan and *P. aeruginosa* produced rhamnolipid to extend the shelf-life of sweet orange through improving the microbial quality and physiological attributes of the coated oranges. These findings are incongruent with the highlights of our study that led to an improvement in both microbiological and physiological qualities of *C. sinensis*.

Also, various previous studies stated that the quality and shelf-life extension of fruits were improved, due to the treatment with various edible coatings (Maftoonazad & Ramaswamy, 2019; Anjum et al., 2020; Jafarzadeh et al., 2021). The mechanisms of action or bioactivity of the edible coatings in improving the microbial and physiological quality of the treated fruits include production of antimicrobial, antioxidative enzymes, pathogenesis inducing protein, and other bioactive components to suppress microbial pathogens and also maintain the fruits physiological integrity (Nair et al., 2020; Tkaczewska, 2020).

Our study further highlighted that there was minimal weight loss due to the application of the potential bioactive edible coatings on the orange fruits as compared to the uncoated control fruits. This result agrees with earlier studies of Abd El-Razek et al. (2019) and Abd-Elkader et al. (2021) where they reported and attributed the prevention of dehydration, loss of water, and fruit shrinkage to edible coatings of fruits. In addition, the application of the potential bioactive edible coatings significantly ($P<0.05$) improved the pH, ascorbic acid, and the total soluble solid content of the orange fruits. This was due to the modifications of the internal environmental conditions of the coated fruits, thereby minimizing the exchange of gases due to the edible coatings and subsequently delaying the ripening of the fruits. These results are in harmony with reports of previous studies (Sharma et al., 2019; Parven et al., 2020; Xing et al., 2020; Hajebi Seyed et al., 2021; Hasan et al. 2021).

Conclusion

In conclusion, this preliminary investigative study on potential bioactive edible coatings that was formulated from a combination of carboxyl methylcellulose and fungal cultured metabolites led to significant improvement in the microbiological and physiological qualities of postharvest treated sweet orange fruits. The potential bioactive edible coatings led to a reduction in the microbial load of the citrus fruits, as well as improving the physiological qualities of the orange fruits. This implies that the potential bioactive edible can be deployed in enhancing the quality and storage life of sweet oranges (*Citrus sinensis*). Therefore, this study is an addition to the growing knowledge of using readily available natural bioactive ingredients to improve the shelf-life of very important orange fruit, thereby minimizing wastes, and ensuring the safety and security of fruits in our society. However, more questions are needed to be answered in further studies to comprehend the safety and mechanisms of action of this potential bioactive edible coating.

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EOSINOPHIL CATIONIC PROTEIN AND IRON STATUS IN PATIENTS INFECTED WITH *Enterobius vermicularis*

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ABSTRACT

The primary goal of this study was to determine the function of eosinophil cationic protein in iron status in patients infected with *Enterobius vermicularis*. For this, a total of 583 suspected patients and thirty healthy people of the same age who have visited the AL-Zahra maternity and pediatrics laboratory, AL-Hakeem hospital, AL-Sajad hospital AL-Fruit al-Awsat hospital, and AL-Munadira hospital in AL-Najaf province from July 2020 to June 2021 were screened. The presence of *E. vermicularis* eggs was estimated by using the saline wet mount technique from faeces samples of all respondents. Blood samples were collected from the 60 positive and 30 healthy control group and centrifuged at 3000 rpm for 5 minutes to separate serum, which was then collected in sterile tubes. Each serum sample was divided into three parts and stored in the deep freezer at -20°C until the serological test was performed. The level of ECP, iron and ferritin in enterobiasis patients was estimated from the isolated blood serum. According to the Manufacturer Company instructions, the concentration of two biomarkers (ECP, Ferritin) in serum samples was determined using the ELISA technique (Human reader, Germany). While the concentration of iron was evaluated using a colourimetric method. In comparison to the control group, the concentration of ECP was reported significantly higher ($P<0.05$) in the *E. vermiculris* infected patients while the concentration of serum iron and ferritin was significantly decreased. The results of the current study can be concluded that E. vermicularis infection changes the serum ECP, iron, and ferritin concentration.

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1 Introduction

According to the World Health Organization, intestinal parasite infection is a major public health concern (Stoltzfus et al., 1997). According to Park et al. (2009), intestinal parasites infect more than 24% of people worldwide and harm approximately one billion people socially and economically. Parasites can invade the human intestine and cause anemia by impairing micronutrient absorption such as iron in the duodenum and jejunum that leads to iron deficiency anemia. Studies have been adopted on the tissue evidence such as eosinophils' effectiveness, the effectiveness of the eosinophils was tested in vitro and found to have the ability to kill parasites; the mechanism that eosinophils use against parasites is not fully understood but generally includes the production of antibodies. In most of the cases, it appears to involve antibody-induced release, complement-induced release, or both of toxic granule proteins and reactive oxygen intermediates by activation eosinophil; the acidity of the ability to kill parasites varies between the different stages of the life cycle of the parasite itself. Adults, children, and both sexes are all affected by *E. vermiculris* infections (Belete et al., 2019). Even though bowel disease is not so much harmful, but it is commonly seen as a parasitic infection, particularly in nations with a high infant mortality rate (Lohiya et al., 2000). Enterobiasis affects 200 million individuals worldwide (Brooker et al., 2006), and the disease's nature makes it easier to spread throughout families and educational institutions (Brooker et al., 2006). Pinworms can be found throughout the world, but they are most common in temperate regions (Kang et al., 2006). Pinworms are worms that exist worldwide and are difficult to control because they can easily be transmitted from person to person, these are mostly transferred through contaminated hands, direct finger and contact is usually an easy way to move between people with appropriate conditions such as primary schools, orphanages, refugee camps, and nurseries can also spread through the air by inhalation resulting in egg ingestion and infection (Burkhart & Burkhart, 2005). In the colon, adult worms are paired, after this, the pregnant females travel to the perianal area, where they deposit eggs, especially at night. Each female produces approximately 10,000 eggs (Ashford et al., 1988). Because the worms are also transmitted by autoinfection, transfer of infectious eggs by hand into the mouth from the site of itching, and the time between swallowing the infected eggs and laying eggs by adult females is 30 days, the female worms cause severe itching during their migration. Pinworms are diagnosed by placing a cellophane tape over the anus area in the early morning before defecating and it is microscopically examined (Garcia & Lynne, 2009). At severe infection conditions, worms can also be reported from the female genitalia, where they can cause diseases like vaginitis and damage to other organs, including the kidneys (Cateau et al., 2010). *E. vermicularis* cause a pelvic abscess to produce peritoneal inflammation in the peritoneal cavity (Das et al., 2001) while it is seen in urinary tracts of man's (Zahariou et al., 2007).

Because intestinal parasites use the source of food from their hosts, that's why these worms share various nutrients such as carbohydrates, minerals, vitamins, fats, and other essential energy sources of the host. These elements create serious physical ailments, so it is necessary to conduct routine parasite screening at regular intervals to detect the presence of infection with parasitic worms and to take appropriate treatment to eliminate the infection.

Minerals like magnesium, iron, copper, and zinc are essential for children's growth and development (Hesham et al., 2004). To achieve optimal growth and mental development in children, intestinal parasite infections must be detected and treated at an early stage (Pegelow et al., 1997). Olivares et al. (2003) recorded that trace elements influence metabolic pathways and the immunological response and suppress the occurrence of many diseases. Iron, copper, magnesium, and vitamin B12 are the most significant critical elements in the human body. Zinc is essential for immune system processes, and deficiency has reduced lymphocyte and thymus function. Zinc deficiency causes severe diarrhea in neonates due to its participation in immune system processes (Culha & Sangun, 2007). Copper is required to develop red blood cells, hemoglobin, iron absorption, and the action of several enzymes (Demirci et al., 2003). Copper and zinc are cofactors for cytosolic superoxide dismutase, and a reduction in the levels of these cofactors affects the functioning of this enzyme (Yousef et al., 2002; Demirci et al., 2003). In this manner, zinc is an important cofactor for many biological reactions.

Parasitic worms stimulate an intense Th2 response, which leads to the production of Interleukin-4 and Interleukin-5, which leads to the synthesis of immune globulin E (Al-Daoody & Al-Bazzaz, 2020). In this manner, the presence of parasitic worms causes an increase in Th2, IgE, and eosinophilia cytokines. In response to this, helminths suppress the host's inflammatory response to Th2 and boost T and B cells (Rashad & Aljanaby, 2021). On the other hand, macrophages create cytokines including IL-10 and TGFß, as well as IgG4 antibodies that block IgE (McSorley & Maizels, 2012; Abd-Aljabar & Aljanaby, 2021). Eosinophile play an essential role in the immune response. The eosinophile and eosinophil granular products such as Eosin cationic protein (ECP), eosinophil-derived neurotoxin (EDN), eosinophil peroxidase (EPO) were found to be efficient in worm management under in vitro conditions (Taliaferro & Sarles, 1939). The role of eosinophils in the organism's body is more complicated. Due to changes in the organism or the organism's immune response, many studies have been conducted about the role of eosinophils against different types of parasites (David et al., 1980). This study aimed to estimate the levels of serum eosinophil cationic protein (ECP), ferritin, and iron in the children (before 12 years old) suffering from enterobiasis disease, and to establish a relation between the level of these biomarkers and *E. vermiculris* infection.

2 Materials and Methods

The occurrence of acute anal itching, especially at night (the most prevalent), as well as apparent clinical indications such as irritability, lack of appetite, abdominal pain, urinary tract infections, yellowing of the face, and weight loss, are the important symptoms of *E. vermicularis* infection (Garcia & Lynne, 2009). The current study was conducted on 583 suspected patients from July 2020 to June 2021; stool samples were collected from these suspected patients and diagnosed by light microscope using wet mount preparation for the detection of adult pinworms or eggs at the microbiological laboratory, department of biology, college of science, Al-Kufa University. To detect the presence of *E. vermicularis* eggs, all faeces samples were examined using the saline wet mount technique, which involved placing a drop of saline (0.9 percent) on a glass slide. For this, a small amount of faeces samples were spread over the glass slide, mixed with a saline drop, covered with a coverslip, and observed at 100 X and 400 X, magnifications under a light microscope (Al-Hadraawy et al., 2019).

2.1 Serum biomarker detection

For the immunological analysis, 5 ml blood samples were taken from 60 *E. vermicularis* positive individuals and 30 healthy children (controls) and maintained at room temperature for 30 minutes (Al-labban et al., 2021; Azeez et al., 2021). The blood samples were centrifuged at 3000 rpm for 5 minutes and serum sample was collected in sterile tubes. Each serum sample was divided into three parts and stored in the deep freezer at -20°C until the serological test was performed. According to the Manufacturer's Company, the concentration of biomarkers (ECP in Ferritin) in serum was determined using the ELISA technique (Human reader, Germany). BTLAB provided these biomarkers units with Cat.No. E1391Hu and Cat.No. E1702Hu, respectively, while iron concentration was quantified using a colourimetric method as per the standard protocol (Al-Hadraawy et al., 2016; Al-Hadraawy et al., 2019; Mohy et al., 2019).

2.2 Statistical analysis

The healthy and control groups were compared using Graph-pad prism version 10-computer software (T-test). A statistically significant P-value of less than 0.05 was used (Al-Hadraawy et al., 2016).

3 Results and Discussion

In the current investigation, the concentration of (ECP) in *E. vermicularis* infected males and females was significantly higher (P=0.05) than in the control group. As shown in table 1, the concentration of ECP was reported 32.48ng/ml and 34.42ng/ml in males and females respectively while in the case of total patients this value was reported 33.35ng/ml. While iron and ferritin concentration was significantly lower (P=0.05) in *E. vermicularis* infected males and females as compared to the control. In the case of total patients, *E. vermicularis* infected patients have 62.23 (µg/ml) and 72.16 (ng/ml) iron and ferritin concentration while this value was reported 86.63 (µg/ml) and 79.19 (ng/ml) in control respectively. Overall *E. vermicularis* influenced the body's immune system and enhance the secretion of ECP.

Table 1 Effect of *E. vermicularis* infection on the selected biological markers

Respondents	Biological Markers		
	Iron (µg/ml)	Eosinophil Cationic Protein(ng/ml)	Ferritin (ng/ml)
Male (Patients)	62.29±0.82	32.48±1.16	72.52±0.69
Male (Control)	87.33±1.05	1.65±0.59	78.89±1.76
Female (Patients)	62.17±0.84	34.42±0.81	71.87±1.15
Female (Control)	85.93±1.28	1.79±0.04	79.95±1.35
Total Patients	62.23±0.58	33.35±0.83	72.16±0.67
Total Control	86.63±0.82	10.70±0.39	79.19±1.09

Results of this study demonstrate that males and females infected with *E. vermicularis* have significantly higher concentrations of ECP compared to the control group. This outcome could be due to worm infection, which triggers an immune response that leads to a sequence of cellular and molecular interactions with mast cells, which are involved in the stimulation of immunoglobulin E (Bethony et al., 2006). This increasing number of eosinophils enhances the production of leukocytes and pellets which kills or expels parasitic worms from the body (Reimert et al., 1991; Reimert et al., 2006). This might be a possible explanation of higher ECP levels in people suffering from *E. vermicularis* infection as compared to healthy people. Among the proteins secreted by eosinophils ECP, EDN, and EPO are the most common. Among these, EPO might play an important role in the parasite defense mechanism (Hamann et al., 1990). These results are in agreement with the findings of Tischendorf et al. (2000). These researchers reported a higher value of ECP with median levels ranging from 46 to 98 g/l in persons infected with parasites. These results are also confirmed by the findings of Amoani et al. (2019) and Hassan et al. (2002), those who reported higher ECP levels in hookworm and helminthic infected participants as compared to the healthy individuals respectively. Similarly, the findings of Tischendorf et al. (2000) refer to EDN's high value in parasite-infected individuals. At the same time, the results of the current study are contradictory to the findings of Amoani et al. (2019) those who suggested that serum ECP levels do not rise

during malaria infection. ECP and EDN/EPX levels were reported to be higher in filariae and schistosome infections linked to eosinophilia (Tischendorf et al., 1996; Iskra et al., 2020).

Compared to the control group, the ferritin concentration in males and females having *E. vermicularis* infections was considerably lower. This might be because of worm infections because Osazuwa et al. (2011) suggested that worm infection is more sensitive in identifying anaemia and was linked to helminth infection in the intestine. The findings of this study accord with those of Adebara et al. (2011) those who identified a link between intestinal helminthiasis and serum ferritin levels in Nigerian school children and observed that ferritin levels were lower in hookworm infected children. Jafer et al. (2015) also reported that ferritin levels increased considerably after hookworm therapy. Similarly, Bhargava et al. (2003) found that serum ferritin levels were low in children infected with intestinal parasites such as Ascaris and Trichuris. Le et al. (2007) and Bayoumy et al. (2018) explained the substantial relationship between serum ferritin values and parasites and suggested that patients with parasitic illness have low serum ferritin levels.

The results demonstrate that iron concentrations in males and females infected by *E. vermicularis* have significantly lower than in the control group; this could be because infections hindered iron absorption from the gastrointestinal tract (Stoltzfus et al., 1997; Li et al., 2007). These findings are comparable to the results of Al-Daoody & Al-Bazzaz (2020) those who found that the mean blood total protein and serum iron levels were considerably lower in the enterobiasis-positive groups than the enterobiasis-negative group's. Olsen et al. (1998) reported that the presence of intestinal parasites generally accompanied the emergence of iron deficiency anaemia. Similarly, In a study by Kuvibidila et al. (1995) children, with parasite and protozoa infections had a higher rate of iron deficiency anaemia (IDA) than those who were not affected. An essential link between parasitic intestinal infection and iron deficiency anaemia among school children in rural Vietnam was discovered in Southeast Asia. Results of this study are contradictory to the findings of Da Silva et al. (2016); Darlan et al. (2018) and Khazaal et al. (2020) those who could not discover an association between serum iron levels and intestinal parasite infection. Similarly, Ngui et al. (2014) could not discover any link between anaemia and parasitic intestinal illness in elementary school pupils in a study conducted in Brazil.

Conclusions

Results of the study can be concluded that *E. vermiculris* infection has a significant effect on the serum ECP, ferritin, and iron concentration. Higher ECP concentration might be an attempt of the human body against worm infection. While the lower iron concentrations are showing malfunctioning of the intestine.

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ANTI-PROLIFERATIVE POTENTIAL OF *Carica papaya* LEAVES ON BREAST CANCER CELLS – MCF-7

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ABSTRACT

The study's objective is to identify the phytoconstituents and determine the anti-cancer potential of *Carica papaya* leaves against the MCF 7 cell line. Chloroform, ethyl acetate, and methanol extracts of *C. papaya* leaves were prepared by cold maceration method and qualitative phytochemical analysis was performed. The anti-proliferative effect of these extracts was determined by 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and apoptotic assay by acridine orange/ethidium bromide staining method on MCF 7 cells. The effect of the extracts, with different concentrations, on DNA fragmentation, was also performed on MCF 7 cells. Qualitative analysis revealed the presence of alkaloids, flavonoids, terpenoids, steroids, saponins, tannins, glycosides, phenols, anthraquinones, proteins, and carbohydrates. Chloroform, methanol, and ethyl acetate extracts of *C. papaya* leaves were observed with potential DPPH free radical scavenging activity with 72%, 75%, and 78% respectively. Of these extracts, the chloroform extract (72%) was found to possess a more free radical scavenging effect against DPPH and also showed a dose-dependent effect, the maximum at 100µg/ml, on DNA fragmentation in MCF 7 cells. Further, chloroform extract showed a maximum anti-proliferative effect on MCF-7 cells with IC_{50} at 22±1.5µg/ml, whereas methanol and ethyl acetate extract at 30±0.5 µg/ml and 28±0.5 µg/ml respectively. Increased apoptosis in MCF 7 cells was observed with an increased concentration of chloroform extract of *C. papaya.* From the results of this study, it can be concluded that leaf extract of *C. papaya* found to possess an anti-proliferative effect and antioxidant potential and it could be due to the presence of rich secondary metabolites of the plant.

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1 Introduction

Breast cancer is among the most common type of cancer in women around the globe (Yahyea et al., 2019). It is developed due to the inheritance of proto-oncogenes such as BRCA1, BRCA2 and by a genetic mutation. Most breast cancer is contributed by carcinoma, while sarcoma is rarely associated with this cancer (Feng et al., 2018). If the tumor is diagnosed early, it could be treated easily (Fadi & Ferguson, 2020). Although it is seen primarily in women, men can also get breast cancer (Farr et al., 2017). Chemotherapy and radiation therapy are the commonly used treatment to treat breast cancer. But, these treatments produce adverse side effects (Alam et al., 2013). The tumor development can be reversed or inhibited by synthetic drugs. Plant extract might play an essential role in preventing and curing breast cancer (Levitsky & Dembitsky, 2015). Medicinal plants are well recognized and have been used in the preparation of traditional medicines with fewer side effects to treat various diseases especially cancer (Richard et al., 2015). The active phytochemical constitutes of herbal plants are carotenoids, flavonoids, ligands, terpenoids, and sulfides stimulate glutathione-transferase that helps protect the cell and prevent the proliferation of the cell (Shareef et al., 2016).

Carica papaya belongs to the family of Caricaceae (Figure 1). It is diploid, dicotyledon, and polygamous plants. The papaya is consumed in the form of jam, juices, or dry fruit. The ripe fruit of *C. papaya* is rich in Vitamin C, A, and calcium. The different product of *C. papaya* is used to produce a variety of commercial products. Chymopapain and papain are made from latex, stem, leaves, and fruit. For many centuries, papaya leaves have been used as folk medicine. Recently researchers proved the wound healing, anti-inflammatory, anti-oxidant, immune-modulatory, and anti-tumor effect of this plant (Saurabh et al., 2016). Hence, the present study aims to analyze the phytoconstituents and to evaluate the anticancer potential of *C. papaya* leaves against the breast cancer cell line MCF 7 cells.

Figure 1 *Caria papaya* leaves

2 Materials and Methods

2.1 Collection of Plant material

The leaves of *C. papaya* were collected from Kumbakonam, Thanjavur District, Tamil Nadu, India. The leaves were shade-dried, powdered with an electrical blender, and stored in an airtight container till use.

2.2 Preparation of plant extract

About 25g of powder was immersed in 100 ml of chloroform, ethyl acetate, and methanol separately for 72 hours with occasional shaking at room temperature. The extract was collected after filtering using Whatman No1 filter paper and stored at 4°C till use. Then, the extracts were condensed and used for experiments (Kiruthika et al., 2020).

2.3 Qualitative analysis of phytochemical constituents

Qualitative analysis of phytochemical constituents was carried out by the methods of Harbone et al. (1998). The detail of the used method for qualitative analysis was given in table 1.

Table 1 Qualitative analysis of various phytochemical constituents

S.No	Phytochemical Constituents	Appeared colour/ Characteristics	Reference
1.	Alkaloids	Creamy white precipitate	Mustikasari & Ariyani, 2010
2.	Flavonoids	Intense red color	Chang et al., 2002
3.	Terpenoids	Reddish-brown color	Indumathi et al., 2014
4.	Steroids	Blue-green color	Silas et al., 2019
5.	Saponins	Appearance of froth	Kokate et al., 2001
6.	Tannins	Green-blue is formed	Schanderl,1970
7.	Glycosides	Formation of Green color	Kokate et al., 2001
8.	Phenols	Development of Blue color	McDonald et al., 2001
9.	Anthraquinones	Appearance of Rose pink color	Silas et al., 2019
10.	Proteins	Violet color is formed	Padmapriya & Maneemegalai, 2014
11.	Carbohydrates	Appearance of violet color	Padmapriya & Maneemegalai 2014

2.4 In-vitro anti-oxidant activity

The anti-oxidant assay was carried out by the method of Mensor et al. (2001). In these assays, DPPH was used as the standard free radical, when it reacts with anti-oxidants, gets reduced to DPPHH. Due to the reduction reaction, the absorbance of DPPHH decreases in comparison to DPPH. The anti-oxidant activity of

compounds or extracts is indicated by the degree of discoloration in terms of hydrogen donating ability. The anti-oxidant activity of the fraction was measured *in vitro* by 1, 1 diphenyl- 2-picrylhydrazyl (DPPH) assay (Mensor et al., 2001). In brief, 0.5 mM DPPH was prepared in 95% methanol, and 1 ml of this solution was added to the tubes containing 3ml of leaf extracts (chloroform, methanol, and ethyl acetate) at different concentrations. The contents of the tubes were mixed well, left at room temperature for 30 minutes, and the absorbance was measured at 515 nm in a spectrophotometer. The antioxidant activity was expressed as:

$$\% \text{ of disappearance} = \frac{\text{control} - \text{sample}}{\text{control}} \times 100 \quad \text{(Control - Sample)}$$

2.5 Cell line and culture conditions

The human breast cancer cells (MCF-7) were purchased from the National Center for Cell Sciences (NCCS), Pune, India. The cancer cells were maintained in MEM medium (MEM+FCS) supplemented with 2mM/L glutamine and balanced salt solution (BSS) adjusted to contain 1.5 g/L Na_2CO_3, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate,2 mM /L glutamine, 1.5 g/L glucose, 10 mM (4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid) (HEPES) and 10% fetal bovine serum (GIBCO, USA). Penicillin and streptomycin (100 IU/100µg) were adjusted to 1mL/L. The cells were maintained at 37°C with 5% CO_2 in a humidified CO_2 incubator (Saravanan et al., 2017).

2.6 MTT assay

MTT [3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay was used to evaluate the Inhibitory Concentration (IC_{50}) of the plant extracts (Joseph et al., 2020). The MEM (1×10^4cells/well) was used to grow the cells in a 96-well plate for 48 hours in 85% confluence. The medium was replaced with a fresh medium containing a serially diluted compound. The cells were then incubated for 48 hours. 100µL of the MTT [3-(4, 5-dimethylthiozol-2-yl)-3,5-diphenyl tetrazolium bromide] solution was added to each well after the removal of the culture medium. Incubate the mixture at 37°C for 4 hours. After removing the supernatant, 50 µL of DMSO/ isopropanol was added to each of the wells and incubated for 10 minutes to solubilize the formazan crystals. The absorbance was measured at 620 nm in an ELISA multi-well plate reader (Thermo Multiskan EX, USA). The recorded absorbance was used to calculate the percentage of viability using the following formula:

$$\% \text{ of viability} = \frac{\text{OD value of the experimental sample}}{\text{OD value of the experimental control}} \times 100$$

2.7 Apoptotic analysis by Fluorescent Microscopy

Fluorescence assay was carried out by the methods of Engin et al. (2011). For this, 0.9 ml of cell suspension (1×10^5 cells/mL) was mixed with 1µL of a dye mixture having 100 mg/mL acridine orange (AO) and 100 mg/mL ethidium bromide (EtBr) in distilled water on a clean coverslip. The phosphate-buffered saline (PBS) (pH 7.2) was used to wash pre-treated cancer that was collected. Then, using 10µL of AO/EtBr, the cells were stained; they were incubated for 2 minutes. After incubation, the cells were washed twice with PBS (5 min each) and visualized under a fluorescence microscope (Nikon Eclipse, Inc, Japan) at 400× magnification with an excitation filter of 580nm. The cells were treated with complex for 2 hrs by placing it on a glass coverslip in a 6-well plate. Using 2% Triton X-100 (50µl), the fixed cells were permeabilized for 10 min at room temperature and incubated for 3min with 10µl of DAPI by placing a coverslip over the cells to enable uniform spreading of the stain. The cells were observed under (Nikon Eclipse, Inc, Japan) fluorescent microscope. The MCF-7 cells treated without plant extract were used as a control (Engin et al., 2011).

2.8 DNA Fragmentation assay

DNA fragmentation assay was carried out by the method of Ramar et al. (2012). In 10mM of Tris-Hcl and 10mM EDTA (pH 8.0), MCF-7 (1×10^6cells) were suspended independently. Then, the cells were treated with 10 mM Tris-HCl, 10mM EDTA (pH 8.0), 2% SDS, and 20 mg/mL proteinase K, and the mixture was kept at 37ºC for 3h. Later, 9the mixture was extracted with phenol: chloroform: isoamyl alcohol solution in the ratio of 25:24:1, respectively. The extracted DNA was treated with DNase-free RNase at 20 mg/mL concentration at 4ºC for 45 min and precipitated with 100 mL of 2.5 M sodium acetate and 3 volumes of ethanol. About 10µg of isolated DNA was taken from control and treated cells and electrophoresed on a 2% agarose gel containing ethidium bromide.

3 Results

Results presented in table 2 revealed the phytoconstituents of chloroform, methanol, and ethyl acetate extract of *C. papaya* leaves. The chloroform extract of the *C. papaya* leaves was found to contain alkaloids, saponins, tannins, glycosides, proteins, and carbohydrates. Similarly, methanol extract of the *C. papaya* leaves contains flavonoids, terpenoids, steroids, tannins, glycosides, proteins, and carbohydrates. The ethyl acetate extract of *C. papaya* leaves contains alkaloids, saponins, tannins, glycosides, phenols, anthraquinones, proteins, and carbohydrates.

Figures 2, 3, 4 represent the anti-oxidant activity of the chloroform, methanol, and ethyl acetate extract of the *C. papaya* leaves respectively. Of these extracts, chloroform extract exerted more DPPH free radical scavenging activity than methanol and ethyl acetate extracts, but lesser with the standard ascorbic acid.

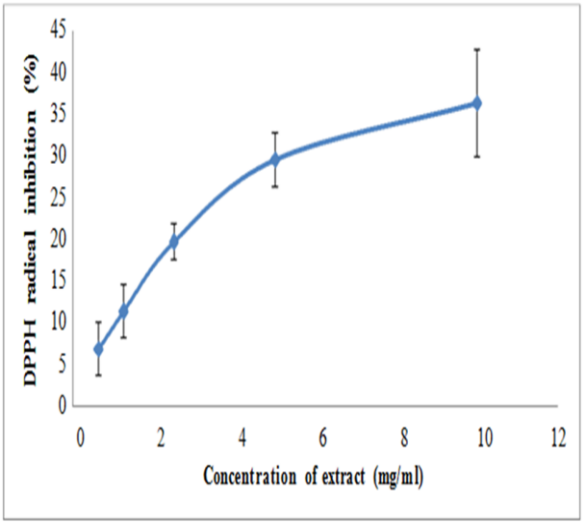

Figure 2 DPPH scavenging activity of chloroform extract of *C. papaya* leaves

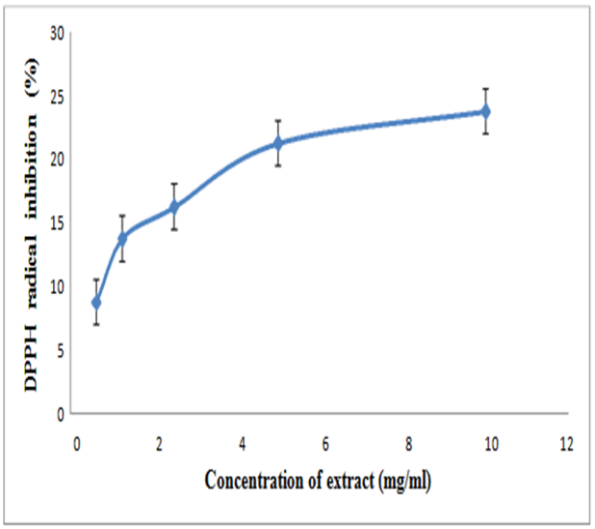

Figure 3 DPPH radical scavenging activity of methanolic extract of *C. papaya* leaves

Table 2 Phytochemical screening of the various extracts of the *C. Papaya* leaves

Phytoconstituents	Chloroform extract	Methanol extract	Ethyl acetate extract
Alkaloids	+	-	+
Flavonoids	-	+	-
Terpenoids	-	+	-
Steroids	-	+	-
Saponins	+	-	+
Tannins	+	+	+
Glycosides	+	+	+
Phenols	-	-	+
Anthraquinones	-	-	+
Protein	+	-	+
Carbohydrates	-	+	+

(+) Presence (-) Negative

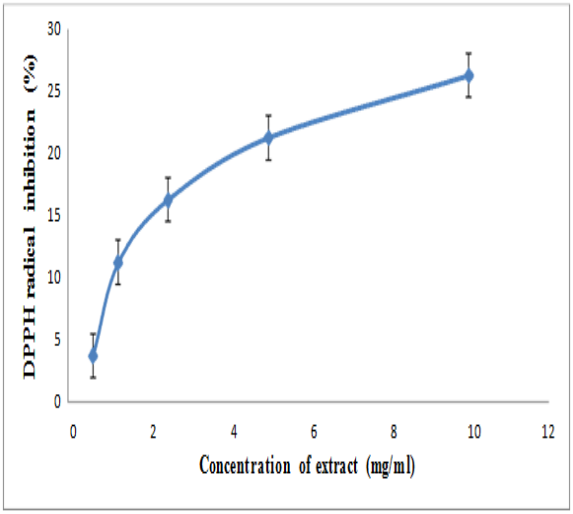

Figure 4 DPPH radical scavenging activity of ethyl acetate extract of *C. papaya* leaves

Figures 5, 6, 7 depict the cytotoxic effect of chloroform, methanol, and ethyl acetate of *C. papaya* leaves at 10µg/ml, 20µg/ml, 40µg/ml against MCF-7 cells. The percentage of viability was analyzed by MTT assay after treatment of *C. papaya* leaves extracts. The IC_{50} values of chloroform, methanol, ethyl acetate extracts on MCF-7 cells was found to be 22±1.5 µg/ml, 30±0.5, and 28±0.5 µg/ml, respectively. Of these three extracts, chloroform extract showed a good cytotoxic effect on MCF-7 cells (22±1.5 µg/ml).

Figure 8 represents the apoptotic effect of chloroform extract of *C. papaya* leaves at 10µg/ml, 20µg/ml, and 40µg/ml on MCF-7 cells and analyzed through fluorescence microscopy. With the increasing concentration of plant extract, a gradual shift from green to red fluorescence was observed in MCF 7 cells, the shift is considered as an index of apoptosis. This is further, supports the apoptotic effect of chloroform extract of *C. papaya* on MCF-7 cells.

Figure 9 represents the DNA fragmentation assay of chloroform extract of *C. papaya* leaves on MCF-7 cells. In the image, "M" represents the marker DNA. The concentrations of the sections were 10µg, 20µg, 30µg, and 100µg, respectively. The DNA fragmentation has increased with the increasing concentration of the extract. Thus, results showed that 100µg of chloroform extract of *C. papaya* leaves exhibit more DNA fragments.

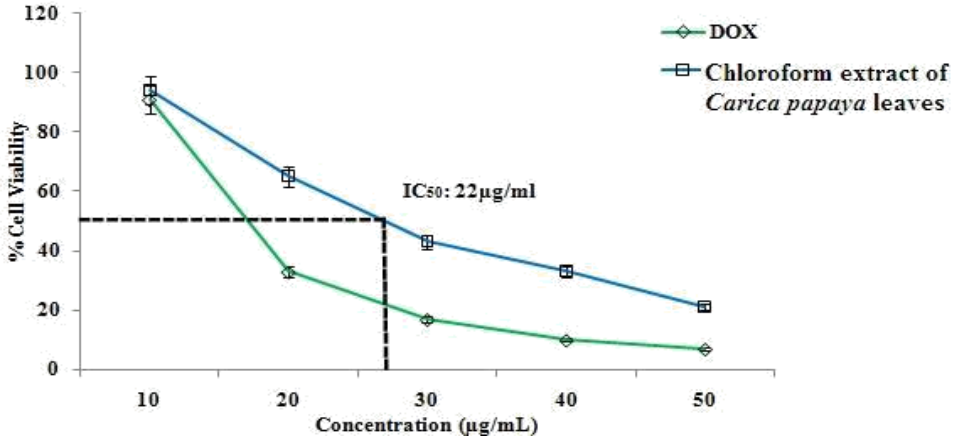

Figure 5 Cytotoxic effect of chloroform extract of *C. papaya* leaves on MCF-7 (MTT Assay)

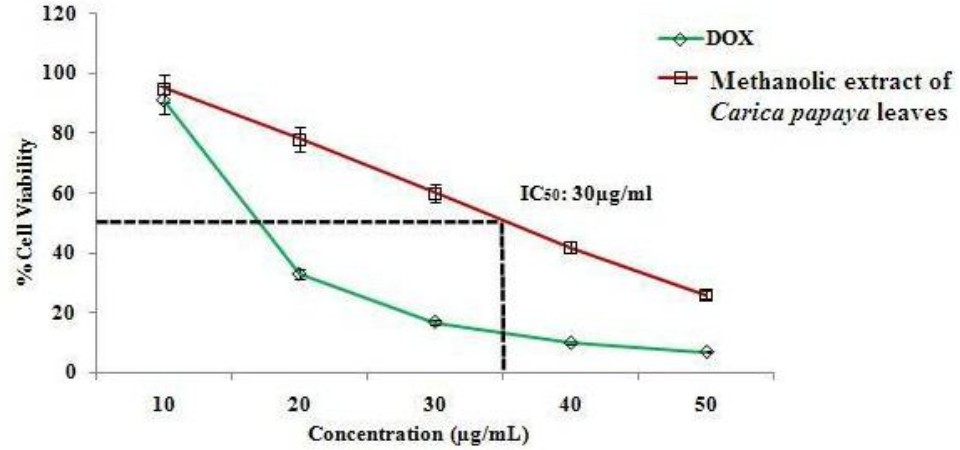

Figure 6 Cytotoxic effect of methanolic extract of *C. papaya* leaves on MCF-7 (MTT Assay)

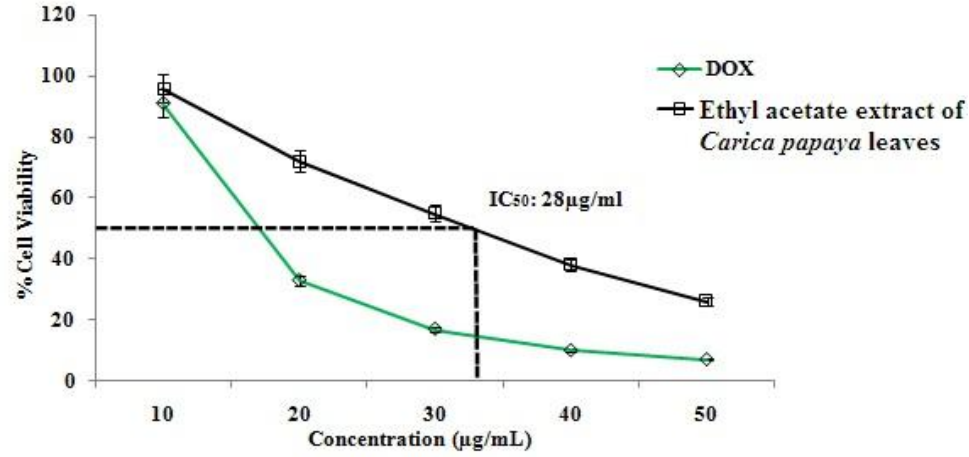

Figure 7 Cytotoxic effect of ethyl acetate extract of *C. papaya* leaves on MCF 7 cells (MTT Assay)

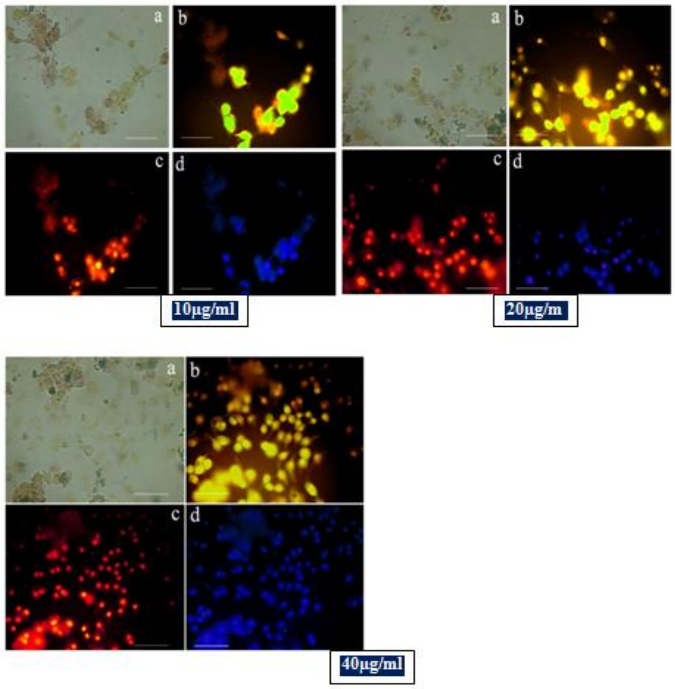

Figure 8 Fluorescence microscopic analysis of chloroform extract of *C papaya* leaves on MCF-7 (a - Phase contrast image of MCF-7-cells; b - Acridine orange and Ethidium bromide image of MCF-7 cells; c - Propidium Iodide of MCF-7 cells; d- DAPI image of MCF-7 cells)

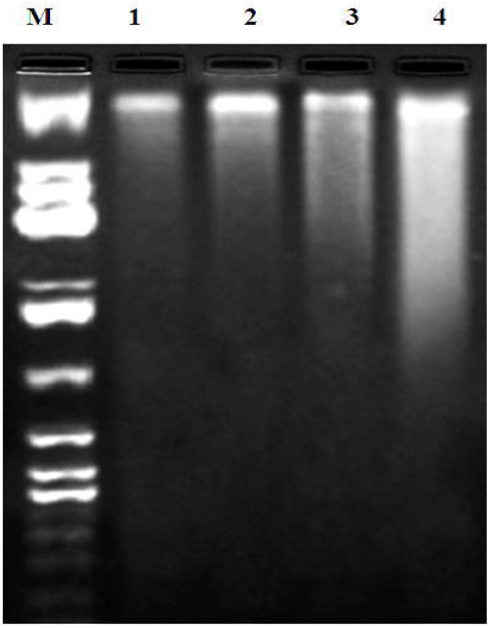

Figure 9 DNA fragmentation analysis of chloroform extract of *C. papaya* leaves on MCF-7 cells (M - Marker DNA; 1 - 10µg/ml treated MCF-7 cells; 2 - 20µg/ml treated MCF-7 cells; 3 -30µg/ml treated MCF-7 cells 4) 100µg/ml treated MCF-7 cells)

4 Discussion

Plants have been used for medical purposes since time immemorial and are the basis of modern medicine. Phytochemicals, secondary metabolites, derived from aerial portions of the plants such as leaves, roots, flowers, seeds, barks, and pulps used to combat several disorders such as asthma, arthritis, cancer, etc. (SahiraBanu & Cathrine, 2015). In this study, leaves of *C. papaya* were found to

possess several secondary metabolites, and it is in line with Javed et al. (2017). It has also been reported that they activate proteins and signaling pathways against cancer cell progression.

Secondary metabolites have been gaining attention as an alternative therapeutic agent against cancer as they act particularly on cancer cells without disturbing normal cells (Rastogi et al., 2016; Javed et al., 2017). Evidence indicates that the free radicals stimulate oxidative damage to biomolecules which ultimately causes aging, diabetes mellitus, inflammation, atherosclerosis, AIDS, cancer, and numerous degenerative diseases in humans. Flavonoids and free radical scavengers that prevent oxidative cell damage possess good anticancer action (Efferth, 2017). In this study, a similar free radical scavenging effect was observed with *C. papaya* leaves and this could be due to the presence of the flavonoids in the plant.

Administration of antioxidants for patients with cancer was found to enhance the therapeutic efficacy and longevity of the patients (Singh et al., 2018). Free radical scavenging ability of chloroform extract was found to be more than methanol and ethyl acetate extracts of *C. papaya* leaves.

Further, the anti-proliferative effect of chloroform extract against MCF 7 was also found to be promising as compared with the other two extracts. Thus, the plant extract possesses antioxidant and cytotoxic effects, researchers suggest that increased free radical scavenging activity, in turn, enhances the cytotoxic effect of plant extract (Suman et al., 2012; Sammar et al., 2019).

Studies on the effect of plant extracts represented major morphological changes, apoptotic characteristics such as shrinkage of the cell, membrane blebbing, and cell density reduced which imply the anticancer activity of the plant extracts (Elumalai et al., 2012; Thangama et al., 2012). The data obtained from the study is in line with previous studies. Similarly, Lu et al. (2014) reported the apoptotic effect of curcumin on MCF-7 cells and observed the characteristic changes of apoptosis. These staining results suggest that the extract triggered apoptosis-mediated cell death in MCF-7.

The DNA fragments generated as the result of apoptosis were visualized after electrophoretic separation. From the results, a 'ladder' pattern of DNA fragments was observed and this is in line with Harshitha et al. (2019). An increased DNA fragment from the MCF 7 cells treated with chloroform extracts further supports the ant-cancer potential of the leaves extract. Previous studies reported that anti-cancer potential may be attributed to either apoptosis induction or DNA fragmentation or caspase -3 activation of poly (ADP) ribose polymerase cleavage caused by these extracts (Desai et al., 2008)

Conclusion

In this study, *C. papaya* leaves extract was selected and screened for its anti-proliferative effect against MCF 7 cells. Qualitative analysis of different extracts revealed the presence of several secondary metabolites which were proven anti-cancer phytoconstituents. From the data obtained, it may be concluded that the *C. papaya* could be used as an alternate anticancer agent and as a good antioxidant.

Conflict of Interest

Nil

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In vitro ANTI-INFLAMMATORY AND ANTIOXIDANT EVALUATION OF AN INDIGENOUS MEDICINAL PLANT – *Pterospermum rubiginosum*

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ABSTRACT

The current study was carried out to determine the antioxidant potential, anti-inflammatory activity, and cellular viability of *Pterospermum rubiginosum* (PR), a tropical tree endemic to the Western Ghats. The antioxidant activities of the PR bark methanolic (PRME) and aqueous extract (PRAQ) were tested using ABTS as well as superoxide, nitric oxide, and hydroxyl radical assays. Total antioxidant activity was evaluated by adopting the colorimetric method and correlation with their antioxidant activities was derived by Pearson co-efficient analysis. The PRME showed the highest ABTS radical scavenging activity, EC_{50} (46.09µg/ml) followed by PRAQ (52.08µg/ml). Furthermore, the PRME exhibited the highest scavenging activity against superoxide, nitric oxide, and hydroxyl radicals. The MTT assay results revealed good cellular viability up to a concentration of 100µg/ml with an EC_{50} (106.869µg/ml). The inflammatory mediators such as Cox-2, IL-1β, IL-6, and NF-kB were reduced during the treatment of PRME in LPS stimulated RAW cells. The stress marker in rat liver cells such as glutathione reductase (GR), glutathione peroxidase (GPx), and reduced glutathione (GSH) levels was found in normal levels when compared to the untreated group of rats. The antioxidant enzyme superoxide dismutase and catalase also exhibited notable bioactivity in PRME treated groups up to a concentration of 1000µg/ml. The present study showed excellent *In vitro* and *In vivo* antioxidant activity; the potent anti-inflammatory ability of PRME in reducing the LPS induced inflammation in cell culture conditions.

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1 Introduction

Reactive oxygen species (ROS) is a biologically harmful group of free radicals formed in our body during cellular metabolism, under the influence of various secondary metabolites and coenzymes (Pizzino et al., 2017). Any change in metabolic equilibrium will lead to the excess production of free radicals, including ROS, which is a sensitive trigger for oxidative stress mechanism. Various food materials are a rich source of natural antioxidants and are found to be very effective for scavenging unwanted radicals, thereby minimizing the complications related to degenerative diseases and aging (Shields et al., 2021). The oxidation induced by ROS such as nitric oxide radical, superoxide radical, hydroxyl radical, hydrogen peroxide (H_2O_2), and singlet oxygen (O_2), resulting in cell membrane disintegration, protein damage, and mutation, which may further lead to diseases such as diabetes, cancer, liver injury and cardiovascular disease (Srivastava et al., 2017). During mitochondrial respiratory chain reaction, superoxide anion is produced as natural by-products of cellular metabolism. The main sources of extracellular ROS are UV and other ionizing radiation and pollutants (Pizzino et al., 2017).

Oxidative stress occurs when the critical balance between ROS production and the endogenous antioxidant defense gets altered due to severe oxidative damage. For maintaining the oxidant status and to repair the redox equilibrium, cells undergo a self-adjustment to homeostatic changes; otherwise, we have to supplement antioxidants (Kurutas, 2015). Antioxidants have a synthetic or natural origin that reacts with free radicals and reduces their lethal effects in our body by neutralizing them. The imbalance between oxidants and antioxidants in the body leads to the damage of macromolecules such as enzymes and proteins in various regulatory and metabolic pathways and diverse health problems including lifestyle disorders. Therefore, homeostasis between antioxidants and oxidants is considered to be a crucial mechanism that reduces ROS and helps to maintain a healthy biological status (Hasanuzzaman et al., 2020).

Due to the limited availability of natural antioxidants, several synthetic molecules have been used as an alternative in the pharmaceutical industry, but long-term use of these chemicals can result in severe side effects including DNA damage, induce premature senescence, and other related complications (Kornienko et al., 2019). Due to ease of availability, synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are commonly used in food preservation and recent animal toxicity (*In vivo*) studies have validated that, these compounds are highly lethal and can induce apoptosis and promote tumor growth (Xu et al., 2021). Strict implementation of dietary restrictions is necessary to minimize systemic disorders, by European Food Safety Authority (EFSA) & Food and Drug Administration (FDA) (Lourenço et al., 2019). So finding novel safe antioxidants from natural sources, especially plant derivatives, is of great interest in food and pharmaceutical applications. Plant extracts have been widely used as effective remedies for the prevention and treatment of multiple health issues for centuries by almost every known culture. The traditional medicinal system of India, Ayurveda has been practiced for more than 5,000 years and decoctions and extracts from medicinal plants remain an essential component of its practice (Subhose et al., 2005; Babich et al.. 2020). There is a diverse variety of secondary metabolites isolated from different medicinal plants exhibiting excellent anti-inflammatory, anticancer, analgesic, antibacterial, antiviral activities and it is mainly due to the presence of novel antioxidants (Tungmunnithum et al., 2018).

Pterospermum rubiginosum (Sterculiaceae), "Ellooti" bark, or "Ellooripatta" is a tropical tree endemic to the Western Ghats, Kerala, and has been previously identified by Kanikaran tribes to exert beneficial effects on bone fractures preventing bone loss and promoting bone health (Anish et al., 2021; Vijayan et al., 2007). There is still a lack of knowledge regarding the exact mechanism behind the bioactivity of *P. rubiginosum* and it requires further study for potential therapeutic applications. Other than traditional knowledge, there have been no detailed reports on the antioxidant activities of this plant; hence the present work investigates the complete antioxidant free radical scavenging profile of methanolic and aqueous fractions from *P. rubiginosum* bark. Best of our knowledge, there has been no previous literature being available on the anti-inflammatory and animal experimental studies of *P. rubiginosum* methanolic bark extract.

2 Materials and Methods

2.1 Plant Materials and reagents

The bark of *P. rubiginosum* was collected from the Kottur forest range, Thiruvananthapuram district of Kerala with the help of tribals. The plant voucher specimen is kept in the herbarium of the Department of Botany, University of Kerala, India with a voucher number of KUBH 6189. All the reagents, methanol, and organic solvents (Merck Millipore, Germany) were analytical grade. The experiment was conducted using cell culture media, antibiotics (Sigma Aldrich, USA) & inflammatory markers (Santa Cruz, USA).

2.2 Sample extraction

After removing exfoliated outer bark, the inner bark of *P. rubiginosum* was shade dried for 3-4 weeks. The dried bark was powdered, and 500g of the powdered material was extracted with methanol in a soxhlet apparatus for 72 hrs. The extract was then concentrated with the help of a rotary evaporator and qualitative analysis of their active ingredients was carried out using standard

procedures (Azmin & Nor, 2020). For water extraction, 100g powdered bark was mixed with 1L distilled water and extracted with the help of a magnetic stirrer.

2.3 Total Antioxidant Activity

The total antioxidant activity (TAA) of both methanolic and aqueous extracts of *P. rubiginosum* was determined by adapting the method used by Subhasree et al. (2009) with slight modifications. Different concentrations of both the extracts (12.5-500µg/ml) from a stock concentration of 1mg/ml were mixed with 3ml of reagent solution (0.6M H_2SO_4, 28mM sodium phosphate, and 4mM ammonium molybdate) in capped test tubes. The tube containing the reaction solutions was incubated in a boiling water bath at 95°C for 90 minutes. After cooling to room temperature, the absorbance of the solution was measured at 695nm against a blank (0.3ml of methanol was used in place of PRME). TAA was determined based on the number of gram equivalents of ascorbic acid.

$$\% \text{ Inhibition} = \frac{(\text{Control} - \text{Test})}{\text{Control}} \times 100$$

2.4 ABTS Assay

The radical mono-cation of 2, 2'azinobis 3 ethyl benzothiazoline 6-sulfonic acid (ABTS) was generated by oxidation of ABTS with potassium persulfate (a blue chromogen) and is reduced in the presence of hydrogen donating antioxidants, following the method of Re et al (1999). ABTS (20 mM) and potassium persulfate (17 mM) were prepared as the stock solution and kept overnight at room temperature in the dark. The stock solution was diluted by mixing 1 mL of ABTS solution with 60% methanol to obtain an absorbance of 0.708±0.001 units at 734nm. 1ml of distilled water was added to 0.2ml of 5 different concentrations of the sample (5-250µg/ml from a stock concentration of 2mg/ml) and 0.16ml of ABTS solution was added and made up to the final volume of 1.36 ml. A tube with an equivalent amount of distilled water but without the test sample served as a control. The decrease in absorbance was taken after 20min at 734nm; against ascorbic acid as standard. The following formula was used to calculate the percentage inhibition:

$$\% \text{ Inhibition} = \frac{(\text{Control} - \text{Test})}{\text{Control}} \times 100$$

2.5 Nitric Oxide Scavenging Activity

In aqueous solutions at physiological pH, sodium nitroprusside spontaneously produces nitric oxide, which interacts with oxygen to create nitrite ions, which can then be measured with the Griess reagent (Patel et al., 2010). A solution of sodium nitroprusside ($5 mmol L^{-1}$) was mixed with different concentrations of extracts (5-250µg/ml) from a stock concentration of 2mg/ml and incubated at 25°C for 30 minutes. A control without test compound, but an equivalent amount of distilled water was taken. The incubated solution (after 30 minutes) was diluted with Griess reagent (1% sulphanilamide, 2% phosphoric acid, and 0.1% N-1-naphthyl ethylene diamine dihydrochloride). The chromophore generated in this study was measured at 546nm and with reference standards of gallic acid was quantified in terms of its scavenging activity.

$$\% \text{ Inhibition} = \frac{(\text{Control} - \text{Test})}{\text{Control}} \times 100$$

2.6 Super Oxide Scavenging Assay

NADH oxidation generates a superoxide anion in the riboflavin-NADH system, which is detected by reducing NBT to blue formazan products (Valento et al., 2002). From the stock solution of 2mg/ml, 5 different concentrations of the sample (5 -250µg/ml) mixed with 0.05 ml of riboflavin (0.12 mM), 0.2 ml of EDTA solution (0.1 mM), and 0.1 ml of NBT solution (1.5 mM) were taken in test tubes, reaction mixtures were mixed with phosphate buffer (0.067M), a control without test compound, but distilled water in an equivalent amount. Triggering of reaction was done by illuminating the above solutions for 5mins using a fluorescent lamp. After 30 minutes, the absorbance was measured at 560 nm using a UV-visible spectrophotometer, percentage of scavenging superoxide anion generation was calculated as

$$\% \text{ Inhibition} = \frac{(\text{Control} - \text{Test})}{\text{Control}} \times 100$$

2.7 Hydroxyl Radical Scavenging Activity

The assay was based on a method to quantify ribose degradation by condensation with TBA (Elizabeth & Rao, 1990). Hydroxyl radical was generated by the Fe^{3+} - ascorbate - EDTA - H_2O_2 system (Fenton reaction). From the stock concentration of 2mg/ml, 5 different concentration of extracts were mixed with 500µl reaction mixture {2 deoxy 2 ribose (2.8mM), $FeCl_3$ (100µm), EDTA (100µm), H_2O_2 (1.0mM), ascorbic acid (100µm) in KH_2PO_4 - KOH buffer (20 mM; pH 7.4)} was made up to a final volume of 1 ml. Control was taken without a test sample but in an equivalent amount of distilled water. After incubation for one hour at 37°C, 2.8% TCA and 1ml of 1% aqueous TBA were added and reacted at 90°C for 15 minutes for color development. The absorbance of the cooling solution was measured against a blank solution at 532nm. Hydroxyl radical scavenging was calculated as:

$$\% \text{ Inhibition} = \frac{(\text{Control} - \text{Test})}{\text{Control}} \times 100$$

2.8 Cell culture

Mouse monocyte macrophage (Raw264.7) cells were initially procured from National Centre for Cell Sciences (NCCS), Pune, India, and maintained Dulbecco's modified Eagles medium, DMEM (Sigma Aldrich, USA). The cell lines were grown in DMEM with 10% FBS, L-glutamine, sodium bicarbonate, and the antibiotic solution containing Penicillin (100U/ml), Streptomycin

(100g/ml), and Amphotericin B (2.5g/ml). The cultured cell lines were kept at 37ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany). Two days old confluent monolayers of cells were trypsinized, suspended in a 10% growth medium, seeded in 96 wells of a tissue culture plate at 37°C in a humidified 5% CO_2 incubator, and then incubated for 24 hours. 1mg of bark extract was dissolved in 1mL DMEM using a cyclomixer (stock sample), and then the sample was filtered using a 0.22 µm Millipore syringe filter to ensure sterility. After incubation, growth medium was discarded and freshly prepared stock samples in 5% DMEM were serially diluted (100µg, 50µg, 25µg, 12.5µg, 6.25µg in 500µl of 5% DMEM). 100µl of diluted samples were added to corresponding wells (in triplicates) and incubated in a humidified 5% CO_2 incubator at 37°C. The cell viability was examined using an inverted phase-contrast microscope (Olympus CKX41 camera equipped with an Optika Pro5 CCD), followed by MTT assays. Microplate readers were used to measure absorbance values at a wavelength of 570 nm. The formula was used to calculate the percentage of growth inhibition,

$$\% \text{ cell viability} = \frac{\text{Mean OD Samples}}{\text{Mean OD of control}} \times 100$$

2.9 ELISA estimation of inflammatory mediators

The ELISA estimation is normally performed to quantify the level of different inflammatory markers (antigens) using a specific antibody (Engvall & Perlman, 1971). The confluent raw 246.7 cells were treated with a sample (PRME) at a stock concentration of 1mg/ml and kept at 37°C for 24 hrs in a CO_2 incubator. The supernatant was collected for protein estimation and transferred 100µL to 96 well plates and incubated at 37°C overnight for cell lysate coating. The cell lysate coated wells were blocked using blocking buffer containing 0.2% gelatin in 0.05% Tween 20 in PBS and incubated for 1hour at room temperature, washed twice with PBS TWEEN20 and 100µl of Cox-2, IL1-β, IL-6, and NF-kB primary antibodies (Santa Cruz, USA) was added and left for 2 hours at room temperature, washed again with PBS TWEEN (2 times). 100µL of secondary antibody (HRP Conjugate, Santa Cruz, USA) was added and kept at room temperature for 1 hour, washed with PBS TWEEN two times, and added 200µL chromogen; O-dianizdine hydrochloride, incubated at room temperature for 30 minutes. The reaction was terminated by adding 50 µl 5N HCl and the absorbance was read at 450 nm after 5-30 minutes in an ELISA reader (Islam & Jones, 1988).

$$\text{Activity of Antibody} = \frac{\text{OD Value}}{\text{Protein concentration}}$$

2.10 Animal studies

Healthy female Sprague Dawley rats of 2-3month age and 200-250g body weight, procured from the animal house, Department of Biochemistry, University of Kerala were used for the study. Animals were treated as per CPCSEA guidelines and the experimental protocol was approved by the Institutional animal ethical committee order number as follows; IAEC-2-KU-01/2018-19-BCH-AAR and IAEC-KU-09/2018-19-BCH-AAR; the dose-dependent toxicity study was sanctioned to be conducted in agreement with OECD guidelines. Rats were grouped into five groups of six animals each, group: I - control rats (normal saline) and group (II, III, IV, V) were administered with different doses of PRME 50, 300, 500, 1000mg/kg/day respectively and kept under observation for 14 days. Animals were scarified on the 15th day; blood was collected for biochemical investigations and stress marker studies. SOD activity was determined by the method described by Kakkar et al. (1984). The catalase activity was determined by the method of Maehly & Chance (1954). Glutathione reductase was assayed by the method of David & Richard (1983). The glutathione peroxidase was estimated by the method of Lawerence & Burk (1976) as modified by Agergaard & Jense (1982). The glutathione content of the tissues were determined by the procedure described by Patterson & Lazarow (1955).

2.11 Statistical analysis

Graph Pad Prism 5 software (GraphPad Software Inc.,) was used to calculate the Standard deviation, Two-way analysis of variance (ANOVA), and EC_{50} values of different antioxidant assays. The Pearson correlation coefficient and p values < 0.05 were regarded as significant. Values expressed are means of three replicate determinations ± standard deviation.

3 Results and discussion

With the help of a single assay, we cannot understand the complete antioxidant profile of any natural product because the bio-potentiality of plant extracts is influenced by various physicochemical factors in a symbiotic pattern and most of the natural antioxidants are multifunctional due to the presence of abundant phytochemicals. So it is very essential to study the complete antioxidant profiling, to understand the antioxidant potential and its interactive mechanisms to eliminate free radical initiated toxicity in various disease conditions and also in metabolic pathways (Gan et al., 2010).

3.1 Antioxidant activity by ABTS and Total antioxidant assay

The ABTS radical scavenging and total antioxidant activity of PRME and PRAQ were estimated and compared with standard (ascorbic acid). The highest scavenging activity was exhibited by PRME followed by PRAQ (Figure 1). Being simple and reliable antioxidant measurement, the total antioxidant assay has been commonly used to evaluate the antioxidant capacity of plant extracts. The method is based on the reduction of Mo^{6+} to Mo^{5+} by

the antioxidants present in natural products and results in the formation of green phosphomolybdenum complex with the maximum absorption being calorimetrically measured at 695nm. The transfer of electrons from antioxidants to neutralize toxic radicals depends on the structure and binding moieties of antioxidant molecules (Lobo et al., 2010). The PRME showed a higher reduction (61.09g AA/100g extract) followed by PRAQ (57.96g AA/100g extract). The antioxidant activity confirmed the medicinal importance of both plant extracts as naturally occurring antioxidants.

ABTS is a protonated radical having a characteristic maximum absorbance at 734 nm and decreases with the scavenging of the $ABTS^{+}$ radicals. The plant extract possessing ABTS free radical scavenging activity indicated that it can act as a hydrogen donor, which converts free radicals to more stable products and thereby terminating the oxidation process (Ilyasov et al., 2020). The high molecular weight phenolics have an excellent capacity to scavenge $ABTS^{+}$ free radicals. Since both the extracts PRME and PRAQ can quench free radicals, thereby lowering the lipid oxidation process via chain-breaking reaction, these molecules can be considered as novel antioxidants based on detailed biological studies. This might be due to the presence of hydroxyl groups in ortho, meta, and para positions of the phenolic compounds present in these extracts (Mathew et al., 2015). The studies on the antioxidant activity of different medicinal plants have indicated that the phenolic compounds are key players to scavenge ABTS radicals. The results showed that PRME with an EC_{50} value of 46.09µg/ml followed by PRAQ fractions (52.08ug/ml) showed good ABTS scavenging capacity, which can lower the oxidation process by reducing free radicals in a dose-dependent manner ($p<0.05$). The correlation analysis showed good Pearson co-efficient relation between standard AA (r=0.8988) and PRME (r=0.8585) and PRAQ with r value 0.9160 respective.

3.2 Antioxidant activity by Nitric oxide and Superoxide radical scavenging assay

The nitric oxide and superoxide radical scavenging ability of both methanolic and aqueous bark extracts were compared with the ascorbic acid standard. The PRME showed better radical scavenging activity than PRAQ and is shown in figure 2. Nitric

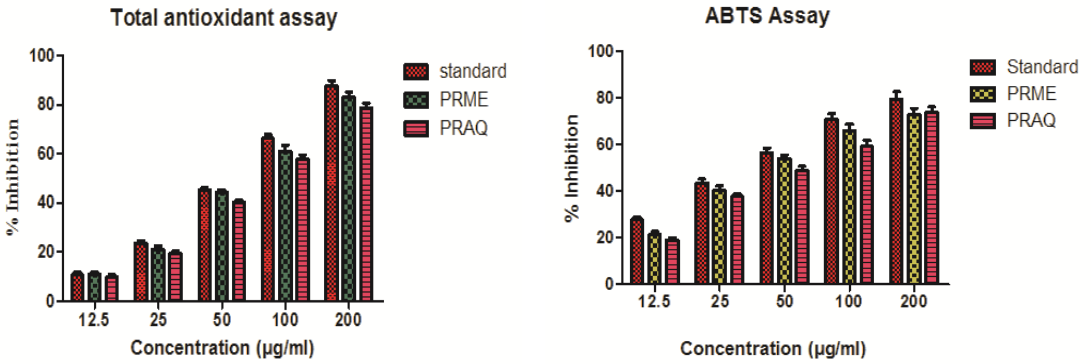

Figure 1 The PRME extract gave the highest TAA with EC_{50} value 81.85µg/ml followed by aqueous EC_{50} value 86.26µg/ml. ($p < 0.05$). Values are expressed as mean ± standard deviation (n=3).

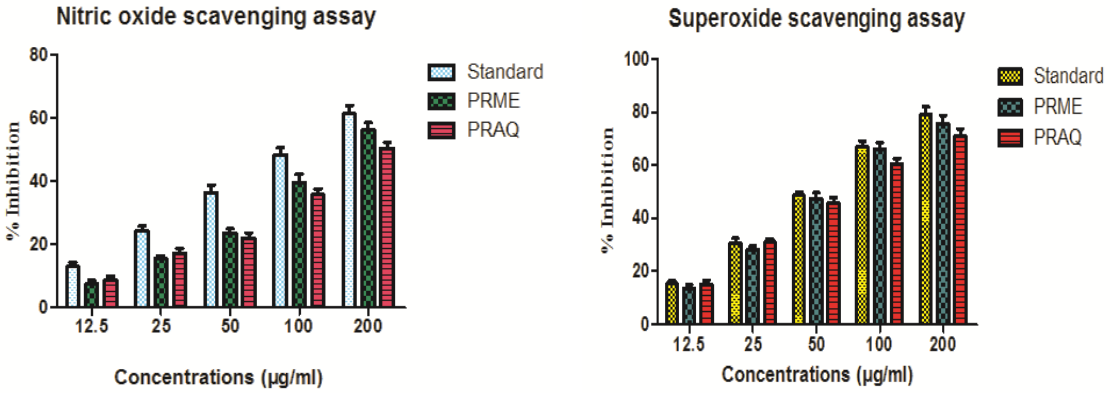

Figure 2 Shows the Nitric oxide and Superoxide radical scavenging activity of PRME and PRAQ ($p < 0.05$). Values are expressed as mean ± standard deviation (n=3).

oxide is an important chemical mediator involved in inflammatory mechanisms. Reactive nitrogen species (RNS) are free radicals that are formed due to the interaction of NO with oxygen, which leads to the production of different intermediates such as stable nitrates, NO_2, N_2O_4, and N_3O_4 (Ozcan & Ogun, 2015). The singlet oxygen (O_2) and NO radicals are directly involved in the lipid peroxidation process and chronic inflammatory cascades (Valko et al., 2007). The results showed a dose-response increase in the capacity to quench hydroxyl radicals for all the concentrations studied (Figure 2). A concentration-dependent scavenging of NO radicals by the PRME was significant at $p<0.05$ ($r= 0.9786$, $p=0.01$), followed by PRAQ ($r=0.9778$) on correlation analysis. The PRME fraction showed the highest OH scavenging potential (EC_{50} value of 177.21µg/ml) in a concentration-dependent manner followed by PRAQ fraction 197.82µg/ml.

The superoxide scavenging ability as measured by the riboflavin-NBT-light system indicates that the anion radicals produced due to the photochemical reduction of riboflavin lead to a decrease in blue formazan solution absorbance at 560 nm. The O_2 anion radical was inhibited in a dose-dependent manner as shown in figure 2. The EC_{50} values of PRME and PRAQ were 76.24 µg/ml & 82.98 µg/ml respectively. These results indicated that PR bark fractions, especially methanolic fractions showed significant inhibition of superoxide radical, compared to positive control ascorbic acid (EC_{50} values 75.18 µg/ml).

3.3 Hydroxyl radical scavenging assay

The hydroxyl radical scavenging capacity of both PRME and PRAQ, compared with standard gallic acid, is summarised in table 1. Hydroxyl radical is one of the dangerous reactive oxygen species in a biological system having the capacity to damage most of the biomolecules in our body. There is no specific enzyme or molecules available in the human body to eliminate or minimize their harmful effects (He et al., 2017). This free radical can combine with unwanted DNA nucleotides, resulting in a mismatch of base sequences and leading to ageing, genetic damages, and carcinogenesis (Sharifi-Rad et al., 2020). Hence eliminating the hydroxyl radical is a very crucial defence mechanism to keep our body healthy and the ability of PRME to reduce hydroxyl radical is considered to be good for preventing the lipid peroxidation process. The results showed a dose-response increase in the capacity to quench hydroxyl radicals for all the concentrations studied (Table 1). Inhibition of hydroxyl radicals by the methanol extract was found to be significant at $p<0.05$ ($r= 0.9009$, $p=0.01$) with gallic acid standard ($r=0.9161$), followed by aqueous ($r=0.8615$) on correlation analysis. The PRME fraction showed the highest OH scavenging potential (EC_{50} value of 95.11 µg/ml) in a concentration-dependent manner followed by PRAQ fraction 181.29 µg/ml.

3.4 Antioxidant assays by correlation analysis

The results of the different antioxidant assays were compared, correlated with each other, and summarized in table 2. Significant correlations (*p-value < 0.001*) were observed for all the antioxidant assays. The TAA assay values showed good correlation coefficient 'r' values for ascorbic acid standard, PRME, and PRAQ with 0.9509, 0.9522, and 0.9560 respectively, Superoxide radical assay with ascorbic acid ($r=0.9150$), PRME ($r=0.9107$), and PRAQ ($r=0.9032$). In hydroxyl radical scavenging assay also standard ($r=0.9161$) showed close value to PRME ($r=0.9009$). The correlation values fall in a range of ($r=0.8585$) to ($r=0.9786$), and this is due to the antioxidant activity of plants extracts which are influenced by various compounds like alkaloids, flavonoids, phenolics & glycosides (Tungmunnithum et al., 2018).

Table 1 Hydroxyl radical scavenging activity of PRME and PRAQ

Concentrations (µg/ml)	Gallic acid	PRME	PRAQ
25	16.50±1.43	17.16±1.06	13.50±1.46
50	31.36±1.53	34.05±1.34	33.24±1.57
100	40.70±1.67	43.59±173	38.62±1.43
200	56.58±2.46	52.73±1.95	45.81±1.81
400	65.66±2.35	63.11±2.47	55.16±2.85

Values are expressed as mean±standard deviation (n=3).

Table 2 Comparative correlation analysis of different antioxidant assays

Antioxidant assays	Pearson co-efficient values (r)		
	Standard	PRAQ	PRME
Total antioxidants assay	0.9509	0.9560	0.9522
Superoxide radical assay	0.9150	0.9032	0.9107
Nitric oxide radical assay	0.9427	0.9786	0.9778
Hydroxyl radical assay	0.9161	0.9009	0.8615
ABTS radical assay	0.8988	0.9160	0.8585

Values are expressed as mean±standard deviation (n=3).

3.5 MTT assay

The MTT assay is used to evaluate the cellular toxicity of any drug or plant material in *In vitro* culture. During plant material characterization, preliminary quantifying the toxicity in cell lines is a crucial step before moving to higher level molecular studies. The MTT assay showed good cellular viability of PRME up to a concentration of 100µg/ml (Figure 3). The anti-inflammatory effect of PRME on the LPS-induced inflammation in RAW 264.7 cells (murine macrophage cell line) was assessed using MTT assay and the entire plate was observed after 24 hours of treatment in an inverted phase contrast tissue culture microscope. The morphological changes in cells including shrinkage, rounding,

granulation, and cytoplasmic vacuolization were regarded as the symptoms of cytotoxicity. The results showed that PRME does not influence the viability of RAW 264.7 cells and the cellular competency was selected for detailed study. LC50 Value of PRME was found to be 106.869µg/ml and the LC50 concentration was selected for evaluating the inflammatory mediators (Figure 3).

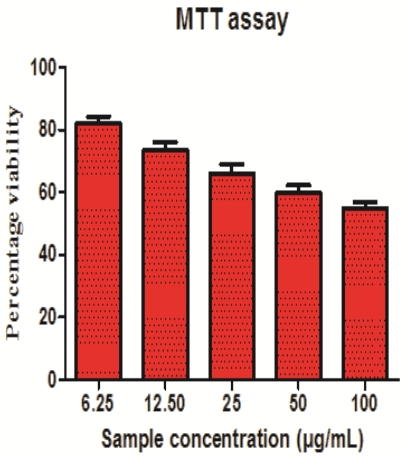

Figure 3 Effect of PRME on RAW cells. The graph represents the percentage cell viability on treatment with different concentrations of the extract. The values are represented as mean ± standard of three experiments.

3.6 Inflammatory marker study

The anti-inflammatory activity of PRME was evaluated using the ELISA method. On LPS treatment, the RAW cells shows the increased activity of inflammatory mediators, after the addition of PRME significantly reduced the activity of cyclooxygenase-2, interleukins - 1β and 6, and nuclear factor-kB (Table 3). The LPS treated wells showed the antibody activity of Cox-2, IL-1β, IL-6, and NF-kB in the order respectively 1.34996, 1.569258, 1.520547, and 1.624434, and this activity is significantly reduced after PRME treatment and incubation period for 24hrs as 1.15089, 1.084416, 1.03618 and 1.102227 (Table 3). During LPS treatment, the macrophages get activated and various inflammatory mediators and cytokines (Cox-2, IL-1β, IL-6) are synthesized, which enhance the microenvironment for inflammation and further aggravate the conditions. The interleukins IL-1β, IL-6 plays an important role in enhancing autoimmune diseases, bacterial infections, metabolic syndromes, malignant disorders, acute and chronic inflammation (Kany et al., 2019). These mediators along with other subsidiary factors activate the transcription factor NF-kB, a crucial checkpoint of inflammatory pathways because NF-kB controls nearly 150 genes involved in inflammation pathways, immune defense as well as in cell proliferation and differentiation mechanisms (Panday et al., 2016). During the LPS activation, NF-kB translocates from the cytoplasm to the nuclear region and acts as a transcription factor for the synthesis of inflammatory enzymes and cytokines. Any agents which can inhibit or reduce the level of inflammatory mediators are considered good anti-inflammatory agents. The result demonstrated that PRME can inhibit the activity of inflammatory enzyme Cox-2 along with the interleukins, thereby reducing the production of pro-inflammatory cytokines, including IL-6 and IL-1β in RAW 264.7 cells stimulated by LPS; which directly or indirectly suppress the activation of transcription factor NF-kB. These results suggest that PRME significantly improves LPS induced inflammatory conditions in RAW cells by inhibiting the overproduction of inflammatory mediators.

Table 3 Inflammatory marker study

Inflammatory markers	Activity Units/mg protein	
	Control	Sample
Cox-2	1.34996	1.15089
IL-1β	1.569258	1.084416
IL-6	1.520547	1.03618
Nf-Kb	1.624434	1.102227

Values are expressed as mean ± standard deviation (n=3).

The antioxidant enzymes and stress markers are investigated from the liver tissue obtained from animal acute toxicity study for 14 days. After the PRME treatment, the animals were scarified and quantified the levels of different stress mediators in liver tissue are summarised in table 4. The first line antioxidant enzyme defense mechanism is from superoxide dismutase (SOD), catalase, and glutathione peroxidize, which convert the toxic superoxide and hydrogen peroxide radicals into water (Ighodaro & Akinloye, 2018). The PRME treated animals up to a concentration of 1000µg/ml did not show any significant variation in antioxidant enzymes when compared to normal rats. The enzyme catalase and SOD of the treated group showed good linearity with control rats, the glutathione peroxidize values exhibited a constant increase in the antioxidant activity in PRME treated animals in the range, (35.71± 1.05 to 39.73 ± 1.72) from table 4, which indicated the potent antioxidant activity of PRME, and it may be due to the presence of high phenolic content in bark extracts. Manna et al. (2009) reported that the bark extract of *P. acerifolium* significantly reduced alcohol-induced ulceration on oxidative damages in adult Charles foster rats, pre-treatment elevated the enzymes SOD, CAT, and GSH content when compared to the control group animal.

The oxidoreductase enzyme, glutathione reductase (GR) enhances the reduction of oxidized glutathione to reduced glutathione (GSH) in the presence of NADPH as a co-factor. The antioxidant enzyme (GR) and cellular thiol antioxidant (GSH) play a major role in cellular defense against drug and toxin-induced hepatotoxicity, associated with oxidative stress (Espinosa-Diez et al., 2015). Being a sensitive indicator of toxicity, both the values of GSH and GR in PRME treated groups were compared with normal control rats, the

Table 4 Stress marker study in liver tissue of rats

Parameters	Group I	Group II	Group III	Group IV	Group V
GPx(U/mg protein)	32.56 ± 1.38	32.69 ± 1.34	34.88 ± 1.34	34.80 ± 2.02	33.85 ± 1.79
GSH (U/mg protein)	82.86 ± 1.86	82.93 ± 2.07	82.65 ±1.91	84.033 ± 1.95	83.78 ± 2.06
GRd(µmole of glutathione reduced/ min/mg protein)	144.67 ± 4.23	143.61 ± 3.31	146.62 ±4.84	145.06 ± 4.49	143.22 ± 4.12
SOD (U/mg protein)	2.41 ± 0.19	2.45 ± 0.123	2.48 ± 0.12	2.57 ± 0.18	2.51 ± 0.26
Catalase (µmol of H_2O_2 consumed/ min/mg protein)	7.43 ± 0.38	7.57 ± 0.25	7.53 ± 0.21	7.59 ± 0.28	7.56 ± 0.237

Values are expressed as mean ± standard deviation (n=6).

PRME treated group showed a slight increase in the values of GSH, a strong non-enzymatic antioxidant in 500 and 1000µg/ml treated groups, at the same time the levels of GR are comparatively high in all treated groups when compared to control rats 147.65 ± 4.08 (Table 4). From this study, we concluded that the PRME is rich in antioxidants, compared to PRAQ, and showed excellent antioxidant activity under *In vitro* condition; stress marker studies in rat liver cells confirmed the non-toxicity of PRME up to a concentration of 1000µg/ml for 14 days of treatment. MTT assay and inflammatory marker studies revealed the anti-inflammatory activity of PRME in reducing the LPS induced inflammation in RAW246.7 cells.

Conclusion

The present study revealed that PRME possesses excellent antioxidant and anti-inflammatory properties and could serve as free radical inhibitors, scavengers, or primary antioxidants. A positive correlation was detected for phenolic content and free radical scavenging activity of PRME. Toxicity evaluation studies on female Spargue Dawley rats showed that PRME up to a concentration of 1000µg/ml for 14 days is not harmful, indicating the prospects for more advanced investigations to better understand PRME's mechanism as a pharmacological agent.

Author Contributions

The authors contributed to this work as follows; extraction, antioxidant assays, anti-inflammatory analyses, and animal experiments were performed by R J Anish under the supervision of Arun R Rauf. Both the authors contributed to statistical and data analysis, manuscript preparation, reviewed the article, edited, and approved the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Abbreviations

PRME: *P. rubiginosum* methanolic extract; PRAQ: *P. rubiginosum* aqueous extract; ROS: reactive oxygen species; ABTS:2, 2'azinobis 3 ethyl benzothiazoline 6- sulfonic acid; TAA: total antioxidant activity; (MTT) : 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; cox-2: cyclooxygenase-2; IL-1β: interleukins-1β; IL-6: interleukins-6; NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells; GR: glutathione reductase; GPx: glutathione peroxidise; GSH: reduced glutathione.

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