MANGROVE PLANTS: A SOLUTION TO BACTERIAL, FUNGAL AND CANCEROUS GROWTH.

THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY (SCIENCE)

IN MICROBIOLOGY

By

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AFFILIATED TO UNIVERSITY OF CALCUTTA

2022

St. Xavier's College (Autonomous), Kolkata
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Mangrove Plants: A solution to bacterial, fungal and cancerous growth.

Ph.D Thesis

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(Submitted on: April, 2022)

This thesis is dedicated to my late grandfather,
Md. Safiruddin, who has been my inspiration, both in his presence and absence.

This thesis is also sincerely dedicated to my parents and sister, who are my greatest source of strength and unconditional support.

Acknowledgements

This thesis has been submitted for the degree of Doctor of Philosophy (Ph.D) in Microbiology (Science). The work was done (from June 2018 – October 2021) at the Department of Microbiology, at St. Xavier's College (Autonomous), Kolkata, under the supervision of Dr. Arup Kumar Mitra and Dr. Satadal Das.

My sincere acknowledgement to **Rev. Dr. Dominic Savio, SJ**, Principal, St. Xavier's College, Kolkata for providing me the privilege and opportunity to carry out my research work in this premier institute as a research scholar.

This work would not have been possible without the help and support of many people. Completing this research work has been an extremely challenging as well as rewarding experience, and has changed my outlook towards my life and career ahead, mainly because of my mentors. Throughout the tenure, I have learnt not only about my research topic from them, but developed a sound knowledge of microbiology and its applications and have learnt the importance of patience, work ethics, team work and coordination, which I believe will be of great value in my future as well.

First and foremost, I am deeply indebted to **Dr. Arup Kumar Mitra**. Throughout the doctoral studies, he has not only been my principal supervisor, but has been my teacher, guide and guardian, and has never ceased to motivate me at any step of my research. His motivation toward scientific research and educational goals has taught me a lot and inspired me. I am indebted to him for teaching me everything from the scratch and has taught me to progress by transforming failures into stepping stones of success. My sincere gratitude and due acknowledgements to my Ph.D joint supervisor, **Dr. Satadal Das**, Consultant Microbiologist and Professor, Peerless Hospital & B.K. Roy Research Centre, Kolkata, for guiding me regarding scientific research approach, research methodologies, designing of experiments and analysis of results. His valuable advice would help me strive ahead in life. His support, kind words and constructive work-related discussions, helped me develop a zeal and passion towards my work every single day during this journey.

I express my heartfelt gratitude to Dr. Sudeshna Shyam Choudhury, Head, Department of Microbiology, for extending her support in conducting research in the department, ensuring that research scholars perform their work in a smoothly. Though officially not my Ph.D mentor/supervisors, my acknowledgement addresses the immense support provided by each and every professor of the department who will always remain my mentors for life-

Dr. Sudeshna Shyam Choudhury, Dr. Mahashweta Mitra Ghosh, Dr. Madhumita Maitra, Dr. Riddhi Majumder, Dr. Anindita Banerjee, Dr. Jaydip Ghosh, Dr. Kasturi Sarkar, Prof. Debjani Dutta and **Prof. Lopamudra Roy**- each one of them have been a great support to me in developing an optimistic view in the field of my work and above all, have guided me in every matter. They have always inspired me to improve as a researcher every day and have appreciated every little accomplishment I have been able to make. Above all, my mentor Dr. Arup Kumar Mitra and all my professors, have always been concerned about by health, well-being and have inculcated good humanitarian values within me, and taught me to become a good human being in life.

I would also like to acknowledge **Dr. Tapati Dutta**, Dean of Science, for her constant motivation. My sincere regards to **Dr. Samrat Roy**, Coordinator of the Ph.D cell, for ensuring smooth progress of every researcher and organizing all the chain of events and processes required in our Ph.D journey and accomplishment of our goals. I sincerely thank all the RAC members for enabling me to improve my work through constructive and fruitful discussions.

Many thanks to my juniors of UG and PG departments for their constant motivation and I also thank my fellow research scholars Puja Agnihotri, Bikram Dhara, Meesha Singh and Debanjana Sengupta for being an excellent team to work with and providing a healthy working and studying environment. My acknowledgements are also addressed to all the laboratory technicians and non-teaching staff of St. Xavier's College, Kolkata and Peerless Hospital, Kolkata, for their support and help.

This work would not have been possible without the financial support from the Department of Biotechnology, Government of West Bengal (WB-DBT) [Sanc. No.:71/(Sanc.)-BT/ST/P/S&T/2G-45/2017], which is very much acknowledged. The technical support from Peerless Hospital and B.K.Roy Research Center, Kolkata for antimicrobial assays, IIT-Bombay for LC-MS and Rajiv Gandhi Centre for Biotechnology, Kerala for Cellular imaging analysis, are duly acknowledged.

Last but not the least-indeed most importantly- I deeply thank my mother for the love and support during the most hectic moments of work. I believe it is her selfless hard work that has enabled me to grow from a toddler- teaching me the values and principles of life- that has ultimately made it possible for me to become capable of submitting my doctoral thesis. I thank my father for extending ceaseless support in all possible ways. My younger sister played a pivotal role in being a great source of strength and positivity, providing me with supportive advice. I thus, thank my parents- **Mrs. Sufia Khatun** and **Mr. Md. Babar Ali**, and my younger sister, **Suhana Sultana**, for their support throughout the journey of my education. Their silent prayers and good wishes have been my armour against all the odds that have come in the way. Me being a reason for their happiness shall always be my greatest accomplishment.

I am deeply indebted to my late grandfather, **Md. Safiruddin**, whose vision and motivation enabled me to take the step ahead in this field of study.

You all were a big and significant part in helping me complete my doctoral research work and I thank each and every one of you from the core of my heart.

Above everything, all praise belongs to the Almighty.

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Kolkata.
April, 2022.

CONTENTS

List of Abbreviations

- *A.alba*: *Avicennia alba*
- *A.flavus*: *Aspergillus flavus*
- *A.rotundifolia*: *Aegialitis rotundifolia*
- AAE: Ascorbic Acid Equivalent
- ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
- AMPH: Amphotericin B
- AMR: Antimicrobial Resistance
- AMX: Amoxicillin
- ATCC: American Type Culture Collection
- *B.gymnorhiza*: *Bruguiera gymnorhiza*
- *B.subtilis*: *Bacillus subtilis*
- *C.parapsilosis*: *Candida parapsilosis*
- *C.tropicalis*: *Candida tropicalis*
- CDDEP: Center for Disease Dynamics, Economics & Policy
- CDK: Cyclin dependent kinase
- CFP: Cyan fluorescent protein
- CIP: Ciprofloxacin
- CLSI: Clinical and Laboratory Standards Institute
- DAD: Diode array detection;
- DCFH-DA: 2', 7' dichlorofluorescein diacetate
- DEVD: Asp-Glu-Val-Asp
- DMSO: Dimethyl sulfoxide
- DNA: Deoxyribonucleotide
- DNS: Dinitrosalicylic acid
- DPPH: 1,1-diphenyl-2-picrylhydrazyl
- DTNB: 5, 5'-dithio 2-nitro benzoic acid
- *E.aerogenes*: *Enterobacter aerogenes*
- *E.agallocha*: *Excoecaria agallocha*
- *E.coli*: *Escherichia coli*
- EBV-EA: Epstein-Barr Virus- Early Antigen
- EDTA: Ethylenediamine tetra acetic acid
- EGFP: Enhanced green fluorescent protein
- ESBL: Extended-spectrum beta-lactamases
- ESBL-EC: Extended-spectrum beta-lactamase *Escherichia coli*
- ESI: Electron Spray Ionization
- F1: Eluted fraction 1 of ethanolic extract of *Excoecaria agallocha*
- F2: Eluted fraction 2 of ethanolic extract of *Excoecaria agallocha*
- F3: Eluted fraction 3 of ethanolic extract of *Excoecaria agallocha*
- FAD: Flavin adenine dinucleotide
- FIGO: International Federation of Gynaecology and Obstetrics
- FRAP: Ferric Reducing Antioxidant Power

- FRET: Fluorescence resonance energy transfer
- FSI: Forest Survey of India
- GASS: Global Antimicrobial Resistance and Use Surveillance System
- GFP: Green fluorescent protein
- GNB: Gram-negative bacilli
- GR: Glutathione reductase
- GSH: Glutathione
- HPLC: High Performance Liquid Chromatography
- HPV: Human Papilloma Virus
- HR LCMS: High resolution liquid chromatography mass spectroscopy
- IARC: International Agency for Research on Cancer
- IC50: Half- maximal inhibitory concentration
- ICMR: Indian Council of Medical Research
- IUCN: International Union for Conservation of Nature and natural resources
- *K.pneumoniae*: *Klebsiella pneumoniae*
- KCZ: Ketoconazole
- LC3: Light chain 3
- MDR: Multidrug resistant
- MDR-TB: Multidrug resistant-tuberculosis
- MIC: Minimum Inhibitory Concentration
- MRSA: Methicillin Resistant *Staphylococcus aureus*
- MTT: (4,5- dimethylthiazol- 2- yl)- 2, 5- diphenyltetrazolium bromide
- NADH: Nicotinamide adenine dinucleotide
- NADPH: Nicotinamide adenine dinucleotide phosphate
- NBT: Nitro Blue Tetrazolium
- NDM: New Delhi metallo-β-lactamase
- NLS: Nuclear localization sequence
- NMR: Nuclear Magnetic Resonanace
- OD: Optical density;
- Omp: Outer membrane protein
- p- NA: para- nitroaniline
- *P.aeruginosa*: *Pseudomonas aeruginosa*
- *P.chrysogenum*: *Penicillium chrysogenum*
- PBMC: Peripheral blood mononuclear cells
- PBS: Phosphate buffer saline
- PDB: Protein Data Bank
- PE: Plant extract
- PI: Propidium iodide
- PMS: Phenazine methosulfate
- Q-TOF: Quadrupole Time of Flight
- R_f: Retardation factor
- RFP: Red fluorescent protein
- ROS: Reactive Oxygen Species

- RPMI 1640: Roswell Park Memorial Institute-1640 medium;
- RR-TB: Rifampicin resistant-tuberculosis
- *S.aureus*: *Staphylococcus aureus*
- *S.typhi*: *Salmonella typhi*
- SAP: Secreted aspartic proteinase
- SD: Standard deviation
- SDS- PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
- SMAC: Second mitochondria- derived activator of caspase;
- SMILES: Simplified Molecular Input Line Entry System
- SOD: Superoxide dismutase
- TAC: Total antioxidant capacity
- TAE: Tannic acid equivalent
- TBA: Thiobarbituric acid
- TCA: Trichloroacetic acid
- TET: Tetracycline
- TFC: Total flavonoid content
- TLC: Thin layer chromatography
- TPC: Total phenolic content
- TPTZ: 2,4,6- tri(2-pyridyl)-S-triazine
- TTC: Total tannin content
- UNESCO: United Nations Educational, Scientific and Cultural Organization
- UV: Ultraviolet
- WHO: World Health Organization
- YFP: Yellow fluorescent protein

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Chapter 1:

Introduction

Introduction

The increase of pollution and anthropogenic activity in the recent years has resulted in the increase of bacterial and fungal outbreaks drastically along with the incidence of cancer. These pose a serious threat to human life as well as to the global health system. Microbial infections are one of the major issues and a serious problem in the healthcare systems all across the globe. Pathogenic microorganisms are transforming into multi drug resistant species due to inappropriate and uncontrolled antibiotic and drug use, especially in the developing countries like India. Antibiotic resistant bacteria emergence has been reported in hospitals throughout the world. Some 'nosocomial' infections may be resistant to a number of clinically efficacious antibiotics and turn out to become less effective even at a considerably high dosage of new generation of antibiotics. Therefore, the emergence of bacteria resistant to antibiotics is a severe problem and cost of treating these infections has become a burden to the common people. These complications in health not only adversely affect the patient but result in longer hospital stays, more expensive medications and treatments that have huge financial impact and challenge for those impacted. Fungal infections have also reached a level of crisis in immunocompromised individuals in recent years. Simultaneously, emergence of drug- resistant fungal infections is exasperating the already difficult treatment situation.

Traditional medicine plays a significant role in the healthcare system of developing countries, especially in Asia including India. Medicinal plants have been used for centuries as remedies for treating human diseases because they possess constituents of therapeutic value. In this present context of microbial drug resistance, it is very important to fall back to the natural resources of plants to identify potential compounds that may be of significant importance in the pharmaceutical and biomedical world. As stated by WHO in 2019, "the clinical pipeline of new antimicrobials is dry". Hence, effective antimicrobials and chemotherapeutic agents need to be developed, probably with novel mode of action, to overcome these health problems.

Indian Sundarban mangrove ecosystem, which encompasses a major area in the deltaic complex of the rivers Ganga, Brahmaputra and Meghna, are known to have diverse natural products with remarkable pharmaceutical importance and have been reported to exhibit potent antimicrobial, antiviral, antilarval, anticancer and anti-plasmodial activity. Limited scientific investigation has been conducted on unravelling the anticancer, antimicrobial and antioxidant aspects of the mangrove plant species of the Indian Sundarbans. The investigation pertaining to this thesis was one such initiative and the findings may serve as a stepping stone to shortlist the mangrove plant of choice that could be further exploited to identify and characterise biologically active compounds for efficient drug development with promising antibacterial, antifungal and anticancer activity with simultaneous study of the associated mechanism of action. The results would plausibly contribute value to already existing experimental evidences and elicit the qualitative standards of natural compounds extracted from mangrove plants to be applied for therapeutic and clinical purposes. Hence, this investigation was undertaken with the following objectives:

Objectives of the study:

1. Collection of mangrove leaf samples, physicochemical analysis of the dry leaf powder extracts along with study of phytochemical, antioxidant and antimicrobial properties of the crude leaf extracts.
2. Characterisation of different bioactive principles present in the selected crude leaf extracts with optimum antioxidant and antimicrobial activity.
3. Selection and purification of the most potent bioactive crude leaf extract, evaluation of antimicrobial activity its purified fractions against selected bacterial and fungal strains with subsequent selection of fraction with optimum antimicrobial activity and study of mechanism of antimicrobial action.
4. Evaluation of anticancer activity of the purified fractions, selection of fraction with optimum anticancer activity and study of mechanism of action.
5. Identification and characterisation of bioactive principles present in the purified fraction and *in-silico* molecular docking study.

Chapter 2:

Literature Review

2.1. Mangroves

Mangroves comprise of characteristic halophytic plants that particularly grow in the intertidal regions of tropical and subtropical coastal areas. The mangrove ecosystems are productive habitats that luxuriantly proliferate in the coastal areas, back water areas and also along river estuaries, containing a substratum that is muddy in nature with variable consistency and depth, which in a way, facilitate their growth (Spalding *et al*., 2010; Xu *et al*., 2014).

2.1.1. Mangroves and their geographical distribution in the world

The mangroves are broadly categorised into two major geographical regions – firstly, mangroves of eastern area (areas of which stretch from west as well as central Pacific region and continues till the southern tip of the African continent) and secondly, mangroves of the western area (areas spanning western coastline of Africa and both the coasts of the American continents). According to the World Atlas of Mangroves, the eastern region harbours more mangrove species in comparison to the western region (Lopez *et al*., 2018). Of the total estimated length of coastline in the world, 25% of them are covered by mangrove vegetation over an approximate area of 181,000 square kilometres, distributed in around 112 countries. The largest reserve of the world's mangroves is found in Asia (42%). They also widely grow across Africa (21%), followed by Central and North America (15%) as well as in Oceania (12%) and in South America (11%) (Patra and Mohanta, 2014). In Asia, the highest percentage of mangrove cover has been observed in Indonesia (around 27- 29%), while the Indian Sundarbans (part of the mangrove forest formed by the Sundarbans National Park situated in India and Sundarbans Mangrove forests located in Bangladesh) is the longest (approximately 10,000 km^2) continuous stretch of mangrove forests, as reported by UNESCO in 2016 and has the fourth largest global mangrove cover (Ghosh *et al*., 2015).

2.1.2. Ecological significance of mangroves

Mangroves are a significant part of the ecosystem, since they belong to one of the highly productive and diverse ecological communities (Saenger, 2002; Hogharth, 2007). They are located at the interface of sea and land and therefore protect the coastal areas against the damaging effects of natural calamities such as floods, tsunamis and cyclones (Dahdouh-Guebas *et al.*, 2005; Danielsen *et al*., 2005; Kathiresan and Rajendran, 2005; Williams, 2005). Ecologically, mangroves are vital filters and recyclers of nutrients present in the environment, assist in mitigation of floodwater and prevent the intrusion of seawater into the coastal areas. Mangrove ecosystem also provide support to offshore waters that in turn, enable phytoplanktons to increase their photosynthetic activity. A robust growth of seagrass beds, coral reefs and reef fish is usually observed in and around the mangrove vegetation. The mangrove forests also act as natural carbon sinks, where carbon may be temporarily stored in the form of organic peat or in the dissolved form at depths of the ocean bed, offsetting the effect of greenhouse gases for a longer period of time (Mumby and Hastings, 2008; Kristensen *et al.*, 2008). The diverse variety of flora and fauna of the mangrove system is itself indicative of the fact that it serves as a refuge and habitat to these organisms. These features in mangroves enable them to possess potential economic value (Costanza *et al*., 2014). However, mangroves are becoming an increasingly threatened community of the ecosystem with an annual global loss of around 1-2%, due to various climatic and anthropogenic activities. The mangrove habitat has been witnessing destruction at alarming levels worldwide. Ecologists anticipate that if such reckless mangrove destruction continues, then by the end of 21st century, there will be no mangrove forests remaining in the environment, that will create ecological imbalance at a huge scale globally with adverse consequences (Ellison, 2002; Duke *et al.*, 2007). Such incidence of habitat fragmentation and forest destruction have raised concerns among environmentalists and ecologists worldwide as a result of which, steps to conserve the diversity of mangrove plants are being adopted locally, nationally and also on global levels.

Mangroves belong to higher group plants that are significantly high levels of salt tolerance (Mandal and Naskar, 2008) that mainly occur along coastal lagoons, estuaries and backwaters. During high and low tides, they are inundated and exposed continuously; they are consequently fed by marine water of the coasts and also by freshwater, contributed from natural rainfall and inland drainage system. This is mainly due to the continuous high and low tides that occur as a result of which they are inundated and exposed respectively (Banerjee *et al*., 2002). Mangrove vegetation comprises of plants that exhibit convergent evolution, wherein they show a similarity in their structures and physiological adaptations. Noteworthy features of mangrove species are that they generally have pneumatophores (commonly known as breathing roots, i.e., negative geotropic root), knee roots, stilt roots, xerophyllous leaves, viviparous mode of germination and specialised salt excretory glands to withstand stress conditions (Tomlinson, 1986). This stringent condition has actually resulted in the formation of different secondary metabolites that have often been deposited in the leaves as excretory matter.

2.1.3. Habitat Strategies of Mangrove

The mangrove plants, with their specialised adaptive features to harsh environmental conditions, are considered to be of immense importance across the globe from the viewpoint of exploiting them for the sustainable development of natural products that may be of promising use in the medicine and pharmaceutical world.

- Adaptations to Low Oxygen concentration

Mangroves typically grow in inundated areas in low oxygen availability. They propagate above the water level by developing stilt roots that are specialised roots capable of absorbing oxygen and facilitates the entry of oxygen through pores present on their bark (lenticels). Mangroves are also well known for developing specialized root- like structures, that grow out from the surface of water body, known as pneumatophores that enable them to survive on higher ground overcoming the oxygen deficiency and facilitating the required aeration for respiration in roots (Gray *et al.*, 2010).

- Limiting Salt Intake

Mangroves sequestrate excess salt by their submerged roots. The roots are impermeable in nature that facilitate a mechanism of ultra-filtration to remove excess salts from the plant body and limiting the further uptake of salts (Gray *et al.*, 2010).

- Restricting Water Loss

The availability of freshwater in the intertidal salty regions is extremely limited. Hence, mangrove plants ensure minimal water loss through the leaves by regulating stomatal opening and closing process, or in other words, by a tight regulation to avoid excess water loss through transpiration. In order to avoid harsh sunlight, the mangrove plants also regulate the orientation of leaves by means of which loss of water through evaporation from the surface of leaves is further reduced (Calfo, 2006).

- Nutrient Uptake

Mangrove plants grow in soils that are waterlogged most of the times. So free oxygen available for uptake by the plant is scarce. Further, anaerobic bacteria that dwell in these soils produce gaseous nitrogen, inorganic phosphates, soluble iron compounds, sulfides as well as methane gas, that together reduce the nutrient content of the soil. Hence, to overcome this, mangroves are equipped with conserving methods such as resorption of nutrients before the onset of leaf fall (from senescent tissues), high root/shoot ratios, slowing down plant growth rate and nutrient immobilization (Calfo, 2006).

- Increasing Survival of Offspring

In order to support the survival of their offspring, mangrove plants have evolved specialised mechanisms. Mangrove seeds are buoyant that make them suitable for dispersal through water. Plant seeds generally germinate in soil; however, mangroves (like red mangroves) have the ability of seed germination while remaining attached to the plant body of the parent, i.e., they

are known to be viviparous. Upon germination, either the seedling grows within fruit body or forms a propagule outside that can independently perform photosynthesis. Once the propagule matures, it falls into the water body and transported across long distances. These propagules can stay dormant and withstand desiccation for more than a year before it gets planted at a place with suitable conditions for its growth. When the propagule further matures for rooting, the density of propagule changes and after assuming an elongated structure, it then floats vertically instead of floating horizontally. This shape gives it an advantage of being lodged into the mud for its growth to take place. If, however, the rooting fails, the propagule can accordingly change its density that enables it to drift and flow again to get rooted in a favourable environment (Gray *et al.*, 2010; Calfo, 2006).

2.1.4. True mangroves and mangrove associates

The mangrove forest vegetation is broadly categorised as "true mangroves" and "mangrove associates". According to (Wang *et al*. 2011), “true mangroves are woody plants, facultative or obligate halophytes”. Tomlinson (2016) stated: "True mangroves are plant species that 1) occur only in mangrove forests and are not found in terrestrial communities; 2) play a major role in the structure of the mangrove community, sometimes forming pure stands; 3) have morphological specialisations to the mangrove environment; 4) have some mechanism for salt exclusion”. Other noteworthy specialisations developed by mangrove plants include: developing aerial roots to withstand anaerobic sediments, formation of support structures, for instance pneumatophores, high concentrations of intracellular salts and low water potentials, excretion of salts through leaves and viviparous propagules (Duke *et al*. 1998). Representative images of such adaptive features have been collated in Fig.2.1. According to Tomlinson (2016), “all species of genera *Avicennia, Lumnitzera, Bruguiera, Ceriops, Kandelia, Rhizophora* and *Sonneratia,* plus the species *Nypa fruticans* and *Laguncularia racemose”*, are classified as "true mangroves" and globally, they form the major components of mangrove vegetation or forests. “Other species, such as *Acrostichum aureum, Aegiceras corniculatum, Osbornia octodonta,* are

also true mangroves but considered as minor components of mangrove forests" (Tomlinson 2016). A list of true mangroves and mangrove associates have been enlisted in Tables 2.1 and 2.2 respectively. On a global scale, mangrove forests are immensely threatened (Duke *et al*., 2007) and thus, a thorough literature on the types of mangrove trees, their habitat and ecological role is required to be documented.

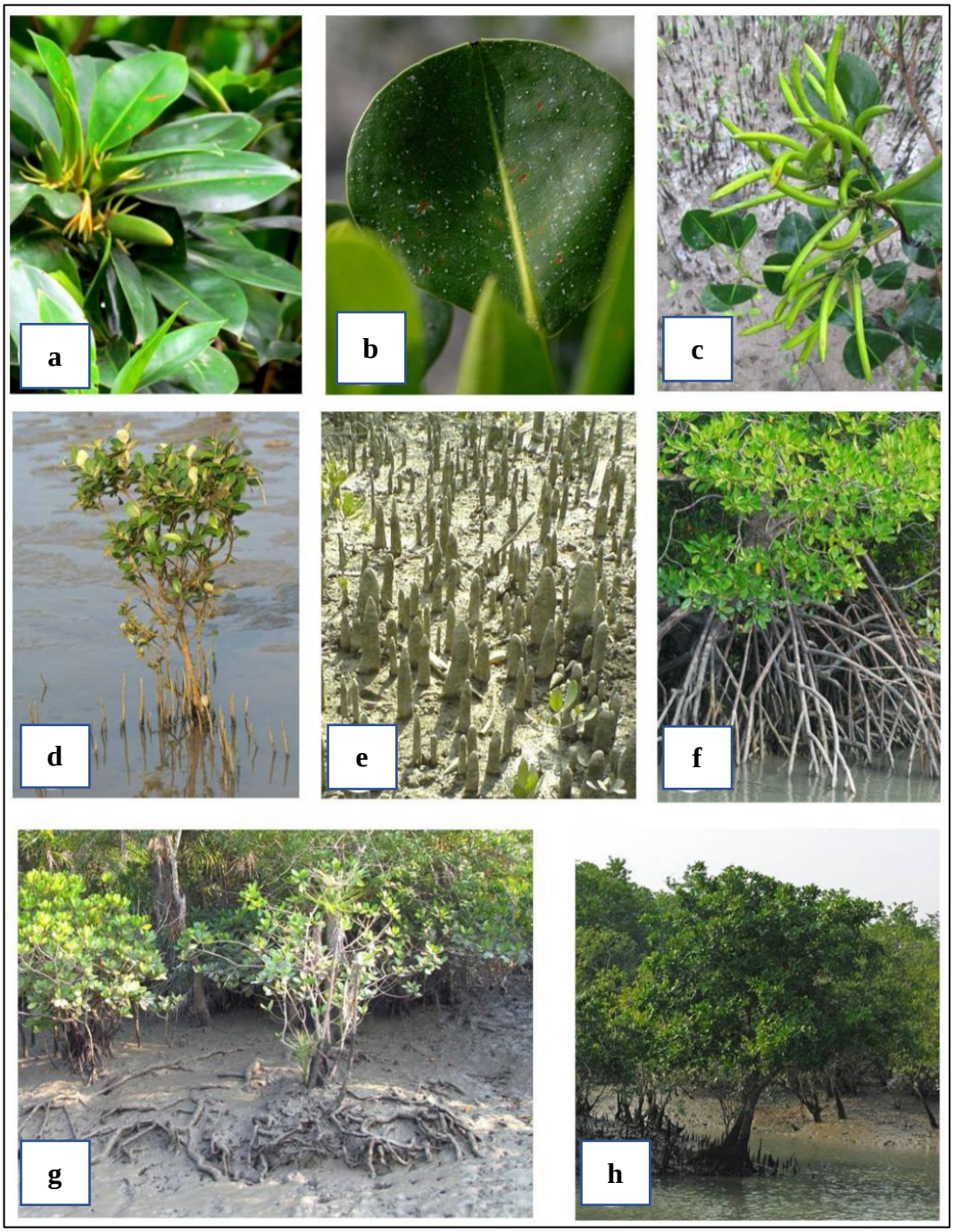


Fig.2.1: Representative images of adaptive features for true mangroves in Indian Sundarbans: (a) Succulent leaves of *Bruguiera sexangula*, (b) Salt crystals on leaves of *Aegialitis rotundifolia*, (c) Viviparous germination in *A.rotundifolia*, (d) Pneumatophores in *Avicennia officinalis*, (e) Knee roots in *Xylocarous mekongensis*, (f) Stilt roots in *Rhizophora mucronata*, (g) Snake roots in *Ceriops decandra*, (h) Root buttress in *X.mekongensis*. (Ref: Barik and Chowdhury, 2014).

The following 57 mangrove species are considered to be “true mangroves” globally:

Table 2.1: List of true mangrove species.

Sl. no	**Name of the plant**	**Family**
1.	*Aegialitis annulate*	Plumbaginaceae
2.	*Aegialitis rotundifolia*	Plumbaginaceae
3.	*Aegiceras corniculatum*	Myricinaceae
4.	*Acanthus ilicifolius*	Acanthaceae
5.	*Acanthus ebracteatus*	Acanthaceae
6.	*Avicennia alba*	Avicanniaceae
7.	*Avicennia bicolor*	Avicanniaceae
8.	*Avicennia eucalyptifolia*	Avicanniaceae
9.	*Avicennia germinans*	Avicanniaceae
10.	*Avicennia integra*	Avicanniaceae
11.	*Avicennia lanata*	Avicanniaceae
12.	*Avicennia marina*	Avicanniaceae
13.	*Avicennia officinalis*	Avicanniaceae
14.	*Avicennia rumphiana*	Avicanniaceae
15.	*Avicennia schaueriana*	Avicanniaceae
16.	*Bruguiera cylindrica*	Rhizophoraceae
17.	*Bruguiera exaristata*	Rhizophoraceae
18.	*Bruguiera gymnorhiza*	Rhizophoraceae
19.	*Bruguiera hainesii*	Rhizophoraceae
20.	*Bruguiera parviflora*	Rhizophoraceae
21.	*Bruguiera rhynchopetala*	Rhizophoraceae
22.	*Bruguiera sexangula*	Rhizophoraceae
23.	*Camptostemon schultzii*	Malvacee
24.	*Ceriops australis*	Rhizophoraceae
25.	*Ceriops decandra*	Rhizophoraceae
26.	*Ceriops tagal*	Rhizophoraceae
27.	*Excoecaria agallocha*	Euphorbiaceae
28.	*Heritiera fomes*	Sterculiaceae
29.	*Heritieria littoralis*	Sterculiaceae

30.	*Kandelia candel*	Rhizophoraceae
31.	*Kandelia obovata*	Rhizophoraceae
32.	*Laguncularia racemosa*	Combretaceae
33.	*Lumnitzera littorea*	Combretaceae
34.	*Lumnitzera racemosa*	Combretaceae
35.	*Nypa fruticans*	Arecaceae
36.	*Osbornia octodonta*	Myrtaceae
37.	*Pelliciera rhizophorae*	Tetrameristaceae
38.	*Phoenix paludosa*	Arecaceae
39.	*Rhizophora apiculata*	Rhizophoraceae
40.	*Rhizophora harrisonii*	Rhizophoraceae
41.	*Rhizophora lamarckii*	Rhizophoraceae
42.	*Rhizophora mangle*	Rhizophoraceae
43.	*Rhizophora mucronata*	Rhizophoraceae
44.	*Rhizophora racemosa*	Rhizophoraceae
45.	*Rhizophora samoensis*	Rhizophoraceae
46.	*Rhizophora stylosa*	Rhizophoraceae
47.	*Sonneratia alba*	Sonneratiaceae
48.	*Sonneratia apetala*	Sonneratiaceae
49.	*Sonneratia caseolaris*	Sonneratiaceae
50.	*Sonneratia griffithii*	Sonneratiaceae
51.	*Sonneratia gulngai*	Sonneratiaceae
52.	*Sonneratia hainanensis*	Sonneratiaceae
53.	*Sonneratia lanceolata*	Sonneratiaceae
54.	*Sonneratia ovata*	Sonneratiaceae
55.	*Xylocarpus granatum*	Meliaceae
56.	*Xylocarpus mekongensis*	Meliaceae
57.	*Xylocarpus moluccensis*	Meliaceae

The following are the enlisted names of 72 plants that are considered as mangrove associates:

Table 2.2: List of mangrove associates

Sl. No.	**Name of the plant**	**Family**
1.	*Acanthus volubilis*	Acanthaceae
2.	*Acrostichum aureum*	Pteridaceae
3.	*Acrostichum speciosum*	Pteridaceae
4.	*Aleuropus lagopoides*	Poaceae
5.	*Aglaia cucullate*	Meliaceae
6.	*Allophlus serratus*	Verbenaceae
7.	*Azima tetracantha*	Salvadoraceae
8.	*Brownlowia tersa*	Tiliaceae
9.	*Caesalpinia bundoc*	Caesalpiniaceae
10.	*Caesalpinia crista*	Caesalpiniaceae
11.	*Canavalia maritima*	Fabaceae
12.	*Cerbera odollam*	Apoceanaceae
13.	*Clerodendron inerme*	Verbenaceae
14.	*Crinum asiaticum*	Amarylidaceae
15.	*Crinum defixum*	Amarylidaceae
16.	*Cryptocoryne ciliata*	Araceae
17.	*Cynometra iripa*	Caesalpiniaceae
18.	*Dalbergia candenatensis*	Fabaceae
19.	*Dalbergia spinosa*	Fabaceae
20.	*Derris heterophylla*	Fabaceae
21.	*Derris scandens*	Fabaceae
22.	*Derris trifolia*	Fabaceae
23.	*Dendropthoe falcate*	Loranthaceae
24.	*Diospyros melanoxylon*	Ebenaceae
25.	*Dolicandrone spathacea*	Bignoniaceae
26.	*Excoecaria indica*	Euphorbiaceae
27.	*Fimbristylis ferruginea*	Cyperaceae
28.	*Flagilaria indica*	Flagillariaceae
29.	*Finlaysonia obovate*	Asclepiadaceae
30.	*Gloriosa superba*	Colchicaceae

31.	*Heliotropium curassavicum*	Boraginaceae
32.	*Hibiscus tiliaceus*	Malvaceae
33.	*Hoya parasitica*	Asclepiadaceae
34.	*Hyrophylax maritime*	Rubiaceae
35.	*Intsia bijuba*	Caesalpiniaceaea
36.	*Ipomea pes-caprae*	Convolvulaceae
37.	*Ipomea tuba*	Convolvulaceae
38.	*Lannea coramandelica*	Anacardiaceae
39.	*Launea sarmentosa*	Asteraceae
40.	*Macuna gigantean*	Fabaceae
41.	*Merope angulata*	Rutaceae
42.	*Myriostachya wighitiana*	Poaceae
43.	*Opuntia dilleniid*	Opuntiaceae
44.	*Pandanus fascicularis*	Pandanaceae
45.	*Pandanus foetidus*	Pandanaceae
46.	*Pandanus odoratissmus*	Pandanaceae
47.	*Pemphis acidula*	Lythraceae
48.	*Pentotropis capensis*	Asclepiadaceae
49.	*Phragmites karka*	Poaceae
50.	*Pongamia pinata*	Fabaceae
51.	*Porteresia coarctata*	Poaceae
52.	*Salacia prinoides*	Celastraceae
53.	*Salicornia brachiata*	Salvadoraceae
54.	*Salvadora persica*	Salvadoraceae
55.	*Sarcolobus carinatus*	Asclepiadaceae
56.	*Sarcolobus globosus*	Asclepiadaceae
57.	*Scaevola plumieri*	Goodeniaceae
58.	*Sesuvium portulacastrum*	Aizoaceae
59.	*Solanum trilobatum*	Solanaceae
60.	*Spinifex squarrosus*	Poaceae
61.	*Stenochlaena palustre*	Blechnaceae
62.	*Suaeda maritima*	Chenopodiaceae
63.	*Suaeda monoica*	Chenopodiaceae
64.	*Suaeda nudiflora*	Chenopodiaceae

65.	*Tamarix troupii*	Tamaricaceae
66.	*Terminalia catappa*	Combretaceae
67.	*Thespesia populnea*	Malvaceae
68.	*Thespesia populneoides*	Malvaceae
69.	*Trianthema portulacastrum*	Aizoaceae
70.	*Tylophora fleuxosa*	A Asclepiadaceae
71.	*Tylophora indica*	sclepiadaceae
72.	*Tylophora tenuis*	Asclepiadaceae

2.1.5. Biological and pharmaceutical significance of Mangrove plants

Mangrove plants regulate unique biosynthetic pathways to synthesize various kinds of secondary metabolites that enable them to grow and sustain in adverse climatic and stress conditions. Traditional folklore medicine of the tribal community of Sundarbans finds the application of mangrove plant extracts for the treatment of various chronic diseases and health disorders (Betoni *et al.*, 2006; Lewis and Ausubel, 2006; Lee *et al.*, 2007). Compounds derived from plants have gained huge importance in the present- day scenario, where cases of multidrug resistance are ever increasing- mangrove plants being one such major candidate (Patra and Mohanta, 2014). The capacity of mangrove plants to produce novel molecules can be exploited for the development of promising drug compounds and therapeutic products. Mangrove plants are thus being extensively studied to identify, purify and consequently develop novel compounds of medicinal and pharmaceutical importance.

Mangrove plants have been utilized by various ethnic and tribal groups in traditional folklore medicine to treat several kinds of diseases (Harbottle *et al.*, 2006; Akram *et al.*, 2007). They have immense ethnopharmacological importance and medicinal properties (Table 2.3) that are widely used by the ethnic communities of the mangrove forest region to treat various symptoms and diseases such as eye infections, rashes and other diseases of the skin, blisters, rheumatism, arthritis, asthma, diabetes and stomach pain or infections (Patra and Thatoi, 2011). Some plant

species belonging to the Avicenniaceae, Euphorbiaceae, Meliaceae and Rhizophoraceae families have exhibited potential antimicrobial property, while other species of Rhizophorae and Meliaceae families were observed to have antimalarial properties (Premanathan *et al.*, 1996; Patra and Mohanta, 2014). Anticancer activity has been recently indicated in some species of the Myrsinaceae and Acanthaceae families (Uddin *et al.*, 2007). However, the scientific evidence is lacking pertaining to the efficacy of the properties reported and any kind of toxicity particularly attributed to these medicinally important mangrove plants could emerge as a serious health problem. Hence, well planned experiments and scientific studies need to be urgently carried out to validate the medicinal uses of plants of the mangrove forests in the developing world. Additionally, mangrove plants may be carefully utilised in bioprospecting projects and programs on a global scale, that are dedicated to explore and identify new and safer alternatives for development of efficient drugs to combat the life- threatening diseases. If medicinal properties are proven in scientific investigations, the use of the plant species in the past, increases the probability of them being considered as potential drug candidates for clinical and therapeutic applications.

These plants are adapted to thrive in harsh environments that contain low oxygen concentration and availability, high salinity and limited uptake of nutrients. These properties may be attributed to their ability to synthesize secondary metabolites (Patra *et al*., 2011; Patra and Mohanta, 2014). These metabolites may be purified and characterised to develop potent compounds of biotechnological and biomedical importance.

It is well known that mangroves grow in marine habitat amidst several abiotic stress factors. Such adversities stimulate the mangrove plants to produce several molecules that help them strive through these environmental stress conditions and facilitate their growth and development. These molecules may be of pharmaceutical importance, as indicated by their popular use in traditional folklore medicine (Bandaranayake, 2002; Patra and Mohanta 2014). During their growth period, these plants produce various chemical substances to withstand the

adverse environmental stress conditions, such as phenols, flavonoids, tannins, glycosides, alkaloids, terpenes and tannins. These compounds have exhibited diverse medicinal properties such as antimicrobial activity and anti- oxidant activity. The local inhabitants dwelling in or around mangrove forest regions have been applying the raw extracts of the various plant parts for therapeutic purposes. This ethnopharmacological knowledge have made the mangrove plants have now become a subject of great interest for scientific research and development of herbal drugs and promising bioactive compounds of pharmaceutical importance.

There are two major factors that substantiate the idea or initiative to study mangrove plants and explore their chemical constituents. Firstly, mangroves are found widely in tropical areas. In conditions where vascular plants are unable to grow, mangrove plants grow efficiently (Patra and Thatoi, 2011). The mangroves thrive and grow under stressful environmental conditions such as violent tropical storms and calamities, high concentration of salt and moisture, with continuous high and low water tides. They bridge the gap between freshwater and marine ecosystem representing a unique ecotone of utmost importance (Patra and Mohanta, 2014). They are adequately equipped with stable and suitable modifications to withstand salt and osmotic stress. Some of their physiological processes are also modified, for instance, synthesis of polyphenols. These physiological alterations might be due to the synthesis of certain specific chemical constituents or compounds that aid in protection and growth of the mangrove plants. Secondly, a large number of mangrove plants have been and are still being used in traditional folklore medicine. So, their transformation to ayurvedic medicine is not ruled out. The potent inhibitory activity against various plant, animal and human pathogens have been exhibited by crude extracts prepared from mangrove plants and mangrove- associates or dependent species. However, it is noted that only limited research investigations have been conducted to characterise and identify the specific metabolites conferring the biological activity (Bandaranayake, 2002; Patra and Thatoi, 2011; Patra and Mohanta, 2014).

Table 2.3: Ethnopharmacological use of selected mangroves in traditional folklore medicine.

Sl.no.	Plant species	Local/Regional name	Plant parts	Therapeutic uses	References
1.	*Acanthus ilicifolius* L.	Harakancha (Oriya)	Leaf, stem, root, flower	Leaves, barks and total plants are used as blood purifier, diuretic and aphrodisiac; it is applied for curing diabetes, leprosy, paralysis, skin disease, snake bite, hepatitis, stomach pain, rheumatism, asthma, etc.	Banerjee *et al*. (2002), Li *et al*. (2011), Thirunavukkarasu *et al*. (2018).
2.	*Aegiceras corniculatum* L. Blanco	Kharsi (Oriya, Bengali), Halsi (Hindi)	Leaf, stem/ bark, root	Leaves are used for curing boil, earache, small pox; seeds and barks are used for curing asthma, diabetes, rheumatism, etc.	Banerjee *et al.* (2002), Agoramoorthy *et al.* (2007).
3.	*Avicennia alba* Bl.	Kala bani (Oriya)	Leaf, stem/ bark, root	Leaves and barks are used in antifertility treatment, skin diseases, ulcers, etc.; it is also used as contraceptive.	Banerjee *et al*. (2002)
4.	*Avicennia marina* (Forsk.) Vierh.	Bani (Oriya)	Leaf, stem/ bark, root	Leaves are used as an astringent and for curing ulcers, small pox, etc.	Thirunavukkarasu *et al.* (2018), Vadlapudi and Naidu (2009), Shanmugapriya *et al*. (2012)
5.	*Avicennia officinalis* L.	Dhala bani (Oriya)	Leaf, stem/ bark, root	Seed, root, barks are used for curing boils, small pox, leprosy, relieving ulcers; it is also used as diuretic and aphrodisiac.	Thirunavukkarasu *et al.* (2018), Vadlapudi and Naidu (2009), Shanmugapriya *et al.* (2012)
6.	*Bruguiera gymnorhiz*a (L.) Lamk.	Bandari (Oriya), Kekra (Oriya), Kankra (Bengali)	Leaf, stem/ bark, root	Bark is used as astringent and also for curing malaria; fruit are also used as astringent; treatment	Banerjee *et al.* (2002).

				of eye disease and as fish poison etc.	
7.	*Bruguiera cylindrica* (L.) Bl.	Kakandan (Hindi)	Leaf, stem/ bark	Leaves are used as cure for hepatitis; it is a good source of tannin.	Agoramoorthy *et al.* (2007)
8.	*Bruguiera parviflora* (Roxb.)	Small-flower *Bruguiera*	Edible pods	Bark is used in constipation; it is also a good antitumor agent.	Banerjee *et al.* (2002)
9.	*Ceriops decandra* (Griff.) Ding	Ghrani (Oriya)	Leaf, stem/ bark, root	Root and bark are used for curing hepatitis, hemorrhage and malaria; fruit paste is used against ulcers.	Banerjee *et al.* (2002), Agoramoorthy *et al.* (2007)
10.	*Excoecaria agallocha* L.	Guan (Oriya), Genwa (Bengali)	Leaf, stem/ bark	Leaves are used for curing epilepsy, ulcers, etc.; roots are used for curing hand and feet swelling, leprosy, toothache, conjunctivitis, dermatitis, etc.; it is also used as uterotonic, purgative, fish poison; milky latex is used against paralysis.	Patra *et al.* (2009), Konishi *et al.* (1998), Konishi *et al.* (2000), Patra *et al.* (2012)
11.	*Heritiera fomes* Buch.-Ham.	Sundari (Oriya, Bengali)	Stem, leaf	Bark is used for healing wound and cuts; seeds are eaten as source of nutrients.	Wangensteen *et al.* (2009)
12.	*Kandelia candel* (L.) Druce	Sindukua (Oriya), Goria (Bengali)	Leaf	Bark mixed with dry ginger in water is used for curing diabetes.	Patra *et al.* (2009)
13.	*Rhizophora apiculata* Bl.	Rai (Oriya)	Leaf, stem/ bark, roots	Leaves are used for curing diarrhea, skin diseases; bark is used for treatment of nausea, hepatitis, vomiting, typhoid; it	Agoramoorthy *et al.* (2007)

				is also used as antiseptic, insecticide, etc.	
14.	*Rhizophora mucronata* Lamk.	Rai (Oriya)	Leaf, stem/ bark, root	Bark is used for curing diabetes, hemorrhage, hepatitis, ulcer, dysentery, etc.; the bark is powerful astringent.	Banerjee *et al.* (2002), Agoramoorthy *et al.* (2008).
15.	*Sonneratia apetala* Buch.Ham.	Keruan (Oriya)	Leaf, stem/ bark, root, seeds	Fruits are edible as source of natural antioxidant; Leaves and roots are used in the treatment of stomach pain, rheumatism, etc.	Banerjee *et al.* (2002), Vadlapudi and Naidu (2009)
16.	*Suaeda maritima* Dumort	Giria saga (Oriya)	Leaf, stem, root	Whole plant is used for curing hepatitis.	Patra *et al.* (2012), Banerjee *et al.* (2002), Tomlinson (2016)
17.	*Xylocarpus granatum* Koenig	Pussur (Hindi), Dhundul (Bengali)	Leaf, stem	Bark is used for treatment of dysentery, diarrhea, febrifuge, malaria, cholera, insect bite, swelling of breast, etc.; it is also used as an astringent.	Vadlapudi and Naidu (2009)

In recent years, there has been a converging interest globally in identifying and characterising natural antioxidants from plant origin so that synthetic antioxidants, reported to have carcinogenicity and toxicity problems, could be replaced with safer alternatives (Gray *et al*., 2010). Various kinds of selected plants have been studied till date and natural compounds or crude extracts derived from these plants have been reported to possess antioxidant property and radical scavenging ability (Robbers *et al.*, 1996; Calfo, 2006). The phenolic compounds and flavonoids are more comprehensively studied in the category of antioxidants due to their large abundance in natural compounds (Gray *et al*., 2010; Patra and Mohanta, 2014).

Several independent research groups have undertaken well-planned scientific investigations for the characterisation and subsequent identification of the biochemical and medicinal properties of different mangrove plant extracts and their antimicrobial activities have also been studied (Asmathunisha *et al.*, 2010; Kayalvizhi, 2012). Several compounds with antimicrobial and therapeutic properties have been discovered from specific mangrove plant species that exhibit a wide range of bioactivities, namely antioxidant, antibacterial, antifungal, antiprotozoal, antiplasmodial etc. Research on an extensive scale has been undertaken with the aim to discover novel natural products and simultaneous assessment of biological activities of selected mangrove plant species (Kathiresan *et al.*, 2013).

Extensive details regarding the biological activities of different mangrove plant species and their associates have been reported and reviewed by (Patra and Thatoi, 2011). Besides conducting research for determining the biological activities, isolation and characterization of bioactive compounds (a few of which have been enlisted in Table 2.4) that confer such properties to these plants are also being studied widely.

Table 2.4: List of chemical compounds isolated from different mangrove plants.

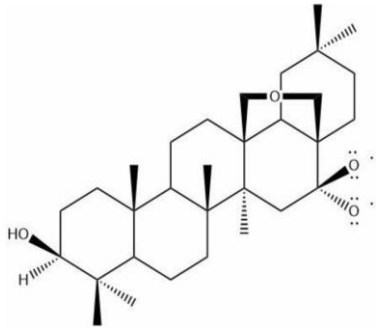
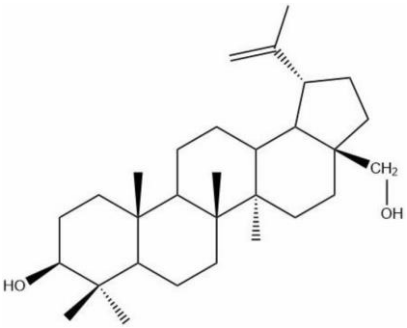
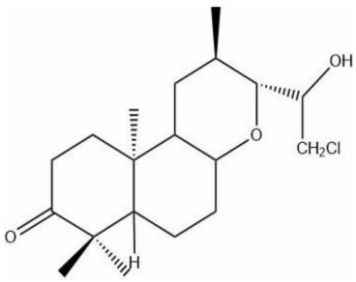
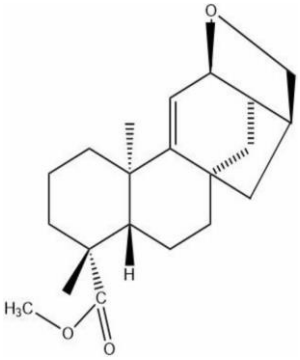
Species	**Compounds**	**References**
Avicennia marina	Naphthoquinones, Avicennones A-G, Avicequinone A, Avicenols Stenocarpoquinone B	Wu *et al.* (2008), Patra *et al.* (2009).
Avicennia officinalis	2-propenoic acid, 3-phenyl ester, 3-acetyl methoxyphenyl Diterpenoids, Seven labdanes, Flavon- Velutin; Hydroxy-4- methoxybenzoic acid, Diethyl phthalate, Oleic acid.	Patra *et al.* (2012)
Aegiceras corniculatum	Dihydroxy-3-tridecyl-1,4-benzoquinone, 2,5-dihydroxy-3-undecyl-1,4 benzoquinone, 5-O-Methylembelin, Genin-A, Aegicerin	Wu *et al.* (2008), Patra *et al.* (2011)

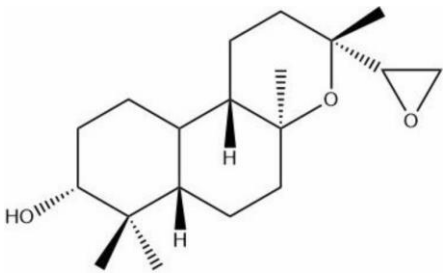
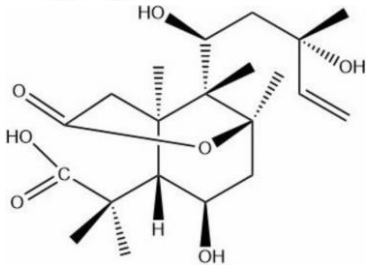
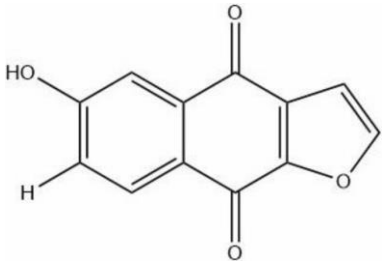
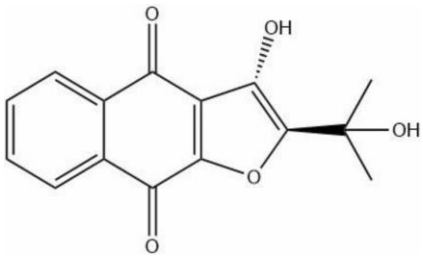
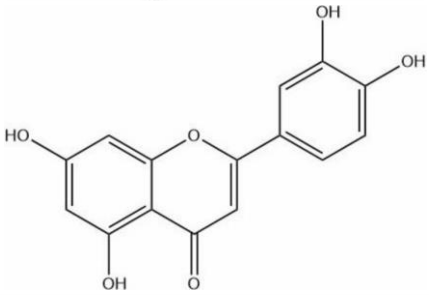
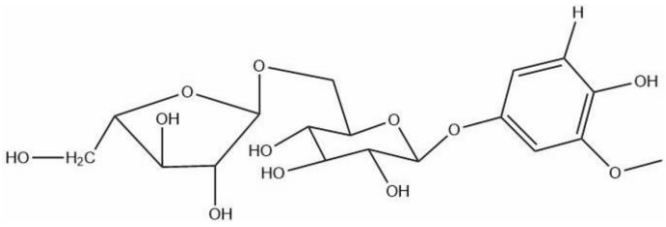
Bruguiera gymnorhiza	Gymnorrhizol, Four ent-kauranes, Pimaranes, Brugierol	Wu *et al*. (2008), Patra *et al.* (2011)
Bruguiera sexangula	Rhyncosides A–D	Wu *et al.* (2008), Patra *et al.* (2011)
Ceriops decandra	Ceriopsin A-G, steviol, methyl ent-16b,17-dihydroxy- 9(11)-kauren-19-oate, ent-16b,17-dihydroxy- 9(11)-kauren-19-oic acid	Wu *et al.* (2008), Patra *et al.* (2011)
Excoecaria agallocha	Phorbol ester, ent-isopimarane diterpenoid, Agallochin A-E, Agallochaone A, 8,13-epoxy-14-1abden-3-one and Excoecarin A,B	Wu *et al.* (2008), Patra *et al.* (2011, 2012)
Laguncularia racemosa	Triacontanol, taraxerol, b-amyrin, betulin, b-sitosterol, friedelin	Wu *et al.* (2008), Patra *et al.* (2011)
Rhizophora apiculata	Taraxerol careaborin, pyrethrin, Taraxeryl cis-p- hydroxycinnamate, gallic acid, rutin, ascorbic acid, quercetin, kaempferol	Premanathan *et al.* (1996), Abeysinghe (2010), Vadlapudi and Naidu (2009), Patra *et al.* (2011).

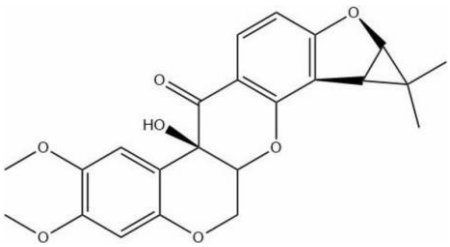
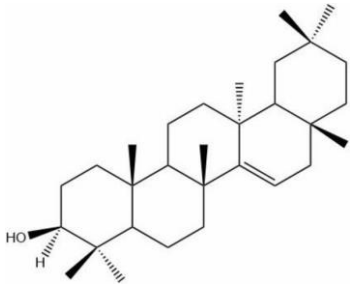
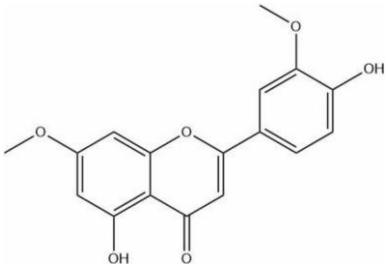
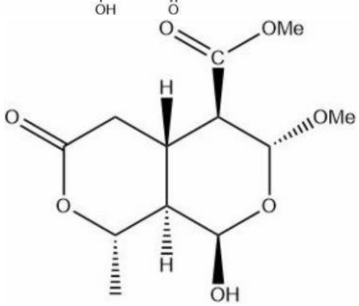
Rhizophora mucronata	Rhizophorin A-E, 1,4-Benzenediol; 2-Furancarboxaldehyde, 5- hydroxymethyl 4-hydroxy benzenesulfonic acid	Premanathan *et al.* (1996), Abeysinghe. (2010), Vadlapudi and Naidu (2009), Patra *et al.* (2011).
Sarcolobus globosus	Sarcolobin,Tephrosin, 12aα-hydroxydeguelin, 11- hydroxytephrosin,12a-hydroxyrotenone, 6aα,12aα-12a-hydroxyelliptone, 6a,12a-dehydrodeguelin, Barbigerone, 6,7-dimethoxy-2,3-dihydrochromone, Genistin, Vanillic acid, 4-O- β-D-glucoside, Glucosyringic acid, Tachioside, Isotachioside.	Wangensteen *et al.* (2009).
Sonneratia caseolaris	Luteolin, luteolin 7-O-β-glucoside	Uddin *et al.* (2007), Wangensteen *et al.* (2009)
Xylocarpus granatum	Catechin, Epicatechin, Procyanadin B1, Procyanidin trimer, Procyanidin pentamer, Xyloccensin O, Xyloccensin P, Gedunin	
Xylocarpus moluccensis	Xylomollin, Catechin, Epicatechin, Procyanidin B1, Procyanidin trimer, Procyanidin Pentamer, Procyanidin hexamer, Procyanidin decamer, Procyanidin, Undecamer, 12a-Hydroxyrotenone, 12aα- Hydroxyrotenone,	

The chemical structures of the isolated and characterised phytochemicals are also being widely studied to have further understanding of the chemical interactions that may be responsible for the various biochemical activities being exhibited by the bioactive compounds being isolated and characterized from mangrove plants. The representative chemical structures of the various phytochemicals are represented in Table 2.5.

Table 2.5: Some representative phytochemical compounds of mangroves and their chemical class and structure (Ref.: Patra et al. 2011).

Phytochemical class	Compound	Structure
Triterpene	Aegicerin	
	Betulin	
Diterpenoid	Agallochin A	
	Ceriopsin E	

	Excoecarin A	
	Rhizophorin A	
Naphthoquinone	**Avicennone D**	
	Avicequinone A	
Flavonoid	**Luteolin**	
Phenolic glycosides	**Rhyncoside A**	

Rotenoid	**Sarcolobin**	
Triterpenoid	**Taraxerol**	
Flavone	**Velutin**	
Monoterpene	**Xylomollin**	

Nowadays, sophisticated purification and isolation techniques like high performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), X-ray crystallography and nuclear magnetic resonance spectroscopy (NMR), etc. are being utilised for studying and characterising antimicrobial compounds derived from mangrove plants (Chomnawang *et al.*, 2005; Cos *et al.*, 2006). With the advent of bioinformatics, advanced software and tools may be explored for natural product discovery that would enable the elucidation of the structural aspect of the identified bioactive compounds and alterations in structure may be studied to enhance the biological activity of the compounds (Muthukala *et al.*, 2015). In silico study and computer aided drug discovery is paving a new avenue for studying the drug- target mechanisms involved and providing concrete theoretical evidence regarding the metabolism of the biologically active compounds (Zaman, 2012; Senthilraja *et al.*, 2013).

The mangrove forest cover has specific plants that have been proved to have therapeutic properties of immense importance. A compound identified as Brugierol has been isolated from *Bruguiera conjugate* that had promising antibacterial property (Bandaranayake, 2002). According to Wu *et al.* (2008) and Bamroongrugsa (2008), the mangrove species *Bruguiera gymnorhiza* is a valuable source of diverse kinds of antimicrobial compounds. Bandaranayake (2002) reported that the extract of *Acrostichum aureum* leaves exhibited potent antibacterial activity. Latex of the mangrove plant, *Excoecaria agallocha*, contains toxic substances and chemicals that have inhibitory and biocidal effects on phytoplankton and marine organisms. In rice field crab, this plant has been reported to induce metabolic depression (Konishi *et al.*, 1998). The stem and leaf extracts of *E. agallocha* have also exhibited remarkable antibacterial activity against different kinds of both Gram-positive and Gram-negative bacteria. The antibacterial activity of *E. agallocha* has been studied and reported by Patra, *et al*. (2009) and Konishi, *et al.* (2000). Investigations conducted by Agoramoorthy *et al.* (2007) have been concluded with the inference that the fatty acid methyl ester extract obtained from *E. agallocha* leaf showed significant inhibitory activity against some potentially pathogenic bacteria and fungi.

Similarly, research has been conducted to explore the biological properties of the *Xylocarpus* species, wherein it was reported that compounds such as gedunin, was responsible for conferring antimicrobial activity to extracts of *Xylocarpus* species (Wu *et al.*, 2008; Bickii *et al.*, 2000, Omar *et al.*, 2003; Nathan *et al.*, 2005). The crude extract from bark of *Xylocarpus moluccensis* exhibited moderate antimicrobial activity against a broad range of microorganisms (Uddin *et al.*, 2007). Extract from the leaves of *Thespesia populnea*, a mangrove-associated plant, has been reported to have antibacterial activity (Hewag *et al.*, 1998). According to the inference drawn by Choudhury, *et al* (48) the results of their investigation indicated that stem and leaf extracts of *Aglaia cucullata, Aegiceras corniculatum, Cynometra iripa* and *Xylocarpus granatum* (mangroves studied from Bhitarkanika mangrove forest of Orissa, India) exhibited species-specific inhibitory activity against selected virulent microorganisms. Leaf extracts of

Finlaysonia obovata possess commendable antibacterial activity against bacteria that act as pathogens for fish in freshwater (Mishra and Sree, 2007). *Heritiera littoralis* was reported to have significant inhibitory action against pathogenic fungi, a property plausibly contributed by the presence of a triterpene ester compound (Bandaranayake, 2002). The compound gedunin has also been isolated from *Cedrela odorata*, a mangrove-associate, that showed strong antifungal property, as confirmed by investigational reports of Wu, *et al* (2008). Extracts of mangrove *Avicennia* species have been reported to exhibit broad spectrum antimicrobial activity against pathogenic microorganisms such as *Candida albicans, Mycobacterium aurum, Mycobacterium vaccae, Mycobacterium fortuitum, Mycobacterium smegmatis* and *Staphylococcus aureus* (Wu *et al*., 2008; Han *et al*., 2007). Another recent investigational finding suggests that bark and leaf extracts of mangrove plants such as *Sonneratia apetala, Sonneratia alba* and *Sonneratia caseolaris* also possess significant antimicrobial activity against some microorganisms that are pathogenic to humans (Han *et al.*, 2007, Saad *et al.*, 2012; Milon *et al*., 2012). *Ceriops decandra*, a mangrove plant species, which is abundantly distributed in almost every mangrove forest area, has the ability to synthesize numerous antimicrobial compounds that have effective inhibitory action against potential pathogens (Wu *et al.*, 2008; Vadlapudi and Naidu, 2009). Sett *et al.* (2014) studied a few selected mangrove plants of the Indian Sundarbans and have reported that leaf extract of *Aegialitis rotundifolia* has significant antibacterial and antifungal activity against a wide range of pathogens. The mangrove plants belonging to the Rhizophoraceae family have also exhibited potential antimicrobial activity (Abeysinghe, 2010). Thus, all these findings of scientific investigations suggest that every part of a mangrove plant has important biological activity and these must be studied further to discover and develop novel compounds that have potent antimicrobial and chemotherapeutic properties that could be subsequently purified to develop efficient drug compounds of pharmaceutical and biomedical importance.

2.2. The Indian Sundarbans

The Indian Sundarbans is classified as a habitat type 12.7 Marine- Intertidal- Mangrove Submerged roots and 1.7 Forest- Sub- tropical/Tropical mangrove vegetation above high tide level under the Habitats Classification system of IUCN (Version 3.1) and is a part of the Bay of Bengal Marine Ecoregion of the World (Spalding *et al*., 2010). It is a habitat to a wide variety of animals, birds and reptiles and has a rich reserve of diverse variety of plants. It is majorly covered by the mangrove forests that have immense role in conserving the biodiversity and maintaining ecological balance of the nature.

2.2.1. Geographical span

Among the various mangrove forests found in Asia, the Sundarban mangrove forests are noteworthy in terms of its rich reserve of flora and fauna. According to Forest Survey of India, the coastline area of the country has an area of about 4,87,100 ha covered by mangrove wetlands of which, 60% is situated along the eastern coast, 27% along the western coastal region and 13% is situated in the Andaman and Nicobar islands (FSI, 2009; Pillai and Harilal, 2018). Reports also suggest that 80% of the total Indian mangrove forests are occupied by the Sundarbans of West Bengal and mangrove forests of the Andaman and Nicobar islands. Situated on the borders of India and Bangladesh, the Sundarban mangrove forest is a dynamic zone that is a habitat for a diverse variety of macro and micro biotic communities, in which mangrove flora occupy a central position (Guha Bakshi *et al*., 1998). The Sundarban mangrove forest is located in the deltaic region formed by the rivers Ganga, Brahmaputra and Meghna, that flow into the Bay of Bengal. The major rivers in this region are the Hooghly, Mooriganga, Thakuran, Matla, Saptamukhi, Haribhanga and Gosaba. Raimangal divides the border of the two countries (Fig.2.2). Within the estuaries, the tidal amplitude generally ranges from 3-4 meters, accompanied by a seasonal variation of around 1-6 metres. The Sundarbans is located within the longitudes 21°32’to 22°40’ N and latitudes 88°05’ to 89°5’E. It approximately covers a wide area of 10,000 square kilometres of which a large portion lies in the Bangladesh region (62%)

and a remarkably wide area (38%) in West Bengal, forming the largest contiguous mangrove forests on the Earth (Ghosh *et al*., 2015; Spalding *et al*., 2010).

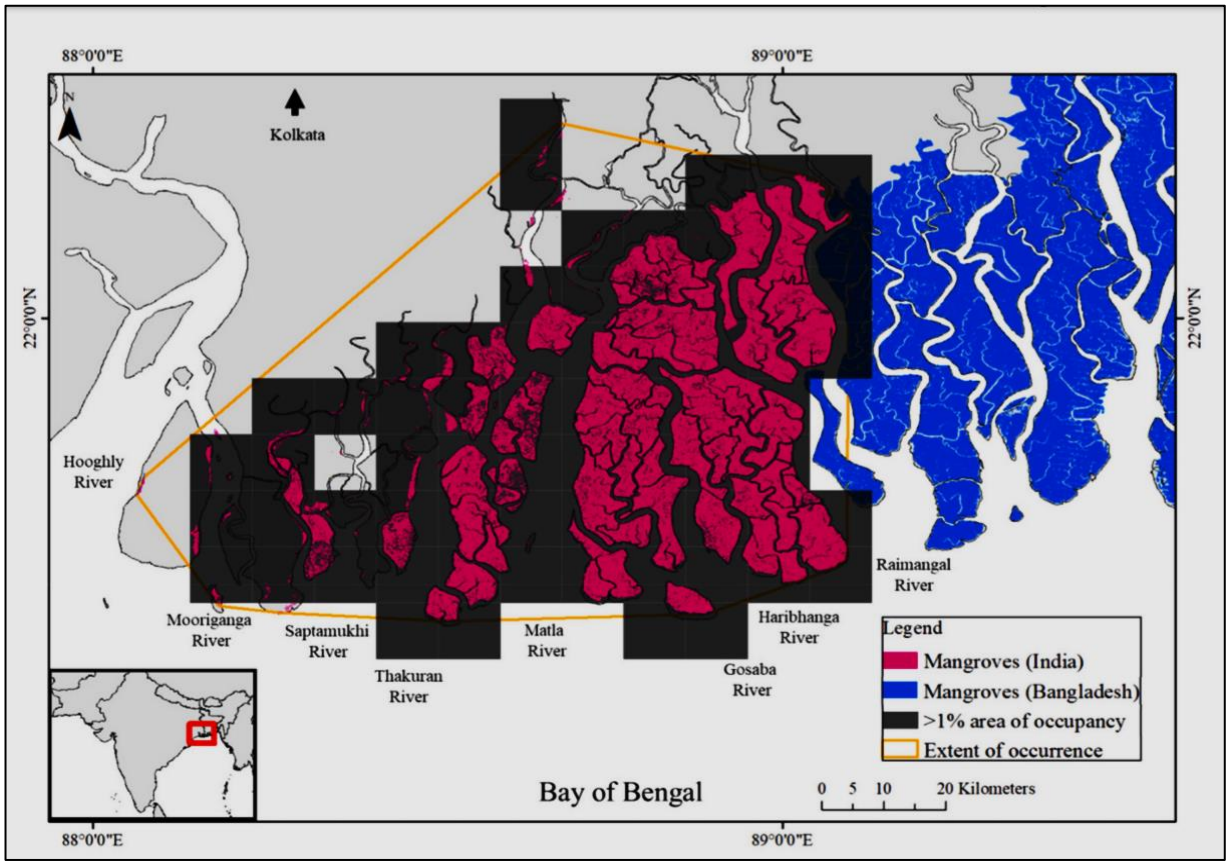


Fig.2.2: Geographical distribution of the Indian Sundarbans spanning regions of India and Bangladesh. (Ref.: Forest Survey of India, 2009)

2.2.2. Biodiversity of the Indian Sundarbans

The Sundarbans floristically are a part of the mangrove province of the Indo-Andaman region which is situated within Indo-West Pacific region, well known for its species- richness and diversity. According to Barik and Choudhury (2014), there are 24 true mangrove species, found in the Indian Sundarbans, that broadly belong to nine different families. *Heriteria fomes* (known as Sundari trees), *Aegialitis rotundifolia, Sonneratia griffithii* or *S. apetala* are some important endemic species of this region. *Avicennia alba, A. marina* and *Bruguiera cylindrica* grow luxuriantly in the lower coastal area. On the other hand, *Bruguiera gymnorhiza, Rhizophora mucoronata* and *Ceriops decandra* grow profusely in the upper coastal regions. *Heriteria fomes, Sonneratia caseolaris* and *Excoecaria agallocha* are considered to be comparatively less salt-

tolearnt and grow mostly in the eastern region. Besides being a huge reserve of mangrove plants, Sundarbans are known as one of the biodiversity hotspots of the world that is an abode to 200 species of other plants apart from mangroves, more than 42 species of mammals, over 35 retile species, more than 300 species of birds and 400 species of fishes along with innumerable invertebrates that belong to benthic habitat, microorganisms such as bacteria, fungi etc. The Sundarbans is the residence for the Royal Bengal tiger (*Panthera tigris* ssp. *tigris*) and a large variety of other animals and migratory birds are also spotted as visitors in this mangrove region.

2.2.3. The Mangrove cover of the Indian Sundarbans

Several researchers and ecologists have considered different parameters and approaches to categorise the mangrove species in different mangrove areas of the world, including the Indian Sundarbans delta. The opinions and views pertaining to the ecological distinction and species composition of mangrove vegetation in the Indian Sundarbans have widely varied. Several classifications have been reported, viz. 60 species categorised into 41 genera and 29 families (Rao 1986), 56 species of true, euryhaline mangrove and mangrove associates (Naskar and Guha Bakshi 1987) and 69 species under 49 genera and 35 families Mandal and Naskar (2008). Further, there exists ambiguity, specifically at the generic levels, in the classification and categorisation of true mangrove plants from minor and mangrove-associated species. Researchers Mandal and Nandi (1989) reported the occurrence of 22 true mangroves species in the Indian Sundarbans; on the other hand, Chaudhuri and Choudhury (1994) have reported 36 true mangrove species from the same region. However, in a recent reporting by Mandal and Naskar (2008), 28 species of true mangroves have been identified in the Indian Sundarbans. The same study also mentioned that the Indian Sundarbans are more diverse with regard to its species composition than the other mangrove vegetations found in other parts of coastal India (Barik and Chowdhury, 2014). Thereafter, several researchers have made an attempt to revise the reported list of mangrove species found in Indian Sundarbans, taking into consideration their

morpho-physiological characters for adapting to the tidal-saline environment in this deltaic region.

2.3. Emergence of Antimicrobial resistance

Antimicrobial resistance has emerged as one of the principal public health problems of the 21st century that threatens the effective prevention and treatment of an ever-increasing range of infections caused by bacteria, fungi, viruses and parasites no longer susceptible to the common medicines or drug compounds used to inhibit their growth or kill them (Prestinaci *et al*., 2015). In other words, antimicrobial resistance occurs when microorganisms develop resistance to antimicrobial medications (Lu *et al*., 2020). Specifically, causal microorganisms of diseases such as tuberculosis, malaria, HIV, influenza, typhoid and some fungal infections are developing resistance all over the world (Cassini *et al*., 2019), leading to higher healthcare costs, increase in the risk of the disease spreading as well as a longer duration of illness. The global consumption of antibiotics and antimicrobial drugs has been estimated at more than 35 billion daily doses in the year 2015 (Klein *et al*., 2018), of which over-the-counter purchase and consumption of antibiotics and consequent overdose or irregular dosage of medicines have been proved to be detrimental. Thus, understanding the burden of AMR and the leading pathogen-drug combinations contributing to it, is crucial to develop health policy decisions, specifically about infection control and prevention programmes, access to essential antibiotics, and research and development of new antimicrobial compounds.

2.3.1. Antimicrobial drug resistance- a global threat

Antimicrobial resistance (AMR) is an issue of concern that has emerged to be a threat to the global public health system (O' Neill, 2016). WHO has expressed its concern and appeals for multisectoral intervention to combat the emerging cases of antimicrobial resistance. AMR occurs when microorganisms such as bacteria, fungi, viruses and parasites undergo modifications with gradual elapse of time and render the existing antimicrobial agents or medicines ineffective thereby, causing difficulty in treating infections caused by these

microorganisms and also increases the risk of transmission of infection or the disease, illness and increased mortality (WHO report, 2021). Emerging cases of antimicrobial resistance poses a threat to the medical fraternity of the world, weakening the treatment strategies of microbial diseases in humans. The situation across the globe is becoming even more worse due to the spread of multidrug and pan- resistant bacteria (referred to as "superbugs") that cause serious infections which show no response to the treatment done with existing antibiotics (de Kraker, 2016). The reason for emergence and acceleration in AMR cases are multiple. The genetic changes or mutations that occur in microorganisms naturally with passage of time is beyond human control and is one of the causes for the emergence of AMR. However, the main cause of AMR is attributed to the overuse and misuse of antimicrobial drugs. Taking antibiotics without clinical supervision or prescription, self- medication and not completing the specific dosage and course of antibiotics further complicate the scenario (Prestinace *et al,* 2015). Poor hygiene and sanitary conditions, lack of accessibility to clean drinking water, lack of access to medicines, vaccines and diagnostic facilities, lack of knowledge and awareness regarding causes, modes of transmission and symptoms of infectious diseases and poor legislation of healthcare facilities also lead to AMR, especially in developing countries (Lim *et al,* 2016).

2.3.2. Mechanisms adopted by microorganism to develop AMR

The antimicrobial drug resistance may be developed by microorganisms by a myriad of mechanisms that enable them to acclimatise and survive the lethal, biostatic or toxic effect of antimicrobial drugs. Emergence of novel resistant genes have been revealed by comparative genomics supplemented with functional studies (Sekyere, 2018). Horizontal gene transfer, reduced permeability of bacterial outer membrane, multidrug- efflux pumps that consequently lead to limiting of drug uptake, modification or inactivation of a drug target and drug efflux (Fig.2.3, Table 2.5) are the foremost contributing mechanisms for development of antimicrobial resistance (Reygaert *et al*., 2018).

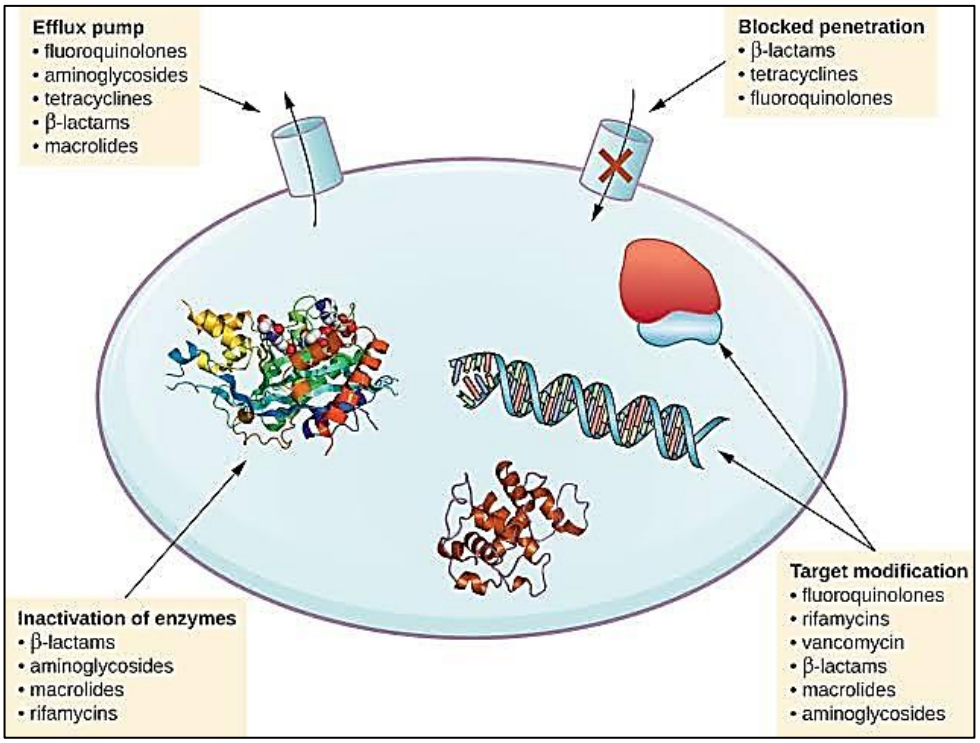


Fig.2.3: General mechanism of resistance to antibacterial drugs observed in bacteria (Ref.: Reygaert *et al*., 2018).

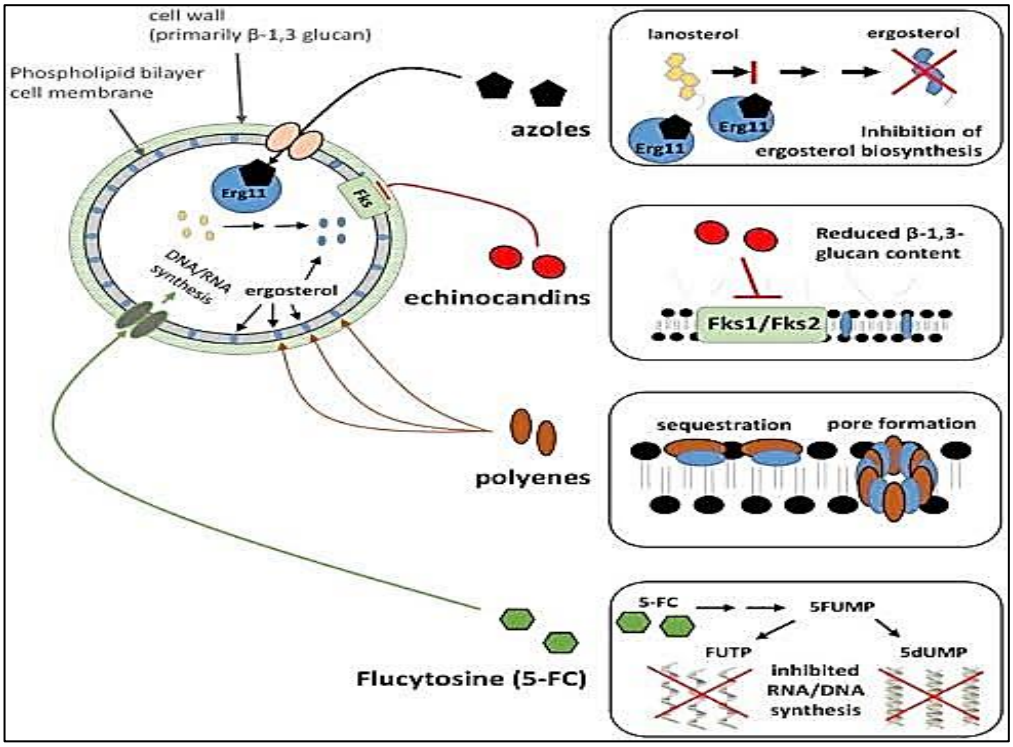


Fig.2.4: General mechanism of resistance to antifungal drugs observed in fungi (Ref.: Cowen *et al*., 2015).

Mechanisms of antifungal drug resistance are largely due to modification of ERG11 gene or ergosterol biosynthetic enzymes, altered affinity for antifungal drug, overexpression of certain drug efflux pumps (Fig.2.4), modification in sterol biosynthesis pathway, biofilm formation (Table 2.6) and decreased intracellular concentration of specific target enzymes (Cowen *et al*., 2015).

Table 2.6: Major antibacterial drugs, their mode of action and mechanism of resistance.

Antibiotic class	**Examples**	**Mode of action**	**Mechanism of resistance to antibiotics**	**Reference**
Aminoglycosides	Ampicillin, arbekacin, gentamicin, tobramycin, streptomycin	Inhibition of protein and cell membrane synthesis	Modification of enzymes such as acetyltranferases, nucletidyl transferases or adenyl transferases, phosphotransferases	Richard and Yitzhak, 2014.
β-lactams	Cephalosporins, carbapenems, monobactams, β-lactam inhibitors	Interference with cell wall synthesis	Production of β-lactamases, such as extended-spectrum β-lactamases (ESBLs), plasmid-mediated AmpC enzymes and carbapenem-hydrolyzing β-lactamases, and through production of ESBL genes (bla ctx-m, bla shv, bla tem)	Shlaes, 2010.
Chloramphenicol	Azidamphenicol, thiamphenicol	Inhibition of protein synthesis	Enzymatic inactivation via, acetylation mediated by chloramphenicol acetyltransferases (CATs). Additional effects include target site modification or mutation, permeability barriers, efflux pumps.	Schwarz *et al*., 2004

Glycopeptide	Teicoplanin, vancomycin	Peptidoglycan units	Inhibit cell wall biosynthesis in Gram-positive bacteria by binding the terminal D-Ala-D-Ala dipeptide of peptidoglycan units sterically inhibiting their use as substrates for penicillin-binding proteins (PBPs) and transglycosylases. Efflux-mediated resistance have been also reported.	Lina *et al*., 1999
Quinolone	Cinoxacin, nalidixic acid, ciprofloxacins	Topoisomerase I and II	Target site modification by mutations to genes gyrA and parC; efflux pump mediated resistance.	Aldred *et al*., 2014
Sulfonamide	Sulfamethoxazole, sulfamethizole, sulfisoxazole	Inhibit dihydropteroate synthase which condenses pteroate and p-aminobenzoic acid (PABA) to form dihydropteroate from which folic acid is produced.	Increased production of PABA); adoption of alternative pathway for folate metabolism.	Richard and Yitzhak, 2014.
Tetracycline	Tigecycline	30S ribosomal subunit	Resistance due to ABC efflux pumps or by ribosomal modification. A tetracycline inactivating enzyme, TetX, has also been reported to confer resistance.	Schaack *et al*., 2010

Carbapenems	Ertapenem, faropenem, imipenem, meropenem	Penicillin binding proteins	Resistance to carbapenems in some species is intrinsic, as in metallo-beta-lactamase (MBL) L1. In clinically important bacteria, resistance mainly occurs due to mutational events or gene acquisition via horizontal gene transfer. Tripartitite efflux pumps; enzyme-mediated resistance (β-lactamases, carbapemases).	Sánchez, 2015; Meletis, 2016.
Colistin	Polymyxin B and E	LPS (lipopolysaccharide layer of bacteria)	Polymyxins, which are polycationic, displace stabilizing magnesium and calcium ions to electrostatically interact with the anionic LPS of gram-negative cell membranes. This disrupting interaction leads to increased cell membrane permeability, cellular content leakage and rapid cell death.	Yu *et al*., 2015; Liu *et al*., 2016)

Table 2.7: Major antifungal drugs, their mode of action and mechanism of resistance.

Antifungal class		**Examples**	**Mode of action**	**Mechanism of resistance to antifungals**	**Reference**
Fluorinated pyrimidine analogues		5-fluorocytosine	Disruption of RNA and DNA synthesis by misincorporation of 5-fluorouracil	Mutation in FUR1 (uracil phosphoribosyl transferase encoding genes)	Hope *et al*., 2004
Polyenes		Nystatin, natamycin Amphotericin B	Disruption of plasma membrane integrity by binding to ergosterol; oxidative damage	Induction of low membrane ergosterol due to defects in ERG3 gene.	Kathiravan *et al*., 2012
Azoles	Imidazoles	Miconazole, clotrimazole, ketoconazole	Inhibition of Erg11p, involved in ergosterol biosynthesis; conversion of Erg11p substrate into toxic methylated sterols	Mutations in Erg11p; induced overexpression of Erg11p; efflux via ABC and MFS transporters; tolerance to methylated sterols via mutation in ERG3	Nascimento *et al*., 2003; Ferrari *et al*., 2011.
	Triazoles	Fluconazole, itraconazole	Cell membrane permeability (ergosterol biosynthesis pathway by targeting 14 α-lanosterol demethylase		
Echinocandins		Caspofungin, micafungin, anidulafungin	Inhibition of (1,3) β-D-glucan synthase	Mutation in (1,3) D-glucan synthase	LaFayatte *et al*., 2010
Allylamines		Terbinafine, naftifine	Inhibit the ERG1 gene of ergosterol biosynthesis	Mutations in squalene epoxidase gene	Gaurav *et al*., 2021.

2.3.3. Global emergence of multidrug resistance in bacteria and fungi

Bacterial infections mainly include urinary tract infections, diarrhoea, sepsis and infections caused by sexual transmission. However, the causative bacterial strains of these infections are increasingly becoming resistant to the standard antibiotics used for the treatment purpose. According to report published by the Global Antimicrobial Resistance and Use Surveillance System (GASS), in certain countries, the rate of bacterial resistance to ciprofloxacin, the clinically approved antibiotic used to treat urinary tract infections, has increased from 8.4% to 92.9% in case of *Escherichia coli* while for *Klebsiella pneumoniae*, the resistance has increased from 4.1% to 79.4% (WHO, 2019). *K. pneumoniae* is a major causal agent of nosocomial or hospital- borne infections namely pneumonia, bloodstream infections, infections in neonates and patients admitted in intensive care units (Paczosa and Mecsas, 2016). To treat such life-threatening infections, carbapenem antibiotics are the drug of choice. But resistance to this last resort antibiotic has spread in an alarming manner where some countries report that more than 50% of patients show no response on treatment with carbapenems rendering them ineffective. Bacterial resistance to the antibiotic colistin, another last resort antibiotic, has been widespread (Nordman *et al*, 2011). Occurrence of E.coli resistance to fluoroquinolones has been accelerating worldwide. Extended- spectrum beta lactamase (ESBL) *E. coli*, as the terminology indicates, are strains of *E. coli* that produce ESBLs that have the ability to breakdown antibiotics such as cephalosporins, ampicillin and most beta- lactam antibiotics, rendering the strains extremely resistant to the most advanced antibiotics (Lim *et al*, 2016; Temkin *et al*, 2018). In 2017 alone, there were 197, 400 new cases of ESBL-EC infections and 9100 deaths worldwide (Singh *et al*, 2017). The incidence of ESBL *E. coli* is on a dramatic rise. Methicillin- resistant Staphylococcus aureus (MRSA) also poses a major threat to global health system and treatment strategies need to be carefully designed because patients with MRSA infecitons are 64% more susceptible to die than patients with drug- sensistive infections (Delorme *et al*, 2017; WHO, 2019). In 2019, the observed median rate for bloodstream infections caused by MRSA was

12.11% and infections caused by *E. coli* resistant to third generation cephalosporins was almost 36% (Kourtis *et al*, 2019). Neisseria gonorrhoeae has exhibited widespread resistance to tetracyclines, macrolides, sulphonamdes, penicillins and fluoroquinolones (Unemo *et al*, 2019; Lovett and Duncan, 2019). *Salmonella* is one of the primary causative agents for food borne infections, typhoid and paratyphoid fever. Researchers have reported that globally, the annual occurrence of nontyphoidal *Salmonella* infections have increased by an estimated 40% with clinically significant cases of resistance in 2016-17 compared to the previous years (Majowicz *et al*, 2010; Stanaway *et al*, 2017) Cases of multidrug resistance pertaining to *Salmonella* strains have been reported worldwide where antibiotics such as ampicillin, ceftriaxone and ciprofloxacin are being rendered ineffective (Balasubramanium *et al*, 2019). In 2018, WHO reported that there were an estimated half million new cases, globally, of rifampicin- resistant TB (RR-TB) of which a huge majority are infected with multidrug- resistant TB (MDR-TB). In the same year, it was reported that around 3.4% new cases of TB and 18% of cases that were previously treated has RR-TB/MDR-TB (Seung *et al*, 2015; Bastos *et al*, 2017). The global population has also witnessed an upsurge in the incidence of multidrug resistance in *Pseudomonas aeruginosa*, a major contributor of nosocomial infections, where antibiotics such as ciprofloxacin, imipenem, ceftazidime and gentamicin are failing to treat the severity of the infection or disease (Poole, 2011; Pang *et al*, 2019).

Increasing incidence of antifungal drug resistance is exasperating the already existing difficult treatment situation. Drug resistant *Aspergillus, Candida albicans*, *C. krusei*, *C. tropicalis*, *C. parapsilosis* and *C. auris* have become widespread with resistance being exhibited to antifungal drugs such as fluconazole, amphotericin B, voriconazole and emerging resistance to Caspofungin has also been reported (Martinez-Rossi *et al*, 2008; Weiderhold, 2017).

According to WHO, a list has been published in 2019, categorising the drug resistant bacteria and fungi, categorising them according to the severity of health threat imposed. They are as follows- (a) Urgent threat: includes carbapenem-resistant *Acinetobacter*, carbapenem- resistant

Enterobacterales, drug resistant *Neisseria gonorrheae, Clostridiodes difficle* and *Candia auris*; (b) Serious threats: includes ESBL producing Enterobacterales, multidrug resistant *Pseudomonas aeruginosa*, drug-resistant non-typhoidal *Salmonella*, drug- resistant *Salmonella* serotype typhi, methicillin resistant *Staphylococcus aureus*, drug resistant Tuberculosis, drug-resistant *Shigella*, vancomycin-resistant *Enterococci* and drug resistant *Candida*; (c) Concerning threats: includes erythromycin- resistant Group A *Streptococcus* and clindamycin-resistant Group B *Streptococcus*; (d) Watch list: the last category includes azole- resistant *Aspergillus fumigatus*, drug resistant *Mycoplasma genitalium* and drug resistant *Bordetella pertussis*. The following table shows the list of antibacterial and antifungal drugs that have become ineffective in the treatment of bacterial and fungal infections respectively.

2.3.4. Antimicrobial drug resistance in India- a grave situation

Antibiotic resistance is a global public health threat (Laxminarayan *et al*., 2013), but nowhere is it as stark as in India (Ganguly *et al*., 2011). The crude infectious disease mortality rate in India today is 416.75 per 100,000 persons and is twice the rate prevailing in the United States when antibiotics were introduced (roughly 200 per 100,000 persons) (Laxminarayan and Chaudhury, 2016). A combined result of poor public health systems and hospital infection, high rates of infectious disease and expensive antibiotics along with increased cost of treatment is together leading to increase prevalence of resistant pathogens and is increasing the burden of untreatable neonatal sepsis and health-care-associated infections.

New Delhi metallo-β-lactamase (NDM) enzymes, first reported in 2008, are now found worldwide (Nordmann *et al*.,2011). In India, *Escherichia coli* (n=1,815) isolated from the community showed high overall resistance to ampicillin, nalidixic acid, and co-trimoxazole (75%, 73% and 59%, respectively) between 2004 and 2007 (Holloway *et al*., 2009). Nearly a third of isolates are resistant to injectables like aminoglycosides (represented by gentamicin). From 2008 to 2013, *E.coli* resistance to third generation cephalosporins increased from 70% to 83%, and fluoroquinolone resistance increased from 78% to 85%. Ten percent of *E. coli* isolates

were resistant to carbapenems in 2008, increasing to 13% in 2013 (CDDEP, 2015). Among *Klebsiella pneumoniae* isolates, third-generation cephalosporin resistance decreased from 90% to 80%, and fluoroquinolone resistance increased from 57% to73% (CDDEP, 2015). Carbapenem resistance among *K.pneumoniae* increased from 2% in 2002 to 52% in 2009 in one tertiary-care hospital in New Delhi (Datta *et al*., 2012). Resistance to fluoroquinolones among invasive *Salmonella typhi* isolates in India increased from 8% in 2008 to 28% in 2014. However, resistance in 2014 to two older antibiotics—ampicillin, 5% and cotrimoxazole,4%—is decreasing, possibly because of a decline in consumption of these two drugs, and is much lower than rates of resistance to fluoroquinolones. Resistance to nalidixic acid in *S. typhi* is increasing (resistance is about 20%–30%) because of widespread use of other quinolones and not because of nalidixic acid use per se. Among *Enterococcus faecium* isolates, 11% were vancomycin resistant (CDDEP, 2015). Surgical site infections are a problem and are predominantly related to Gram-negative pathogens. A study from Mumbai reported a 1.6% rate of surgical site infections, with 66% caused by Gram-negative bacilli (GNB) (Chatterjee *et al*., 2015). With diminishing options for treating multidrug resistant *Acinetobacter baumannii* and other resistant infections, colistin use is increasing, but resistance to colistin is on the rise (Chatterjee *et al*., 2015). Gram-positive infections are also a problem. High rates of methicillin-resistant *Staphylococcus aureus* (MRSA) in clinical isolates from various studies in India have been documented, with rates as high as 54.8% (ranging between 32%and 80%) recorded (Van Boeckel *et al*., 2014). A recent report records a steep increase in MRSA, from 29% of S. aureus isolates in 2009 to 47% in 2014 based on data from a large private laboratory network (CDDEP, 2015). The overall burden of resistance is hard to assess for the general population but is likely focused on neonates and the elderly, both of whom are more prone to infections and vulnerable to ineffective treatment. Although accurate estimates of the overall burden of resistance are not available, it is estimated that 58,000 neonatal deaths are attributable to sepsis caused by drug resistance to first-line antibiotics each year (Laxminarayan *et al*., 2015).

2.4. Cancer

In most organs and tissues of a mature animal, a balance between cell renewal and cell death is maintained. Under normal circumstances, production of new cells is regulated, so that number of any particular type of cell remains constant. Occasionally, though, cells arise that no longer respond to normal growth control mechanisms. These cells give rise to clone of cells, that can expand to a considerable size, producing a tumor. A tumor that is not capable of indefinite growth and does not invade the healthy surrounding tissue extensively is termed as benign. A tumor that continues to grow and becomes progressively invasive is known as malignant. In addition to uncontrolled growth, malignant tumors exhibit metastasis- a process in which small clusters of cancerous cells dislodge from a tumor, invade the blood or lymphatic vessels and are carried to other tissues, where they continue to proliferate. Homeostasis in normal tissue is maintained by a highly regulated process of cellular proliferation balanced by cell death. If there is an imbalance, either at the stage of cell proliferation or at the stage of cell death, then a cancerous state will develop. Oncogenes and tumor suppressor genes have been shown to play an important role in this process by regulating the cellular proliferation or cell death.

2.4.1. Cancer – the global picture

Cancer till date remains the leading cause of death worldwide and an important detrimental factor in the biomedical field where it is poses an obstruction to increase the life expectancy of the population across the globe (Sung *et al*., 2021). According to the reports of 2019, published by WHO, cancer is the first (or second) leading cause for causing mortality before attainment of the age of 70 in about 112 out of 183 countries and in further 23 countries, it ranks as the third or fourth leading cause (WHO, 2020). A survey conducted by the International Agency for Research on Cancer (IARC), the cancer surveillance agency of WHO, reported the occurrence of 19.3 million new cancer cases and around 10 million mortality cases that occurred in the year 2020 alone (Sung *et al*., 2021). Even more alarmingly, reports suggest that for both genders combined, half of all cases and about 58.3% of cancer death cases occurred in Asia in

the year 2020, where almost 59.5% of the population of the world resides (Ferlay *et al*., 2020). On one hand, one- third of the cancer cases are due to sedentary lifestyle that includes consumption of excessive alcohol, use of tobacco, lack of physical activity and low fruit and vegetable intake; on the other hand, microbial infections, for instance human papillomavirus (HPV) and hepatitis virus, are responsible for contributing to around 30% of cancer cases in countries with low- and lower- middle income (Bagnardi *et al*., 2015; Gupta *et al*., 2018). Diagnosis of the cancer at a delayed stage and lack of accessibility with regard to diagnostic facilities are also common in such countries that leads to an overall impact in the economy of a country besides leading to adverse health effects and financial burden on the patients (Sung *et al*., 2021). Cancer incidence as well as mortality is growing worldwide that needs attention and adoption of strong measures to prevent the cases from escalating. Sustained efforts to develop proper infrastructure for the dissemination and awareness of cancer prevention measures and developing affordable treatment strategies at national as well as local levels is crucial for cancer control at a global level.

2.4.2. Cancer incidence and upsurge in India

A survey conducted under the National Cancer Registry Programme of ICMR showed that cancer incidence in India for the year 2020- among males, it was 679,421 (94.1 per 100,000) and among females, it was reported to be 712,758 (103.6 per 100,00) (Mathur *et al*., 2020). According to its report, the figures also highlight that 27.1% of the cancer cases were lung cancers (mainly due to tobacco consumption), followed by 19.7% of gastrointestinal cancers. Among women, breast cancers incidence is around 14.8% while the incidence of cervical cancer being 5.4% (Behera and Patra, 2018; Mathur *et al*., 2020). The increasing trend in breast and cervical cancer among women has been observed globally (Fig.2.5) which is mainly due to diagnosis at a very delayed stage that eventually led to metastases, causing failure of standard treatment strategies. Earlier, cervical cancer incidence and malignancy was reported mainly from the rural areas of the nation. However, present scenario highlights that the incidence of

cervical cancer is increasing continuously both in rural areas as well as in metropolitan cities (Tewari *et al*., 2018). Taken together, cervical cancer is emerging as a major problem in the public health context in India, taking a toll on the health of women besides overburdening them and their families with economic impact due to the cost involved in treatment of cervical cancer in India.

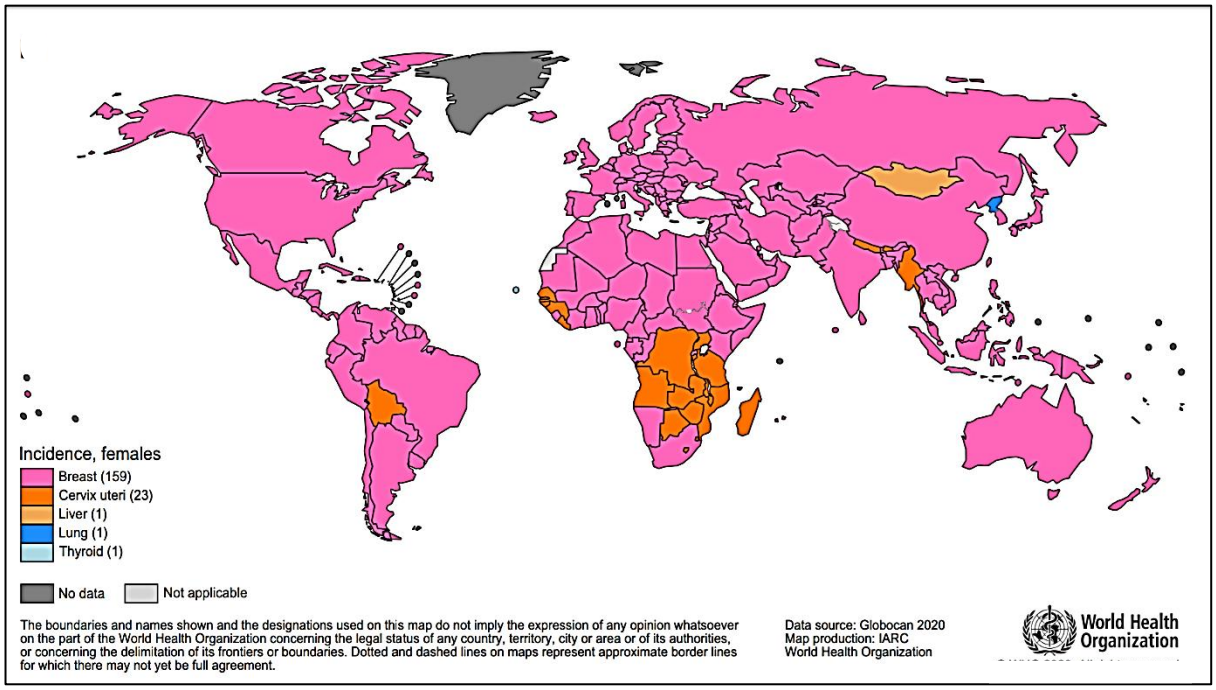


Fig.2.5: Global incidence of common types of cancer among women (Source: IARC, 2020).

2.4.3. Cervical cancer incidence and its cause

At a global level, around 570,000 women were diagnosed with cervical cancer and approximately 311,000 women died in 2018 (WHO, 2018). In India, about 96,9222 new cervical cancer cases (9.2%) have been reported and 60,078 deaths (8.4%) in the year 2018 (NICPR, 2018). Cervical cancer contributes to almost 14% of all female cancer cases reported among women in India. Among the total reported cases of cervical cancer, almost 99% cases are linked to a viral infection by the Human Papillomavirus (HPV) that is generally transmitted through sexual contact (Chatterjee *et al*., 2016).

Cervical cancer, if detected at an early stage and provided with treatment, is curable. However, it still remains the fourth most common cancer among women at a global level and second most common form of cancer among women in India (Tewari *et al*., 2018). The huge incidence of

cervical cancer cases and the mortality related to this type of cancer is undoubtedly a consequence of neglect by the women themselves as well as by the global health community (Mohanty and Ghosh, 2015). Moreover, early-stage cancer of the cervix does not generally produce any sign or symptom, making the screening process even more difficult. But in the advanced stage, signs and symptoms do arise that may include watery, bloody discharge from the vagina, vaginal bleeding or pelvic pain (Bobdey *et al*., 2016). Reports indicate that women in the age group of 15 to 44 years have the risk of developing cervical cancer owing to the lack of awareness or negligence in adopting preventive measures during sexual intercourse and poor sense of hygiene (Sreedevi *et al*., 2015) that ultimately affects their reproductive health, making the conditions favourable for the development of cancer in the cervix.

2.4.4. Acute chemo radiotherapy toxicity and chemoresistance in cervical cancer treatment

WHO approves the use of specific drugs for the treatment of cervical cancer, namely- bevacizumab, topotecan hydrochloride and tisotumab vedotin, or combination drugs such as carboplatin-taxol and cisplatin-gemcitabine (WHO 2018, 2019). However, combinatorial drug regimen often leads to the emergence of drug toxicity, infertility and chemoresistance (Chuang *et al*., 2016). The International Federation of Gynaecology and Obstetrics (FIGO) categorises cervical cancer into various stages (Fig.2.6) and treatment strictly depends on the cancer stage of the patient (Marth *et al*., 2017). The early-stage cancers are treated surgically in maximum cases, locally advanced stages require application of chemoradiation. Recurrent or metastatic stage treatment may be often salvaged with pelvic exenteration or may be palliated with systemic chemotherapy along with administration of a target specific chemotherapeutic drug, such as cisplatin, paclitaxel or bevacizumab (Kalaghchi *et al*., 2016). In short, concurrent chemoradiotherapy is applied that may include chemotherapy with external beam radiotherapy and subsequent application of brachytherapy. In all cases, radiotherapy can cause acute radiation toxicity. 84% of patients, who undergo radiotherapy treatment for cervical cancer, experience some form of radiation toxicity (Dutta *et al*., 2015). Manifestations are generally

gastrointestinal, hematological or genitourinary toxicity. Though the intensity of toxicity depends on various factors such as age, radiation dose and technique and its duration of application, stage of cancer, genetic factors, comorbidities et., the occurrence of the toxicity is prevalent in some form or the other (Liberman *et al*., 2014; Hernandez-Moreno *et al*., 2015). Often, this acute toxicity leads to chronic toxicity that leads to chemoresistance with subsequent increase in the costs involved in the treatment procedure. Moreover, an issue of major concern is the effect on the female reproductive system (Kuku *et al*., 2013; Qin *et al*., 2013). If radiation toxicity incidence is not properly addressed, the patient might suffer from a number of adverse effects such as infertility, premature deliveries, tumor recurrence, vaginal bleeding, pelvic pain or pain due to bone metastases (Meng *et al*., 2016). Further, increasing the dosage of the drug have also been reported to cause severe consequences by adding on to the effects of toxicity and eventually leading to chemoresistance and stalling of the treatment procedure (Radojevic *et al*., 2020).

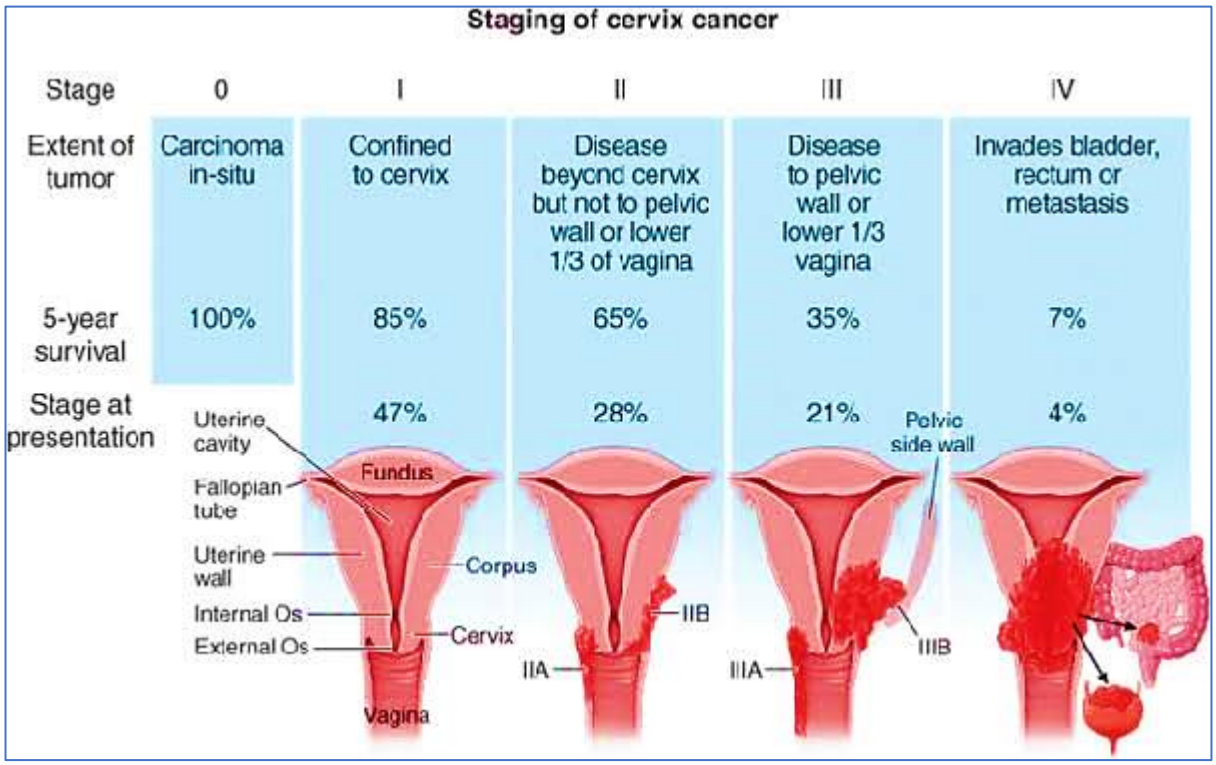


Fig.2.6: Various stages of human cervical cancer (Source: FIGO, 2018).

2.5. Mangroves of Sundarbans as a source of biologically active compounds to combat antimicrobial drug resistance and cancer incidence

Literature studies and research findings clearly indicate the potential of mangrove plants being used and applied for clinical purposes, to fight various problems that presently exist in the global health system (Bandarnayake, 1998; Cos *et al*., 2006; Patra and Mohanta, 2013; Gray *et al*., 2010; Patra and Thatoi, 2011). Till date, qualitative studies have been mainly carried out to analyse the antimicrobial and antioxidant activity of mangrove plants of the Indian Sundarbans. Detailed study of biologically active compounds present in the plant extracts and their associated mechanism of action is yet to be understood clearly. Moreover, limited research has been done to explore the antimicrobial activity of mangrove plant extracts against multidrug resistant microorganisms. The existing expensive chemotherapy procedures for cancer treatment that are increasingly being accompanied by cases of chemoresistance, cannot be replaced if the research findings do not convince the biomedical fraternity. Hence, there is a growing need to assess mangrove plant extract for anticancer activity so that they can be used in combination with existing therapies to minimise toxicity in normal healthy cells and arrest tumour progression. Previous studies have already made it evident that undoubtedly the mangrove plants are a repertoire of biologically active compounds that have been responsible for conferring the reported antimicrobial, antimalarial, antioxidant, antinociceptive, anti-inflammatory and other such properties (Alam *et al*., 2006; Li *et al*., 2007; Wangensteen *et al*., 2009; Nurdiani *et al*., 2012). These investigations further necessitate the initiative to identify, characterise and purify biologically active compounds and demarcate the associated mode of action, so that effective pharmaceutical compounds can be designed.

Since mangroves of the Indian Sundarbans has not yet been explored completely with respect to the above-mentioned properties, there might be a chance to discover novel and potential compounds that may add immense value to the existing pharmaceutical and biomedical reserve of drugs. Sparse literature exists on the medicinal aspect of the mangrove plants of the Indian

Sundarbans mainly because of the difficulty in approaching the estuaries and localization of these plants to the estuarine region. As residents of Kolkata, a city located at just a few-kilometres away from the Sundarbans area, we are blessed and have the opportunity to study these plants and exploit the different medicinal properties of these plants.

This investigation is aimed towards the exploration of some of these secondary metabolites from mangrove plants of Sundarbans which will be the future tool for treating microbial infection and cancer.

Chapter 3:

Materials and Methods

- **List of chemicals and reagents used:**

A list of all the chemicals and reagents used in this investigational study has been enlisted in Table 3.

Table 3: List of chemicals and reagents used

Sl. No.	Chemicals/Reagents (with manufacturer's specifications)
1.	Absolute ethanol (HiMedia)
2.	ABTS (CAS 30931-67-0, HiMedia)
3.	Acetyl acetone (CAS 123-54-6, Merck Millipore)
4.	Acrylamide (CAS 79-0-1, Sigma-Aldrich)
5.	Alpha amylase (CAS 9000-90-2, Sigma-Aldrich)
6.	Aluminium chloride anhydrous (CAS 7446-70-0, Merck Millipore)
7.	Ammonium acetate (CAS 631-61-8, Merck Millipore)
8.	Ammonium molybdate (ammonium heptamolybdate tetrahydrate, CAS 12054-85-2, Merck Millipore)
9.	Antibiotic rings (HiMedia)
10.	Antifungal discs (HiMedia)
11.	Ascorbic acid (L-Ascorbic acid, CAS 50-81-7, HiMedia)
12.	Bergenin commercial grade (Sigma Aldrich, Batch no. 80479, HPLC purified)
13.	Bromophenol blue (CAS 115-39-9, Thermo Fisher)
14.	Calcofluor White stain kit (F3543, Sigma-Aldrich)
15.	Caspase 3 assay kit (BioVision K106-25)
16.	Chloroform (CAS 67-66-3, Merck Millipore)
17.	Conc. Nitric acid (69%) (Product code: 101799, Merck Millipore)
18.	Conc. Sulphuric acid (98%) (CAS 7664-93-9, Merck Millipore)
19.	Coomassie Blue R250 (Thermo Fisher)
20.	DCFH-DA (Thermo Fisher)
21.	Dimethylsulfoxide (MB058, HiMedia)
22.	DNS (3,5-Dinitrisalicylic acid, CAS 609-99-4, HiMedia)
23.	DPPH (CAS 1898-66-4, HiMedia)
24.	EDTA(Disodium salt, dihydrate, molecular biology grade, CAS 6381-92-6,
25.	Merck Millipore)
26.	Egg albumin (hydrolysate, RM6379, HiMedia)
27.	Egg homogenate (Egg yolk emulsion, HiMedia)
28.	Ethanol (MB228, HiMedia)
29.	Ethyl acetate (CAS 141-78-6, Merck Millipore)
30.	Ferric chloride anhydrous (CAS 7705-08-0, Merck Millipore)
31.	Ferrous ammonium sulfate [Ammonium iron(II) sulfate hexahydrate, CAS 7783-85-9, Merck Millipore)
32.	Folin-Ciocalteu reagent (Phenol reagent, LR, HiMedia)
33.	Gallic acid (CAS 149-91-7, Sigma-Aldrich)
34.	Glacial acetic acid (MB052, HiMedia)

35.	Glutathione (GSSG, CAS 27025-41-8, Sigma-Aldrich)
36.	Glycerol (CAS 56-81-5, Merck Millipore)
37.	Griess reagent (G-7921, Thermo Fisher)
38.	Hoechst dye 33258 (Thermo Fisher)
39.	Hydrogen peroxide (35%, Code: 107298, Merck Millipore)
40.	Hydroxylamine hydrochloride (CAS 5470-11-1, Sigma-Aldrich)
41.	Methanol (CAS 67-56-1, HiMedia)
42.	MTT cell proliferation kit (CyQUANT, Thermo Fisher)
43.	Mueller Hinton Agar (M173, HiMedia)
44.	NADH (disodium salt, CAS 606-68-8, Merck Millipore)
45.	NADPH (tetrasodium salt, CAS 2646-71-1, Merck Millipore)
46.	n-butanol (CAS 71-36-3, Merck Millipore)
47.	Nitroblue tetrazolium chloride (NBT) (CAS 298-83-9, HiMedia)
49.	Phenazine methosulfate (PMS) (CAS 299-11-6, Sigma-Aldrich)
50.	Phenol (CAS 108-95-2, Merck Millipore)
51.	Potassium acetate (CAS 127-08-2, HiMedia)
52.	Potassium ferric cyanide (Code: 702587, Sigma-Aldrich)
53.	Potassium permanganate (CAS 7722-64-7, Merck Millipore)
54.	Potassium persulphate 9CAS 7727-21-1, HiMedia)
55.	Propidium iodide (TC252, HiMedia)
56.	Proteinase K (Thermo Fisher)
57.	Quercetin (quercetin=95 HPLC, solid 117-39-5, Sigma Aldrich)
58.	RNase A (PureLink^{TM}, Thermo Fisher)
59.	Sabouraud agar (GM063, HiMedia)
60.	Sodium dodecyl sulfate (CAS 151-21-3, Merck Millipore)
61.	Silica gel (60-120 Mesh for column chromatography, Sigma-Aldrich)
62.	Sodium acetate (CAS 127-09-3, Merck Millipore)
63.	Sodium carbonate anhydrous (CAS 497-19-8, Merck Millipore)
64.	Sodium chloride (CAS 7647-14-5, Merck Millipore)
65.	Sodium nitroprusside (CAS 13755-38-9, HiMedia)
66.	Sodium nitroprusside (dihydrate, CAS 13755-38-9, HiMedia)
67.	Sodium phosphate (CAS 96-7601-54-9, Sigma-Aldrich)
68.	Sodium sulfite (CAS 7757-83-7, Merck Millipore)
69.	Starch (CAS 9005-84-9, Sigma-Aldrich)
70.	Tannic acid (powder form, pure, HiMedia GRM7541)
71.	TBA (RM1594, HiMedia)
72.	Toluene (CAS 108-88-3, Merck Millipore)
73.	TPTZ (CAS 3682-35-7, HiMedia))
74.	Trichloroacetic acid (CAS 64-19-7, HiMedia)
75.	Vanillin reagent (CAS 121-33-5, Sigma-Aldrich)
76.	β-mercaptoethanol (CAS 60-24-2, HiMedia)

3. Materials and Methods

3.1. Collection and preservation of plant samples

Fresh leaf samples of *Bruguiera gymnorhiza, Excoecaria agallocha, Avicennia alba* and *Aegialitis rotundifolia* were collected from Bali Island of the Indian Sundarbans (near 30°24´-30°28´ N latitude and between 77°40´- 77°44´ latitude in the South 24 Parganas, West Bengal) during the month of June, 2018. The plant samples were washed with distilled water stored at 4˚C after collection and utilized within 7 days for extract preparation (Sett *et al*., 2014).

3.2. Extract preparation with different solvents

The leaf samples were oven dried at 60˚C till crisp and ground to fine powder using mortar and pestle. About 1 g of each of the finely powdered plant leaf material was soaked in 10ml of solvents (70% ethanol, 50% methanol, water and 1% dimethyl sulfoxide [DMSO]) for a period of 1 week at room temperature. Then the extracts were filtered and concentrated by rotary vacuum evaporator (RotaVap). The final concentration was adjusted to 1mg/ml for screening the antimicrobial activity (Sett *et al*, 2014).

3.3. Powder preparation and pharmacognosy

The leaf samples were oven dried at 60˚C till crisp and ground to fine powder using mortar and pestle. The fine powder obtained was used in microscopic and physicochemical analysis (Bandaranayake *et al*., 1998; Khandewal *et al*., 2005). Different physicochemical parameters such as loss on drying, acid insoluble ash, water soluble ash, water soluble extractive values and ethanol soluble extractive values were measured for the different plant extracts.

3.3.1. Loss on drying

Loss on drying is the loss of mass expressed as per cent w/w. It determines both water and volatile matter in the crude drug. Moisture is an inevitable component of crude drug, which must

be eliminated as far as possible. An accurately weighed quantity of about 2 g of powdered drug was taken in a tared glass Petridish. The powder was distributed evenly. The petridish kept open in vacuum oven and the sample was dried at a temperature between 100° to 105°C for 2 h until a constant weight was recorded. Then it was cooled in a desiccator to room temperature, weighed and recorded. % Loss on drying was calculated using the following formula:

$$\% \text{ Loss on drying} = \frac{\text{Loss in weight of the sample}}{\text{Weight of the sample}} \text{ X } 100$$

3.3.2. Determination of Ash values

Ash values are helpful in determining the quality and purity of a crude drug, especially in the powdered form. The objective of ashing crude drugs is to remove all traces of organic matter, which may otherwise interfere in an analytical determination.

a) Total Ash value :

It is the total amount of material remaining after ignition. This includes both physiological ash, which derived from the plant leaf tissue itself, and non-physiological ash, which is residue of the extraneous matter. Total ash value was found out after putting the powdered sample (about 2 gm) in crucible by using furnace at temp. of 600°C for 2hr. The percentage yield of ash value, with reference to air dried sample, was calculated.

b) Acid-Insoluble Ash :

It is residue obtained after boiling the total ash with dilute hydrochloric acid, and igniting the remaining insoluble matter. The ash was boiled for 5 to 10 minutes with 25ml of dilute hydrochloric acid, the insoluble matter was collected in a crucible on an ash less filter –paper, ignited, and weighed. The percentage yield of acid-insoluble ash was calculated with reference to the air-dried sample, using the following formula:

$$\%\,\text{Acid insoluble ash value} = \frac{\text{Wt. of acid insoluble ash}}{\text{Wt. of crude drug taken}} \times 100$$

c) Water soluble Ash value:

The total ash was boiled for five minutes with 25 ml of water; the soluble matter was collected in a crucible, ignited, and weighed. The percentage or water-soluble ash with reference to air dried sample (crude drug), was calculated using the following formula:

$$\text{\% Water soluble ash value} = \frac{\text{Wt. of total ash} - \text{Wt. of water insoluble ash}}{\text{Wt. of crude drug taken}} \times 100$$

3.3.3. Determination of extractive values

Determination of extractive values is useful for evaluation of crude drug. It gives idea about the nature of the chemical constituents present in a crude drug.

a) Alcohol soluble extractive value

5 gm accurately weighed coarse powdered plant sample was mascerated with 100 ml of alcohol (90%v/v) in a stoppered flask for 24 h, shaking frequently during first 6 h. Then it was filtered rapidly through filter paper taking precaution against excessive loss of alcohol. 25 ml of alcoholic extract was evaporated to dryness in a tared dish and weighed. The percentage w/w of alcohol soluble extractive was calculated with reference to the air-dried drug using following formula:

% Alcohol soluble extractive value = 80 X (Weight of residue)

b) Water soluble extractive value

The procedure as above was followed using deionized water instead of alcohol.

3.4. Phytochemical screening of the plant extracts

Test for cardiac glycosides: The solvent extract (100 µl) was mixed with of glacial acetic acid (500 µl) containing 10% $FeCl_3$. Then, 1 ml of concentrated H_2SO_4 was added to the above mixture and observed for a greenish-yellow colour at the interface (Thiruvukkarasu *et al*, 2018).

Test for saponins*:* The solvent extract (100 µl) was mixed with distilled water (500 µl), shaken vigorously and observed for a stable persistent froth. Then, 3 drops of olive oil were added, shaken vigorously and observed for the formation of an emulsion (Umaru *et al*, 2018).

Test for flavonoids: The extract (2ml) was mixed with dil. HCl (3ml) followed by addition of conc.H_2SO_4 (1ml). Yellow colouration development showed the presence of flavonoids (Umaru *et al*., 2018)

Test for terpenoids*:* The solvent extract (100 µl) was mixed with chloroform (500 µl) to which concentrated H_2SO_4 was added carefully to form a layer and observed for a reddish-brown coloration of the interface (Thiruvukkarasu *et al*, 2018).

Test for tannins: The solvent extract (100 µl) was mixed with few drops of 0.1% $FeCl_3$ and observed for brownish-green coloration (Umaru *et al*, 2018).

Test for proteins and xanthoproteins*:* To the solvent extract (500 µl), few drops of concentrated nitric acid were added and observed for yellow coloration (Umaru *et al*, 2018).

Test for steroids: The solvent extract (100 µl) was dissolved in chloroform (900 µl). Then, 1 ml concentrated H_2SO_4 was added from the side of the test tube and observed for a brown ring at the interface (Jayashree D., 2013).

3.8. Determination of Total Phenolic Content of the plant extracts

The amount of protein in the four different extracts were determined by Folin Ciocalteu reagent, according to the method using 1% gallic acid as a standard phenolic compound (Alhakmani *et al*, 2013) 1.0 ml of extract solution containing 1.0 g extract in a conical flask was diluted with 46 ml of distilled water in methanol. 1.0 ml of Folin-Ciocalteau reagent was added and mixed thoroughly. After three minutes, 3.0 ml of 2% sodium carbonate was added and the mixture was allowed to stand for 3 h with intermittent shaking. The absorbance of the blue colour that developed was read at 760 nm. The concentration of total phenols was expressed as mg/g of dry extract (Maswada *et al*, 2013). All determinations were performed in triplicates.

3.5. Determination of Total Flavonoid content of the plant extracts

Aluminium chloride colorimetric method was used with some modifications to determine the flavonoid content. 1 ml of plant extracts were mixed with 3 ml of methanol, 0.2 ml of 10% aluminium chloride, 0.2 ml of 1M potassium acetate and 5.6ml of distilled water and was allowed to stand at room temperature for 30 min. The absorbance was measured at 420 nm. Quercetin was used as standard (1mg/ml). All the tests were performed in triplicates. Flavonoid content was determined from the standard curve and expressed as quercetin equivalent (mg/g of extracted compound) (Zhishen *et al*, 1999).

3.6. Determination of total tannin content

The quantitative tannin content in various leaf extracts was evaluated by the method reported by Price and Butler (1977). About 0.5 ml of the sample was mixed with 1.0 ml of 1% potassium ferric cyanide and 1.0 ml of 1% ferric chloride and the final volume was made to 10.0 ml with distilled water. The reaction mixture was kept at room temperature for 5 min, after which its absorbance was read at 720 nm against a reagent blank. Tannic acid (1mg/ml) was used as standard. The tannin content was expressed as milligram of tannic acid (TAE) equivalence per gram of extract.

3.7. Determination of Total antioxidant activity

The total antioxidant activity was evaluated by phosphomolybdenum method described by Prieto *et al*. (1999). The extract (1 ml) was mixed with 1.0 ml of the standard reagent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95°C for 90 min. After cooling to room temperature, the absorbance was measured at 695 nm against a reagent blank. Ascorbic acid (0.1%) was used as the standard. The total antioxidant capacity was expressed as milligram of Ascorbic Acid Equivalence (AAE) per gram of extract.

3.8. *In vitro* antioxidant activity

3.8.1. DPPH free radical scavenging activity

The stable radical DPPH (1,1-diphenyl-2-picrylhydrazyl) was used to assess the free radical scavenging activity of the different solvent extracts as a direct value of their anti-oxidant activity (Venugopal and Devarajan, 2011). To 900 µl of each test sample (100 mg/ml), 100 µl of 95% methanol and 1 ml of freshly prepared DPPH solution in 95% methanol (1 mM) were added, mixed well and incubated at dark for 30 min. After 30 min, the absorbance was measured at 517 nm using methanol (95%) and de-ionized water with DPPH solution as reference and control respectively. The ability to scavenge the DPPH radical was measured using the following equation: % DPPH scavenged = {(Ac – At)/Ac} x 100, where Ac is the absorbance of the control and at is the absorbance of the sample (solvent extracts). The antioxidant activity was expressed as IC_{50}.

3.8.2. ABTS [2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)] assay

$ABTS^{\cdot}$ solution was prepared by mixing 7mM of ABTS and 2.45 mM of Potassium persulphate in water, which was incubated for 12 hours in dark at room temperature. Before use, the ABTS solution was diluted with ethanol to get an absorbance of 0.7± 0.002 at 734 nm. Briefly, to 5µl

of the plant extract, 4 ml of ABTS solution was added. The samples were mixed thoroughly, incubated for 30 minutes at room temperature and absorbance was recorded at 734 nm (Pattanayak *et al*, 2012).

3.8.3. Ferric Reducing Antioxidant Power (FRAP) assay

The method is based on the reduction of Fe^{3+} TPTZ complex (colourless complex) to Fe^{2+} tripyridyltriazine (blue coloured complex) formed by the action of electron donating antioxidants at low pH. The FRAP reagent is prepared by mixing 300mM acetate buffer, 10ml TPTZ in 40Mm HCl and 20Mm $FeCl_3$.6H20 in the proportion of 10:1:1 at 37°C. Freshly prepared working FRAP reagent is pipetted (3ml) and mixed with 5 µl of the plant sample and mixed thoroughly. An intense blue colour complex is formed when ferric tripyridyl triazine (Fe3+ TPTZ) complex is reduced to ferrous (Fe2+) form and the absorbance is recorded at 593 nm. A blank is also prepared by adding FRAP to water (Pattanayak *et al*, 2012).

3.9. *In vitro* Reactive Oxygen Species (ROS) scavenging assays

3.9.1. Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity of the extracts was determined according to the method reported by Klein *et al*. (1981). The reaction mixture contained 1.0 ml of different concentration of extracts (2-10 mg/ml), 1.0 ml of iron-EDTA solution (0.13% ferrous ammonium sulphate 0.26% EDTA), 0.5 ml of 0.018% EDTA, 1.0 ml of DMSO (0.85 % in 0.1 M phosphate buffer pH7.4) and 0.5 ml of 0.22% ascorbic acid. The tubes were capped tightly and heated in a water bath at 80-90ºC for 15 minutes, the reaction was terminated by adding 1.0 ml of ice-cold TCA (17.5 %). To the above reaction mixture 3.0 ml of Nash reagent (75.0 g of ammonium acetate, 3.0 ml of glacial acetic acid and 2.0 ml of acetyl acetone were mixed and distilled water was added to a total volume of 1 L) was added and incubated at room temperature for 15 minutes for color development. The intensity of the yellow colour formed was measured at 412 nm against a reagent blank. Ascorbic acid was used as standard. % inhibition = [(Control-Test)/control] ×100

3.9.2. Hydrogen peroxide scavenging assay

The ability of the plant extracts to scavenge hydrogen peroxide was estimated according to the method reported by Ruch *et al*. (1989) with minor modification. A solution of hydrogen peroxide (43 mM) is prepared in phosphate buffer (1 M pH 7.4). Different concentration of sample (2-10 mg/ml) was added to a hydrogen peroxide solution (0.6 ml, 43 mM). Absorbance of hydrogen peroxide at 230 nm was determined after 10 minutes against a blank solution containing phosphate buffer without hydrogen peroxide. Ascorbic acid was used as standard. The free radical scavenging activity was determined by evaluating % inhibition as described in the preceding section.

3.9.3. Nitric oxide radical scavenging assay

Nitric oxide generated from sodium nitroprusside interacts with oxygen to produce nitrite ions which was measured by the Griess reaction. This assay was done by the procedure described by Green *et al*. (1982). The reaction mixture contained 3.0 ml of 10 mM sodium nitroprusside in phosphate buffered saline (pH 7.4) and various concentration of (2-10 mg/ml) extracts. The resulting solution was then incubated at 25ºC for 60 minutes. To the incubated sample 5.0 ml of Griess reagent was added and the absorbance of the chromophore formed was measured at 546 nm against a reagent blank. Percentage inhibition of the nitrite ions generated is observed. The standard ascorbic acid was used for comparison. The free radical scavenging activity was determined by evaluating % inhibition as in earlier sections.

3.9.4. Superoxide anion radical scavenging assay

The superoxide anion radical scavenging activity of the mangrove leaf extracts were assessed using the method described by Fontana *et al*. (2001) with slight modification. To various concentrations of the extracts (2-10 mg/ml), 1.0 ml of phosphate buffer (0.1 M, pH 7.2), 1.0 ml

of NADH (2 mM), 1.0 ml of NBT (0.5 mM) and 0.1 ml of PMS (0.03 mM) were added. After 5 minutes incubation at room temperature, the absorbance was read at 562 nm against a reagent blank to determine the quantity of formazan generated. Gallic acid was used as the standards. The % inhibition was determined as mentioned earlier.

3.10. *In vitro* activity of ROS scavenging enzymes

3.10.1. Superoxide dismutase activity

The assay was carried according to the method of Beauchamp and Fedovich (1976). To 0.5 ml of plant extract, 1 ml of 125 mM sodium carbonate, 0.4 ml of 25 μM NBT and 0.2 ml of 0.1 mM EDTA were added. The reaction was initiated by adding 0.4 ml of 1 mM Hydroxylamine hydrochloride and the absorbance was read at 560 nm using spectrophotometer at 1 min intervals. Units of SOD were expressed as amount of enzyme required for inhibiting the reduction of NBT by 50%. The specific activity was expressed in terms of units per mg of protein.

3.10.2. Catalase activity

Catalase activity was determined by the titrimetric method described by Chance and Maehly (1995). To 1 ml plant extract, 5 ml of 300 μM phosphate buffer (pH 6.8) containing 100 μM hydrogen peroxide (H_2O_2) was added and left at 25°C for 1 min. The reaction was arrested by adding 10 ml of 2% H_2SO_4, and residual H_2O_2 was titrated with potassium permanganate (0.01 N) till pink colour was obtained. Enzyme activity was estimated by calculating the decomposition of μM H_2O_2 per min per mg protein.

3.10.3. Ascorbate oxidase activity

Ascorbate oxidase activity was measured according to Diallinas *et al*. (1997). The sample was homogenised [1:5 (w/v) with phosphate buffer (0.1 mol/L, pH 6.5)] and centrifuged at 3000xg

for 15 mins at 50°C. The supernatant obtained was used as enzyme source. To 3 ml of the prepared substrate (8.8mg vitamin C in 300ml phosphate buffer, pH 5.6), 0.1 ml of the enzyme extract was added and the absorbance at 265nm was recorded for every 30 s for a period of 5mins. One enzyme unit is equivalent to 0.01 OD change per min.

3.10.4. Glutathione reductase activity

The activity of glutathione reductase (GR) was assayed using the method that was described by Carlberg and Mannervik (1985). The GR assay was performed in a cuvette that contained 1M Tris-HCl buffer + 5mM EDTA (pH 8.0), 0.033 M GSSG, 2 mM NADPH, and a 20 µl plant extract in a final volume of 1.0 ml. The decrease in absorbance, which reflects the oxidation of NADPH during reduction of GSSG by GR present in the sample, was monitored spectrophotometrically at 340 nm. Results were expressed as units of GR activity/mg cell protein.

3.10.5. Assay of Glutathione

1ml of the plant extract was precipitated with 10% TCA (trichloro acetate) and centrifuged at 3000rpm. To the supernatant, 2ml of PBS (phosphate buffer saline) and 0.5ml of DTNB (5, 5'-dithio 2-nitro benzoic acid) were added and final volume was made 5ml with distilled water. The resulting yellow colour of the mixture was measured at 412 nm by spectrophotometer. (Optizen Pop, Mecasys Co. Ltd.). Commercial grade glutathione was used as standard and expressed in µM (Alisik *et al*., 2019).

3.11. *In vitro* assay for inhibition of alpha amylase activity, albumin denaturation and lipid peroxidation

3.11.1. *In vitro* inhibition of alpha amylase activity

Alpha amylase was dissolved in phosphate buffer saline (PBS, 0.02 mol/L, pH 6.8) at a concentration of 0.1 mg /ml. Various concentrations of sample solutions (0.25ml) were mixed with alpha-amylase solution (0.25ml) and incubate at 37°C for 5 min. Then the reaction was initiated by adding 0.5ml 1.0% (w/v) starch substrate solution to the inoculation medium. After incubation at 370C for 3 min, the reaction was stopped by adding 0.5 ml DNS reagent (1% Dinitrosalicylic acid, 0.05% Na_2SO_3 and 1% NaOH solution) to the reaction mixture and boiling at 100oC for 5 min. After cooling to room temperature, the absorbance (Abs) at 540 nm was recorded by a spectrophotometer (Banerjee *et al*, 2017). Blank was prepared by replacing the enzyme solution with distilled water. Control was prepared without extract. The inhibition percentage was calculated by following equation:

Percentage inhibition = [(Abs1 –Abs2)/Abs1] × 100

(Abs1: absorbance of control, Abs2: absorbance of test sample)

3.11.2. Inhibition of albumin denaturation

The reaction mixture consisted of 1ml of egg albumin (1mM), 3ml of phosphate buffered saline (PBS, pH-6.4), 1ml of test extracts. Similar volumes of PBS without extracts served as control. Then the mixtures are incubated at 37°C± 2°C for 15 mins and heated for 10 mins at 70°C. After cooling, their absorbance was measured at 660 nm against blank (Kaur *et al*, 2018). The percentage inhibition of protein denaturation was calculated by using the following formula:

Percent inhibition= (control- test/control) x 100

3.11.3. Inhibition of lipid peroxidation

Egg homogenate (0.5 ml, 10% in distilled water) and 0.1 ml of each fraction were mixed separately in a test tube and the volume was made up to 1 ml, by adding distilled water. Finally, 0.05 ml FeSO4 (0.07M) was added to the above mixer & incubated for 30min to induce lipid peroxidation. Thereafter, 1.5 ml of 20% acetic acid & 1.5 ml of 0.8% TBA (w/v) in 1.1 SDS and 0.05 ml 20% TCA was added, vortexed and then heated in a boiling water bath for 1 hr. After cooling, 5 ml of butanol was added to each tube and centrifuged at 3000 rpm for 10 mins. The absorbent of the organic upper layer was measured at 532 nm (Badmus *et al*, 2013). Control used was ascorbic acid (0.1mg/ml).

3.12. Antimicrobial assays

3.12.1. Antibacterial screening assay

Petri plates were prepared by pouring 20 ml of Mueller Hinton agar and allowed to solidify for the use in susceptibility test against bacteria. Plates were dried and 0.1 ml of standardized inoculum suspension was poured and uniformly spread. The plates were allowed to dry for five minutes. After drying, wells were made on the plate with sterile cork borer and gently pressed. The wells were then filled with the plant extracts. Standard antibiotics were used as reference. The plates were incubated at 37°C for 24 hours (Bhalodia *et al*., 2011). The zone of inhibition was observed and measured in millimetres. Each assay in these experiments was repeated three times for concordance and the mean values were recorded.

3.12.2. Antifungal screening assay

Petri plates were prepared by pouring 20 ml of Sabouraud’s dextrose agar and allowed to solidify for the use in susceptibility test against fungus. Plates were dried and 0.1 ml of standardized inoculum suspension was poured and uniformly spread. The plates were allowed to dry for five minutes. After drying, wells were made on the plate with sterile cork borer and gently pressed.

The wells were then filled with the extracts. Standard antifungal solutions were used as reference. The plates were incubated at 28°C for 48 hours (Bhalodia *et al*., 2011). The zone of inhibition was observed and measured in millimetres. Each assay in these experiments was repeated three times for concordance and the mean values were recorded.

3.12.3. Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) assay was carried oit by serial dilution method, using 96 well plate, and plate reader (Erba Lisa Scan II Tranasia Mannheim, Germany).

100µL of Mueller Hinton broth (Hi Media, India) was dispensed in all the wells of the plate. 100µL of stock concentration of extract was added to the first well of each column. Serial dilution was done till the eighth well. Finally, 10µL of 0.5 McFarland opacity culture was added to each well of the plate. The microbes used were *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213 and *E. coli* ESBL (extended-spectrum beta-lactamases) strain. The plate was then gently shaken to mix the contents properly and immediately a baseline absorbance reading at 620nm was taken. Then, the plates were kept for incubation for 16-18 hours at 37˚C and another absorbance reading at 620nm was recorded (Perumal S., 2012).

3.13. Thin Layer Chromatography of crude extracts

The crude plant extract was freshly prepared and filtered for TLC profiling. The solvent system used was Toluene: Ethyl acetate in 9:1 ratio (standardized by trials). Silica gel 60 F254 plate (Merck) of uniform thickness of 0.2 mm was used a stationary phase (Mundekad and Padmaja, 2014). 10µl of the extract was applied on the TLC plate and developed in the solvent system in a closed glass chamber to a height of about 8cm. The plate was sprayed with Vanillin spray reagent (0.5gm Vanillin in 100ml ethanol and 1.5 ml of conc. Sulphuric acid) and the R_f values of each band was recorded according to the formula:

Retention factor (R_f) = Distance travelled by the plant extract/Distance travelled by the solvent

3.14. HR-LCMS of the crude ethanolic plant extracts

The crude leaf extracts were subjected to High Resolution Liquid Chromatography and Mass Spectroscopy (HR- LCMS) with DAD (Diode Array Detection) for characterisation and identification of the bioactive compound present in this fraction. HR-LCMS of the fraction was carried out in Sophisticated Analytical Instrument Facility (SAIF), Powai, IIT Bombay. [Instrument specification-Make: Agilent Technologies, USA; Model: 1290 Infinity UHPLC system, 6550 iFunnel Q-TOFs; Mass range : 50-3200 amu; Column details: Syncronis C18 100x 2.1, particle size 1.7µ; acquisition time-30mins; flow rate 0.3 ml/min].

3.15. Column Chromatography of crude plant extract (of *E. agallocha*)

Based on all the previous experimental findings, the crude ethanolic extract of *E. agallocha* was inferred to be the most potent in terms of antimicrobial and antioxidant activity. Hence, the extract was further purified for identification. The chromatographic procedure was performed according to methods described by Patra *et al* (2012). The ethanol extract of the leaf sample of *E. agallocha* was adsorbed onto silica gel (purchased from Sigma Aldrich) by triturating in a mortar and left for about 10 hours to dry. The column (2 cm x 25 cm) was packed with a solution of silica gel with n-butanol using the wet slurry method. This involves preparing a solution of silica gel, with n-butanol in this case, in a beaker and subsequently adding this into the column till it is about three-fourths filled. The solution was stirred for dispersal and quickly added to the column before the gel settles. This method was used to prevent the trapping of air bubbles. A ball of wool was pushed into the column to settle atop the packed silica gel. A substantial amount of n-butanol: acetic acid: water (4:1:1) was poured continuously into the column and allowed to drain but prevented from reaching the cotton wool. The quantity collected was poured back into the column. Periodically, a piece of rubber tubing was used to agitate the column to allow for the escape of trapped air bubbles. 12 fractions are eluted and collected in dry glass bottles. The column fractions were again tested with TLC chromatogram and the R_f values were determined.

The fractions with similar R_f value were combined together and kept for bioautography screening (Arockiamary and Vijayalakshmi, 2014).

3.16. TLC of the purified plant fractions eluted from Column Chromatography

The 12 fractions eluted from column chromatography were subjected to TLC. Silica gel 60 F254 plate (Merck) of uniform thickness of 0.2 mm was used a stationary phase (Mundekad and Padmaja, 2014). 10µl of the fraction was applied on the TLC plate and developed in the solvent system in a closed glass chamber to a height of about 8cm. The plate was sprayed with Vanillin spray reagent (0.5gm Vanillin in 100ml ethanol and 1.5 ml of conc. Sulphuric acid) and the R_f values of each band was recorded according to the formula:

Retention factor (R_f) = Distance travelled by the plant extract/Distance travelled by the solvent

3.17. Antimicrobial activity of the purified plant fractions.

The three bioactive fractions eluted from the column chromatography procedure were subjected to antimicrobial assay and subsequent determination of MIC, as described earlier. Clinical isolates of *Salmonella typhi* and *Candida parapsilosis* were used as test bacteria and fungi respectively for the assay. The fraction with the maximum inhibitory action was selected for further study.

3.18. Antibacterial and antifungal drug sensitivity test with the purified plant fraction against MDR bacteria

Antibiotic sensitivity test was performed on eight different cultures isolated, following the Kirby-Bauer Method (Bauer *et al*, 1966). Antibiotic hexa-ring (Hexa Gminus 5, HIMEDIA) was taken having 6 different types of discs of antibiotics- Cefotaxime, Levofloxacin, Aztreonam, Imipenem, Ampicillin and Ceftazidime. Similarly, for antifungal drug sensitivity, Amphotericin B, Caspofungin, Fluconazole, Flucytosine, Micafungin and Voriconazole were used (HiMedia). The discs were carefully kept on the spread culture plates of F2 treated and untreated *S. typhi* and *C.parapsilosis* respectively, and plates were incubated at 37°C for 24 hours. Next day the plates were observed for antibiotic sensitivity of the bacteria by checking

for the zone of inhibition (if any). The results were interpreted according to the guidelines of CLSI (CLSI performance standards for antimicrobial susceptibility, 2013).

3.19. Study of mechanism of antimicrobial action

3.19.1. Study of membrane pore formation and damage by propidium iodide staining

The effect of plant extract on the membrane integrity of the microbial cells was observed using propidium iodide staining, that selectively crosses damaged cell membrane (Crowley *et al*, 2016). The microbial cells were incubated with the MIC dosage of the selected plant extract fraction for 3 hours. Then, the cells were treated with 10^{-3}M of propidium iodide (PI) (tagged with RFP) and incubated in dark for 30 minutes. After incubation, the cells were washed with 0.15M NaCl or PBS thrice, to remove unbound PI and cells were then observed under fluorescence microscope with an excitation and emission wavelength of 490 nm and 520 nm respectively.

3.19.2. Study of ROS overproduction by DCFH-DA staining

DCFH-DA (2', 7' dichlorofluorescein diacetate) was used to assess the intracellular ROS production (Kim and Xue, 2020). The microbial cells were treated with the MIC dosage of the selected plant extract fraction with suitable positive (antimicrobial drug) and negative (only microbial cell) controls in each case. After incubating for 3 hours, cells were harvested and washed thrice. Then, the cells were treated with 10µM of DCFH-DA (tagged with GFP) for 30 minutes in dark. The cells were then washed thrice with sterile PBS and fluorescence intensity of cells were observed using a fluorescence microscope (Olympus System BX 60, Japan) with an excitation and emission wavelength of 488nm and 525 nm respectively.

3.19.3. Study of mechanism of action of the purified bioactive extract fraction by observing effect on genomic DNA of MDR bacteria and fungi.

a) Bacterial genomic DNA isolation: Genomic DNA isolation of the bacteria was carried out according to the methods described by Sambrook *et al*. (1989). Briefly, a suspension of overnight MDR strain of *S. typhi* (around 1.5 ml) (with various treatment combinations of the purified fraction at 15µg/ml concentration and without treatment, as indicated in Table 1, Figure 4) was kept overnight. The final mixture was taken and the cells were harvested by centrifugation at 10,000 rpm for 10 mins. After centrifugation, the supernatant was discarded and the cell pellet was suspended in 500µl of Tris EDTA buffer to get a homogeneous cell suspension. 50 µl of 10% SDS and 5µl of Proteinase K was added to the mixture and then incubated at 37°C for 1 hour. Then 500 µl of tris saturated phenol was added to the mixture and then it was centrifuged at 10,000 rpm for 10 mins. The aqueous phase was collected in a fresh Eppendorf tube and a 1:1 mixture of tris saturated phenol and chloroform was added in equal volume. Centrifugation was performed at 10,000 rpm for 10 mins. Aqueous phase was separated and collected in a fresh centrifuge tube and equal volume of chloroform was added to remove trace amount of phenol present (if any). The contents were again centrifuged at 10,000 rpm for 10 mins and the aqueous phase was separated and collected in a fresh tube.0.3M sodium acetate and 2.5 volume chilled absolute ethanol was added to facilitate the precipitation of nucleic acids. After 1 hour incubation at -20°C, centrifugation was done at 12,000 rpm for 20 mins. The supernatant was discarded and the pellet was treated with 500µl of 70% alcohol. This final pellet was air dried and dissolved in 30µl of tris EDTA buffer (pH 8). The isolated genomic DNA of the treated and untreated cells were run on agarose gel and viewed in the UV gel documentation machine (Vilber Lourmat, Mega Bio-Print, 1100/20M).

b) Fungal genomic DNA isolation: Thirty mg of freeze-dried mycelium was ground to a fine powder in an Eppendorf tube in liquid nitrogen using a pre-cooled pestle. The ground mycelium was resuspended and lysed in 500ml of lysis buffer (40mmol/l Tris-acetate, 20mmol/l sodium

acetate, 1mmol/l EDTA, 1% w/v SDS pH7·8) until the viscosity of the suspension was significantly reduced and the formation of froth indicated the detachment of DNA from polysaccharides. Five cycles of gentle pipetting were performed. RNase A (2ml of 10mg/ml; Sigma) was added and the mixture was incubated for 5min at 37°C. To facilitate the precipitation of most polysaccharides, protein and cell debris, 165ml of 5mol/l NaCl solution was added and the components mixed by inverting the tube several times. The suspension was centrifuged at 13000 rev/min for 20min at 4°C, the supernatant was immediately transferred to a fresh tube and 400ml of chloroform and 400ml of phenol were added. The solution was mixed by gently inverting the tube until the solution became milky (usually-50 times). After centrifugation for 20min, the aqueous phase was removed and extracted with an equal volume of chloroform. The DNA in the aqueous supernatant was precipitated with two volumes of 95% ethanol. To free the DNA from polysaccharide the precipitate was resuspended in 500ml of lysis buffer and mixed by gentle pipetting. Then 165ml of 5mol/l NaCl was added and the tube gently inverted several times. The suspension was then chloroform-extracted as described above. Usually, after centrifugation for 10min, the aqueous phase was clear and the DNA was precipitated with 95% ethanol. On rare occasions the aqueous phase was still cloudy, in which case it was re-extracted with one volume of chloroform before the DNA was precipitated. The precipitated DNA was washed three times with 70% ice-cold ethanol, dried and dissolved in 50 ml TE buffer (10mmol/l Tris-HCl, 0·1mmol/l EDTA pH7·8) and stored at −20°C.

DNA was determined by a fluorometric assay, using Hoechst dye 33258 (Brunk *et al.* 1979) and a TKO 100 Mini Fluorometer (Thermo Fisher Scientific).

3.19.4. Effect on protein profile of bacteria and fungi

a) Observing effect on protein of *S. typhi* by SDS-PAGE protein profiling.

The whole cell lysates of *Salmonella* for SDS PAGE analysis were prepared essentially (as described by Aksakal, 2010). Briefly, one colony of the resistant strain of *S. typhi* was transferred from MacConkey agar plate to100 ml of Tryptic Soy Broth and incubated overnight at 37°C. Subsequently, the broth culture was centrifuged at 15000 rpm for 15 min at 4°C. The sediment was resuspended in 10 ml of Phosphate Buffer Solution (PBS, pH 7.2). One ml of the suspension was transferred into 1.5 ml microcentrifuge tubes and centrifuged at 15000 rpm for 15 min at 4°C. The sediment was then suspended in 10 µl of 10% SDS and an equal volume of loading buffer [0.125M Tris (hydroxymethyl) aminomethane, 4% SDS, 10% 2-mercaptoethanol, 0.2% bromophenol blue] was added. After vigorous shaking by vortex, the prepared samples were boiled for 10 min at 100°C, centrifuged for 1 min (15000 rpm at 20°C) and the supernatants were stored at -20°C for further use. SDS-PAGE was carried out and the gel was analysed for any protein followed by analysis of the results obtained.

Protein samples were heated with 1X SDS loading buffer (50mM Tris- Cl; pH-6.8), 2.5% β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol at 95C for 10 minutes to bring out the extract. The samples were then centrifuged at 10000 rpm for 2 minutes at 4C and then resolved by SDS-PAGE. Samples were run in SDS-gel running buffer using an electrophoresis cell. SDS-PAGE was run at 40mA and 280 volts to resolve the components (BIORAD gel apparatus was used) (Aksakal *et al*, 2010). The first well was loaded with the molecular weight marker (Thermo Fisher).

b) Observing effect on protein of *C.parapsilosis* by SDS-PAGE protein profiling.

C.parapsilosis culture was grown on Sabouraud dextrose media, incubated in a shaking machine at 37°C. The cells were harvested at logarithmic growth phase and washed three times in 0.85% sterile saline solution. Yeast cells (200 mg + 500 ml of distilled water) maintained in liquid nitrogen (-196°C), were powdered for three minutes, centrifuged at 3000g for 5 min and the supernatants

were stored at -80°C. The preparation of proteins for electrophoresis was made according to method described by Bruneau and Guinet (1989).

SDS-PAGE was performed according to the method described by Laemmli (1970). Samples containing 30 µg of the fungal protein were separated by SDS-PAGE in 8% acrylamide gel at pH 8.5 and stained with Coomassie blue R250.

3.19.5. Secreted aspartyl proteinases assay

To induce the secretion of secreted aspartyl proteinases (Sap), 200 µl of *C.parapsilosis* suspension ($1x10^8$ cells/ml) was added to 20ml of BSA-Remold medim [2% glucose, 0.1% KH2PO4, 0.5% MgSO4, 0.7% yeast nitrogen base (without (NH4)2SO4 and amino acids), and 1% BSA]. Yeast cells were cultivated for 7 days at 27°C, according to the method described by Dostal *et al*. (2003). Thereafter, the yeast cells were removed and supernatant was filtered. For the assay, test tubes contained 750 µl of 0.2 mol/L sodium citrate-HCl buffer, 750 µl of fresh substrate solution (1% BSA in the same buffer, 250 µl of supernatants and 250 µl of the plant extract fraction in different concentrations. A similar set of reaction mixtures was prepared with Amphotericin B instead of plant fractions. The mixtures were incubated at 37°C for 60 min (T60) and the reaction was stopped with 500 µl of trichloroacetic acid. For each reaction mixture, an additional control was prepared by adding all ingredients plus 20% trichloroacetic acid simultaneously (T0). All samples were centrifuged at 3000g for 30 min at 4°C. A total of 160 µl of each clear supernatant was added to 40 µl of dye reagent concentrate (Coomasie Brilliant Blue G-250) and the OD at 595 nm was measured. Activity was calculated as the difference in the OD:T60-T0.

3.19.6. Extracellular lipase activity

Accodring to the method described by Dostal et al. (2003), *C.parapsilosis* cells were washed twice with PBS and adjusted to $5x10^6$ cells/ml in 0.6% (m/v) yeast nitrogen base containing 2.5% (v/v) Tween 20 in the presence or absence of varying concentration of the test compounds/drug. The flasks were incubated in shaker at 37°C for 8 h. The lipolytic activity in filtrated culture supernatants

was determined by a plate assay on olive oil agar that contained 0.7% yeast nitrogen base, 2% olive oil (v/v), 0.001% rhodamine B (m/v) and 2% agar.

3.19.7. Calcofluor white staining

The *C.parapsilosis* cells were stained with Calcofluor white stain according to protocol provided in the kit (Sigma-Aldrich, F3543). Briefly, the specimen to be examined is put on a clean glass slide. One drop of Calcofluor White stain and one drop of 10% KOH is added. A coverslip is placed over the specimen and allowed to stand for 1 min. The slide is examined under UV light (355 nm) at 100x to 400x magnification of microscope (Olympus).

3.20. Toxicity against healthy mammalian cell line and cancer cell line.

The evaluation of cytotoxicity of the purified plant fraction (F2) of ethanolic extract of *E. agallocha* was done on cervical cancer cell line (SiHa HPV 16+) and healthy peripheral mononuclear cells (PBMC) by using MTT assay (Heckenkamp *et al*., 1999; Lupitha *et al*, 2020), at RGCB, Kerala. Briefly, the cells were cultured in 96-well plates at density of 2.5x 10^4 cells per well in the presence of the fraction F2. After incubation for 48 h, MTT dissolved in PBS was added to each well at a final concentration of 5mg/ml and then incubated at 37°C and 5% CO_2 for 2h. The water- insoluble dark blue formazan crystals that formed during MTT cleavage in actively metabolizing cells were dissolved in DMSO. The optical density was read by a microplate reader at a wavelength of 570 nm. Results were expressed as the mean of three replicates as a percentage of control (taken as 100%). The extent of cytotoxicity was defined as the relative reduction of optical density (OD), which correlated to the number of viable cells in relation to cell control (100%). The cell viability was plotted in a graph and the IC_{50} was calculated accordingly to determine the optimum dosage of the purified extract for further studies.

3.21. Effect on human cervical cell line by Cellular Imaging Analysis – effect on autophagy, mitophagy, apoptosis and proteasome inhibition.

The fraction containing the anti-cancer activity indicated from MTT assay was considered for these assays. For live cell imaging analysis, the fraction was preserved in lyophilized form before use.

During experiment, it was dissolved in distilled water to obtain the aqueous extract with a stock concentration of 100µg/ml. At RGCB, the methodologies have been standardized for all the assays. For the live cell imaging analysis, the SiHa cells were incubated with the purified plant extract in different concentrations 7.5, 15 and 30µg/ml for 24 hr in 5 % CO_2 at 37^{o} C. After 24 hr incubations, cells were imaged viewed under Inverted fluorescent microscope EclipseTi2 (Nikon, Japan) equipped with Spectra X light source from Lumencore (Lupitha *et al*, 2020).

Autophagy study: SiHa LC3- Green fluorescent protein (GFP) cells were used. The GFP was excited using a 470/24 nm filter and Red fluorescent protein (RFP) at 575/35 nm filter. The emission of GFP 536/40 and RFP 593/40 was collected using specific filters placed on the external wheel. The images were captured with an Electron multiplying charge coupled device (EMCCD) camera (AndoriXON 897) using NIS element software (Nikon). Cisplatin (at its IC_{50} dosage) was used as reference standard.

Mitophagy study: SiHa-MitoKeima (SiHa labeled with mtKeima) cells were imaged using excitation at 440 nm and emission range of 590 to 630 nm. Ratio of 561 nm/458 nm was calculated using NIS element software (Nikon).

Apoptosis study: SiHa Smac- mCherry cells were used. The fluorescent protein Smac was used to visualize the mitochondrial permeabilization event during apoptosis. Increase in red fluorescence was determined using fluorescence microscopy. The images were captured with an EMCCD camera (AndoriXON 897) using NIS element software (Nikon).

Study of proteasome inhibition: SiHa cells expressing proteasome sensor (GFP-dgn) stable cells were used. Proteasomal inhibition was monitored in cells treated with plant extracts along with untreated cells. The GFP was excited using a 470/24 nm filter and emission of GFP 510/20 was collected using specific filters placed on the external wheel. The images were captured with an EMCCD camera (AndoriXON 897) using NIS element software (Nikon).

3.22. Caspase- 3 activity by colorimetric assay

Caspase-3 Colorimetric assay kit (BioVision K106-25) was used and the protocol described in the kit. Briefly, Caspase-3/CPP32 Colorimetric Assay Kit provides a simple and convenient means for assaying the activity of caspases that recognize the specific sequence Asp-Glu-Val-Asp (DEVD). The assay is based on spectrophotometric detection of the chromophore *p*-nitroaniline (*p*NA) after cleavage from the labeled substrate DEVD-*p*NA. The *p*NA light emission can be quantified using a spectrophotometer or a microtiter plate reader at 400- or 405-nm. Comparison of the absorbance of *p*NA from an apoptotic sample, induced with the plant extract for 24hrs, with an uninduced control allows determination of the fold increase in caspase activity (ID K106-25, BioVision Inc.; Mountain View, USA). Cisplatin (at IC50 dosage value) was used as reference standard.

3.23. Cell cycle analysis by flow cytometry and determination of protein expression analysis by Western Blotting

For cell cycle analysis, SiHa cells at $1x10^6$ cells density was cultured in 96-well plates. After 16 hours, the cells were treated with different concentrations of the plant extract for 48 hours. After treatment, the cells were washed with PBS (phosphate buffer saline) and harvested by adding 300µL of trypsin-ethylenediamine tetraacetic acid (EDTA). The harvested cells were washed in ice- cold PBS and centrifuged at 5000rpm for 5 minutes. Further, the cells were resuspended and fixed with ice cold 70% ethanol for 2 hours. After fixing, the cells were washed with ice cold PBS and centrifuged at 5000 rpm for 5 minutes. The pellets were resuspended in 250 µL PBS containing Propidium Iodide (PI) (10µg/ml), RNase A (20µg/ml) and incubated for further 30 minutes in the dark (Pozarowski and Darzynkiewicz, 2004). Finally, the cells were analyzed by flow cytometry (BD FACSArya III, BD Biosciences, USA) and dot plot diagrams were generated using BD software (BD Biosciences, USA).

Western Blot analysis was done using a kit-based assay (Fast Western Blot Kit, Thermo Fisher Scientific). SiHa cells (1.0 x 10^6cells/60) were detached, washed in ice-cold cold PBS, and suspended in 100 1L of lysis buffer. The suspension was kept on ice for 20 min and then centrifuged at 5000 rpm for 20 minutes at 4 °C. The protein extracts were resuspended in sample buffer and this mixture was boiled for about 5 minutes. 50 µg of the proteins were loaded into each lane, resolved on a 10% Sodium dodecyl- sulfate (SDS)polyacrylamide gel (PAGE) and then transferred to nitrocellulose membrane (Saha *et al*, 2015) for western blot using the required antibodies against cyclinB1, cyclin D1, Cdc2, p21 and p53.Proteins of interest were visualized by using Trans-Blot Turbo Transfer system (Bio-Rad).

3.24. High Resolution Liquid Chromatography-Mass Spectrometry (HR LC- MS) analysis of isolates

The bioactive fraction exhibiting potent anticancer activity against SiHa cells was subjected to High Resolution Liquid Chromatography and Mass Spectroscopy (HR- LCMS) with Diode Array Detection (DAD) for characterization and identification of the bioactive compound present in this fraction. DAD also enable us to know the wavelength at which the compound with highest abundance is present. HR-LCMS of the fraction was carried out in Sophisticated Analytical Instrument Facility (SAIF), Powai, IIT Bombay. [Instrument specification: Agilent Technologies, USA; Model: 1290 Infinity UHPLC system, 6550 iFunnel Q-TOFs; Mass range: 50-3200 amu; Column details: Syncronis C18 100x 2.1, particle size 1.7µ; acquisition time-30mins; flow rate 0.3 mL/min].

3.25. Molecular docking using Bioinformatics

Molecular structures of p53 (1A1U), E6 (6SJV) and E6AP (1D5F) were retrieved from Protein Data Bank (Martinez-Zapien *et al*, 2016). Structure of Bergenin was obtained by Avogadro Molecule Viewer using the using SMILES ID) [Simplified molecular- input line- entry system]. The molecular docking of bergenin with the p53-E6-E6AP complex was analysed using CB Dock Server (https://clabshare.cn/cb-dock/php), an automated server that uses a cavity search logarithm to detect cavities and then AutoDock Vina was used to dock the molecule into those cavities.

Statistical analysis

All experimental results are expressed as mean± standard deviation from at least three independent experiments performed in triplicates. Statistical analysis was done using ANOVA test of Microsoft Excel (Version 2016) data analysis tool. When $p < 0.05$, the result was considered statistically significant.

Chapter 4:

Results

4. Results

The results of the experiments, according to the objectives of this investigation, are as follows:

4.1. OBJECTIVE- 1: Collection of mangrove leaf samples, physicochemical analysis of the dry leaf powder extracts along with study of phytochemical, antioxidant and antimicrobial properties of the crude leaf extracts.

4.1.1. Collection and preservation of mangrove leaf samples

- Fresh leaf samples of *Bruguiera gymnorhiza, Excoecaria agallocha, Avicennia alba* and *Aegialitis rotundifolia* were collected from Bali Island of the Indian Sundarbans (near 30°24′-30°28′ N latitude and between 77°40′- 77°44′ latitude in the South 24 Parganas, West Bengal) during the month of June, 2018 (Fig.4.2-4.5). The identification of the plants was verified by Dr. Pradeep Vyas, Former Chief, Wildlife Warden and IFS officer (Retd.) and also by researcher Dr. Sourav Sett, Department of Marine Science, University of Calcutta.
- The plant leaf samples were preserved and deposited at Department of Microbiology, St. Xavier's College (Autonomous), Kolkata with voucher numbers MCB2018/2G-45/001, MCB2018/2G-45/005, MCB2018/2G-45/006 and MCB2018/2G-45/008 respectively, for future reference (represented in Fig.4.1).

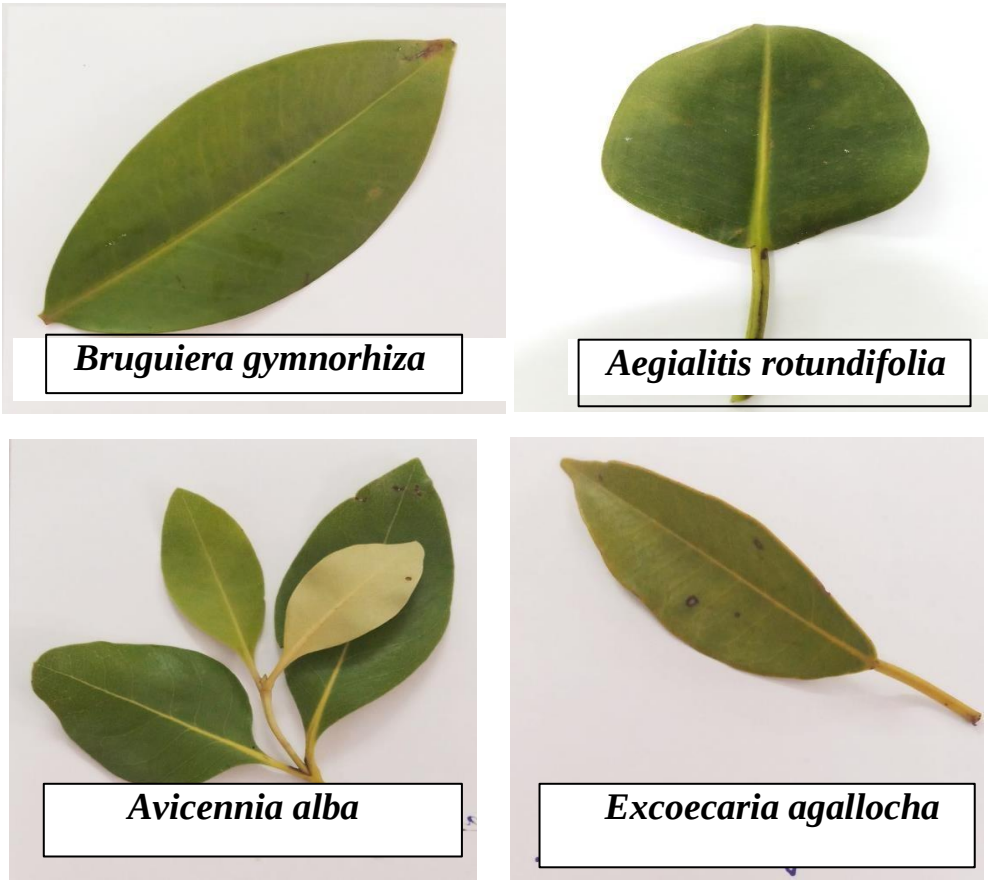


Fig. 4.1: The mangrove plant leaves collected from the Sundarbans for the present investigation.

Identifying features and morphological characteristics of the collected plants are as follows:

1. Aegialitis rotundifolia

Fig.4.2: *Aegialitis rotundifolia* plant in the Bali islands of Sundarbans.

Family: Plumbaginaceae.

Common/local name: Tora

Identifying features: A soft wooded shrub that grows up to a height of 4 m, with a club-shaped stem.

Roots and stem: Tap roots sunken, but do not penetrate much deep. Aerial roots are absent. Swollen trunk base with spongy tissue structure. Bark is fissured and dark grey or blackish grey in colour. Straight stems with inconspicuous leaf scars on shoot.

Leaves: Simple, alternate, broad ovate, coriaceous, slightly fleshy, deep green, dorsiventral, dorsal side shining, entire, obtuse, cuneate at base.

Flowers: Inflorescence a raceme, branched. Flowers curved, up to 1 cm long and 0.8 cm broad, green. Sepals 5, green, persistent. Petals 5, white. Stamens 5; filaments whitish. Styles 5, terminal; stigma absent.

Fruit: Quadrangular, upto 5 cm long.

Flowering and Fruiting season: August - January.

Habitat: True riverine mangrove zone species.

2. *Avicennia alba*

Fig.4.3: *Avicennia alba* plant in the Bali islands of Sundarbans.

Family: Plumbaginaceae.

Common name: Kala Baen, Dudhi Baen.

Identifying features: Tall trees, upto 25 m tall.

Roots: Pencil-like pneumatophores emerge above ground from long shallow underground roots.

Bark: Dark greyish, lenticellate, smooth, not fissured.

Leaves: Simple, lanceolate, 12 - 15 x 2.5 - 3.5 cm, usually pointed apically, green above, silvery grey or white beneath.

Flowers: Yellow, 1-1.5 cm diam., several together, forming a cross-shaped inflorescence.

Fruits: Capsule conical, ca 4 cm long. Seeds flattened, ca 3.2 cm long.

Flowering and fruiting season: November - April.

Habitat: True riverine mangrove zone species.

3. *Bruguiera gymnorhiza*

Fig.4.4: *Bruguiera gymnorhiza* plant in the Bali islands of Sundarbans.

Family: Rhizophoraceae.

Common name: Burma / Oriental / Large - leaved Orange Mangrove, Kankra.

Identifying features: Trees up to 30 m tall, with much spreading branches. Stem and branches marked with leaf-scars and stipules.

Roots: Short or shallow buttress like aerial roots, thickened at trunk base. Knee roots (45 cm high) with buttresses present.

Bark: Brownish black. Leaves: Simple, at the end of branches, opposite-decussate, 8-22 x 5.7.5 cm, exstipulate, petiolate, ellipticoblong, acute at apex, or bluntly pointed, coriaceous, dark green.

Flowers: Solitary, axillary. Calyx ribbed, campanulate, large (3 - 4 cm); sepals that are red in the sun and yellowish in the shade. Petals 1.2 - 1.6 cm long, tips acute.

Fruits: The seed germinates in the fruit forming a cigar-shaped seedling 10-20 cm long.

Flowering season: Throughout the year.

Habitat: In riverine mangrove zone.

4. *Excoecaria agallocha*

Fig.4.5: *Excoecaria agallocha* plant in the Bali islands of Sundarbans.

Family: Euphorbiaceae.

Common name: Blind Your Eye, Milky Mangrove, Genwa.

Identifying features: Evergreen trees upto 12 m high, with acrid milky juice.

Bark: Grey, rough covered with brown corky pores.

Leaves: Simple, alternate, 6-10 cm long, coriaceous, glabrous, base cuneate or rounded, pointed at tips; secondary nerves 5-8, rather obscure; petiole 1.2-3 cm, 2 glandular just below the blade. The young leaves are pinkish. Old leaves turn bright red when they are about to drop off.

Flowers: Male and female flowers occur on separate trees. Male flowers yellow, in catkins. Female flowers green, few in raceme. Petals persistent. Style simple; stigmas 3.

Fruits: Small, about 1 cm diam., tricoccus, in clusters. Seeds float on water.

Flowering and fruiting season: Almost throughout the year.

Habitat: In riverine mangrove zone.

4.1.2. Extract preparation with different solvents

The plant leaves collected were dried and grinded to fine powder form. The powdered form was then dissolved in different solvents, namely, ethanol, methanol, DMSO and water to prepare the crude extracts. A brief outline of the method of extraction is represented in Fig.4.6.

The leaf samples were oven dried at 60°C till crisp and ground to a fine powder using mortar and pestle.

Coarsely ground powder of the plant leaves using mortar and pestle

1. **ETHANOLIC EXTRACT**
2. **METHANOLIC EXTRACT**
3. **DMSO (DIMETHYLSULPHOXIDE) EXTRACT**
4. **AQUEOUS EXTRACT**

The extracts were concentrated by rotary vacuum evaporator (RotaVap).

Fig.4.6.: Crude leaf extract preparation with different solvents.

Inference: The concentrated extracts were prepared with the four different solvents with a final concentration of 1mg/ml.

4.1.3. Pharmacognostic study and microscopic analysis of powdered leaf extract

The pharmacognostic study of the powdered leaf extracts was carried out and the observations are represented in Table 4.1 and Fig.4.7 (a-d).

Table 4.1: Microscopic observations of the powdered leaf extracts

PLANT SAMPLE	**OBSERVATIONS**
Bruguiera gymnorhiza	Rosette and prismatic crystals of calcium oxalate Rectangular stomatal fragments (length of 500-1250 µm)
Excoecaria agallocha	Epidermal cell fragments (2000 µm length) Spherical starch granules, very few (diameter around 10 µm) Spherical and rectangular crystals (very few) Astro-sclereid, sparsely present. Globular cells, large, abundantly present (diameter 30 µm)
Avicennia alba	Astro-sclereids- few in number, sparsely present. Branched sclereids- uniformly present Oval and rectangular crystals (10-30 µm length)
Aegialitis rotundifolia	Branched sclereids Spherical oil globules (around 15-30 µm diameter) Xylem vessel fibres, occurring singly as well as in clusters.

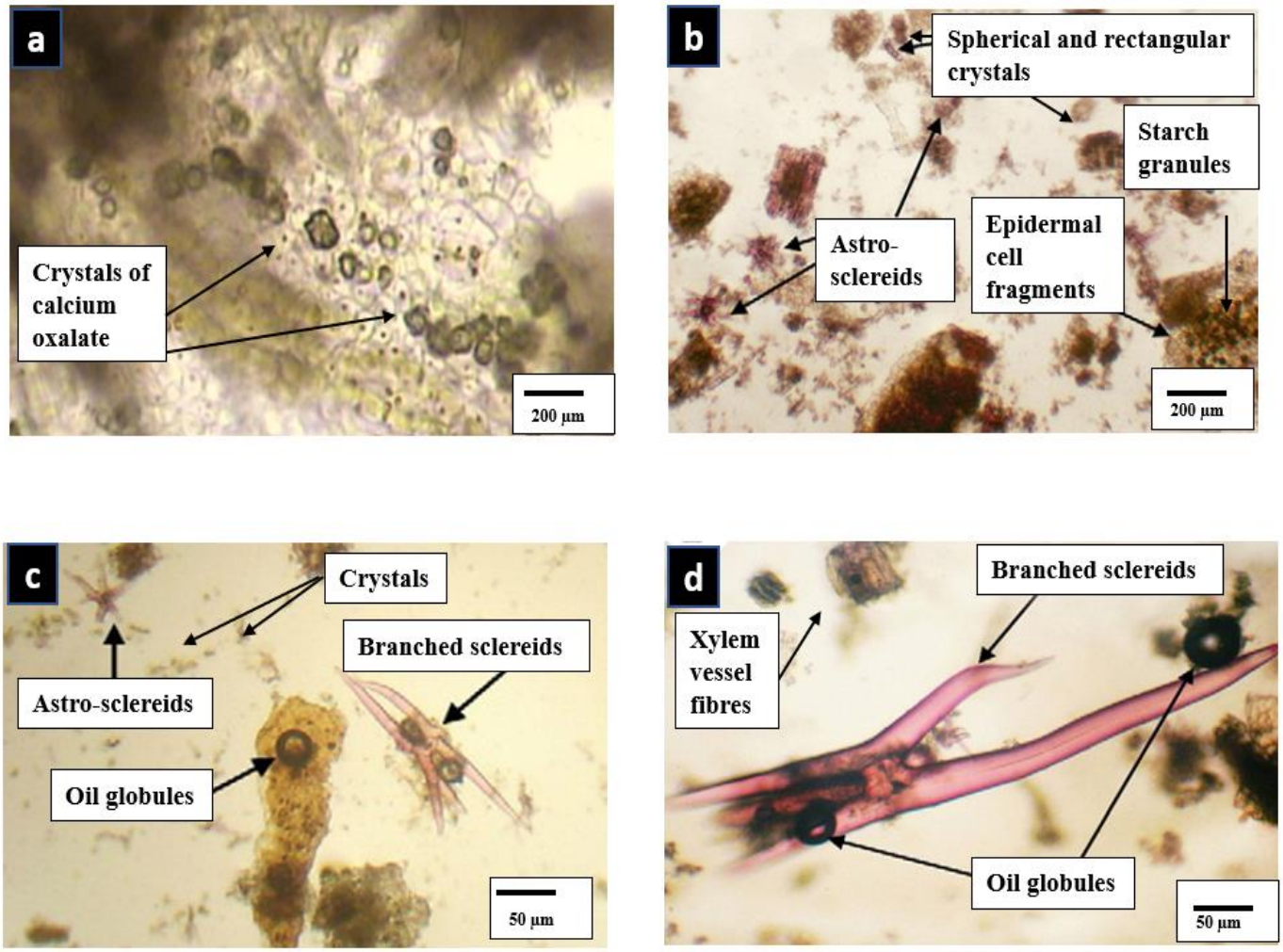


Fig.4.7: Microscopic analysis (400x magnification) of the powdered leaf extracts of (a) *Bruguiera gymnorhiza* (b) *Excoecaria agallocha* (c) *Avicennia alba* and (d) *Aegialitis rotundifolia.*

Inference

- Pharmacognosy study was conducted to detect various identifying features that may be useful in the differentiation of different substituents of the drug, that may be supplied in the form of dried powder.
- It provides a preliminary idea regarding the nature and disposition of various cellular components and cell inclusions, and thus helps understand where the compounds of interest may be located.
- The study revealed the presence of calcium oxalate crystals and stomatal fragments in *B. gymnorhiza*, epidermal cell fragments and globular cells in *E. agallocha*, astro-sclereids, branched sclereids, oval and rectangular crystals in *A. alba* and oil globules in *A. rotundifolia.*

4.1.4. Physicochemical analysis of leaf extracts

The physico-chemical parameters total ash content, acid insoluble and water-soluble ash, pH, water and ethanol extractive values were determined for dry leaf powder extracts. Results are tabulated in Table 4.2.

Table 4.2: Physicochemical analysis of powdered leaf extracts

PLANT SAMPLE	PARAMETERS	VALUE
Bruguiera gymnorhiza	1. Loss on drying 2. Total ash 3. Acid insoluble ash 4. Water soluble ash 5. Water soluble extractive value 6. Ethanol soluble extractive value	2.8% 7.63% 4.31% 3.07% 10.43% 9.37%
Excoecaria agallocha	1. Loss on drying 2. Total ash 3. Acid insoluble ash 4. Water soluble ash 5. Water soluble extractive value 6. Ethanol soluble extractive value	3.7% 3.43% 3.783% 5.82% 8.12% 10.15%
Avicennia alba	1. Loss on drying 2. Total ash 3. Acid insoluble ash 4. Water soluble ash 5. Water soluble extractive value 6. Ethanol soluble extractive value	4.3% 7.43% 2.561% 3.11% 7.64% 11.23%
Aegialitis rotundifolia	1. Loss on drying 2. Total ash 3. Acid insoluble ash 4. Water soluble ash 5. Water soluble extractive value 6. Ethanol soluble extractive value	9.7% 5.7% 1.233% 3.742% 23.4% 21.43%

Inference:

- The physico-chemical analysis of the powder extract showed characteristic pattern of each extract. Moisture content varied between 2.8-9.7%; The parameter of loss on drying helps to determine the moisture content of a drug. It aids to the decomposition of the drugs either due to chemical change or microbial contamination. This parameter met the prerequisite for drug development, i.e. <10%. Water content of more than 10% can cause deterioration of the drug components and make it unsuitable for long term storage. Here, the values indicate the suitability of the drug for storage on a long-term basis.
- Total ash ranged between 3.43-7.63%; water soluble ash was between 3.07-5.82%, highest being in the case of *Bruguiera gymnorhiza.* Determination of total ash content is the primary criterion to evaluate the purity of drugs. The residue remaining after incineration is the ash content of the drug. These could be inorganic salts such as carbonates, sulphates, phosphates, silicates etc. naturally occurring in the drug or adhered to it or deliberately added to it in order to adulterate the drug. Since the leaf samples were collected personally and directly from their natural habitat, there was no scope for adulteration.
- Acid insoluble ash content varied from 1.2-4.3%, highest being in the case of *Excoecaria agallocha.* Acid insoluble ash or water-soluble ash content is the residue obtained after boiling the total ash either with dilute hydrochloric acid or water, which measures the amount of sand and silica matter present in the drug.
- Water soluble extraction values and ethanol soluble extraction value were both markedly higher in *A. rotundifolia* powder extract with values of 23.4% and 21.43% respectively than others. Determination of extractive value measures the nature of the chemical constituents present in the crude drug.

4.1.5. Phytochemical analysis of plant leaf extracts

The presence of various phytochemicals was determined for the crude plant extracts that had been prepared in different solvent systems.

Table 4.3: Results of the phytochemical tests conducted with the crude leaf extracts

Phytochemical Tests	***Bruguiera gymnorhiza***				***Excoecaria agallocha***			
	A	E	M	D	A	E	M	D
Cardiac glycosides	-	+	-	-	-	+	-	-
Saponins	-	+	+	-	-	+	+	-
Flavonoids	-	-	+	-	+	+	+	-
Terpenoids	+	+	+	+	+	+	+	-
Tannins	-	+	-	+	-	-	+	-
Proteins and Xanthoproteins	+	-	+	+	+	+	+	+
Steroids	+	-	+	-	+	-	-	-

A- Aqueous; E- Ethanolic; M- Methanolic; D- DMSO

Phytochemical Tests	***Avicennia alba***				***Aegialitis rotundifolia***			
	A	E	M	D	A	E	M	D
Cardiac glycosides	+	-	-	-	-	-	-	-
Saponins	-	-	+	+	-	-	+	-
Flavonoids	-	-	-	-	+	-	-	+
Terpenoids	+	-	+	-	+	+	+	-
Tannins	-	+	-	+	-	-	+	+
Proteins and Xanthoproteins	+	-	+	-	+	+	+	-
Steroids	+	-	+	-	+	-	-	-

A- Aqueous; E- Ethanolic; M- Methanolic; D- DMSO

Inference:

- The ethanolic extract of *E. agallocha* was found to be rich in terpenoids, saponin, flavonoids, glycosides, proteins and xanthoproteins. The aqueous and methanolic extract was observed to contain terpenoids besides the other phytochemicals observed in the ethanolic extract.
- Ethanolic extract of *B. gymnorhiza* was also found to be more enriched in phytochemicals than the other solvent extracts.

4.1.6. Determination of Total phenolic, Total flavonoid, total tannin content and total antioxidant capacity (TAC) of the plant extracts

The total phenolic content, total flavonoid content, total tannin content and total antioxidant capacity were determined using the different solvents [gallic acid, quercetin, tannic acid and ascorbic acid were used as positive controls (standards) respectively while the respective solvents were used as negative controls for each extract)] and the results are given in Table 4.4.

Table 4.4: Results of Total phenolic (TP), Total flavonoid (TF), Total tannin (TT) and Total antioxidant capacity (TAC) of the crude leaf extracts.

Extract/solvent/standard	Total phenolic content (mg/g dry wt.)	Total flavonoid content (mg/g dry wt.)	Total tannin content (mg TAE/g extract)	TAC (mg AAE/g extract)
E. agallocha (EE)	342.56±0.1	245.56±0.2	115.67±1.1	89.27±1.5
B. gymnorhiza (EE)	312.65±0.5	207.33±0.1	106.45±0.7	71.12±0.1
A.rotundifolia (EE)	236.16±1.1	186.64±2.5	89.78±0.01	79.51±0.8
A.alba (EE)	214.62±0.4	143.45±1.6	88.75±0.4	45.44±0
Ethanol	8.06±0.5	11.01±0.2	6.45±1.7	8.47±0.7
E. agallocha (ME)	248.18±0.7	221.44±0.5	95.66±1.3	68.43±1.8
B. gymnorhiza (ME)	215.44±2.4	198.15±0.4	116.75±0.5	55.86±0.4
A.rotundifolia (ME)	134.08±1.3	177.42±1.6	96.45±1.8	58.10±0.5
A.alba (ME)	178.53±0.7	187.34±3.2	98.31±1.1	37.87±1.7
Methanol	6.48±1.2	2.85±0.1	10.04±0.4	2.1±0.4
E. agallocha (AqE)	230.22±0.4	198.16±1.3	103.25±1.2	86.15±2.3
B. gymnorhiza (AqE)	208.95±1.7	167.37±0.4	97.28±0.4	81.67±3.5
A.rotundifolia (AqE)	126.64±1.1	185.76±0.4	88.31±1.3	77.08±0.4
A.alba (AqE)	201.04±0.8	156.76±1.2	88.43±0.3	58.33±1.6
Water	10.42±0.5	13.67±0.3	4.51±1.4	12.36±0.8
E. agallocha (DE)	246.06±0.5	205.64±1.8	108.89±0.5	64.08±3.1
B. gymnorhiza (DE)	189.65±1.8	199.46±0.4	77.42±3.4	70.03±0.4
A.rotundifolia (DE)	196.54±2.2	167.20±2.2	90.71±0.7	69.66±0.1
A.alba (DE)	207.66±0.8	168.32±0.4	92.45±1.1	38.87±1.3
DMSO	3.34±1.7	10.66±1.1	7.18±0.5	2.04±0.8
Gallic acid	273.32±0.4	-	-	-
Quercetin	-	276.78±1.4	-	-
Tannic acid	-	-	162.98±2.2	-
Ascorbic acid	-	-	-	88.74±3.5

EE: ethanolic extract; ME: methanolic extract; AqE: aqueous extract; DE: DMSO extract.

Inference:

- Total phenolic content was observed to be highest in ethanolic extract of *E. agallocha* (342.56 mg/g of dry weight).
- Total flavonoid content was observed to be highest in ethanolic extract of *E. agallocha* (243.56 mg/g of dry weight). The ethanolic extract of *E. agallocha* exhibited the highest concentration (118.75 mg TAE/g of extract) of total tannin content in comparison to other plant extracts.
- The total tannin content was remarkably more in case of the *E. agallocha* extracts.
- As clearly inferred from the graphical representation, the ethanolic and aqueous extract of *E. agallocha* indicated the maximum antioxidant capacity (around 90 mg AAE/g of extract), providing a qualitative idea of the antioxidant capacity of the plant extract for further investigation.
- Thus, as inferred from the observations (Fig.), ethanolic extract of *E.agallocha* is a potent source of flavonoids, phenolic compounds, tannins and antioxidant compounds.

4.1.7. Determination of antioxidant activity of the plant extracts

The antioxidant activity of the plant extracts was evaluated using the DPPH, ABTS and FRAP methods (using ascorbic acid as positive control in all cases) and the results are represented in Table 4.5.

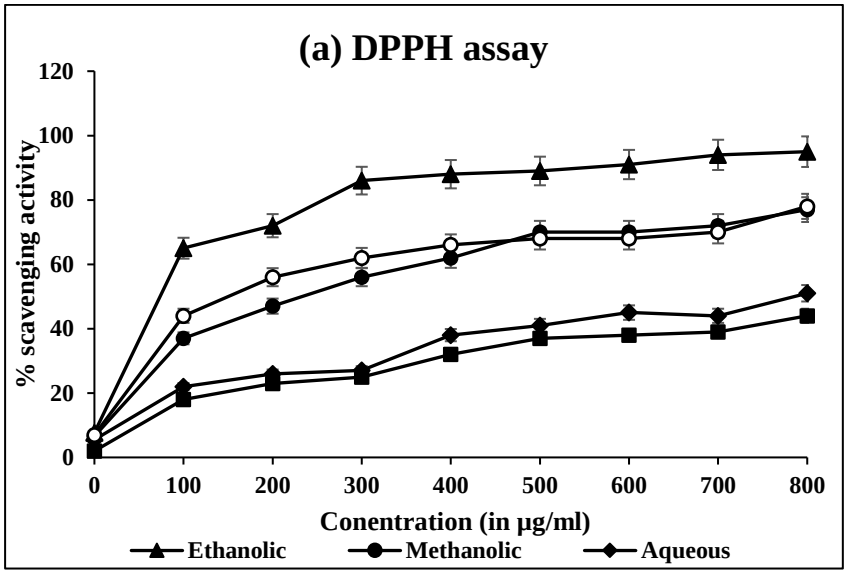
Table 4.5: Results of DPPH, ABTS and FRAP assay for determination of antioxidant activity of the crude leaf extracts.

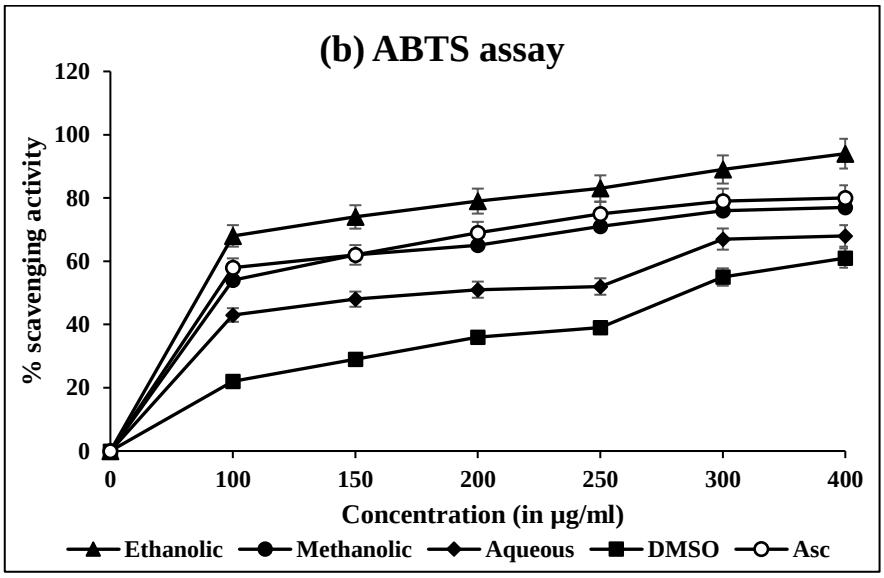
	DPPH scavenging (%)	**ABTS scavenging (%)**	**FRAP (OD, 700nm)**
E. agallocha (EE)	75.55±0.2	78.53±1.6	1.235±2.3
B. gymnorhiza (EE)	64.53±2.1	67.83±0.7	1.021±0.3
A.rotundifolia (EE)	47.45±3.1	50.22±1.1	0.982±1.5
A.alba (EE)	53.42±2.7	56.41±2.5	0.767±1.1
Ethanol	8.92±0.3	15.12±1.0	0.102±0.4
E. agallocha (ME)	46.12±0.6	66.03±1.2	1.117±0.6
B. gymnorhiza (ME)	32.27±0.3	59.76±0.5	1.018±2.4
A.rotundifolia (ME)	28.34±1.4	42.26±0.7	0.615±1.5
A.alba (ME)	28.08±1.5	23.51±3.2	0.243±0.8
Methanol	9.72±0.5	13.42±0.8	0.014±1.1
E. agallocha (AqE)	31.54±1.7	60.14±0.8	1.121±0.7
B. gymnorhiza (AqE)	45.04±2.4	60.05±1.4	1.031±3.3
A.rotundifolia (AqE)	41.32±0.7	47.34±1.5	0.095±0.6
A.alba (AqE)	38.67±2.5	28.15±2.4	0.067±1.5
Water	10.03±1.5	11.63±1.7	0.002±2.0
E. agallocha (DE)	59.04±0.7	42.35±0.5	1.076±1.1
B. gymnorhiza (DE)	54.13±1.4	37.13±0.2	1.083±1.7
A.rotundifolia (DE)	34.76±1.8	33.25±1.5	1.004±0.5
A.alba (DE)	38.52±0.5	17.16±0.7	0.013±2.0
DMSO	8.6±0.4	9.45±2.6	0.008±2.5
Ascorbic acid	88.45±0.6	88.76±1.7	1.42±3.5

EE: ethanolic extract; ME: methanolic extract; AqE: aqueous extract; DE: DMSO extract.

Inference:

- It can be inferred that ethanolic extract of *E. agallocha* has highest DPPH scavenging activity (75.55%) and thus, can be used a s a potent antioxidant agent.
- ABTS scavenging activity was observed to be the highest in ethanolic extract of *E. agallocha* (78.53%) and this activity further substantiates its antioxidant activity.
- The reducing power was found to be highest in ethanolic extract of *E. agallocha* and it might be -used as an effective antioxidant agent.





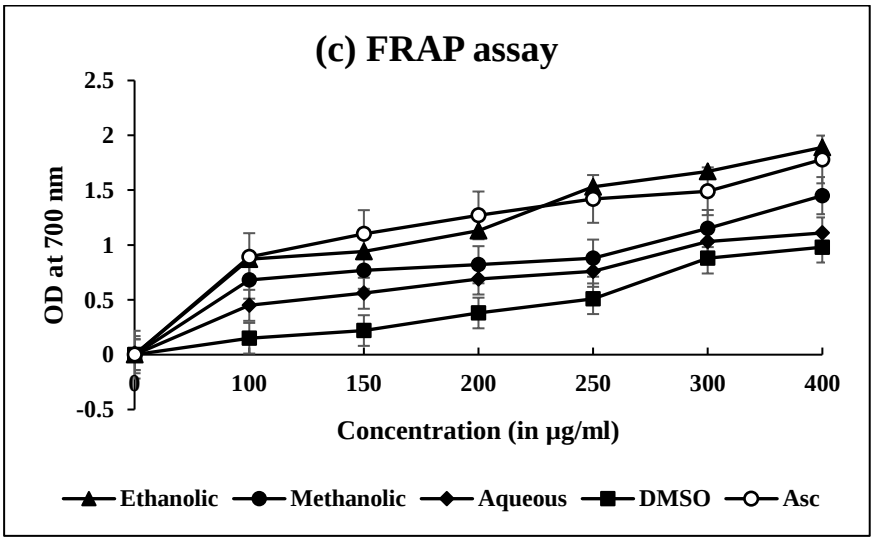


Fig.4.8: Antioxidant activity of *E. agallocha* extracts as estimated by (a) DPPH (b) ABTS and (c) FRAP assay.

4.1.8. *In vitro* radical scavenging assay of the plant extracts

The *in vitro* reactive oxygen species or radical scavenging capacity of the plant extracts was carried out and the results are depicted in Table 4.6.

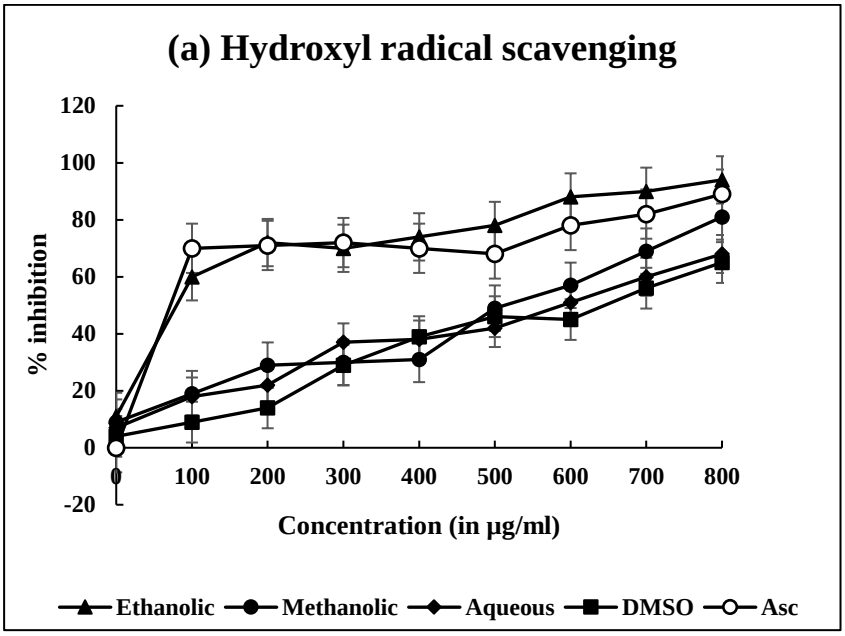
Table 4.6: *In vitro* radical scavenging assay of the crude leaf extracts

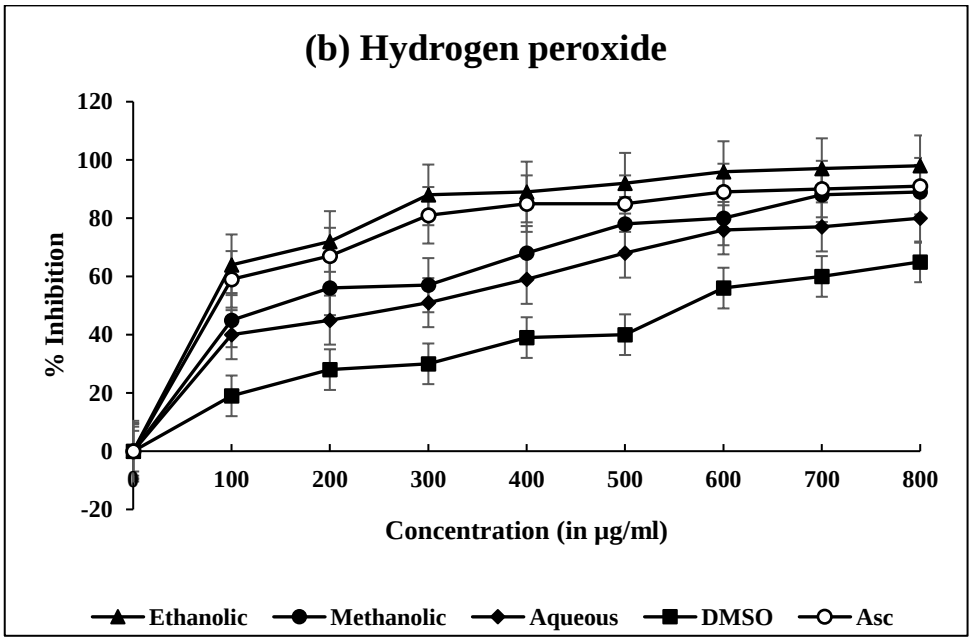
	Radical scavenging (%)			
	OH	**H_2O_2**	**Superoxide**	**NO**
E. agallocha (EE)	58.14±1.5	39.13±2.5	36.03±2.0	19.17±0.4
B. gymnorhiza (EE)	48.43±3.1	26.33±0.2	38.70±3.1	19.06±1.4
A.rotundifolia (EE)	47.41±3.0	23.41±1.6	32.03±1.6	17.67±1.4
A.alba (EE)	41.77±2.5	26.15±2.1	27.24±1.1	14.44±0.3
Ethanol	11.20±0.2	7.26±0.4	17.07±1.1	5.47±0.7
E. agallocha (ME)	49.43±0.7	22.34±0.4	31.16±1.4	18.02±1.3
B. gymnorhiza (ME)	47.51±1.5	21.05±1.5	33.36±0.2	17.92±2.8
A.rotundifolia (ME)	43.55±1.1	21.73±1.5	28.34±0.1	16.05±0.6
A.alba (ME)	40.13±1.6	27.03±1.8	31.76±0.8	11.24±0.5
Methanol	8.15±1.5	8.26±0.4	12.05±1.5	7.18±0.2
E. agallocha (AqE)	51.07±0.3	37.15±0.4	36.67±0.7	21.55±2.5
B.gymnorhiza (AqE)	48.02±2.5	24.17±1.4	34.81±1.4	20.03±0.3
A.rotundifolia (AqE)	38.15±0.8	24.55±2.8	28.76±1.8	18.63±1.4
A.alba (AqE)	36.14±1.1	24.78±0.3	32.03±0.1	17.12±0.2
Water	13.69±0.7	12.54±1.2	19.43±1.7	9.31±1.1
E. agallocha (DE)	42.05±1.3	20.01±1.1	32.61±1.1	16.66±0.2
B. gymnorhiza (DE)	40.37±0.5	20.07±2.4	29.74±2.4	20.07±1.5
A.rotundifolia (DE)	37.71±0.1	11.21±2.6	30.92±3.1	16.96±1.2
A.alba (DE)	19.02±1.2	9.76±1.7	26.15±0.1	15.35±0.7
DMSO	7.56±0.4	7.13±1.7	5.64±0.2	6.69±2.1
Ascorbic acid	79.43±1.2	80.03±2.5	73.18±0.8	61.12±1.2

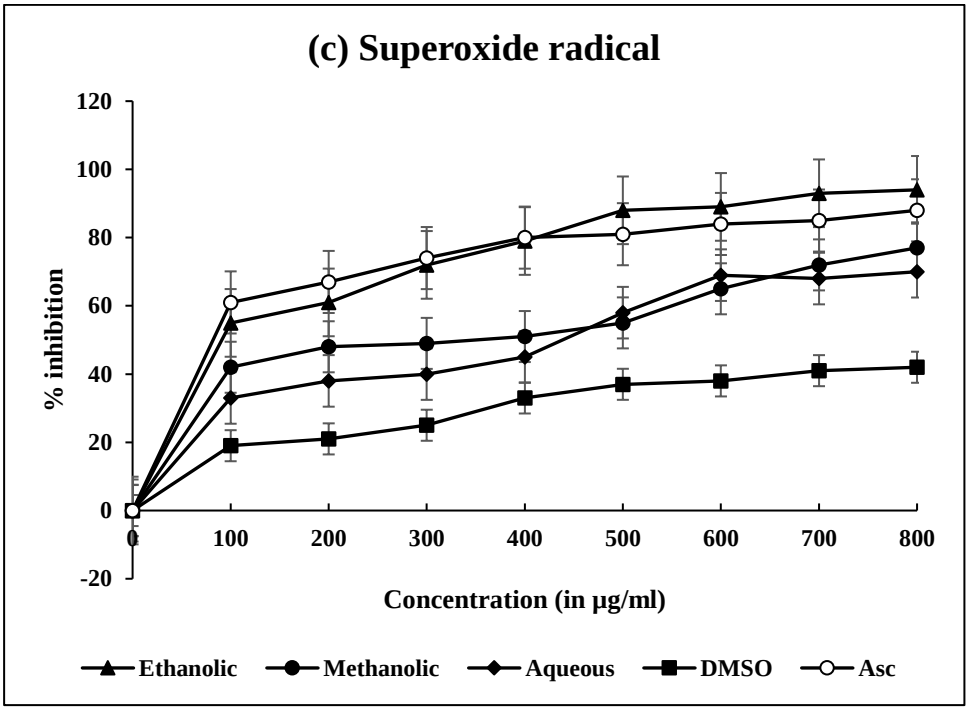
EE: ethanolic extract; ME: methanolic extract; AqE: aqueous extract; DE: DMSO extract.

Inference

- Ethanolic extract of *E. agallocha* exhibited a significant hydroxyl radical scavenging activity with 82.5% inhibition of free radical generation at 8mg/ml concentration. This further substantiates the antioxidant activity of this plant extract as observed in prior experimentation procedures.
- Ethanolic extract of *E. agallocha* exhibited a marked increase in hydrogen peroxide radical scavenging activity with increase in extract concentration. Almost 43% inhibition of free radical generation was noted the ethanolic extract of *E. agallocha.* This indicates that ROS generation is significantly reduced in the presence of this plant extract.
- In this radical scavenging assay, aqueous extract of *E. agallocha* showed a more pronounced nitric radical generation inhibition in comparison to the ethanolic extract of *E. agallocha*. A 37% inhibition was noted in case of its aqueous extract while only 29% inhibition was observed in case of ethanolic extract. A possible explanation may be that the free radicals in ethanolic extract undergo further chain reactions that reduce its scavenging potential.
- In accordance with hydroxyl radical and hydrogen peroxide radical scavenging assays, ethanolic extract of *E. agallocha* exhibited the maximum potent superoxide anion radical scavenging activity of with around 59% inhibition of free radical generation. It can thus, be inferred, that the ethanolic extract of *E. agallocha* has a promising antioxidant activity with the capacity to reduce the free radical generation (*in vitro*). Superoxide is generated as a by-product of oxygen metabolism and if not regulated, causes many types of cellular damage.


(a) Hydroxyl radical scavenging
% inhibition
120
100
80
60
40
20
0
-20
0
100
200
300
400
500
600
700
800
Concentration (in µg/ml)
Ethanolic
Methanolic
Aqueous
DMSO
Asc


(b) Hydrogen peroxide
% Inhibition
120
100
80
60
40
20
0
-20
0
100
200
300
400
500
600
700
800
Concentration (in µg/ml)
Ethanolic
Methanolic
Aqueous
DMSO
Asc



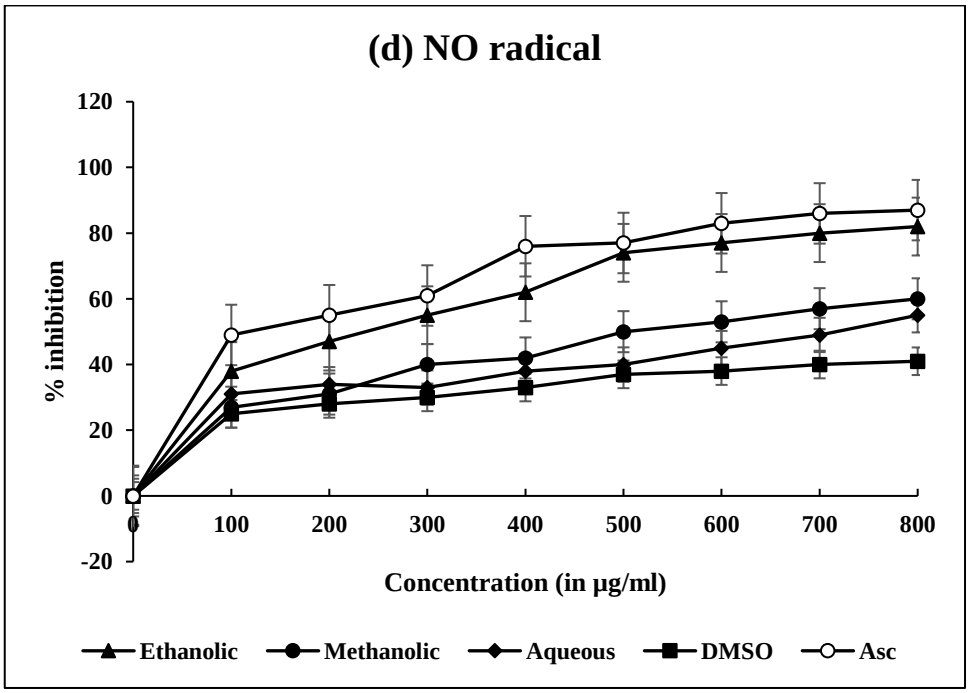


Fig.4.9: *In vitro* radical scavenging activity of *E. agallocha* extract exhibiting (a) hydroxyl radical (b) hydrogen peroxide free radical (c) superoxide anion and (d) nitric oxide radical scavenging activity.

4.1.9. *In vitro* enzyme activity of ROS scavenging enzymes

The *in vitro* content of ROS scavenging enzymes in the different plant extracts was carried out and the results are represented in Table 4.7.

Table 4.7: *In vitro* activity of ROS scavenging enzymes in the crude leaf extracts.

	SOD (U/mg plant tissue)	**Catalase (U/mg plant tissue)**	**Ascorbate oxidase (U/mg plant tissue)**	**Glutathione reductase (U/mg plant tissue)**	**GSH (µM)**
E. agallocha (EE)	64.17±1.1	58.74±1.7	192.46±1.3	301.14±1.1	2.65±1.1
B. gymnorhiza (EE)	58.11±0.3	42.36±0.6	164.18±0.8	278.47±0.2	1.17±2.5
A.rotundifolia (EE)	47.35±1.2	44.81±1.5	147.64±1.7	259.18±2.6	1.78±2.7
A.alba (EE)	46.33±1.5	40.01±0.6	140.05±0.7	268.25±3.1	1.12±0.1
Ethanol	5.67±0.2	15.02±0.8	22.63±2.8	42.08±0.7	0.17±1.3
E. agallocha (ME)	60.04±0.1	47.70±2.8	182.24±2.5	278.18±2.3	2.33±2.8
B. gymnorhiza (ME)	51.28±1.2	40.62±0.6	160.72±0.7	262.02±0.4	2.01±0.3
A.rotundifolia (ME)	48.05±2.4	33.17±1.5	140.50±1.5	237.55±0.8	1.18±2.5
A.alba (ME)	38.16±1.5	37.33±0.7	123.07±3.5	238.18±1.1	1.23±2.7
Methanol	3.34±2.5	10.05±1.7	17.63±2.7	29.64±2.2	0.82±1.1
E. agallocha (AqE)	72.11±3.2	58.23±2.2	190.26±0.5	308.25±2.6	3.34±0.3
B.gymnorhiza (AqE)	61.07±0.2	40.03±2.5	181.42±0.4	284.16±1.8	2.05±1.5
A.rotundifolia (AqE)	51.16±1.5	37.63±3.5	142.08±1.8	263.71±3.1	1.69±0.7
A.alba (AqE)	49.01±1.6	38.17±0.1	126.15±2.4	242.08±0.2	1.18±1.2
Water	10.36±0.6	22.71±1.3	27.02±2.2	51.34±1.2	0.94±0.2
E. agallocha (DE)	58.72±1.7	37.11±1.6	168.17±2.1	218.20±0.1	1.16±0.8
B. gymnorhiza (DE)	49.17±0.4	38.23±0.7	148.53±1.7	202.16±2.4	1.23±1.7
A.rotundifolia (DE)	39.36±2.5	28.05±1.5	116.28±0.2	189.18±1.6	0.58±0.6
A.alba (DE)	35.04±0.3	25.47±1.5	120.33±0.7	183.25±1.5	0.47±2.1
DMSO	11.01±3.5	13.82±2.2	15.91±1.7	22.79±3.4	0.21±1.1
Ascorbic acid	79.02±2.6	67.44±0.2	208.14±1.4	332.54±0.8	6.15±0.5

EE: ethanolic extract; ME: methanolic extract; AqE: aqueous extract; DE: DMSO extract.

Inference

- Ethanolic extract of *E. agallocha* shows the maximum SOD enzyme activity (71 U/mg of plant extract). We know, superoxide dismutase is an enzyme that alternately catalyses the dismutation of the superoxide radical into ordinary molecular oxygen and hydrogen peroxide.

- Thus, this enzymatic activity can be utilised to further investigate the antioxidant activity of the plant extract in abrogating the effect of ROS generation in vivo. Also, the result is indicative of the pharmacological property of the plant extract and the possible antioxidant defense role that it may play in human body.

- The catalase activity was measured in terms of decomposition of μM H_2O_2 per min per mg plant extract. The hydrogen peroxide free radical has damaging effects and is monitored by degradation with the help of the enzyme catalase. This enzyme decomposes hydrogen peroxide to water and oxygen, thereby, protecting the cell from oxidative stress and associated damage by reactive oxygen species (ROS) generation. In case of ethanolic extract of *E. agallocha*, 52 μM H_2O_2 per min per mg plant extract was degraded which indicates that this plant extract might be exploited for its antioxidant activity in mammalian system as well.

- Ethanolic extract of *E. agallocha* exhibited maximum ascorbate oxidase activity (27.46 U/mg plant tissue). Ascorbate oxidase is a potential free radical scavenger that helps in tolerating cellular stress conditions. This enzyme is known to protect cellular lipids and proteins against salinity or drought-induced oxidative adversaries, which is quite evident in case of mangrove plants. This property can be utilsed to screen the role of the extract in producing oxidative defence potential in vivo, thereby contributing to its antioxidant

property.

- In accordance with other *in vitro* antioxidant assays, ethanolic extract of *E. agallocha* exhibited a promising Glutathione reductase activity (315 GR activity/mg of plant tissue). This enzyme catalyzes the reduction of glutathione disulfide (GSSG) to the sulfhydryl form glutathione (GSH), which is a critical molecule in resisting oxidative stress and maintaining the reducing environment of the cell. GR functions as a dimeric disulfide oxidoreductase and utilises FAD prosthetic group and NADPH to reduce one molar equivalent of GSSG to two molar equivalents of GSH. Thus, the potential of this extract can be utilised to monitor its role in protecting cells from cellular damage in vivo.

- The glutathione content was observed to be the highest in case of ethanolic extract of *E. agallocha* (19.65 μM). it is an important antioxidant found in plants that is capable of preventing damage to important cellular components by reactive oxygen species such as free radicals, peroxides, lipid peroxides etc. moreover, glutathione also participates in thiol protection and redox regulation of cellular thiol proteins under oxidative stress. Thus, this finding further validates the antioxidant capacity of the plant extract that can be further purified and investigated for its antioxidant effect in vivo.

4.1.10. *In vitro* inhibition of amylase activity, albumin denaturation and lipid peroxidation

The above mentioned *in vitro* assays were carried out and the results are represented in Table 4.8 and Fig.4.10 (a-c).

Table 4.8: *In vitro* inhibition of amylase activity, albumin denaturation and lipid peroxidation exhibited by crude leaf extracts.

	Percentage inhibition (%)		
	Alpha amylase inhibition	**Albumin denaturation inhibition**	**Lipid peroxidation inhibition**
E. agallocha (EE)	64.61±2.5	74.32±0.4	64.35±2.5
B. gymnorhiza (EE)	43.13±1.7	56.43±2.3	47.83±1.3
A.rotundifolia (EE)	52.23±1.7	42.33±1.7	50.24±0.2
A.alba (EE)	23.34±0.8	45.04±2.2	36.47±1.1
Ethanol	4.03±3.3	1.12±0.2	0.96±2.4
E. agallocha (ME)	46.12±1.8	66.03±2.3	58.32±1.8
B. gymnorhiza (ME)	32.27±0.3	59.76±1.4	40.11±2.1
A.rotundifolia (ME)	28.34±2.2	42.26±0.8	37.25±3.5
A.alba (ME)	28.08±1.6	23.51±1.1	27.04±0.7
Methanol	0.42±0.2	1.27±1.9	0.02±0.1
E. agallocha (AqE)	31.54±1.6	60.14±2.2	55.21±1.9
B. gymnorhiza (AqE)	45.04±2.2	60.05±1.5	25.14±1.6
A.rotundifolia (AqE)	41.32±1.4	47.34±2.2	29.44±2.8
A.alba (AqE)	38.67±3.1	28.15±2.5	21.07±0.3
Water	2.04±0.6	1.62±0.4	1.67±1.7
E. agallocha (DE)	49.04±0.2	42.35±1.5	47.02±2.2
B. gymnorhiza (DE)	54.13±2.3	37.13±1.1	27.47±1.7
A.rotundifolia (DE)	34.76±1.1	33.25±0.5	20.36±0.4
A.alba (DE)	38.52±1.4	17.16±1.4	11.57±3.1
DMSO	0.18±2.2	0.03±0.4	0.85±0.5
Ascorbic acid	88.34±1.6	90.23±1.7	71.23±3.5

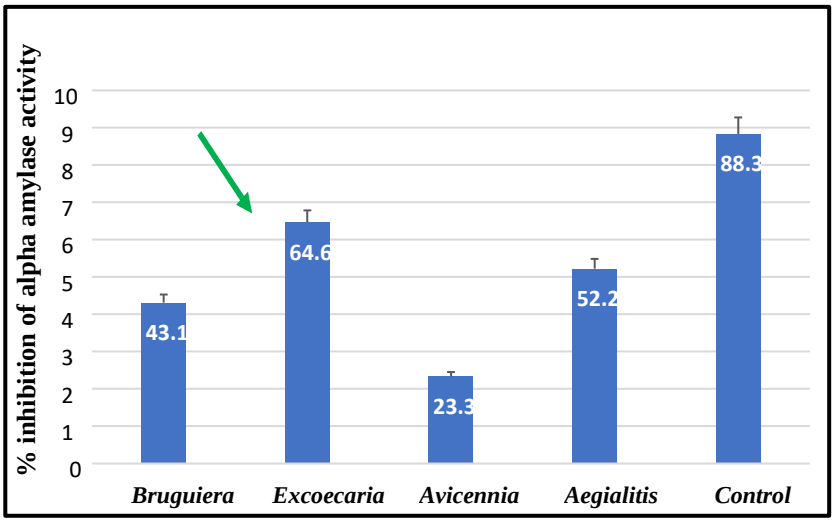


Fig.4.10.a: *In vitro* anti-diabetic activity of the ethanolic plant extracts denoted by the inhibition of alpha amylase activity.

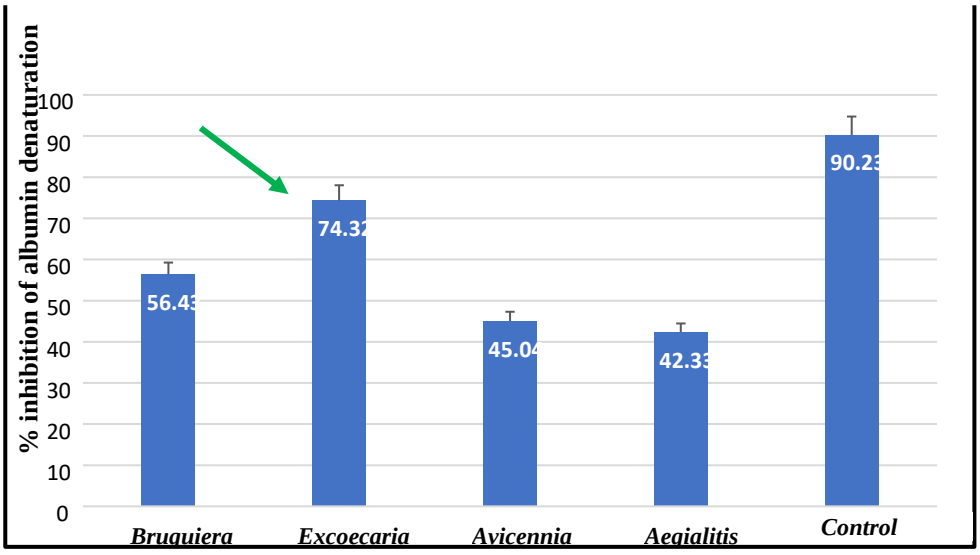


Fig.4.10.b: *In vitro* anti-inflammatory activity of the ethanolic plant extracts denoted by inhibition of albumin denaturation.

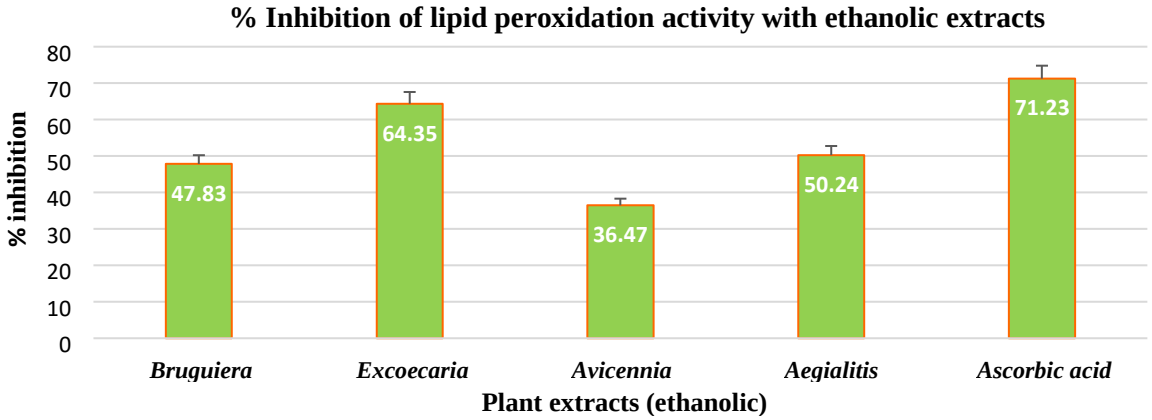


Fig.4.10.c: Graphical representation of the percentage of lipid peroxidation inhibition facilitated by the ethanolic plant extracts.

Inference:

- Ethanolic extract of *E. agallocha* exhibited the highest inhibitory activity (64.61%) against alpha amylase. These plant extracts might be used as starch blockers since it prevents or slows down the absorption of starch into the body mainly by blocking the hydrolysis of 1,4-glycosidic linkages of starch and other oligosaccharides into maltose, maltotriose and other simple sugars.

- As a part of the investigation, the ability of extract protein denaturation was studied. Maximum inhibition (74.32%) was observed from extract of *E. agallocha.* The result provides evidence of protein denaturation inhibition as an additional mechanism of action apart from antimicrobial activity. This effect may possibly inhibit release of lysosomal content of neutrophils at the site of inflammation and hence, this finding is of medicinal importance.

- Peroxidation in the egg homogenate is induced by Fe3+ generation that attacks the biological material. This leads to the formation of Malondialdehyde and other aldehydes which form a pink chromogen with TBA, absorbing at 532nm. The highest peroxidation inhibitory activity exhibited by the ethanolic extract of *E. agallocha* may have a protective role in biological system by inhibiting peroxidation and consequent damage caused due to lipid peroxidation.

4.1.11. Study of antimicrobial property of the crude leaf extracts.

The antibacterial activity of the crude plant extracts was preliminarily checked against standard Gram positive and Gram-negative bacteria to analyse whether the plant extracts were more effective specifically against Gram positive or negative bacteria or both. Antifungal activity was also checked against common laboratory strains of fungi. The MIC values were observed to evaluate the efficacy of the plant extracts against specific laboratory strains of bacteria and fungi. The results are represented in Tables 4.9 (a-d).

Thereafter, the antimicrobial activity was checked against antimicrobial resistant, pathogenic bacterial and fungal strains (isolated and maintained at Department of Microbiology, Peerless Hospital and B.K. Roy research Centre) and the MIC value was determined by 96- well micro titre broth dilution method to specifically ascertain the most potent plant extract in terms of antimicrobial activity. The results are graphically represented in Fig.4.11-4.17.

4.1.11a. Antimicrobial screening assay

The crude plant extracts in the different solvents were tested for their efficacy against inhibiting the growth of different bacteria and fungi using the disc-diffusion method and the results are represented in Tables 4.10.a, b, c and d.

Table 4.9.a: Zone of inhibition (in mm) produced by the ethanolic plant extracts against bacteria and fungi

	Solvent control	*E. agallocha*	*B. gymnorhiza*	*A. alba*	*A. rotundifolia*	AMX (30)	CIP (15)	TET (30)	AMPH (10)	KCZ (10)
B. subtilis MTCC 2049	0	11.33±1.15	8.16±0.28	6.4±0.20	13.46±0.04	26.43±0.16	28.53±0.12	26.8±0.16	-NA-	-NA-
S. aureus MTCC 2408	0	15±1.00	8.96±0.45	5.43±0.21	10.43±0.16	22.36±0.04	29.13±0.28	19.2±0.26	-NA-	-NA-
E. coli MTCC 443	0	22.66±1.15	18.56±0.60	10.56±0.15	12.20±0.24	29.13±0.28	35.50±0.37	34.06±0.16	-NA-	-NA-
K. pneumoniae MTCC 109	0	14.33±0.57	12.1±0.40	7.56±0.15	9.7±0.21	22.40±0.08	31.36±0.28	33.10±0.14	-NA-	-NA-
P. aeruginosa MTCC 2408	0	22.66±0.57	16.46±0.25	13.63±0.21	15.7±0.08	30.66±0.71	35.06±0.33	34.06±0.09	-NA-	-NA-
E. aerogenes MTCC 2615	0	11.67±2.08	9.6±0.65	7.73±0.11	5.66±0.12	23.2±0.16	33.03±0.20	35.03±0.12	-NA-	-NA-
A.flavus MTCC 13062	0	20.33±1.52	16.3±0.26	10.6±0.10	14.5±0.24	-NA-	-NA-	-NA-	24.06±0.09	24.43±.012
P. chrysogenum MTCC 5108	0	15.66±1.15	11.4±0.26	5.53±0.21	8.3±0.14	-NA-	-NA-	-NA-	21.63±0.12	23.10±0.08

AMX: Amoxicillin; CIP: Ciprofloxacin; TET: Tetracycline; AMPH: Amphotericin B; KCZ: Ketoconazole.

Table 4.9.b: Zone of inhibition (in mm) produced by the methanolic plant extracts against bacteria and fungi

	Solvent control	*E.agallocha*	*B.gymnorhiza*	*A. alba*	*A.rotundifolia*	AMX (30)	CIP (15)	TET (30)	AMPH (10)	KCZ (10)
B. subtilis MTCC 2049	0	9.12±1.05	6.78±0.21	2.4±0.14	7.06±0.04	26.43±0.16	28.53±0.12	26.8±0.16	-NA-	-NA-
S. aureus MTCC 2408	0	10.17±0.06	5.96±0.24	6.43±0.71	8.20±0.28	22.36±0.04	29.13±0.28	19.2±0.26	-NA-	-NA-
E. coli MTCC 443	0	18.08±0.40	12.56±0.57	11.63±0.21	12.50±0.37	29.13±0.28	35.50±0.37	34.06±0.16	-NA-	-NA-
K. pneumoniae MTCC 109	0	12.66±0.15	7.1±0.40	4.7±0.40	8.6±0.24	22.40±0.08	31.36±0.28	33.10±0.14	-NA-	-NA-
P. aeruginosa MTCC 2408	0	20.66±0.50	12.86±0.12	9.40±0.14	12.12±0.33	30.66±0.71	35.06±0.33	34.06±0.09	-NA-	-NA-
E. aerogenes MTCC 2615	0	8.65±0.08	5.6±0.08	6.63±0.45	6.75±0.15	23.2±0.16	33.03±0.20	35.03±0.12	-NA-	-NA-
A.flavus MTCC 13062	0	16.33±0.57	10.3±0.14	7.36±0.33	11.15±0.12	-NA-	-NA-	-NA-	24.06±0.09	24.43±.012
P. chrysogenum MTCC 5108	0	8.66±1.10	5.4±0.25	3.46±0.26	7.6±0.57	-NA-	-NA-	-NA-	21.63±0.12	23.10±0.08

AMX: Amoxicillin; CIP: Ciprofloxacin; TET: Tetracycline; AMPH: Amphotericin B; KCZ: Ketoconazole.

Table 4.9.c: Zone of inhibition (in mm) produced by the aqueous plant extracts against bacteria and fungi

	Solvent control	*E.agallocha*	*B.gymnorhiza*	*A. alba*	*A.rotundifolia*	AMX (30)	CIP (15)	TET (30)	AMPH (10)	KCZ (10)
B.subtilis MTCC 2049	0	7.4±0.07	5.42±0.11	0.00	6.12±0.12	26.43±0.16	28.53±0.12	26.8±0.16	-NA-	-NA-
S. aureus MTCC 2408	0	6.8±1.15	3.09±0.78	1.17±0.28	3.47±0.33	22.36±0.04	29.13±0.28	19.2±0.26	-NA-	-NA-
E. coli MTCC 443	0	13.5±0.21	10.05±0.02	7.26±0.16	9.48±0.21	29.13±0.28	35.50±0.37	34.06±0.16	-NA-	-NA-
K. pneumoniae MTCC 109	0	9.86±0.0.16	7.82±0.16	7.06±0.21	8.4±0.28	22.40±0.08	31.36±0.28	33.10±0.14	-NA-	-NA-
P. aeruginosa MTCC 2408	0	14.12±0.12	10.36±0.08	11.01±0.14	10.07±0.05	30.66±0.71	35.06±0.33	34.06±0.09	-NA-	-NA-
E. aerogenes MTCC 2615	0	6.54±0.20	5.53±0.11	4.08±0.12	4.05±0.21	23.2±0.16	33.03±0.20	35.03±0.12	-NA-	-NA-
A.flavus MTCC 13062	0	12.30±1.15	9.36±0.55	2.40±0.16	4.5±0.04	-NA-	-NA-	-NA-	24.06±0.09	24.43±.012
P. chrysogenum MTCC 5108	0	6.42±0.26	7.26±0.15	3.09±0.05	4.86±0.28	-NA-	-NA-	-NA-	21.63±0.12	23.10±0.08

AMX: Amoxicillin; CIP: Ciprofloxacin; TET: Tetracycline; AMPH: Amphotericin B; KCZ: Ketoconazole.

Table 4.9.d: Zone of inhibition (in mm) produced by the DMSO plant extracts against bacteria and fungi

	Solvent control	*E. agallocha*	*B.gymnorhiza*	*A. alba*	*A.rotundifolia*	AMX (30)	CIP (15)	TET (30)	AMPH (10)	KCZ (10)
B. subtilis MTCC 2049	0	5.63±0.02	2.41±1.15	0.95±0.15	4.58±0.33	26.43±0.16	28.53±0.12	26.8±0.16	-NA-	-NA-
S. aureus MTCC 2408	0	5.12±0.21	5.52±0.21	1.12±0.28	5.08±0.21	22.36±0.04	29.13±0.28	19.2±0.26	-NA-	-NA-
E. coli MTCC 443	0	8.75±0.16	10.03±0.33	1.56±0.03	6.25±1.15	29.13±0.28	35.50±0.37	34.06±0.16	-NA-	-NA-
K. pneumoniae MTCC 109	0	6.36±1.15	6.14±0.78	2.25±0.16	5.58±0.05	22.40±0.08	31.36±0.28	33.10±0.14	-NA-	-NA-
P. aeruginosa MTCC 2408	0	10.51±0.21	5.36±0.21	4.45±0.78	6.47±0.37	30.66±0.71	35.06±0.33	34.06±0.09	-NA-	-NA-
E. aerogenes MTCC 2615	0	7.12±1.15	8.01±0.04	4.05±0.15	4.45±0.78	23.2±0.16	33.03±0.20	35.03±0.12	-NA-	-NA-
A.flavus MTCC 13062	0	11.25±0.28	6.42±1.15	2.57±1.15	8.17±0.20	-NA-	-NA-	-NA-	24.06±0.09	24.43±.012
P. chrysogenum MTCC 5108	0	5.83±0.16	4.31±0.27	2.28±0.33	4.97±0.05	-NA-	-NA-	-NA-	21.63±0.12	23.10±0.08

AMX: Amoxicillin; CIP: Ciprofloxacin; TET: Tetracycline; AMPH: Amphotericin B; KCZ: Ketoconazole.

4.1.11b. Determination of Minimum Inhibitory Concentration (MIC) against pathogenic microbes

The MIC of the different plant extracts against different bacteria and fungi was evaluated and the results are represented in Fig. 4.9- 4.15.

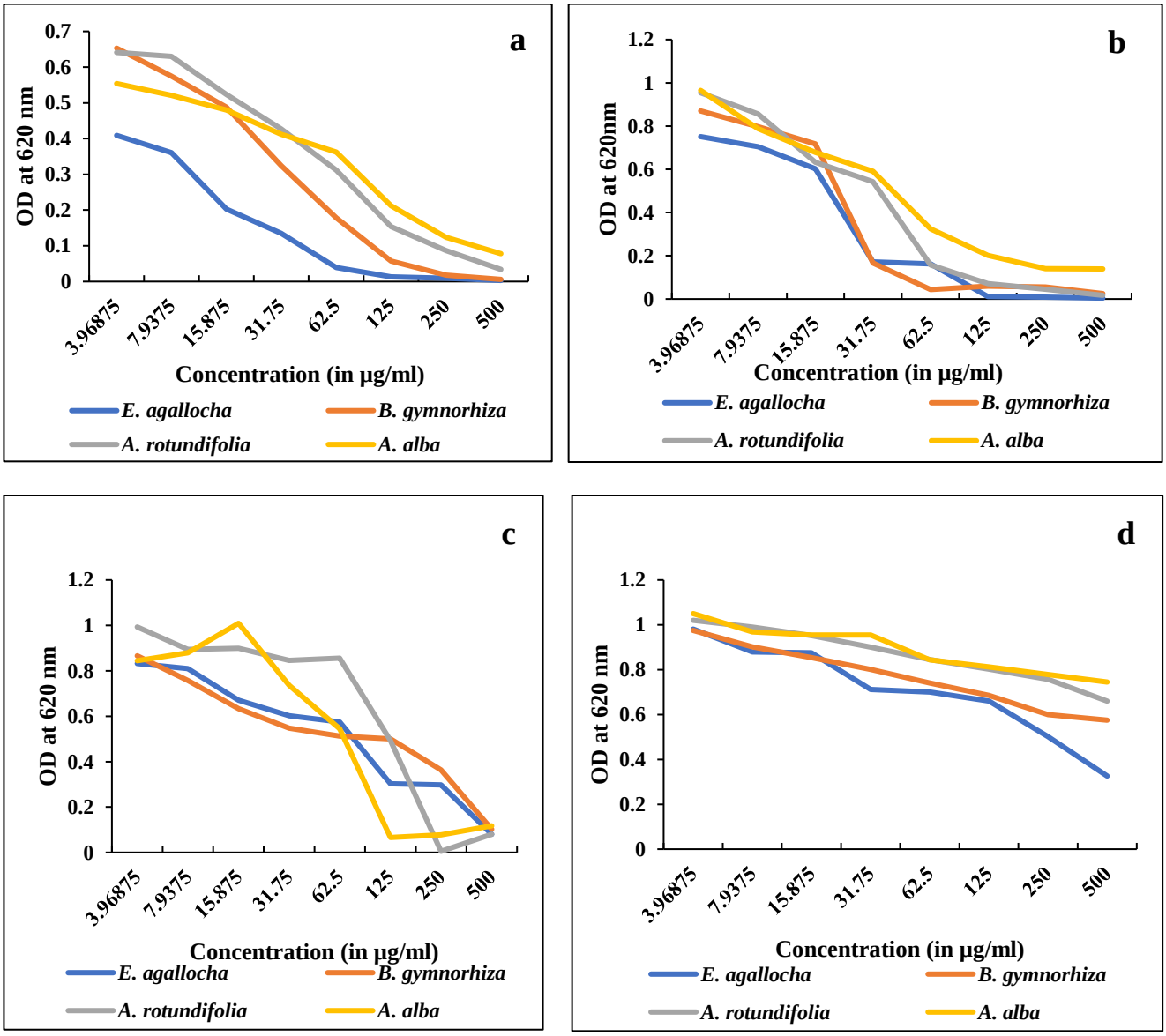


Fig. 4.11: MIC of the (a) ethanolic (b) methanolic (c) aqueous and (d) DMSO plant extracts against *Pseudomonas aeruginosa.*

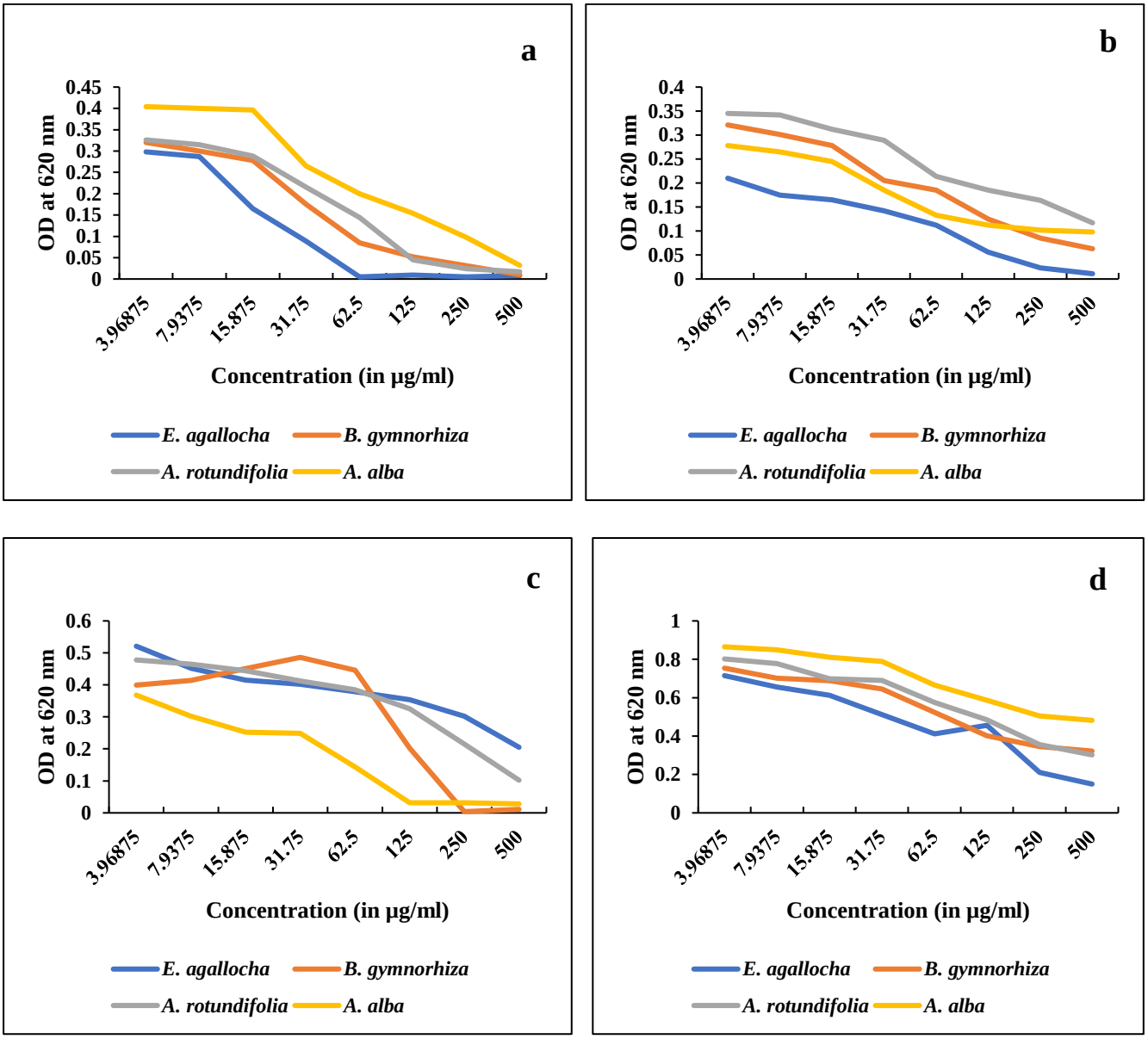


Fig. 4.12: MIC of the (a) ethanolic (b) methanolic (c) aqueous and (d) DMSO plant extracts against ESBL *Escherichia coli.*

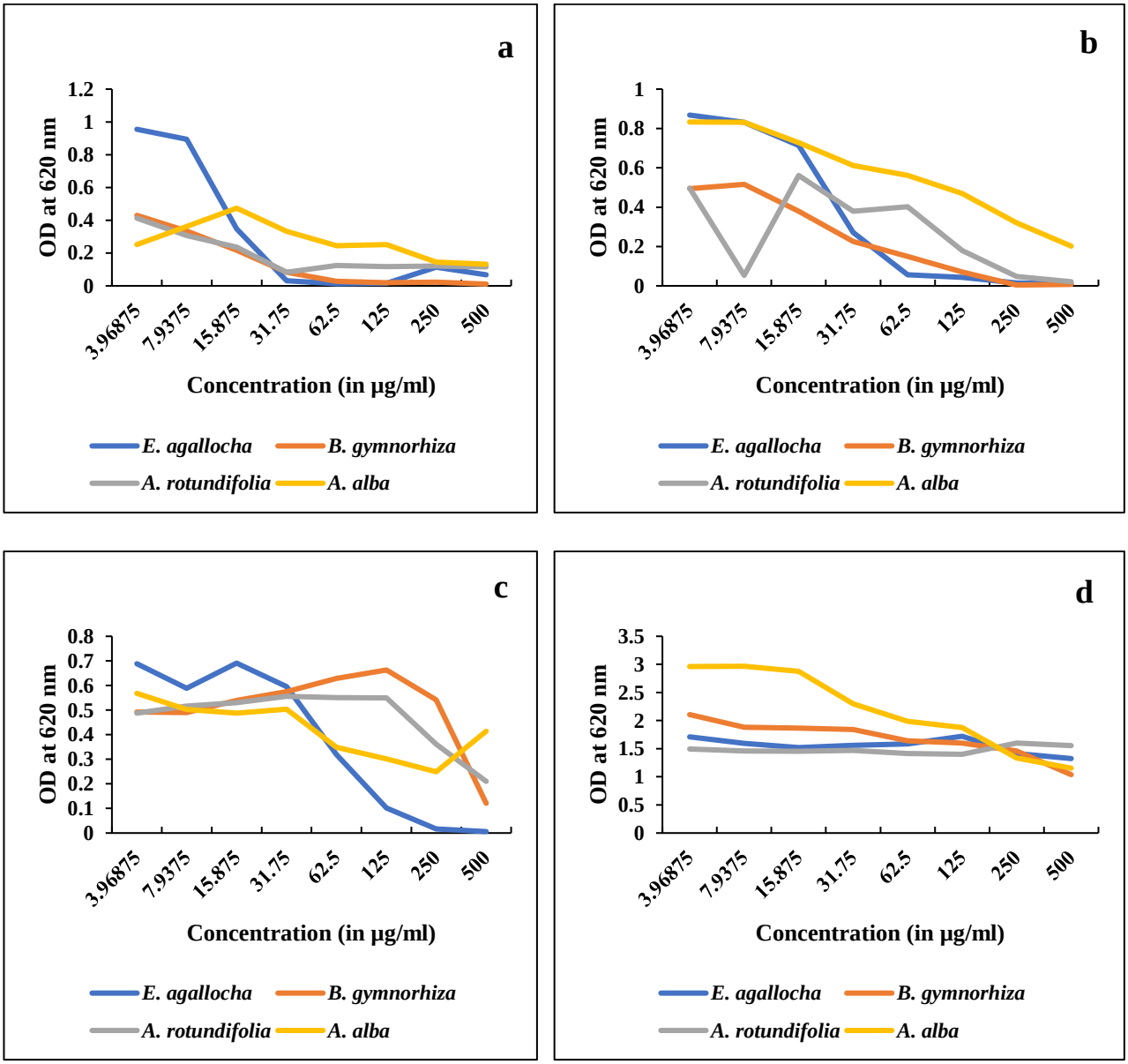


Fig. 4.13: MIC of the (a) ethanolic (b) methanolic (c) aqueous and (d) DMSO plant extracts against *Escherichia coli.*

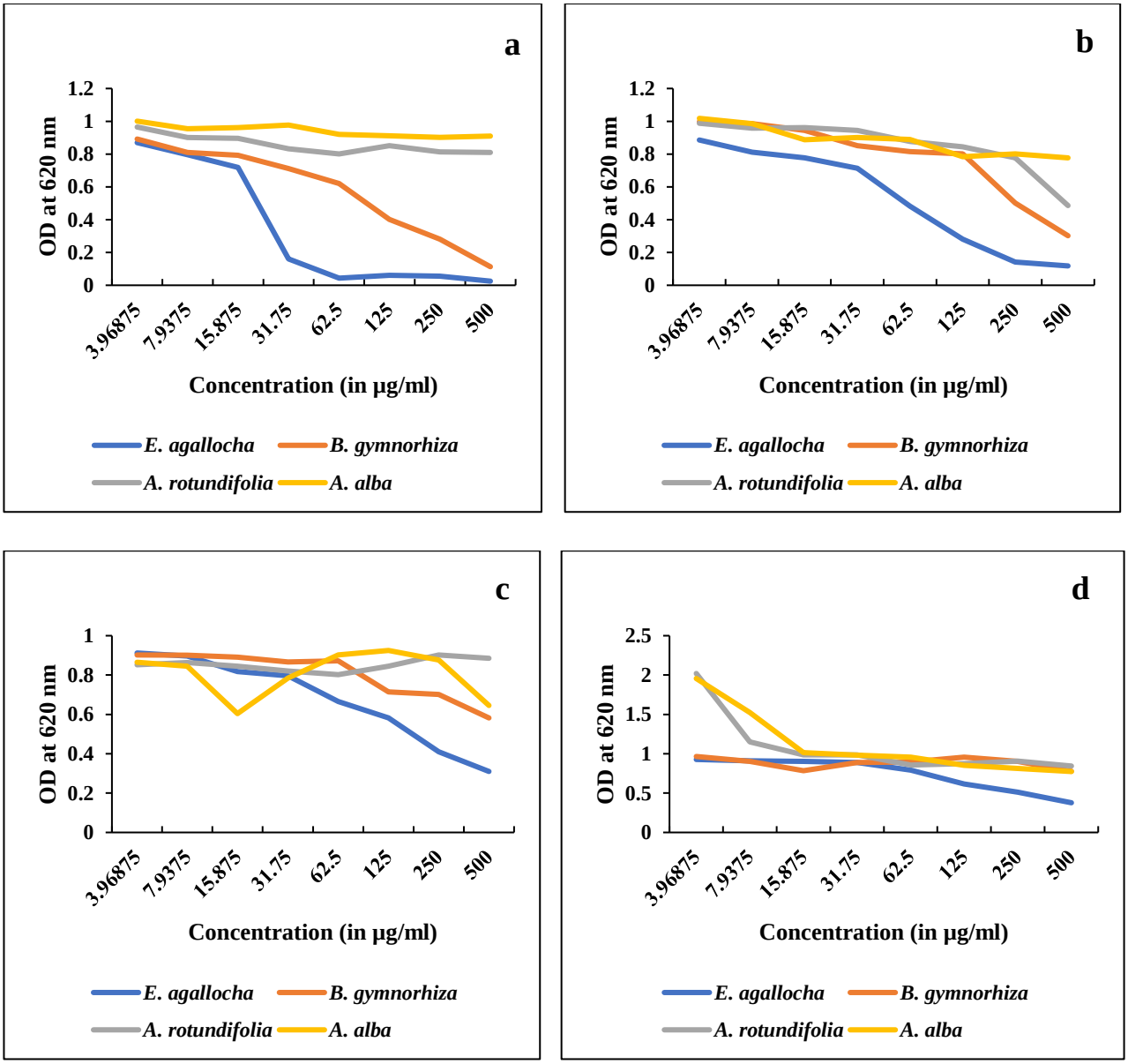


Fig. 4.14: MIC of the (a) ethanolic (b) methanolic (c) aqueous and (d) DMSO plant extracts against *Salmonella typhi.*

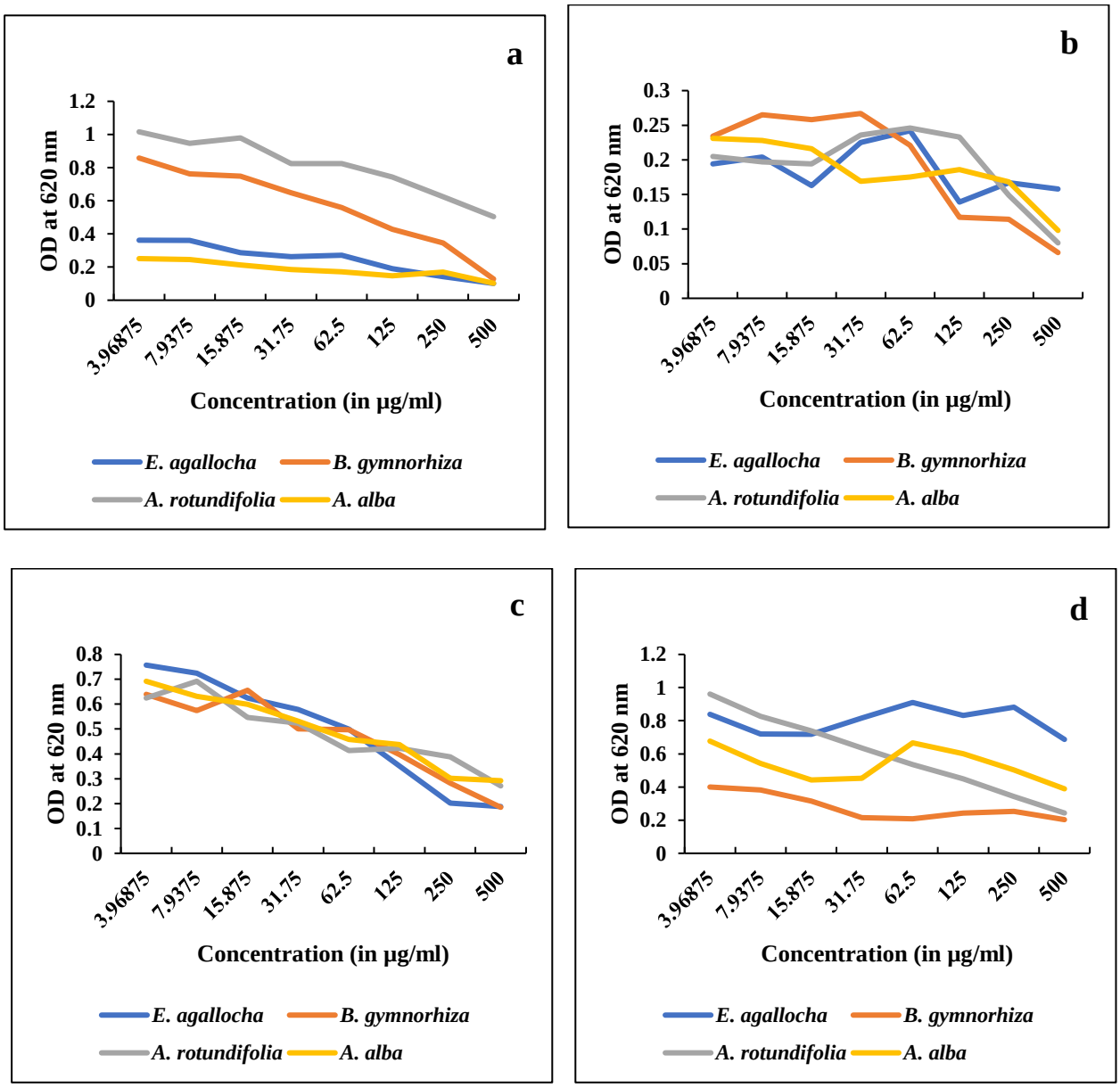


Fig. 4.15: MIC of the (a) ethanolic (b) methanolic (c) aqueous and (d) DMSO plant extracts against *Staphylococcus aureus.*

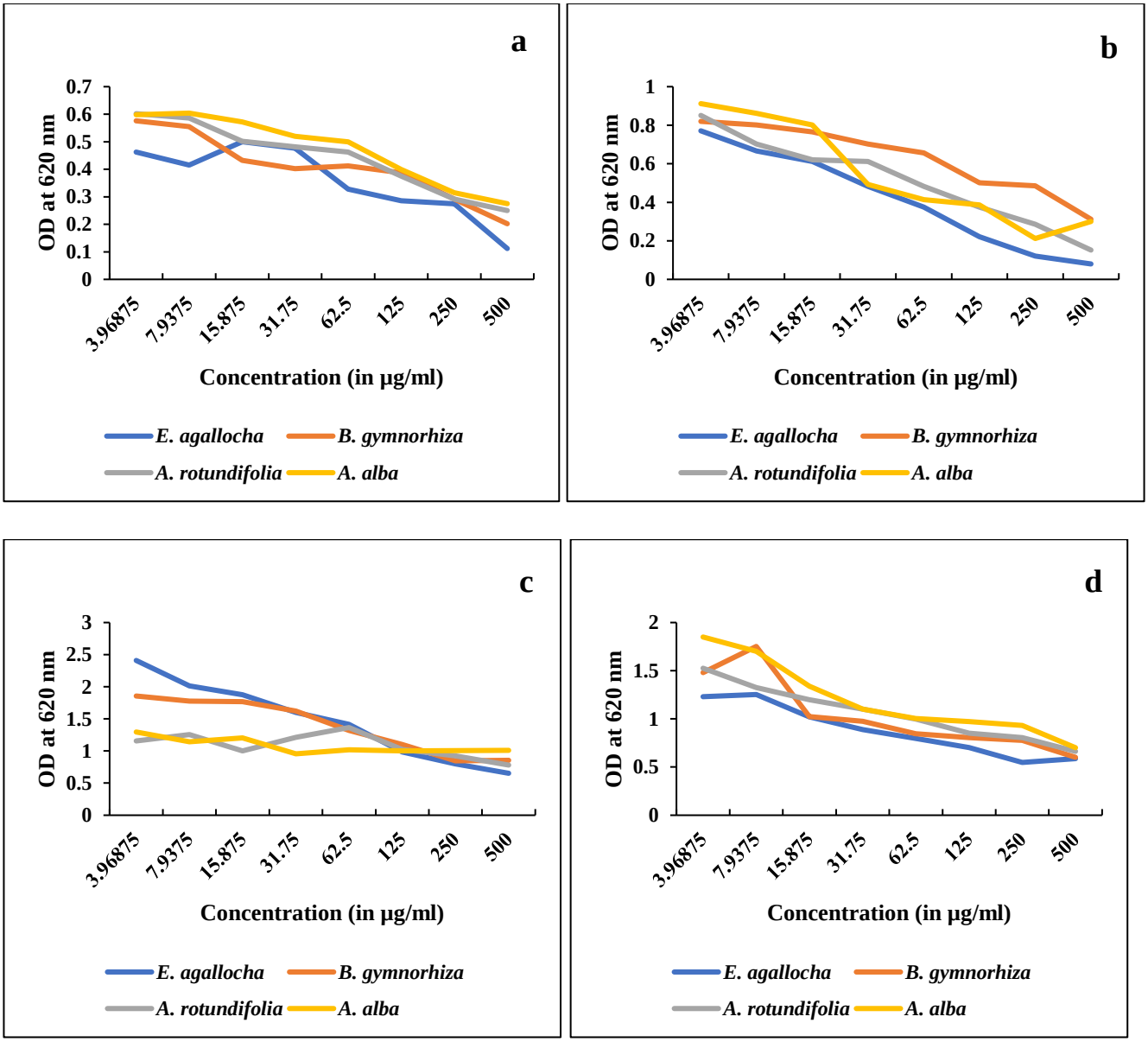


Fig. 4.16: MIC of the (a) ethanolic (b) methanolic (c) aqueous and (d) DMSO plant extracts against *Candida tropicalis.*

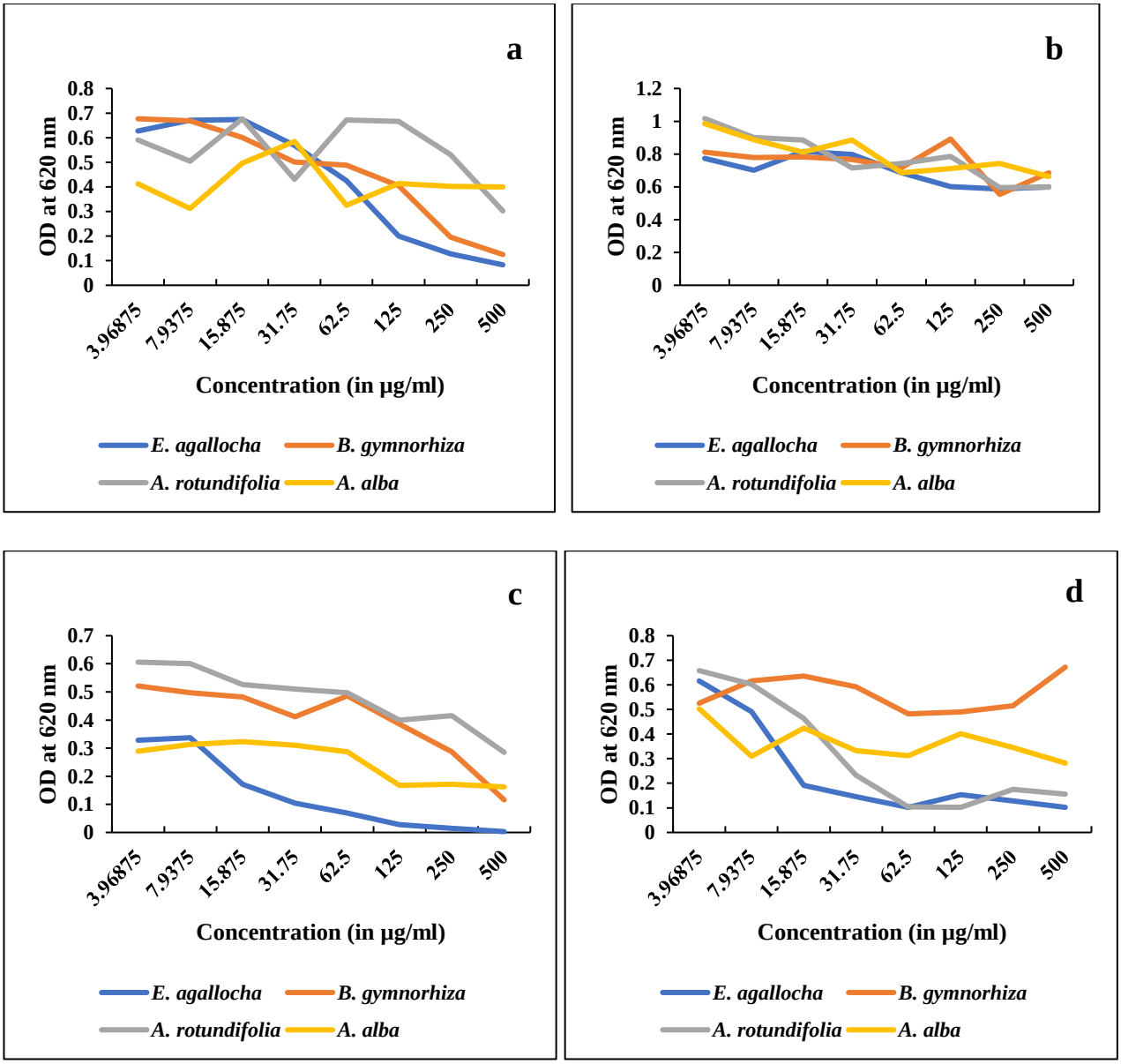


Fig. 4.17: MIC of the (a) ethanolic (b) methanolic (c) aqueous and (d) DMSO plant extracts against *Candida parapsilosis.*

Inference:

- Ethanolic extract of *B. gymnorhiza* has been inferred to be the most effective extract which has promising antibacterial activity against MDR strain of *P. aeruginosa* ATCC 27853 and *E. coli* ESBL strain with an MIC value less than 3.96µg/ml in both the cases.
- Another very important finding in this study is the antimicrobial activity of DMSO extract of *B. gymnorhiza* against *E. coli* ESBL strain. However, in the case of *E. coli* ESBL strain, a sharp increase in absorbance is noted which may be due to the formation of bacterial cell aggregate.

- Ethanolic extract of *E. agallocha* inhibited the growth of *P. aeruginosa* with an MIC value of around 7.937µg/ml while its methanolic extract exhibited an MIC value of 15.875µg/ml.

- Methanolic extract of *E. agallocha* showed antifungal activity against a hospital strain of *Candida parapsilosis* with an MIC value of around 180 µg/ml. Ethanolic extract of *E. agallocha* exhibited a property of inhibition of biofilm formation by *Pseudomonas*.

- DMSO extract of *A. alba* exhibited mild antibacterial action by inhibiting the growth of MDR strain of *E. coli* ATCC 25922 and MDR strain of *P. aeruginosa* ATCC 27853 with an MIC value of less than 3.96µg/ml.
- On the other hand, ethanolic extract of *A. rotundifolia* was observed to have a mild antibacterial action against the same bacterial strain of *Pseudomonas* with an MIC value slightly more than 3.968µg/ml.

- Ethanolic extract of *E. agallocha* inhibited the growth of *a multidrug resistant strain of Salmonella typhi* with an MIC value 15.5µg/ml. Fungal inhibitory effect was also noted against Candida parapsilosis. However, the antimicrobial action was more pronounced in case of *S.typhi*.

4.2. Objective – 2: Characterisation of different bioactive principles present in the selected crude leaf extracts with optimum antioxidant and antimicrobial activity

4.2.1. Thin Layer Chromatography

The chemical nature of the bioactive components present in the plant extracts were analysed and separated by TLC (using 9:1 toluene: ethyl acetate as mobile phase) wherein distinct bands were observed in the TLC plate after derivatization.

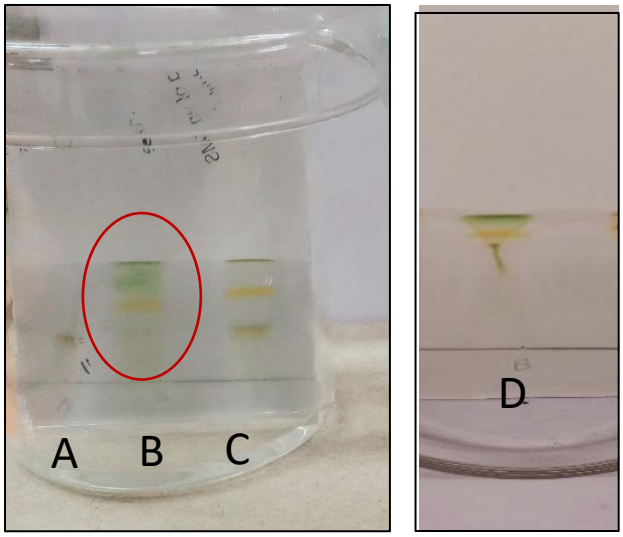


Fig. 4.18: TLC of ethanolic leaf extracts-plates after derivatization with Vanillin spray reagent (Mobile phase- Toluene: Ethyl acetate=9:1)
A: ***A. rotundifolia*****, B:** ***E. agallocha*****, C:** ***B. gymnorhiza*****, D:** ***A. alba***

Inference:

- Ethanolic extract of *E. agallocha* separated distinctly into its constituents when subjected to Thin Layer Chromatography with distinct R_f values of 0.485, 0.471, 0.442. 0.328, 0.228, 0.185 and 0.142. A pinkish coloration was observed after derivatization indicating the presence of terpenoid compounds and phenolic derivatives.

4.2.2. LC-MS of the extracts

The LCMS study of the crude plant extracts were carried out by HR-LCMS at IIT Bombay. [Instrument specification-Make: Agilent Technologies, USA; Model: 1290 Infinity UHPLC system, 6550 iFunnel Q-TOFs; Mass range: 50-3200 amu; Column details: Syncronis C18 100x 2.1, particle size 1.7µ; acquisition time-30mins; flow rate 0.3 ml/min]. The main constituents identified are enlisted in Table 4.10 (a-d).

Table 4.10.a: LC-MS data of *Excoecaria agallocha (ethanolic)* extract

SL. NO.	COMPOUND NAME	FORMULA	MASS (g/mol)	RT	REL. INTENSITY (%)
1.	Hexanoylglycine	$C_8 H_{15} N O_3$	173.1051	1.183	11.39
2.	Nicotinamide mononucleotide	$C_{11} H_{15} N_2 O_8 P$	334.0551	1.506	27.48
3.	1-L-Leucyl-L-Proline	$C_{11} H_{20} N_2 O_3$	228.1472	1.555	3.57
4.	3-(4-Hydroxyphenyl) pyruvic acid	$C_9 H_8 O_4$	180.0421	3.567	6.47
5.	Chorismic acid	$C_{10} H_{10} O_6$	226.0473	4.672	37.67
6.	6-Phosphogluconic acid	$C_6 H_{13} O_{10} P$	276.0268	5.038	13.15
7.	N-Deacetylketokonazole	$C_{24} H_{26} Cl_2 N_4 O_3$	488.1357	5.88	45.47
8.	Tyramine	$C_8 H_{11} N O$	137.084	2.158	11.21
9.	Methyl jasmonate	$C_{13} H_{20} O_3$	224.1409	4.947	22.69
10.	Bergenin	$C_{14} H_{16} O_9$	328.0808	4.809	88.23
11.	Dihydromyricetin	$C_{15} H_{12} O_8$	320.0526	6.692	27.10
12.	Khivorin	$C_{32} H_{42} O_{10}$	586.2782	19.09	26.57
13.	Khayanthone	$C_{32} H_{42} O_9$	570.2835	19.376	22.36
14.	Chlorogenic acid	$C_{16} H_{18} O_9$	354.0963	3.474	22.28
15.	S, S, S, -Tributylphosphotrithioate	$C_{12} H_{27} O P S_3$	314.1017	4.74	10.41
16.	Alpha,4-Dihydroxytriazolam	$C_{17} H_{12} Cl_2 N_4 O_2$	374.0292	5.338	27.89
17.	Ellagic acid	$C_{14} H_6 O_8$	302.0079	6.601	19.63

Table 4.10.b: *Bruguiera gymnorhiza (ethanolic)*

SL. NO.	COMPOUND NAME	FORMULA	MASS (g/mol)	RT	REL. INTENSITY (%).
1.	4-Hydroxyclobazam	$C_{16} H_{13} Cl N_2 O_3$	316.0611	5.741	18.13
2.	Bilirubin	$C_{33} H_{36} N_4 O_6$	584.2631	18.478	24.14
3.	Harderoporphyrin	$C_{35} H_{36} N_4 O_6$	608.2614	18.478	11.24
4.	Khayanthone	$C_{32} H_{42} O_9$	570.2835	19.395	41.92
5.	(3a, 5b, 7b, 12a) -(1,3-dihydro-5-nitro1,3-dioxo-2H-isoindol-2-yl) methyl ester3,7,12-trihydroxy-Cholan	$C_{33} H_{44} N_2 O_9$	612.2945	19.85	15.45
6.	Harderoporphyrinogen	$C_{35} H_{42} N_4 O_6$	614.3114	20.789	17.69
7.	Hexacosanedioic acid	$C_{26} H_{50} O_4$	426.3718	24.713	16.13
8.	Teasterone	$C_{28} H_{48} O_4$	448.354	25.341	67.50
9.	Quercitrin	$C_{21} H_{20} O_{11}$	448.1013	5.399	14.51
10.	Demeclocycline	$C_{21} H_{21} Cl N_2 O_8$	464.0968	6.141	12.65
11.	Hederagenin	$C_{30} H_{48} O_4$	472.3562	18.405	20.67
12.	3-alpha, 6-alpha, 7-alpha, 12 -alphaTetrahydroxy-5beta-cholest-24-en-26-oic acid	$C_{27} H_{44} O_6$	464.3163	22.25	15.25

Table 4.10.c: ***Aegialitis rotundifolia (ethanolic)***

SL. NO.	COMPOUND NAME	FORMULA	MASS (g/mol)	RT	REL. INTENSITY (%)
1.	Isoamyl nitrite	$C_5 H_{11} N O_2$	117.0785	2.11	44.32
2.	2-Aminopropiophenone	$C_9 H_{11} N O$	149.0836	9.225	14.80
3.	Lecanoric acid	$C_{16} H_{14} O_7$	318.0736	9.305	19.43
4.	Bilirubin	$C_{33} H_{36} N_4 O_6$	584.2619	17.708	12.43
5.	Khivorin	$C_{32} H_{42} O_{10}$	586.2782	18.873	42.24
6.	Khayanthone	$C_{32} H_{42} O_9$	570.2835	19.438	50.25
7.	Rescinnamine	$C_{35} H_{42} N_2 O_9$	634.2759	19.905	14.96
8.	Dihydrogambogic acid	$C_{38} H_{46} O_8$	630.3048	20.307	11.03
9.	Trandolapril glucoronide	$C_{30} H_{42} N_2 O_{11}$	606.2818	20.787	44.88
10.	Harderoporphyrinogen	$C_{35} H_{42} N_4 O_6$	614.3085	20.797	15.71
11.	2,4,6-trimethyl-2, 15-tetracosadienoic acid	$C_{27} H_{50} O_2$	406.3816	23.806	20.06
12.	2-chloro-4-(1-piperazinyl)-phenol	$C_{10} H_{13} Cl N_2 O$	212.0726	4.22	25.14
13.	Quercitrin	$C_{21} H_{20} O_{11}$	448.1009	6.703	22.47
14.	Pinocembrin	$C_{15} H_{12} O_4$	256.0731	11.646	10.37
15.	3-Deoxy-3-azido-25-hydroxyvitamin D3	$C_{27} H_{44} N_3 0$	426.3491	15.128	170.7

Table 4.10.d: ***Avicennia alba (ethanolic)***

SL. NO.	COMPOUND NAME	FORMULA	MASS (g/mol)	RT	REL. INTENSITY (%)
1.	Isoamyl nitrite	$C_5 H_{11} N O_2$	117.0785	2.112	56.32
2.	Lecanoric acid	$C_{16} H_{14} O_7$	318.0736	6.56	18.58
3.	8-hydroxy-13Z-octadecene-9,11-diynoic acid	$C_{18} H_{26} O_3$	290.1876	10.811	11.59
4.	Picrotin	$C_{15} H_{18} O_7$	310.1063	12.336	36.63
5.	Isotectorigenin, 7-methyl ether	$C_{18} H_{16} O_6$	328.0932	12.984	17.50
6.	1-heptadecanoyl-2-(9Z-tetradecenoyl)sn-glycero-3-phosphoserine	$C_{37} H_{74} N_2 O_{10} P$	737.5052	17.982	18.81
7.	Khivorin	$C_{32} H_{42} O_{10}$	586.2782	19.272	69.60
8.	Khayanthone	$C_{32} H_{42} O_9$	570.2835	19.776	35.11
9.	Maltotriitol	$C_{18} H_{34} O_{16}$	506.1777	8.979	12.09
10.	11-hydroxyperoxy-12,13-epoxy-9octadecenoic acid	$C_{18} H3_2 O_5$	328.2246	8.988	25.09
11.	9S, 10S, 11R- trihydroxy-12Zoctadecenoic acid	$C_{18} H_{34} O_5$	330.2398	9.564	10.64
12.	Koparin 2'-methyl ether	$C_{17} H_{14} O_6$	314.0786	11.959	21.47
13.	3-deoxy-3-azido-25-hydroxyvitamin D3	$C_{27} H_{44} N_3 O$	426.3466	14.808	18.76

Inference:

- A number of novel compounds had been detected by LC-MS.

- LC-MS is an analytical technique that involves physical separation of target compounds (or analytes) followed by their mass-based detection. Its sensitivity, selectivity and accuracy have made it a technique of choice for detecting microgram or even nanogram quantities of a variety of analytes such as drug metabolites and natural compounds of pharmaceutical importance.

- In the crude ethanolic extract of *E.agallocha,* the compounds chorismic acid, methyl jasmonate, bergenin, khayanthone and chlorogenic acid were observed to be present as major constituents. Chorismic acid is an important biochemical intermediate in plants and precursor for salicylic acid, alkaloids and other aromatic metabolites. Bergenin is an acid glycoside and a natural secondary metabolite. Khayanthone is a limonoid compound. Limonoids are also known as tetranortriterpenoids. They occur mainly in the Meliaceae, Rutaceae, and Cneoraceae families. Chlorogenic acid is a phenolic compound. It releases glucose slowly after meals and it has got antihypertensive anti-inflammatory effects.

- The abundance of the compounds khayanthone and teasterone was noted to be highest in case of the ethanolic extract of *B.gymnorhiza*. Teasterone is a phytosteroid while khayanthone is a limonoid compound.

- LC-MS data of ethanolic extract of *A.rotundifolia* revealed the presence of the compounds Isoamyl nitrite, khayanthone and trandolapril glucuronide in greater abundance in comparison to other constituents. While isoamyl nitrite is a nitrite ester, trandolapril glucuronide is a non-sulfhydryl angiotensin-converting enzyme (ACE) inhibitor with antihypertensive activity. It is converted into its active form, trandolaprilat, in the liver, which competitively inhibits ACE, blocking the conversion of angiotensin I to angiotensin II. It also decreases the secretion of aldosterone by the adrenal cortex. Trandolapril may improve survival in clinically stable myocardial infarction patients with left ventricular dysfunction, as an adjunct treatment, it is used in congestive cardiac failure, and it slows the progression of kidney damage in hypertension associated with diabetes mellitus and micro-albuminuria.

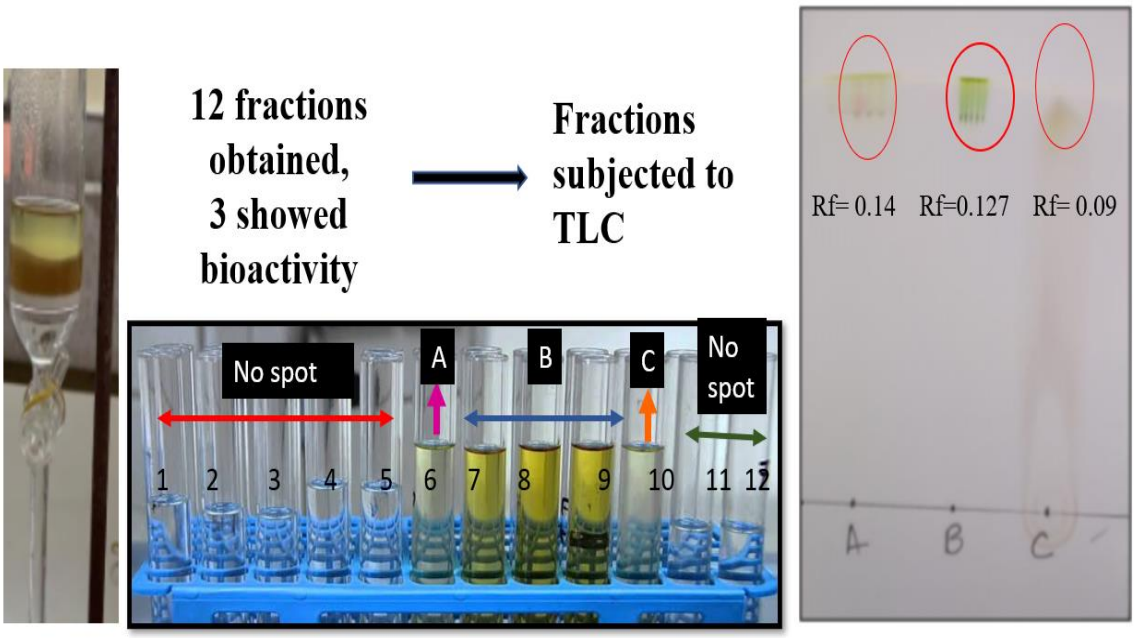
- On the other hand, in the ethanolic extract of *A.alba*, compounds such as isoamyl nitrite, picrotin and khayanthone were present in higher abundance relative to other minor constituents indicated by the LCMS data. It is to note that picrotin is a poisonous crystalline plant compound and is the saturated form of plant toxin known as picrotoxin.

4.3. Objective-3: Selection and purification of the most potent bioactive crude leaf extract, evaluation of antimicrobial activity its purified fractions against selected bacterial and fungal strains with subsequent selection of fraction with optimum antimicrobial activity and study of mechanism of antimicrobial action.

In accordance with the results obtained from the previous sections, the best and efficacious plant extract was purified and further analysed for its antimicrobial and antioxidant properties.

4.3.1. Column chromatography of plant fraction

The ethanolic extract of *E.agallocha* was subjected to silica gel column chromatography (Fig.4.19 a-b) and the eluted fractions were analysed by TLC (Fig.4.19.c).



(a) (b) (c)

Fig.4.19- (a) Separation of the crude ethanolic plant extract of *E.agallocha* using silica gel column chromatography; (b) Fractions eluted and bioactivity evaluation by TLC (c) TLC of the three bioactive fractions.

- The ethanolic extract of *E. agallocha* subjected to column chromatography yielded 12 fractions.
- Fractions with same R_f were pooled down.
- Out of the 12 eluted fractions, 3 exhibited distinct R_f values of 0.14, 0.127 and 0.09, (denoted as F1, F2 and F3 respectively) indicating the probable bioactivity of the fractions.
- These three fractions were evaluated for further bioactivity to demarcate the specific fraction with potential antimicrobial and anticancerous properties

4.3.2. Antimicrobial activity of the eluted plant fractions F1, F2 and F3.

The eluted fractions F1, F2 and F3 with R_f values 0.14, 0.127 and 0.09 respectively, were further used in various combinations (to consider synergistic activity, if any) to study their antimicrobial activity against two selected nosocomial, multidrug resistant strains of *Salmonella typhi* and *Candida parapsilosis.* Thereafter, the most potent fraction was selected for further investigations. The results are depicted in Fig. 4.20-4.23.

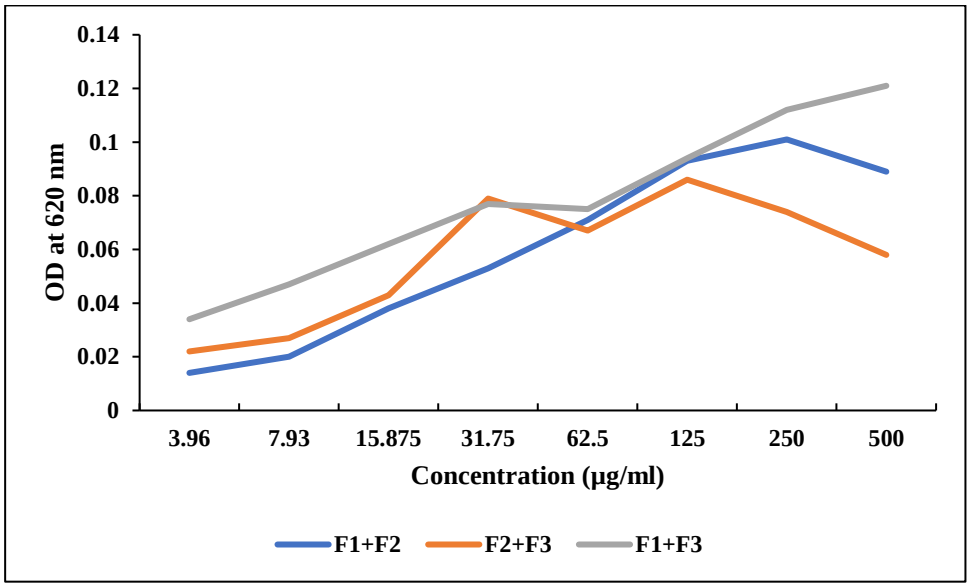


Fig.4.20: Graph representing the antibacterial activity of the eluted purified extract fractions of *E. agallocha* in various combinations against a multidrug resistant strain of *Salmonella typhi.*

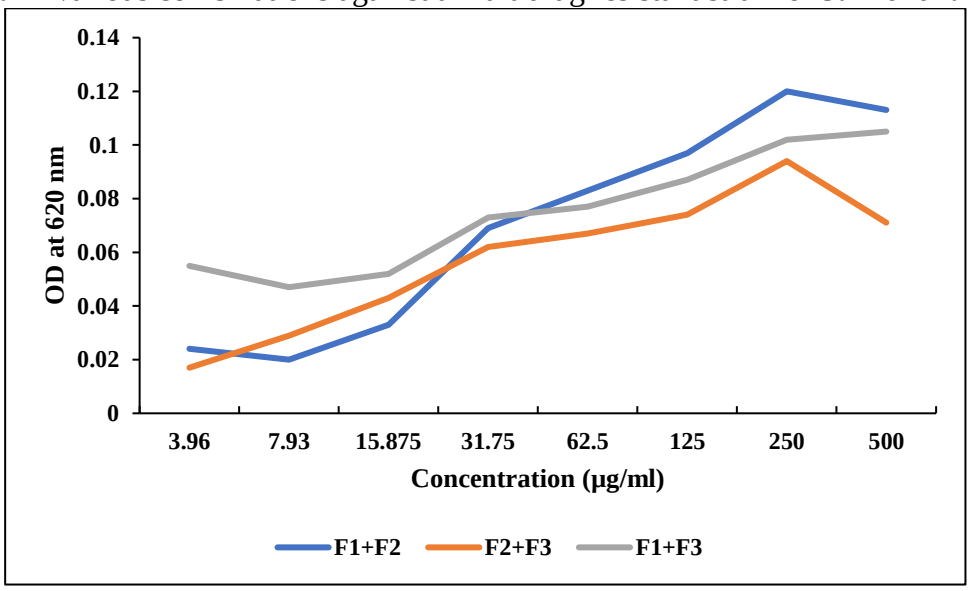


Fig.4.21: Graph representing the antifungal activity of the eluted purified extract fractions of *E. agallocha* in various combinations against a multidrug resistant strain of *Candida parapsilosis.*

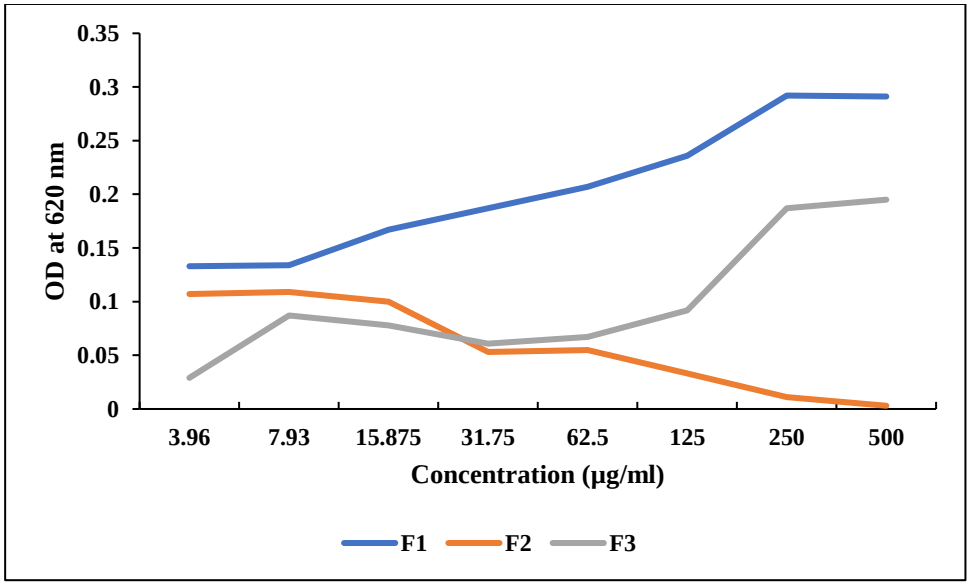


Fig.4.22: Graph representing the antibacterial activity of the individual eluted purified extract fractions of *E. agallocha* against the multidrug resistant strain of *S.typhi.*

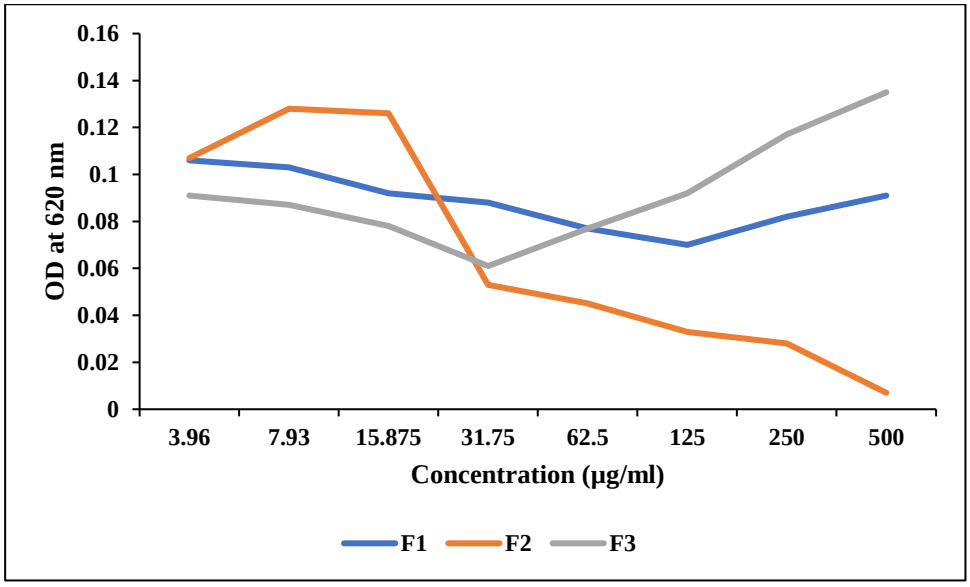


Fig.4.23: Graph representing the antifungal activity of the individual eluted purified extract fractions of *E. agallocha* against the multidrug resistant strain of *C.parapsilosis.*

Inference:

- The three fractions-F1, F2 and F3, in various combinations as well as individually-were tested against *S. typhi* and *C. parapsilosis*. When treated in combination, fractions 2 and 3 produced more pronounced inhibitory antimicrobial activity. Hence, further assessment was required to be specific about the fraction that possessed the maximum bioactivity.

- Thereby, the individual fractions were tested similarly. It was noted that fraction 2 produced significant antibacterial and antifungal effect in *S. typhi* and *C. parapsilosis* with MIC values of 8µg/ml and 14.5µg/ml respectively, which was quite remarkable.

- Also, this fraction produced a significant inhibitory action against an MDR strain of *S. typhi* [resistant to Cefotaxime (30 µg), Levofloxacine (30 µg), Aztreonam (30 µg), Imipenem (10 µg), Ampicillin (30 µg) and Ceftazidime (30 µg) with an MIC value of 15 µg/ml. Inhibitory activity against the resistant strain of *C.parapsilosis* was also observed. However, the MIC was slightly higher in this case (31 µg/ml).

- This indicates that the purified fraction F2 has promising bioactivity that may be exploited to monitor its associated mechanism of action followed by its characterization and identification.

Hence, hereafter, the **fraction F2** was further analysed to understand its mechanism of antimicrobial action.

4.3.3. Antibiotic and antifungal drug susceptibility of *S.typhi* and *C.parapsilosis* respectively, after treatment with eluted fraction F2

The antibiotic sensitivity assay was carried out using disc diffusion method after treatment of *S.typhi* and *C.parapsilosis* with the eluted fraction F2 of ethanolic extract of *E.agallocha*. The results are depicted in Fig.4.24 and Fig.4.25, with antibiotic and antifungal drug sensitivity pattern represented in Table 4.11 and 4.12 respectively.

Fig.4.24: Effect on antibiotic sensitivity of *S.typhi* after treatment with F2 fraction of *E.agallocha.*

Table 4.11: Change in MIC of antibacterial drugs against *S. typhi* strain treated with F2.

Sl. No.	**Antibacterial drug**	**MIC (in µg/ml) of *S. typhi***	
		Without plant extract treatment	**With plant extract treatment**
1.	Ampicillin	>32 (R)*	30.25±1.40 (SSD)*
2.	Aztreonam	>16 (R)	8.13±1.38 (SSD)
3.	Cefotaxime	>4 (R)	2.43±0.50 (IS)*
4.	Ceftazidime	>16 (R)	34±29.28 (R)
5.	Chloramphenicol	>32 (R)	28.28±1.1 (IS)
6.	Imipenem	>4 (R)	3.21±0.25 (SSD)

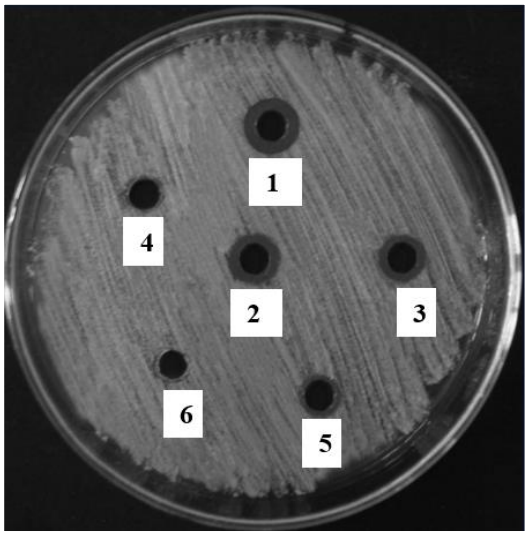


Fig.4.25: Effect on antifungal drug sensitivity of *C.parapsilosis* after treatment with F2 fraction of *E.agallocha.*

Table 4.12: Change in MIC of antifungal drugs against *C.parapsilosis* strain treated with F2.

Sl. No.	**Antifungal drug**	**MIC (in µg/ml) of C. parapsilosis**	
		Without plant extract treatment	**With plant extract treatment**
1.	Amphotericin B	>5 (R)*	3.8±1.40 (S)*
2.	Caspofungin	>11.5 (R)	8.13±1.18 (SSD)
3.	Fluconazole	>10.8 (R)	10.45±0.50 (R)*
4.	Flucytosine	>35 (R)	32±1.5 (R)
5.	Micafungin	>10 (R)	16.24±1.28 (R)
6.	Voriconazole	>2.5 (R)	4.25±0.3 (SSD)

Inference:

- It was previously seen that F2 effectively lowers the MIC against *S. typhi* and *C. parapsilosis*.
- To check its effectivity in synergism with antibiotics, the test microorganisms were first treated with the F2 fraction for 24 hrs and then subjected to antibiotic treatment.
- It was interesting to note that on treating the *S. typhi* with the MIC of the plant extract, the bacteria became intermediately susceptible to a few antibacterial drugs as observed by MIC values (Table 2).
- The interpretations were made according to CLSI standards wherein a bacterial strain is said to be "susceptible-dose dependent" when it is sensitive to a given antibacterial drug and its growth is inhibited with a probability of therapeutic success but multiple approved dosing options have to be checked while "intermediate sensitivity" means that the bacteria is inhibited *in vitro* by a concentration of the antibacterial drug that is associated with an uncertain therapeutic effect.
- Though none of the strains became absolutely sensitive to the antibacterial drugs but became susceptible to a certain extent depending upon the dose of the extract and the antibacterial drug taken together.
- The antifungal drug sensitivity was comparably less pronounced than the antibacterial drug sensitivity. However, it is of notable importance that *C.parapsilosis,* that was highly resistant to all these drugs earlier, became sensitive to Amphotericin B and also to caspofungin and voriconazole to a certain extent.

4.3.4. Study of mechanism of antimicrobial action of the bioactive plant fraction F2.

In accordance with the results obtained from the antimicrobial activity assay, the eluted fraction F2 was observed to have the most pronounced inhibitory action against *Salmonella typhi* and *Candida parapsilosis*. Hence, further study was undertaken to understand the mechanism of action of the antimicrobial activity being exhibited by F2 against these specific microbes. This was mainly carried out by observing the effect of F2 on the membrane permeability and ROS generation in the bacterial and fungal cells, effect on genomic DNA, effect on protein profile. The antifungal mechanism of action was further studied by observing effect on secreted aspartyl proteinases and lipolytic activity followed by observing the effect on fungal biofilm formation. In all the cases, the MIC dosage of F2 against the respective microbes (determined previously) were used for the various treatments. Antibacterial/antifungal drug and F2, when used in combination, (to examine synergistic effect, if any) were used in the ratio 1:1 (v/v).

(I) Effect on membrane by propidium iodide staining

The membrane damage was observed after propidium iodide staining of *S.typhi* and *C.parapsilosis* cells treated with the respective MIC dose of F2 fraction of ethanolic extract of *E.agallocha* for 24 hours. The observations are depicted in Fig.4.26 and Fig.4.27 respectively.

(I.a) Effect of F2, Ampicillin and F2+Ampicillin on membrane permeability of *S.typhi*

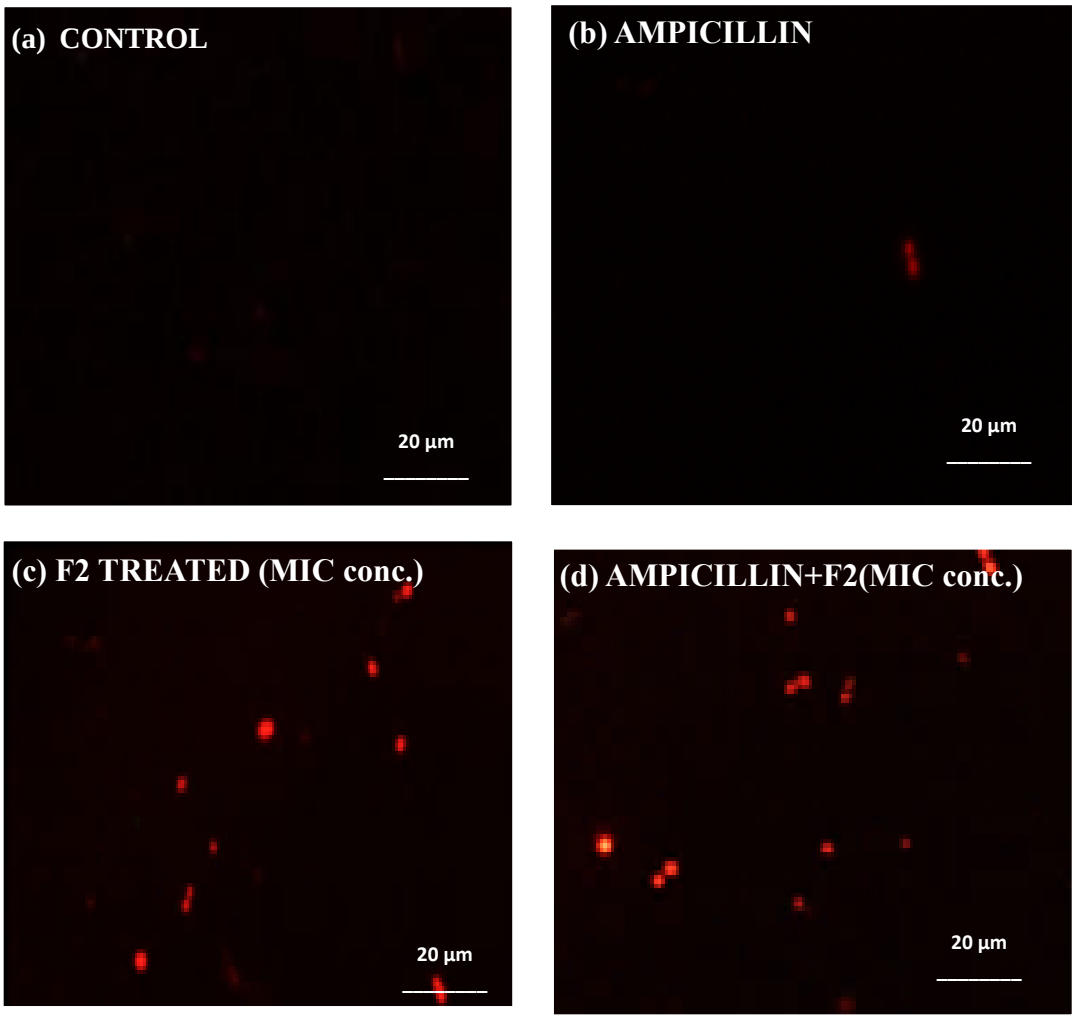


Fig.4.26: Membrane damage in *S.typhi* with (a) no treatment (b) Ampicillin treatment (c) F2 treatment and (d) F2+Ampicillin treatment, as observed by Propidium iodide staining method.

- Control cells showed no fluorescence, confirming the membrane was intact in the absence of plant fraction/drug being tested. The F2 plant fraction (at MIC conc. of 30 µg/ml) was able to induce damage in the membrane, to allow PI to pass through the membrane and emit fluorescence.
- As the image (in Fig.4.26) indicates, Ampicillin alone was not able to produce pronounced bacterial membrane damage. However, when applied along with the plant fraction, produced increased fluorescence. Thus, it could be said that the plant fraction F2 may synergistically act with the antibiotic to induce bacterial membrane damage and make it susceptible to the antibiotic.

(I.b) Effect of F2, Amphotericin B and F2+Amphotericin B on membrane permeability of *C.parapsilosis*

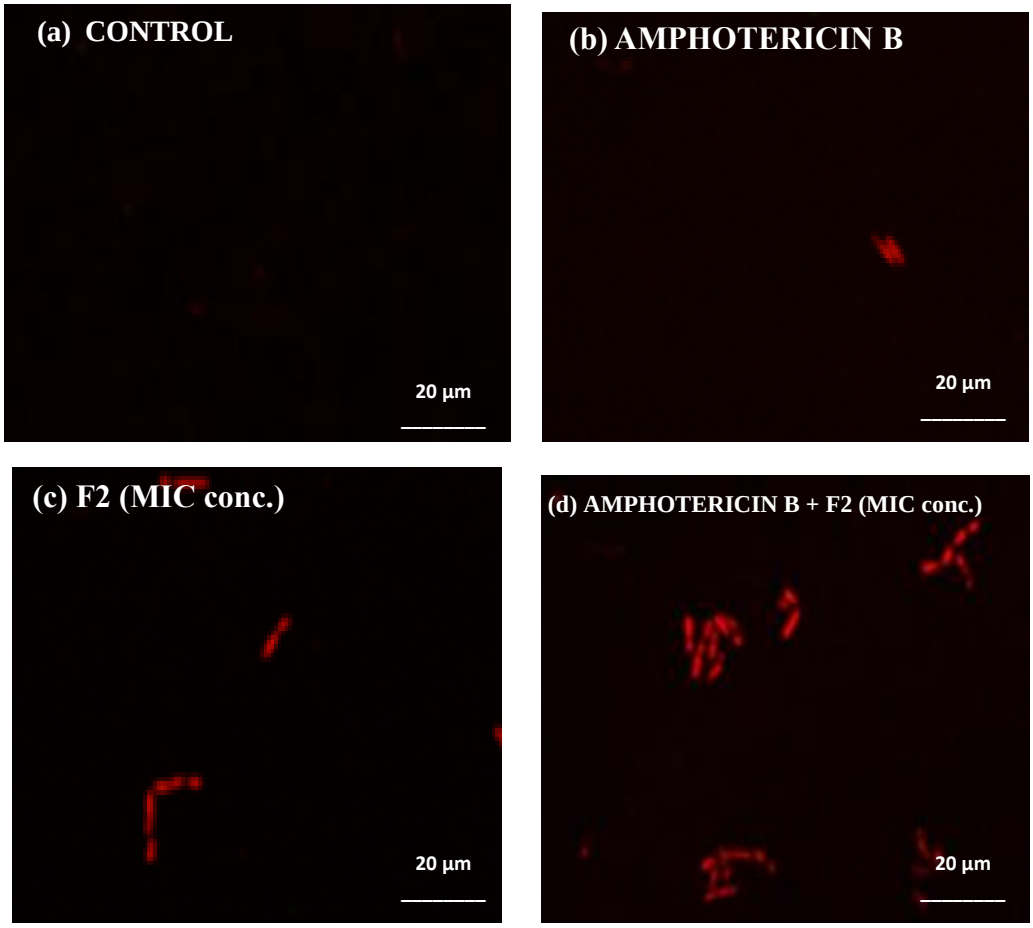


Fig.4.27: Membrane damage in *C.parapsilosis* with (a) no treatment (b) Amphotericin B treatment (c) F2 treatment and (d) F2+Amphotericin B treatment, as observed by Propidium iodide staining method.

- Control cells showed no fluorescence, confirming the membrane was intact in the absence of plant fraction/drug being tested.
- The F2 plant fraction (at MIC conc.of 30 µg/ml) was able to induce damage in the membrane, to allow PI to pass by the membrane and emit fluorescence. The F2 fraction probably reduces the thickness of the fungal cell membrane.
- As the image (Fig.4.27) indicates, Amphotericin B alone was not able to produce pronounced bacterial membrane damage. However, when applied along with the plant fraction, produced increased fluorescence. Thus, it could be said that the plant fraction may synergistically act with the antibiotic to induce bacterial membrane damage. Thereby, F2 may facilitate and enhance the antifungal action of Amphotericin B.

(II) ROS Mediated cellular damage by DCFH-DA staining

The effect of the plant extract fraction F2 on the production of ROS in the bacterial and fungal cells was observed by DCFH-DA staining method and the results are depicted in Fig.4.28 and Fig.4.29 respectively.

(II.a) Effect of F2, Ampicillin and F2+Ampicillin treatment on ROS production in *S.typhi*

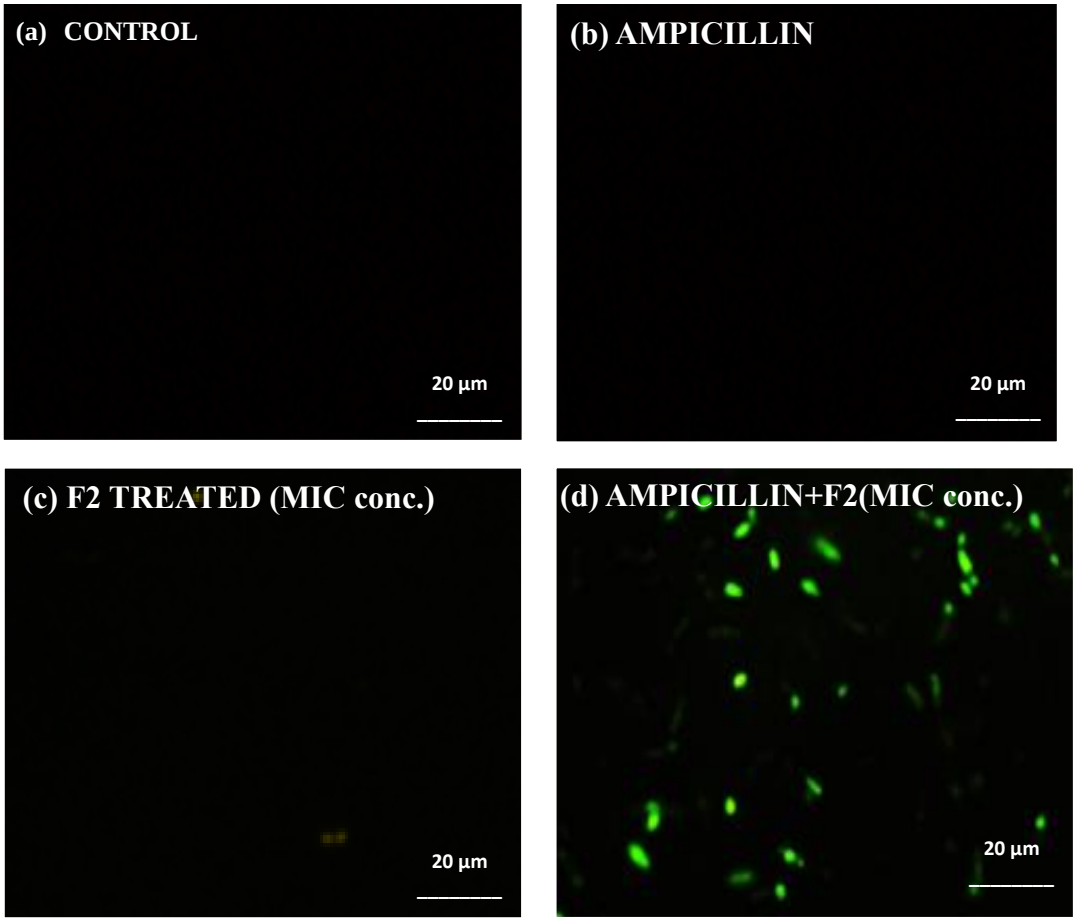


Fig.4.28: ROS production in *S.typhi* with (a) no treatment (b) Ampicillin treatment (c) F2 treatment and (d) F2+Ampicillin treatment, as observed by DCFH-DA staining method.

Inference

- Control cells showed no fluorescence indicating no ROS generation. Ampicillin treatment also did not produce observable fluorescence indicating absence of ROS production.
- F2 plant fraction treatment (30 µg/ml) produced a very weak fluorescence, while when treated along with Ampicillin, produced prominent green fluorescence, indicating the production of ROS within the F2 treated bacterial cell. ROS-mediated damage was thus, more pronounced when F2 and Ampicillin were treated together.

(II.b) Effect of F2, Amphotericin B and F2+Amphotericin B treatment on ROS production in *C.parapsilosis*

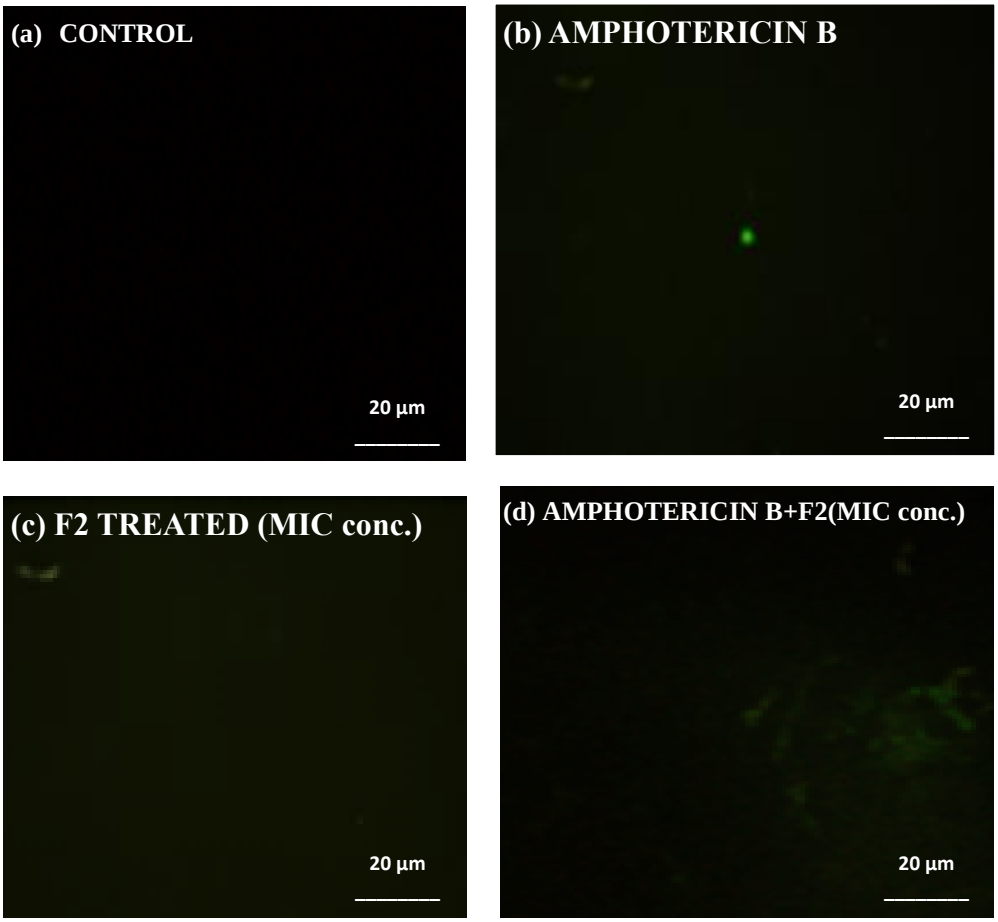


Fig.4.29: ROS production in *C.parapsilosis* with (a) no treatment (b) Ampicillin treatment (c) F2 treatment and (d) F2+Ampicillin treatment, as observed by DCFH-DA staining method.

Inference

- No significant ROS production observed when the fungal cells were treated with the plant fraction/antifungal agent individually or in combination, thereby indicating that the antifungal action may be facilitated by some other mechanism.

(III) Effect on genomic DNA

The effect on the genomic DNA of *S.typhi* and *C.parapsilosis*, on treatment with the plant extract fraction F2 treatment and antimicrobial agents, was observed by standard genomic DNA isolation methods, to check whether ROS generation or membrane damage caused some detrimental effect on the microbial genomic DNA. The results were observed by agarose gel electrophoresis, depicted in Fig.4.30 and Fig.4.31 respectively.

IIIa. Effect of F2 treatment on genomic DNA of *S.typhi*

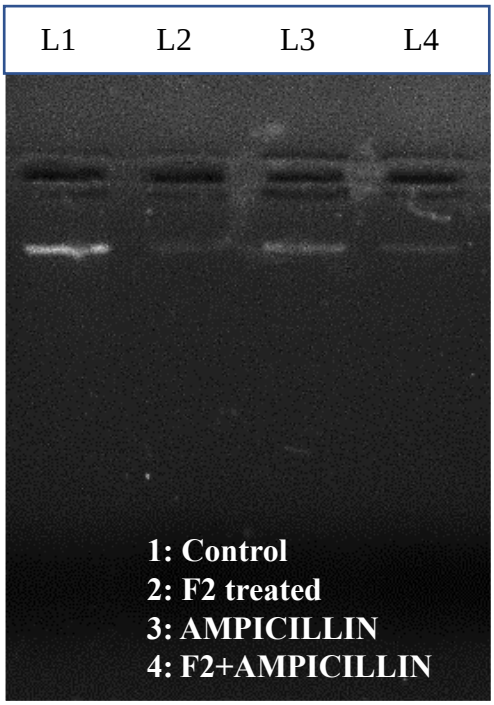


Fig.4.30: Genomic DNA of *S.typhi* visualized by agarose gel electrophoresis after treatment with F2 and Ampicillin.

Inference

- There was no significant effect on genomic DNA of the treated bacteria was observed. However, when treated with the plant fraction and in combination with the antibiotic, the bands produced were comparatively of lower intensity than the control.

IIIb. Effect of F2 treatment on genomic DNA of *C.parapsilosis*

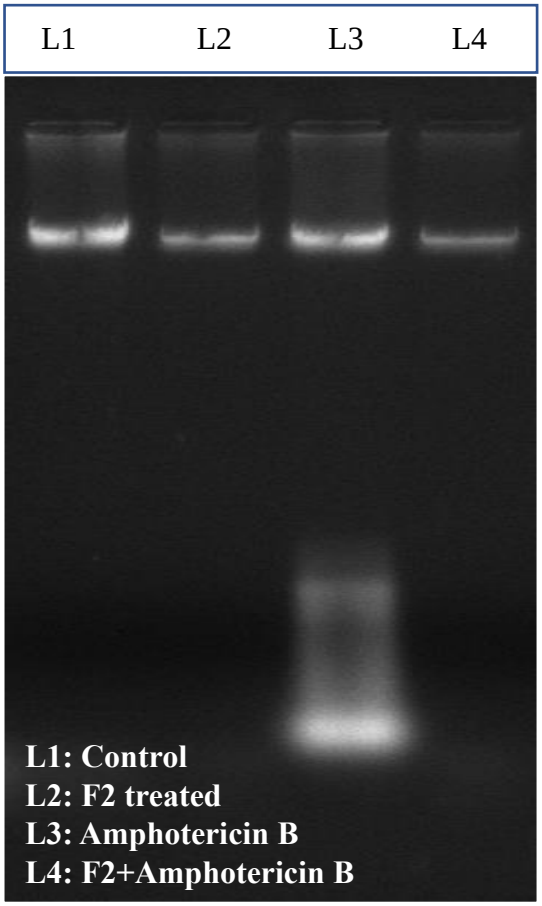


Fig.4.31: Genomic DNA of *C.parapsilosis* visualized by agarose gel electrophoresis after treatment with F2 and Ampicillin.

Inference

- There was no significant effect on DNA was observed as the band obtained were distinct without any damage or smearing.
- Hence, it can be said that the extract probably had no detrimental effect on the fungal genomic DNA.

(IV) Effect on protein profile of *S.typhi* and *C.parapsilosis*

The effect on protein profile of the microbes under study, was observed to check whether membrane damage or ROS generation caused any significant change in the expression of any protein(s), that would aid in better understanding of the antimicrobial mode of action exhibited by F2.

IVa. Effect on protein profile of *S. typhi* by SDS-PAGE analysis.

The effect on the protein profile of *S.typhi* was studied by SDS-PAGE and the observations are depicted in Fig.4.32.

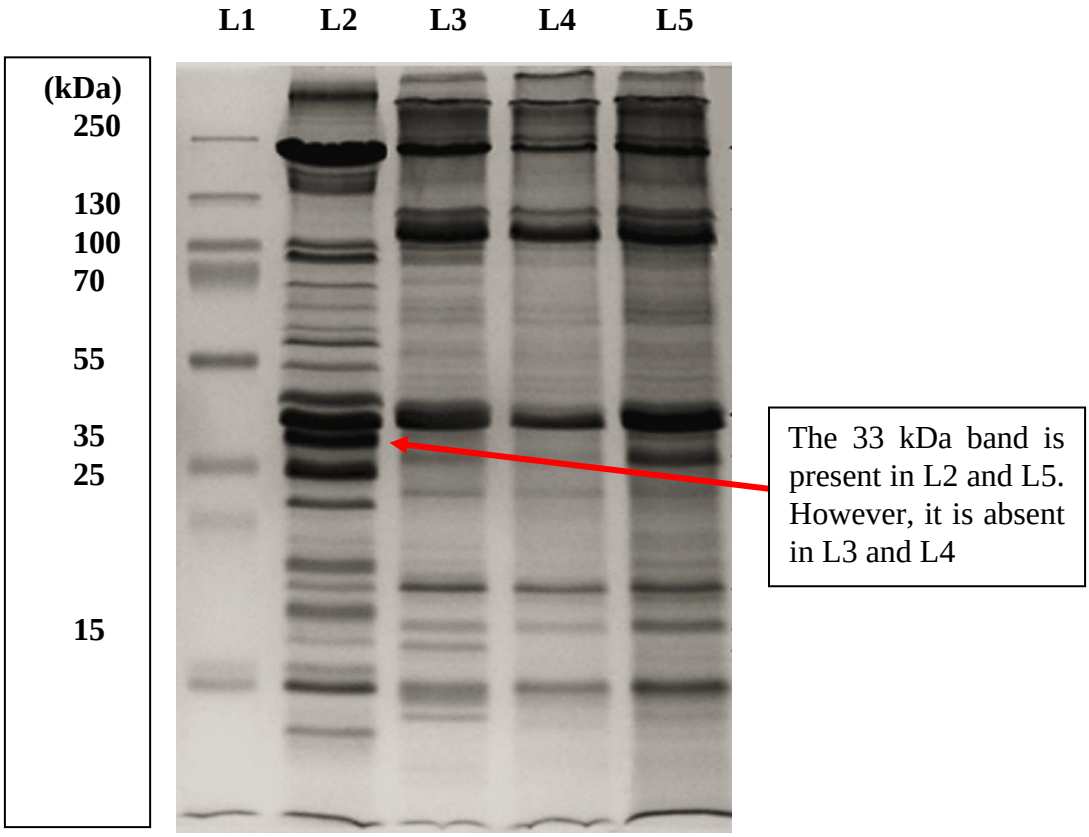


Fig.4.32: Effect of F2 fraction of ethanolic extract of *E.agallocha* on protein profile of *S.typhi*. L1:Marker; L2: control (untreated *S.typhi*); L3: F2 treated; L4: F2 and Ampicillin treated; L5: Ampicillin treated (all treatments for 24 hrs.)

Inference:

- The total protein of *S.typhi* was isolated and was analyzed by SDS PAGE and the bands were studied.
- The resistant strain shows the presence of a 33kDa band in Lane 1 (Fig. 4.32) and it is thought to confer antibiotic resistance by increasing the membrane integrity and escalating drug efflux.
- When treated with F2 fraction for 24 hours, this protein band was missing in both the cases (L3 and L4), indicating that the bioactive plant fraction is disrupting the membrane integrity by downregulation of the 33kDa protein expression, and probably this effect might might be leading to the sensitivity of the bacteria to the antibiotics.

IVb. Effect on protein profile of *C. parapsilosis* by SDS-PAGE analysis.

The effect on the protein profile of *C.parapsilosis* was studied by SDS-PAGE and the observations are depicted in Fig.4.33.

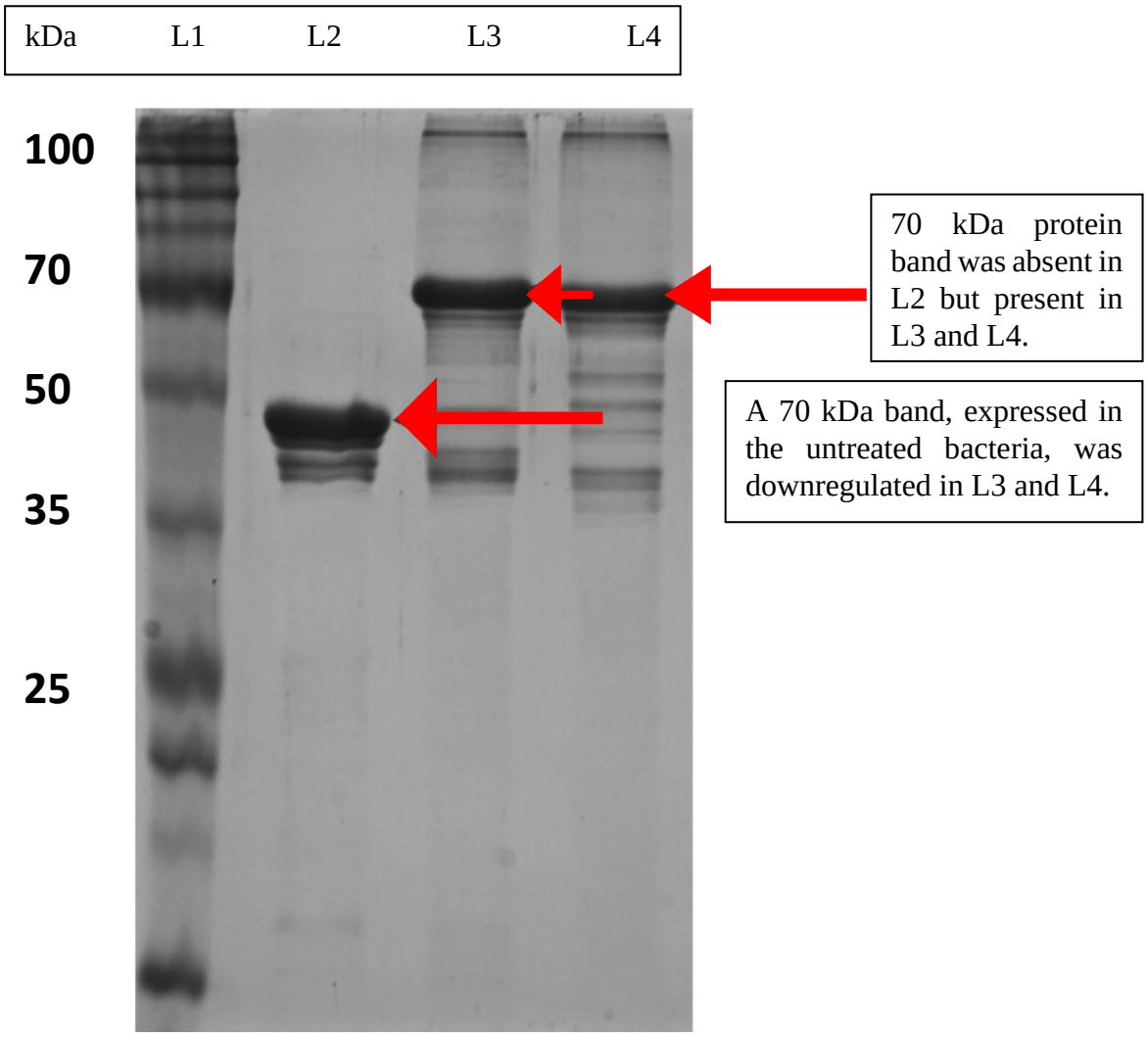


Fig.4.33: Effect of F2 fraction of ethanolic extract of *E.agallocha* on protein profile of *C.parapsilosis.* L1: Marker; L2: F2 treated; L3: Amphotericin treated; L4: No treatment (control) (all treatments for 24 hrs)

Inference:

- The 70 kDa band is missing in lane L1 while it is present in lanes L3 and L4.
- It might be said that downregulation of this protein may play a role in increasing the sensitivity of *C.parapsilosis* towards Amphotericin B, as was observed in the antimicrobial assay.

(V) Effect of F2 plant fraction on fungal Sap and Lipase activity

The effect of PE and antifungal agent Amphotericin B was observed on fungal extracellular enzymes Sap (secreted aspartyl proteinase) and lipase, as these extracellular enzymes are thought to confer virulence to pathogenic strains of most fungi. The results are graphically expressed in Fig.4.34 (a) and (b).

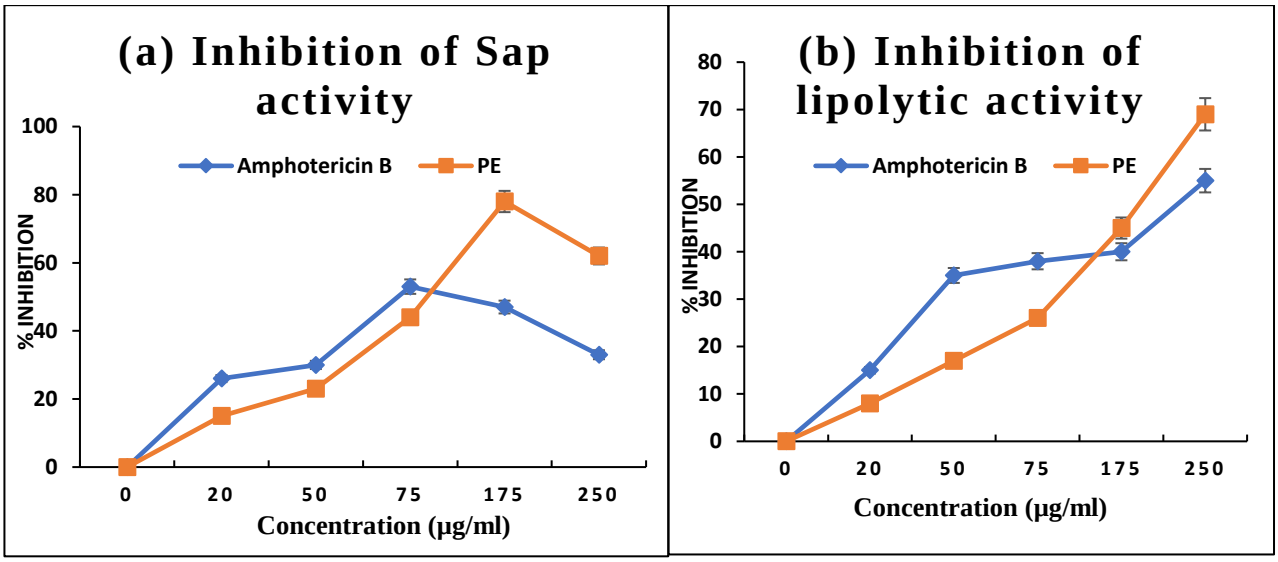


Fig.4.34: Inhibition of Sap and lipase activity in *C.parapsilosis* after treatment with F2. (PE refers to plant extract fraction F2).

Inference

- Aproximately, 75% inhibition of Sap (secreted aspartly proteinase) activity was observed at a concentration of around 175 µg/ml, when treated with the plant extract.
- At the same time, it could be inferred that this effect was not strictly dose dependent since at higher concentrations, the Sap activity was reduced only to a little extent. Amphotericin B, on the other hand, caused only a 55% reduction in Sap activity.
- The cultivation of *C. parapsilosis* with the plant extract at varied concentrations exhibited a dose dependent inhibition in lipase activity. The results thereby indicate that the inhibition of lipase activity may have a role in preventing *C.parapsilosis* from invading the host, its survival inside the host as well as induction of inflammatory responses.
- Overall, the present results suggest that the *in vitro* inhibition of such extracellular enzyme activity of *C.parapsilosis* might be realted to decreased pathogenecity and thereby, increased susceptibility to antifungal compounds, aiding the treatment process.

(VI) Effect on biofilm formation by Calcofluor staining method

The effect of F2 and Amphotericin B was tested on the fungal biofilm formation by Calcofluor white staining method and the results are depicted in Fig.4.35

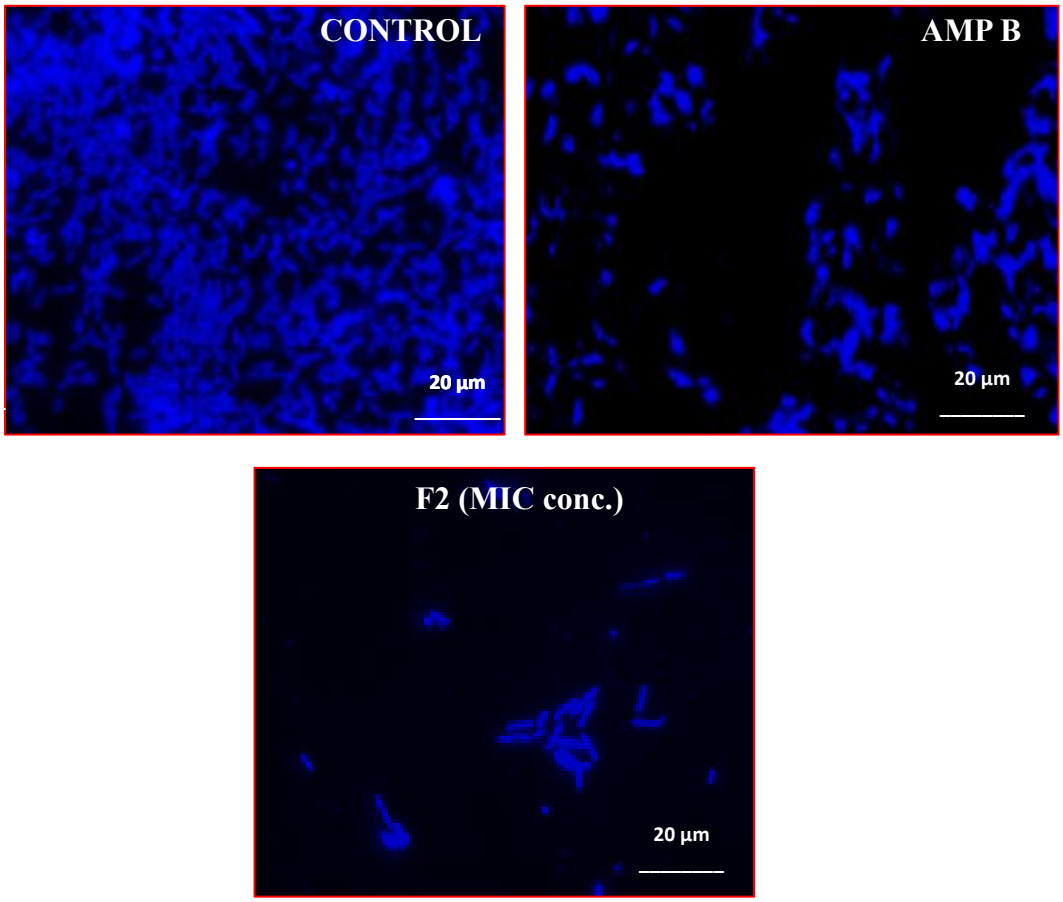


Fig.4.35: Effect of F2 and Amphotericin B on fungal biofilm formation (of *C.parapsilosis*) observed by Calcofluor white staing method (400X magnification).

Inference

- Calcofluor white staining method is a useful fluorescence microsopy method to enhance visualization of fungal elements in specimens for microscopic examination.
- The CFW non specifically binds to the chitin and cellulose components of the fungal cell wall and produces bright blue fluorescence. Used with Evans Blue as counterstain, the fungal elements fluoresce blue against a dark red/brown background (340-380 nm excitation, suppression filter 430 nm).
- In this study, Calcofluor staining observations revealed that the plant extract produced remarkable inhibition of biofilm formation in *C. parapsilosis*, because the overall fluorescence decreased on treatment with the plant extract fraction with respect to the untreated fungal cells.
- The result simultaneously indicates that the density of chitinous components of the fungi has been significantly reduced hinting that the biofilm framework has been destabilized. Amphotericin (30 µg/ml), an antifungal agent, also inhibited fungal biofilm formation to a certain extent. However, inhibition was more prominently observed when the fungal cells were treated with the plant extract fraction F2 (30 µg/ml).

Objective- 4: Evaluation of anticancer activity of the purified fractions, selection of fraction with optimum antimicrobial activity and study of mechanism of action.

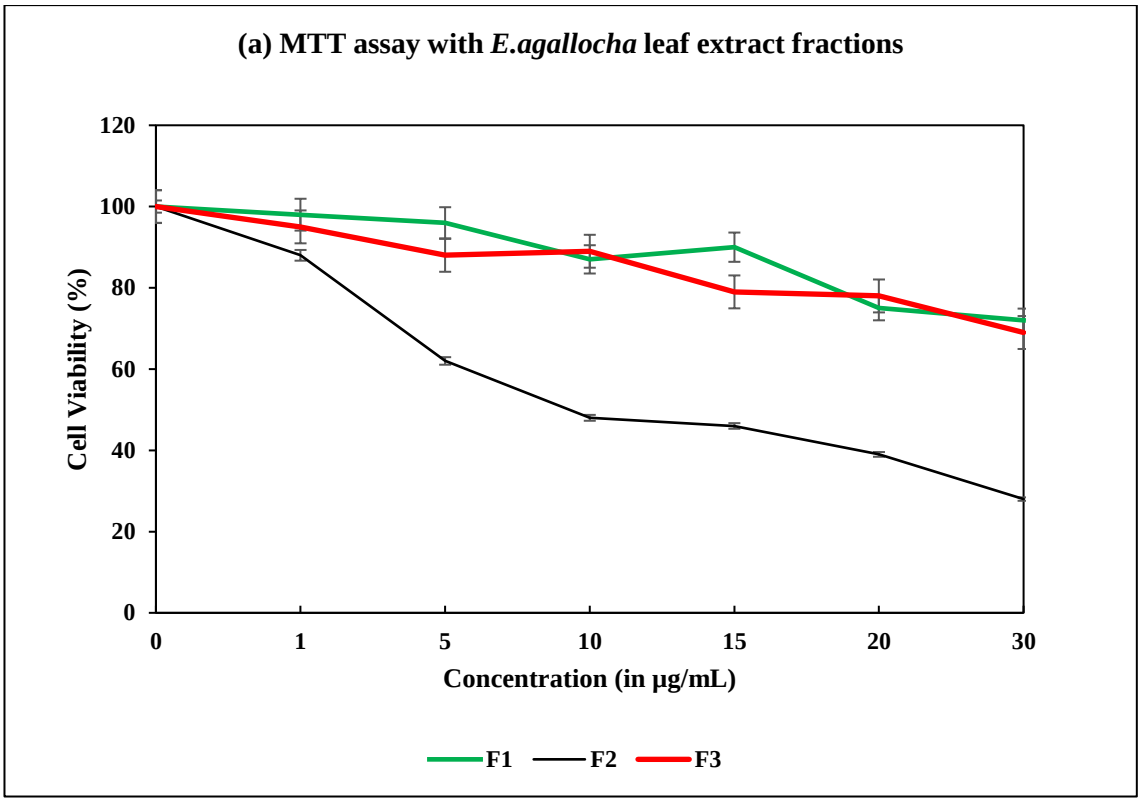
The anticancer activity of the F2 fraction (lyophilized before use and dissolved in water for anticancer study; stock concentration used was 1 mg/ml) was tested on SiHa cell line by MTT assay, autophagy, mitophagy, apoptosis, caspase-3 activity, proteasome inhibition and cell cycle analysis. The results are given in the following sections.

4.4.1. MTT assay: The *in vitro* antiproliferative potential of the bioactive plant fraction was determined against human SiHa cell line by MTT assay. The results are given in Table 4.13. and Fig.4.33 and 4.34.

Table 4.13: IC50 value of plant fractions F1, F2 and F3 along with cisplatin, against SiHa cell line

Compound	IC50 value
1. Control- Cisplatin, against SiHa cells	5.122 ± 0.004 µg/ml
2. *E. agallocha* fraction 1 against SiHa cells	> 30-40 µg/ml
3. *E. agallocha* fraction 2 against SiHa cells	15.538 ± 0.577 µg/ml
4. *E. agallocha* fraction 3 against SiHa cells	> 30- 35 µg/ml
5. *E. agallocha* fraction 2 against healthy PBMC cells	No toxicity; IC50 may lie much beyond > 250 µg/ml

Thus, based on the MTT result, F2 was studied for further analysis of its anticancer mechanism of action exhibited against SiHa cells.



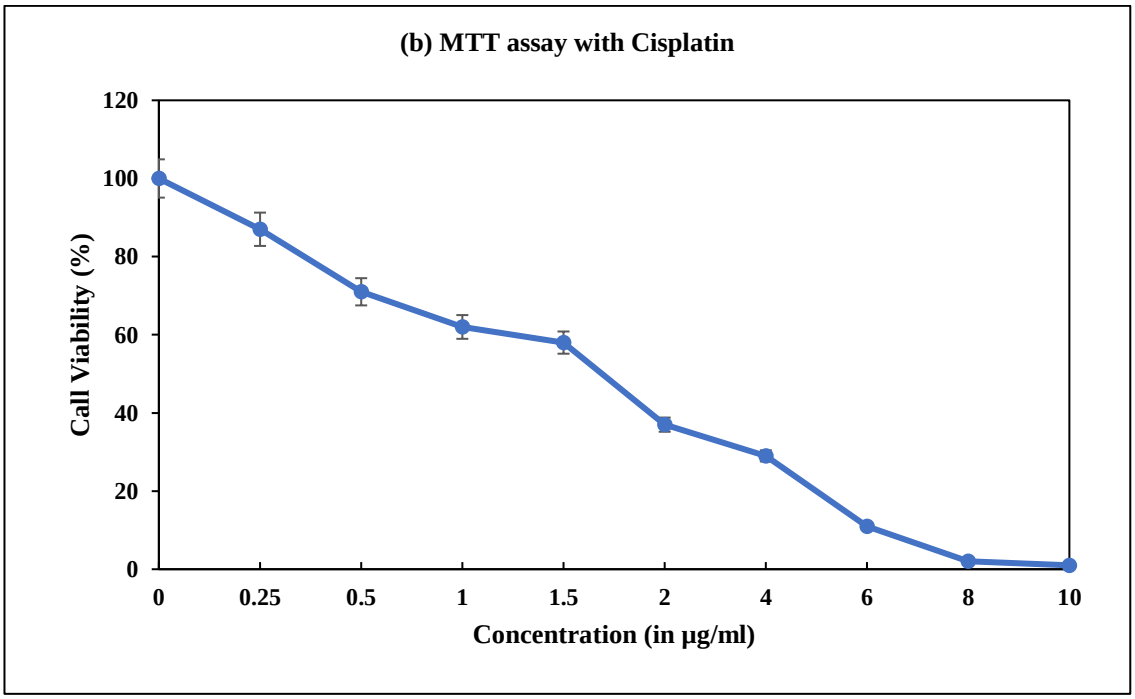


Fig.4.36: Graphs depicting the anticancer potential of the plant extract fractions by MTT assay of (a) *E.agallocha* extract fractions F1, F2 and F3 and (b) anticancer potential of Cisplatin.

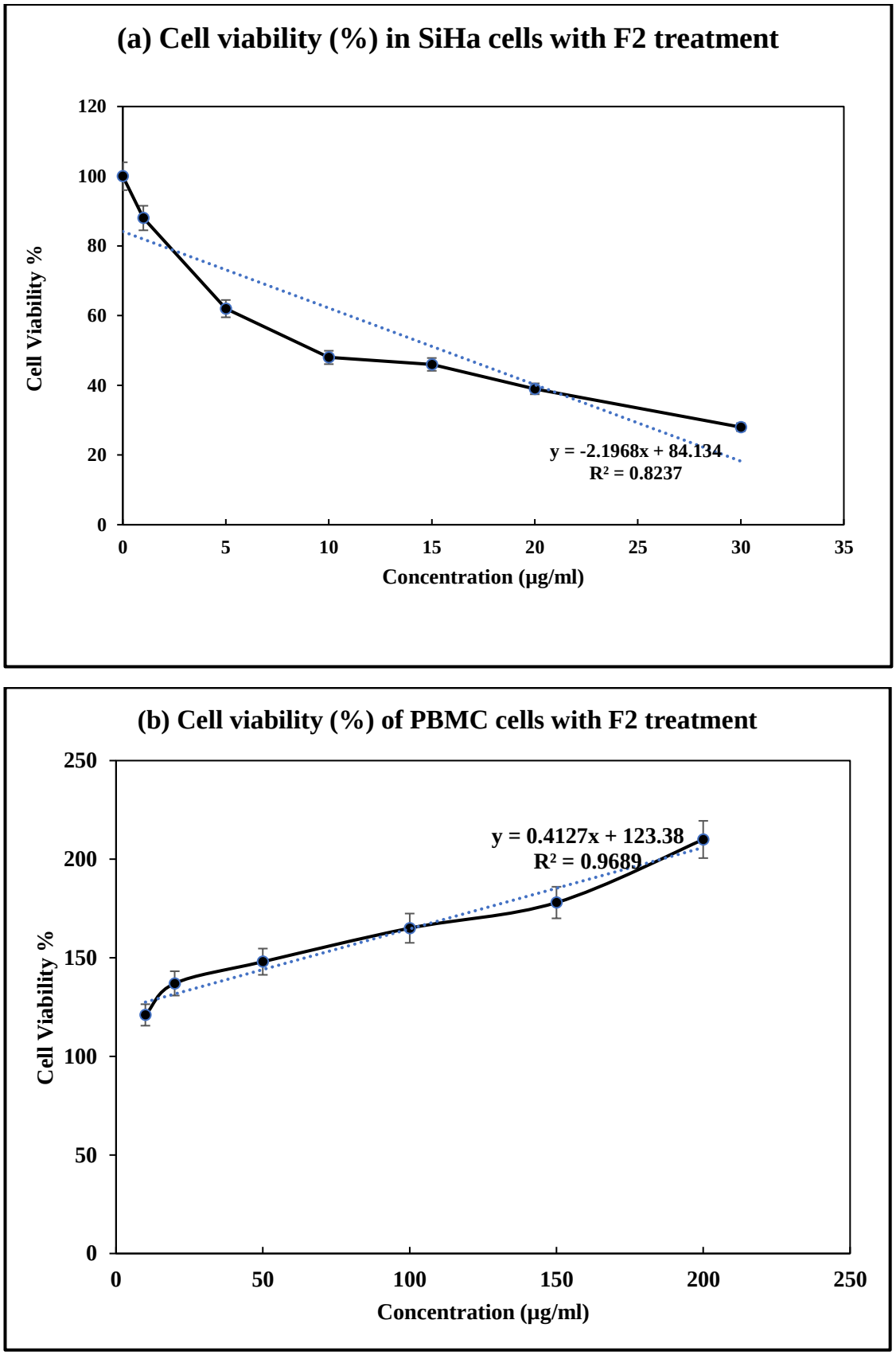


Fig.4.37: Cell viability of cancerous (a) (SiHa) and (b) non- cancerous (PBMC) cells on treatment with plant extract fraction F2.

Inference

- MTT assay results exhibited a remarkable cytotoxic activity of F2 against the SiHa cell line (Fig.4.36) while the other two fractions were not cytotoxic against SiHa cells within the range of concentration studied (Table 4.13).
- Cisplatin used as standard reference drug was observed to have an IC_{50} value of 5.122 ± 0.004 µg/ml (Fig.4.36b). The results of the MTT assay indicated that the half- maximal inhibitory concentration (IC_{50}) value of the F2 plant extract was about 15.538 µg/ml± 0.577 µg/ml (Fig.4.36a).
- Moreover, the F2 fraction induced a significant increase in cell proliferation when treated on healthy PBMC cells (Fig.4.37b), which is indicative of its selective toxicity. Thus, this extract would produce no toxicity in normal healthy cells in the applied dosage concentration (15µg/ml). This potent fraction possessing cytotoxic activity was thereafter subjected to LC-MS for identification of the bioactive compounds present.

4.4.2. Induction of autophagy indicated by LC3 puncta formation in SiHa cells

The puncta formation, a morphological character of cells undergoing apoptosis, was studied using fluorescence microscopy and the results are represented in Fig.4.38.

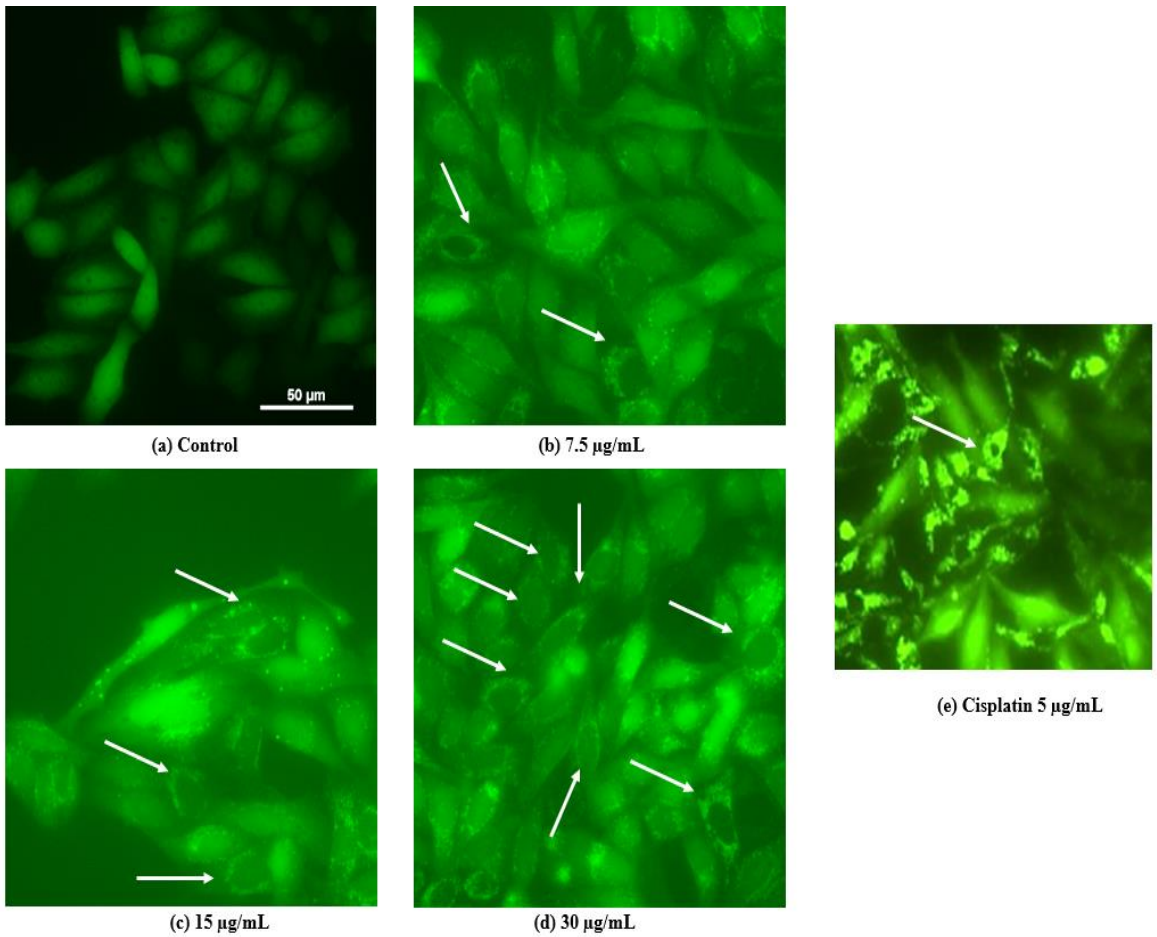


Fig.4.38: Induction of autophagy by F2, indicated by LC3 puncta formation (white arrow) in SiHa cells (400X magnification).

Inference

- The control cells (SiHa cells without plant extract treatment) showed absence of LC3 puncta formation while 24 hrs treatment of aqueous plant extract (7.5, 15, 30 µg/ml) showed increased LC3 Puncta formation in SiHa LC3- GFP cells in a dose dependent manner (hole/ puncta formation in cells, indicated by arrows in Fig. 4.38) clearly denotes the onset of autophagy in the SiHa cells, with increasing green fluorescence. Cisplatin also exhibits increased fluorescence but puncta formation is relatively lower, which further indicated more efficiency of the plant fraction F2 in inducing autophagy in SiHa cells.

4.4.3. Detection of Mitophagy through determination of MitoKeima ratio in SiHa cells

The Mt-Keima ratio was determined by fluorescence microscopy that exhibits a clear corelation with mitophagy induction. The results are represented in Fig.4.39.

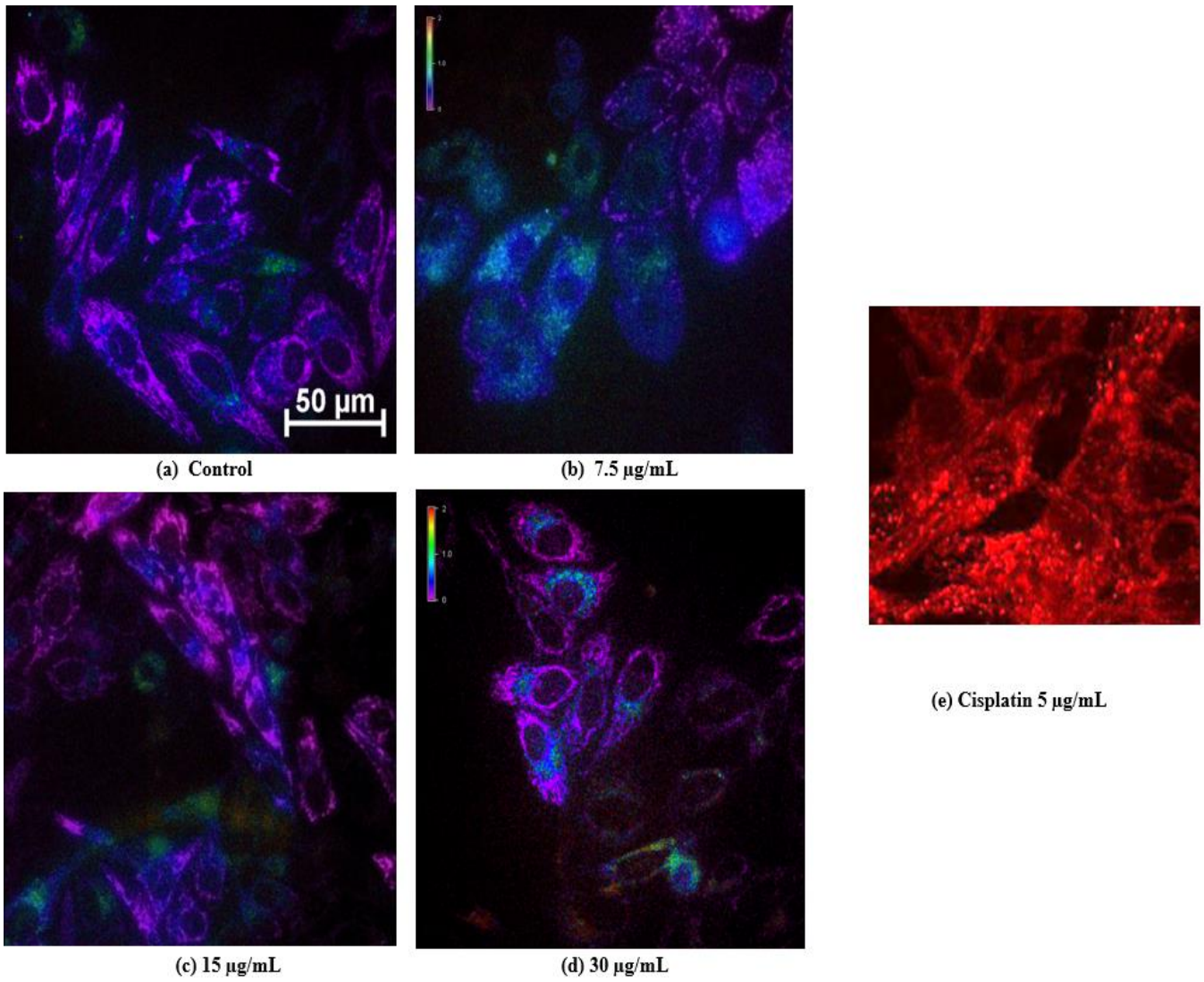


Fig.4.39: Detection of Mitophagy induced by F2 treatment, through determination of MitoKeima ratio in SiHa cells (400X magnification).

Inference

- The MitoKeima ratio (560nm/440 nm) is observed for inferring the results. Low MitoKeima Ratio indicates absence of mitophagy while a higher ratio implies the occurrence of mitophagy.

- The control cells showed low MitoKeima ratio. After 24 hrs treatment with aqueous plant extract (7.5, 15, 30 µg/ml), there was increased mitokeima ratio in SiHa MitoKeima cells in comparison to control cells, indicating the induction of mitophagy and permeabilization of the mitochondrial membrane in the SiHa cells (Fig.4.39).

- The activity of cisplatin is relatively much higher as observed by the increased MitoKeima ratio that showed almost all the cells undergoing mitophagy, marked by red fluorescence.

4.4.4. Smac- induced cytochrome c dependent activation of apoptosis

The Smac (Second Mitochondria-derived activator of caspases) induced cytochrome C dependent activation of apoptosis was observed in SiHa cells by fluorescence microscopy and the results are represented in Fig. 4.40.

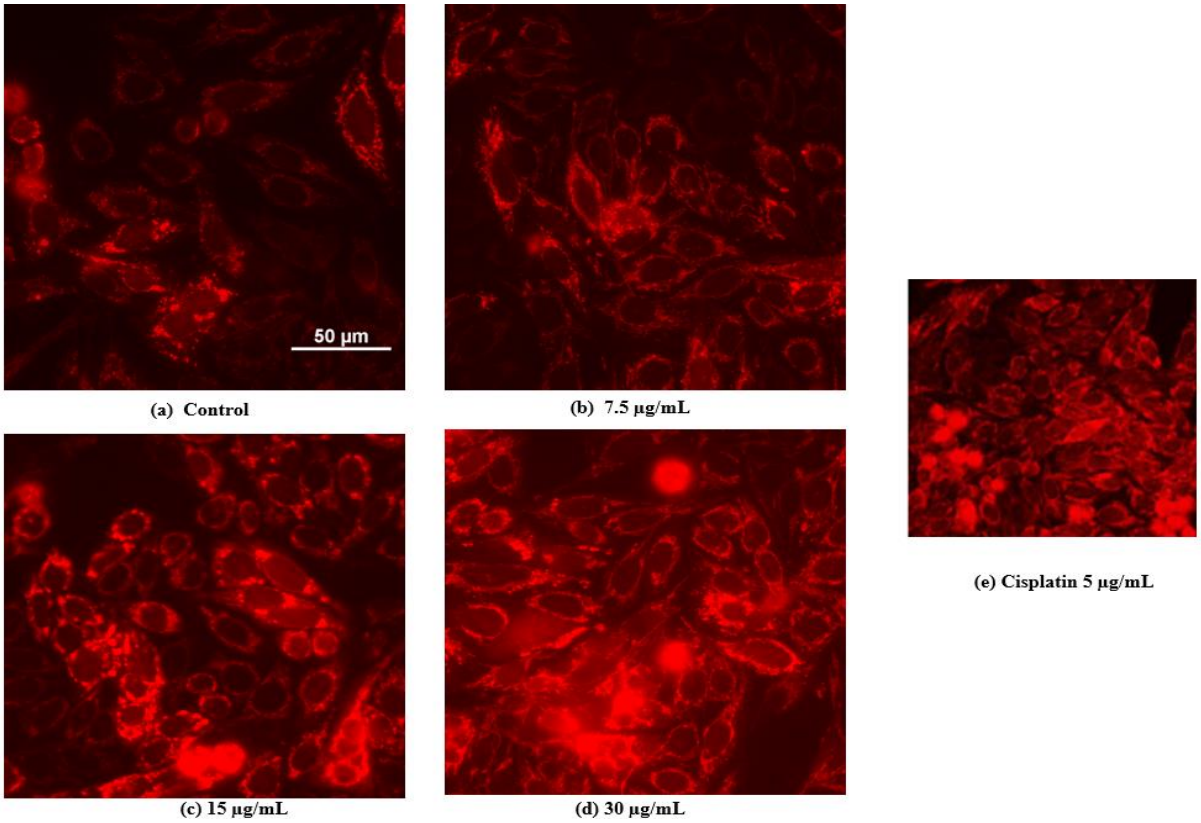


Fig.4.40: Induction of apoptosis by F2 treatment, visualized in smac-mCherry SiHa cells by fluorescence microscopy (400X magnification).

Inference

- The Smac (Second Mitochondria-derived activator of caspases) protein had been fluorescently labeled in order to trace its release, which in turn indicates the activation of apoptosis.
- Control cell showed intact mitochondria without Smac release. 24 hrs treatment of plant extracts (7.5, 15, 30 µg/ml) showed mild increase in Smac release when compared to normal SiHa Smac- mCherry Cells (Fig.4.40), indicating that autophagy and mitophagy consequently might lead to the apoptotic death of cells.

4.4.5. Caspase-3 assay by colorimetric assay

Caspase-3 activation was studied *in vitro* by a kit-based (BioVision Caspase-3 kit) colorimetric assay and the results are represented in Fig.4.41.

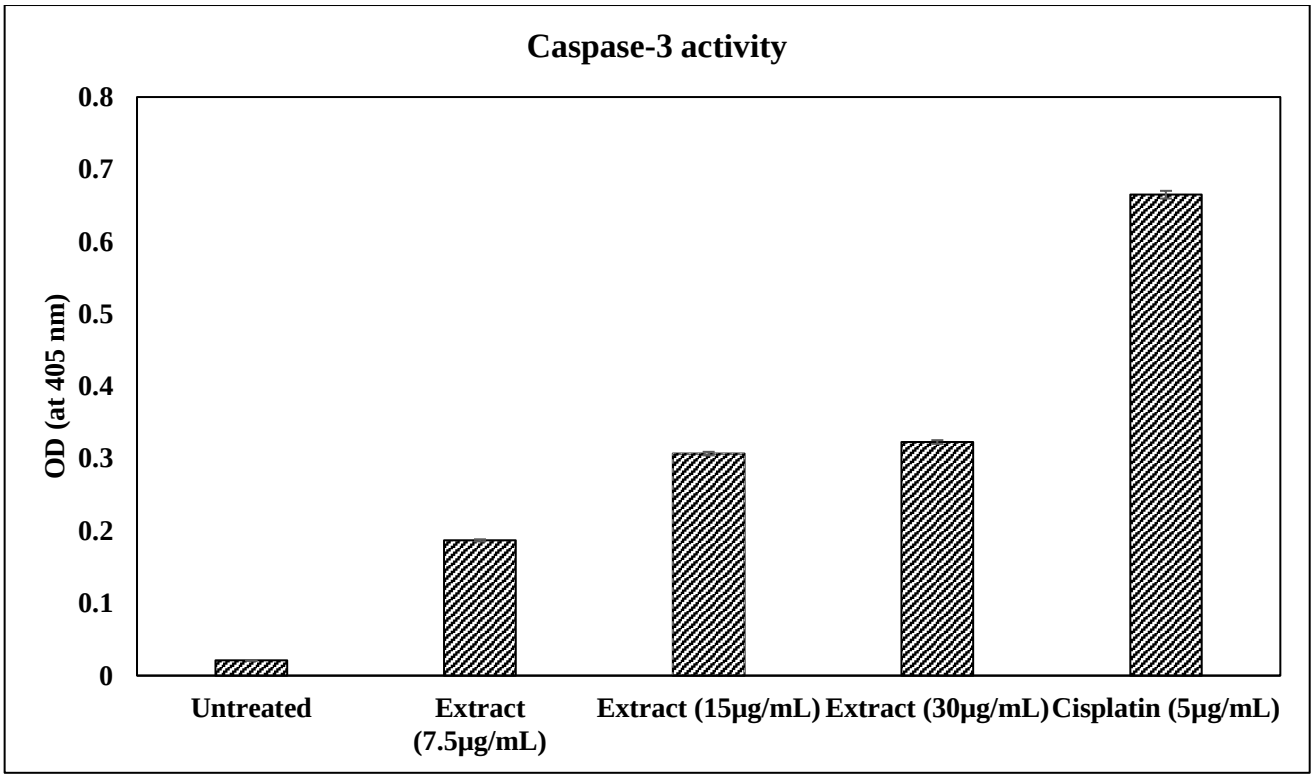


Fig.4.41: Caspase-3 assay results in SiHa cells treated with variable doses of F2.

Inference

- The results indicate that the caspase-3 content increases in SiHa cells in a dose-dependent manner (Fig.4.41), validating the imaging analysis observations.
- Induction of apoptosis by the plant extract indicates an increase in the DEVD-dependent protease activity that ultimately leads to apoptosis of the cancer cells, as activated caspase-3 in apoptotic cells cleave the substrate into free pNA which is determined spectrophotometrically at 405 nm.

4.4.6. Proteasome inhibition analysis

The proteasomal degradation of a GFP-dgn (degron composed of a specific region of p53 proetin) in SiHa cells is traced by fluorescence microscopy. Increase in fluorescence correlates to degradation of p53 and simultaneous tumor progression, while a decrease in fluorescence indicates inhibition of p53 degradation and thus, inhibition of tumor progression or proliferation (Fig.4.42).

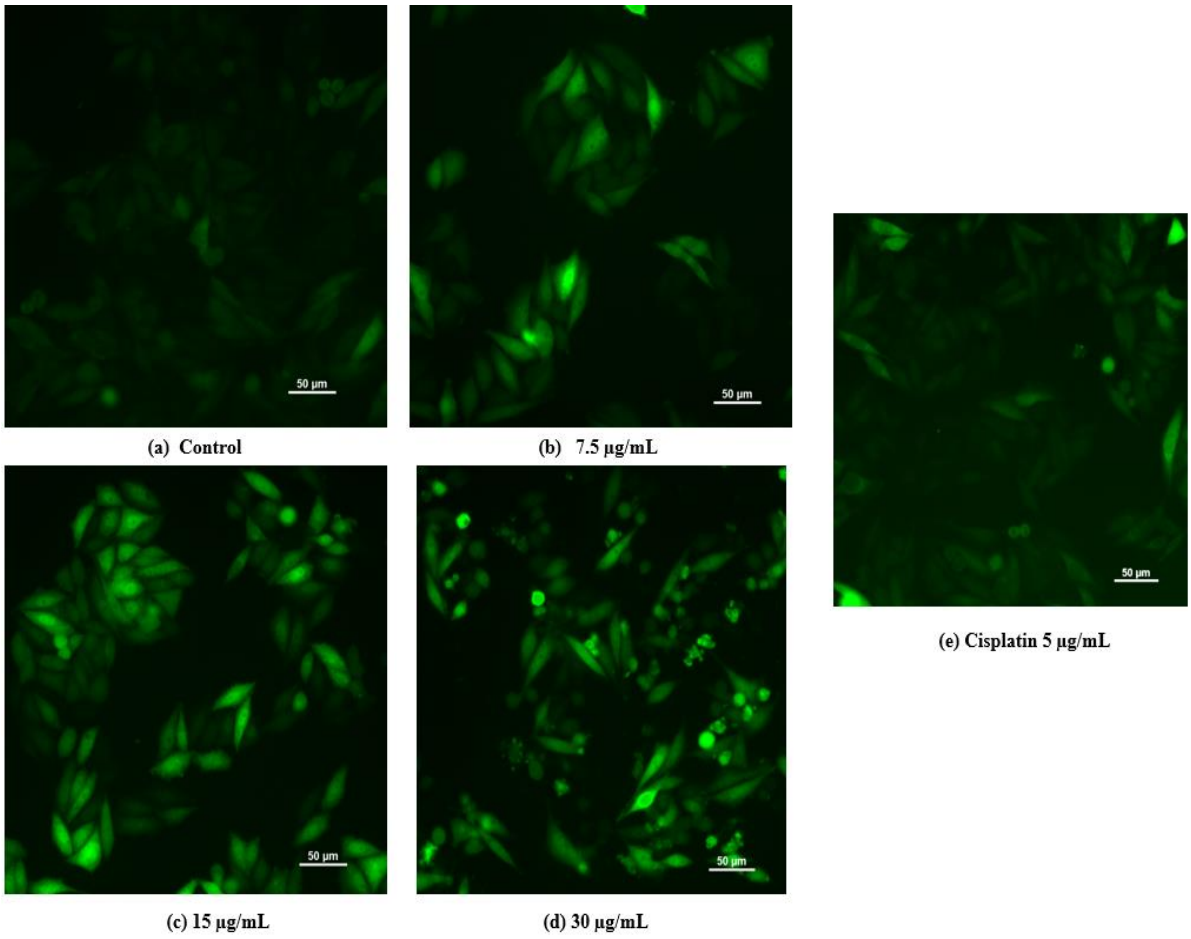


Fig.4.42: Inhibition of proteasomal degradation in GFP-dgn SiHa cells by F2 (400X magnification).

Inference

- The GFP-dgn fusion protein contains a specific sequence which is targeted for proteasomal degradation, which corresponds to a decrease in GFP fluorescence signal.
- Treatment of SiHa cells with aqueous plant extract (7.5, 15, 30 µg/ml) for 24 hours showed clear proteasomal inhibition of the protein in a dose dependent manner (Fig.4.42), indicated by the increased amount of fluorescence detected.

4.4.7. Cell cycle analysis

The cell cycle analysis of SiHa cells treated with F2 was analyzed by flow cytometry and the results are depicted in Fig.4.43 and Table 4.14.

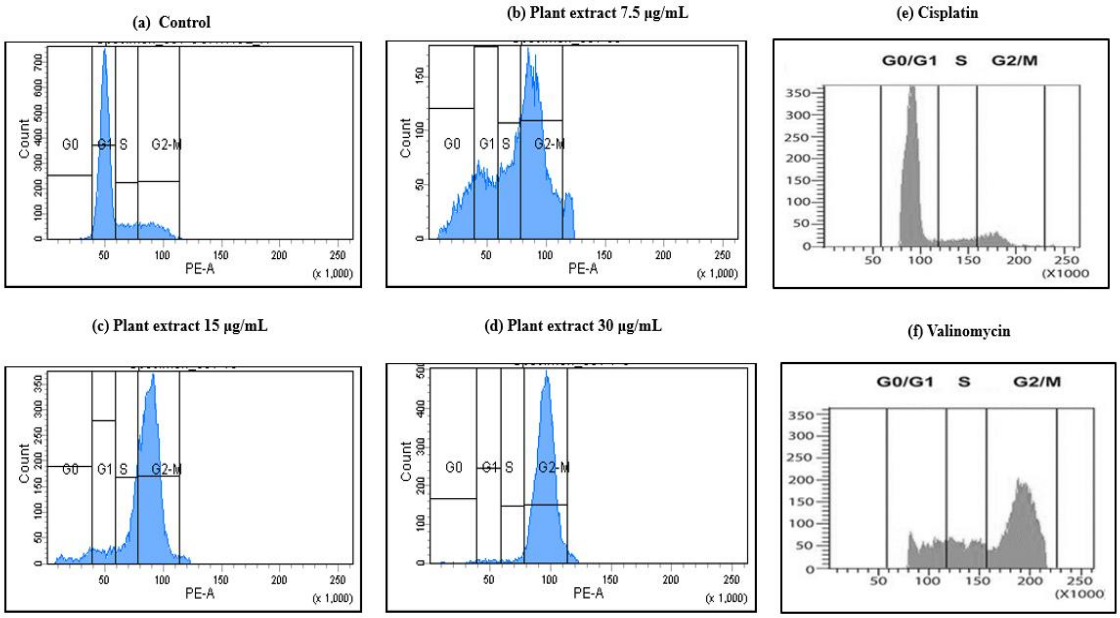


Fig.4.43: Cell cycle analysis of SiHa cells after treatment with F2, by flow cytometry

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Table 4.14: Cellular distribution (in %) in different cell cycle phases of SiHa cells after treatment with plant extract.

Conc. of extract (µg/ml)	Cellular distribution (in %) in the different cell cycle phases			
	G0	G1	S	G2/M
0 (no treatment)	1.0±0.15	40.5±1.0	6.4±0.02	8.9±0.15
7.5	4.6±0.35*	5.6±0.30*	7.0±0.04	16.8±0.25*
15	2.3±0.24	2.6±0.07*	7.2±0.20	30.8±0.30*
30	0.5±0.01	0.8±0.01*	1.1±0.01*	41.8±0.40*
Cisplatin (5µg/ml)	1.3±0.02	55.7±0.15*	13.2±0.06	3.8±0.025
Valinomycin (2µg/ml)	2.4±0.02	3.6±0.07	5.3±0.24	24.8±0.01*

Results are presented as mean ± SD of three sets of experiments.

*Significant difference in comparison to the control group ($p < 0.05$)

Inference

- The observations suggest that *E. agallocha* plant extract fraction F2 exposure to SiHa cells resulted in accumulation of cells in the G2/M phase in a dose- dependent manner (Fig.4.43).

- Results also indicated (Table 4.14) that increase in the percentage of cells in the G2/M phase was associated with simultaneous decrease in the G1 phase cells. Thus, it can be inferred that the *E. agallocha* aqueous extract caused inhibition of cell growth in cervical cancer cells (SiHa) by arresting cell growth at the G2/M phase.

  Cisplatin, on the other hand, arrested growth at G0/G1 phase. Hence, valinomycin (IC50 2.2 µg/ml against cervical cell line, that induces arrest of cell growth at G2/M phase, was used as reference standard for further validation of the results. The IC50 value of valinomycin for SiHa cells was pre- determined and standardized in the laboratory.

4.4.8. Effect of *E. agallocha* extract on the expression of cyclin A, cyclin B1, Cdc2, p21 and p53.

The effect of F2 was observed on the expression of cyclins A, B1, Cdc2 and also on p21 and p53 by western blot method and the observations are reported in Fig.4.44.

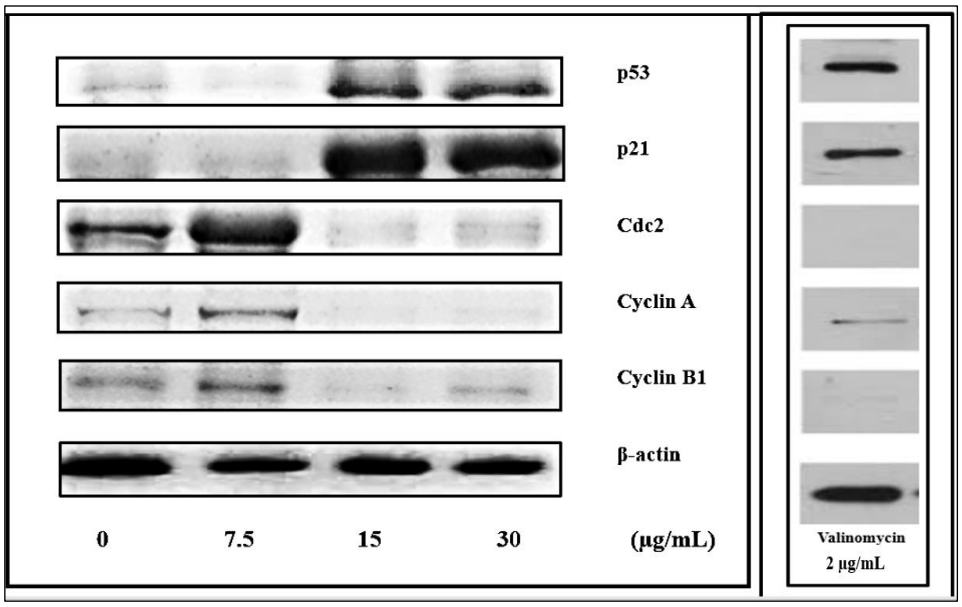


Fig.4.44: Effect of extract fraction F2 on the expression of cyclin A, cyclin B1, Cdc2, p21 and p53.

Inference

- The observations indicated that there was marked decrease in the expression of mitotic cyclins B1 and D1, mitotic- cyclin dependent kinase Cdc2 with simultaneous increase in the expression of p21 and p53 in a dose-dependent manner (Fig.4.44).

- Valinomycin also induced a similar change in protein expression of the treated SiHa cells, further validating the fact that the plant extract causes cell cycle arrest at G2/M phase. β-actin was used as loading control.

4.5. Objective-5: Identification and characterisation of bioactive principles present in the purified fraction and in-silico molecular docking study.

The bioactive fraction F2 was subjected to HR-LCMS for characterisation of its constituent bioactive principles. A study was also undertaken to observe the drug-target interaction with the compound with relatively higher abundance by computer aided methods of bioinformatics.

4.5.1. Identification of the bioactive compound by HR-LCMS

The DAD (Diode array detector) analysis firstly confirmed that the fraction was devoid of any major impurities and hence, was subjected to HR-LCMS for further analysis. HR-LCMS of F2 was conducted at IIT, Bombay [Instrument specification -Make: Agilent Technologies, USA; Model: 1290 Infinity UHPLC system, 6550 iFunnel Q-TOFs; Mass range: 50-3200 amu; Column details: Syncronis C18 100x 2.1, particle size 1.7µ; acquisition time-30mins; flow rate 0.3 ml/min]. The results are represented in Table 4.15 and 4.16 and Figures 4.45-4.47. TLC of the F2 fraction was done with the commercial grade standard analyte of the compound that was present in highest abundance.

Table 4.15: DAD and LC analysis of the *E. agallocha* plant extract fraction at 280 nm.

Compound name	**Formula**	**Mass (g/mol)**	**Relative intensity (%)**
ESI POSITIVE ION MODE			
Bergenin	$C_{14}H_{16}O_9$	328.0808	97.344
Chorismic acid	$C_{10}H_{10}O_6$	226.0473	46.842
N- Deacetylketoconazole	$C_{24}H_{26}Cl_2N_4O_3$	488.1357	52.085
Nicotinamide mononucleotide	$C_{11}H_{15}N_2O_8P$	334.0551	61.483
ESI NEGATIVE ION MODE			
Khayanthone	$C_{32}H_{42}O_9$	570.2835	72.634
Khivorin	$C_{32}H_{42}O_{10}$	586.2782	71.092
Alpha-4-dihydroxytriazolam	$C_{17}H_2Cl_2N_4O_2$	374.0292	56.154
Chlorogenic acid	$C_{16}H_{18}O_9$	354.0962	28.771

Table 4.16: DAD and LC-MS analysis of the compound Bergenin.

Compound lbel	**m/z**	**Formula**	**RT (Retention time)**	**Algorithm**	**Mass (g/mol)**
BERGENIN	333.0595	C14H16O9	5.809	Auto MS/MS	328.0808

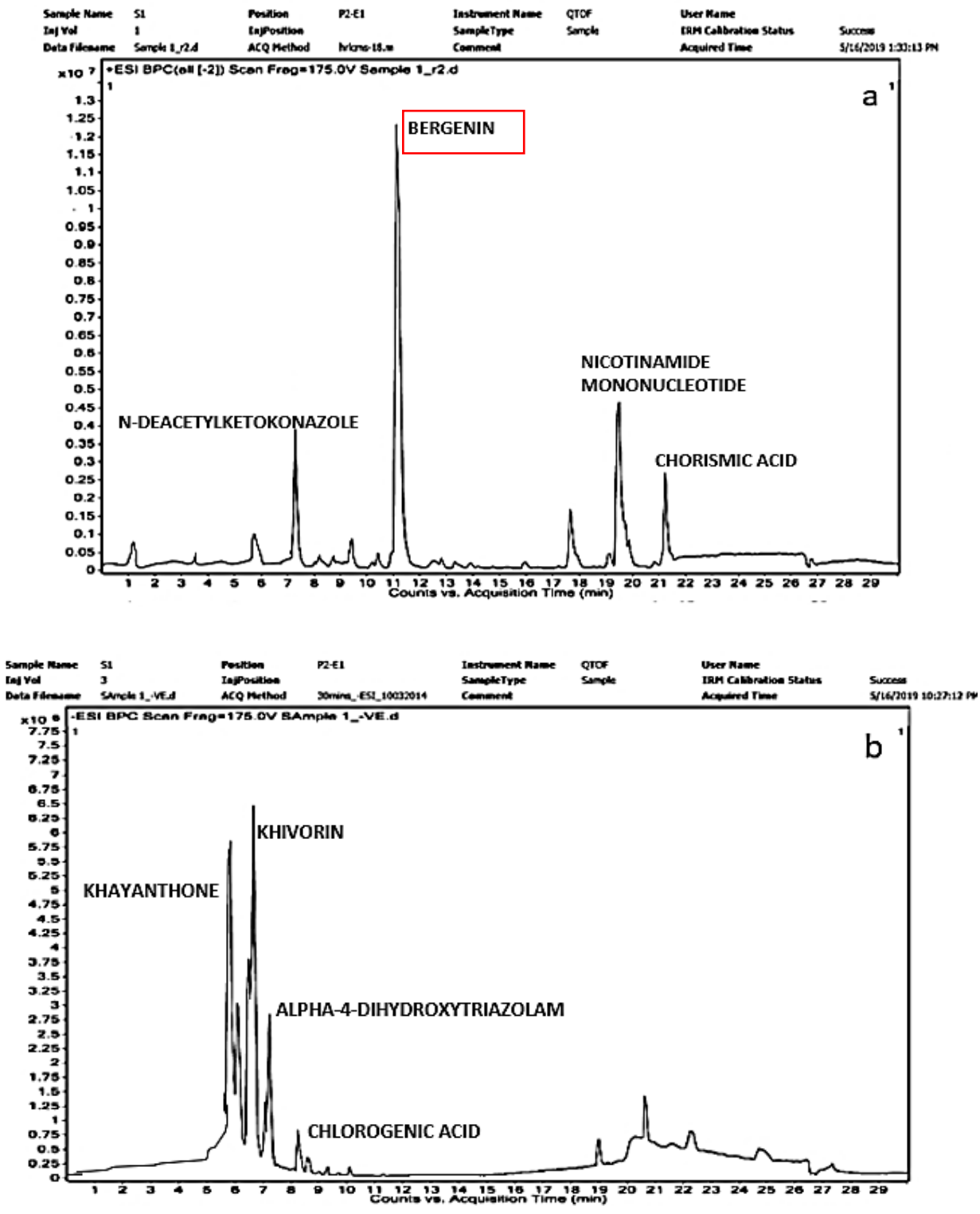
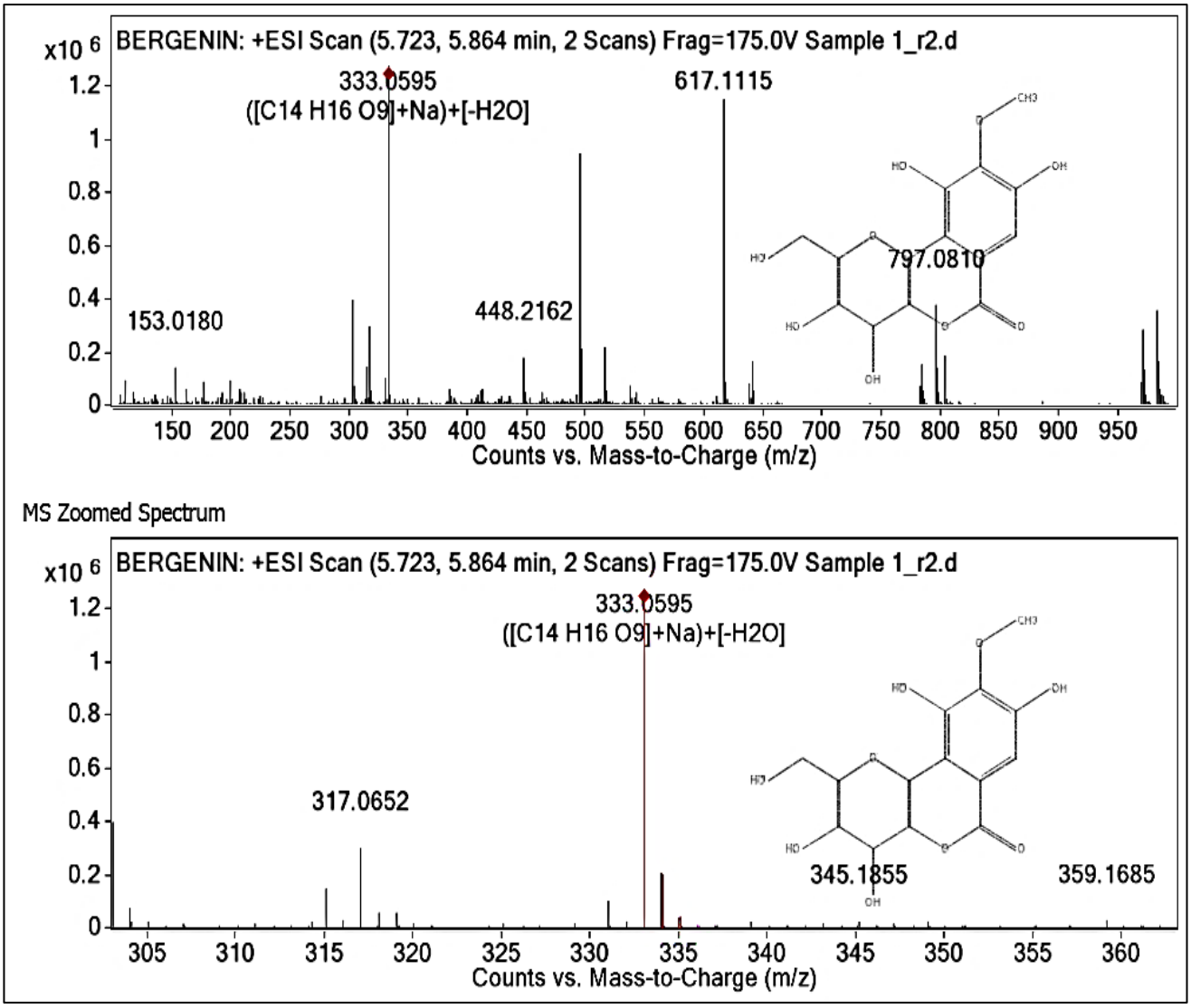
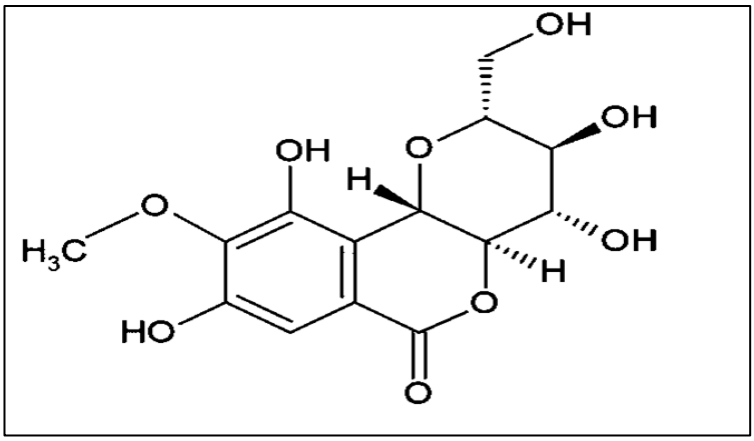


Fig.4.45: ESI scan results of the *E. agallocha* F2 fraction in (a) positive ion mode (b) negative ion mode.



(a)



(b)

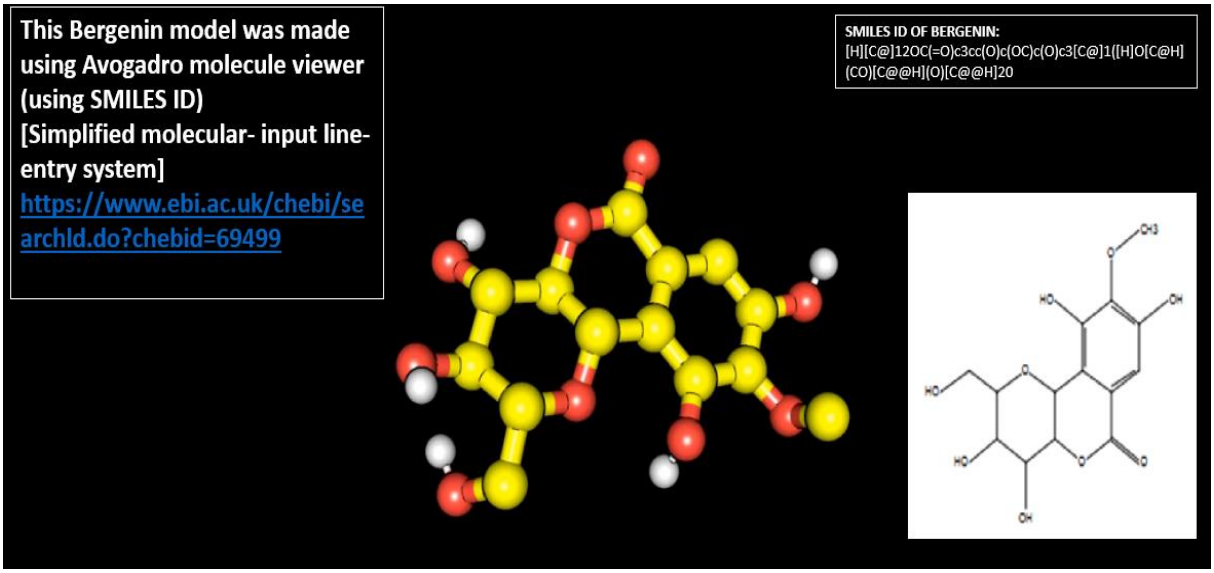
Fig.4.46: (a) LC-MS analysis (MS- spectra) of F2 fraction indicated the presence of Bergenin (m/z= 333.0595) in the highest abundance. (b) The chemical structure of Bergenin.

Inference

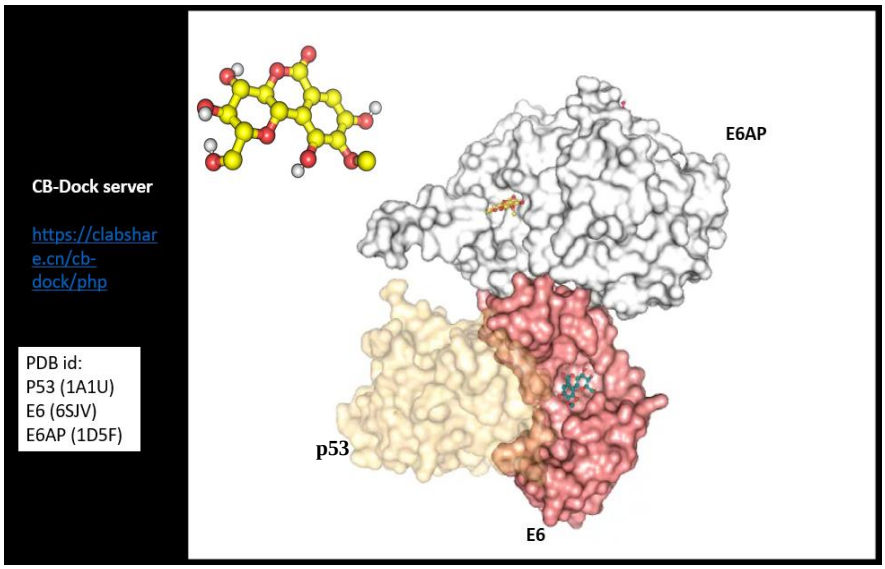
- HR-LCMS results of F2 fraction indicated the presence of certain major compounds at 280 nm scan (Table 4.15).
- The electron spray ionization (ESI) (positive and negative mode scan) analysis of the plant extract has been represented (in Fig. 4.45) where Bergenin and other pharmaceutically important compounds were detected in the positive ion mode.
- Khivorin, khayanthone, alpha-4-dihydroxytriazolam and chlorogenic acid were majorly detected in ESI negative ion mode while Bergenin, Chorismic acid, N- Acetyl ketoconazole and Nicotinamide mononucleotide were detected in the positive ion mode (Fig.4.45.a and 4.45.b).
- Out of these compounds identified, Bergenin (328.08 g/mol) was present in the highest abundance (Table 4.16), hinting its contribution individually or in synergistic association with the other compounds, for the anti-cancer activity observed against SiHa cells.
- The detailed MS spectra (Fig.4.46a) with the molecular formula determined (Table 4.16) further validated the DAD analysis results and the structure of the compound Bergenin was elucidated by HR LC-MS (Fig.4.46b).
- TLC of the F2 fraction was done along with commercial grade Bergenin, where both had R_f values of 0.127 and 0.130 respectively, which were quite comparable. This further confirmed the purity of the eluted fraction and validated the results of the HR-LCMS analysis.

4.5.2. Computer aided molecular docking

The compound bergenin interacts with the E6-E6AP-p53 complex of the HPV infected SiHa cells, thereby hinting its role in inhibition of tumour progression by protecting the p53 protein from proteasomal degradation. The structure of bergenin was made using Avogadro molecule viewer (using SMILES ID), structures of p53, E6 and E6AP were retrieved from Protein Data Bank repository and the molecular docking interaction was studied using CB Dock Server. The observations are represented in Fig.4.47 and 4.48.



(a)



(b)

Fig.4.47: Chemical structure of Bergenin obtained by Avogadro molecule viewer and SMILES ID; (b) the structural interaction of E6-E6AP-p53 complex of SiHa cells visualized by CB-Dock Server.

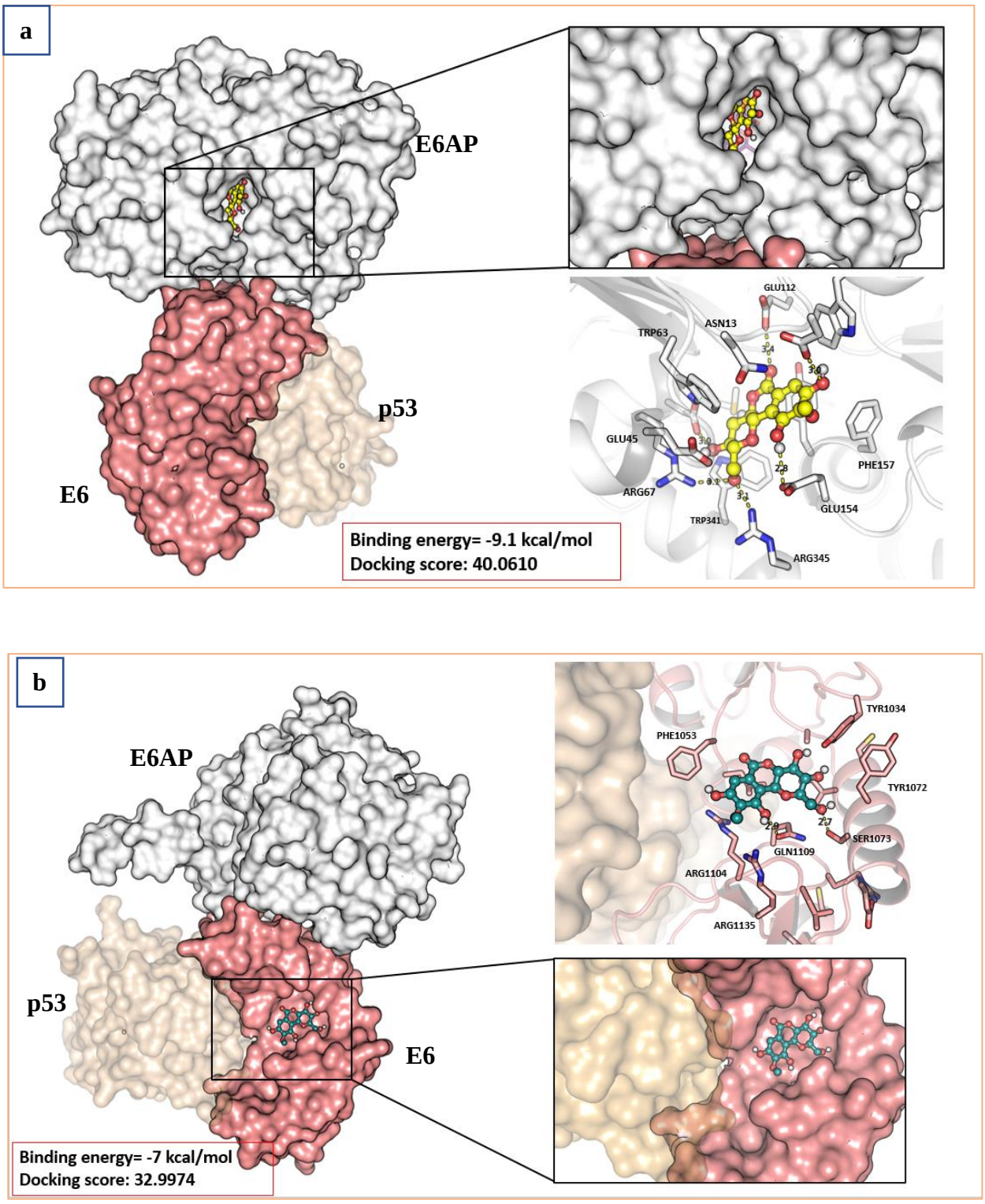


Fig.4.48: Bergenin binding at two specific positions of the E6-E6AP-p53 complex may inhibit the proteasomal degradation of p53. Binding energies of (a) -9.1 kcal/mol and (b) -7 kcal/mol indicate stable complex formation between bergenin and the complex being studied.

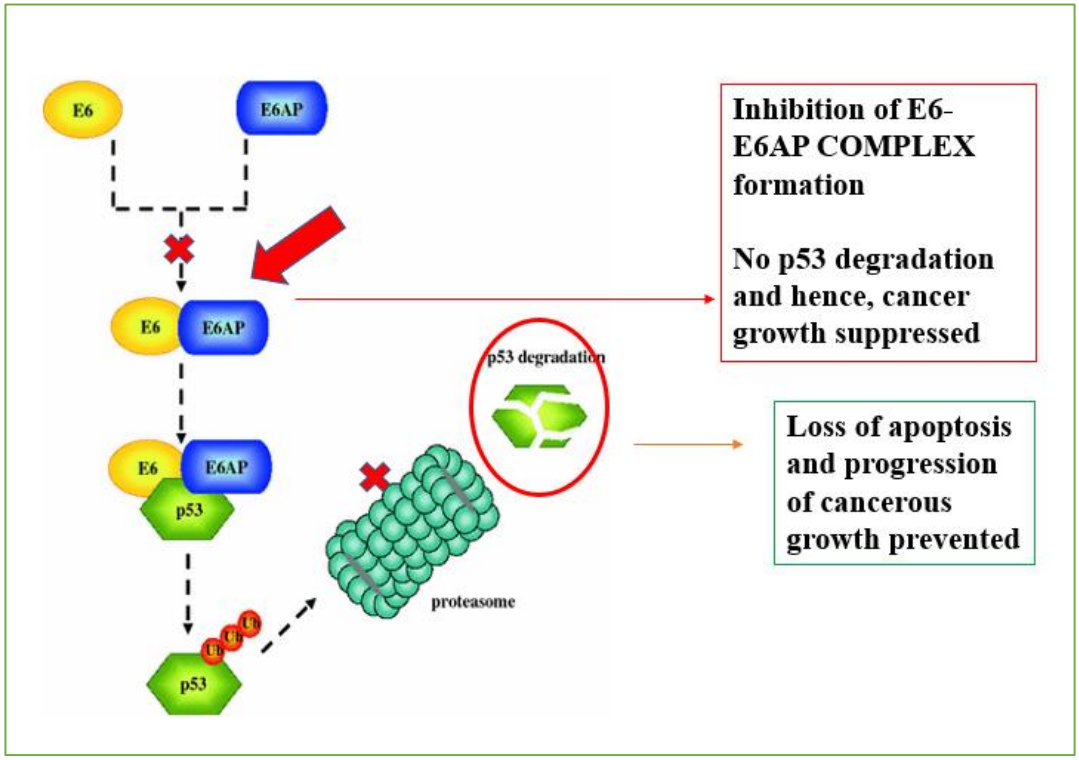


Fig.4.49: Schematic pathway summarizing the probable drug-target interaction.

Inference

- Bergenin, *in silico*, is observed to bind to the E6-E6AP complex with binding energy of -9.1 kcal/mol, which is considerably stable. Moreover, bergenin is observed to interact with certain amino acid residues of E6AP [Glutamine (45), Arginine (67) and Tryptophan (63) (numbers in brackets indicate position)] that might interfere with the interaction of E6 with E6AP (Fig.4.48a). Eventually, this may lead to an alteration in the orientation of p53 in the complex, that may protect it from proteasomal degradation. Thereby, this probable drug-target interaction (Fig.4.49) might have a possibility of inhibiting proteasomal degradation of p53 and thus, help in prevention of tumor progression in HPV infected SiHa cells.

Chapter 5:

Discussion

5. Discussion

For many years, mangroves have been utilized by various ethnic groups for the treatment of various diseases. Ethnopharmacological uses of different mangrove species have been very diverse and have included treatment for eye problems, skin diseases, rheumatism, blisters, arthritis, haemorrhage, asthma, throat infections, stomach disorders and diabetes (Lopez *et al*., 2018). Some species from Avicenniaceae, Meliaceae, Rhizophoraceae and Euphorbiaceae families have shown significant antimicrobial property, while other species belonging to Meliaceae and Rhizophoraceae families exhibited antimalarial properties. Selected members of Acanthaceae and Myrsinaceae families have been reported to possess anticancer property. Based on ethnopharmacological studies and existing literature pertaining to medicinal properties of mangrove plants, four plants were selected for the present study, namely- *Aegialitis rotundifolia, Avicennia alba, Excoecaria agallocha* and *Bruguiera gymnorhiza*. Extracts of these plant samples were prepared in ethanol, methanol, water and DMSO. All the extracts were screened for antimicrobial activity against a range of laboratory-maintained and pathogenic as well as antimicrobial drug-resistant strains of bacteria and fungi. The observations of the present investigation were promising, supported with scientific validations. The ethanolic extract of *E. agallocha* was deciphered to be the most potent plant extract with excellent antimicrobial, antioxidant and anti-cancerous properties. Characterization of the crude plant extract and subsequent purification revealed the presence of various bioactive principles present in this plant extract, of which bergenin was observed to be present in maximum abundance.

Antimicrobial activity of E. agallocha extract

The *E.agallocha* extract exhibited promising antimicrobial activity against Gram negative bacteria and selected fungal strains. Based on the preliminary observations, we mainly focused our study on two important human pathogens - *S. typhi* and *C. parapsilosis,* isolated from blood and urine sample of patients at Peerless Hospital and B.K.Roy Research Centre, Kolkata. This is because these two microbes recently acquired antimicrobial resistance to a great extent, and they become

emerging pathogens in many countries throughout the globe. Only a few studies are present on antimicrobial activity of mangrove plants against *S. typhi*. In one study dried leaf samples of *E. agallocha* collected from Muthupettai mangrove forest of Thiruvarur district of Tamil Nadu, India showed good antimicrobial activity against *S. typhi* (Prakash and Sivakumar, 2013). In another study ethanolic extract of leaves of *Sonneratia alba* collected from Chorao island, Goa, India, showed good antimicrobial activity against *S. typhi* (Sahoo *et al*, 2012). Although anti *Candida* activities of some common terrestrial plants are well known (Pesando and Caram, 1984; McFadden, 1995; Prabhakar *et al*, 2008; Sahoo *et al*, 2012), but there is only few studies on this activity in relation to mangrove plants (Shanmugapriya *et al*, 2012). Again, it is important to note that some yeasts such as *Candida* spp. and *Kluveromyces aestuarii* are also present in mangrove ecosystems (Meyers *et al*, 1971; Naumova *et al*, 2004), where they play important role in the detritus food web (Meyers *et al*, 1975), possibly involving marine invertebrates and zooplanktons. Some *Candida* spp. is also frequently found in mangrove ecosystem (de Araujo *et al*, 1995). However, the role of yeasts in mangrove ecosystem is largely unknown. Among different *Candida* spp., *C. tropicalis* is most commonly found in this ecosystem as observed in an important study in China (Chi *et al*, 2012). In one study leaves of *Avicennia officinalis*, collected from the mangrove forest of Mahanadi delta region of Odisha coast, India showed good antifungal activities against *Candida albicans* and *C. krusei* with MIC values of 200 and 100 μg/ml respectively (Das *et al*, 2018). In this study although *E. agallocha* crude extract showed excellent antimicrobial activity against *S. typhi*, however, antifungal activity against *C. parapsilosis* was not that prominent in comparison to the antibacterial activity. This is possibly due to coexistence of *Candida* spp. with mangrove plants in this ecosystem.

Incidence of antimicrobial resistance has escalated in the past years at an alarming rate, demanding the need to develop new chemotherapeutic agents from plants. Herbal medicines are

gaining attention in the present scenario due to the multi-potent biological activities exhibited by the phytochemicals. The Sundarbans are a huge reserve of such medicinally important plants, that may be explored to combat the issues such as antimicrobial resistance and chemoresistance. *E. agallocha* was collected from the Sundarbans in West Bengal and studied for its potential against antibacterial drug resistant *Salmonella typhi* and its effect on human colon cancer cell line.

The results of the present investigation show that a specific fraction of ethanolic *E. agallocha* extract has significant antibacterial activity against the MDR strain of *Salmonella* under study. The MIC was determined to be 15.7±0.16 µg/ml, which is quite remarkable. HR LC- MS revealed that this fraction had two major bioactive compounds namely, bergenin and hexanoyl glycine. Bergenin was present in a relatively larger amount and has not been reported to be extracted from *E. agallocha* till date. PI staining gave us a qualitative idea that the extract permeabilized and disrupted the membrane of the bacteria. To further validate this finding, protein profiling and analysis was done by visualizing the different proteins in SDS PAGE. The results clearly show the absence of a 33 kDa band in the extract treated bacteria which is thought to contribute to antibacterial drug resistance. Further, on treatment with the plant extract, the bacterial strain became moderately susceptible to a few antibacterial drugs it was resistant to earlier. The outer membrane of Gram- negative bacteria such as *S. typhi* contain an intricate framework consisting of lipopolysaccharides, phospholipids and porins (Henderson *et al*., 2016). This membrane also helps in inhibiting the transport of various compounds including antibacterial drugs and bile acids that are detrimental for the bacteria. (O'Shea and Moser 2008; Pages *et al*. 2008). This characteristic of membrane often serves as an obstacle in developing novel antimicrobial drugs, that target such pathogens leading to failure of treatment strategies and multidrug resistance. (Lee *et al*. 2013). Among the various membrane proteins of *S. typhi*, outer membrane protein A (OmpA) is a non-specific porin, through which only selected chemicals can pass through by passive diffusion (Sugawara and Nikaido 1992; Iyer *et al*. 2018). OmpA and OmpF are closely related and mutations or change in expressions of these proteins have

been reported to be the contributing factors for antibacterial drug resistance in various Gram-negative bacteria. Antibacterial drugs such as β-lactams and fluoroquinolones penetrate the bacterial cell through the porin OmpF (Mach *et al*. 2008; Delcour 2009). Thus, the ompF mutant strains have been reported to be resistant to several β- lactam antibacterials in some Gram-negative pathogens, including *Escherichia coli* (Nikaido *et al*. 1983; Ziervogel and Roux 2013), *Klebsiella pneumoniae* (Sugawara *et al*. 2016), *Serratia marcescens* (Moya-Torres *et al*. 2014), *Pseudomonas aeruginosa* (Okamoto *et al*. 2001), and *Enterobacter aerogenes* (Bornet *et al*. 2000). On the other hand, mutation, downregulation or deletion of OmpA have been reported to be associated with susceptibility to a number of antibacterial drugs including β-lactams in *Acinetobacter baumannii* (Smani *et al*. 2014). This feature may be attributed by the effect of OmpA, that is known to maintain bacterial cell membrane integrity. Disruption of the membrane structure or impaired membrane integrity can increase the diffusion of certain antibacterial drugs. OmpA is known to affect membrane integrity through noncovalent interaction of its C-terminal periplasmic domain with peptidoglycan (Samsudin *et al*. 2016). The role of the C-terminal domain appears to be pivotal for the role of OmpA in antibacterial drug resistance as well as the maintenance of membrane integrity. Thus, the results of this investigation are in accordance with previous findings and the fraction obtained from *E. agallocha* extract is not only effective individually but may be used synergistically with antibacterial drugs in combination therapy to combat the problem of multidrug resistance in *S. typhi* and the infections associated with it.

Similarly, a 70 kDa protein, in *C. parapsilosis* was downregulated when the fungi was treated with the eluted and purified extract. Though the ROS production or membrane damage produced was not significant in the presence of the plant extract but the inhibition of Sap activity and lipolytic was remarkably increased when it was treated with E. agallocha extract. Calcofluor staining clearly indicated that the biofilm production was markedly reduced when the fungus was treated with the MIC concentration of the *E. agallocha* extract. Taken together, the mechanism of action against this

fungus was mainly by inhibition of biofilm formation coupled to decreased Sap and lipolytic activity. Analyzing the results closely, it could be said that this plant extract, both in its crude form as well as in its eluted and purified form has potential antibacterial and antifungal activity against life threating and potentially antimicrobial resistant strains. Also, the antimicrobial property of the plant extract has been observed to be enhanced when administered in synergism with specific concentration of antibacterial and antifungal agents, indicating its promising aspect in the field of clinical and *in vivo* application in the future.

Anticancer activity of E. agallocha extract

In recent years, use of natural products from plants or herbal medicines in cancer treatment has received much attention due to the huge reservoir of phytochemicals that exhibit dynamic biological activity. The plant collected from the Sundarbans of West Bengal was analyzed for the presence of phyto chemicals, antibacterial, antifungal and antioxidant activity that have been reported in our earlier investigations (Sultana *et al*, 2019, 2020). Evaluation of the anticancer activity was the next objective that has been evaluated in this investigation.

Recent studies on anticancer activity of *E. agallocha* extracts of different plant parts indicate a few interesting findings. Recently conducted studies have reported that ethanolic stem extract of *E. agallocha* exhibits cytotoxic effect onvarious cell lines such as Miapaca-2, BxPC-3, PANC-1 and Capan-1, with IC_{50} values> 0.11 µg/ml. Researchers have concluded that the cytotoxic effect exhibited by the stem extracts might be due to the action of phytochemicals such as phenolic derivatives, glycosides and saponin present in the extracts of *E. agallocha,* as indicated by chromatographic results (Patil *et al*, 2012). Konoshima *et al*. (2001) have reported that *E. agallocha* wood contained biologically important compounds namely, diterpenoids, that under *in vitro* condition exhibited potent inhibitory action against Epstein-Barr virus early antigen (EBV-EA).

Further, asecolabdane-type diterpenoid was identified from the extract that had potent anti-tumour effect when analyzed by *in vivo* Two-Stage Mouse Skin Carcinogenesis Test with promoter (12-O-tetra decanoyl phorbol acetate) and an initiator 7,12-dimethylbenz[a]anthracene. It is interesting to note that certain flavanol glycosides extracted from *E. agallocha* block the action of GLI-related protein (Glioma associated oncogene homolog). GLI is a transcriptional effector involved in tumour development; thus, blocking its action consequently leads to the inhibition of its translocation in to the nucleus. Hence, it acts as an effective inhibitor of the Hedgehog signaling pathway in cancer therapy (Kinzler and Vogelstein, 1990; Rifai *et al*, 2011). In 2012, anticancer activity on human lung cancer cell lines of the ethanol stem extract were reported by Patil *et al*. where the result showed potent cytotoxic activities in a dose dependent manner (Rifai *et al*, 2011). However, as evident from literature studies and recent research findings, not much is known about the anticancer activity of its leaf extract with regard to human cervical cell lines, especially SiHa cells. The present study emphasizes on the aqueous extract so as to minimize toxicity factors and easy administration on cell lines because many a times, solvent system also culminates into the anticancer activity of a plant extract. The MTT assay allows to assess any intrinsic toxicity of the plant extract and helps to determine a safe dosage concentration administration. The results clearly indicate the anticancer activity of Fraction II against SiHa cells with an IC_{50} value of 15. 35 ± 0.577 µg/ml and no cytotoxic activity against normal healthy cells. Cisplatin has been used as reference standard drug since it is widely used clinically, for the treatment of lung, cervical and ovarian cancers. Hence, it is used for *in vitro* evaluation of cytotoxic potential of various potent chemotherapeutic drug candidates. The HR LC-MS indicates the presence of the compound Bergenin in the highest abundance in this fraction which is a novel finding since Bergenin has never been reported to be isolated from *E. agallocha.* Further, Bergenin is a trihydroxybenzoic acid glycoside and thus, this result is in accordance with the TLC observation, where the R_f value indicated the presence of acid glycoside or glycosidic derivatives.

Purification of E.agallocha extract and identification of bioactive principles

Isolation of bioactive compounds using column chromatographic method is a widely adopted method and involves preparation of sample, packing of silica gel column, running the column with suitable stationary and mobile phase, elution of fractions and analysis of each eluted fraction using TLC (Bajpai *et al*, 20016). The LC-MS results relates to the discovery of a new natural source of Bergenin, which is also known as Cuscutin, Ardisic acid B or Vakerin (IUPAC name is (2R,3S,4S,4aR,10bS)-3,4,8,10-tetrahydroxy-2-(hydroxymethyl)9-methoxy-3,4,4a,10b-tetrahydro-2H-pyrano[3,2-c] isochromen-6-one. Its chemical formula is C14H16O9 and has a molecular mass of 328.27 g/mol). Experimentally, Bergenin is known to have anti-inflammatory (Patil *et al*, 2012), antioxidant (GuoCai *et al*, 2008), antiulcerogenic, neuroprotective, immunomodulatory, antiarrhythmic (Pozharitskaya *et al*, 2007), hepatoprotective (Sridhar *et al*, 2006) and antiretroviral (Kimura *et al*, 2007) activities. However, anticancer activity on SiHa cells have not been reported earlier. In the known studies, there are also many other natural sources of Bergenin. Bergenin has been reported to be present in many plants –*Aconophora compressa*, *Ardisia japonica*, *Astilbe chinensis* , *Astilbe rivularis*, *Astilbe thunbergii*, *Bergenia crassifolia*, *Caesalpinia digyna*, *Corylopsis spicata*, *Dryobalanops aromatic*, *Ficus racemosa*, *Flueggea leucopyrus*, *Flueggea virosa*, *Mallotus japonicus*, *Mallotus philippensis*, *Mallotus roxburghianus*, *Peltophorum africanum*, *Pentaclethra macrophylla*, *Sacoglottis gabonensis*, *Sacoglottis uchi*, *Saxifraga melanocentra*, *Shorea robusta*, *Teramnus labialis*, *Tridax procumbens* etc. (de Araujo *et al*, 1995; Varshney and Dayal, 2006; Patra *et al*, 2009; Sivaperumal *et al*, 2010; Chi *et al*, 2012; Das *et al*, 2012) but has not been reported to be present in any mangrove plant till date. The LCMS results of this investigation, suggesting that this chemical is present in significantly high amount in *E. agallocha* extract, is a new finding and thus it appears to be an easy source of this chemical in West Bengal, India. Other compounds detected in the potent fraction may also contribute to the cytotoxic activity of this plant extract. Chorismic acid is present at a key branching point in aromatic acid

biosynthesis. It is the precursor of tryptophan, tyrosine, and phenylalanine. It helps the biosynthesis of vitamin K and folate in plants and microorganisms. It can modulate t-RNA and is also a precursor of salicylic acid (Widermuth *et al*, 2001). N- Deacetylketoconazole, a derivative of ketoconazole, is an antifungal compound used to treat and prevent fungal infections. It works by inhibiting an enzyme required for the synthesis of ergosterol and ultimately altering the fungal cell membrane and is primarily fungistatic in nature. It is lipophilic in nature, which leads to accumulation in fatty tissues, and is best adsorbed at the highly acidic level. In conventional treatment, ketoconazole is usually prescribed for infections such as ringworm, candidiasis, etc. The decrease in testosterone caused by the drug makes it useful for treating prostate cancer and for preventing postoperative erections following penile surgery (Rodriguez and Miranda, 2000). On the other hand, Nicotinamide mononucleotide is a nucleotide derived from nicotinamide and ribose. It is a derivative of niacin, and in the human body, it is converted to nicotinamide adenine dinucleotide (NAD) (Irie *et al*, 2020). Khayanthone and khivorin are limonoids formed from apotirucallane after loss of four terminal carbons. Limonoids are also known as tetranortriterpenoids. Chlorogenic acid is a cinnamate ester and a tannin, having anti-inflammatory and anti-hypertensive properties. Alpha-4-dihydroxytriazolam is a benzodiazepine derivative and has anticonvulsant properties (Tajik *et al*, 2017). All the compounds detected are of pharmacological importance; however, only bergenin has been reported to have anti-cancer property. Though the other compounds do not have a direct role in inducing cytotoxicity in cancer cells, they might facilitate the overall survival of healthy normal cells while bergenin ceases the proliferation of cancer cells. All these compounds may be acting in a concerted manner, with Bergenin being the major contributor, for the observed selective cytotoxic potential of the plant extract against SiHa cells with minimal toxicity production in healthy PBMC cells.

E. agallocha extract in combatting human cervical cancer, through live cell imaging analysis and other diagnostic tools.

The mechanism of action of the bioactive fraction exhibiting anticancer activity against SiHa cells in this study has been assessed through live cell imaging analysis. It is usually considered that loss of apoptosis and increased autophagy lead to cancer progression (Bandaranayake, 2002). Apoptosis inducers and autophagy inhibitors are widely preferred in formulating anticancer strategies. However, autophagy is often triggered along with apoptosis as a pro survival mechanism (Kondo *et al*, 2005). Some drug molecules such as chloroquine and hydroxychloroquine induce autophagic changes in treated cells before induction of apoptosis (Li *et al*, 2010; Shen *et al*, 2011; Levy *et al*, 2017). It might happen that when a cell is undergoing the death process, both apoptosis and autophagy may occur simultaneously because a certain level of cellular stress might initiate the autophagy process that leads to apoptosis. To have a better understanding of the associated events, real time imaging methods to detect and monitor both the process of autophagy as well as apoptosis within the same cell is crucial. There are a number of standardized methods in this regard; however, the best real time compatible method is LC3 puncta formation (Verbaanderd *et al*, 2017). In order to track and follow the cellular event *in vivo*, labeling of phagophores and autosomes with simultaneous visualization is important. Only few proteins are uniquely associated with autophagic vesicles or precursors, out of which only LC3 labels autophagic structures, both before and after fusion with lysosomes. Recruitment of LC3 to autophagosomes is observed as puncta formation, where small pores are formed and followed by late mitochondrial permeabilization (Bampton *et al*, 2005; Chen *et al*, 2010). The delivery of mitochondria to lysosomes has been traced by using mt-Keima (Keima with a mitochondria-targeting sequence). Mt-Keima has pH dependent excitation profile and has resistance to lysosomal protease. When mitochondrial damage occurs, the membrane potential changes which leads to a change in its excitation level (Lupitha *et al*, 2020, Mizushima *et al*, 2020). Fluorescent fusion of intermembrane

proteins such as Smac was used to screen the mitochondrial permeabilization process during apoptosis (Muonoz-Pinedo *et al*, 2006). Further, caspase-3 activation was confirmed by colorimetric assay that showed an increase in the Caspase-3 activity in a dose-dependent manner. The method adopted for proteasomal inhibition study allows a simple, rapid and efficient way to study the ubiquitin-proteasome activity in living cells. A stable overexpression of the GFP reporter substrate has been designed to develop a GFP-dgn that rapidly undergoes proteasomal degradation in cancer cells, which can be observed with decreasing fluorescence (Greussing *et al*, 2012). The cell cycle in cancer is dysregulated or disrupted and hence, is a widely studied target for developing new and more potent anti-cancer drugs. Various independent research findings have reported that G2/M phase cell growth arrest is a plausible mode of action by which certain cytotoxic drugs work (Carnero *et al*, 2002; Kaina, 2003). In this investigation,"flow cytometry analysis reveals that the *E. agallocha* extract exhibited a pronounced effect on the cell cycle regulation of SiHa cells, wherein accumulation of cells in the G2/M phase was observed. The cell growth arrest was accompanied by decrease in the expression of Cyclin A and B1, along with reduction in Cdc2 expression, with simultaneous increase in the expression of p21 and p53. Cdc2 kinases are mainly activated with activation of cyclin B1 during the G2/M phase (Wolgemuth *et al*, 2004; Stark and Taylor, 2004). When the Tyr15 of Cdc2 is phosphorylated, the activity of Cdc2/cyclin A and B1 kinase complex is suppressed, thus, acting as a determining step for entry into mitotic phase. The p53 detects DNA damage and stimulates DNA repair machinery or pro- apoptotic factors that leads to an increase in the susceptibility of the cells to undergo apoptosis. Further, p21 enable the arrest and maintenance of cells in the G2/M phase by inactivation of the cyclin B1/Cdc2 complex (Guillot *et al*, 1997). The results also indicate that reduction in expression of cyclin A, cyclin B1, Cdc2 and upregulation of p21 and p53 result in growth arrest in SiHa cells at G2/M phase, as has been reported by standard reference drugs such as Valinomycin, that induce cell cycle arrest at G2/M phase. Thus, by analyzing the observations in this investigation, it may be inferred that the SiHa cell proliferation

inhibition on treating with the *E. agallocha* plant extract may be due to the G2/M phase growth arrest. While the mode of action of the plant extract in case of inducing apoptosis, autophagy and mitophagy is comparable to that of cisplatin to a certain extent, the pattern of inducing cell cycle arrest and changes in protein expression (summarized in Fig. 13) are similar to the effects observed with treatment with valinomycin *in vitro*. Hence, a further intricate elucidation of signaling pathways and molecular biology analytical methods may be undertaken to precisely infer the mode of action of the purified plant extract fraction.

Computer Aided Drug designing – an attempt to study the interaction between bergenin and p53 binding site of HPV E6 and E6AP complex of SiHa HPV+ cells

The E6-E6AP-p53complex represents a prototype of viral hijacking of both the ubiquitin-mediated protein degradation pathway and the p53 tumor suppressor pathway (Ghittoni *et al*, 2010; Martinez-Zapien *et al*, 2016; Kolluru *et al*, 2019). Molecular docking studies, in this investigation, using bioinformatics revealed that bergenin is capable of forming a satisfactory stable complex (-9.1 kcal/mol) with E6-E6AP complex, that is responsible for binding to p53 and its degradation that eventually leads to cancer progression. If this interaction is proved to be stable *in vitro* and *in vivo*, then this observation may have a significant role in protecting p53 degradation, and thus, inhibiting cancer progression in SiHa cells. The observation here presents a probable structure interaction that may serve as a framework for the design of inhibitory therapeutic strategies of bergenin against oncogenesis and cervical cancer progression mediated by human papilloma virus.

Thus, based on the observations, it may be inferred that the ethanolic extract of *E.agallocha* is a repertoire of biologically important compounds. The purified fraction, denoted as F-II in this investigation, of this extract has notable antimicrobial and anticancer activities with the compound Bergenin being present in highest abundance in comparison to other bioactive principles. This

compound has been reported to be present in a mangrove plant extract for the first time hinting its potential role in the exhibited antibacterial, antifungal and anticancer properties. *In silico* computer aided molecular docking study also indicates a significant role of bergenin in inhibition of proteasomal degradation of p53, thus, contributing as a lead for drug development, that may be further explored *in vitro* as well as *in vivo* to design newer and safer alternatives to overcome the problem of chemoresistance, especially with regard to human cervical cancer.

Summary & Conclusions

Summary

Objective-1: Collection of mangrove leaf samples, physicochemical analysis of the dry leaf powder extracts along with phytochemical, antioxidant and antimicrobial properties of the crude leaf extracts.

- Mangrove leaf samples of *Bruguiera gymnorhiza, Excoecaria agallocha, Avicennia alba* and *Aegialitis rotundifolia* were collected from Bali Island of the Indian Sundarbans (near 30°24′-30°28′ N latitude and between 77°40′- 77°44′ latitude in the South 24 Parganas, West Bengal).
- Crude leaf extracts were prepared in ethanol, methanol, water and DMSO as solvents.
- Pharmacognosy and microscopic analysis of dry leaf powder extracts were conducted since it provides a preliminary idea regarding the nature and disposition of various cellular components and cell inclusions, and thus helps understand where the compounds of interest may be located. The study revealed the presence of calcium oxalate crystals and stomatal fragments in *B. gymnorhiza*, epidermal cell fragments and globular cells in *E. agallocha*, astro-sclereids, branched sclereids, oval and rectangular crystals in *A. alba* and oil globules in *A. rotundifolia.*
- Ethanolic extract of *E.agallocha* exhibited highest total phenolic content (342.56 mg/g of dry weight), total flavonoid content 243.56 mg/g of dry weight) as well as highest tannin content (118.75 mg TAE/g of extract), in comparison to other plant extracts. This extract also exhibited the highest total antioxidant capacity (around 90 mg AAE/g of extract), providing a qualitative idea of the potential antioxidant capacity of the plant extract for further investigation.
- In accordance with the phytochemical analysis, the ethanolic extract of *E. agallocha* has also showed highest DPPH scavenging activity (75.55%), ABTS radical scavenging activity (78.53%) and highest ferric reducing antioxidant potential, hinting its probable role in being used as an effective antioxidant agent.

- Ethanolic extract of *E. agallocha* exhibited a significant hydroxyl radical scavenging activity with 82.5% inhibition of free radical generation at 8mg/ml concentration. The extract also exhibited a marked increase in hydrogen peroxide radical scavenging activity with increase in extract concentration. Almost 43% inhibition of free radical generation was noted the ethanolic extract of *E. agallocha.* This indicates that ROS generation is significantly reduced in the presence of this plant extract. A pronounced nitric oxide radical generation inhibition was observed with aqueous extract of *E. agallocha,* in comparison to ethanolic extract. A 37% inhibition was noted in case of its aqueous extract while only 29% inhibition was observed in case of ethanolic extract. A possible explanation may be that the free radicals in ethanolic extract undergo further chain reactions that reduce its scavenging potential.
- In accordance with hydroxyl radical and hydrogen peroxide radical scavenging assays, ethanolic extract of *E. agallocha* exhibited the maximum potent superoxide anion radical scavenging activity of with around 59% inhibition of free radical generation. It can thus, be inferred, that the ethanolic extract of *E. agallocha* has a promising antioxidant activity with the capacity to reduce the free radical generation (in vitro). Superoxide is generated as a by-product of oxygen metabolism and if not regulated, causes many types of cellular damage.
- Ethanolic extract of *E. agallocha* shows the maximum SOD enzyme activity (71 U/mg of plant extract), catalase enzyme activity (52 μM H2O2 per min per mg), exhibited maximum ascorbate oxidase activity (27.46 U/mg plant tissue) and a promising Glutathione reductase activity (315 GR activity/mg of plant tissue). The glutathione content was observed to be the highest in case of ethanolic extract of *E. agallocha* (19.65 μM). it is an important antioxidant found in plants that is capable of preventing damage to important cellular components by reactive oxygen species such as free radicals, peroxides, lipid peroxides etc. Moreover, glutathione also participates in thiol protection and redox regulation of cellular thiol proteins under oxidative stress. Thus, this finding further validates the antioxidant capacity of the plant

extract that can be further purified and investigated for its antioxidant effect in vivo.

- In comparison to other extracts, ethanolic extract of *E. agallocha* exhibited the highest in vitro inhibitory activity (64.61%) against alpha amylase, maximum albumin denaturation inhibition (74.32%) and lipid peroxidation inhibition, indicating its probable role as an anti-diabetic, anti-inflammatory and peroxidation inhibitor of lipids, thereby, having protective biological roles.
- Ethanolic extract of *E. agallocha* inhibited the growth of *a multidrug resistant strain of Salmonella typhi* with an MIC value 15.5µg/ml. Fungal inhibitory effect was also noted against *Candida parapsilosis*. However, the antimicrobial action was more pronounced in case of *S.typhi.*

<u>Objective – 2</u>: Characterisation of different bioactive principles present in the selected crude leaf extracts with optimum antioxidant and antimicrobial activity

- Ethanolic extract of *E. agallocha* separated distinctly into its constituents when subjected to Thin Layer Chromatography with distinct R_f values of 0.485, 0.471, 0.442. 0.328, 0.228, 0.185 and 0.142. A pinkish coloration was observed after derivatization indicating the presence of terpenoid compounds and phenolic derivatives.

- In the crude ethanolic extract of *E.agallocha*, the compounds chorismic acid, methyl jasmonate, bergenin, khayanthone and chlorogenic acid were observed to be present as major constituents. Chorismic acid is an important biochemical intermediate in plants and precursor for salicylic acid, alkaloids and other aromatic metabolites. Bergenin is an acid glycoside and a natural secondary metabolite. Khayanthone is a limonoid compound. Limonoids are also known as tetranortriterpenoids. They occur mainly in the Meliaceae, Rutaceae, and Cneoraceae families. Chlorogenic acid is a phenolic compound. It releases glucose slowly after meals and it has got antihypertensive anti-inflammatory effects.

<u>Objective-3:</u> Selection and purification of the most potent bioactive crude leaf extract, evaluation of antimicrobial activity its purified fractions against selected bacterial and fungal strains with subsequent selection of fraction with optimum antimicrobial activity and study of mechanism of antimicrobial action.

- Silica gel column chromatography of crude ethanolic extract of *E.agallocha* led to the elution of 12 fractions initially. Out of the 12 eluted fractions, 3 exhibited distinct R_f values of 0.14, 0.127 and 0.09, (denoted as F1, F2 and F3 respectively) indicating the probable bioactivity of the fractions. These three fractions were evaluated for further bioactivity to demarcate the specific fraction with potential antimicrobial and anticancerous properties.
- The three fractions-F1, F2 and F3, in various combinations as well as individually-were tested against multidrug-resistant strains of *S. typhi* and *C. parapsilosis*. When treated in combination, fractions 2 and 3 produced more pronounced inhibitory antimicrobial activity. Hence, further assessment was required to be specific about the fraction that possessed the maximum bioactivity.

- Thereby, the individual fractions were tested similarly. It was noted that fraction 2 produced significant antibacterial and antifungal effect in *S. typhi* and *C. parapsilosis*.
- This fraction (F2) produced a significant inhibitory action against the MDR strain of *S. typhi* (resistant to Cefotaxime, Levofloxacine, Aztreonam, Imipenem, Ampicillin and Ceftazidime) with an MIC value of 15 µg/ml.
- The growth of a multidrug resistant strain of *Candida parapsilosis* was also significantly reduced by treatment with F2. The MIC value in this case was around 31 µg/ml.

- It was interesting to note that on treating the *S. typhi* with the MIC of the plant extract, the bacteria became intermediately susceptible to a all the antibacterial drugs it was resistant to, earlier, as observed by MIC values. The antifungal drug sensitivity was comparably less pronounced than the antibacterial drug sensitivity. However, it is of notable importance that *C.parapsilosis,* that was highly resistant to all these drugs earlier, became sensitive to Amphotericin B and also to caspofungin and voriconazole to a certain extent.

- While studying the mechanism of action, it was observed that F2 mainly caused membrane disruption of the bacteria, as observed by PI staining and also caused remarkable increase in ROS generation in the bacterial cells, as indicated by DCFH-DA staining. The membrane damage and ROS-mediated cellular damage of *S.typhi* was further enhanced when the bacterial cells were treated with F2 and Ampicillin in combination. Effect on protein profile indicates the downregulation of a 33 kDa protein (Omp family protein) that may be plausibly responsible for disrupting membrane integrity and facilitating the ROS-mediated damage by F2.

- No major effect on membrane damage or ROS generation was observed in *C.parapsilosis* cells. However, inhibition of lipolytic activity and secreted aspartyl proteinases, on treatment with F2, was observed, that may have a role in reducing the adherent property of the fungal cells as well as decrease its virulence and pathogenicity. Downregulation of a 70 kDa protein was observed in the *C.parapsilosis* cells treated with F2, that might be correlated to the inhibition of biofilm formation by F2 observed by Calcofluor white staining.

Objective- 4: Evaluation of anticancer activity of the purified fractions, selection of fraction with optimum antimicrobial activity and study of mechanism of action.

- The results of the MTT assay indicated that the half- maximal inhibitory concentration (IC_{50}) value of the F2 plant extract was about 15.538 µg/ml± 0.577 µg/ml.
- Moreover, the F2 fraction induced a significant increase in cell proliferation when treated on healthy PBMC cells, which is indicative of its selective toxicity. Thus, this extract would produce no toxicity in normal healthy cells in the applied dosage concentration (15µg/ml). This potent fraction possessing cytotoxic activity was thereafter subjected to LC-MS for identification of the bioactive compounds present.
- The control cells (SiHa cells without plant extract treatment) showed absence of LC3 puncta formation while 24 hrs treatment of aqueous plant extract (7.5, 15, 30 µg/ml) showed increased LC3 Puncta formation in SiHa LC3- GFP cells in a dose dependent manner, indicating its ability to induce autophagy in SiHa cells.
- After 24 hrs treatment with F2 (7.5, 15, 30 µg/ml) there was increased mitokeima ratio in SiHa MitoKeima cells in comparison to control cells, indicating the induction of mitophagy and permeabilization of the mitochondrial membrane in the SiHa cells by F2.
- 24 hrs treatment of increasing dosage of F2 (7.5, 15, 30 µg/ml) showed mild increase in Smac release when compared to normal SiHa Smac- mCherry Cells, indicating that autophagy and mitophagy consequently might lead to the apoptotic death of cells.
- Colorimetric assay of caspase-3 revealed that its content increases in SiHa cells treated with F2 in a dose-dependent manner, validating the apoptotis results of imaging analysis observations.
- Treatment of SiHa cells with increasing dosage of F2 (7.5, 15, 30 µg/ml) for 24 hours showed clear proteasomal inhibition of the GFP-dgn(p53) protein in a dose dependent manner,

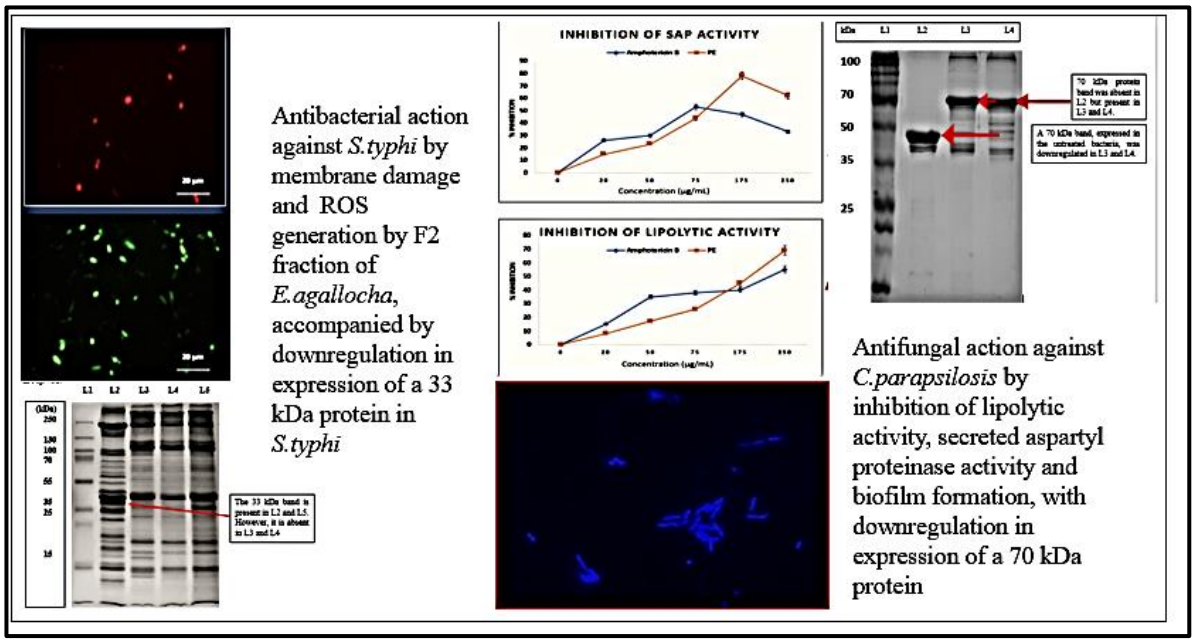
indicated by the increased amount of fluorescence detected. This consequently indicates that F2 may have a protective role in preventing proteasomal degradation of p53 protein, thereby leading to inhibition of tumor progression and cancer metastases.

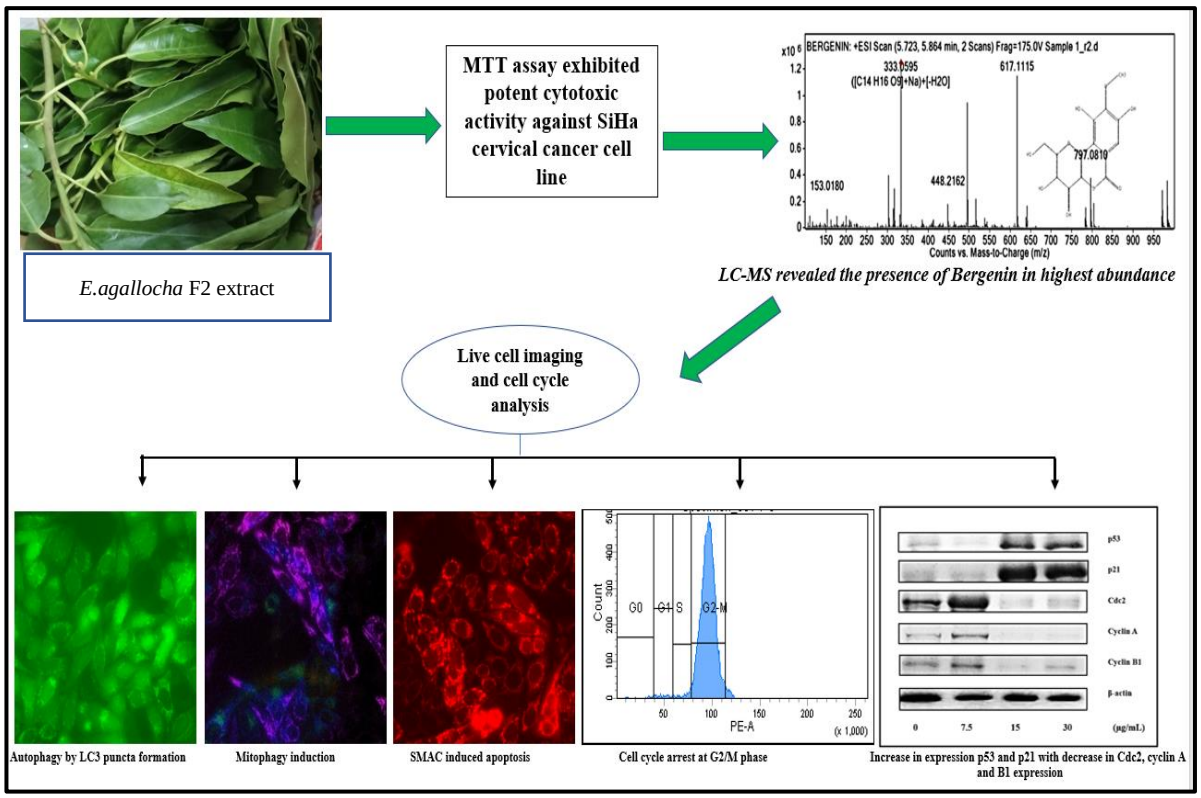
- Flow cytometry results clearly indicate exposure of SiHa cells to F2 resulted in accumulation of cells in the G2/M phase in a dose- dependent manner.
- Further, Western Blot analysis showed marked decrease in the expression of mitotic cyclins B1 and D1, mitotic- cyclin dependent kinase Cdc2 with simultaneous increase in the expression of p21 and p53 in a dose-dependent manner.

<u>Objective-5:</u> Identification and characterisation of bioactive principles present in the purified fraction and in-silico molecular docking study.

- The electron spray ionization (ESI) (positive and negative mode scan) analysis of the plant extract fraction F2 has been analysed where Bergenin and other pharmaceutically important compounds were detected in the positive ion mode.
- Khivorin, khayanthone, alpha-4-dihydroxytriazolam and chlorogenic acid were majorly detected in ESI negative ion mode while Bergenin, Chorismic acid, N- Acetyl ketoconazole and Nicotinamide mononucleotide were detected in the positive ion mode.
- Out of these compounds identified, Bergenin (328.08 g/mol) was present in the highest abundance, hinting its contribution individually or in synergistic association with the other compounds, for the anti-cancer activity observed against SiHa cells.
- Bergenin has been reported for the first time to be present in any mangrove plant extract.
- In silico molecular docking study provides a preliminary and probable role of bergenin in interfering with E6 and E6AP complex, thereby hinting its probable role in protecting p53 from proteasomal degradation and consequently, preventing tumor progression in HPV infected SiHa cells.

Graphical summary of the major findings





***Excoecaria agallocha*-mangrove with a solution to bacterial, fungal and cancerous growth.**

Conclusion

In conclusion, it may be said that F2 fraction eluted from ethanolic extract of *E.agallocha* is an unexplored source of Bergenin that may be developed to a commercially and pharmaceutically important product and serve as a solution to bacterial, fungal and cancerous growth. F2 fraction, being a source of bergenin, may be developed as a potential treatment option, in combatting antimicrobial resistance and chemoresistance. Presence of bergenin in the purified fraction of *E.agallocha* is a novel finding and further research may be undertaken in the future to develop it as a pharmacologically important product, that may be incorporated in combinatorial treatment regimen with antibacterial drugs, antifungal drugs as well as chemotherapeutic agents.

▪ Awards and Achievements

1. Awarded the Best Oral Presentation for the topic entitled in a two-day national webinar on "Cytogenetics, *in vitro* culture and phytochemistry of plants and microbes for sustainable use," organized by Department of Botany, Visva Bharati, on 22nd-23rd January, 2022 **[BEST ORAL PAPER PRESENTATION]**.

2. Oral Presentation at IACS, Kolkata:
Secured 3rd position in paper presentation at "Women in Science and Technology- present scenario" organized by the Indian Association for the Cultivation of Science in collaboration with Kolkata Nivedita Shakti, on 8th March, 2020.

3. Poster presentation at Visva Bharati University:
Poster entitled "Antimicrobial action of Mangrove plants *Bruguiera gymnorhiza* and *Excoecaria agallocha*- in *in vitro* conditions" was presented in the National Seminar on "Utilization of Plants and Microbes" under UGC-SAP-DRS programme held on 28th March, 2019 in the Department of Botany, Visva-Bharati, Shantiniketan. An abstract volume was also published.

4. Poster presentation at St. Xavier's College (Autonomous), Kolkata:
Poster entitled "An Observation on Differential Action of some Mangrove Plants of Sundarbans" was presented in the two-day National Seminar "Modern Trends in Microbiology: Chapter XV" organized by St. Xavier's College (Autonomous), Kolkata held on 10th -11th October, 2018. Also, won the 1st Prize in the Poster Presentation Competition in the Research Scholars' category **[BEST POSTER PRESENTATION]**.

▪ Publications

1. Sultana, T., Mitra, AK. and Das, S. (2022) Evaluation of anti-cancer potential of *Excoecaria agallocha* L. leaf extract on human cervical cancer (SiHa) cell line and assessing the underlying mechanism of action. *Future Journal of Pharmaceutical Sciences*. 8:3 https://doi.org/10.1186/s43094-021-00389-y **[SPRINGER, I.F:3.075 (UGC CARE enlisted journal)]**

2. Sultana, T., Mitra, AK. and Das, S. (2021) An *in vitro* approach to combat multidrug resistance in *Salmonella typhi* and human colon cancer with *Excoecaria agallocha* L. extract. *Bulletin of the National Research Centre*. 45:210. https://doi.org/10.1186/s42269-021-00668-x **[SPRINGER, I.F:2.12]**

3. Sultana, T., Mitra, AK. and Das, S. (2021). Antimicrobial Action of Mangrove plant extracts against *Salmonella typhi* and *Candida parapsilosis* characterized by their Antioxidant potentials and Bioactive compounds. *International Journal of Pharmaceutical Science and Research.* 12 (9): 4774-89. **[I.F:2.04]**

4. Sultana, T., Das, S. and Mitra, A. K. (2019), A Preliminary Observation on an Explicit Antimicrobial Action of Mangrove Plants on Pseudomonas aeruginosa. Asian Journal of Pharmaceutical and Clinical Research, Vol. 12, (5), pp. 226-230 (E-ISSN: 2455-3891, PISSN: 0974-2441). [**UGC CARE enlisted journal**]

▪ Patent

1. ***Excoecaria agallocha* is a natural source of Bergenin.**
Indian patent filed on 10/07/2020
CBR No.: 11750, Application no.: 202031029308
Tamanna Sultana, Satadal Das and Arup Kumar Mitra

Published in the IPR journal on January 14, 2022.

Publication

Sultana *et al.*
Future Journal of Pharmaceutical Sciences (2022) 8:3
https://doi.org/10.1186/s43094-021-00389-y

Future Journal of Pharmaceutical Sciences

RESEARCH Open Access

Evaluation of anti-cancer potential of *Excoecaria agallocha* (L.) leaf extract on human cervical cancer (SiHa) cell line and assessing the underlying mechanism of action

Tamanna Sultana, Arup Kumar Mitra and Satadal Das*

Abstract

Background: The incidence of cervical cancer is increasing at an alarming rate in many countries and presently, it is the most common form of malignant cancer being reported among women in India. Development of novel approach for cervical cancer therapy, sparing healthy normal cells overcoming the limitations of prevailing therapies is of prime importance. Mangroves constitute a significant repository of medicinally important plants. Thus, in this study, we aimed to determine the anticancer activity of the mangrove *Excoecaria agallocha* L. leaf extracts on human cervical cancer (SiHa HPV 16+) cell line with subsequent characterization of the bioactive compounds conferring the anticancer activity and studying the probable underlying mechanism of action of the purified plant extract.

Results: The plant extract was subjected to silica gel column chromatography and the fractions obtained were analyzed for cytotoxic activity against SiHa cells by MTT assay. One out of the three eluted fractions exhibited selective toxicity against SiHa cells with an IC_{50} value of 15.538 ± 0.577 µg/mL, while it had no cytotoxic effect on normal healthy human peripheral blood mononuclear cells. High-resolution liquid chromatography mass spectroscopy, coupled to electron spray ionization and diode array detection analysis, led to the structure elucidation and identification of a few pharmacologically important compounds, with Bergenin being present in the highest abundance. Fluorescence microscopy results revealed that the plant extract fraction induced LC3 puncta formation, in EGFP- SiHa cells indicating the onset of autophagy, with simultaneous stimulation of mitophagy. The plant extract also inhibited proliferation of the SiHa-smac-mCherry cells by second mitochondria-derived activator of caspase (SMAC)—induced cytochrome c dependent apoptosis, that was further confirmed with Caspase-3 activation by colorimetric assay. The GFP-dgn in SiHa cells was remarkably protected from proteasomal degradation that might upregulate the survivability of the cells significantly. Flow cytometry followed by Western blot analysis further asserted the ability of the plant extract fraction to cause cell cycle arrest of SiHa cells in the G2/M phase by significantly reducing protein expression levels of cyclin B1 and D1, decreasing Cdc2 level and simultaneously increasing p21 and p53 levels.

Conclusion: It could be inferred that the aqueous extract of *E. agallocha* successfully decreased the proliferation of SiHa cervical cancer cells through induction of autophagy and apoptosis in a concerted manner, with simultaneous stimulation of mitophagy and G2/M phase cell cycle arrest, hinting at Bergenin being the major compound

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Publication

Sultana *et al.*
Bulletin of the National Research Centre (2021) 45:210
https://doi.org/10.1186/s42269-021-00668-x

Bulletin of the National Research Centre

RESEARCH Open Access

An in vitro approach to combat multidrug resistance in *Salmonella typhi* and human colon cancer with *Excoecaria agallocha* L. extract

Tamanna Sultana, Arup Kumar Mitra and Satadal Das*

Abstract

Background: The incidence of antibacterial resistance and colon cancer is increasing in India. Antibacterial resistance and chemoresistance demand the need of developing herbal or natural chemotherapeutic agents. Our study thus, aims to determine the antibacterial and anticancer activities of the leaf extracts of the mangrove *Excoecaria agallocha*.

Results: Liquid chromatography–mass spectroscopy analysis of the ethanolic *E. agallocha* extracts revealed the presence of Bergenin. The plant extract fraction containing Bergenin had potent antibacterial action against a resistant strain of *Salmonella typhi* with an MIC value of 15.7 ± 0.04 µg/mL. Treatment of the bacteria with the plant extract made it moderately susceptible to the antibacterial drugs ampicillin, aztreonam, cefotaxime, chloramphenicol and imipenem. The plant extract caused membrane damage and disrupted the expression of a 33 kDa outer membrane protein (OmpA) in *S. typhi*. It was plausibly due to this mechanism of the plant extract that made the bacteria susceptible to the antibacterial drugs to a certain extent. Further, fluorescence microscopy analysis revealed the anticancer property of the extract against a human colon cancer (DLD-1) cell line by activation of Caspase-3followed by subsequent apoptosis and exhibited cytotoxicity against the cancerous cell line with an IC50 value of 17.99 ± 1.12 µg/mL. Caspase-3 activity was observed to increase in a dose-dependent manner as determined by spectrophotometric assays. Moreover, the expression of the metalloproteinase-7 (MMP-7) was significantly reduced in plant extract treated DLD-1 colon cancer cells.

Conclusion: The results indicate that *E. agallocha* is a novel source of Bergenin, and the plant extract fraction under study may be used in combination therapy along with antibacterial drugs to combat antibacterial resistance of *S. typhi* and also to alleviate the risks of colon cancers in human. However, further investigations may be undertaken for its therapeutic application and to explore its potential bioactivity against other bacterial strains and human cancer cell lines.

Keywords: Antibacterial resistance, Bergenin, Caspase-3, Colon cancer, *Excoecaria agallocha*, Mangrove, *Salmonella typhi*

Background

Bacterial infections pose a serious threat worldwide and are a leading cause of deaths reported every year in almost all countries. Though antibacterial drugs are routinely used to treat bacterial infections, the incidence of increasing resistance against antibacterial drugs is a matter of concern (Harbottle et al. 2006). Multidrug resistance in bacteria is emerging as a global threat that is prompting researchers to discover potential bioactive compounds from natural sources and plants.

The World Health Organization (WHO) enlists numerous bacterial pathogens that cause severe infectious diseases. Among them, *Staphylococcus aureus, Klebsiella pneumoniae, Haemophilus influenzae, Salmonella typhi*

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Publication

Sultana et al., IJPSR, 2021; Vol. 12(9): 4774-4789. E-ISSN: 0975-8232; P-ISSN: 2320-5148

IJPSR (2021), Volume 12, Issue 9 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 07 September 2020; received in revised form, 02 February 2021; accepted, 23 May 2021; published 01 September 2021

ANTIMICROBIAL ACTION OF MANGROVE PLANT EXTRACTS AGAINST *SALMONELLA TYPHI* AND *CANDIDA PARAPSILOSIS* CHARACTERISED BY THEIR ANTIOXIDANT POTENTIALS AND BIOACTIVE COMPOUNDS

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Keywords:

Mangrove plants, *Excoecaria agallocha*, Antimicrobial, Antioxidant, Natural bioactive agents, Liquid chromatography-mass spectroscopy

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ABSTRACT: Plants present in the mangrove ecosystem are underexplored for their natural bioactive agents, including a neglected scope of inventing newer antimicrobials, to combat global crisis mediated by MDR microorganisms. In this study, we investigated four plants-*Excoecaria agallocha, Bruguiera gymnorhiza, Avicennia alba* and *Aegialitis rotundifolia* of the Sundarbans, world's largest mangrove ecosystem in West Bengal, India, for their antimicrobial activities against *Salmonella typhi* and *Candida parapsilosis*, in addition to their important bioactive resources including antioxidants. Ethanolic, methanolic, and DMSO extracts of leaves of these plants were studied by antimicrobial screening, determination of total phenolic and flavonoid contents, DPPH free radical scavenging activity, ABTS [2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)] assay, ferric reducing antioxidant power (FRAP), lipid peroxidation inhibition, thin layer chromatography, and liquid chromatography-mass spectroscopy (LC-MS). *E. agallocha* extracts showed excellent antimicrobial activities against *S. typhi*, while antifungal activity against *C. parapsilosis* was almost lacking. MIC values of all the extracts against *S. typhi* was as low as 3.96 µg/mL, however, growth inhibition was most with *E. agallocha* extract. Again total phenolic content (>300 mg/g), DPPH scavenging activity (75.55%), ABTS scavenging (78.53%), lipid peroxidation inhibition (64.35%) activities were found highest with *E. agallocha* extract. All chemicals retrieved by LC-MS of *E. agallocha* were found bioactive, among them hexanoylglycine, chorismic acid, tyramine, methyl jasmonate, khayanthone, chlorogenic acid were found particularly important. This study undoubtedly pointed out a good quality natural reservoir of important antimicrobials, antioxidants, and bioactive chemicals in the mangrove plants studied by us, predominantly in *E. agallocha*, which emerged a candidate mangrove plant for industrial development for such chemicals.

INTRODUCTION: Antibiotics are regularly used for the therapy of bacterial infections. However, overuse of antibiotics has become the major risk factor for the emergence of multi-drug resistant (MDR) strains of microorganisms [1].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.12(9).4774-89

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.12(9).4774-89

The worldwide emergence of *Escherichia coli, Klebsiella pneumoniae, Haemophilus influenzae,* and many other β-lactamase producers has become a major therapeutic problem.

Water and food-borne infections caused by *Salmonella* and *Vibrio* is a global threat that needs to be controlled. Multi-drug resistant strains of *E. coli* and *K. pneumoniae* are widely distributed in hospitals and are increasingly being isolated from community-acquired infections [2, 3]. *S. typhi* is a clinically important bacterium which causes typhoid fever, while many other *Salmonella* spp.

Publication

ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 12, Issue 5, 2019

Online - 2455-3891
Print - 0974-2441

Research Article

A PRELIMINARY OBSERVATION ON AN EXPLICIT ANTIMICROBIAL ACTION OF MANGROVE PLANTS ON *PSEUDOMONAS AERUGINOSA*

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Received: 13 March 2019, Revised and Accepted: 08 April 2019

ABSTRACT

Objectives: Due to emerging drug-resistant microorganisms throughout the world, newer antimicrobial agents should be looked for. Plants are enriched with different bioactive chemicals. In this study, we searched antibacterial activities of some mangrove plant extracts against *Pseudomonas aeruginosa*, *Staphylococcus aureus,* and *Escherichia coli.*

Methods: In the present study, the antimicrobial activity of the leaves of *Bruguiera gymnorhiza, Excoecaria agallocha, Avicennia alba,* and *Aegialitis rotundifolia* was evaluated against a few reference pathogenic bacterial strains, namely, *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *S. aureus* ATCC 29213, and multidrug-resistant bacterial strains *E. coli* extended-spectrum beta-lactamases strain. Aqueous, ethanolic, methanolic, and dimethyl sulfoxide (DMSO) extracts were studied. The antimicrobial activities of the organic solvent extracts on the various test microorganisms were investigated using agar well diffusion technique followed by determination of minimum inhibitory concentration values by serial dilution in a microtiter plate.

Results: Ethanol and DMSO extracts of *B. gymnorhiza* exhibited promising antimicrobial activity followed by extracts of *A. alba* and *E. agallocha*. Among all microorganisms studied, *P. aeruginosa* ATCC 27853 showed significant growth inhibition with ethanol and DMSO extracts.

Conclusion: Extracts of some mangrove plants, particularly, *B. gymnorhiza* showed very good antimicrobial activities against common microbial agents causing human infections and in general mangrove plants appear to act better on *P. aeruginosa.*

Keywords: *Bruguiera gymnorhiza, Excoecaria agallocha, Avicennia alba, Aegialitis rotundifolia, Pseudomonas aeruginosa*, Antimicrobial activity.

 DOI: http://dx.doi.org/10.22159/ajpcr.2019.v12i5.32975

INTRODUCTION

In the recent years, over-exploitation of antibiotics has caused the emergence of multidrug-resistant (MDR) microbes, and therefore treatment of such MDR microbial diseases demand a search for novel alternative and effective therapeutic compounds [1]. The major search focus has, thus, been shifted toward exploring the natural flora with known antimicrobial and antioxidant properties.

Historically, plants have been proved to be a source of inspiration for novel drug compounds as plant-derived medicines have made large contributions to human health and well-being. Their role is two-fold in the development of new drugs: First, they may become the base for the development of a medicine, a natural blueprint for the development of new drugs, or second: A phytomedicine to be used for the treatment of various diseases. It is estimated that today, plant materials are present in or have provided the models for 50% Western drugs [2]. The primary advantage of using plant-derived medicines is that they are relatively safer than synthetic alternatives, offering profound therapeutic benefits, and more affordable treatment.

Several research works to date have suggested the potential of mangrove floral community in traditional medicines [3-5]. For centuries, the tribal population employed mangrove plant extracts as their traditional folk medicine for healing several health disorders [6-8]. However, unlike various herbs, seaweeds and higher medicinal plants, the use of mangroves as alternative medicine has been comparatively less explored [9-14].

The Indian Sundarbans, one of the most taxonomically diverse and physicochemically dynamic ecosystems of the Indian subcontinent, sustains some 34 species of true mangroves among which members of the Avicenniaceae family in which our present study plants present, rank second in terms of prevalence [15,16]. In comparison to the normal terrestrial flora, this halophytic mangrove community gets exposed to high and low tides twice in every 24 h [17], and therefore, has developed a unique mode of adaptation, which could have enriched their phytochemical repertoire of medicinal importance. Wu *et al.* showed that *Avicennia marina*, a true mangrove floral species (commonly known as gray mangrove), contains few quinone derivatives with therapeutic and antimicrobial properties [18].

Mangroves are widespread in tropical and subtropical regions, growing in the saline intertidal zones of sheltered coastlines and contain biologically active antimicrobial compounds. Previous studies on mangrove plant parts and its chief chemical classes exhibited various levels of biological activities such as antibacterial, antifungal, cytotoxic, hepatoprotective, and free radical scavenging activities [19-25,]. Mangrove plant parts have been used for centuries as popular medication for various health disorders. Numerous studies have been conducted on various natural products screening their antimicrobial property [20,26]. The present study made an attempt to investigate the antibacterial properties of four mangrove plants, namely, *Bruguiera gymnorhiza, Excoecaria agallocha, Avicennia alba,* and *Aegialitis rotundifolia* against pathogenic and antibiotic-resistant bacterial strains.

METHODS

Collection of plant samples

Fresh leaf samples of *B. gymnorhiza, E. agallocha, A. alba,* and *A. rotundifolia* were collected from Bali Island of the Indian Sundarbans during the month of June 2018. The samples were stored at 4°C after collection and utilized within 7 days.

Patent

(12) PATENT APPLICATION PUBLICATION
(19) INDIA
(22) Date of filing of Application :10/07/2020

(21) Application No.202031029308 A
(43) Publication Date : 14/01/2022

(54) Title of the invention : EXCOECARIA AGALLOCHA IS AN IMPORTANT NATURAL SOURCE OF BERGENIN

(51) International classification	:C07D0305140000, G01N0033000000, A61K0047610000, G06K0009620000, A61K0036835000
(31) Priority Document No	:NA
(32) Priority Date	:NA
(33) Name of priority country	:NA
(86) International Application No	:NA
Filing Date	:NA
(87) International Publication No	: NA
(61) Patent of Addition to Application Number	:NA
Filing Date	:NA
(62) Divisional to Application Number	:NA
Filing Date	:NA

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(57) Abstract :
This invention relates a new natural source of a biologically active chemical known as Bergenin. Although there are many other natural sources of this chemical, we claim a new natural source of this chemical in Excoecaria agallocha, which is a common mangrove plant in the Sundarbans - a mangrove forest of West Bengal. In Excoecaria agallocha, this chemical is present in large amounts and it can be extracted with ethanol and purified to HPLC grade, thus, the extracted Bergenin from Excoecaria agallocha will be more pure and will be less costly than the available Bergenin in the market.

No. of Pages : 11 No. of Claims : 4

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