IN VITRO STUDIES ON THE ANTI - INFECTIVE PROPERTIES OF FLOWER AND FRUIT EXTRACTS OF SELECTED MEDICINAL PLANTS AGAINST ENTEROPATHOGENS.

Submitted by

MUHSINA A. S.

(2014-09-122)

THESIS

Submitted in partial fulfilment of the

requirement for the degree of

B. Sc. – M. Sc. (INTEGRATED) BIOTECHNOLOGY

Faculty of Agriculture
Kerala Agriculture University, Thrissur

B.Sc. - M.Sc. (INTEGRATED) BIOTECHNOLOGY
DEPARTMENT OF PLANT BIOTECHNOLOGY
COLLEGE OF AGRICULTURE
VELLAYANI, THIRUVANANTHAPURAM- 695 522
KERALA, INDIA

2019

DECLARATION

I hereby declare that this thesis entitled '***In vitro*** **studies on the anti-infective properties of flower and fruit extracts of selected medicinal plants against enteropathogens'** is a bonafide record of the research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

By

Place: Vellayani

MUHSINA A. S.

Date: 29-11-2019

(2014-09-122)

CERTIFICATE

Certified that thesis entitled **"*In vitro* studies on the anti-infective properties of flower and fruit extracts of selected medicinal plants against enteropathogens"** is a record of the research work done independently by **Mrs. Muhsina A. S.** (2014-09-122) under my guidance and supervision and that this has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

Place: Palode
Date: 29-11-2019

Dr. Shiburaj Sugathan
(Chairperson, Advisory Committee)
Senior Scientist
Department of Microbiology
JNTBGRI, Palode
Thriruvananthapuram-695562

CERTIFICATE

4

We, the undersigned members of the advisory committee of Ms. Muhsina A. S. (2014-09-122) a candidate for the degree of B. Sc. - M. Sc. (Integrated) Biotechnology, agree that the thesis entitled **"*IN VITRO* STUDIES ON THE ANTI-INFECTIVE PROPERTIES OF FLOWER AND FRUIT EXTRACTS OF SELECTED MEDICINAL PLANTS AGAINST ENTEROPATHOGENS"** may be submitted by Ms. Muhsina A. S. in partial fulfilment of the requirement for the degree.

Dr. Shiburaj Sugathan
(Chairman, Advisory Committee)
Senior Scientist
Department of Microbiology
JNTBGRI, Palode
Thriruvananthapuram-695562

Dr. K. B. Soni
(Member, Advisory Committee)
Professor and Head
Department of Plant Biotechnology
College of Agriculture, Vellayani
Thiruvananthapuram- 695522

Dr. Swapna Alex
(Member, Advisory Committee)
Professor and Course Director
B. Sc. - M. Sc. (Integrated)
Biotechnology Course
Department of Plant Biotechnology
College of Agriculture, Vellayani
Thiruvananthapuram- 695522

Dr. Vipin Mohan Dan
(Member, Advisory Committee)
Scientist
Department of Microbiology
JNTBGRI, Palode
Thriruvananthapuram-695562

Dr. T. S. Swapna
(External Examiner)
Professor and Head
Department of Botany
University of Kerala, Kariavattom
Thiruvananthapuram- 695581

ACKNOWLEDGMENT

The successful outcome of this research work would not have materialized if not for the guidance, critical evaluation and support of a group of remarkable people. First and foremost, I must acknowledge and thank the God Almighty for blessing, protecting and guiding me throughout this period.

I am indebted to my supervisor, Dr. Shiburaj Sugathan of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode, Thiruvananthapuram for his guidance, support and endeavour in making this research possible. I would also like to express my sincere gratitude to Dr. Shiburaj Sugathan for the continuous support of my research study, for her patience, motivation, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis.

I am thankful to Dr. Prakash Kumar Director JNTBGRI for providing me all facilities for carrying out this work and giving me an opportunity to work at JNTBGRI for my thesis.

I extend my sincere gratitude to the members of advisory committee Dr. Vipin Mohan Dan, Scientist, Division of Microbiology, Dr. Swapna Alex, Professor and Head, Department of Plant Biotechnology, College of Agriculture, Vellayani, Dr. K. B. Soni, Course Director, Integrated Biotechnology Course for their valuable counselling and constructive suggestions that were helpful throughout my project.

I take immense pleasure to express my thanks to Mr. Nandu T.G, Mrs. Praseetha Sarath, Dr. Rachana Mol R. S, Dr. Reji S. R, Dr. Swaroop S. Kumar, Ms. Gayathri V, Ms. Reshma R. Anilkumar, Mrs. Soumya K. M, Mrs. Apsara Lekshmi M, Ms. Viji Das M. S, Mr. Nitheesh P of Microbiology Division for being there for me always, rendering whatever help I needed during my all experiments and for providing me suggestions and encouragement throughout my research work.

I express my heartfelt thanks to Dr. Anil kumar DEAN College of Agriculture Vellayani, for the facilities provided.

I thankfully remember my friends Anjana, Reshma T.K., Hasmi, Parveen, Adithya, Reshma A, Amiya, Neethu, Akitha, Lekshmi, Linet, Keerthana, Rajani, Jyothi, Sree,Vishnu, Alif who have rendered helping hands and support during my research work.

I thank all my seniors and juniors for their support and guidance in my research.

My parents deserve special mention for their inseparable support and prayers. My Father, in the first place is the person who showed me the joy of intellectual pursuit ever since I was a child. My Mother is the one who sincerely raised me with her caring and gentle love. I would like to express my heartfelt thanks and great debts of gratitude to my Father in law, Mother in law, Brother in law and Sister in law for being supportive and caring. Words elude me to express my appreciation to my son Aabid Ali and my husband Saril P. A. whose dedication, love and persistent confidence in me, has taken the load off my shoulder. He helped me in different ways & with whom I shared my joys & sorrows.

Besides this, there are several other people who have knowingly or unknowingly helped me in the successful completion of this project. I thank all those people for every ounce of efforts they contributed, with my sincere apology, if I could not mention anyone. Above all, I bow my head before the ***Almighty God*** *with whose grace and beatitude, I moved through this venture.*

MUHSINA A. S.

Dedicated to Almighty, my Family, Friends and Teachers

TABLE OF CONTENTS

9

LIST OF TABLES

10

LIST OF FIGURES

TABLE OF APPENDICES

12

LIST OF ABBREVIATIONS AND SYMBOLS USED

%	Percent
°C	Degree Celsius
µg	Microgram
µL	Microliter
µM	Micromolar
AA	Ascorbic acid
AMR	Anti microbial resistance
Ag NPs	Silver nanoparticles
Cm	Centimeter
DNA	Deoxyribonucleic acid
EDTA	Ethylene Diamine Tetra Acetic Acid Disodium salt
et al.	And other co workers

etc.	Etcetera
$FeCl_2$	Ferric chloride anhydrous
$FeSO_4$	Ferrous sulphate
G	Gram
GTP	Guanosine triphosphate
H	Hour
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HGT	Horizontal gene transfer
IPTG	Isopropyl β-D-1 thiogalactopyranoside
KCL	Potassium Chloride
Kg	Kilogram
L	Litre
M	Molar
MBC	Minimum bactericidal concentration
MBIC	Minimum biofilm inhibitory concentration
Mg	Milligram
Min	Minute

mL	Millilitre
Mm	Millimetre
mM	Millimolar
MIC	Minimum inhibitory concentration
N	Normality
Ng	Nanogram
NIH	National Institute of Health
Nm	Nanometer
Nmol	Nanomole
NPs	Nanoparticles
OD	Optical Density
OH	Hydroxyl
pH	Potential of hydrogen
QS	Quorum sensing
Rpm	Rotation per minute
RB	Round Bottom

15

RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
STD	Standard
TCA	Trichloro Acetic acid
TPC	Total Phenolic Content
UV	Ultraviolet
w/v	Weight/Volume
WHO	World Health Organization

Introduction

CHAPTER 1
INTRODUCTION

Infectious diseases are the leading cause of death worldwide that are caused by bacteria, fungi and viruses. It is well known that infectious diseases are responsible for a high proportion of health problems, especially in developing countries. The World Health Organization (WHO) recently warned that "Infectious diseases are emerging faster and spreading more rapidly than ever before". The battle against emerging infectious diseases has a long history and deep roots. Bacterial infections are the most common cause of diarrhoea, often spread through the fecal-oral route. This is when infected feces is accidentally spread from one person to the next (such as through a handshake), by coming into contact with a contaminated surface or utensil, or ingesting contaminated food or drink. There are also non-infectious sources of diarrhoea, often related to medical conditions affecting the digestive, immune, or endocrine (hormone) system. Among these are irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), celiac disease, hyperthyroidism, and lactose intolerance. If the diarrhoea is accompanied by blood; it is referred to as dysentery.

Gastroenteritis, also known as infectious diarrhoea, is characterized by the inflammation of the stomach and gastrointestinal tract and can lead to vomiting, diarrhoea, fever, and abdominal distress. Infection is more common when there is a shortage of adequate sanitation and hygiene and safe water for drinking, cooking and cleaning. *Escherichia coli* and *Vibrio cholerae*, are the two most common etiological agents of moderate-to-severe diarrhoea in developing countries. Other pathogens such as *Staphylococcus aureus, Salmonella enteritis* serotype Typhi and *Enterococcus feacalis* species may also be important. Location-specific etiologic patterns also need to be considered. *Vibrio cholerae,* the etiologic agent for the diarrhoeal disease cholera, continues to be an important cause of morbidity and mortality in many areas of Asia, Africa, and Latin America. The WHO describes cholera as a tragedy because this theoretically "most preventable disease" is one of the top causes of human morbidity and

mortality in the world. Many diarrhoeal diseases are known to be treated with herbal remedies throughout the history of humankind. Even today, plant materials continue to play a major role in primary health care as therapeutic remedies in many developing countries (Zakaria, 1991). For centuries, many secondary compounds of fruits are found to have an outstanding role in medicine. Most of these compounds belong to a few families of substances such as alkaloids, terpenoids and phenolics (Herbal Medicine Research Centre, 2005). The WHO estimates that 80% of the population living in rural areas use or depend on herbal medicine for their health needs (WHO, 2003). Therefore, globally, various species of tropical flowering plants have medicinal properties and this has made traditional medicine cheaper than modern that can be used to treat chronic as well as infectious diseases (Hertog *et al.*, 1993; Dash *et al.*, 2007; Odugbemi, 2006). The control of bacterial infection has been remarkably effective since the discovery of antibiotics. However, some of the pathogens rapidly become resistant to many of the first discovered effective drugs. The development of drug-resistance as well as appearance of undesirable side effects of certain antibiotics (WHO, 2002) has led to the search of new antibacterial agents from medicinal plants. The screening of plant extracts has been of great interest to scientist for the discovery of new drug effective in the treatment of several diseases (Dimayuga & Garcia, 1991).

There is an urgent need for new therapeutic strategies to counteract the global threat of antibiotic resistance, which has become, in recent years, one of the major public health concern (Schillaci *et al.,* 2017). Compounds targeting novel targets and agents acting without selection pressure are promising therapeutic approaches, that can reduce emergence of drug-resistant bacteria, The giant leap in genome sequencing technology enabled scientific community to hunt for novel drug targets that can act as alternative to classical antibiotic targets. The whole genome sequence (WGS) of bacteria revealed several drug targets crucial for bacterial survival. Information generated from WGS opened a new avenue for target based drug antibiotic discovery. The overuse of antibiotics against classical drug targets inserted selection pressure on bacteria and resulted in emergence of drug-resistant strains. Compounds acting on novel targets may result in development of novel antibiotics that effective on drug-

resistant bacteria. The survival ability of bacteria (virulence) within harsh host environment helps bacteria to establish infection and spread systemically. Virulence factors play a crucial role in intra-host survival of pathogens. Compounds targeting virulence factors reduce bacteria's ability to establish infection and survival. These agents act on bacteria without any selection pressure and they are less prone to development of drug-resistance. Thus, anti-virulent agents can play crucial role humankind's defence against drug-resistant bacteria.

An important contribution to the microbial survival in hostile environments has been given by the capability of pathogens to form sessile communities able to adhere to biotic or abiotic surfaces, known as biofilms (Li X *et al* ., 2017). Biofilm formation remains one of the leading cause of antibiotic treatment failure so that it is an ideal target for the development of new anti-infective agents able to interfere with the bacterial adhesion of enteropathogens. Unfortunately, no anti-biofilm compound has reached the clinical use and the most promising candidates are still in early stages of drug development, this is mainly due to the lack of *in vivo* studies. (Rabin *et al* ., 2015). Because of that the treatment of serious biofilm-associated infections are remain a significant problem. The second target is used in this study is FtsZ protein, which plays prime role in bacterial cell division. It orchestrates cell division by recruiting other cell division proteins. Targeting bacterial cell division is believed to be critical in new antibiotic development because cell division is an essential process for bacterial survival and the bacterial divisome possesses a complex set of biochemical machinery that contains many proteins as potential drug targets. Among these proteins, filamenting temperature-sensitive mutant Z (FtsZ) has been identified as a very critical protein that can influence bacterial cell division and it is highly conserved in a wide range of bacteria (Margolin., 2000)

Phytochemicals are biologically active, powerful group of naturally occurring chemicals that are derived from plants, which contribute health benefits for human. They protect the plants from disease and damage caused from environmental hazards such as pollution, stress, UV exposure and pathogenic attack. Phytochemicals are concentrated in different parts of the plants, such as in the roots, stems, leaves, flowers, fruits, tubers or seeds. These are secondary me-

tabolites that are produced by plants to protect themselves, and have biological properties like modulation of detoxification enzymes, antioxidant property, antimicrobial effect, immune system stimulation, anticancer property, hormone metabolism modulation and decrease of platelet aggregation (Saxena *et al.*, 2013). Among plant secondary metabolites, phenols are the largest group of phytochemicals which is abundantly distributed through the plant kingdom (Singh *et al.*, 2007). They are complex in structure. Antioxidant properties of phenols are beneficial to human health. They protect human tissues from oxidative damage by scavenging harmful free radicals. (Walton *et al.*, 2003). Fresh and processed fruits and there products contain high levels of a diverse range of phytochemicals of which polyphenols including hydrolyzable tannins [ellagitannins (ETs) and gallotannins] and condensed tannins (proanthocyanidins), and anthocyanins and other flavonoids make up a large proportion.

Ellagic acid is a naturally occurring phenolic lactone compound found in a variety of plant species, especially fruits. It is present in plants in the form of hydrozable tannins called ellagitannins as the structural components of the plant cell wall and the cell membrane. Ellagitannins are esters of glucose with ellagic acid which, when hydrolyzed, yield ellagic acid. Ellagic acid is seen at high concentrations in many berries including strawberries, raspberries, cranberries and grapes (Marwan and Nagel 1986; Chen *et al.*, 2001). Other sources of ellagic acid include walnuts, pecans (Daniel *et al.*, 1989) and distilled beverages (Goldberg *et al.*, 1999). Recent studies have indicated that ellagic acid possesses antimutagenic, antioxidant and anti-inflammatory activity in bacterial and mammalian systems (Kaur *et al.*, 1997) Gallic acid, ellagic acid and punicalagin, in addition to their free radical-scavenging properties, also possess antibacterial activities against intestinal flora, particularly enteric pathogens, i.e., *Escherichia coli, Salmonella spp*, *Shigella* spp., as well as *Vibrio cholerae* (Aviram *et al.*, 2008)

In silico tools simplifies complicated procedures of drug discovery. *In silico* tools enable to screen large compound library to identify biologically active compounds (hits), improving drug likeness of lead compound by increasing

biological activity or optimizing pharmacokinetic properties and designing new compounds (Sliwoski et al. 2014). *In silico* methods include quantitative structure-activity relationships, similarity search, pharmacophores, homology models, and other molecular modeling instruments, network analysis instruments and computer-using data analysis instruments. Such methods have seen frequent use in the discovery and optimization of novel molecules with affinity to a target, the clarification of absorption, distribution, metabolism, excretion and toxicity properties as well as physicochemical characterization (Ekins *et al.*, 2007).

Investigation of anti-infective properties of fruit and flower extracts of medicinal plants against enteropathogens, may lead to the development of new plant based drugs. Therefore it is of great to carryout antibacterial studies of fruits and flowers, in order to validate their use in modern medicine. Household fruits and their flowers of Kerala, available commonly, are selected in the present study for evaluating their medicinal roles. The main aims and objectives of the present study are:

-Screening of medicinal plant extracts for anti infective property against enteropathogens and identification of lead compounds.
-Targeted efficacy testing of selected compounds against FtsZ proteins through *in vitro* and *in silico* approaches.
-Demonstrating the effect of these compounds on virulence factors and biofilm formation of enteropathogens.

Review of Literature

CHAPTER 2

REVIEW OF LITERATURE

2.1 TARGETING ANTIMICROBIAL RESISTANCE USING PLANT COMPOUNDS

Antimicrobial resistance (AMR) is one of the ultimate fears to the health of humans and animals worldwide. Use of antimicrobial drugs in humans or animals results in the emergence and dissemination of resistant bacteria, and overuse or abuse of them makes this situation worse. Thus, it is important to simultaneously preserve effective antimicrobials as long as possible as well as continue to employ them for the service of human and animal health (Chang *et al.*, 2015). The dissemination of AMR has not been paralleled by the development of novel antimicrobials. This is due to that the process of drug discovery and clinical trials of new antimicrobials takes a long time, and only a few new agents have recently been approved for use. These situations prompt the efforts to develop alternatives to traditional antimicrobials, as described in our previous review (Cheng *et al.*, 2014). However, some of the alternatives are only used for the prevention of bacterial infections (e.g., vaccines); some show indirect effect against pathogens (e.g., immunomodulators, feed enzymes); some are of complex composition (e.g., probiotics, plant extracts), thus the effects vary greatly; and the antimicrobial peptides, such as one of the bacteriocins, antibiotics, have been reported causing bacterial resistance (Draper *et al.*, 2015).

We concentrated on old and new antimicrobials against AMR in this review. There are two levels of AMR, cellular (mutation and horizontal gene transfer (HGT) of resistance determinants) and community (biofilm and persistent cells) resistance (Penesyan *et al.,* 2015). The studies examined suggest that only the rational use of old antimicrobial drugs and the combination of anti- resistance or antibiofilm strategies with antimicrobials and the continued development of new antimicrobial agents can combat AMR.

2.1.1 MECHANISMS OF AMR

24

AMR includes two levels of resistance, the cellular level resistance and the community level resistance (Penesyan *et al.*, 2015). Cell resistance develops due to endogenous gene mutations and resistance determinants from other microorganisms via HGT. A group of bacteria can also tolerate the environmental stress that individual cells cannot, which is referred to as resistance at the Community level. Such tolerance can lead to increased antimicrobial resistance (Penesyan *et al.,* 2015). For example, the resistance of biofilm microorganisms can be up to 1000 times higher than their planktonic counterparts, which affects the treatment of clinical treatment with biofilm - related infections (Lebeaux *et al.*, 2014; Penesyan *et al.*, 2015). The presence of persistent cells is the main mechanism for explaining this tolerance. Persists can escape the lethal action of antimicrobials by entering a physiological state that does not kill antimicrobials (Maisonneuve and Gerdes, 2014). In addition, the resistance levels of cellular and community can be synergistic, significantly improving the overall AMR of the microbial community. (Penesyan *et al.*, 2015)

2.1.2 REVISIT TO ANCIENT ANTI - MICROBIAL PRODUCTS

As AMR increases in commonly used antimicrobial drugs, older antimicrobials are "revived" and attract attention. These old antimicrobials are a new alternative to AMR control (Pulcini *et al.*, 2012). Cassir *et al.* (2014) collected microbiological and clinical data on potentially useful older antimicrobials for the successful treatment of multidrug– resistant (MDR) Gram negative bacterial infections (e.g., polymyxins, fosfomycin, mecillinam, temocillin, and nitrofurantoin), MDR Gram-positive infections (e.g., trimethoprim-sulfamethoxazole, tetracyclines, chloramphenicol, clindamycin, pristinamycin, rifampicin, and fusidic acid), and MDR tuberculosis (e.g., clofazimine, amoxicilline-clavulanate, trimethoprim-sulfamethoxazole, and minocycline). Since older antimicrobials have rarely been subject to current drug development

procedures, practical guidelines take less account of them. Its efficacy and safety must therefore be reassessed in order to optimize therapy.

A number of antimicrobials discovered decades ago that have unique chemical scaffolds and/or use new ways of interacting with bacterial targets such as ribosomes (Arenz and Wilson, 2016). For example, in a small ribosomal subunit, dityromycin, a cyclic antibiotic decapeptide produced by *Streptomyces sp.*, can uniquely bind to ribosomal protein S12, a different mode of action than any other known translation inhibitor (Bulkley *et al.*, 2014). These "forgotten" compounds often show cytotoxicity to eukaryotic cells and have therefore been abandoned (Arenz and Wilson, 2016). However, recent structure - function analysis gives us a better understanding of the similarities and differences between bacterial targets and their eukaryotic counterparts, leading to the development of more specific and less toxic inhibitors in the future. Revisiting the known antimicrobials is helpful in the exploration of the next generation of antimicrobial drugs with an increased understanding of AMR mechanisms.

Antimicrobial registration procedures have significantly improved. The EU (EMA, 2013) and the US authorities (FDA, 2010) have published numerous guidelines and addressed the growing need for antimicrobials active against MDR bacteria. The guidance documents contain recommendations for dosing regimes based on the relationship between pharmacokinetics (PK) and pharmacodynamics (PD). PK / PD provides a universal framework for relationships between exposure and response, and the responses include effectiveness, toxicity and resistance (Muller *et al.*, 2015). In the area of PK profiling in patients, there are knowledge gaps for these revived antimicrobials and PD targets derived from pre - clinical and clinical studies (Muller *et al.*, 2015). Although the regulatory requirements for new antimicrobials have become increasingly strict, updates to the product information for old antimicrobials are either missing or insufficient, which would pose significant risks to patients with potential harm. In addition, companies are not motivated to develop antimicrobials when the cost and time of drug approval far exceeds commercial interests, even if there is a clear medical need.

The current antimicrobials, mainly derived from natural sources, inhibit cellular processes such as cell wall biosynthesis, DNA replication, and protein synthesis. With the worldwide emergence of AMR, there is renewed interest in the investigation of alternative essential cellular processes, including bacterial central metabolic pathways, as the drug targets for the next generation of antimicrobials (Murima *et al.*, 2014). For examples, bedaquiline is an antitubercular drug targeting the F0F1 ATP synthase (Andries *et al.*, 2005). Like bedaquiline, Q203, an optimized imidazopyridine amide compound, selectively inhibits the respiratory cytochrome bc1 complex in mycobacteria regardless of architectural conservation of the bc1 complex in many species (Pethe *et al.*, 2013). The inhibition of the bacterial divisome, mainly by targeting the central cell division mediator FtsZ, has been accepted as a promising strategy for antimicrobial attack by either interfering with the natural dynamics and functions of FtsZ during the cell cycle or activating a bacterial protease to degrade FtsZ, thus causing bacterial death in a suicidal manner (Sass and Brötz - Oesterhelt, 2013). The mode of action of alkyl gallate is a combination of direct targeting of FtsZ and permeabilization of bacterial membranes, which is a promising hit for the further development of antibacterials (Król *et al.*, 2015). Recent efforts have also been devoted to developing drugs that interrupt the assimilation of iron by bacteria, a process that is vital to cellular homeostasis (Foley and Simeonov, 2012). The unique asymmetric outer membrane in Gram-negative bacteria, which acts as a permeability barrier that protects the cell from external stresses such as the presence of antimicrobials, has become an attractive drug target. A novel β - hairpin macrocyclic peptide, JB-95, exhibits an ability to selectively disrupt the outer membrane through interactions with selected β - barrel outer membrane proteins including Bam A and Lpt D, but not the inner membrane of *E. coli* (Urfer *et al.*, 2016). Furthermore, the bacterial protein secretion pathway is a target for eliminating or disarming pathogens. Targeting the Sec-pathway for novel antimicrobial agents focuses on two key components: Sec A, the ATP-driven motor protein responsible for driving pre proteins across the cytoplasmic

membrane and theType I signal peptidase which is responsible for the removal of the signal peptide to allow the release of the mature protein from the membrane (Rao *et al.*, 2014). Targeting resistance, usually used in combination with the traditional antimicrobials, is another strategy to fight against AMR. Among the four general resistance mechanisms, which include target alteration (Hooper and Jacoby, 2015), drug inactivation (Shaw *et al.*, 1993; Bush and Fisher, 2011), decreased permeability (Nikaido, 2003) and increased efflux (Sun *et al.*, 2014; Li *et al.*, 2015), drug extrusion by multidrug efflux pumps serves as an important mechanism of MDR. Efflux pumps also have physiological functions in response to various of environmental and physiological signals (Sun *et al.*, 2014). Recent studies have tried to reverse the resistance phenotype conferred by efflux pump activation (Opperman and Nguyen, 2015; Venter *et al.*, 2015). It was observed that the addition of efflux pump inhibitors partially restored drug susceptibility in vitro and in vivo in the antimycobacterium tuberculosis therapy (Pule *et al.*, 2016). The class of zinc dependent hydrolases, metallo-β-lactamase, can confer bacteria with extended spectrum β-lactam resistance. The design of compounds with the β-lactam core scaffold is an attractive approach to the development of metallo-β-lactamase inhibitors. Some promising inhibitors, including cephalosporin derivatives, penicillin derivatives, carbapenem derivatives, cyclobutanone β-lactam analogs, thiol derivatives, succinic acid derivatives, mercaptoacetic acid thioester derivatives, pyrrole-, pyridineand triazole-containing compounds, and DNA aptamer, have been thoroughly reviewed by King and Strynadka (King and Strynadka, 2013). Targeting the resistance mechanism itself by a vaccine is an interesting but hitherto unexplored approach (Henriques-Normark and Normark, 2014). Vaccination directed against the resistance mechanism can be possible when resistance is mediated by an enzyme whose activity can be inhibited by neutralizing antibodies. Except for the above inhibitors targeting resistance, drugs in already-known classes such as new β-lactams, quinolones, aminoglycosides, and tetracyclines have been designed to escape from many of the known resistance mechanisms. BAL30072, a siderophore monosulfactam similar to aztreonam, exhibits antibacterial activity against most species of aerobic Gram-negative bacteria (Page

et al., 2010). It is stable toward metallo-β-lactamases and is a poor substrate for many serine carbapenemases. Several new quinolones, such as avarofloxacin, delafloxacin, finafloxacin and the desfluoroquinolone nemonoxacin, which show enhanced activity against fluoroquinolone-resistant Gram-positive bacteria including MRSA are in clinical development (Page and Bush, 2014). A modified aminoglycoside, plazomicin, has been demonstrated activity against both Gram-negative and Gram-positive bacterial pathogens (Zhanel *et al.*, 2012). Modified tetracyclines, such as tigecycline, omadacycline and eravacycline are of interest for their activity against many MDR Enterobacteriaceae and *Acinetobacter* sp., including isolates expressing tetracycline-specific efflux and ribosomal protection proteins (Sutcliffe, 2011).

2.1.4 APPROACHES TO COMBAT BIOFILMS

The approaches to combat biofilms are extensively reviewed by Beloin *et al.* (2014) During the biofilm development, the bacteria initially adhere to a surface that ultimately leads to colonization and infection by pathogenic bacteria. Therefore, reducing adhesion is a strategy to prevent biofilm formation and related infections (Veerachamy *et al.*, 2014). Recently, non specific inhibition of adhesion . Targeting specific adhesions has been developed to reduce bacterial adhesion. Quorum sensing (QS), which controls many important physiological processes such as biofilm development, is a widespread intercellular for of communicating and cooperative activities of bacteria at the population level, and it depends on the production, secretion, and detection of small diffusible autoinducers, such as acylhomoserine lactones, auto-inducing peptides and autoinducer 2 (Zhang and Li, 2016). Cyclic di-GMP is a second messenger that acts to regulate a wide range of functions including developmental transitions, adhesion and biofilm formation (Caly *et al.*, 2015). Targeting these signaling pathways is also a strategy to prevent biofilm development. Moreover, using enzymes or chelating agents can hydrolyze biofilm components or destabilize biofilm matrix. On the other hand, persister cells have recently been subjected to an intensive research in order to limit biofilm associated antimicrobial toler-

ance. The formation of persister cells depends on the ubiquitous bacterial regulatory nucleotides tetra and penta-guanosine phosphate (ppGpp) that activate inhibitors of cell growth (Germain *et al.*, 2015). Therefore, interfering ppGpp could inhibit the formation of persister cells. The deep research on the mechanism of biofilm formation leads to the emergence of numerous promising antibiofilm approaches. However, the conversion of experimental data into clinical settings is time-consuming and to some extent unsatisfactory. Non-biocidal antiadhesive or antivirulence strategies face the diversity of bacterial phenotypes and may only be active against a sub population of bacteria encountered in clinical practice, therefore limiting their overall efficacy. *In vitro* biofilms are probably structurally different from *in vivo* biofilms (Lebeauxetal ., 2013). Currently, due to the diversity of the *in vivo* conditions leading to the complexity of clinical biofilm situations, the diversity of persister phenotypes is unknown. Most of the studies use rodent models, but these *in vivo* models may not properly replicate real clinical state. Furthermore, as for clinical trials, rigorous statistical analysis is compulsory in order to avoid any false positive results. Most importantly, molecules identified in vitro should be validated using in vivo models not only for the antibiofilm activity but also non-toxicity.

2.1.4.1 Natural antibiofilm agents:

Reserpine (1) was found extensively in Rauwolfia genus plants and was effective in *Klebsiella pneumoniae* biofilm inhibition at 0.0156 mg / mL, 64 times lower than its MIC (Magesh *et al.*, 2013) Bakuchiol (2) from *P. corylifolia* Linn seeds showed bactericidal effects with MICs below 4 µg / mL on numerous oral bacterial species. It also displayed inhibitory effects on adherence of *S. mutans* JCM 5175 at 100 µg/mL (Katsura *et al.*, 2001) Piperine (3) an alkaloid found in *P. longumdboth* which is a widely distributed plant species in India, showed the minimum biofilm inhibitory concentration (MBIC) against *S. mutans* of 0.0407 mg/mL (Dwivedi *et al.*, 2016) . Prabu *et al.*, found that guaijaverin (4) a flavonoid isolated from the leaves of *Psidium guajava* Linn., had the ability to prevent the adherence of *S. mutans* to smooth surfaces with 83.7% inhibition

against CLSM 001 at 500 µg/mL. Isosteviol (5) isolated from the medicinal plant *Pittosporum tetraspermum* also showed anti-biofilm activity against *P. aeruginosa* at 100 µg/mL (Al-Dhabi *et al.*, 2015). Xanthorrhizol (6) isolated from the rhizome of an edible plant (*C. xanthorrhiza Roxb.*), has the ability to remove *S. mutans* biofilm in a dose-dependent manner. Using biofilms in different stage xanthorrhizol could completely inhibit the biofilm formation in the adherent stage at the concentration of 5 µM. In mature biofilms, the inhibitory activities could reach 76% when treated with xanthorrhizol at the concentration of 50 µM with an exposure time of 60 min (Rukayadi *et al.*, 2006). Curcumin (7) showed a biofilm reduction of 83.2% at 1/2MIC (8 µg/mL) against *Helicobacter pylori* (Pattiyathanee *et al.*, 2009) Pinostrobin (8) has been found in honey and in some plants, and it has been used as a natural food supplement. Pinostrobin displayed a significant antibiofilm effect against *E. coli* and *P. aeruginosa* at 0.5 µg/mL (Christena *et al.*, 2015). A very popular fruit is *Syzygium cumini* (Myrtaceae), commonly known as Indian blackberry and widely distributed in most parts of India.In Gopu's study, malvidin and anthocyanin from *S. cumini* could significantly inhibit biofilm formation, violacein production and Extra cellular polymeric substance production in *K. pneumoniae* by more than 70%. These findings suggest that this plant may be used to control food borne disease caused by *K. pneumoniae* (Gopu *et al.*, 2015) There is enough evidence to show that many plant extracts, fractions and phytochemicals have antimicrobial and anti-biofilm activities, but it is not yet known whether these activities are clinically meaningful.

2.1.5 METALLIC NANO PARTICLES (NPs)

Physiochemical properties of nanomolecules as antimicrobial agents are widely used in human and veterinary medicine. Metallic NPs are of great interest for use as potential antimicrobial agents because of their unique optical, electronic, and magnetic properties (Kandi and Kandi, 2015). The electrostatic interaction of NPs with negatively charged bacterial surfaces draws the particles to the bacteria and promotes their penetration into the membrane, causing membrane dis-

ruption, bacterial flocculation and a reduction in viability. The generation of reactive oxygen species (ROS) is also a mechanism of antibacterial activity of NPs (Thekkae Padil and ˇCerník, 2013). Further actions of NPs as antimicrobial agents include disrupting DNA during the replication and cell division of microorganisms, compromising the bacterial membrane integrity via physical interactions with the microbial cell, and releasing toxic metal ions and causing lysis of cells (Franci *et al.*, 2015). Recently, the silicon dioxide NPs (Si-NP) were engineered to target the signaling molecules (i.e., acylhomoserine lactones) used for QS in order to halt bacterial communication (Miller *et al.*, 2015). The bactericidal activity of NPs depends on size, stability and concentration in the growth medium (Tillotson and Theriault, 2013). The applications of nanomolecules in medicine have recently been evaluated in reports highlighting the in vitro antimicrobial activities of NPs , and also the possible potential adverse effects of nanomolecules on human health and the environment (Kandi and Kandi, 2015).

As various metallic NPs and their oxides have already been used as potent antimicrobial agents, silver or ionic form is most toxic for microorganisms when compared to other metals (Seil and Webster, 2012). This makes silver of particularly interests. Silver NPs (AgNPs) probably have multiple mechanisms of antibacterial action (Markowska *et al.*, 2013). For example, (1) AgNPs can affect bacterial membrane permeability by attaching to the cell membrane surface and modifying the cell potential; (2) AgNPs can causes oxidative damage by production of ROS (Kim *et al.*, 2007; Xu *et al.*, 2012); (3) AgNPs can interact with the SH-groups of bacterial membrane proteins and intracellular proteins, and also can interact with the phosphate residues in DNA, thus to interfere with protein synthesis and function and cell division (Durán *et al.*, 2016). However, due to the current lack of knowledge, the exact basis for the activity of AgNPs is still uncharacterized.

The antibiofilm activity of AgNPs has also been demonstrated in a number of studies . Most of the AgNPs are within the range of 1~100 nm. Although smaller AgNPs may have greater biological activity, it is important to note that differences in the chemical and physical properties of AgNP may cause varia-

tion in its antimicrobial and antibiofilm efficacy (Markowska *et al.*, 2013). AgNPs can also enhance the antibacterial and antibiofilm activity of conventional antimicrobials. There are reports describing synergistic activity between AgNPs and e.g., ampicillin, kanamycin, streptomycin or vancomycin against *E. coli* and *P. aeruginosa* (Wolska *et al.*, 2012). Some AgNPs have been subjected to clinical trials (Franci *et al.,* 2015). Although metallic NPs have great potential in the future to become antimicrobial agents, the local and systemic toxic complications and the deleterious effect they have on beneficial bacteria in humans and animals may be a cause for concern (Zhang *et al.*, 2010; Prabhu and Poulose, 2012). NPs have the ability to spread throughout the body, cross the blood-brain barrier, cause haemolysis, and may result in degradation products which have toxic effects and influence blood coagulation pathways (Kandi and Kandi, 2015). Most studies have not conclusively evaluated the exact mechanism by which nanomolecules contribute to toxic complications, and many of the interactions of the AgNPs with the human or animal body are still poorly understood (dos Santos *et al.*, 2013). It has been observed that the larger size of NP, the greater is the risk of adverse health effects (De Jong and Borm, 2008). Research is necessary to clearly understand the interaction of nanomolecules with living cells, the extent of their distribution in the body, and their specific organ toxicity.

2.1.6 TARGETING THE ESSENTIAL CELL DIVISION PROTEIN FtsZ

The essential cytoskeletal cell division protein FtsZ (named after the filamenting temperature-sensitive mutant Z) is an essential GTPase structurally related to eukaryotic tubulins (de Boer *et al.*, 1992) and highly conserved in bacteria and archaea (Wang *et al.*,1996) During cell division, FtsZ forms a ring like structure at the site of division and functions as a scaffold for the assembly of a multiprotein complex (referred to as the"divisome") essential for cell viability. Not surprisingly, FtsZ, as well as proteins that interact directly with and regulate the activity of FtsZ, has emerged as a prime target for antibacterial development. The use of FtsZ as an antibacterial drug target has been reviewed

and its structural biology (Addinall *et al.*, 2002) and inhibition with small molecules have been discussed. Specifically, targeting FtsZ with small molecules as a defense against tuberculosis has also been extensively reviewed.(Ojima *et al.*,2014)

2.1.6.1 FtsZ protein and Cell Morphology:

The peptidoglycan layer of bacterial cell walls consists of a heteropolymer of polysaccharides cross-linked with short peptides that function as the load-bearing material to resist mechanical and physical forces (e.g., osmotic pressure) on cells. During the growth of rod-shaped cells, peptidoglycan is assembled in two distinct regions of the cell: (1) along the cylindrical body of cells, which is required for cell elongation, and (2) at the site of cell division, which creates a new curved pole for the two daughter cells. FtsZ is required for septal/cell-division-associated peptidoglycan growth and remodeling due to its essential role in recruiting cell-division-specific peptidoglycan synthesis enzymes (Typas *et al.*, 2012) However, recent research suggests that the regulatory role of FtsZ on peptidoglycan synthesis during cell division extends beyond its ability to recruit proteins to the mid-cell. Particularly, it was recently shown that the intrinsically disordered C-terminal linker region of FtsZ is important for regulation of enzymes involved in peptidoglycan metabolism in *C. crescentus*.(Sundararajan *et al.*,2015) In addition to septal/cell-division-associated peptidoglycan growth mediated by the divisome, rod-shaped bacteria have other cellular machinery mediating lateral peptidoglycan synthesis along the length of the cell. This multiprotein complex named the "elongasome" is organized by the ancestral homologue of actin, MreB (Typas *et al.*, 2012) Until recently, the role of FtsZ has been thought to be restricted to participating in peptidoglycan assembly and remodeling at the division site. However, recent studies have demonstrated that FtsZ may also play a role in elongation-associated cell wall growth in rodshape bacteria (Aaron *et al.*,2007)

2.1.6.2 Mechanisms of action of inhibitors of FtsZ

Several characteristics validate FtsZ as a target for the development of new antibiotics to selectively combat bacterial infections. Hydrolysis of GTP requires assembly of two FtsZ monomers to complete the catalytic site. This innate step in catalysis can be modulated by targeting either the T7 loop of the "upper" monomer or the nucleotide-binding pocket of the "lower" monomer (Lowe *et al.*1999) Alternatively, an allosteric site on FtsZ may modulate its ability to form proto filaments. In this sense, the tightly regulated division process could be halted by several mechanisms, including (1) overly stabilizing protofilaments, which cannot disassemble as GDP is produced by GTP hydrolysis; (2) destabilizing protofilaments; and (3) preventing polymerization. The chemical inhibitors of FtsZ reported to date can be classified into three main groups: (1) natural products and their derivatives; (2) nucleotide analogs; and (3) molecules that emerged from high-throughput screening. Below, we provide an overview of the inhibitors that were a starting point for further development of structurally related compounds or assays for their activity as inhibitors of cell division (Katherine *et al.*, 2016)

2.1.6.3 FtsZ Inhibitors from Natural Products:

Sanguinarine (1) is a polycyclic alkaloid that inhibits FtsZ protofilament assembly by decreasing FtsZ polymerization;(Beuria *et al.*, 2005) it also inhibits eukaryotic tubulin, which complicates its use as an antibiotic. Berberine (2) is a structurally related alkaloid that inhibits GTPase activity and decreases FtsZ polymerization. It is predicted to bind in the vicinity of the GTP binding pocket and overlaps with several hydrophobic residues located in the GTP binding site (Domadia *et al.*, 2008) Although allegedly indifferent to tubulin, it has since been described as a promiscuous binder of different proteins. (Czaplewski *et al.*, 2012) Plumbagin (4) inhibits the GTPase activity of FtsZ and increases the lag phase of FtsZ assembly (i.e., adversely affects the nucleation rate). Berberine 2 (3) was designed to have an extended alkyl group in place of one of

the methyl groups on 2; the in vitro GTPase inhibition activity of berberine 2 was measured to be approximately 38 μM against *S. aureus* FtsZ (Sun *et al.*, 2014) Resveratrol (6) has been screened many times due to its known antimicrobial activity, which has been attributed to inhibiting Z-ring formation and suppressing the expression of FtsZ mRNA.(Bhattacharya *et al.*,2013)(Katherine *et al.*,2016) Targeting cell division with small molecules can leverage many of the different biochemical steps that are connected to FtsZ and cytokinesis. There are a variety of in vivo assays that can be used (or new methods that can be engineered) to qualitatively or quantitatively measure the inhibition of cell division and are compatible with highthroughput screening methods. Bacterial cell division remains an active area of fundamental research, and inhibitors of specific proteins that participate in this process may be important tools for studying the biochemical and biophysical mechanisms that are involved.

2.1.7 NEED FOR THE STUDY

The outbreak of antimicrobial resistance, together with the lack of newly developed antimicrobial drugs, represents an alarming signal for both human and animal healthcare worldwide. Selection of rational dosage regimens for traditional antimicrobial drugs based on pharmacokinetic/pharmacodynamic principles as well as development of novel antimicrobials targeting new bacterial targets or resistance mechanisms are key approaches in tackling AMR. In addition to the cellular level resistance (i.e., mutation and horizontal gene transfer of resistance determinants), the community level resistance (i.e., bilofilms and persisters) is also an issue causing antimicrobial therapy difficulties. Therefore, anti-resistance and antibiofilm strategies have currently become research hotspot to combat antimicrobial resistance. Although metallic nanoparticles can both kill bacteria and inhibit biofilm formation, the toxicity is still a big challenge for their clinical applications. In conclusion, rational use of the existing antimicrobials and combinational use of new strategies fighting against antimicrobial resistance are powerful warranties to preserve potent antimicrobial drugs for both

humans and animals. Compounds showing profound anti-resistance and antibiofilm effects are in research hotspot, but they still have limitations. Combining existing antimicrobials with compounds that inhibit their specific resistance mechanisms would be a good choice. Although metallic NPs can both kill bacteria and inhibit biofilm formation, the toxicity is still a big challenge for their clinical applications (dos Santos *et al.*, 2013). With single-drug therapy, there is always a selective advantage to resistance; specific combinations of drugs can inhibit bacterial growth while disfavoring resistance to the individual components. These approaches can be used to invert the selective advantage of resistant bacteria competing with their sensitive cousins, or even drive a resistant bacterial population back toward drug sensitivity (Baym *et al.*, 2016). Besides, screening and developing multiple-target inhibitors as "resistance-resistant" antimicrobials can reduce the effects of target mutation (Oldfield and Feng, 2014). The natural products have also been a prolific and unsurpassed source for new lead antimicrobial compounds, but target identification and validation has remained a major bottleneck. (Farha and Brown, 2016)

Materials and Methods

CHAPTER 3

MATERIALS AND METHODS

The present study entitled **"*In vitro* studies on the anti-infective properties of flower and fruit extracts of selected medicinal plants against enteropathogens"** was carried out in the Microbiology division of Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, during 2018-2019 academic years.

3.1 Samples Description

The nutritional and medicinal values of fruits and flowers are highly beneficial to human for survival. Many fruits contain biologically active phytochemicals having multiple health protecting qualities. Some of such fruits and flower description are given below:

3.1.1 Pomegranate *(Punica granatum* L.)

In Ayurveda and in folk medicine it is used in different applications. It is effective for controlling thridoshas - 'vatha', 'pitha' and 'kapha'. The fruit helps in digestion. It removes fatigue and thirst. (Nesamony, S., 1998) Pomegranate juice is a good antioxidant agent because it is rich in antioxidant, the *'ellagitannin-punicalagin'* (Cerda *et al.*,2002; Gil *et al.*, 2003; Singh *et al.*, 2000; Chidambara *et al* .,2000) The efficiency of pomegranate fruit in chemoprevention of cancer was extensively studied. Reports suggest that the pomegranate fruit therapy can prevent breast cancer, colon cancer and other kinds of tumor-genesis (Mehta, R., and Lansky, E.P., 2004; Adams *et al* .,2006; Malik *et al* .,2006; Lansky *et al* .,2007) Peel extract has wound healing properties (Chidambara *et al* ., 2004) It reduces blood sugar level by rejuvenating beta cells of pancreas. (Khalil, E.A.M., 2002; Das *et al.*, 2001) It possesses immunomodulatory (Gracious *et al.*, 2001) and gastro protective (Gharzouli *et al.*, 1999) activities.

3.1.2 Rose apple (*Syzygium jambose* (L.) Alston)

Jambos is an evergreen tree with a regular shaped, dense crown of wide-spreading branches; it can grow 6 - 10 metres tall. It provides food, medicines and a range of commodities for the local population. An attractive tree with showy cream-coloured flowers and dark-green foliage, it is often grown as an ornamental and hedge plant in tropical garden. The fruit is somewhat to very juicy and has a delicate, rose-water fragrance. In India, the fruit is regarded as a tonic for the brain and liver. An infusion of the fruit acts as a diuretic. A sweetened preparation of the flowers is believed to reduce fever. The seeds are employed against diarrhoea, dysentery and catarrh. In Nicaragua, it has been claimed that an infusion of roasted, powdered seeds is beneficial to diabetics. They say in Colombia that the seeds have an anaesthetic property. (World Agroforesty Centre - Tropical Plants Database, 2019)

3.1.3 Custard apple *(Annona squamosa L.)*

The sugar-apple or custard apple is the fruit of *Annona squamosa*, the most widely grown species of *Annona* and a native of the tropical Americas and West Indies. It is also known as Shareefa in India and Pakistan. The fruit is spherical-conical, 5–10 cm (2.0–3.9 in) in diameter and 6–10 cm (2.4–3.9 in) long and weighing 100–240 g (3.5–8.5 oz), with a thick rind composed of knobby segments. The color is typically pale green through blue-green, with a deep pink blush in certain varieties, and typically has a bloom. It is unique among *Annona* fruits in being segmented, and the segments tend to separate when ripe, exposing the interior. The flesh is fragrant and sweet, creamy white through light yellow, and resembles and tastes like custard. It is found adhering to 13 to 16-millimetre long (0.51 to 0.63 in) seeds forming individual segments arranged in a single layer around a conical core. It is soft, slightly grainy, and slippery. The hard, shiny seeds may number 20–40 or more per fruit and have a brown to black coat, although varieties exist that are almost seedless. (Morton ., *1987)* Most of the species of *Annona* are medicinally valuable. For example, 'Annonaceous acetogenins' are a new class of biologically ac-

tive compounds present in the seeds of various *Annona species*. These compounds possess cytotoxic, pesticidal, antimalarial, antiproliferative, antiparasitic and antimicrobial properties. (Chih *et al.*, 1997; Xiao-Feng *et al.*, 2001; Bories *et al.*, 2002) Methanolic extracts of seeds of *Annona muricata* and *Annona cherimola* have anti- parasitic activity (Oberlies *et al* ., 1991) Fruit pericarp and seeds of *Annona squamosa* possess antitumor activity. (Pardhasaradhi *et al.*, 2005; Joy, B., and Remani, P., 2008)

3.1.4 Koovalam *(Aegle marmelos* L.)

Aegle marmelos L., commonly known as bael also Bengal quince, golden apple, Japanese bitter orange, stone apple or wood apple, is a species of tree native to the Indian subcontinent and Southeast Asia. The flowers are 1.5 to 2 cm, pale green or yellowish sweetly scented, bisexual, in short drooping unbranched clusters at the end of twigs and leaf axils. They usually appear with young leaves. The calyx is flat with 4(5) small teeth. The four or five petals of 6–8 mm overlap in the bud. Many stamens have short filaments and pale brown, short style anthers. The ovary is bright green with an inconspicuous disc. The bael fruit typically has a diameter of between 5 and 12 cm. It is globose or slightly pear-shaped with a thick, hard rind and does not split upon ripening. The woody shell is smooth and green, gray until it is fully ripe when it turns yellow. Inside are 8 to 15 or 20 sections filled with aromatic orange pulp, each section with 6 (8) to 10 (15) flattened-oblong seeds each about 1 cm long, bearing woolly hairs and each enclosed in a sac of adhesive, transparent mucilage that solidifies on drying. It takes about 11 months to ripen on the tree and can reach the size of a large grapefruit or pomelo, and some are even larger. The shell is so hard it must be cracked with a hammer. The fibrous yellow pulp is very aromatic. It has been described as tasting of marmalade and smelling of roses. Boning (2006) indicates that the flavor is "sweet, aromatic and pleasant, although tangy and slightly astringent in some varieties. It resembles a marmalade made, in part, with citrus and, in part, with tamarind." Numerous hairy seeds are encapsulated in slimy mucilage.

3.1.5 Vannilla (*Vanilla planifolia* L.)

The main species harvested for vanilla is *V. planifolia*. Although it is native to Mexico, it is now widely grown throughout the tropics. Indonesia and Madagascar are the world's largest producers. Vanilla grows as a vine, climbing up an existing tree, pole, or other support. It can be grown in a wood (on trees). Flowers are greenish-yellow, with a diameter of 5 cm (2 in). They last only a day, and must be pollinated manually, during the morning, if fruit is desired. The plants are self-fertile, and pollination simply requires a transfer of the pollen from the anther to the stigma. If pollination does not occur, the flower is dropped the next day. In the wild, there is less than 1% chance that the flowers will be pollinated, so in order to receive a steady flow of fruit, the flowers must be hand-pollinated when grown on farms. Fruit is produced only on mature plants, which are generally over 3 m (10 ft) long. The fruits are 15–23 cm (6–9 in) long pods (often incorrectly called beans). Outwardly they resemble small bananas. They mature after about five months, at which point they are harvested and cured. Curing ferments and dries the pods while minimizing the loss of essential oils. Vanilla extract is obtained from this portion of the plant.

3.2 MATERIALS

3.2.1 Microbial strains and growth media

Antimicrobial activity of the extract towards enteropathogens **(Table: 1)** were kindly provided by Microbial type culture collection and gene bank (MTCC), CSIR-IMTECH, Chandigarh. All bacterial cultures are maintained on Mueller Hinton Agar (MHA) by periodical sub culturing. Glycerol stock of all microbial strains used for experiments were prepared and stored at – 80 °C. The microbial strains were pre-cultured for 24 hours to achieve exponential phase of growth.

Table :1 Bacterial strains used for preliminary screening test

SL.No:	Bacterial Species	Gram Reaction	MTCC Strain No:
1	*Vibrio cholerae*	Gram negative	3906
2	*Escherichia coli*	Gram negative	443
3	*Staphylococcus aureus*	Gram positive	3160
4	*Enterococcus faecalis*	Gram positive	439
5	*Salmonella Typhi*	Gram negative	733

*MTCC: Microbial Type Culture Collection and Gene Bank, CSIR-IMTECH, Chandigarh; BGSC: Bacillus Genetic Stock Centre, Columbus, USA

3.2.2 Chemicals

Ethanol (95%), Magnesium chloride (HIMEDIA, Mumbai), Calcium chloride (SRL, Mumbai), Pottassium chloride (HIMEDIA, Mumbai), Tris base (Sigma Aldrich), DMSO (HIMEDIA, Mumbai), Malachite Green phosphate assay kit (Sigma Aldrich), GTP (Sigma Aldrich), Ampicillin (HIMEDIA, Mumbai), Ciprofloxacin (HIMEDIA, Mumbai), Ellagic acid (purity $\geq$ 95%) were supplied by Sigma Aldrich Standard solutions of each substance were prepared in the recommended solvent according to supplier and stored at -20 ° C, plasmid DNA ($_{P}$CXZ) -pet11b, Toluene(SRL, Mumbai), Ethyl acetate(SRL, Mumbai), Formic acid (SRL, Mumbai), Methanol (SRL, Mumbai)

3.2.3 Equipments

Incubator Shaker-Orbitech (Scigenics Biotech, India) , Rotary vacuum evaporator (Heidolph, India), Hot air oven (KEMI, Cochin), Electronic balance (Sartorius, Japan), Cooling Centrifuge (HERMLE Z 323K, Mumbai), Spectrophotometer (Agilent- Cary 100, Hungary), pH Meter (Cyber Scan, Germany), Centrifuge (SIGMA), Digital Water Bath (Beston Industries, Cochin), GelDoc (UVP, USA) , Soxhlet apparatus (Borosil, India)Phase contrast Microscopy (

Nikon ECLIPSE, Japan) and Microtitre Plate Reader (TECAN SPARK10M) were used.

3.3 METHODS

3.3.1 Sample collection

Fruit and flower of the plant samples (*Punica granatum* L, *Syzygium jambos* L. Alston, *Annona squamosa* L, *Aegle marmelos* L, *and Vanilla planifolia* L) are collected. **(Table: 2)** Samples were shade dried, powdered and hot and cold solvent extractions were made with ethanol using soxhlet apparatus and shaker respectively.

All the plants were collected in different seasons from various locations of Thiruvanandapuran and Idukki district; Kerala, India and majority of them were collected from Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode. The plant material was identified and authenticated by Dr. E. S. Santhosh Kumar, Technical Officer, Plant Genetic Resources, and JNTBGRI.

Table: 2 Details the choice of plant samples

Sl. No:	Samples	Common Name	Geographical Area	Family
1	*Punica granatum* L	pomegranate	Vattiyoorkkavu, Thiruvananthapuram	Lythraceae
2	*Syzygium jambos* (L.) Alston	Roseapple	Thodupuzha, Idukki	Myrtaceae
3	*Annona squamosa* L	Custardapple	TBGRI, Palode, Thiruvananthapuram	Annonaceae
4	*Aegle marmelos* L	Koovalam	TBGRI, Palode, Thiruvananthapuram	Rutaceae
5	*Vanilla planifolia* L	Vanilla	Vagamon, Idukki	Orchidaceae

3.3.2 Preparation of extraction

3.3.2.1 Hot ethanol extraction

Soxhlet method makes use of high temperature extraction. In this method, 5g of the powdered plant sample was taken and wrapped in a good quqlity filter paper and put it in a soxhlet apparatus for approximately 6 hours. The resulting ethanol extract was filtered through Whatmann paper No.1 and concentrated under reduced pressure at 45°C using the Heidolph Rotavapour R-200 to obtain a crude residue.

3.3.2.2 Cold ethanol extraction

For preparing crude cold extract, 5g of the fine powdered plant part was weighed into 250ml conical flask. 150ml ethanol was added to the flask and its mouth was tightly closed. The conical flask containing the plant powder wetted with ethanol was kept in rotary shaker at 100 rpm for 3 days. The cold crude extract collected was concentrated by using rotary evaporator at room temperature.

3.3.3 Antimicrobial testing

Prior to sensitivity testing, each of the bacterial strains were cultured onto Mueller Hinton Agar (MHA) plate and incubated for 18 to 24 hours at 37°C. A single colony was then cultured in 5 ml Luria Broth for 4 hours at 37°C. The density of bacteria culture required for the test was adjusted to 0.5 McFarland standards. (1.0 x 108 CFU/ml) Different assays like disc diffusion assay, well diffusion assay, determination of minimum inhibitory concentration and minimum bactericidal concentration are often used for measuring the antimicrobial activity of crude extract. In the preliminary screening, antimicrobial activities

of 5 hot crude ethanol extract against 5 enteropathogens was performed with both agar well diffusion and disc diffusion methods; preliminary results indicated that well diffusion method was more efficient as it yielded reliable and consistence results.

3.3.3.1 Disc Diffusion Method

Disc diffusion method for antimicrobial susceptibility testing was carried out according to the standard method by Bauer *et al.*, 1966 to assess the presence of antibacterial activities of the plant extracts. A bacteria culture (which has been adjusted to 0.5 McFarland standard), was used to lawn Muller Hinton agar plates evenly using a sterile swab. The plates were dried for 15 minutes and then used for the sensitivity test. The discs which had been impregnated with a series of plant extracts were placed on the Mueller Hinton agar surface. Each test plate comprises of six discs. One positive control, which is a standard commercial antibiotic disc, one negative control, and four treated discs. The standard antibiotic disc was Ciprofloxacin 5µg/disc. The negative control was DMSO (100%). Besides the controls, each plate had four treated discs placed about equidistance to each other. The plate was then incubated at 37°C for 18 to 24 hours depending on the species of bacteria used in the test. After the incubation, the plates were examined for inhibition zone. The inhibition zone were then measured using scale and recorded. The tests were repeated three times to ensure reliability.

3.3.3.2 Well Diffussion Method

The antimicrobials present in the plant extract are allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. The resulting zones of inhibition will be uniformly circular as there will be a confluent lawn of growth. The diameter of zone of inhibition can be measured in millimeters. A bacterial culture (which has been adjusted to 0.5 McFarland standard), was used to lawn Muller Hinton agar plates evenly using a sterile swab. The plates were dried for 15 minutes and then used for the sensitivity test.

Then, a hole with a diameterof 4 to 6 mm is punched aseptically with a sterile cork borer and 15-20µl volume of the plant crude extract of six samples at desired concentration is introduced into the well. Each test plate comprises of three wells. One positive control, which is a standard commercial antibiotic disc, one negative control, and two treated wells. The standard antibiotic discs were ciprofloxacin 5µg. The negative control was DMSO (100%). Besides the controls, each plate had two treated wells placed about equidistance to each other. The plate was then incubated at 37°C for 18 to 24 hours depending on the species of bacteria used in the test. After the incubation, the plates were examined for inhibition zone. The inhibition zones were then measured using scale and recorded. The tests were repeated three times to ensure reliability.

3.3.3.3 Minimum Inhibition Concentration Determination (MIC)

Minimum Inhibition Concentrations (MIC's) was determined by the Clinical and Laboratory Standards Institute (CLSI) guidelines for broth microdilution. It is done by carrying out the diffusion test with different concentration of the plant extracts similar to the concentration used in the sensitivity tests against the five bacterial strains mention earlier (Guerin-Faublee *et al.*, 1996). The MIC was quantified using Luria Broth (LB) in 96 well microplates as well. 2µl of plant extract (50 - 500µg/ml) by keeping the concentration of DMSO as 1% was added to 196µl of LB broth. 2µl of the diluted overnight culture were added microplate containing broth. The broth is containing 1% DMSO used as control. The microtitre plate was incubated at 37^0C for 16-18 hours and OD_{600} of the culture were measured. The lowest concentrations that inhibit the growth of bacteria were noted and considered as the MIC value for each of the bacteria strain.

3.3.3.4 Minimum Bactericidal Concentration Determination (MBC)

The method used and described below is an amended version of the procedure described in the BSAC Guide to Sensitivity Testing and can be adapted for de-

termining the minimal bactericidal concentration (MBC) of different concentration of the plant extracts similar to the concentration used in MIC determination against the five bacterial strains mention earlier. After MIC determination of the plant extract , an aliquot of 10 µl from all tubes in which no visible bacterial growth was observed were seeded in MHA plates .The plates were then incubated for overnight at 37°C. The MBC endpoint is defined as the lowest concentration of antimicrobial agent that kills > 99.9% of the initial bacterial population where no visible growth of the bacteria was observed on the MHA plates.

3.3.4 Chromatographic studies

Chromatography is a separation process which depends on the differential distribution of the components of a mixture between a mobile bulk phase and an essentially thin film stationary phase. This technique is used as analytical tool to establish the complexity of mixtures and the purity of samples, and as preparative tools for the separation of mixtures into individual components.

3.3.4.1 Thin Layer Chromatography (TLC)

TLC is based on adsorption chromatography in which separation depends on the selective adsorption of the components of a mixture on the surface of solid. The stationary phase was in the form of a thin layer adhering to a suitable form of backing material over which the mobile phase was allowed to ascend by capillary action. Traditionally analytical TLC has found application in the detection and monitoring of compound through a separation process.

TLC of the fruits (*Punica granatum* and *Syzygium cumini*) hot extracts with strong antimicrobial activity was carried out. Different solvent systems were used for different classes of compounds based on the polarity of the organic solvents. TLC was performed on 20 cm×20 cm TLC Silica gel 60 F254 plates from Merck (Darmstadt, Germany), which were cut into segments of 10 cm×10 cm. Standards and samples were applied with a capilliary tube, 10 mm from the

left edge and 1cm from the bottom of the plate. About 2 ml of plant extract was applied to the TLC chromatogram. Ellagic acid and Gallic acid were used as control. Solvent systems used were (**Table: 3)** Toluene: Ethylacetate: Formic acid, 6:4:1 solvent is the best. The plates were developed up to 7 cm in an un-saturated normal developing chamber (Camag) for 10 cm×10 cm plates. After development, the plates were dried for 2 min in room temperature. Individual R_f for each spot was measured. TLC spots were visualized under UV light and adequate TLC reagents were used to detect the phytoconstituent.

The R_f value is the constant and characteristic of the substance which indicates its movement relative to the solvent front in a given chromatographic system.

R_f = Distance travelled by compound / Distance travelled by solvent

R_f value depends on number of variables such as

- The particle size of different batches of adsorbent.
- The solvent composition and the degree of saturation of the tank atmospheres with solvent vapours.
- Prior activation and storage condition of the plates.
- The thickness of adsorbent layer

Table: 3 Solvents used for TLC

SL.NO:	SOLVENTS	RATIO
1	Chloroform and Methanol	8:2
2	Chloroform and Methanol	1:1
3	Ethylacetate and Methanol	1:1
4	Chloroform: Acetone: Methanol	5:3:2
5	**Toluene : Ethylacetate : Formic acid**	**6:4:1**

3.3.4.2 TLC - bioautography

Bio-autography is a useful technique to determine bioactive compound with antimicrobial activity from plant extract. TLC bioautographic methods combine chromatographic separation and in situ activity determination facilitating the localization and target-directed isolation of active constituents in a mixture. Traditionally, bioautographic technique has used the growth inhibition of microorganisms to detect anti-microbial components of extracts chromatographed on a TLC layer. This methodology has been considered as the most efficacious assay for the detection of anti-microbial compounds (Shahverdi, 2007). Bio-autography localizes antimicrobial activity on a chromatogram using three approaches: (i) direct bio-autography, where the micro-organism grows directly on the thin-layer chromatographic (TLC) plate, (ii) contact bio-autography, where the antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact and (iii) agar overlay bio-autography, where a seeded agar medium is applied directly onto the TLC plate (Hamburger and Cordell, 1987; Rahalison *et al.*, 1991). The inhibition zones produced on TLC plates by one of the above bioautographic technique will be use to visualize the position of the bioactive compound with antimicrobial activity in the TLC fingerprint with reference to R_f values (Homans and Fuchs, 1970).

For direct bioautographic assay, agar overlay assay as described by Slusarenko *et al.* (1998) was used with minor modification. *V. cholerae* and *E. coli* are used as test strain. About 1ml of plant extract was spotted on preparative Merck chromatographic silica gel plates. Only one solvent system (Toluene: Ethylacetate: Formic acid, 6:4:1) was used. The developed chromatogram was cut it into different pieces and placed in sterilized Petri plates. The bacteria culture (which has been adjusted to 0.5 McFarland standard), was added to Muller Hinton agar, mixed and poured over the chromatograms as a thin layer. Plates were incubated at 37°C for 24 hour. The zone of inhibition of bacterial growth could be seen around the active chromatogram spot.

3.3.4.3 Column Chromatographic studies

50

The stationary phase is in form of a packed column through which a mobile phase is allowed to flow under gravity, or under pressure applied to the top of the solvent reservoir. The choice solvent for transferring the mixture to be chromatographed to the column depends upon the solubility characteristics of the mixture. Frequently the most non-polar solvent for introducing the mixture on to the column and the initial solvent for chromatogram development. Eluting solvent with increasing the polarity applied for solvent selection, all these solvents have sufficient low boiling points to permit ready recovery of eluted material. The solvents include chloroform, ethylacetate and methanol.

The column assembly was set for the separation of compounds from methanolic extract. The silica gel 100 – 200 mesh LR was used for column packing. The slurry method was adopted for filling of the column. Slurry was made with chloroform. The adsorbent was allowed to settle evenly and free of air bubbles, by with gentle tapping of the column with a wooden rod. The top of the column was frequently covered with a circle of filter paper to prevent disturbances of the surface during subsequent loading. Semisolid methanolic extract was poured through a funnel into the packed column. The solvent level was maintained 2.5 cm above the extract. For continuous supply of solvent at the constant rate the reservoir was set and filled with the solvent. The elutes were collected drop by drop in 10 ml volumetric flasks at the bottom of column. TLC was carried out for the identification of constituents which were eluted by different eluent solvent systems. Active fractions 5 to 7 were pooled and separated by sub column. Eluted fractions which have same R_f on the TLC were pooled. Afterwards other fractions were collected and subjected to thin layer chromatographic studies. . The active areas were scraped from the plates, and the substance eluted from the silica with methanol. Eluted samples were further purified using the above preparative chromatography method. Finally, the components were identified by HPLC **(Figure: 1)**

3.3.4.4 HPLC (High performance liquid chromatography) and analysis

Purification of the compound of interest using HPLC is the process of separating or extracting the target compound from other (possibly structurally related) compounds or contaminants. Each compound should have a characteristic peak under certain chromatographic conditions. Depending on what needs to be separated and how closely related the samples are, the chromatographer may

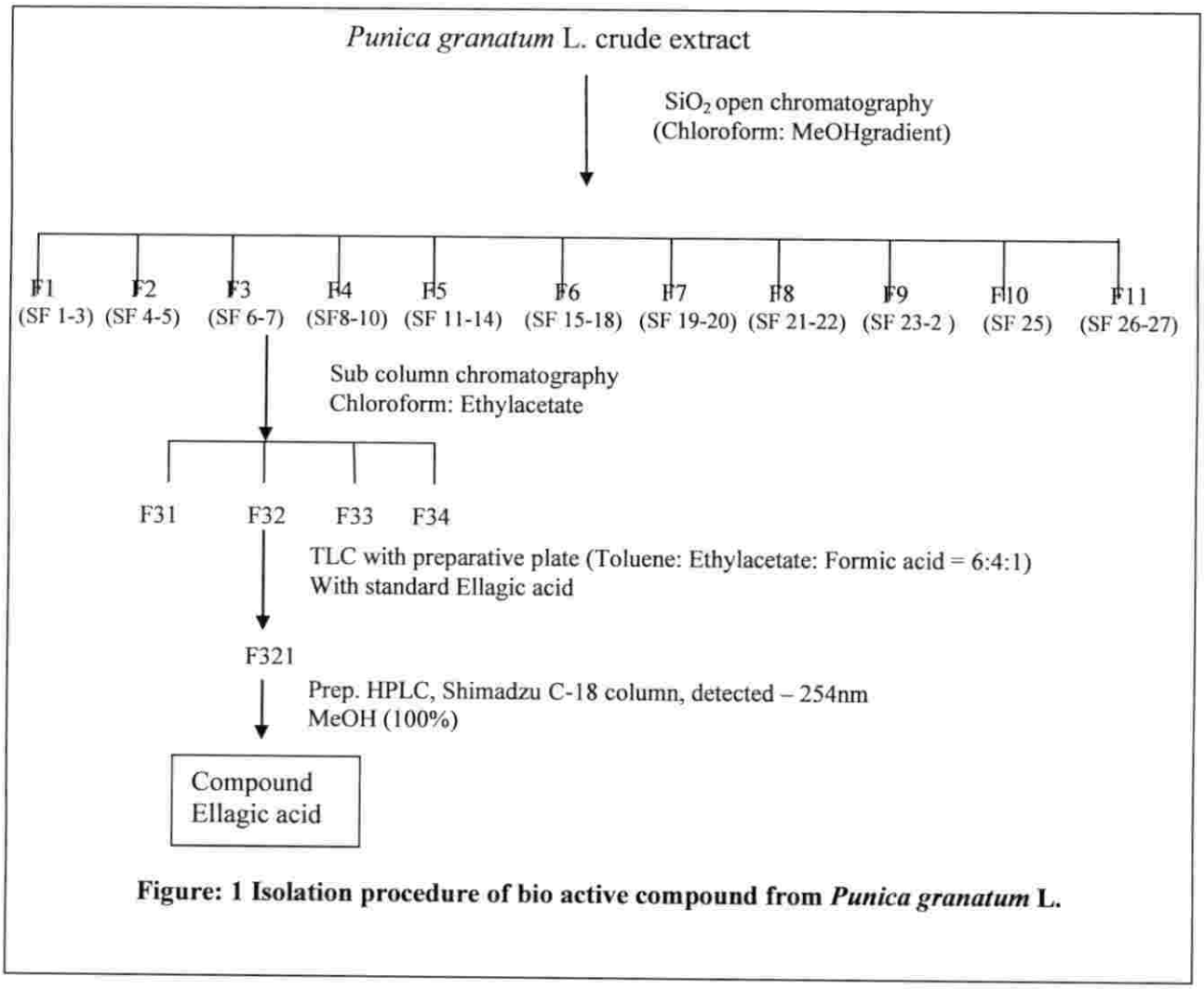


Figure: 1 Isolation procedure of bio active compound from *Punica granatum* L.

choose the conditions, such as the proper mobile phase, flow rate, suitable detectors and columns to get an optimum separation. The processing of a crude source material to provide a sample suitable for HPLC analysis as well as the choice of solvent for sample reconstitution can have a significant bearing on the overall success of natural product isolation.

The given plant extracts were analysed using HPLC Shimadzu Japan. The system attached a UV/VIS Detector (Prominance SPD M20 A diode array detector). Data acquisition and instrumental control were performed using Shimadzu Lab solution version 5.73. Separation of the compounds was performed on a general purpose Shimadzu C-18 column (250 x 4.6 mm, 5 µm particle size, 5 µm) and the isocratic mobile phase consisted of 100% methanol (Spectrochem, India - HPLC grade). The flow rate was 1.0 mL/min and the injection volume was 10 µL. Pump consists of (LC 6 AD) system interface (CBM-20A) and a high pressure adjustable volume dynamic mixer. The analysis was performed at room temperature (25°C) and the compound was detected at 254 nm. The samples for analysis were separately filtered using a 0.22 µm filter (Millipore) before injecting. At the same retention time of standard the peak area of the extracts were measured.

3.3.5 Transformation and Expression of Bacillus subtilis FtsZ protein in E.coli

3.3.5.1 Preparation of competent cells using Calcium chloride ($CaCl_2$) method

- An overnight culture of *E.coli* DH5α prepared in 1mL LB medium.
- 1 mL of overnight culture added to 100mL of fresh LB broth and kept for incubation for 3-4 hours to reach the OD 0.4-0.6
- The culture was aliquoted to sterile Oakridge tubes and kept it in ice for 30 minutes.
- The culture was centrifuged at 4000 rpm for 10 minutes and the supernatant was discarded.
- The pellets were resuspended in a sterile ice cold 20mL of 0.1M $CaCl_2$ and incubated for on ice for 30 minutes.
- Culture was centrifuged at 4^0C at 4000 rpm for 10 minutes.

- The supernatant was discarded and resuspend the cells in 2mL of 0.1M $CaCl_2$ with 20% glycerol.
- The competent cells were aliquoted to 200µL suspension in eppendorf tube and kept in -80^{0}C

3.3.5.2 Transformation by heat shock

- The competent cells were thawed by keeping on ice for 10 minutes.
- Add 3 or 4µL of plasmid DNA ($_P$CXZ) to tubes containing competent cells and mix by gentle mixing and incubated in ice for 30 minutes.
- Tubes were transferred to the 42^{0}C waterbath for 90s for heatshock and quickly transferred to ice and incubated for 10 minutes.
- Bacterial cells were transferred to 800µL of LB medium to Eppendorf slowly and incubated at 37^{0}C for I hour.
- 200µL of culture was transferred to LB Agar containing 100µg/mL of Ampicillin.
- Plate were incubated at 37^{0}C for 12-16 hours and determined for colony formation after incubation.

3.3.5.3 Expression of recombinant FtsZ protein

Select any two colonies from the successful transformation were inoculated in 6 mL of Ampicillin containing LB medium.The cells were incubated at 37^{0}C overnight. 500mL of LB medium containing 100µg/mL Ampicillin was inoculated with 6mL of initial culture grown at 37^{0}C and keep till OD become (0.6). A stock solution of IPTG was then added to the culture to make a final IPTG concentration of 1mM and incubate for 4 hours at 37^{0}C. The cells were taken by centrifuging at 8000 rpm for 10 minutes and the pellet was washed with 10mM Tris HCl buffer. Add 10mg/mL lysozyme about 100µL into the above solution and keep for half an hour in ice. Sonication of the chilled cell broke *E.coli* cells. The membrane fraction was removed using centrifuge at 14000 rpm for 30 minutes. A 30% ammonium sulfate (16.6g/100mL of extract) was then slowly added to the supernatant with stirring of 20 minutes and kept for 1

hour. The ammonium sulfate precipitate was collected using centrifugation and resuspended in buffer (50mM Tris HCl, pH 7.9; 50mM KCl; 1mM EDTA, 10% glucerol). The sample was dialyzed using dialysis membrane overnight. The dialysed ammonium sulfate fraction was loaded. Fractions of 4 mL were collected and examined by SDS-PAGE (Laemmli, 1970). The eluted fractions containing pure FtsZ were mixed and stotred at -80^{0}C (Anderson *et al.*, 2012).

3.3.6 In silico docking studies and homology modelling of FtsZ

3.3.6.1 Homology modeling of FtsZ

Homology modeling predicts the three-dimensional structure of proteins. Homology modeling was done using Swiss model server. The three-dimensional structure of *E.coli* FtsZ (Target proteins) were predicted using X-ray Crystal structure of *P. aeruginosa (*PDB ID: 2VAW; Res. 2.9 Å) as a template protein.

3.3.6.2 Receptor and Ligand preparation

Homology model of constructed using Swiss model server using the X-ray crystal structure of *P. aeruginosa* FtsZ (PDB ID: 2VAW, 2.9 Å). The quality of the homology model was analysed using the Ramachandran plot. Ligand and receptor for docking were prepared using Dock Prep tool of UCSF Chimera. AM1-BCC and Gasteiger charges were added to receptor and ligand, respectively.

3.3.6.3 Receptor grid generation and molecular docking

Molecular docking was done using AutoDock Vina 1.1.1. Receptor grid generated at GDP binding site. Ligand molecule excluded from receptor grid. Grid maps were constructed using 124 x 116 x 126 points, with a grid spacing of 0.375 A˚, and a Vina docking exhaustiveness of 10 was used. The best conformation between receptor and ligand was selected based on the highest binding affinity.

3.3.7 In vitro biochemical studies

3.3.7.1 Cell Elongation Assay

55

Overnight *B. subtilis* culture was inoculated in test tube containing 3mL LB medium and grown at 37◦C. The culture was diluted to A600 of 0.5. 10 mL of LB medium taken into a conical flask and 100 µl of the overnight *Bacillus subtilis* culture were added and incubated in a shaker for 2 hours at 37^0C. After incubation, add 100µL plant compounds and incubate 4 hours at 37^0C, 20 µL culture samples were transferred to glass slides containing small piece of 2% agarose for microscopy. Cell morphology was assessed by phase-contrast light microscopy.

3.3.7.2 Malachite green assay for FtsZ GTPase activity.

The Effect of compound on the GTPase activity of recombinant FtsZ was measured in 96-well microplates using Malachite green assay according to an optimized protocol. *Bacillus subtilis* FtsZ (6 µM) was pre-incubated with vehicle (1% DMSO) or different concentrations of each test compound in 50 mM Tris buffer (pH 7.4) for 10 minutes at 25^0C. Then 100 mM of MgCl2 and 2M of KCl were added. Reactions were started with the addition of 1 mM GTP and incubated at 30◦C in waterbath for 20 minutes. After 20 minutes, the reactions were quenched by adding 20 µL of malachite green reagent to 80µL of sample. Inorganic phosphate was quantified by measuring the absorbance at 650 nm with a microplate reader.

3.3.7.3 FtsZ Polymerization Assay

Light scattering assay has done to understand the effect of plant compounds on polymerization. The test compound at different concentration added to assay buffer containing 6µM *B. subtilis* FtsZ. The assay mix contains (Tris-HCl 50mM, (pH 7.4); KCl 2M; $MgCl_2$ 10mM; $CaCl_2$ 10mM) and the compound, the mix is centrifuged at 14500 rpm at 20minutes. 6µM of FtsZ protein (*B. subtilis*) was added to the mix and it was transferred into microtiter plates, incubated for

10 minutes for the base line establishment. 2mM GTP was also added to each well and incubated for 1 hour. The absorbance was observed at 340nm (kaul *et al.*, 2013)

3.3.8 Anti - Biofilm Assay

3.3.8.1 Determination of Minimum Biofilm Inhibition Concentration (MBIC)

The minimum biofilm inhibitory concentration (MBIC) value determines the sensitivity of bacteria in their biofilm phase (sessile phase) of development. Biofilm quantification was done using crystal violet assay for biofilms. The cells which are attached to the surfaces were stained purple with CV whereas abiotic surfaces are not stained. In the assay the CV gives a reliable method for biofilm quantification without any disruption during process of quantification.

MBIC was determined by a broth dilution method on 24-well polystyrene plates with 1mL LB medium. 1% inoculum of standard cell suspension of *Vibrio cholerae* and *Escherichia coli* strains was treated with increasing concentrations (100-500µg/mL) of plant compounds and incubated at 37^0C for 24 hours. After incubation planktonic cells were gently removed from the microplate wells and the wells were washed twice with distilled water. After rinsing, 1ml of the crystal violet reagent (0.2%) were added to all the well in the plate and the suspension was incubated for 10 minutes at 37°C.Wash 3 times using distilled water and allow to complete dry. After that 1mL of 20% acetic acid is added to the entire well. Take the reading on microtitre plate reader at 570nm. The minimum biofilm inhibitory concentration (MBIC), defined as the lowest concentration of an antimicrobial agent required to inhibit the formation of biofilms was determined by observing the inhibition of growth in the microplate wells. Non-biofilm forming DMSO was used as negative control.The bioassay was performed in triplicate and MBIC of the extract was taken for further assays Percentage of biofilm inhibition was calculated by;

Percentage of inhibition = [(Control OD_{570}nm – Test OD_{570}nm) / Control OD_{570}nm] × 100

3.3.8.2 Cell surface hydrophobicity assay (CSH)

A CSH assay was performed to assess the effect of the EA on the cell surface hydrophobicity of *V. cholerae.* The culture was grown in the absence and presence (16µg/mL) of EA for 24 hour at 37^0C in THYG broth. The cultures were diluted to obtain OD 0.4 at 600 nm. One millilitre of toluene was added individually to 1 mL of the diluted cultures, vortexed thoroughly and allowed to stand at room temperature for aqueous/solvent phase separation. The cells retained in the aqueous phase were quantified by measuring absorbance at OD 600nm. The percentage of hydrophobicity was calculated by the formulae:

% hydrophobicity = [1 - (OD 600nm after vortexing / OD 600nm before vortexing)] ×100

3.3.8.3 Protease assay

The total secreted protease of the control and the ellagic acid treated (16 µg/mL) *V. cholerae was* quantified using an azocasein assay as described by Hollands *et al.,* 2008. The *V. cholerae* 3906 strain were centrifuged at 12000 rpm and the supernatants were filter-sterilized through a 0.2 micron nylon membrane filter. An equal volume of activation buffer (1mM EDTA; 20mM DTT in 0.1 M sodium acetate buffer, pH 5.0) was added to the cell free culture supernatants and incubated at 40^0C for 30 minutes. Then an equal volume of 1% (w/v) azocasein was added to the samples and incubated for 1 h at 40^0C. Subsequently, trichloroacetic acid (10 %) was added and mixed thoroughly to precipitate the protein and thereby stop the reaction. The samples were centrifuged at 12000 rpm for 5 minutes and the supernatants were read at 440 nm. Activity was expressed as the change in absorbance value.

3.3.8.4 Extracellular polymeric substance (EPS) estimation

EPS, the cementing material of biofilms, is mainly composed of polysaccharides. Hence, the effect of ellagic acid (EA) on EPS was assessed by measuring the total carbohydrate using the phenol-sulphuric acid method. The *V. cholerae* strain were grown in the absence and presence (16µg/mL) of EA for 24 hour at 37^0C in THYG broth. Following incubation, the cells were harvested by centrifugation (10000 rpm for 10 minutes) and washed thrice with sterile PBS and resuspended in 200 µL of the same. Subsequently, 200 µL of a reaction mixture containing an equal volume of 5% phenol and five volumes of concentrated sulphuric acid containing 0.2% hydrazine sulphate was added to the cell suspensions, mixed well and incubated in the dark at room temperature for 1 hour. The samples were centrifuged at 10000 rpm for 10 minutes, and the absorbance of the supernatants was measured at 490nm (Viszwapriya *et al.*, 2016)

Results

CHAPTER 4

RESULTS

60

4.1 Antimicrobial testing

In this study, hot and cold ethanol extracts fruit and flower were tested against *Escherichia coli, Enterococcus faecalis, Salmonella typhi, Staphylococcus aureus* and *Vibrio cholerae* using well diffusion assay to determine the anti-bacterial activity. The antibacterial activity was scored in terms of diameter of inhibition zone (mm) and results of the alcoholic extracts are presented in **Table 4 and Table 5.** The hot ethanol extracts was showed activity against all bacterial strains tested, while the cold extract detected no activity. According tofruit and flower extracts; fruit extracts were showed progressing activity against bacterial strains tested. Highest activity of *P. granatum* L. fruit hot extract against *E. coli* with an average value of 20.0 mm and lowest activity against *E. faecalis* (10.2 mm). *A. squamosa* L. Fruit hot extract showing activity against all bacterial strains tested ; *S. jambos* hot extract showing very less activity against bacterial strain.

Table 4: Antibacterial activity of fruit ethanol extracts against enteropathogens.

MTCC Strains	Zone of inhibition in mm (Mean ± S. D.)									
	P. granatum A	*P. granatum B*	*A. squamosa A*	*A. squamosa B*	*S. jambos A*	*S. jambos B*	*A. marmelos A*	*A. marmelos B*	*V. planifolia A*	*V. planifolia B*
E. coli 443	20 ±0.2	–	8 ± 0.76	–	9.2 ± 0.98	1.3± 0.011	–	–	–	–
V. cholerae 3906	12 ±1.1	–	15.2±1.50	–	–	–	–	–	–	–
S. aureus 3160	19 ±1.7	2±0.02	18.1±1.79	–	1.5±0.013	–	–	–	1.1 ±0.01	–
E. faecalis 439	10.2±0.98	–	4 ± 0.32	–	–	–	–	–	–	–
S. typhi 733	10 .8±0.96	–	11.8 ±1.16	–	8 ± 0.76	–	–	–	–	–

A-Hot ethanol extract; B- Cold ethanol extract; (-) –no activity

Table 5: Antibacterial activity of flower ethanol extracts against enteropathogens.

MTCC Strains	Zone of inhibition in mm (Mean ± S. D.)									
	P. granatum A	*P. granatum B*	*A. squamosa A*	*A. squamosa B*	*S. jambos A*	*S. jambos B*	*A. marmelos A*	*A. marmelos B*	*V. planifolia A*	*V. planifolia B*
E. coli 443	–	–	–	–	–	–	–	–	–	–
V.cholerae 3906	–	–	–	–	–	–	–	–	–	–
S. aureus 3160	–	2 ± 0.02	–	–	1.2±0.011	–	–	–	–	–
E. faecalis 439	–	–	–	–	–	–	–	–	–	–
S. typhi 733	–	–	–	–	–	–	–	–	–	–

A-Hot ethanol extract; B- Cold ethanol extract; (-) –no activity

The minimum inhibitory concentration (MIC) of hot ethanol extract against different bacterial strains was determined by micro-dilution method. The result showed that, in *Punica granatum* L. fruit hot extract against *Vibrio cholerae, Enterococcus faecalis* and *Salmonella typhi* had the highest MIC (64 µg/mL) and MBC (64 µg/mL), while the lowest MIC of 32 µg/mL was shown by *Escherichia coli* and *Staphylococcus aureus* respectively.But in *A.squamosa* L. fruit hot extract against *Staphylococcus aureus* and *Salmonella typhi* had the highest MIC (128 µg/mL) and MBC (128 µg/mL), while the lowest MIC of 32 and 64 µg/mL was shown by *Vibrio cholerae, Escherichia coli* and *Enterococcus faecalis* respectively **(Table : 6)**

Table 6: MIC and MBC of *Punica granatum* L. and *Annona squamosa* L.

Organisms	*Punica granatum* L.		*Annona squamosa* L.	
	MIC µg/ml	MBC µg/ml	MIC µg/ml	MBC µg/ml
***Vibrio cholera* 3906**	64	64	32	64
***Escherichia coli* 443**	32	64	64	64
***Staphylococcus aureus* 3160**	32	32	128	128
***Enterococcus faecalis* 439**	64	64	64	128
***Salmonella* Typhi 733**	64	64	128	128

4.2 Chromatographic studies

4.2.1 TLC studies

Traditionally analytical TLC has found application in the detection and monitoring of compound through a separation process. Precoated plates are also available for TLC technique in which backing material may be either of aluminium foil or a solvent resistant polyester sheet. These sheets can be cut to the desired size and activated if necessary. Thin layer chromatographic studies are shown in **figure 4.2**

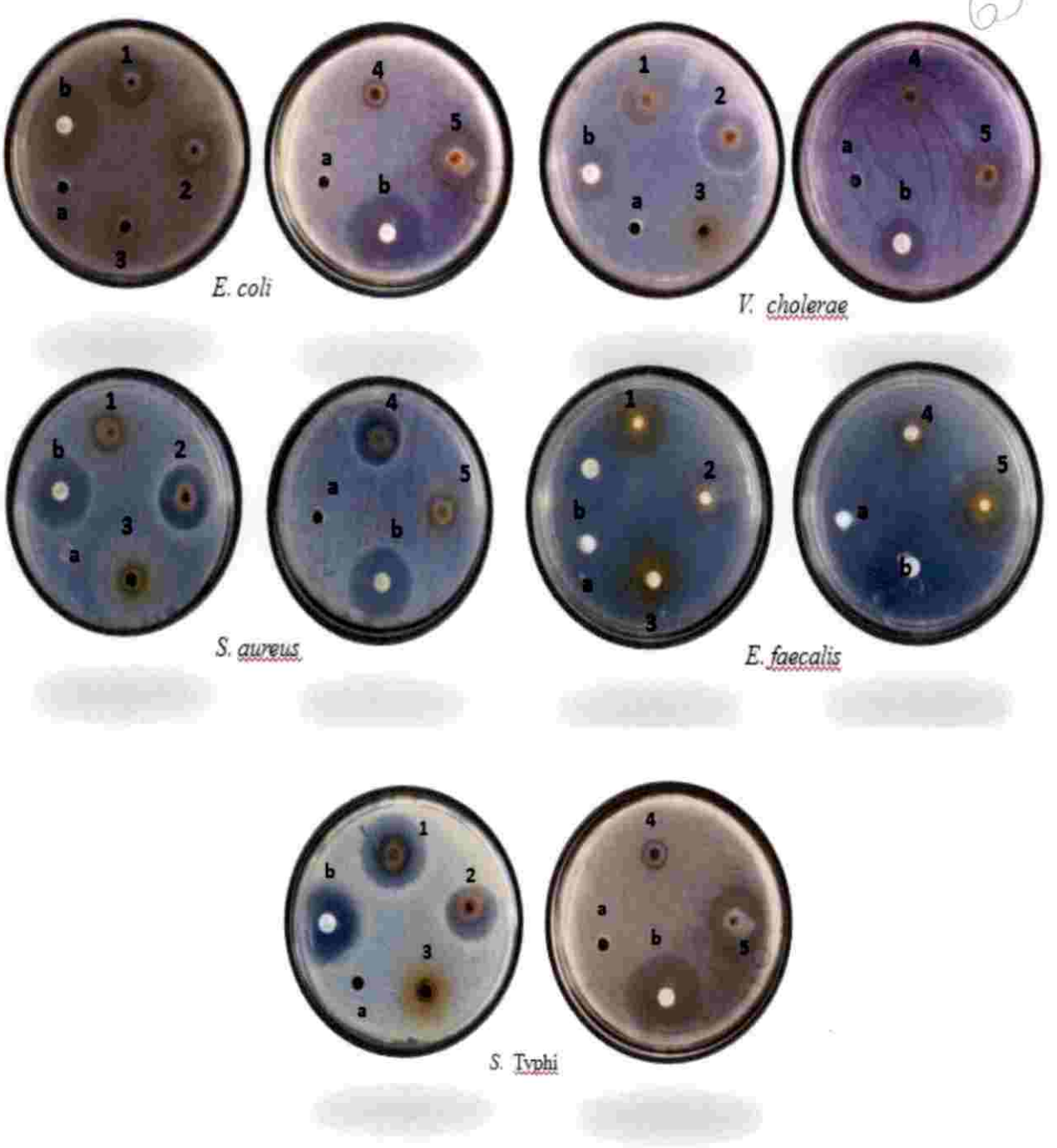


Figure 4.1: Anti bacterial activity of 5 fruit hot extract against 5 strains.
1- *P. granatum;* 2- *A. squamosa* ; 3- *S. jambose* ; 4- *A. marmelos* ; 5- *V. planifolia*
a- negative control DMSO; b- positive control Ciprofloxacin 5mcg

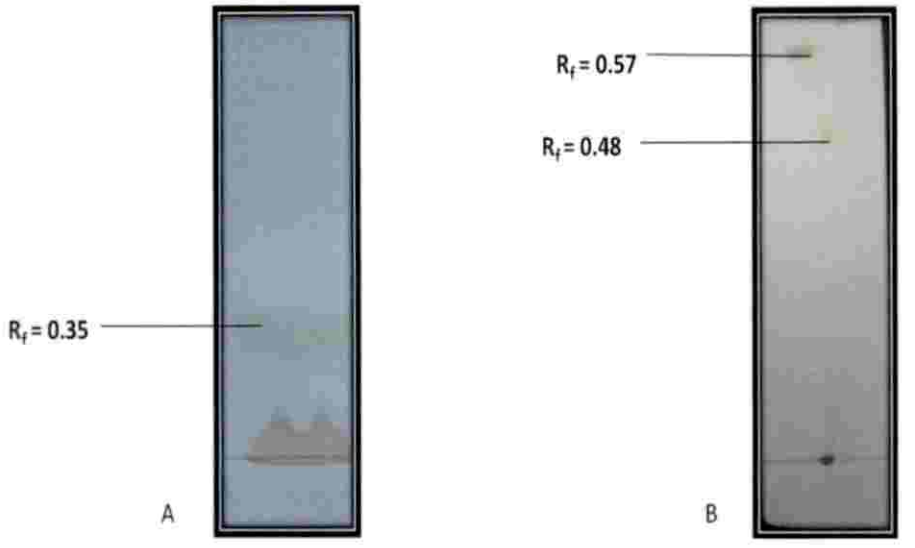


Figure 4.2: TLC of fruit hot extracts in visible region (A) *Punica granatum* L. (B) *Annona squamosa* L.

4.2.2 TLC bioautography

The zone of inhibition of bacterial growth could be seen around the active chromatogram spot of *P. granatum* and *A. squamosa* **(Table: 7)**

Table 7: Diameter of the zone of inhibition of TLC bioautography

MTCC Strains	*Punica granatum* L.	*Annona squamosa* L.
Escherichia coli 443	**2.5mm ± 0.023**	–
Vibrio cholerae 3906	**1.7mm ± 0.0156**	–
Staphylococcus aureus 3160	–	–
Enterococcus faecalis 439	–	–
Salmonella Typhi 733	–	–

Values are expressed as mean ± S.D.; (-)- no activity

4.2.3 Column chromatographic studies

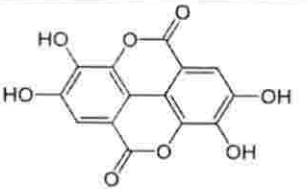
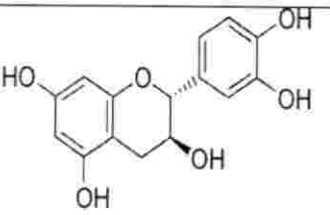
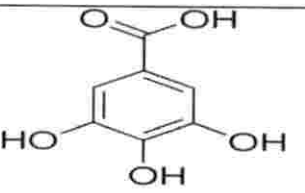
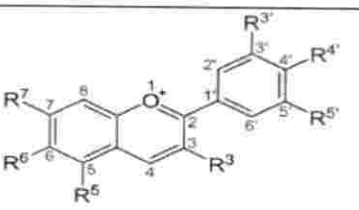
Column chromatography of hot ethanol extract of *Punica granatum* L. fruit **(Table: 8)** In this study, E1, E2,and E3 are chloroform eluent fractions; E4 is chloroform : methanol (9:1) as the eluent. In these, E4 (21-30) fractions were collected and subjected to thin layer chromatographic studies. At last the column was Fractions showing same number of compounds and R_f values were combined and concentrated. Concentrated fractions were purified by re-column chromatography using chloroform: ethylacetate (9:1) as eluent which was used for their separation from respective extracts. Afterthat preparative TLC showing active band according to standard ellagic acid. Numerous phytochemical constituents have been reported to be present in different parts of the pomegranate plant making it pharmacologically precious (Prakash., 2011). Barzegarl *et al.* (2007) studied the peel extract of *P. granatum* and reported substantial amounts of polyphenols such as ellagic tannins, ellagic acid and gallic acid. According to literature studies, to determine the docking score of bio active compounds from *Punica granatum* L **(Table:9).** Ellagic acid has more docking score than other compounds present in the *P. granatum* L.

Table 8: Consolidated details of fractions eluted by the column chromatography

Sl. No :	**Eluent solvent**	**Fractions**	**TLC solvent system (R_f) Toluene: Ethylacetate: Formic acid (6:4:1)**
1	chloroform	E1 (1-11)	0. 74,0.75
2	chloroform	E2 (12-18)	0.95 ,0.97
3	chloroform	E3 (19-20)	0.80,0.82
4	**Chloroform : methanol (9:1)**	(21-30)	0.33, **0.35**,0.39

Table 9: docking scores of bioactive compounds of *Punica granatum* L. fruit

66

Sl. No:	Bioactive Compounds of *P.granatum* L.	Structure	Docking score
1	Ellagic acid		- 8.9
2	Catechin		-6.4
3	Gallic acid		-7.2
4	Anthocyanin		-6.5

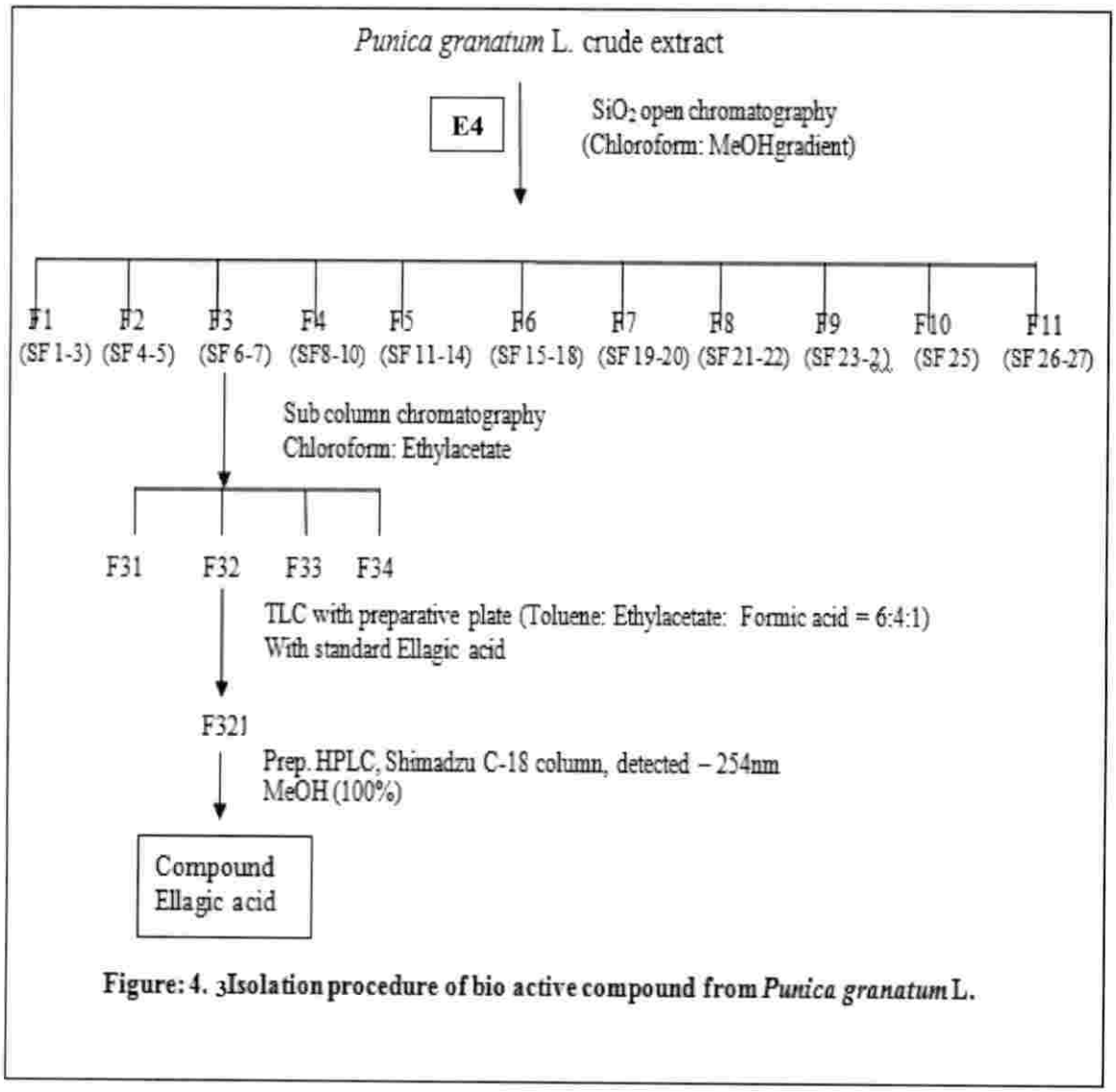
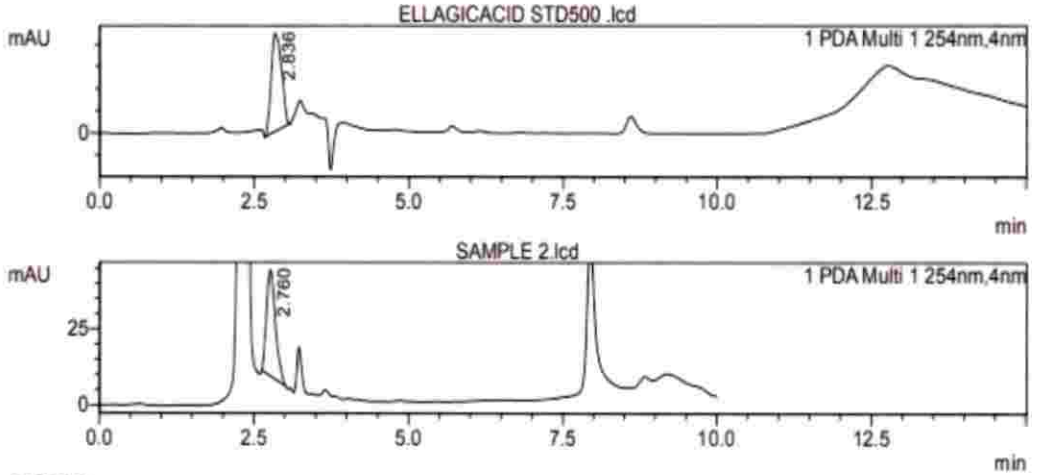


Figure: 4. 3Isolation procedure of bio active compound from *Punica granatum* L.

Figure 4.3 shows the isolation procedure of pure compound from *Punica granatum* L. fruit crude extract.

Figure 4.4: TLC plate showing re-spotted F321 fraction eluted from column chromatography of *P. granatum* Fruit crude hot ethanol extract shown antibacterial activity against test organisms with Rf value of 0.35 (A), Standard Ellagic acid (Rf=0.35) (B)



<< PDA >>

ID#1 Compound Name: ELLAGIC ACID

Data File Name	Sample Name	Sample ID	Ret. Time	Area	Height	Conc.
ELLAGICACID STD500 .lcd	ELLAGICACID	STD500ug	2.836	515411	44288	500.000
SAMPLE 2.lcd	SAMPLE 2	TLC SEPERATE	2.760	351741	34828	341.224
Average			2.798	433576	39558	420.612
%RSD			1.900	26.692	16.911	26.692
Maximum			2.836	515411	44288	500.000
Minimum			2.760	351741	34828	341.224
Standard Deviation			0.053	115732	6690	112.271

Figure 4.5: HPLC profile of TLC separated active fraction of Ethanol hot extract of *P. granatum* Fruit showing Ret. Time of 2.760 (E) and Standard Ellagic acid with Ret. Time of 2.836 (F).

4.3 In silico docking studies and homology modelling of FtsZ

An *in-silico* docking study was conducted to find bacterial cell division inhibition potential of ellagic acid using *E. coli* FtsZ protein homology model as substrate using Autodock Vina 1.1.2 software. The results revealed that the ellagic acid blocks a significant portion of the GTP binding site of the FtsZ protein, in a low-energy orientation characterized with optimal interactions **(figure 4.6).** Ellagic acid binds through hydrogen bonds to amino acid residues of FtsZ, particularly at GLY21 and ASN165, forms hydrophobic interaction with PHE182 and ARG142, which in turn decrease affinity of GTP with FtsZ. Higher negative binding score indicates more favourable binding of the ligand to the protein. The calculated binding energies of Ellagic acid-FtsZ receptor interaction (−8.9Kcal/mol) are comparable to that of standard compound berberine (−7.6Kcal/mol) and the data supported the *in vitro* inhibitory activity.

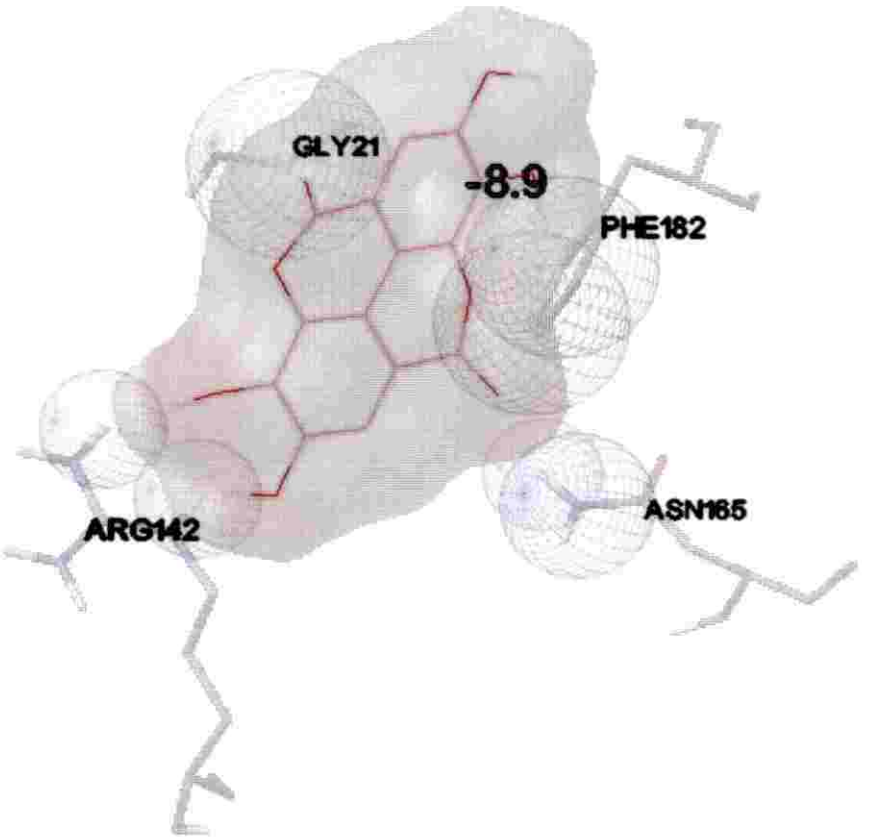


Figure 4.6: *In silico* analysis of Ellagic acid using Autodock Vina 1.1.2. Molecular Docking with *E. coli* FtsZ protein.

In vitro biochemical studies

Cell elongation assay

Cell Division inhibition property of hot ethanol extract of *P. granatum* and purified Ellagic acid was demonstrated using *Bacillus subtilis* 168 strain. 100 µg of ellagic acid inhibited cell division of this strain as shown in the **figure 4.7** Increased bacterial cell length is indicative of FtsZ inhibition. *P. granatum* and Ellagic acid treated cells showed increased cell length comparing to control conditions.

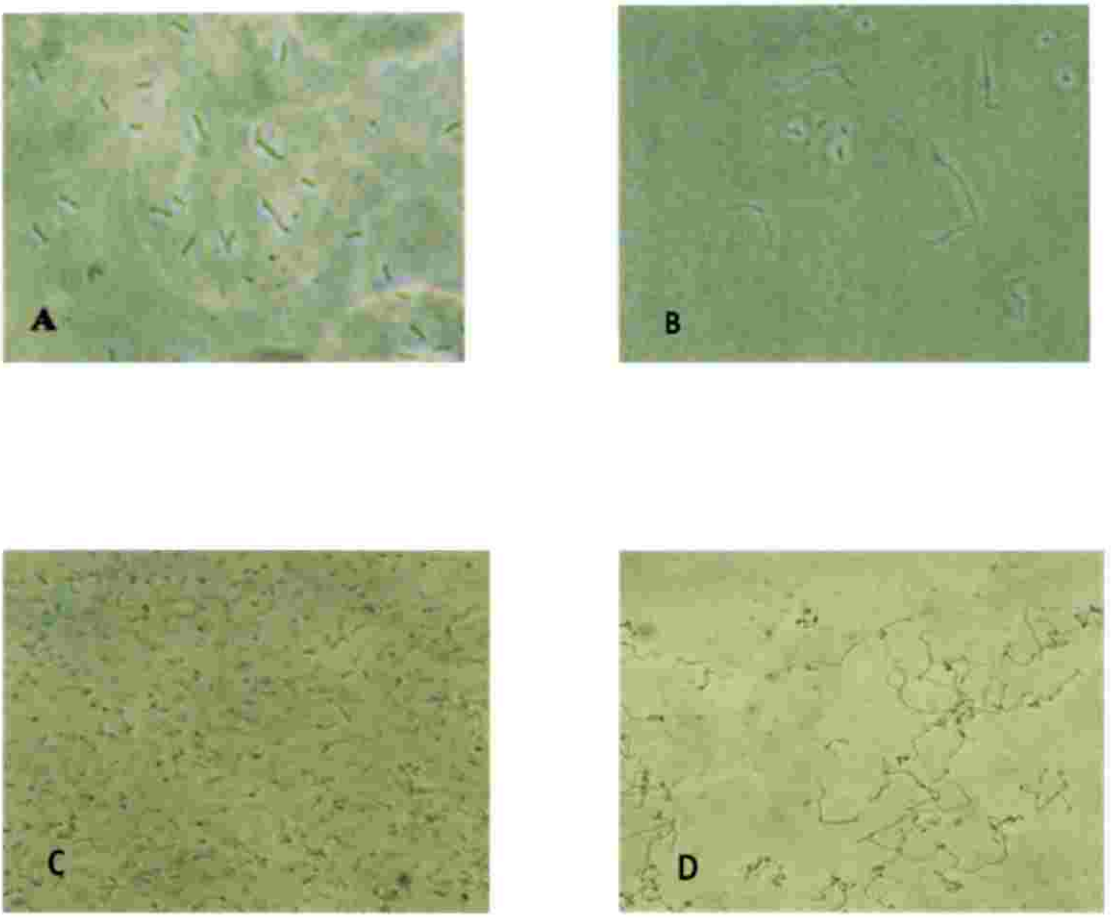


Figure 4.7: Phase contrast microscopy showing the comparison of Cell elongation of *Bacillus subtilis* 168 cells (BGSC, USA) on treated with *P. granatum* Ethanol hot extract and purified Ellagic acid compared with Control (DMSO treated). A) *Bacillus subtilis 168* cells treated with 1% DMSO (Control), B) treated with 100µg/mL of *P. granatum* Ethanol extract in 1% DMSO, C) Control and D) Treated with Ellagic acid 10µg/mL in 1% DMSO.

Malachite green assay for FtsZ GTPase activity.

The dose dependant inhibition of FtsZ GTPase by Ellagic acid was analysed by nonradioactive malachite green-phosphomolybdate assay. GTPase activity is crucial for the proper functioning of the FtsZ protein. The results shows that Ellagic acid with a concentration of 80 µg/ml is exhibiting potential FtsZ GTPase activity with more than 70% of inhibition **(figure 4.8).**

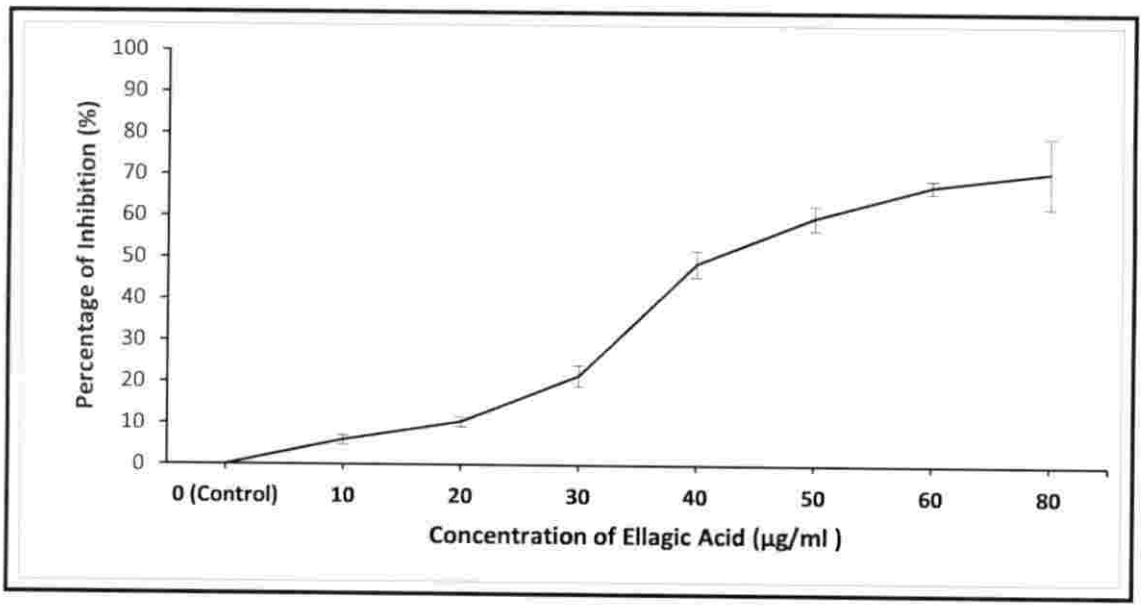


Figure 4.8: Chart showing dose dependent GTPase activity against *E. coli* FtsZ *in-vitro.*

FtsZ polymerization assay

The effect of Ellagic acid from *P. granatum* on the FtsZ polymerization dynamics was assessed using light scattering in a thermostatically (30 °C) controlled fluorescence spectrophotometer in which changes in FtsZ polymerization are reflected by corresponding changes in absorbance at 340 nm. **Figure 4.9** shows time-dependent polymerization of *E. coli* FtsZ in the absence and presence of

ellagic acid at 40 and 80 µg/mL. Ellagic acid (80 µg/mL) displayed complete inhibition of FtsZ polymerization.

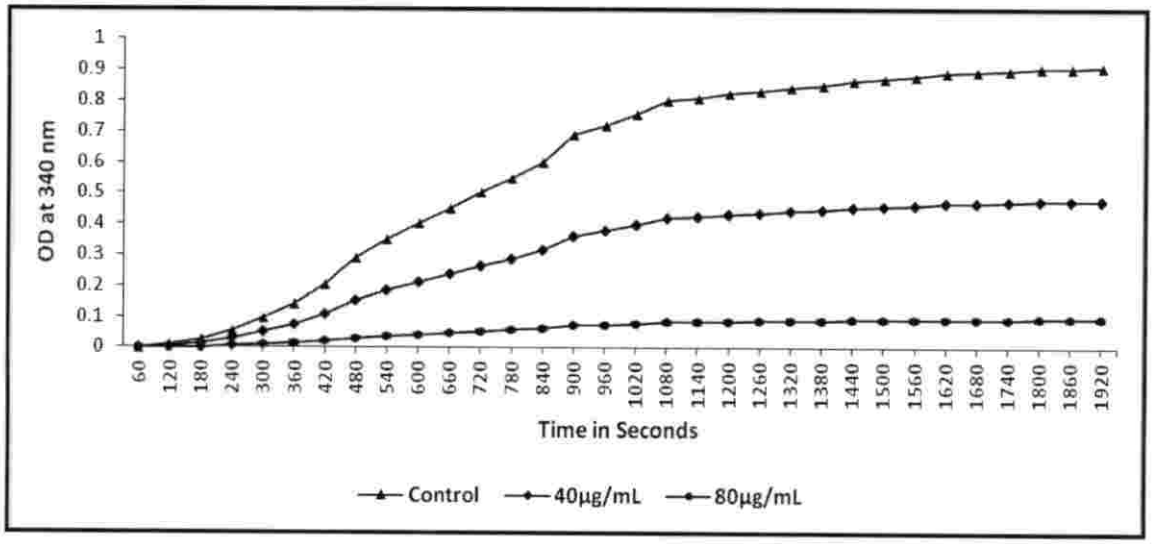


Figure 4.9: Polymerization activity of *E. coli* FtsZ in the presence and absence of Ellagic acid

Anti - Biofilm Assay

Minimum bio film inhibitory concentration (MBIC) of *Punica granatum* is 50µg and Ellagic acid is 20µg **(Table:10).**

Table 10: MBIC of *Punica granatum* and Ellagic acid

	10µg T1	20µg T2	30µg T3	40µg T4	50µg T5	SAMPLE
BLANK 0.085726	0.015512 85.3%	**0.014169 86.6%**	0.012166 88.5%	0.008605 91.8%	0.00499 95.3%	**Ellagic acid**
CONTROL 0.106171	0.018664 82.4%	**0.011001 89.6%**	0.0023 97.8%	0.000664 99.3%	0.000653 99.3%	**Duplicate**
BLANK 0.06821	0.030742 73.07%	0.028313 75.19%	0.021671 81%	0.021224 81.4%	**0.00952 86.6%**	***Punica granatum***
CONTROL 0.114164	0.03549 68.9%	0.026332 76.9%	0.021635 81.04%	0.000577 84.6%	**0.003272 87.1%**	**Duplicate**

Protease assay

The effect of ellagic acid (16 μg /mL) on the production of the extracellular cysteine protease (major secreted protease) of S. pyogenes was evaluated qualitatively using the azocasein assay, wherein the amount of coloured azo dye released into the suspension after proteolytic cleavage of the substrate (azocasein) was measured. The amount of dye released directly reflects the amount of protease production. Ellagic acid was not found to significantly enhance the protease production of the M serotypes.

Extracellular polymeric substance (EPS) estimation

In order to confirm the reduction in EPS production upon ellagic acid treatment, EPS was quantified using the phenolsulphuric acid method. Ellagic acid treatment (16 μg/mL) significantly not reduced the EPS production of all the M serotypes tested. Because cell surface hydrophobicity plays a key role in biofilm formation, this was also measured. Ellagic acid treatment considerably reduced the cell surface hydrophobicity of all the M serotypes.

Discussion

CHAPTER 5
DISCUSSION

Traditional medicine has given importance to crude forms of medicine to cure general diseases such as diarrhoea, vomiting, fever, constipation, cough, liver related problems and blood bom diseases. Later, development of modem science made revolutionary changes in medicine. Through various observations, modem medicine has come to know that natural products are the more powerful means in the fight against diseases than synthetic drugs. This led to extensive, thorough and scientific investigations on various plants or their products to develop them as effective agents against various diseases. During the last few decades, great emphasis has been given to fruits for identifying their medicinal properties. This is because, fruits are one of the common sources of human diet and their ingredients are biologically active and are well absorbed by the body without any side effects. Therefore, diverse studies have been conducted all over the world to elucidate the medicinal role of fruits. All the studies are pointing to the fact that fruits are essential for the man's well being and his healthy life.

World Health Organisation in its comprehensive report on antimicrobial resistance highlighted the urgency for taking immediate steps and strategies against fast emerging threat of AMR (WHO, 2014). This highlights the imperative need for alternative, cost-effective antimicrobial agents with novel mode actions. One of the main drug targets identified for the discovery of novel antibacterial agents is the bacterial cell division protein FtsZ, a highly conserved, essential part of bacterial cell replication machinery (Kusuma *et al.*, 2019).

Gastrointestinal diseases, particularly diarrhoea is one of the major concerns in many African and Asian countries including India, and a major cause of childhood mortality. Use of herbal preparations is widely practiced in Traditional medicines to cure gastrointestinal ailments. Ethnobotanical information

has a crucial role in the discovery of novel therapeutic molecules (Hong *et al.*, 2015) Therefore, this study was planned to investigate the *in vitro* studies on the anti infective properties of flower and fruit extracts of selected medicinal plants against enteropathogens.

In this study, hot and cold ethanol extracts fruit and flower were tested against *Escherichia coli, Enterococcus faecalis, Salmonella typhi, Staphylococcus aureus* and *Vibrio cholerae* using well diffusion assay to determine the antibacterial activity. Ethanol soxhlet extract showed inhibitory effect over growth of all the strains of bacteria such that zone of inhibition was detected, and minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) were determined.

The available literature on the phytochemical constituents revealed the presence of many saponins, quinones, terpenoids, flavonoids, phenols, and Coumarins in the fruit peel of *P. granatum.* It also contains substantial amounts of polyphenols such as ellagic tannins, ellagic acid and gallic acid (Yasoubi *et al.*, 2007). Activity guided thin layer chromatography on silica gel plates, then column chromatography followed by HPLC analysis leads to the identification of the active principle as Ellagic acid and the same was purified following standard method. According to literature studies, to determine the docking score of bio active compounds from *Punica granatum* L. So that ellagic acid was high docking score (-8.9)

In the present study, the ligand Ellagic acid was docked into *E. coli* FtsZ molecule using Auto Dock Vina 1.1.2 software. Ellagic acid was readily placed within the GTP binding pocket of FtsZ, in a low-energy orientation characterized with optimal interactions. *In vitro* studies also confirmed the FtsZ inhibitory property of Ellagic acid.

Filamentous temperature sensitive mutant Z (FtsZ) is the prokaryotic analogue of eukaryotic tubulin. It polymerizes into dynamic protofilaments and assembled as ring in the site cell division by GTP hydrolysis (Margalit *et al.*, 2004). The FtsZ inhibitory activity of small molecules can be visualised the induction of an enlarged phenotype of vegetatively growing cells of *Bacillus subtilis* 168 (Araújo-Bazán *et al.*, 2016). Formation of elongated cells in the presence of 100 µg/ml of Ellagic acid demonstrated its FtsZ inhibition activity. *In silico* screening, by docking ligand molecules to target protein is an effective method of screening bioactive molecules (Nandu *et al.*, 2017).

GTPase activity is crucial for the proper functioning of the FtsZ protein and in the present study Ellagic acid displayed increased GTPase activity, corroborates with a reduction in FtsZ polymer formation at a concentration of 80µg/ml. Earlier findings suggested that the anti-diarrhoeal effect of pomegranate rind may be due to its inhibitory effect on Gram-positive bacteria, as well as the astringent effect of tannins (Panichayupakaranant et al., 2010). Our findings suggested that the antibacterial property of ethanolic extracts of *P. granatum* is due to FtsZ inhibitory property of the active compound Ellagic acid.

Summary

CHAPTER-6
SUMMARY

The thesis entitled '*In vitro* studies on the anti-infective properties of flower and fruit extracts of selected medicinal plants against enteropathogens'was carried out in the Microbiology Division of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode, Thiruvananthapuram during the academic year 2018-2019. The objective of the study was to screening of medicinal plant extracts for anti infective property against enteropathogens and identification of lead compounds, targeted efficacy testing of selected compounds against FtsZ proteins through *in vitro* and *in silico* approaches and demonstrating the effect of these compounds on virulence factors and bio-film formation of enteropathogens.

In the present study, five fruits and their flowers are primarily selected for identifying anti infective property against enteropathogens. Hot and cold ethanol extracts of fruit and flower were tested against standard MTCC entero bacterial strains. Preliminary results showed that out of five fruits and flowers tested , two fruits of them – *Punica granatum* L. and *Annona squamosa* L. have powerful activity against all the tested stains. As various authors have already studied about the fruits of *Punica granatum* L. and *Annona squamosa* L. was selected for searching its medicinal properties like antibacterial, antifungal and antiprotozoal properties. Since , the crude hot ethanolic extract showed antibacterial activity and minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) were determined. it was subjected to purification of active compound through chromatographic techniques. Hot ethanol extract of *Punica granatum* L.fruit showing progressive activity of TLC bio autography. Activity guided thin layer chromatography on silica gel plates followed by column chromatography and HPLC analysis leads to the identification of the active principle as Ellagic acid and the same was purified following standard method.

In the present study Ellagic acid displayed increased GTPase activity, corroborates with a reduction in FtsZ polymer formation at a concentration of 80µg/ml. Ellagic acid does not possess any anti bio film propery so we concluded that the antibacterial property of ethanolic extracts of *P. granatum* is due to FtsZ inhibitory property of the active compound Ellagic acid.

However, in the present investigation only *in-vitro* and *in-silico* approaches were conducted. That means, the above compound showed various antibacterial activities in an invitro condition. Therefore, *in-vivo* experiments are inevitable to assess the *in-vivo* efficiency of Ellagic acid as a broad spectrum antibacterial antibiotic. Previous reports show that this compound is non toxic to animal cell. However detailed pharmacological and cytotoxic studies are necessary before developing this compound as antidiarrhoeal agent.

References

CHAPTER 7
REFERENCE

Ali, K., Ahmed, B., Dwivedi, S., Saquib, Q., Al-Khedhairy, A. A., and Musarrat, J. (2015). Microwave accelerated green synthesis of stable silver nanoparticles with eucalyptus globulus leaf extract and their antibacterial and antibiofilm activity on clinical isolates. PLoS ONE 10:e0131178.

Allison, K. R., Brynildsen, M. P., and Collins, J. J. 2011. Metabolite-enabled eradication of bacterial persisters by aminoglycosides. *Nature* 473: 216–220.

Andries, K., Verhasselt, P., Guillemont, J., Göhlmann, H. W., Neefs, J. M., Winkler, H., et al. 2005. A diarylquinoline drug active on the ATP synthase of Mycobacterium tuberculosis. Science 307: 223–227.

Aramwit, P., Bang, N., Ratanavaraporn, J., and Ekgasit, S. 2014. Green synthesis of silk sericin-capped silver nanoparticles and their potent anti-bacterial activity. Nanoscale Res. Lett. 9:79.

Arenz, S., and Wilson, D. N. 2016. Blast from the past: reassessing forgotten translation inhibitors, antibiotic selectivity, and resistance mechanisms to aid drug development. *Mol. Cell* 61: 3–14.

Aviram, A., Ahmed, A. S., Oves, M., Khan, M. S., Habib, S. S., and Memic, A. 2008. Antimicrobial activity of metal oxide nanoparticles against Grampositive and Gram-negative bacteria: a comparative study. *Int. J. Nanomedicine* 7: 6003–6009.

Azizi, S., Ahmad, M., Mahdavi, M., and Abdolmohammadi, S. 2013. Preparation, characterization, and antimicrobial activities of ZnO nanoparticles/cellulose nanocrystal nanocomposites. *Bioresources* 8 :1841–1851.

Balamurugan, P., Hema, M., Kaur, G., Sridharan, V., Prabu, P. C., Sumana, M. N., et al. 2015. Development of a biofilm inhibitor molecule against multidrug resistant Staphylococcus aureus associated with gestational urinary tract infections. *Front. Microbiol.* 6: 832.

Banin, E., Brady, K. M., and Greenberg, E. P. 2006. Chelator-induced dispersal and killing of Pseudomonas aeruginosa cells in a biofilm. Appl. *Environ. Microbiol.* 72: 2064–2069.

Baym, M., Stone, L. K., and Kishony, R.. 2016. Multidrug evolutionary strategies to reverse antibiotic resistance. *Science* 351: 3292.

Beloin, C., Renard, S., Ghigo, J. M., and Lebeaux, D. 2014. Novel approaches to combat bacterial biofilms. *Curr. Opin. Pharmacol.* 18: 61–68.

Bernier, S. P., Lebeaux, D., DeFrancesco, A. S., Valomon, A., Soubigou, G., Coppée, J. Y., et al. 2013. Starvation, together with the SOS response, mediates high biofilm-specific tolerance to the fluoroquinolone ofloxacin. PLoS Genet. 9:e1003144.

Bibbs, R. K., Harris, R. D., Peoples, V. A., Barnett, C., Singh, S. R., Dennis, V. A., et al. 2014. Silver polyvinyl pyrrolidone nanoparticles exhibit a capsular polysaccharide influenced bactericidal effect against Streptococcus pneumoniae. *Front. Microbiol.* 5:665.

Blázquez, J. 2003. Hypermutation as a factor contributing to the acquisition of antimicrobial resistance. *Clin. Infect. Dis.* 37: 1201–1209.

Briers, Y., Walmagh, M., Grymonprez, B., Biebl, M., Pirnay, J. P., Defraine, V., et al. 2014. Art-175 is a highly efficient antibacterial against multidrugresistant strains and persisters of *Pseudomonas aeruginosa. Antimicrob. Agents Chemother.* 58: 3774–3784.

Bulkley, D., Brandi, L., Polikanov, Y. S., Fabbretti, A., O'Connor, M., Gualerzi, C. O., et al. 2014. The antibiotics dityromycin and GE82832 bind protein S12 and block EF-G-catalyzed translocation. *Cell Rep.* 6: 357–365.

Bush, K., and Fisher, J. F. 2011. Epidemiological expansion, structural studies, and clinical challenges of new beta-lactamases from gram-negative bacteria. *Annu. Rev. Microbiol.* 65: 455–478.

Caly, D. L., Bellini, D., Walsh, M. A., Dow, J. M., and Ryan, R. P. 2015. Targeting cyclic di-GMP signalling: a strategy to control biofilm formation? *Curr. Pharm. Des.* 21:12–24.

Cassir, N., Rolain, J. M., and Brouqui, P. 2014. A new strategy to fight antimicrobial resistance: the revival of old antibiotics. *Front. Microbiol.* 5:551.

Chang, Q., Wang, W., Regev-Yochay, G., Lipsitch, M., and Hanage, W. P. 2015. Antibiotics in agriculture and the risk to human health: how worried should we be? *Evol. Appl.* 8: 240–247.

Chauhan, A., Bernardin, A., Mussard, W., Kriegel, I., Estève, M., Ghigo, J. M., et al. 2014. Preventing biofilm formation and associated occlusion by biomimetic glycocalyxlike polymer in central venous catheters. *J. Infect. Dis.* 210: 1347–1356.

Chauhan, A., Lebeaux, D., Ghigo, J. M., and Beloin, C. 2012. Full and broadspectrum in vivo eradication of catheter-associated biofilms using gentamicinEDTA antibiotic lock therapy. *Antimicrob. Agents Chemother.* 56: 6310–6318.

Cheng, G., Hao, H., Xie, S., Wang, X., Dai, M., Huang, L., et al. 2014. Antibiotic alternatives: the substitution of antibiotics in animal husbandry? *Front. Microbiol.* 5:217

Chen, L., Weir, M. D., Xu, H. H., Antonucci, J. M., Kraigsley, A. M., Lin, N. J.,etal. 2001.Antibacterialamorphouscalciumphosphatenanocompositeswith a quater-

nary ammonium dimethacrylate and silver nanoparticles. *Dent. Mater.* 28: 561–572.

Chiang, W. C., Nilsson, M., Jensen, P. Ø., Høiby, N., Nielsen, T. E., Givskov, M., et al. 2013. Extracellular DNA shields against aminoglycosides in Pseudomonas aeruginosa biofilms. *Antimicrob. Agents Chemother.* 57: 2352–2361.

Chitra, K., and Annadurai, G. 2013. Antimicrobial activity of wet chemically engineered spherical shaped ZnO nanoparticles on food borne pathogen. *Int. Food Res. J.* 20: 59–64.

Christena, L.R.; Subramaniam, S.; Vidhyalakshmi, M.; Mahadevan, V.; Sivasubramanian, A.; Nagarajan, S .2015. Dual role of pinostrobin-a flavonoid nutraceutical as an efflux pump inhibitor and antibiofilm agent to mitigate food borne pathogens. *Rsc Advances,* 5(76): 61881-61887.

Conibear, T. C., Collins, S. L., and Webb, J. S. 2009. Role of mutation in Pseudomonas aeruginosa biofilm development. PLoS ONE 4:e6289

Conlon,B.P.,Nakayasu,E.S.,Fleck,L.E.,LaFleur,M.D.,Isabella,V.M.,Coleman, K., et al. 2013. Activated ClpP kills persisters and eradicates a chronic biofilm infection. *Nature* 503: 365–370.

Dash rouiche, R. O., Mansouri, M. D., Gawande, P. V., and Madhyastha, S. 2007. Antimicrobial and antibiofilm efficacy of triclosan and DispersinB combination. *J. Antimicrob. Chemother.* 64: 88–93.

Daniel Costa, V. M., McGrann, K. M., Hughes, D. W., and Wright, G. D. 1989. Sampling the antibiotic resistome. *Science* 311: 374–377.

De Jong, W. H., and Borm, P. J. 2008. Drug delivery and nanoparticles:applications and hazards. *Int. J. Nanomedicine* 3: 133–149.

de la Fuente-Núñez, C., Reffuveille, F., Haney, E. F., Straus, S. K., and Hancock, R. E. 2014. Broad-spectrum anti-biofilm peptide that targets a cellular stress response. *PLoS Pathog.* 10:e1004152.

Elizabeth, K. and Rao, M. N. 1990. Oxygen radical scavenging activity of curcumin. *Int. J. Pharm.* 58: 237-240.

Ekins Ford R. I. 2007. *The Nature and Status of Ethnobotany* (2nd Ed.). Museum of Anthropology, Ann Arbor, USA, 67 p.

Forman, H. J., Ursini, F. and Maiorino, M. 2014. An overview of mechanisms of redox signaling. *J. Mol. Cell. Cardiology* 73: 2-9.

Gandidasan, R., Thamaraichelvan, A. and Baburaj, S. 1991. Antiinflammatory action of *Lannea coromandelica* by HRBC membrane stabilization. *Fitoterapia* 62: 81-83.

Gerber, M., Boutron-Ruault, M. C., Hercberg, S., Riboli, E., Scalbert, A. and Siess, M. 2002. Food and cancer: State of the art about the protective effects of fruits and vegetables. *Bull. Cancer* 89(3): 293–312.

Goldberg, lamnezhad, Z., Keyhanmanesh, R. and Boskabady, M. H. 1999. Anti-inflammatory, antioxidant, and immunomodulatory aspects of *Nigella sativa* for its preventive and bronchodilatory effects on obstructive respiratory diseases: A review of basic and clinical evidence. *J. Funct. Foods* 17: 910-927.

Gulcin, I., Alici, A. H. and Mehmet Cesur, M. 2005. Determination of *in Vitro* Antioxidant and Radical Scavenging Activities of Propofol. *Chem. Pharma. Bull.* 53(3): 281-285.

Gupta, S. S. 1994. Prospects and perspectives of natural plants products in medicine. *Indian J. Pharmacol.* 26: 1 -12.

Hafeez, A., Jain, U., Sajwan, P., Srivastava, S. and Thakur, A. 2013. Evaluation of Carrageenan induced anti-inflammatory activity of ethanolic extract of bark of *Ficus virens* Linn. in swiss albino mice. *J. Phytopharmacology* 2(3): 39-43.

Halliwell, B. and Gutteridge, J. M. 1984. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J.* 219: 1 – 14.

Harbourne. 1998. *Phytochemical methods: A guide to modern technique of plant analysis* (3rd Ed.). Chapman and Hall, London, pp. 4-227.

Hertog hendra, R., Ahmad, S., Oskoueian, E., Sukari, A., and Shukor, M. Y. 1993. Antioxidant, Anti-inflammatory and Cytotoxicity of *Phaleria macrocarpa* (Boerl.) Scheff Fruit. *BMC Complement. Altern. Med.* 11: 110.

Houck, J. C. and Jacob, R. A. 1960. Chemical dissection of rat skin. *Proc. Soc. Exp. Biol. Med.* 105: 324–326.

Ismail, T. S., Gopalakrishnan, S., Begum, B. H. and Elango, V. 1997. Anti-inflammatory activity of *Salacia oblonga* Wall. and *Azima tetracantha* Lam. *J. Ethnopharmacology* 56 (2): 145-152.

Jagetia, C. G. and Baliga, S. M. 2004. The Evaluation of Nitric Oxide Scavenging Activity of Certain Indian Medicinal Plants *in Vitro*: A Preliminary Study. *J. Med. Food* 7(3): 343–348.

Jaijoy, K., Vannasiri, S., Piyabhan, P., Lerdvuthisopon, N., Boonraeng, S., Khonsung, P., Lertprasertsuke, N. and Sireeratawong, S. 2011. Acute and subchronic toxicity study of the water extract from the fruits of *Piper chaba* Hunter in rats. *Int. J. Appl. Res. Nat. Products* 3(4): 29-35.

Ji, L., Wu, J., Gao, W., Wei, J., Yang, J. and Guo, C. 2011. Antioxidant capacity of different fractions of vegetables and correlation with the contents of ascorbic acid, phenolics and flavonoids. *J. Food Sci.* 76: 1257-1261.

Kaur, Kapewangolo, P., Omolo, J. J., Bruwer, R., Fonteh, P. and Meyer, D. 1997. Antioxidant and anti-inflammatory activity of *Ocimum labiatum* extract and isolated labdane diterpenoid. *J. Inflammation* 12: 4.

Karimi, A. and Moradi, M. 2015. Total phenolic compounds and in vitro antioxidant potential of crude methanol extract and the correspond fractions of *Quercus brantii* L. acorn. *J. HerbMed Pharmacol.* 4(1): 35-39.

Krippner, S. 2003. Models of Ethnomedicinal Healing. *Ethnomedicine Conferences*; 26-27 April and 11-12 October, 2003, Munich.

Kumari, A. and Kakkar, P. 2008. Screening of antioxidant potential of selected barks of Indian medicinal plants by multiple *in vitro* assays. *Biomedical Environ. Sciences* 21: 24-29.

Labiad, M. H., Harhar, H., Ghanimi, A. and Tabyaoui, M. 2017. Phytochemical Screening and Antioxidant Activity of Moroccan *Thymus satureioïdes* Extracts. *J. Mater. Environ. Sciences* 8(6): 2132-2139.

LiX, M., Khoo, S. C., Münch, G., Govindaraghavan, S. and Sucher, J. N. 2017. An *in vitro* study of anti-inflammatory activity of standardised *Andrographis paniculata* extracts and pure andrographolide. *BMC Complement. Altern. Med.* 15: 18.

Majid, M., Khan, R. M., Shah, A. N., Haq, U. I., Farooq, A. M., Ullah, S., Sharif, A., Zahra, Z., Younis, T. and Sajid, M. 2015. Studies on phytochemical,

antioxidant, anti-inflammatory and analgesic activities of *Euphorbia dracunculoides*. *BMC Complement. Altern. Med.* 15: 349.

Marcocci, L., Maguire, J. J., Droy, Lefaix, M. T. and Packer, L. 1994. The nitric oxide scavenging property of *Ginkgo biloba* extract. *J. Biochem. Biophys. Res. Commun.* 201: 748-755.

Mathai, K. 2000. *Krause's Food, Nutrition, and Diet Therapy* (10th Ed.). Philadelphia, Pa.: W.B. Saunders, 271: 274-275.

Mishra, R. N. 2012. Vayasthapak - The Ayurvedic Anti-aging drugs. *Int. J. Res. Pharma. Biomedical Sciences* 3(1): 234-249.

Moharram, A. F. and El-Shenawy, M. S. 2007. Antinociceptive and Anti-Inflammatory Steroidal Saponins from *Dracaena ombet*. *Planta Medica* 73(10): 1101-1106.

Muhammad, N., Muhammad Saeed, M. and Haroon Khan , H. 2012. Antipyretic, analgesic and anti-inflammatory activity of *Viola betonicifolia* whole plant. *BMC Complement. Altern. Med.* 12: 59.

Nagaharika, Y., Kalyani, V. and Rasheed, S. 2013. Anti-inflammatory activity of leaves of *Jatropha gossypifolia* L. by HRBC membrane stabilization method. *J. Acute Dis.* 2(2):156-158.

Oueslati, S., Ksouri, R., Falleh, H., Pichette, A., Abdelly, C. and Legault, J. 2012. Phenolic content, antioxidant, anti-inflammatory and anticancer activities of the edible halophyte *Suaeda fruticosa* Forssk. *Food Chem.* 132 (2): 943-947.

Padal, S. B., Ramakrishana, H. and Devender, R. 2012. Ethomedicinal studies for endemic diseases by the tribes of Munchingiputtu Mandal, Vishakhapatnam district Andhra Pradesh, India. *Int. J. Med. Arom. Plants* 2(3): 453-459.

Patgiri, B., Umretia, B. L., Vaishnav, U. P., Prajapati, P. K., Shukla, V. J. and Ravishankar, B. 2014. Anti-inflammatory activity of *Guduchi Ghana* (aqueous extract of *Tinospora Cordifolia* Miers.). *Ayu.* 35(1): 108–110.

Phillipson, J. D. 2001. Phytochemistry and medicinal plants. *Phytochemistry* 56: 237–243.

Pinchuk, I., Shoval, H., Dotan, Y. and Lichtenberg, D. 2012. Evaluation of antioxidants: scope, limitations and relevance of assays. *Chem. Phys. Lipids* 165: 638–47.

Prieto, P., Pineda, M., and Aguilar, M. 1999. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum Complex: Specific application to the determination of vitamin E. *Analyt. Biochem.* 269: 337-341.

Pushpangadan, P. (1995). *Ethnobiology in India: A Status Report.* Ministry of Environment and Forests, Government of India, New Delhi, 68p.

Rajendran, A., Rao, N. R., Kumar, K. R. and Henry, A. N. 1997. Some medicinal orchids of southern India. *Anc. sci. life* 17(1): 10.

Rajendran, V. and Lakshmi, K. S. 2008. *In vitro* and *in vivo* anti-inflammatory activity of leaves of *Symplocos cochinchinensis* (Lour) Moore ssp Laurina. *Bangladesh J Pharmacol.* 3: 121-124.

Ramachandran, R. and Appukuttan, G. 2016. Phytochemical and pharmacognostic evaluation of leaf and stem extracts of *Gynochthodes ridsdalei* Razafim & B. Bremer (Rubiaceae): An endemic medicinal plant of southern Western Ghats. *J. Pharmacy Res.* 10 (5): 233-239.

Rice-Evans, C. A., Diplock, A. T. and Symons, M. C. 1991. *Techniques in Free Radical Research, Laboratory Techniques in Biochemistry and Molecular Biology*. Techniques in Free Radical Research, Elsevier Science, 22: 3-8.

Richard, M. J., Arnaud, J., Jurkovitz, C., Hachache, T., Meftahi, H., Laporte, F., Foret, M., Favier, A. and Cordonnier, D. 1991. Trace Elements and Lipid Peroxidation Abnormalities in Patients with Chronic Renal Failure. *Nephron* 57: 10–15.

Sakat, S. S., Archana, R., Juvekar, and Manoj, N. 2009. *In vitro* antioxidant and anti-inflammatory activity of methanol extract of *Oxalis corniculata* Linn. *Int. J. Pharmacy Pharm. Sci*. 2: 149-162.

Saleem, T. K., Azeem, A. K., Dilip, C., Sankar, C., Prasanth, N. V. and Duraisami, R. 2011. Anti-inflammatory activity of the leaf extacts of *Gendarussa vulgaris* Nees. *Asian Pac J Trop Biomed* 1(2): 147-149.

Saxena, M., Saxena, J., Nema, R., Singh, D. and Gupta, A. 2013. Phytochemistry of Medicinal Plants. *J. Pharmacognosy and Phytochemistry* 1(6): 168- 182.

Shah, M. S., Shah, M. M., Ahmad, Z., Yaseen, M., Shah, R., Sadiq, A., Khan, S. and Khan, B. 2015. Phytochemicals, in vitro antioxidant, total phenolic contents and phytotoxic activity of *Cornus macrophylla* Wall bark collected from the North-West of Pakistan. *Pak. J. Pharm. Sci.* 28 (1): 23-28.

Sharma, R. A., Yadav, A. and Bhardwaj, R. 2013. DPPH Free Radical Scavenging Activity of Phenolic Compounds in *Argemone mexicana* Linn. *Int. J. Pharmacy Pharma. Sciences* 5(3): 683-686.

Shenoy, S., Shwetha, K., Prabhu, K., Maradi, R., Bairy, K. L. and Shanbhag, T. 2010. Evaluation of antiinflammatory activity of *Tephrosia purpurea* in rats. *Asian Pac. J. Trop. Med.* 3(3): 193-195.

Sies, H. 1997. Oxidative stress: oxidants and antioxidants. *Exper. Physiol.* 82: 291–295.

Singh, R. 2015. Medicinal Plants: A Review. *J. Plant Sciences* 3(1): 50-55.

Singh, G., Marimuthu, P., Heluani, C. S. and Catalan, C. 2005. Chemical constituents and antimicrobial and antioxidant potentials of essential oil and acetone extract of *Nigella sativa* seeds. *J. Sci. Food Agric*. 85: 2297–2306.

Singh, R., Singh, S., Kumar, S. and Arora, S. 2007. Evaluation of antioxidant potential of ethyl acetate extract/fractions of *Acacia auriculiformis* A. Cunn. *Food Chem. Toxicol.* 45(7): 1216-23.

Srikanth, P. and Natarajan, V. 2010. Redox regulation of Nox proteins. *Respiratory Physiology & Neurobiology* 174: 265–271.

Tepe, B., Sokmen, M., Akpulat, H. A. and Sokmen, A. 2006. Screening of the antioxidant potentials of six *Salvia* species from Turkey. *Food Chem.* 95: 200-204.

Uddin, M. N., Ahmed, T., Pathan, S., Al-Amin, M. and Rana, S. 2015. Antioxidant and cytotoxic activity of stems of *Smilax zeylanica* in vitro. *J. Basic Clin. Physiol. Pharmacol.* 26(3): 453-464.

Umapathy, E., Ndebia, E. J., Meeme, A., Adam, B., Menziura, P., Nkeh-Chungag, B. N. and Iputo, J. E. 2010. An experimental evaluation of *Albuca setosa* aqueous

extract on membrane stabilization, protein denaturation and white blood cell migration during acute inflammation. *J. Med. Plant Res.* 4(5): 789-795.

UNESCO. 1996. Culture and Health, Orientation Texts – World Decade for Cultural Development. *Document CLT/DEC/PRO*, Paris, France. 129p.

Vane, J. R. and Botting, R. M. 1995. New insights into the mode of action of anti-inflammatory drugs. *Inflammation Res.* 44 (1): 1-10.

Vinegar, R., Schreiber, W. and Hugo, R 1969. Biphasic development of carrageenan in rats. *J. Pharmacol. Exp. Ther.* 166: 96-103.

Visavadiya, N. P, Soni, B. and Dalwadi, N. 2009. Free radical scavenging and antiatherogenic activities of *Sesamum indicum* seed extracts in chemical and biological model systems. *Food Chem. Toxicol.* 47: 2507-2515.

Vriesman, M. F., Haenen, R. M., Westerveld, G., Paquay, J. B., Voss, H. P. and Bast, A. 1997. A method for measuring nitric oxide radical scavenging activity. Scavenging properties of sulfur–containing compounds. *Pharmacy World Sci.* 19(16): 283–286.

Zakaria, Yasuda, T., Inaba, A., Ohmori, M., Endo, T., Kubo, S. and Ohsawa, K. 1991. Urinary metabolites of gallic acid in rats and their radical scavenging effect on DPPH. *J. Nat. Prod.* 63(10): 1444-1446.

Appendices

CHAPTER 8

APPENDICES

95

APPENDIX I

Phosphate buffer (0.2M, 6.6pH)

a) 0.4 M solution of $Na_2HPO_47H_20$=7.122 g in water and make upto 100 mL

b) 0.4M solution of $NaH_2PO_4.2H_2O$=6.242 g in water and make upto 100 mL.

Mix X mLof (a) with YmL of (b) and dilute to 200 mL of water. X=37.5 mL and Y=62.5 mL (6.6pH).

APPENDIX II

Nutrient agar (in 250ml of distilled water)

a) Peptone crystalline = 1.25g

b) Sodium Chloride = 1.25g

c) Beef extract = 0.75g

d) Agar agar = 5

APPENDIX III

Mueller Hinton Agar (pH- 7.4)

a) Beef infusion form = 3 g

b) Casein hydrolysate = 1.75 g

c) Starch = 0.15 g

d) Agar = 1.5 g

IN VITRO STUDIES ON THE ANTI - INFECTIVE PROPERTIES OF FLOWER AND FRUIT EXTRACTS OF SELECTED MEDICINAL PLANTS AGAINST ENTEROPATHOGENS.

Submitted by

MUHSINA A. S.

(2014-09-122)

ABSTRACT OF THESIS

Submitted in partial fulfilment of the

requirement for the degree of

B. Sc. – M. Sc. (INTEGRATED) BIOTECHNOLOGY

Faculty of Agriculture
Kerala Agriculture University, Thrissur

B.Sc. - M.Sc. (INTEGRATED) BIOTECHNOLOGY
DEPARTMENT OF PLANT BIOTECHNOLOGY
COLLEGE OF AGRICULTURE
VELLAYANI, THIRUVANANTHAPURAM- 695 522
KERALA, INDIA

2019

CHAPTER 9
ABSTRACT

The thesis entitled "*In vitro* studies on the anti-infective properties of flower and fruit extracts of selected medicinal plants against enteropathogens" was carried out in the Microbiology division of Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, during 2018-2019 academic years.The objective of the study was Screening of medicinal plant extracts for anti infective property against enteropathogens and identification of lead compounds, targeted efficacy testing of selected compounds against FtsZ proteins through *in vitro* and *in silico* approaches, demonstrating the effect of these compounds on virulence factors and bio-film formation of enteropathogens.

Diarrhoeal diseases remain a leading cause of preventable death, especially among children under five in developing countries. Acute watery diarrhoea mainly affecting children of developing countries that results in dramatic dehydration and it is most dangerous in the very young. Enterotoxigenic *Escherichia coli* or *Vibrio cholerae* are most common causative agent of acute watery diarrhoea. Diarrhoea also cause huge economic burden on people of impoverished countries. There is need for need for novel anti-infective agents that can prevent disease and economic burden of diarrhoea. Developing countries are rich source of traditional knowledge and natural resources. These resources can be channelized to develop novel anti-diarrhoeal agents that can prevent emergence and dissemination of gastrointestinal pathogen including drug-resistant strains.

Many diarrhoeal diseases are known to be treated with herbal remedies throughout the history of humankind. Even today, plant materials continue to play a major role in primary health care as therapeutic remedies in many developing countries. For centuries, many secondary compounds of fruits are

found to have an outstanding role in medicine. Most of these compounds belong to a few families of substances such as alkaloids, terpenoids and phenolics. Fruits and flowers are ethno medically valuable products of a plant. They have been extensively used in folk and modern medicine for treating various diseases.

We have explored anti-infective activity of fruit and flower extract using microbiological assays. Hot ethanol extracts exhibited antibacterial activity against gastrointestinal pathogens such as *E. coli*, *V. cholera*, *E. faecalis, S.* Typhi *and S. aureus.* It induced filamentation of *Bacillus subtilis* 168 which is an indication of cell division inhibition. Active compound can be used to develop novel anti-diarrhoeal agents.