IN VITRO STUDIES ON ANTI-INFECTIVE PROPERTIES OF LEAF AND ROOT EXTRACTS OF SELECTED MEDICINAL PLANTS AGAINST GASTRO INTESTINAL BACTERIAL PATHOGENS.

Submitted by

ANJANA BABU

(2014-09-119)

THESIS

Submitted in partial fulfillment 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

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2019

DECLARATION

I hereby declare that this thesis entitled '**In vitro studies on the anti-infective properties of leaf and root extracts of selected medicinal plants against gastro intestinal bacterial pathogens'** is a bonafide record of the research work carried out 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.

Vellayan
2-3-2020

Anjana Babu
(2014-09-119)

CERTIFICATE

Certified that thesis entitled '***In vitro* studies on the anti-infective properties of leaf and root extracts of selected medicinal plants against gastro intestinal bacterial pathogens'** is a record of the research work done independently by **Mrs. Anjana Babu** (2014-09-119) 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: 2-3-2020

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(Chairman, Advisory Committee)
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Dedicated to Almighty, my Family, Friends and Teachers

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LIST OF ABBREVIATIONS AND SYMBOLS USED

et al.	And other co workers
Cm	Centimeter
CCE	Cold crude extract
DNA	Deoxyribonucleic acid
DMSO	Dimethyl Sulfoxide
°C	Degree Celsius
E. coli	*Escherichia coli*
etc.	Etcetera
EDTA	Ethylene Diamine Tetra Acetic Acid Disodium salt
EPS	Exopolysaccharide
FtsZ	Filamenting temperature-sensitive protein Z
GI	Gastrointestinal tract
G	Gram
Hr	Hours
HLPC	High-performance liquid chromatography
HCl	Hydrochloric acid
HCE	Hot crude extract
Kg	Kilogram
L	Litre
LB	Luria-Bertani
MBC	Minimal bacterial concentration assay
µg	Microgram

µL	Microliter
µM	Micromolar
min	Minutes
MIC	Minimal inhibitory concentration assay
M	Molar
MDA	Malondialdehyde
Mg	Milligram
MH agar	Muller-Hinton agar
mM	Millimolar
mL	Millilitre
Mm	Millimetre
Nm	Nano meter
ORS	oral rehydration solution
OD	Optical Density
%	Percent
pH	Potential of hydrogen
KCL	Potassium Chloride
rpm	Rotasion per minutes
RB	Round Bottom
R_f	Retardation factor
Sec	Seconds
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
STD	Standard

TLC	Thin-layer chromatography
UV	Ultraviolet
V. cholera	*Vibrio cholera*
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

Gastrointestinal (GI) tract is a complex anaerobic microbial ecosystem that nearly accommodates 400 different bacterial species, moreover because of this diverse inherent microbiota GI is been acknowledged as the most immunologically and metabolically active organ. The coliform organisms among the gastro bacterial pathogens colonize at the upper intestine, this unusual event is a characteristic of certain infectious pathogens such as *Vibrio cholerae* and *Escherichia coli.* These infectious pathogens colonize and produce enterotoxins that in turns stimulates the mucosal cells to secrete fluids, this abnormal event causes health problems like diarrhoea, vomiting and abdominal pain (Sherwood 1996). Diarrhoea is a global health concern leading to higher mortality and morbidity, besides diarrhoea is being the second chief cause of child death (below the age of 5) and it accounts for killing 5,25000 children every year (WHO, 2017). Recent statistical analysis shows that diarrhea has globally killed 1.339 billion children below the age of 5 and 79 thousand neonates between 0 to 27 days (Kamath *et. al.,* 2018). The main causatives of diarrhoea are a variety of bacteria, parasites and viral organisms, which spread infection through contaminated food and drinking water. The pathogens are also been transmitted from person to person (WHO, 2017). Among these etiological agent, *V. cholerae* and *E. coli* are the predominant diarrhoeal pathogens, because they possess an amalgam of virulence factors which are solely responsible for the disease pathophysiology, diagnosis and even treatment protocol choice (Liu *et al*., 2010). Diarrhoea in its early stages is been treated successfully through the prompt administration of oral rehydration solution (ORS), whereas patients dehydrated from diarrhoea are rapidily administrated with intravenous fluids along with appropriate

antibiotics in order to diminish the duration of diarrhoea. Even though antibiotic therapy is not recommended until and unless the illness is very severe. The recent past years has witnessed **enteropathogens** showing increasing resistance towards the first-line antibiotics, particularly fluoroquinolones. This decreased susceptibility of antibiotics is an evolving challenge for clinicians requiring reconsideration of clinical practices guidelines. Thus it is high time to work out alternatives for treating diarrhoea.

The use of herbal drugs for treating diarrhoea is a common practice especially in India and subcontinents. India being one among the pioneers in the use of herbal medicines for manifold ailments and in this regard serious efforts are being made by Indian phytochemists and botanists for exploring the plant diversity in order to identify more effective drugs from them (Akuodor *et al.,* 2011). Besides plants are considered as an economic and renewable source of pharmacologically active compounds that are recognized to produce certain phytochemicals which are toxic to pathogens. These compounds are plant secondary metabolites and are of great clinical importance because of their antimicrobial activity without conferring resistance (Srivastava *et al*., 2014). Plants are identified for their unlimited production of secondary metabolites like glycosides, alkaloids, flavonoids, terpenoids, steroids, saponins, quinones, coumarin and tannins, which are the basis of plant derived drugs (Das *et al.,* 2010). These highly diverse phytochemicals present in plants exhibits antimicrobial activity by interacting with specific targets within the microbial cells (Seow *et al*., 2014). They attack onto the microbial cell targets by which they block critical biochemical pathways or will interrupt the cellular structure. This event eventually leads to the microbial growth inhibition and death via one or more of the following reasons that include quorum sensing inhibition, efflux pump inhibition,, mating factors inhibition, antibiotic synthesis factors inhibition, interrupting riboswitches, terminating lipid biosynthesis, inhibition of virulence factor, biofilm inhibition and cell division inhibition. Among these drug targets biofilm inhibition and cell division protein inhibition of bacterial cells are the most scrutinized these days.

Biofilm is an aggregation of microorganism in which cells are embedded within a self-protected matrix extracellular polymeric substance which adheres to each other or onto a surface (Flemming *et al*., 2016). The organism embedded in the self-protected matrix are prone to develop antibiotic resistance because of its increased competence to receive resistance gene, thus a biofilm inhibiting molecule can prevent the development of drug resistance in organism. The two main leading causatives of diarrhoe, *V. cholerae* and *E. coli*, are highly susceptible for biofilm formation (Shikuma et al., 2010). Understanding cell division has led to the development of new antimicrobial targets as cell division is an essential process for the organism to survive (Ning *et al.,* 2017). Bacterial cell division possesses a complex set of biochemical machinery, which has many proteins as potential drug targets (Ning *et al.,* 2017). Filamenting temperature sensitive protein (FtsZ) is identified to be a critical protein that can influence the cell division process and it is highly conserved in a wide variety of bacteria (Ning et al., 2017). During the time of cytokinesis, FtsZ undergoes GTP dependent polymerization and it assembles into Z-ring, which is highly dynamic scaffold (Oliva *et al.,* 2004). Moreover, FtsZ is responsible for recruiting other proteins for invagination of the cell membrane and septum formation for completing the bacterial cell division (Adams *et al.,* 2009). Many plant antimicrobial compounds are identified that can inhibit FtsZ, some among them are Curcumin, Berberis, Coumarins, Plumbagin, Chrysophaentins Phenylpropanoids, Cinnamaldehyde etc. (Haranahalli *et al*., 2016).

The isolation and purification of bioactive compounds from plants is a technique that has undergone rapid development in recent years. There are precise techniques for the isolation, separation and purification of compounds, many bioactive compounds are isolated and purified by employing thin-layer chromatography and column chromatography method (Ammar *et al.,* 2017). After purification of the bioactive compounds they are structurally determined by employing different range of techniques like UV –visible, Infrared, Nuclear magnetic resonance, mass spectroscopy and HPLC. (Ammar *et al*., 2017). The molecular simulations of bioactive compounds with the different targets in microorganism are determine by

molecular docking methods, this would help in identifying the most efficient compound that could bind most accurately with the target site. Molecular docking of bioactive compounds will support the existing drug development process as the molecular chemical interaction could be scrutinized with the help docking (Biswal *et al.*, 2019).

In the present study the leaf and roots of selected medicinal plants were screened for anti-infective properties against gastro intestinal bacterial pathogens like *V. cholera* and *E. coli*, the two main causatives of diarrhoea. The screening led to identification of the potential fraction in the active extract and also demonstrated the mode of action of the extracts over the cell division protein FtsZ. The study evaluated the effect of lead compound on the virulence factor and biofilm formation of *V. cholera* and *E. coli*. The study examined the in-silico analysis of the mode of action of the bioactive compound over the microbial target. The lead compound identified in this study can be further processed into an herbal formulation for diarrhoeal treatment.

CHAPTER 2
REVIEW OF LITERATURE

2.1 Introduction

Since prehistoric time plants with medicinal value have been identified and used in traditional medicine practices, research proved that even Neanderthals that lived 60,000 years ago were using plants for healing purposes, and those plants are still used in ethno medicine around the globe. It is estimated that there are around 2,50,000 to 5,00,000 species of plants on Earth in which relatively a small percentage that is from 1 to 10 has been used as food by humans and other animals, and possibly more are used for the medicinal purpose (Borris *et al*., 1996). During the late fifth century of BCE around 300 to 400 medicinal plants were mentioned by Hippocrates, and during the first century a Greek Doctor and apothecary named Dioscorides who is also considered as father of pharmacology wrote a document on botany and pharmaceuticals called "De Materia Medica" (Vassiliadis., 2013). This documentation became the prototype for modern pharmacopoeias and later on plants were observed for their efficacy as therapeutic agents and they were frequently prescribed, even if their chemical constituents we re not completely known. But when in 1909's the idea of antibiotics was introduced by Paul Ehrlich, the use of plant derivatives as antimicrobials were virtually non-existent.

Antibiotics are one among the chief therapeutic discoveries in medicinal history, and they have revolutionized the treatments for patients, have brought out reduction in mortality and morbidity. Empirical use of antibiotics lead to emergence of multi-drug resistant bacteria (Tenover., 2006). One of the major problems faced by humanity during the late 20th century is antibiotic resistance. Understanding the fast dissemination of resistance and realization of limited life span of antibiotics are making researchers look for novel anti-infective agents which could effectively fight against resistant microbes (Ginovyan et al., 2017). Plants were also investigated because of their well know traditional use in

medicine practices and they are demonstrated to be one of the most promising sources. Thus, the pace of using plant products in the medicinal field has again increased. Antimicrobial agents derived from plants are considered to be safer than synthetic compounds because of their natural origin (Rajeh et al., 2010, Upadhyay et. al., 2014). Plant-derived compounds have other target sites than traditional antimicrobials and subsequently having different mechanisms of action against microbes.

2.2 Diarrhoea and its causatives

Human abdomen accommodate bacteria that contributes extremely to human biology, moreover the gut is been colonized by an extensive and multiplexed arrays of bacterial cells, that are approximated to be 10 times more than the total body cells (Carman et al, 1993). Nearly all of the bacteria that colonizes in human gut has an anaerobic metabolism while aerobic and facultative anaerobes are grades of magnitude lower than obligate ones (Fanaro et al, 2003). It has been stipulated that in normal adults, obligate anaerobic bacteria are 90% of the predominant organisms and 10% are aerobic in nature (Salminen et al, 2002; Fanaro et al, 2003; Wanga et al, 2004). Traditionally human gut is been considered as the sewage system which is efficient of recycling water through absorption, as well as storing and removing of waste material from Gastrointestinal tract (GI). However, because of the inherent microbiota, GI tract is been acknowledged as the most immunologically and metabolically active organs in human body. GI being a complex anaerobic microbial ecosystem accommodates approximately 400 different gastrointestinal bacterial species, these gastrointestinal bacterial pathogens are the root causatives of a variety of infections. These infection at initial stages may be confined to gastrointestinal tract in its initial stages, before spreading into different parts of the body. Gastrointestinal infections may leads to an inflammation in gastrointestinal tract, such inflammations are called gastroenteritis inflammations. The gastroenteritis inflammation is because of the colonization of pathogenic organism in the intestinal tract, as they colonize they

release an enterotoxin that stimulates mucosal cells to secrete fluids due to the increase in intracellular adenosine monophosphate and these abnormal events cause health problems like diarrhoea, vomiting and abdominal pain (Sherwood 1996). Mostly the gastro infection is associated with the consumption of contaminated food, leading to a condition of food poisoning, in such cases food acts as vehicle to transport these pathogens, besides it provides a stable condition to support the growth of organism.

Among the gastrointestinal tract infection most common one is diarrhoea, normally diarrhoea results because of the fluid increase and the loss of electrolyte from the lumen in order to produce liquid or unformed faces and is been forcibly expelled out the pathogens, there by aids its dissemination. Diarrhoea is a global health concern which leads to higher morbidity and mortality (WHO 2017), it has been considered as a major public health concern because of this high morbidity and mortality rate it causes among all group of ages. Moreover diarrhoea is one among the prominent infectious causes in hospitalizations and outpatients visit in developing and developed countries. In developing countries illnesses that cause diarrhoea is more common and as such in these regions health care is limited, diarrhoea becomes a major health concern that can be life-threatening due to dehydration. It has been reported that on an average the morbidity attack rate of 3.2 episodes per year for a child with diarrhoea, but in some developing countries, this number can go up to as high as 12 episodes per child per year. Research studies show that long term consequences of disease burdened during early childhood has affected physical and mental development of children. Some of the diarrhoeal disease are not diagnosed mainly because they are self-limiting and mild, at the same time the parents do not seek medical attention due to the unavailability of medical and laboratory facilities. One of the major centre for research in diarrhoeal diseases is the International Centre for Diarrhoeal research at Bangladesh. The institute have a record of treating 100,000 patients in a year and this number is increasing year after year. The primary aim of the institute is to conduct research on pathogenesis, prevention and treatment for diarrhoeal diseases.

Diarrhoeal pathogens are mainly transmitted through faecal oral route and are responsible for worldwide inexorable outbreaks from time to time. Infectious diarrhoea is multifactorial in nature and they are associated with a vast array of etiological agents. Different etiological agents are highly affiliated with diarrhoea and they include bacterial pathogens, parasites and viruses. The predominant diarrhoeal pathogenic agents are bacterial pathogens, mainly *Vibrio cholera* and *Escherichia coli* strains (Liu *et al.,* 2010), they possess amalgam of virulence factors that are responsible for the disease pathophysiology. *E. coli* and *V. cholera* are the first line bacterial causatives of acute diarrhoea and which is most dangerous for young malnourished children. Among these diarrhoeal pathogenic agents mentioned above, *E. coli* is an extremely versatile microorganism and also signifies as one of the important leading etiological agent of moderate to severe diarrhoea worldwide. *E.coli* and *V. cholera* are employed as main strain in this study for checking anti-infective properties of the plant crude extracts. Diarrhoeagenic *E.coli* are further catalogued into different pathogenic types based on the occurrence of unique virulence factors that are been determined by the contribution of specific pathophysiology (Miliwebsky *et al*., 2016) some among them are enteropathogenic *E. coli*, enteroaggregative *E. coli*, enterotoxigenic *E. coli*, enteroinvasive *E. coli* and enterohemorrhagic *E. coli* (Nataro *et al*., 1998). These diarrhoegenic *E.coli* pathotypes exhibits special unique virulence arsenal which helps in transforming the predominant repertoire available for therapeutic and diagnostic approaches (Shetty *et al.,* 2012). Various extracellular structures in *E. coli*, like lipopolysaccharide and adhesion helps the organism to establish pathogenicity in the host, they are also found to produce different combinations of heat labile enterotoxins and many different factors needed for its colonization, all these factors together contributes to the virulence of the pathogen (Nataro *et al.,* 1998 and Gaastra *et al.,* 1996).

Vibrio is also an important diarrhoea causing pathogen, responsible for several global pandemics and epidemics (WHO. 2017). Vibrio are member of Vibrionaceae family a gram negative comma shaped aquatic bacterium that

colonizes the small intestine (Levine *et al.,* 1985). The colonization of Vibrio spp. causes profuse watery diarrhoea and if left untreated these diseases become severe and fatal. Symptoms of Vibrio mediated diarrhoea include rice watery stools, severe dehydration, fever and vomit. Vibrio spp. express several virulence factors like toxins, colonization factors like lipopolysaccharide, outer membrane proteins, flagellar components, toxin coregulated pilin, hemagglutinin and protease like hemolysins, thermolysins, cytolysins, metalloproteases and also iron acquisition systems.

Diarrhoea is an easily treatable disease, majority of patients are been treated successfully through prompt administration of oral rehydration solution (ORS) in the initial infection stages. Severely dehydrated patients are at risk of shock and require the rapid administration of intravenous fluids, these patients are also given appropriate antibiotics to diminish the duration of diarrhoea. But the mass administration of antibiotics is not recommended because it may contribute to antimicrobial resistance by gastrointestinal pathogens. Even though antibiotic therapy is not recommended unless the illness is serious or due to cholera. In the past two decades enteropathogens have shown increasing resistance to first-line antibiotics, particularly fluoroquinolones. Thus this decreased susceptibility of preferred antibiotics is an evolving challenge for clinicians requiring interval reconsideration of clinical practice guidelines. Thus it is high time to think and work on alternatives for treating diarrhoea, and in this present study we are scrutinizing traditionaly known medicinal plants for the development of alternative therapeutic systems acting on novel drug targets.

Antimicrobial resistance is represented as one of the highly concerned threats to global health and new range of anti-infective are required to overcome this global issue (Thabit *et al.*, 2015). It is estimated that around 700,000 people is currently dyeing each year because of AMR infection, moreover studies says that this number will be projected to reach 10 million annually by 2050 (O'Neill, 2016). Besides, at present scenario more than ever reinvigorating the pipeline of anti-infective development is more critical, as it is hard to treat bacteria that are

continuing to emerge day by day (Pewtrusts.org., 2018). Overall, novel anti-infective which exhibits new mechanism of action are needed (Schroder *et. al.*, 2017).

2.3 Role of plant natural products as anti-infective

Plants are used in traditional medicinal practices, mostly against infections and are found to inhibit microbial growth and thier virulence factors (Ahmad and Beg, 2001., Kumar *et al*.,2006., Bibi *et al*., 2011.,Cioch *et al*., 2017). The use of traditional medicines are considered as one of the most easily available treatment method and around 80% of the populations in developing countries are using it to meet their primary healthcare needs (WHO,2002 and Maroyi, 2013). Plants are identified to synthesize a diverse array of secondary metabolites as an adaptation for self-defence and communication with other organisms. These metabolites are found to have advantages over anti-infective drug development, mostly in form of bioactive molecule which are drug-like and are harbouring potential for synergy with other secondary metabolites (Harvey *et al.*, 2015).

Herbal medicines are used for treating many infectious diseases throughout the world. Plant extracts are used as antifungal, antibacterial and antiviral agents in traditional medicine mainly because of their wound healing property and ability to treat infectious diseases. Plants like *Vaccinium macrocarpon* (cranberry juice), and *Arctostaphylos uvaursi* (bear-berry) are used to treat urinary tract infections. *Melaleuca alternifolia* (tea tree) essential oil is used to treat cutaneous infections and essential oil from *Hydrastis canadensis* is used in treatment of tuberculosis. Because of the high curative potential of plant extracts, they are investigated for the development of novel drugs, to control bacterial infections. Moreover, antimicrobials derived from plants are considered to be safer than synthetic compounds because of their natural origin (World Health Organization., 2014).

Natural products serves as a critical reservoir of adjuvants of antibiotic in order to overcome the resistance mechanisms and the investigations for alternative anti-

infective action mechanism is opening up newer avenues in drug development (Wright, 2017 and Schroeder *et al.,* 2017). Some of the major research interest these days are bacterial virulence, bacterial quorum sensing inhibition, biofilm formation, inhibition of cell division. In the present study we are considering biofilm inhibition and cell division protein inhibition through the action of selected medicinal plants against major diarrhoea coursing organisms like *E. coli* and *V. cholerae.*

2.4 Alternative therapeutic systems

The development of alternative therapeutic systems acting on novel drug targets and drugs combating microbial biofilms is a high need of the time. Medicinal and aromatic plant based molecules are one the most attractive source of novel bioactive molecules with potential antibacterial activities. The practice of herbalism is becoming a mainstream throughout the world. In spite of great advance observed in modern medicine, plants still make an important contribution to health care. Many plant based compounds are isolated from different plant parts, which shows high rate of antimicrobial activity and these compounds mainly belong to the classes of alkaloids, essential oil, xanthonoids, anthraquinones, flavanoids, isoflavanoids, coumasins, saponins.

With the global emergence of AMR there is an urgent renewed interest on investigating alternative cellular processes as drug targets for the next generation antimicrobials (Murima *et al.,* 2014). Bacterial virulence factor is an ideal target in most studied pathogenic organisms like *S. aureus, E. coli, V. cholerae* and *Mycobacterium tuberculosis* (Otto, 2004 and 2014). Most of these organism's virulence is mediated through quorum sensing. Quorum sensing controllers the pathway that is responsible for the production of virulence factor (Quave *et al*., 2015 and Muhs *et al.,* 2017). Bacterial quorum sensing is also one among the most preferred target for controlling the antibiotic resistance, the classical pharmacological approach to receptor antagonism was employed for studying the

strategy to interfere the bacterial quorum sensing (Dianella *et al*.,2012).The quorum sensing inhibitor molecules are generally of low-molecular mass, and they have the ability to reduce the expression of quorum sensing gene (Dianella *et al.*,2012). Zang *et al* isolated and identified a natural group of compound called halogenated furanones from *Delisea pulchra*, a marine red algae that exhibited an effect on bacterial quorum sensing mechanism. The team also found that the compound had the ability to modify and inactivate S-ribosylhomocysteine lyase, known as LuxS, which is an enzyme that helps in the production of autoinducer that mediate the interspecies quorum sensing in Gram-negative bacteria (Zang *et al*.,2009). Rasmussen *et al* in 2005 identified a number of compounds from bean sprout, *Daucus carota, Matricaria chamomilla*, *Allium sativa*, Capsicum Chinese, Nymphaeaceae, *Capsicum annum* and two compounds from Penicillium fungi, patulin and penicillic acid. Rasmussen in his study verified the ability of patulin and penicillic acid to inhibit quorum sensing by DNA array analysis, which suggested that penicillic acid and patulin targeted the RhlR and the LasR proteins, which are responsible for the production of autoinduceres from executing quorum sensing (Rasmussen *et al*., 2005). Bacterial biofilm formation is also considered as an important target and is highly investigated these days. The biofilm associated infections could be more effectively resolved by inhibiting the formation of biofilms. Moreover bacterial biofilm is proved to play a significant role in pathogenesis, antibiotic resistance and therapeutic treatment failure and hence has garnered more attention lately (Kania *et al*.,2007). Compounds that control the biofilm formation and there by disturbs the growth of organism that are resistant to antimicrobial agents is been identified and isolated by researches. Understanding cell division has critically lead to the development of new antimicrobial targets because cell division is an essential process for the organism to survive (Ning *et al*., 2017). Many plant bioactive compounds like curcumin, coumarins, resveratrol and berberine are identified to inhibit the major cell division protein FtsZ.

2.5 Inhibition of biofilm formation as anoval target

Biofilm can be defined as a self-produced matrix aggregation of microorganism, in which microbes are embedded in and this extracellular self-protective matrix is constructed with extracellular polymeric substance (EPS), that adheres to each other and on to a surface and thus forms a matrix (Flemming *et al*., 2016). Normally bacterial cells exhibits two type of growth pattern, one is the planktonic cell condition and the other is sessile aggregate which is the biofilm formation stage (Flemming *et al*., 2016). Biofilm is the most widely distributed and successful mode of life on Earth and they play a major role in driving the biochemical cycle of most elements in soil, water, sediment and subsurface environment (Stoodley *et al.*, 2003). Bioflims have a major input hand on the pathogenesis, antibiotic resistance and treatment failure and hence garnered more attention these days (Kania *et al*., 2007).

In 2004 Knowles observed Carvacrol, a monoterpene phenol and a natural biocide, with an effect on biofilm formation by *Staphylococcus aureus* and *Salmonella enteric*.Tthe nonbiocidal concentration of this compound disturbed the normal biofilm formation, it prevented the buildup of protein mass and thereby arrested the micro colony. Later in 2008 Barira *et al.,* purified a compound from the extract of Morus Alba that inhibits biofilm formation by reducing the secretion of bacterial extracellular polysaccharide in *Streptococcus mutans*, a major causative of dental caries (Islam *et al.,* 2008). Similarly, another compound belonging to the group of naphthalene family was extracted out from *Trachyspermum ammi* seeds, which also exhibited the same effect of that of 1-deoxynoijirimycin against *Streptococcus mutants*.

Cobrado *et al.* (2011) extracted compound Hamamelitannin belonging to tannin family from *Hamamelis virginiana* bark, which exhibited a significant reduction in biofilm metabolic activity in *S. aureus, S. epidermidis, A. baumanni* (Cobrado *et al.*, 2012 and Dianella 2012). Then in 2012 Artini *et al.*, isolated compounds like Chelerythrine, Sanguinarine, Dihydroxybenzofurane and Pro-antocyanidin

A2 from the roots of plants like *Macleya cordata*, *Krameria lappacea*, *Chelidonium majus* and *Aesculus hippocastanum*. These isolated compounds exhibited antibiofilm activity against *S. epidermidis* RP62A and *S. aureus* 6538P. Among these four compounds Pro-antocyanidin A2 isolated from *Aesculus hippocastanum* and Chelerythrine isolated from *Macleya cordata* exhibited an inhibition on *de novo* biofilm formation without any bacterial activity. Treatments with alkaloids mostly downregulates expression of some proteins belonging to different pathways. Pro-antocyanidin A2 exhibited an action on iron binding protein, thus impairing uptake of iron by the microbe. Iron is known to be an essential micronutrient for microorganism and its scarcity leads to blockage in the process of planktonic cells becoming sessile state of bacteria. The compound also alters the action of autolysin, a penicillin-binding protein, and thus the compound inhibits biofilm formation (Dianella 2012). Coenye *et al*.,(2012) identified that *Propionibacterium acnes*, a microorganism that is responsible for acne vulgaris, can form a biofilm and it showed susceptibility to plant extracts containing Icariin, Salidroside and resveratrol (Coenye *et al*., 2012). Essential oils are also identified to exhibit anti-biofilm action., An essential oil isolated from genus Pogostemon exhibited anti-biofilm activity against pharyngitis causing *Streptococcus pyogenes* (Group A Streptococcus) (Nithyanand *et.al*., 2015) and this essential oil is used as an novel natural resource to combat drug resistant pathogenic biofilms. Fukugiside isolated from *G. travancorica*, was identified to exhibit concentration-dependent biofilm inhibition against *Streptococcus pyogenes*. It was found that Fukugiside also exhibits anti-virulence activity against different M serotypes of *S. pyogenes* and also it is non-toxic, which augurs well for its clinical applicationI(Nandu *et.al.*, 2018).

Currently, Most of the preclinical studies use rodent models to study biofilm, 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.

2.6 Cell divion protein inhibition

Understanding cell division has led to the development of new antimicrobial targets because cell division is an essential process for the organism to survive (Ning *et al.,* 2017). Bacterial cell division possesses a complex set of biochemical machinery, which has many proteins as potential drug targets (Ning *et al*., 2017). Among these cell division proteins, filamentous temperature-sensitive protein (FtsZ) is identified to be a critical protein that can influence the cell division processes, and it is highly conserved in a wide verity of bacteria (Ning *et al*., 2017). FtsZ is a GTP-dependent bacterial cell division protein which is similar to tubulin, an eukaryotic cell division protein. FtsZ during cell division forms a dynamic ring structure at the center of the dividing cell which is called as Z-ring. These Z-rings divides the bacterial cell into daughter cells. Tubulin and FtsZ has 7% similarity and both has two β-sheeted domains, N and C terminals is connected by α-helix, the N-terminal consist of H1-H6 helices and S6-S7 strands. The N-terminal has a nucleotide binding site, and the C-terminal has H8-H10 helices, S7-S10 strands and a T7 loop. GTPase active site is at the nucleotide binding site of one FtsZ together with T7 loop of another FtsZ, the junction between two FtsZ monomers forms the active site. FtsZ Polymerizes and forms protofilaments upon GTP binding. The formed proto filaments aligns at the center of the dividing cells. Only 30% of the FtsZ forms Z-ring, the remaining monomers of FtsZ floats around the cytoplasm and they are been replaced by polymerized FtsZ at the protofilaments of the FtsZ ring.

Curcumin a naturally-occurring compound extracted from *Curcuma longa* inhibits FtsZ in a dose-dependent manner was found by using light scattering assay against *B. subtilis* 168 and 5 *E. coli* K12 MG1655. It was found that curcumin inhibited FtsZ by increasing the GTPase activity, hence destabilizing FtsZ polymers (Rai *et al*., 2008). Similarly, another compound called Coumarins which has a

benzopyrone core and are mostly known for their anticoagulant properties shows inhibition of FtsZ polymerization in a dose-dependent manner in *M. tuberculosis* H37Rv. Resveratrol, a bioactive compound which is a phytoalexin that is produced mainly in plants such as Grapevines, Legumes, and Pines as a defense mechanism in response to attacks by pathogens. Resveratrol has been shown to have antioxidant, antimicrobial, and antiproliferative activities and it was found that it affects the Z-ring formation in E. coli to a great extent thereby causing induced cell elongation in *E. coli*. Berberine an alkaloid has been isolated from various species of Berberis has been reported to target *E. coli* FtsZ (Ec-FtsZ) by inhibiting the FtsZ assembly and GTPase activity in a dose-dependent manner. Computational docking studies revealed that the berberine binding site overlapped with the GTP binding pocket in Ec-FtsZ (Domadia *et al.*, 2008). Another compound Berberine binds to the interdomain cleft of FtsZ in *S. aureus* and thereby inhibiting its growth (Sun *et al.,* 2014). Phenylpropanoids are secondary metabolites produced by plants to protect plants against predators and pathogens; Phenylpropanoids have been shown to possess antibiotic activity and inhibit FtsZ polymerization in *E. coli*. Bedaquiline Q203 is an imidazopyridine amide an optimized compound that has the ability to inhibit respiratory cytochrome complex present in mycobacteria, moreover this compound Bedaquiline is developed into a drug to treat multi-drug-resistant tuberculosis and is sold under the brand name Sirturo (Pethe *et al.*, 2013). One of the most accepted strategy for remediating antimicrobial attack is by interfering the function and natural dynamics of major cell division protein FtsZ and there by disturbing the cell division, leading to the death of the bacterial cell in a suicidal manner.

2.7 Need of the study

Antimicrobial resistance is turning out to be a global concern these days, and many of the bacterial pathogens have developed resistance against most antimicrobial

agents,. Resistance happens when microorganisms are over-exposed to antimicrobials continuously, as a result such agents become ineffective and the infections persist. Thus there is an urgent need to control such a situation.

Plants are a renewable and an attractive source of antimicrobial compounds that demonstrate therapeutic potential. These compounds can be validated as possible therapeutic candidates for clinical setting. The diversity of plants in nature offers a wide chemical diversity and this diversity, is seeking the attention of pharmaceutical companies to find newer options for antimicrobial resistance.

Some of these bioactive compounds inhibit the growth of a resistant organism by acting over a particular target site that helps in controlling or denying the growth of the resistant organism. These compounds either blocks key biochemical pathways or interfere with cellular structures leading to either growth inhibition or bacterial death by targeting specific characteristic of the organism like, virulence factor, transfer of mobile DNA , mating factors, antibiotic synthesis factors, interrupting riboswitches, terminating lipid biosynthesis, biofilm formation and cell division disruption.

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

The present study entitled **"*In vitro* studies on the anti-infective properties of leaf and root extracts of selected medicinal plants against gastro intestinal bacterial pathogens"**, was carried out at the Division of Microbiology, Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode, Thiruvananthapuram during the year 2018-2019. Details regarding the experimental materials used and procedures followed in the study is elaborated in this chapter.

3.1.1 Equipments

The equipments used for the conductance of the present study are listed down in **Table1.**

Table 1: Equipments used throughout the present study

Sl:no	Equipment	Make
1.	Hot air oven	Beston, India
2.	Cooling centrifuge (model:Z323K)	Hermle, Germany
3.	Fluorescent spectrophotometer (Spark 10M)	Tecan, Switzerland
4.	UV spectrophotometer (moel:V-730)	Jasco, USA
5.	Inverted microscope (model: Eclipse TS100)	Nikon, Japan
6.	Microscope digital camera (model: MU 300)	AmScope, USA
7.	GelDoc	UVP, USA

8.	Water bath (model: LWB 211D)	Lab Tech
9.	pH meter	Cyber Scan, Germany
10.	Milli-Q integral Water Purification System for Ultrapure Water (ZRXQ003WW)	Millipore, France
11.	Incubator shaker – Orbitek	Scigenics Biotech, India
12.	Incubator (model:Heraterm)	Termo Scientific, USA
13.	Mini-PROTEAN SDS-PAGE Electrophoresis	Bio-Rad Laboratories, USA
14.	Deep Freezer (-30°C)	Haier, Germany
15.	Deep Freezer (-40°C)	Haier, Germany
16.	Deep Freezer (-80°C)	Haier, Germany
17.	Grinding machine	Philips
18.	Magnetic stirrer (model: C-MAG HS 7)	IKA, Germany
19.	Digital camera (model: D5 100)	Nikon, Japan
18.	Rotta shaker	REMI RS-24BL
19.	Soxhlet apparatus	Borosil
20.	Heating mantel	Sunbim
21.	Rotavapor	Heidolph
22.	Hot air oven	KEMI
23.	Autoclave	Labline Instruments India
24.	Laminar air flow system (model: GS LAF 4X2)	Genesys incorporation, India
25.	Bunsen burner	Aman Scientific Works
26.	Analytical balance (model: BSA423S)	Sartorius, Germany
27.	Inoculating loop	Yashika solutions
28.	Pipettes	Gilson
29.	Sterile disc of diameter 6mm	HiMedia

30.	96-well microtitre plates	HiMedia
31.	Antibiotics disc of 6mm diameter	HiMedia
32.	Forceps	Yashika solutions
33.	Vortex mixer	GeNei, India
34.	Chromatography column	Borosil
35.	TLC silica gel sheets	Merck Millipore
36.	TLC chamber	Borosil
37.	Hot plate (model: HS7)	Iacmg

3.1.2 Plant materials collection

Leaves and roots of *Moringa oleifera, Bixa orellana, Persicaria glabra, Pimenta dioica*, and *Homonia riparia*, were collected from different parts of Kerala (**Table 2** and **Figure 1**). The specimens were prepared for herbarium at JNTBGRI. The samples were cleaned, shade dried, powdered, both hot and cold ethanol extracts were prepared using Soxhlet apparatus and Incubator shaker (orbitek) respectively.

3.1.2.1 : Sample description

3.1.2.1.1 *Moringa oleifera* Lam.

Moringa oleifera Lam. (Drumstick) is a medicinally important tress, it is tropical flowering plant family that has around thirteen diverse species (Shahzad et al., 2013), and it is indigenous to South Asia, parts of Himalayan foothills (Sharma et al., 2011). Moreover the plant is highly potential to serve as high-value food crop, fodder for animals, and medicinal products. Moringa has a wide range of bioremediation and culinary application, also well-known nutritional and medicinal properties. Traditionally moringa leaves are used in treating ailments like malaria, typhoid, arthritis, parasitic diseases, diabetes and diarrhea (Leone et al., 2015). But there are only less published works on these species because

of lacking sufficient scientific based information.

3.1.2.1.2 *Bixa orellana* L.

Bixa orellana L. is a small tree, American native and was first among American plant that was introduced into south Asia and later to Africa and became naturalized to these areas. The plant is indigenous to northwest coast of Mexico and Panama and it is been cultivated in warmer regions of Asia like Sri Lanka, India, and Java mostly for their dye. Bixa is famous for its healing properties and nutritional value, it is also traditionally employed for treating in digestive illness, such as diarrhea, abdominal pain, indigestion and dysentery. Several research groups have also been studying Bixa orellana anticancer and apoptotic properties (Coronado-Cáceres et al., 2014).

Table 2: Passport data of species selected for the study

Sl: no	Botanical name	Family	Common name	Place	District
1.	*Moringa oleifera*	Moringaceae	Moringa, Drumstick tree	Vellayani	Thiruvananthapuram
2.	*Bixa Orellana*	Bixaceae	Achiote	Vellayani	Thiruvananthapuram
3.	*Persicaria glabra*	Polygonaceae	Knotgrass	Rosemala	Kollam
4.	*Pimenta dioica*	Myrtaceae	Allspice	Tripunithura	Ernakulam
5.	*Homonia riparia*	Euphorbiaceae	Willow-leaved water croton	Rosemala	Kollam

3.1.2.1.3 *Persicaria glabra* (Willd.) M.Gomez

Persicaria glabra is a commonly known as prostrate knotweed belonging to family of polygonaceae, itis an effective ayurvedic shrub that is used in the treatment of many disorders. They are a natives of Louisiana, southeast United States and occurs on roadsides, wasted grounds and in soils that are deficient in lime and in urban areas, it occurs in less trampled. *Persicaria glabra* is been traditionally used as an expectorant for cough, bronchitis, also used as an astringent in piles, hemorrhages, and diarrhea, it's also used in expelling urinary tract stones and cures the inflammation of the urinary tract, moreover it is been also employed in improving the health of the blood vessels (plant ayurveda).

3.1.2.1.4 *Homonoia riparia* Lour

Homonoia riparia can be a gregarious, deciduous shrub, or a small, crooked and twisted tree, growing 1 - 4 metres tall and forming a woody, deep and extensive root system. The shrub provides a number of popular local medicines, which are gathered from wild plants. It is planted in Java and Sumatra in order to protect riverbanks and prevent soil erosion, more over the plant is been is claimed to be active against various ailments like constipation, emesis, piles, bladder stones, gonorrhoea, syphilis, toothache, angina, malaria, and wounds caused by scorpion and fish bites.

3.1.2.1.5 *Pimento dioica* (L.) Merr.

Pimento dioica is commonly known as allspice, it is an aromatic, evergreen, profusely-branched tree that usually grows 7 - 10 metres tall but occasionally reaches 20 metres. The tree was used as a spice in the Caribbean before the arrival of the Europeans, besides it has become a popular spice in many areas of the world and is now often cultivated in the American tropics, especially in Jamaica. Allspice berries contain about 4% essential oils, of which 80% is eugenol, proteins, lipids,

the vitamins A, B1, B2 and C, plus minerals are also been identified. The plant is also been employed in improving the digestion, and it is locally antiseptic and anesthetic. It is used internally in the treatment of indigestion, wind and diarrhea.

Figure 1: Samples collected for the present study

Moringa oleifera

Bixa orellana

Persicaria glabra

Pimenta dioica

Homonia riparia

3.1.3 Source of microbial strains

The microbial strains used in the present study (**Table 3**), *Vibrio cholera* 3906, *Enterococcus faecalis* 439, *Salmonella typhi 733*, *Staphylococcus aureus* 3160 and *Escherichia coli* 443 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 strains mentioned above were prepared and stored at −80°C. The microbial strains were pre-cultured for 24hr to achieve exponential phase of growth.

Table 3: List of microbial strains used in the present study

Sl.no:	Microbial strain	MTCC No.
1.	*Vibrio cholera*	3906
2.	*Escherichia coli*	443
3.	*Staphylococcus aureus*	3160
4.	*Enterococcus faecalis*	439
5.	*Salmonella typhi*	733

3.2METHODS

3.2.1 Plant extract preparation

The method employed for preparing the plant crude ethanol extracts were described by Azwanida, 2015. The leaves and roots of the selected plats were harvested, cleaned, and shade-dried for a month and were grounded into fine powder. Ethanol was used as the solvent for extracting the plant crude extracts. Both hot and cold ethanol extracts of the leaves and roots of the selected plats were prepared (Azwanida, 2015).

3.2.1.1 Hot extraction using Soxhlet apparatus

For preparing crude hot extract Soxhlet apparatus method was used, 5g of the fine powdered plant part were weighed out into an extraction thimble made out of filter paper, this thimble was placed into the Soxhlet extractor chamber and 150mL ethanol, the extraction solvent was taken in round bottom flask. Isomantle the heat source, heats the solvent in round bottom flask get heated up, the solvent begins to vaporize and moves through the apparatus into the condenser. The condensed solvent drips into the thimble and gets in contact with the powdered plant sample in it. As the solvent level races in the extraction chamber the solvent reaches the siphon and it is poured back into the round bottom flask through siphon. This extraction process continues for 24hr and the hot crude extract is collected from the round bottom flask. The collated hot crude extract was then condensed by removing the solvent, employing rotary evaporator at room temperature (Edewor *et al.*,2013).

3.2.1.2 Cold extraction method

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 (Roopali *et al.,* 2018). The cold crude extract collected was concentrated by using rotary evaporator at 78.4ºC for 9hr (Edewor *et al.*,2013).

3.2.2 Preparation of bacterial culture

The fresh bacterial cultures of *Vibrio cholera* 3906 and *Escherichia coli* 443, were prepared by sub-culturing the stock culture into freshly prepared LB broth (5mL in each test tube) and incubated at 37ºC for 24hr. 1mL of the incubated culture was transferred into sterile test tubes with 4mL of freshly prepared LB broth and they were standardized to 0.5 Mc Farland turbidity standards

(AppendixIII) by observing absorbance at 600nm, to obtain a cell density of 1.5 $\times 10_8$ cells/mL.

3.2.3 Antimicrobial activity of the crude extract

The methodology used in this study for testing the antimicrobial activity of both hot and cold crude ethanol extract is by well diffusion method, minimal inhibitory concentration assay (MIC), minimal bacterial concentration assay (MBC) and bacterial survival assay. These method was repeated three times, each time the results were recorded and were then statistically analyzed (Matuschek *et al.*,2014).

3.2.3.1 Well diffusion method

The Well Diffusion method is widely used for evaluating the antimicrobial activity of plant extracts, this method used for checking the antimicrobial susceptibility was employed on the basis of the standard method suggested by Perez *et al.,* (1990). The overnight bacterial cultures of *E. coli*, *Enterococcus faecalis, Salmonella typhi*, *Staphylococcus aureus* and *V. cholera*, after been adjusted to 0.5 Mc Farland standard were swabbed evenly over entire MH agar plate using sterile cotton swab. The plates were allowed to dry for 15 min and then used for conducting the assay. Each plate comprises six wells of 6mm diameter, these wells where made by using cork borer, four wells where loaded with 1mg/mL of the concentrated crude ethanol plant extract (both hot and cold extracts of root and leaf), the crude extracts where dissolved in 1% DMSO. Two among the sex wells was loaded with antibiotics ampicillin as positive control and DMSO as negative control respectively (Zaidan *et al*., 2005). The agar plates were inverted, placed in for incubation at 35ºC and allowed to grow for approximately 16-24hr (Matuschek et al. 2014). The growth plates were evaluated after incubation. The zones of inhibition on MH agar plates were read from the back of the plates, against a dark background, a pair of Callipers was

used to make the measurements of zone, the measurements were recorded and analysed.

3.2.3.2 Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) determination.

The MIC and MBC of the crude extracts against *E. coli, Enterococcus faecalis, Salmonella typhi, Staphylococcus aureus* and *V. cholerea* were determined by the broth microdilution method, which was previously described by Eloff (1998) and was modified by Tong *et al* in the year 2010. The lowest concentration of the crude extract where there was a complete inhibition of bacteria was considered as MIC. 96-Well microtitre plates were used to determine MIC. Onto each well in the plate 196µL of LB broth was added. Then 2µL of plant extract (32,64,128,256,512µg/mL) by keeping the concentration of DMSO as 1%, and 2µL of the diluted overnight culture were also added into the microtiter plate. Microtiter plate wells with 196µL LB broth, 2µL plant extract and 2µL microbial culture was considered as test and 1% DMSO in 196µL LB broth was considered as control. The microtiter plate was incubated for 16-18hr at 37ºC. After incubation period, absorbance was taken at 600nm (Guerin-Faublee et al., 1996) with Spectrophotometer. Strong activity was the lowest concentration that inhibit the growth of bacteria were noted and considered as the MIC value for each of the bacteria strain used in present study (Bussmann *et al.,* 2010).

Then briefly the 10µL of suspension of determined MIC was taken and spread onto a MH agar plate and incubated for 37°C for 24hr and the lowest concentration of that produced bacterial growth not more than five colonies on each plate was considered as MBC (Kang et al., 2011).

3.2.3.3 Bacterial survival assay

Overnight culture of *V. cholera* 3906 and *E. coli* 443 were diluted with LB approximately to standardized to 0.5 Mc Farland turbidity standards

(AppendixIII) by observing absorbance at 600nm, to obtain a cell density of 1.5 × 10_8 cells/mL in a volume of LB broth with various concentration of the crude extract, 1 and 2 times the MIC and was incubated for 37°C with shaking for 16hr. the viable count was monitored up to 10hr. the aliquots where taken at appropriate intervals, they were diluted in 10mM phosphate buffer and 100µL of the aliquot was plated onto MH agar plates and incubated at 37°C for 16-18hr and colony forming units (CFU) were counted after incubation.

3.2.4 Identification of potential fraction and bioactive molecule from the crude extract

The potential faction and the bioactive molecule from the ethanolic crude extract were identified by employing techniques like thin-layer chromatography, column chromatography and high-performance liquid chromatography.

3.2.4.1 Thin-layer chromatography

TLC is based on adsorption chromatography in which the separation is based on the selective adsorption of the components of a mixture on the surface of solid, traditionally analytical TLC is applicable for the detection and monitoring of compound through the separation process. Merck Millipore TLC silica gel 60, F254, 25 aluminium sheets of 20×20cm were used as the stationary phase in this study. 20µL of the crude extract completely dissolved in ethanol (1mg/mL) were spoted onto the TLC sheet, 1cm above the base line, by using a capillary spotter tube, capillary spotter is prepared by holding the capillary tube from both the ends in the blue flames of Bunsen burner and when the capillary is softly melted is taken off from burner and is polled in appositive direction to form micro thin tipped capillary spotter for sampling and the spot was allowed to dry in room temperature for 30min (Bipin *et al.*, 2014).

In order to produce maximum separation for the crude extract, immense journals and literature were searched, different solvent system were prepared in appropriate ratio depending upon the polarity of compounds in solvents **(Table**

4). 30mL of the prepared solvent was poured into a TLC chamber and were kept for saturation, in closed condition for 45min. After the saturation of solvent in the TLC chamber, the TLC sheet loaded with the crude extract (20µL) were introduced into the TLC chamber. The chamber is kept undisturbed till the solvent front reaches the maximum end of TLC plate this means sample is fully run on TLC (Bipin *et al.,* 2014). The developed chromatogram provided information about number of compounds in the crude mixture and TLC bands were visualized by using short-wavelength UV (254nm). The Retardation factor (R_f) was calculated using the formula,

R_f = Distance moved by the solute/Distance moved by the solvent

The R_f value determines the characteristics of the substance which indicates its movement relative to the solvent front in the chromatographic system. And it depends on number of variables like the particle size of different batches of adsorbent, solvent composition, degree of saturation of the solvent, thickness of adsorbent layer and storage condition of the crude extract.

3.2.3.2 Column chromatography

In order to isolate the potential fraction containing bioactive molecule from the crude extracts column chromatography technique standardized by Jayaprakasha *et al.,* 1998 was used. The packed column is considered as stationary phase, through which the mobile phase is allowed to flow by gravity.

The column assembly was set for separating the compounds from the ethanolic extract. A column of 60cm 18mm was cleaned by rinsing with chloroform, dried, and aligned in vertical position, slurry method was adopted for filling the column. The slurry of silica gel of mesh size 100-200 was prepared (oxalic acid impregnated) in chloroform and were packed into the column (Figure 2). The absorbent was allowed to settle evenly and by gentle tapping of the column with rubber rod the column was made air bubbles free. In order to prevent disturbance of the surface during the subsequent loading, the top of column is covered with

an aluminium foil. The 500g of semisolid ethanolic extract was mixed with 100-200 mesh sized silica gel using chloroform and made into a slurry, which is the eluent. The eluent was poured into the packed column through a funnel. The isolation and elution of phytoconstituents of the crude was done by gradient elution, using Chloroform, Ethyl acetate, Methanol as solvent system from a ratio of 50:50, 40:60, 30:70, 20:80, 10:90, 0:100. For each solvent system 10mL fraction were collected and they were combined on the basis of TLC profiling. The TLC profiling was conducted at the same conditions in 3.2.3.1 and the combined fractions with same Rf on TLC conducted in 3.2.3.1 were pooled out.

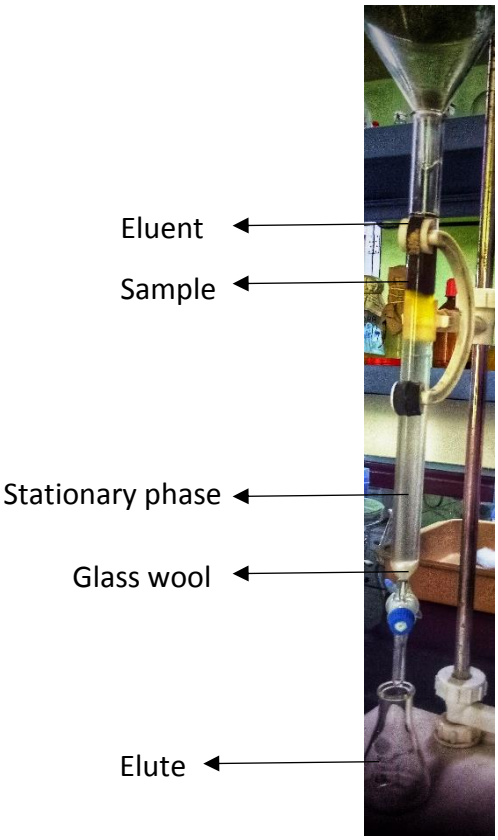
Table 4: Composition of different solvent system used in present study

Sl.No	Solvent	Ratio
1	Acetone : Water : Ammonia	9:0.7:0.3
2	Ethyl acetate : Toluene : Fomic acid	5:5:2
3	Toluene : Ethyl acetate: Formic acid	5:5:2
4	Chloroform : Acetone : Methanol	5:3:2
5	Butanol : Ethanol : Water	4:1:5

3.2.3.3 High-performance liquid chromatography

HPLC is a versatile and widely employed analytical technique for the isolation of natural products. The natural products are been mostly isolated by evaluating the crude extract, but the bioactive entity is often present as a minor component in the extract. The resolving power of HPLC very much suited for processing of such multicomponent samples on both preparative and analytical scales. The separation of compounds is accomplished by using HPLC is by utilizing the basic fact that compounds has different rates of migration over different mobile phases.

Figure 2: Packed column chromatography immediately before elution (0min)



The TLC scraped outs 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.

3.2.4 *In-silico* screening of the identified molecules

For demonstrating the mode of action of the plant crude extract, the identified molecule was screened in-silico by docking of molecule structure with homology modelled FtsZ protein. Autodock Vina 1.1.2 was used to dock the 3D coordinates of the identified compound into *E. coli* FtsZ protein model. Swiss model server was used for Homology model, using X-ray crystal structure of FtsZ (PDB ID: 2VW, 2.9 Å). The quality of the homology model was analyzed with Ramachandran plot by using Dock Prep tool of UCSF Chimera the ligand and receptors for docking was prepared, to them AM1-BCC and Gasteiger charges were added respectively. Then grid maps were constructed with appropriate grid spacing and Vina Docking exhaustiveness was used. The best conformation between the receptor and ligand was selected, based on the highest binding affinity.

3.2.4.1 Homology modeling

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. aeruginoa (*PDB ID:2VAW; Res. 2.9 Å) as a template protein.

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.

3.2.4.2 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.

3.2.5 Functional characterization of FtsZ protein inhibitor

Filamenting temperature-sensitive protein Z (FtsZ) is prokaryotic cell division protein, which is GTP-dependent bacterial cell division protein similar to tubulin present in eukaryotic cell. FtsZ during cell division forms a dynamic ring structure at the center of the cell known as Z-ring these rings intern divided the bacterial cell into daughter cells. In this action of FtsZ the Z-ring formation during the cell division is been employed as a target in order to control the growth of organism that shows resistance to antimicrobial agents, that is the inhibition of the FtsZ protein prevents proper formation of the divisome, which leads to filamentation and eventual cell death of the organism (Haranahalli et.al., 2016).

For characterizing the FtsZ inhibitor, fist the FtsZ protein has to be isolated and purified, then the Functional characterization of FtsZ inhibitors in the crude extract is confirmed by experimental assays like light scattering assay, cell elongation assay and malachite green assay (GTP hydrolysis assay).

3.2.5.1 Isolation and purification of FtsZ protein

E. coli, DH5α cells culture harboring FtsZ protein expressing plasmids and transformed into C41 (*E.coli*) strain.

3.2.5.1.1 Plasmid isolation by Alkaline lysis method

E.coli, DH5α cells culture harboring FtsZ protein cording plasmids "PCXZ", were grown in 3mL Lauria broth (LB) and incubated over night at 37°C for growth. 1.5 mL of the culture was poured into a microfuge tube. The cells where harvested by centrifuging at 12000 rpm at 4°C for two min, the supernatant was carefully removed with a fine-tip pipette and the pellet (cells) was completely resuspended in 200µL of ice-cold Alkaline lysis solution I with vigorous vortexing and made sure that the bacterial pellet was completely dispersedin Alkaline lysis solution I, then this bacterial suspension 400µL of freshly prepared Alkaline lysis solution II was added and the tube was tightly closed, mixed the contents by inverting the tube rapidly five times. Then 300µL of ice-cold alkaline lysis solution III was added. Closed the tube and disperse Alkaline lysis solution III through the viscous bacterial lysate by inverting the tube several times and the tubes were stored in ice for 3 to 5 min. Then the bacterial lysate was centrifuged at 12000rpm at 4°C for 5 min, the supernatant was transferred into a fresh tube and to it equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) mixture was transferred into the supernatant and the separated organic and aqueous phase was mixed by vortexing for 2 min, then was centrifuged at 12000rpm, 2min, 4ºC. Then an equal volume of 100% ethanol was added to the supernatant and contents were mixed by inverting the tube for 6-8 times, then the mix was centrifuged at a speed of 14000rpm for 30min at 25ºC. Then the upper aqueous layer was separated completely using a pipette and the pellet was mixed with 500µL of 70% ethanol and mixed with inversion for several times, then centrifuged at 14000rpm for 5min at 25ºC and the supernatant was completely pipette out, and then the pellet was dissolved in 20µL TE buffer of pH 8 and were stored in -40ºC (Sambrook and Russell,2001 and Bimboim et al., 1979).

3.2.5.1.2 Recovery of plasmid DNA

For recovering the plasmid from the pellet, 2 volume of ethanol was added at room temperature to the tube containing aqueous phase, ethanol will precipitate nucleic acid from the suspension. The tube was mixed with vortexing and allow the mix to stand for 2min at room temperature. The precipitated nucleic acid was collected by centrifugation at 12000rpm 5 min 4ºC in microfuge. The supernatant was gentle aspirated as mentioned above, the tube was placed inverted over a paper towel to allow the fluid to drain away. Then the pellet was resuspented in 1ml of 70% ethanol and the tube was gently inverted for mixing. Then the tube was centrifuged for 2 min at 12000rpm for recovering the DNA. Agene the supernatant was aspirated as mentioned above. Then the ethanol was completely removed from the tube by keeping the tube open at room temperature until the ethanol has evaporated and no flude is visible in the tube (5-10 min) and then dissolved the nucleic acid in 50µL of TE buffer of pH 8 containing 20µg/Ml RNase A (pancreatic RNase), gently vortexes the solution for few seconds and stored the DNA solution in -20°C (Sambrook and Russell., 2001). To conform plasmid is been isolated, the recovered solution was examined in gel electrophoresis (polyacrylamide).

3.2.5.1.3 Preparation of competent E.coli cell (Calcium Chloride treatment cell)

E.coli C41 cells were plated and incubated for 16-20hr at 37°C, from those plates a single bacterial colony was picked and transformed into 100mL LB. The culture was incubated for 3hr at 37°C with vigorous agitation. As the guideline, (at 600nm OD of the culture contains over 109 bacterial cells in it) after incubation the bacterial cells were transferred sterile polypropylene tube and the tube was stored in ice for 10min. The bacterial cells were recovered by centrifuging at 4°C, 4000rpm for 10min. After centrifugation the medium was decanted from the cell pellet, and tubes were incubated in ice for 30 min. Then the pellet was resuspended by gentle vortexing in 0.1M $CaCl_2$ and a gene was incubated in ice for 30 min. Then the cells were recovered by centrifuging at 4000rpm, 10min,

4°C. The supernatant was discarded and the pellet was observed in horse shoe shape. Then the cells were resuspented in 2mL ice-cold $CaCl_2$ solution with gentle vortexing (Sambrook and Russell., 2001).

3.2.5.1.4 Transformation of FtsZ protein in *E. coli* by Heat shock

For transforming the $CaCl_2$ treated cells, 200µL of the competent cell were transferred into sterile, chilled polypropylene tube (17×100 mm). Using a chilled tip 3µL of the isolated plasmid DNA (PCXZ) was added to the transformed cells, mix the tube gentle swirling and was incubated for 30 min in ice. Then the tube was transferred into preheated water bath at 42°C for 90 sec (heat shock step). Then the tubes where rapidly transferred into ice, allowed the cells to chill for 1-2 min. Then 800µL of LB was added gentle through the sides of the tube and incubated for 1hr in shaking incubator. Then the bacterial cells were allowed to grow on SOC medium plates with 200µL of ampicillin, for selecting the transformed bacterial cells. And incubated for 12-16 hours at 37°C (the transformed bacterial cells will grow) (Sambrook and Russell., 2001).

3.2.5.1.5 Expression of FtsZ protein in *E. coli* by IPTG induction method

From the SOC plates with appropriate antibiotics, were the transformed bacterial cells are grown, select 2 colonies and inoculate into 6mL LB containing ampicillin, incubated in shaker apparatus for 12-16hr at room temperature. After incubation 1% of the culture was transferred into a fresh sterile conical flask containing LB with ampicillin and incubated for 1hr in shaker incubator. After incubation 100µL of 1mM IPTG was added to the flask and was incubated for 4hr in shaker apparatus. After incubation culture was transferred into polypropylene tube (17×100 mm) and were centrifuged at 8000rpm for 10min. Then the supernatant was discarded carefully and the pellet was washed in 5mLtris-buffer (two times washed) and centrifuged at 8000 rpm for 10 min. The pellet was transferred into fresh tubes and were stored in -80°C (for storage). For recovering the cells from storage, the cells were thawed in ice and 5mL of tris-

buffer was added to it with vortexing. Then to it 100µL of lysozyme was added and kept in ice for 30min. After incubation cells were sonicated for 5sec, and then centrifuged at 14000rpm for 30min. The supernatant was aspirated out carefully by using a pippet and stored in -80°C. (Sambrook and Russell., 2001).

3.2.5.1.6 FtsZ protein purification by ammonium sulfate precipitation method

The stored supernatant (after expression) was removed using centrifuge at 14000 rpm for 30 min. A 30% ammonium sulfate (16.6g/100mL of extract) was then slowly added to the supernatant with stirring of 20min and kept for 1hr. 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 stored at -800C (Anderson and Heyne.,2012).

3.2.5.2 Light scattering assay

Light scattering assay is done in order to understand the effect of the compound on polymerization of FtsZ, thus the assay is also known as polymerization assay. FtsZ protein polymerization is monitored with microtiter plate based light scattering assay in which changes in light scattering are reflected by the corresponding change in OD at 340nm. The test components at different concentration are added to the assay buffer, assay buffer contains 50mM tris-HCl, 2M KCl, 2M MgCl, 5M CaCl2 and the compound in 30µg/mL and 60µg/mL respectively, the mix is centrifuged at 14500rpm at 20min before adding the protein in order to prevent non-specific binding due to compound aggregation, 10µL of FtsZ protein (*B. subtilis*) was added to the mix, control tube were received 2% DMSO alone. 95µL of it was transferred into microtiter plates,

incubated for 10min for the base line establishment. 4µL of 2mM GTP was also added to each well and incubated for 1 hour for baseline establishment, the polymerization was continuously monitored at 30°C by measuring OD at 340nm in a tecan spark 10M plate reader for different intervals till 2000sec (Kaul et al., 2012).

3.2.5.3 Cell elongation assay

Phase contrast microscopy was done to check the morphological change of the bacterial cells when exposed to the plant compound, cell elongation assay was performed on *Bacillus subtilis* 168. *Bacillus subtilis* 168 culture was inoculated in 5mL LB and incubated for 12-16hr in shaker apparatus at 37ºC. After incubation period 200µL of the culture was transferred in a sterile 100ml conical flask with 10mL LB and incubated in a shaker for 2hr at 37°C. After incubation, 100µL of plant compound was added and DMSO were added to 200µL of culture and maintained as control, incubated for 4hr at 37°C respectively. 20µL culture samples were transferred to agar (0.2%) coated slides for microscopy. Cell morphology was assessed by using phase-contrast light microscopy. The images of bacterial cells was measured using Amscope imaging software.

3.2.5.4 Malachite green assay

The malachite green phosphomolybdate assay was performed according to the modified protocol of Anderson and Heyne., 2012. For assaying the dose-dependent inhibition of FtsZ, the assay buffer (50mM Tris-HCL pH-7.4, 2.5mM MgCl2, 5mM KCL) were prepared and to 196µL of the assay buffer, 4µL of the compound inhibitor was added and mixed for 20min, the mix was centrifuged for 20min at 14500 rpm. To it 2µL of the FtsZ protein isolated from *B. subtilis* was added and made the final volume to 198µL, the reaction mix was incubated for 5min at 30°C. Then 2µL (final volume 200µL) of 1mM GTP was added in order to initiate the GTP hydrolysis, and incubated for 20min at 30°C. 600µL of malachite green working reagent was freshly prepared (0.8mg/mL of malachite

green, 23.2mg/mL of polyvinyl alcohol, 57.2 mg/mL ammonium molybdate), of with 20µL was transferred into each tube with reaction mix and tubes were incubated for 30min at room temperature, then 200µL from each tube was transferred in a microtiter plate and absorbance was read at 630nm (Anderson and Heyne., 2012).

3.2.6 Demonstration of the effect of plant compound on virulence factor and biofilm formation

To demonstrate the effect of plant compounds on virulence factor and biofilm formation was assayed by cell surface hydrophobicity test, protease assay and exopolysaccharide estimation assay.

3.2.5.1 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 microdilution method on 24-well polystyrene plates with 1mL LB broth. To the LB broth 5µL compound (concentrations: 2, 4, 6, 8, 10), 10µL of the selected organism were added. As blank the LB was added in separate well and as control 1mL LB and 10µL organism was added. Then the plate was incubated for 12-16hr. After incubation the rate of planktonic cells formed was read at an absorbance of 650nm, and then the planktonic cells were gently removed from the microplate wells and the wells were washed twice with distilled water after drying 0.2% 1mL crystal violet was added to each well and incubated for 10min at room temperature. Then the plates were washed three times with distilled water to remove the crystal violet stain. Then after complete

drying the plates 20% acetic acid (1mL) was added to each well and the plates where read at an absorbance of 650nm (Nandu et al.,2018).

3.2.5.2 Cell surface hydrophobicity test

The microbial cells adhesion assay was conducted to estimate the effect of the bioactive compound over the cell surface hydrophobicity of the organism. The test organism was grown in the presence and absence of the bioactive compound at its MBIC, for 24hr at 37C in THYG broth, after incubation the culture was diluted to an OD 0.4 at 600nm. The diluted culture were separated 1mL each into new test tubes and 1mL of toluene was added. The tubes were vortexed and allowed stand for the phase separation at room temperature. The cells in aqueous phase were quantified by taking OD at 600nm, and the % of hydrophobicity was calculated, using formula:

% of hydrophobicity = [OD at 600nm after vortexing ÷ OD at 600nm before vortexing]×100

3.2.5.3 Protease content estimation

For estimating the amount of protease secreted by *V. cholerae* in the liquid culture was assessed by estimating the protease content, the organism free culture supernatant were grown in the presence and absence of the bioactive compound at its MBIC concentration and incubated at 37°C for 24hr. After the incubation period the supernatant were collected, to it 200µL of mixed activation buffer was added and incubated for 30min at 4°C, after incubation 400µL of azocasein (2%) and incubated at 37°C for 1hr, in order to terminate the reaction 600µL of trichloroacetic acid (10%) and further incubated for 20min at -20°C. The culture tubes were then centrifuge for 5min at 3000rpm, then 600µL of the supernatant was transferred into a fresh tube and to it 700µL

of NaOH (1M) was added and its absorbance was noted at 440nm (Viszwapriya *et. al*., 2016).

3.2.5.4 Exopolysaccharide content estimation

The influence of the plant compound on the extracellular polymeric substance production is determined by estimating the total carbohydrate surrounding the bacterial cells. The bacterial culture were grown in presence and absence of the compound at a concentration of MBIC, for 24hr 37°C, and centrifuged at 10,000rpm for 10min, the pellets were washed with Phosphate Buffered saline and resuspemdedin 200L of the same. An equal voloume of 5%phenl and 5 volumes of concentrated sulfuric acid containing 0.2% hydrazine sulfate were added, mixed and incubated in dark for 1 hr at room temperature and were centrifuged at 10000rpm for 10min, then absorbance of supernatants were measured at 490nm (Viszapriya *et al.*, 2016).

CHAPTER 4

RESULTS

The present study entitled **"*In vitro* studies on the anti-infective properties of leaf and root extracts of selected medicinal plants against gastro intestinal bacterial pathogens"**, was carried out at the Division of Microbiology, Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode, Thiruvananthapuram during the year 2018-2019. The selected 5 plants were screened for the anti-infective activity against gastro intestinal bacterial pathogens like *V. cholera*, *E. coli*, *E. faecalis, Salmonella typhi, S. aureus* the mode of action of leaf and root extracts targeting on FtsZ protein was demonstrated through *in-vitro* and *in-silico* analysis, and also the lead compound was assessment for its action over virulence factor and biofilm formation.

4.1 Total yield of plant crude extract

The leaf and root of collected plant samples, *Moringa oleifera, Bixa orellana, Persicaria glabra, Pimenta dioica,* and *Homonia riparia,* shade dried and hot and cold ethanolic extracts where obtained. The final yield of ethanolic extracts were calculated and listed in **Table5**.

4.2 Antimicrobial activity of crude extracts

Well diffusion method, Minimal inhibitory concentration assay (MIC), Minimal bacterial concentration assay (MBC) and Bacterial survival assay were employed to scrutinize and screen the crude extract for its antimicrobial activity against *E.*

coli and *V. Cholerae*. The crude extracts exhibiting antimicrobial activity were selected for the following experiments conducted in the present study.

4.2.1 Measuring the inhibition zone diameter

The well diffusion method was employed for evaluating the antimicrobial activity of plant extracts against *V. cholerae* and *E. coli*, zones of inhibition on MH agar plates were read against a dark background, a pair of Calipers was used to make the measurements. The effect was calculated as mean of triplicate test and recorded (**Table 6, Figure 3 and 4)**. Among the leaf ethanolic extracts, the hot ethanolic leaf extract of *Persicaria glabra* and *Homonia riparia* showed zone of inhibition against *E. coli* and *V. cholerae*, whereas the hot ethanolic leaf extract of *Bixa orellana* exhibited activity for *E. coli* and its ethanolic cold leaf extract showed zone for *V. cholerae*, and the hot and cold ethanolic leaf extracts of *Moringa oleifera* and *Pimenta dioica* showed no zone of inhibition. On the other hand none among the root ethanolic extract of the five samples showed zone against *E. coli* and *V. cholerae.*

4.2.2 Determination of MIC and MBC of the crude ethanolic extracts against *V. cholera* and *E. coli.*

The best most promising MIC values where observed for *Persicaria glabra* and *Homonia riparia* hot ethanolic leaf extract, was found to be 128µg/µL against *E. coli*, 64µg/µL and 32µg/µL for *V. cholerae* respectively. To demonstrate MBC value the MIC inoculums were subcultured and promising MBC values were observed for *Persicaria glabra* and *Homonia ripari*, was found to be 256µg/µL against *E. coli* and was 128µg/µL and 64µg/µL against *E. coli* and *V. cholera* respectively (**Table7**).

4.3 Identification of potential fraction and bioactive molecule from the crude extract

The potential faction and the bioactive molecule from the crude extracts that exhibited higher antimicrobial activity were identified by employing techniques like thin-layer chromatography, column chromatography and high-performance liquid chromatography

4.3.1 Thin-layer chromatography

The samples that showed high antimicrobial activity, hot crude leaf extracts of *Persicaria glabra* and *Homonia riparia* were selected for TLC analysis. Among the different solvent system used, Toluene: Ethyl acetate: Formic acid in 5:5:2 ratio exhibited maximum separation of the crude extracts and it was optimized. TLC evaluation indicated the presence of plant compounds as different bands (**Figure 5**). The R_f values were calculated (**Table 8**) and the bands were then scraped out and its antimicrobial activity was observed a zone of inhibition against *E. coli* and *V. cholera* (**Figure 6**). The TLC fraction number 4 (F4) showed zone of inhibition against *E. coli* and *V. cholera*, fraction number (F5) is a fraction of hot leaf extract of *Persicaria glabra*. Whereas other fragments belonging to *Persicaria glabra* and *Homonia riparia* did not show zone of inhibition against *E. coli* and *V. cholera* **(Table 9).**

4.3.2 Column chromatography

The semisolid hot ethanolic leaf extract of *Persicaria glabra* was subjected to column chromatography. Chloroform, Ethyl acetate and Methanol in a ratio of 50:50, 40:60, 30:70, 20:80, 10:90, 0:100 by 100mL volume was employed to obtain the active fraction from the crude. 36 different fractions of 10mL were collected. The column fractions were monitored by TLC with Toluene: Ethyl acetate: Formic acid (5:5:2) as mobile phase and fractions with similar R_f values were combined together (**Figure 7**). The elute number 5, E5 (**Figure 8**) possessed a band with R_f value same as that of the active band identified from the hot ethanolic leaf extract of *Persicaria glabra* by using TLC profiling and TLC chromatogram bioautography. E5 fraction was subjected to recolumn with

Ethyl acetate: Acetonitrile in different ratio of 80:20, 60:40, 40:60, 20:80, 0:100 by 100mL volume. The factions obtained were monitored with TLC in Toluene: Ethyl acetate: Formic acid (5:5:2) as mobile phase (**Figure 9**) and observed that the fraction number E51 is providing a band with R_f value same to that of the active band identified by TLC profiling and TLC chromatogram bioautography of the hot ethanolic leaf extract of *Persicaria glabra.* E51 was suggested for HPLC to identify the active compound.

Table 5: Final yield of the leaf and root crude ethanolic extract of the selected sample

Plant sample	Plant part	Extract	Yield (mg)
Moringa oleifera	**Leaf**	**Hot**	**690**
		Cold	**740**
	Root	**Hot**	**682**
		Cold	**758**
Bixa Orellana	**Leaf**	**Hot**	**653**
		Cold	**730**
	Root	**Hot**	**680**
		Cold	**763**
Persicaria glabra	**Leaf**	**Hot**	**690**
		Cold	**775**
	Root	**Hot**	**673**
		Cold	**770**
Homonia riparia	**Leaf**	**Hot**	**676**
		Cold	**780**
	Root	**Hot**	**680**
		Cold	**774**
	Leaf	**Hot**	**650**

Pimenta dioica		**Cold**	**740**
	Root	**Hot**	**650**
		Cold	**783**

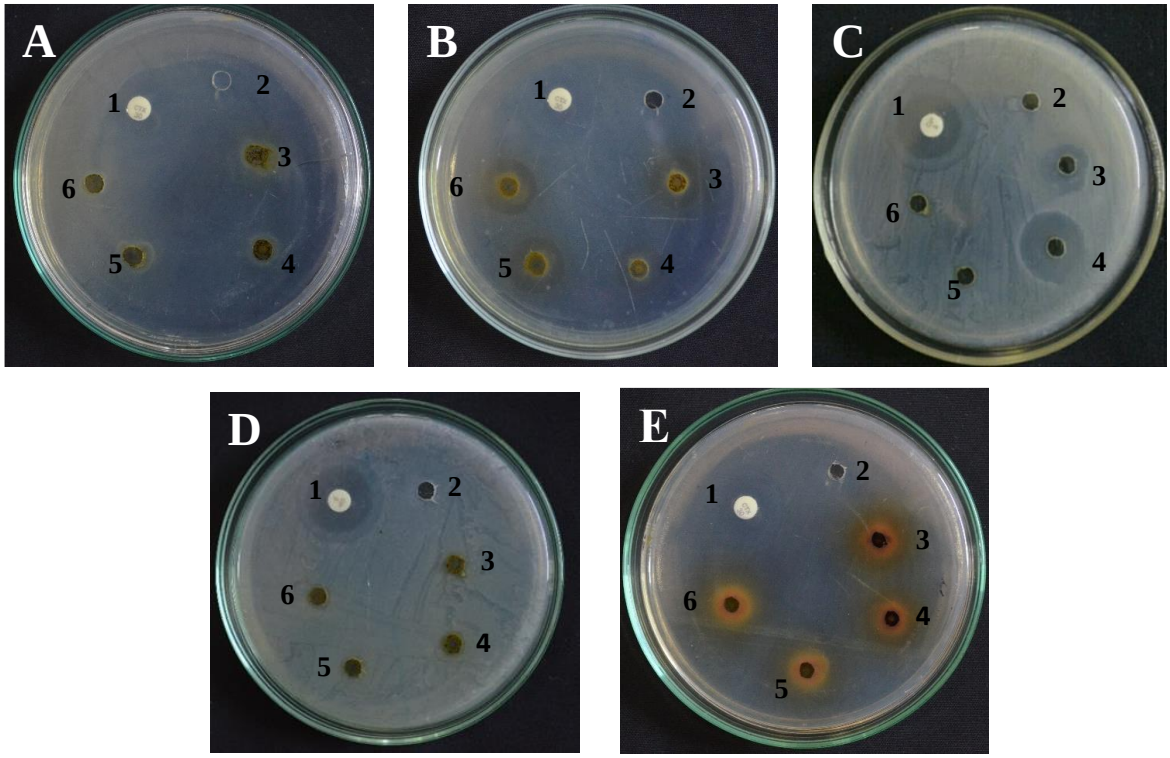


Figure 3 : Antibacterial activity of the plant crude ethanol extracts against *E. coli*

1. Positive control, 2. Negative control, 3. Hot leaf extract, 4. Cold leaf extract, 5. Cold root extract, 6. Hot root extract. A: *Moringa oleifera*, B: *Bixa orellana*, C: *Persicaria glabra*, D: *Pimenta dioica*, E: *Homonia riparia*

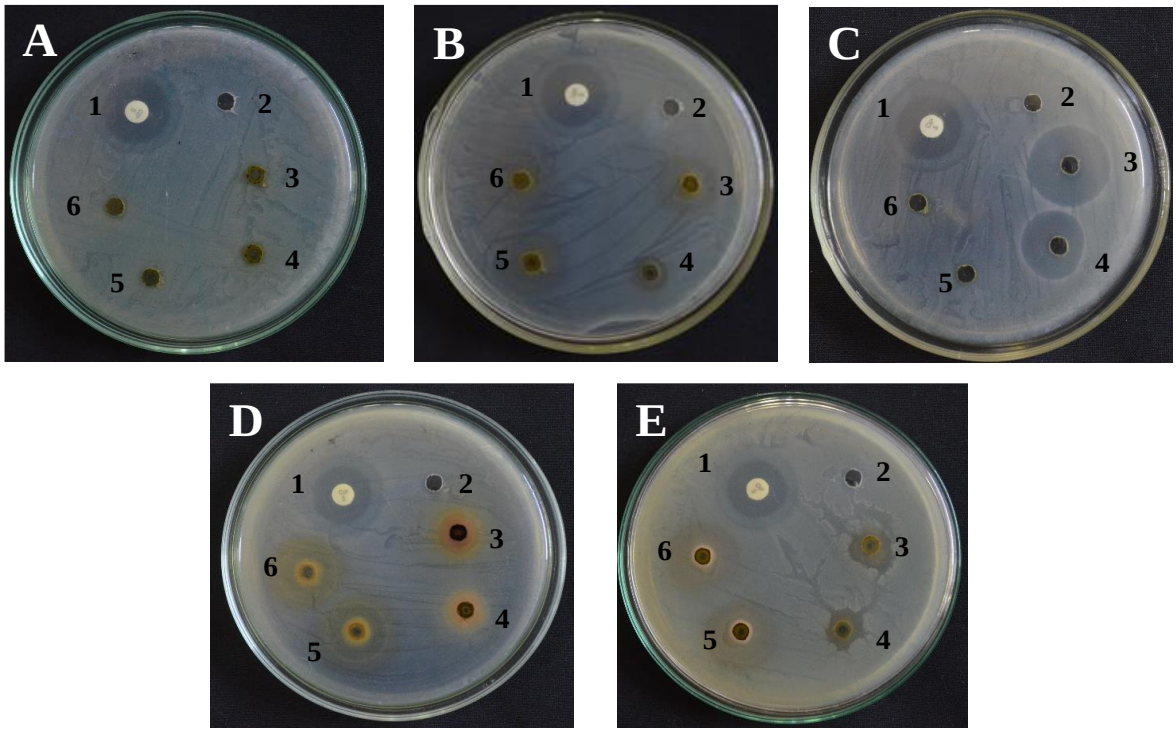


Figure 4: Antibacterial activity of the plant crude ethanol extracts against *V. cholera*

1. Positive control, 2. Negative control, 3. Hot leaf extract, 4. Cold leaf extract , 5. Cold root extract, 6. Hot root extract. A: *Moringa oleifera*, B: *Bixa Orellana*, C: *Persicaria glabra*, D: *Pimenta dioica*, E: *Homonia riparia*

Figure 5: TLC chromatograms of hot ethanolic leaf extract, A: *Homonia riparia*, with TLC fraction numbers TLC F1, TLCF2 B: *Persicaria glabra* with TLC faction number TLCF3, TLCF4, TLCF5

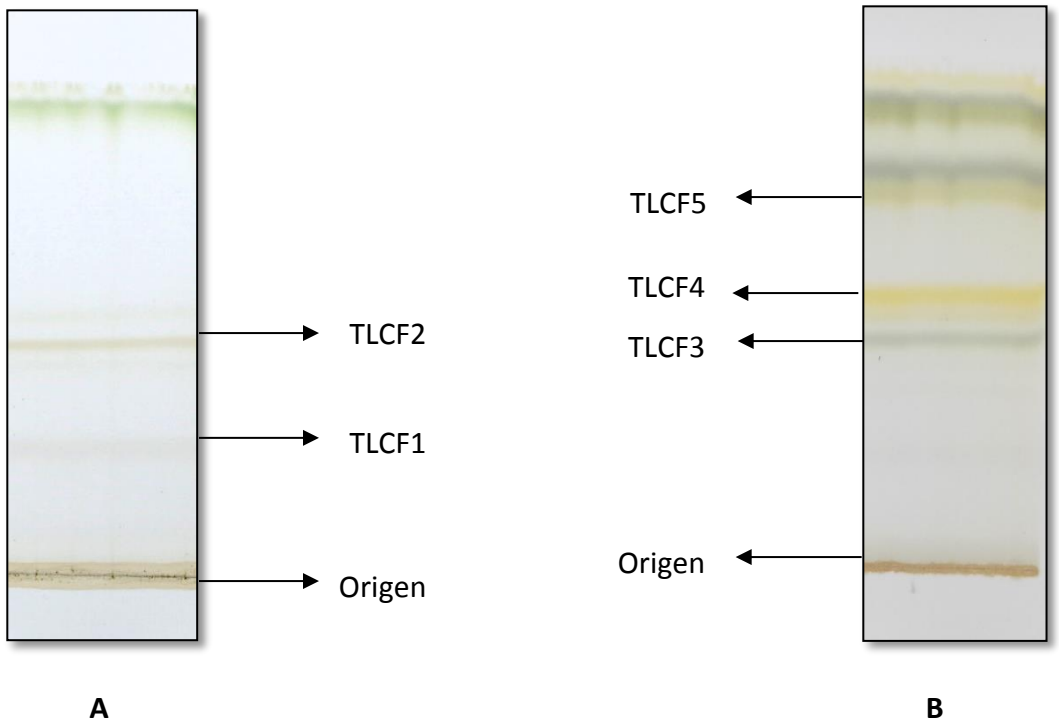


Table 8: R_f values of the separated fragments on TLC chromatograms

TLC Fraction	Distance moved by solute (cm)	Distance moved by solvent (cm)	R_f Value
F1	2	8.5	0.23
F2	3.5	8.5	0.41
F3	3.5	8.5	0.41
F4	4	8.5	0.47
F5	6.5	8.5	0.76

Table 9: Antibacterial effect of TLC fractions of *Persicaria glabra* and *Homonia riparia* against *E. coli* and *V. cholerae*.

TLC fraction number	R_f of the fraction	Diameter of inhibition zone (mm) for *E. coli*	Diameter of inhibition zone (mm) for *V. cholera*
F1	0.23	0	0
F2	0.41	0	0
F3	0.41	0	0
F4	0.47	8±1	10.6±1.3
F5	0.76	0	0

Figure 6: Antimicrobial activity of fraction TLCF4 from TLC chromatogram of *Persicaria glabra.* **A.** *V. cholerae* , **B.** *E. coli,* **1.** Positive control, **2.** Negative control, **3.** TLC band F4

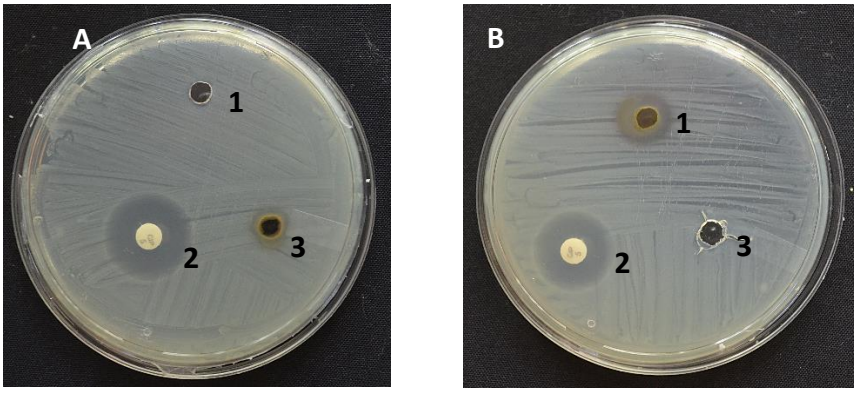


Figure 7: Procedure for identification of active fraction, Eluent number- E 1to 6, Fraction number-F 1 to 36, Sub fraction number- F 37 to 72

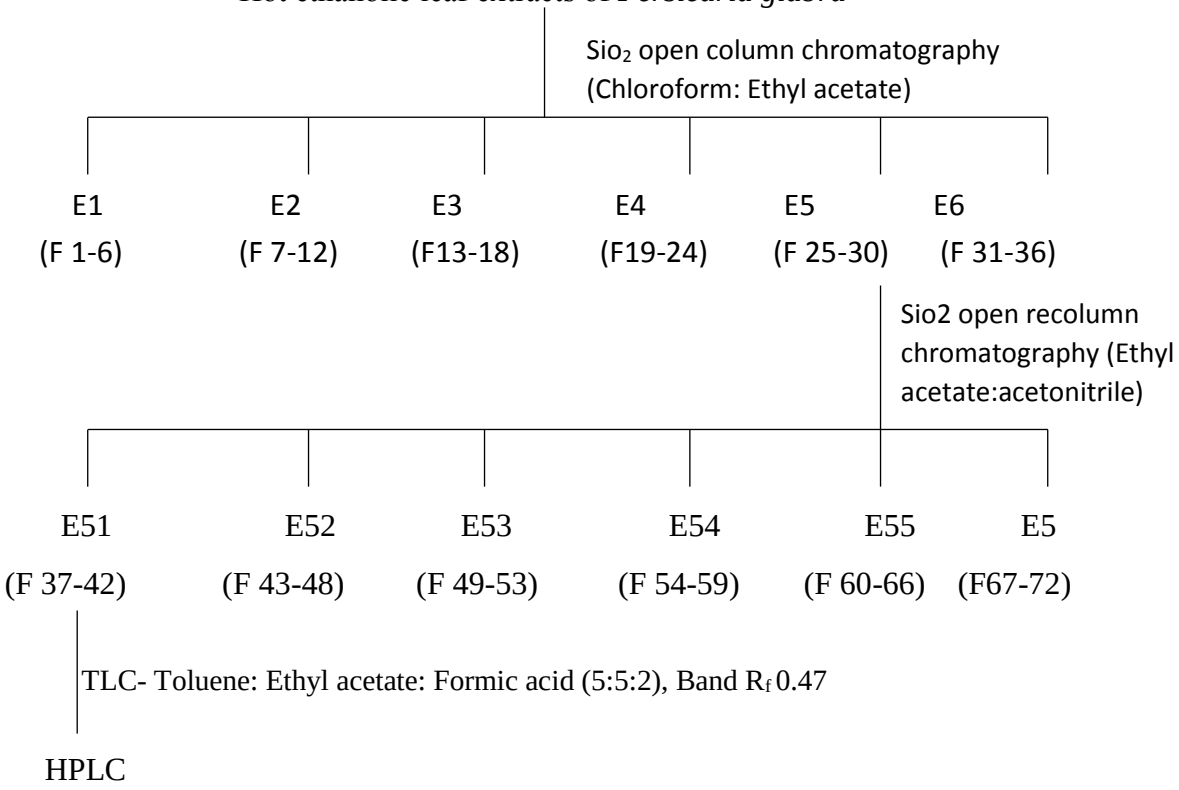


Figure 8: TLC of E5, solvent system Toluene: Ethyl acetate: Formic acid (5:5:2)

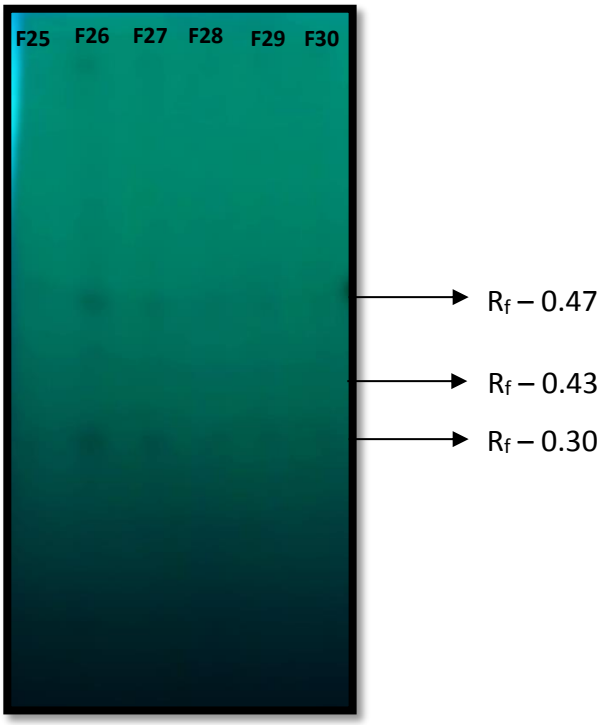
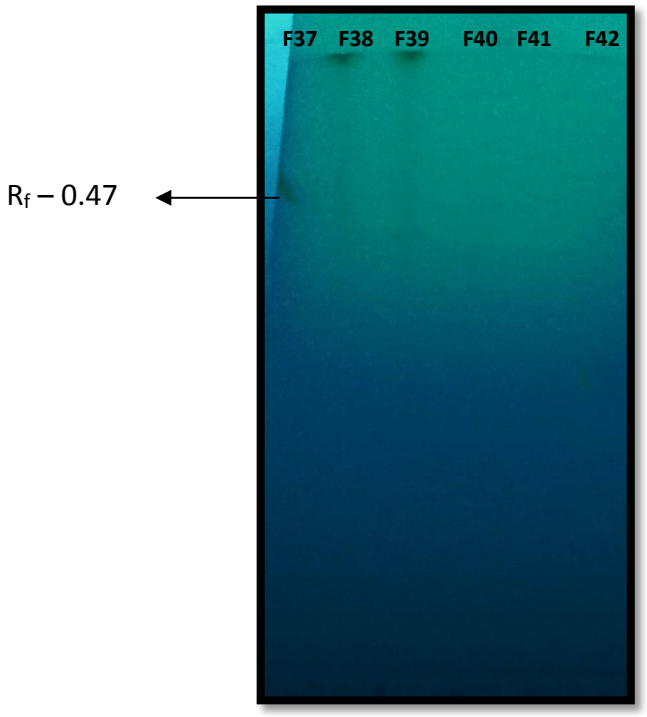


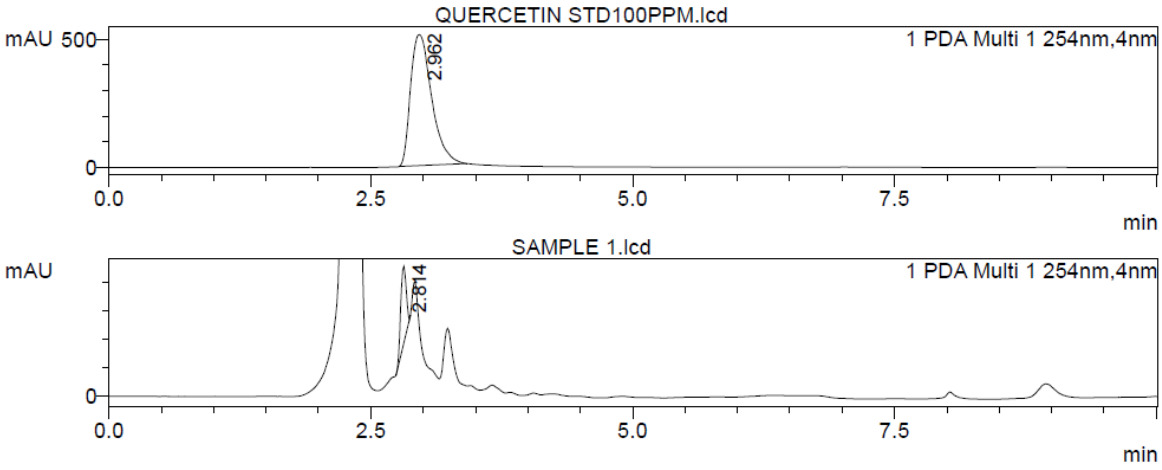
Figure 9: TLC of E51, solvent system Toluene: Ethyl acetate: Formic acid (5:5:2)



4.3.3 HPLC

Active fraction identified by TLC analysis furthere checked Identification of the isolted bioactive compound faction of *Persicaria glabra* was by HPLC, using mobile phase consisting of 100% methanol. The HPLC chromatogram showed a sharp peak with retension time 2.814, this peack is comparatively similar to the peack oberved in standaed (STD) at retenion time 2.962 (**Figure 10**). The compound is thus same as the STD, Quercetin.

Figure 10 : HPLC analysis of TLC and standard compound in mobile phase consisting of 100% methanol.



ID#1 Compound Name: Quercetin

Data File Name	Sample Name	Sample ID	Ret. Time	Area	Height	Conc.
QUERCETIN STD100PPM.lcd	QUERCETIN	STANDARD	2.962	7158426	511059	100.000
SAMPLE 1.lcd	SAMPLE1	TLC SEPERATED	2.814	52575	13607	0.734
Average			2.888	3605501	262333	50.367
%RSD			3.632	139.359	134.086	139.359
Maximum			2.962	7158426	511059	100.000
Minimum			2.814	52575	13607	0.734
Standard Deviation			0.105	5024595	351752	70.191

4.4 COMPUTATIONAL ANNALYSIS

The identefyed molecule was screened in-silico by docking of its molecular structure with homology modelled FtsZ protein, thus demonstrating the mode of action of the molecule with the protein (**Figure: 11, 12** and **Table: 10**).

Figure 11: Structure of quercetin

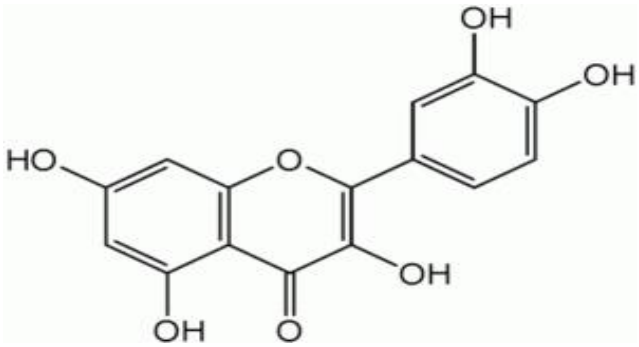


Figure 12: Molecular docking of quercetin with *E. coli* FtsZ protein

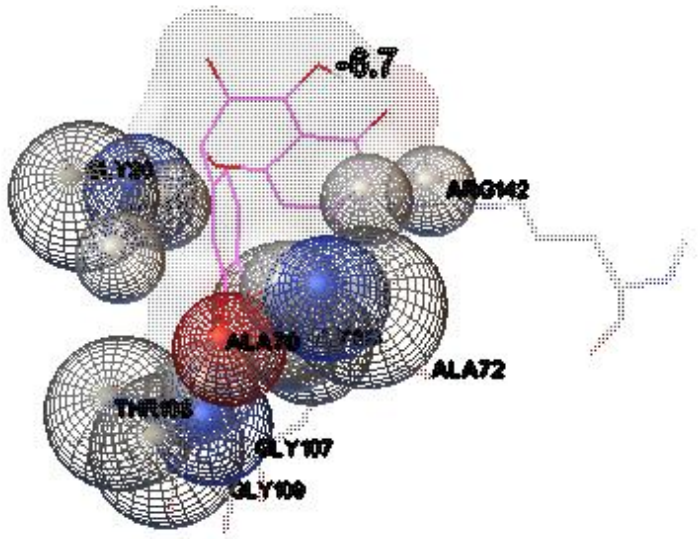


Table 10 : Amino acids involved in FtsZ and quercetin interaction

Amino acid interacting	Bond length Å
Gly-109	2.10
Thr-109	1.66
Ala-71	2.13
Gly-107	2.77
Ala-72	1.86
Arg-142	2.36

4.5 Functonal characterisation of FtsZ protein inhibitor

For characterizing the FtsZ inhibitor, fist the FtsZ protein has to be isolated and purified, then the Functional characterization of FtsZ inhibiton of quercetin is confirmed by experimental assays like light scattering assay, cell elongation assay and malachite green assay (GTP hydrolysis assay)

4.5.1 Isolation and purification of FtsZ protein

FtsZ protein was isolated from *E. coli*, DH5α cells that harboured FtsZ protein expressing plasmids, the plasmid was isolated (**Figure 13**), then transformed and

expressed in *E.coli* C41 strains and then isolated and purified the protein (**Figure 14**). The total volume of protein obtained was 5mg/mL.

Figure 13 : Agarose gel electrophoresis of the isolated plasmid

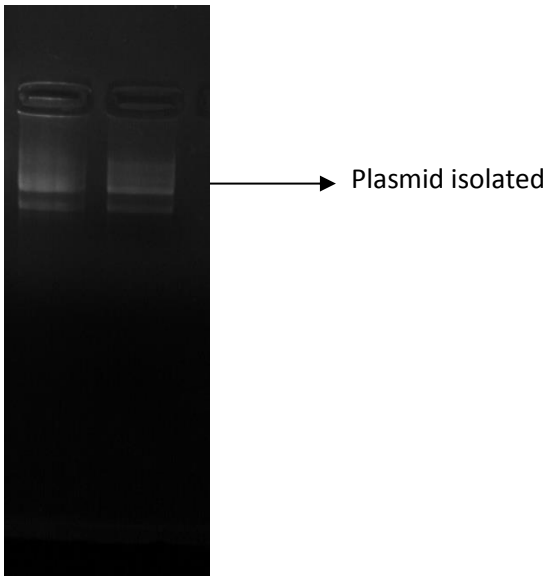
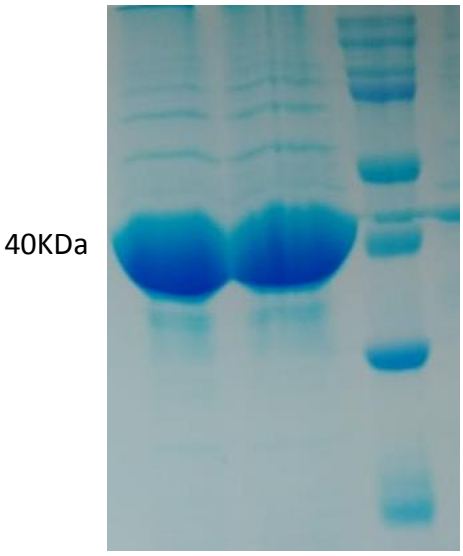


Figure 14: SDS-PAGE of purified FtsZ protein.1 and 2 partially purified protein, 3. Marker

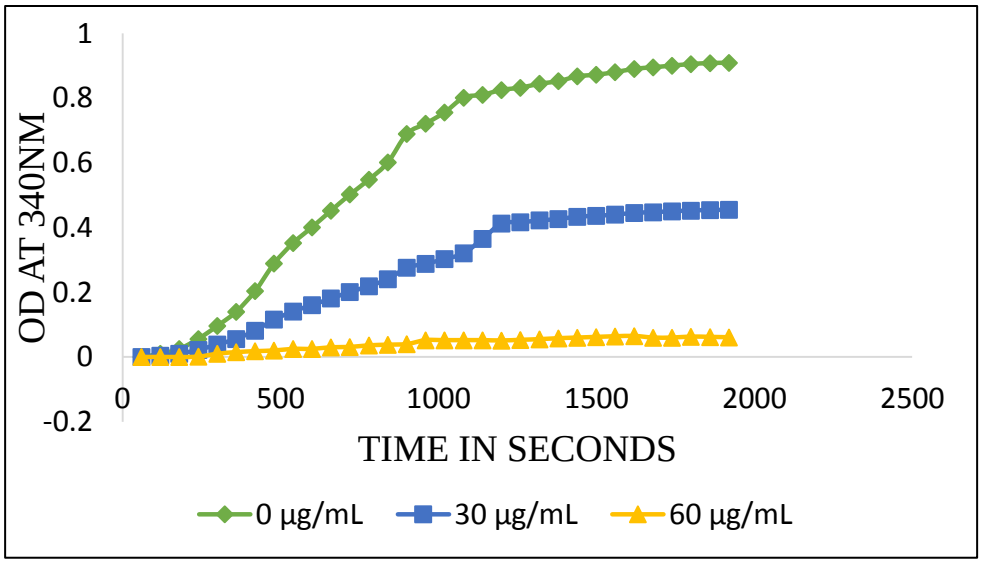


4.5.2 Light scattering assay

The effect of compound quercetin on FtsZ protein polymerization was observed by conducting light scattering assay. The time dependent polymerization of FtsZ

protein was observed in different concentration of compound (30µg/mL, 60µg/mL) and in its absence (0µg/mL). A dose dependent decrease in FtsZ polynerization was observed in presence of compound and was recorded graphically (**Figure 15**).

Figure 15 : Polymerization of FtsZ in a time dependent manner, both in presence and absence of querctin (µg/mL)



4.5.3 Cell elongation assay

The morphological change of the bacterial cell in the presence of quercetin and was checked by using phase contrast microscope. The *Bacillus subtilis* 168 cells were observed to be elongated in the presence of hot crude leaf ethanolic extract (100µg/mL) and quercetin (500µg/mL), which is an indecation of FtsZ inhibition (**Figure 16** and **17**). The non-treated cells was at an average length of 5.25±1.

Figure 16 : Bacterial elongation assay. **A.** *Bacillus subtilis* 168 treated with 1% DMSO **B.** *Bacillus subtilis* 168 treated with 500µg/mL hot crude leaf extract *Persicaria glabra*

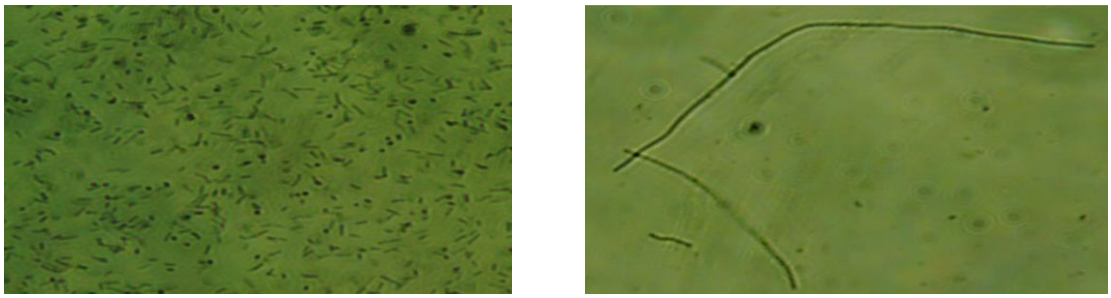
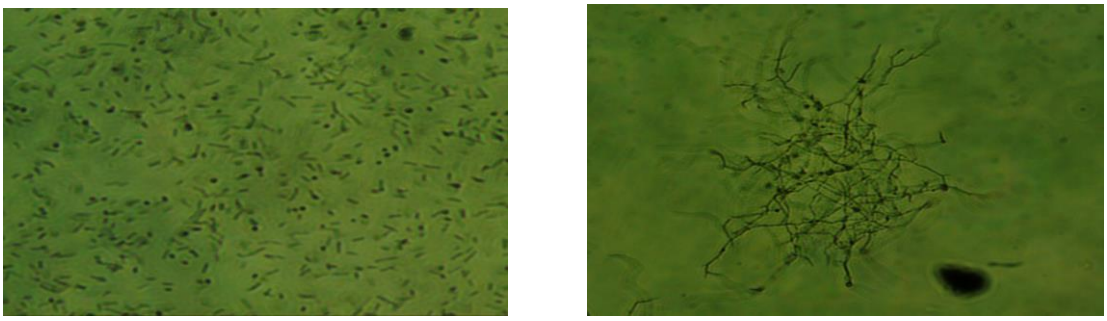


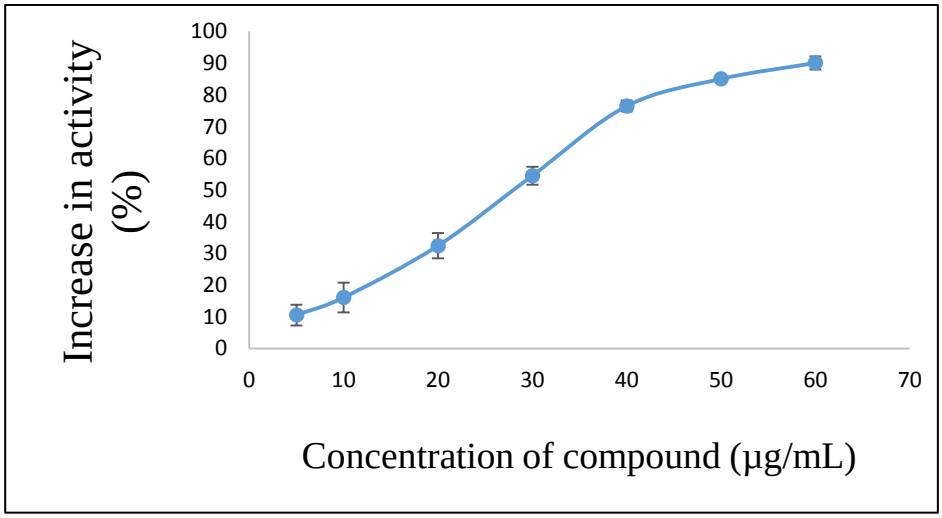
Figure 17: Bacterial elongation assay. **A.** *Bacillus subtilis* 168 treated with 1% DMSO **B.** *Bacillus subtilis* 168 treated with 80µg/mL quercetin



4.5.4 Malachite green assay

The malachite green assay was done to quantify the inorganic phosphate produced by GTP hydrolysis. An increased GTPase activity of FtsZ was observed in the presence of quercetin (**Figure 18**). A dose dependent increase in the production of inorganic phosphate was observed at a concentration from 10µg/mL to 60µg/mL, when compared to control (0µg/mL).

Figure 18: Malachite green assay for FtsZ GTPase activity



4.6 Assessment of crude extract over biofilm rupturing ability

In order to demonstrate the effect of plant compounds on virulence factor and biofilm formation cell surface hydrophobicity test, protease assay and exopolysaccharide estimation assay.

4.6.1 MBCI value determination

Broth microdilution method was used to determine the MBIC of quericitn against *V. cholera*. Among *E. coli* and *V. cholerae*, *V. cholerae* was identified to produce promising biofilm (**Figure 19**). Minimum biofilm inhibitory concentration (MBIC) of *Persicaria glabra* is 50µg and quercetin is 20µg (**Table:11**). The value above 85% at the lowest concentration used is considered as MBIC values.

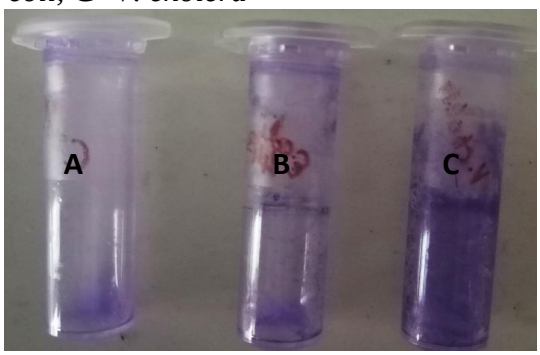
4.6.2 Effect of compound on protease production

The effect of quercetin (18 µg /mL) on the production of the extracellular cysteine protease (major secreted protease) of *S. pyogenes* was qualitatively evaluated using the azocasein dye. 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 proposnal to the amount of protease production. The compound quercetin was not found to enhance the protease level when comparing to the control.

Table 11: MBIC of *Persicaria glabra* and Quercetin

	10µg/µl	20µg/µl	30µg/µl	40µg/µl	50µg/µl	
BLANK 0.069174	0.094029 77%	0.093402 79%	0.09279 79.6%	0.091497 80.2%	0.091536 86%	*Persicaria glabra*
CONTROL 0.11369	0.094031 77%	0.093406 79%	0.09272 79.2%	0.092716 80%	0.086052 86%	Duplicate
BLANK 0.085726	0.091580 85%	0.091416 86.6%	0.091216 88.5%	0.090860 91%	0.090499 95%	Quercetin
CONTROL 0.106171	0.091596 85.9%	0.091100 86%	0.091209 88%	0.008605 91.8%	0.00499 95.3%	Duplicate

Figure 19: Biofilm forming ability of organism. The biofilm formed are stained in blue, **A-** Control, **B**- *E. coli*, **C**- *V. cholera*



4.6.3 Effect of compound on exopolysaccharide content

In order to confirm the reduction in EPS production upon quericetin treatment, EPS was quantified using the phenolsulphuric acid method. Quercetin treatment (16 µg/mL)

significantly not reduced the EPS production of all the M serotypes tested. Cell surface hydrophobicity plays a key role in biofilm formation, this was also measured. Quercetin treatment considerably reduced the cell surface hydrophobicity of all the M serotypes.

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 in modem science has brought about many revolutionary changes in medicines. Through various experiments and observations modem medicine has come to a conclusion that natural products are the most powerful means to fight against diseases than synthetic drugs. This led to scientific investigations on various plants and plant products to develop effective agents against various diseases. During the last few decades, great emphasisment has been given to plant leafs and roots for identifying their medicinal properties. Studies has been conducted all over the world inorder to elucidate the medicinal role of leafs and roots.

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 of actions. FtsZ is one among the main drug targets identified for the discovery of novel antibacterial agents.

Gastrointestinal diseases, particularly diarrhoea is one of the major concerns in many countries world wide, and is a major causeative of childhood death. The use of herbal products are widely practiced in cureing gastrointestinal ailments. Ethnobotanical information plays crucial role in the discovery of novel therapeutic molecules (Hong *et al*., 2015). This study is planned to investigate the *in vitro* studies on the anti infective properties of leafs and root extracts of selected medicinal plants against gastro intestinal bacterial pathogenes.

In this study, hot and cold ethanol extracts of both leaf and root of selected medicinal plants were tested against *Escherichia coli, Enterococcus faecalis, Salmonella typhi, Staphylococcus aureus* and *Vibrio cholerae* using well diffusion assay to determine the antibacterial activity. Ethanolic extract showed inhibitory effect over growth of all the strains of bacteria, which was observed by zone of inhibition, and minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) were also symultanisly determined. Among the different samples evaluated in above mentioned preleminry antimicrobial tests *Persicaria glabra* was identified with higher antimicrobial activity against test organisms.

Persicaria glabra, commonly known as Denseflower Knotweed or Marsh Buckwheat, is a perennial amphibious hurb and are found in moist habitat mostly like river banks, channels tank beds. This wetland plant is erect about 3-4 feet height, with clusters of beautiful little pink flowers which are in paniculate inflorescence of 5-10 cm long and they have oblong-lanceola to narrowly lanceolate shaped leaves of 14-28 cm length and 3-6 cm width, which is a rich source of essential oil. *Persicaria glabra* is considered as an ayurvedic herb and has been employed to treat many disorders like bronchitis, dysentery, haemorrhoids, diarrhoea, piles, healing wounds and bleedings, moreover the whole plant is an astringent, anthelmintic, cardiotonic, febrifuge, cholagogue, diuretic, haemostatic, vulnerary and low grass. Research studies in the past have revealed that plants belonging to Polygonaceae family produce a wide variety of bioactive secondary metabolites that include anthraquinones, flavonoids, steroids, and alkaloids. This interesting plethora of phytochemical composition of *Persicaria glabra* has put the plant as the therapeutic research focus for discovery novel drugs. The present study investigates the remarkable pharmaceutical attributes with a particular focus on finding drug candidates against microbial pathogens, causing diarrhoea. The plant compounds have been reported with minimal side effects so far, furthermore much-branched as in lately, pharmaceutical companies are earmarking natural extracts for the development of

newer therapeutics. The prevalent problem of antibiotic resistance has, in turn, put enormous pressure on researchers to find novel therapeutics (Slama et al., 2010).

The bioactive molecule present in *Persicaria glabra* was identified to be quercetin by employing activity guided thinlayer chromatography, bioautography, column chromatography and the active faction was then evaluated with HPLC and the active compound present in *Persicaria glabra* was identefyed as quercetin. Moreover through *in vitro* studies FtsZ inhibitory property of quercetin was also coformed. Also by employing Auto Dock Vina 1.1.2 software the ligand quercetin was docked into *E.coli* FtsZ molecule, quercetin was readily placed within the GTP binding site of FtsZ with low energy orientation characteized with optimal interactions.

Filamentous temperature sensitive protein 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. Formation of elongated cells in the presence of 80 µg/ml of quercetin 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 quercetin displayed increased GTPase activity, corroborates with a reduction in FtsZ polymer formation at a concentration of 80µg/ml. Our findings suggested that the antibacterial property of ethanolic extracts of *Persicaria glabra* is due to FtsZ inhibitory property of the active compound quercetin.

CHAPTER-6
SUMMARY

The thesis entitled **"***In vitro* studies on the anti-infective properties of leaf and root extracts of selected medicinal plants against gastro intestinal bacterial pathogens"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 this study is to screen medicinal plant extracts for anti infective property against gastro intestinal bacterial pathogens and identification of there 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, the leaf and roots of five medicinal plants *Moringa oleifera, Bixa orellana, Persicaria glabra, Pimenta dioica and Homonia riparia* were primarily selected for identifying there anti infective property against enteropathogens. Hot and cold ethanol extracts of the leaf and root were tested against standard MTCC entero bacterial strains. Preliminary results showed that among the five plants tested, the hot leaf extractes of *Homonia riparia* and *Persicaria glabra* excibited higher activity against the tested stran. Since the hot leaf extracts of *Homonia riparia* and *Persicaria glabra* was showed antibacterial activity and minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) comparitivly in a higher, they were subjected to purification of active compound by employing chromatographic techniques. The hot ethanol leaf extract of *Persicaria glabra* showed 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 quercetin ctand the same was purified following standard method.

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

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 quercetin 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.

CHAPTER 7
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CHAPTER 8

APPENDICES

EQUIPMENTS

Sl:no	Equipment	Make
1.	Hot air oven	Beston, India
2.	Cooling centrifuge (model:Z323K)	Hermle, Germany
3.	Fluorescent spectrophotometer (Spark 10M)	Tecan, Switzerland
4.	UV spectrophotometer (moel:V-730)	Jasco, USA
5.	Inverted microscope (model: Eclipse TS100)	Nikon, Japan
6.	Microscope digital camera (model: MU 300)	AmScope, USA
7.	GelDoc	UVP, USA
8.	Water bath (model: LWB 211D)	Lab Tech
9.	pH meter	Cyber Scan, Germany
10.	Milli-Q integral Water Purification System for Ultrapure Water (ZRXQ003WW)	Millipore, France
11.	Incubator shaker - Orbitek	Scigenics Biotech, India
12.	Incubator (model:Heraterm)	Termo Scientific, USA
13.	Mini-PROTEAN SDS-PAGE Electrophoresis	Bio-Rad Laboratories, USA
14.	Deep Freezer (-30°C)	Haier, Germany
15.	Deep Freezer (-40°C)	Haier, Germany
16.	Deep Freezer (-80°C)	Haier, Germany
17.	Grinding machine	Philips
18.	Magnetic stirrer (model: C-MAG HS 7)	IKA, Germany
19.	Digital camera (model: D5 100)	Nikon, Japan
18.	Rotta shaker	REMI RS-24BL
19.	Soxhlet apparatus	Borosil
20.	Heating mantel	Sunbim
21.	Rotavapor	Heidolph
22.	Hot air oven	KEMI
23.	Autoclave	Labline Instruments India

24.	Laminar air flow system (model: GS LAF 4X2)	Genesys incorporation, India
25.	Bunsen burner	Aman Scientific Works
26.	Analytical balance (model: BSA423S)	Sartorius, Germany
27.	Inoculating loop	Yashika solutions
28.	Pipettes	Gilson
29.	Sterile disc of diameter 6mm	HiMedia
30.	96-well microtitre plates	HiMedia
31.	Antibiotics disc of 6mm diameter	HiMedia
32.	Forceps	Yashika solutions
33.	Vortex mixer	GeNei, India
34.	Chromatography column	Borosil
35.	TLC silica gel sheets	Merck Millipore
36.	TLC chamber	Borosil
37.	Hot plate (model: HS7)	Iacmg

APPENDIX II

Glassware

Glassware used in the present study are of Borosilicate grade, and were purchased from Borosil, India and Schott Duran UK. Before using they were all soacked in chromic acid solution (100g potassium dichromate dissolved in 1L water with 500mL sulphuric acid) for 6hr and then were washed with detergent (Labogent), then they were rinsed in dist.H_2O and dried in hot air oven (160°C, 3hr).

Plasticwares

The plasticwares used in the present study were purchased from Tarson, India and Genaxy, India.

Chemicals

- **Media components**

  All the microbial culture media, including salts used in the present study were purchased from HiMedia, India.

- **Analytical chemicals and solvents**

Solvents, acids and chemicals used in the present study were purchased from Sisco Research Laboratories Pvt. Ltd andSRL, India respectively.

- **Sterilization**

All the glassware and culture media used in the present study were sterilized by autoclaving at 15 Ib pressure at 120°C, 20min.

Other materials and chemicals

1.	Ampicillin	Sigma-Aldrich
2.	IPTG	Sigma-Aldrich
3.	Ethidium Bromide	Sigma-Aldrich
4.	Coomassie Brilliant Blue R-250	HiMedia, India
5.	BSA	HiMedia, India
6.	Whatman No.1 filter paper	Whatman
7.	Parafilm	HiMedia, India

APPENDIX III

Media Composition

1. Lysogeny broth (LB) medium

Tryptone	:	10g
Yeast extract	:	5g
NaCl	:	10g
pH	:	7.2
Distilled water	:	1000mL

2. Mueller-Hinton agar

Beef extract	:	2.0g
Acid hydrolysate of Casein	:	17.5g

Starch	:	1.5g
Agar	:	1.7g
Distilled water	:	1000Ml

Reagent, buffers and procedures

1. **TE buffer**

Tris HCL (pH 8.0)	:	10mM
EDTA (pH 8.0)	:	1mM

2. **Phosphate buffer (PBS) 1X**

NaCl	:	8g
KCl	:	0.2g
Na_2HPO_4	:	1.44g
KH_2PO_4	:	0.24g
Distilled water	:	1000mL
pH	:	7.4

3. **McFarland turbidity standards (0.5 × 108)**

Barium chloride	:	0.05ml of 1.175%
Sulphuric acid	:	9.95ml of 1%

4. **Activation buffer for protease content estimation**

EDTA	:	1Mm
DTT	:	20mM
Sodium acetate buffer (pH 5.0)	:	0.1M

5. **Plasmid isolation (Alkaline lysis method)**

Alkaline lysis solution I

Tris HCL (pH8.0)	:	25mM
EDTA	:	10mM
Glucose	:	50mM

Sterilized by autoclaving, stored at 4°C

Alkaline lysis solution II

NaOH	:	0.2N
SDS	:	1%

Alkaline lysis solution III

Potassium acetate (5M)	:	60mL
Glacial acetic acid	:	11.5mL
Distilled water	:	28.5Ml

3. Transformation buffer solution

$CaCl_2$-$2H_2O$	:	1M
$CaCl_2$ (ice cold)	:	20mM

4. Expression buffer

IPTG (Stored at 4°C)	:	2M
Tris-buffer	:	1X

5. Light scattering assay mix

Tris-HCL (pH 7.4)	:	50mM
KCL	:	2M

MgCl	:	2M
$CaCl_2$	:	0.5M

6. GTP hydrolysis assay buffer

Tris-HCL (pH 7.4)	:	50mM
$MgCl_2$	:	2.5 Mm
KCl	:	5Mm

IN VITRO STUDIES ON ANTI-INFECTIVE PROPERTIES OF LEAF AND ROOT EXTRACTS OF SELECTED MEDICINAL PLANTS AGAINST GASTRO INTESTINAL BACTERIAL PATHOGENS.

Submitted by

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(2014-09-119)

ABSTRACT OF THESIS

Submitted in partial fulfilment of the

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

Faculty of Agriculture
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B.Sc. - M.Sc. (INTEGRATED) BIOTECHNOLOGY
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9. ABSTRACT

The thesis entitled “in vitro studies on anti-infective properties of leaf and root extracts of selected medicinal plant against gastro intestinal bacterial pathogenes” 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 gastro intestinal bacterial pathogens 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 the compounds identified 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 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 metabolites present in leaves are found to have an outstanding role in medical world. Most of these compounds belong to alkaloids, flavonoids, terpenoids, steroids, saponins, quinones, coumarin and tannins. Leafs and roots are been extensively used in folk and modern medicine for treating various diseases.

We have explored anti-infective activity of the leaf and roots ethanol extract of five medicinal plants *Moringa oleifera, Bixa orellana, Persicaria glabra, Pimenta dioica and Homonia riparia* against enteropathogens like *E. coli, V. cholera, E. faecalis, S. Typhi and S. aureus*. Among these extractes hot ethanol extracts of *persicaria glabra* exhibited antibacterial activity against gastrointestinal pathogens such as *E. coli, V. cholera, E. faecalis, S. Typhi and S. aureus.* It also induced filamentation of *Bacillus subtilis* 168 which is an indication of cell division inhibition. The active compound present in *persicaria glabra* extract was identified to be querecetin, this compound can be used to develop novel anti-diarrhoeal agents.