CERTIFICATE

This is to certify that the research work in the dissertation titled "**Exploration and Characterization of endophytic bacteria associated with *Dillenia pentagyna* Roxb.**" has been carried out by **Ms. Lallawmsangi** under my supervision for the full period prescribed under the M.phil ordinance of this University and that this work has not been submitted for any other degree or diploma to any University or Institute.

Dr. H. LALHRUAITLUANGA

Supervisor

DECLARATION

Mizoram University

July, 2020

I, LALLAWMSANGI, hereby declare that the subject matter of this dissertation is the record of work done by me, that the contents of this dissertation did not form basis of the award of any previous degree to me or to do the best of my knowledge to anybody else, and that the dissertation has not been submitted by me for any research degree in any other University/ Institute

This is being submitted to the Mizoram University for the Degree of Master of Philosophy in Biotechnology

(LALLAWMSANGI)

(HEAD) **(SUPERVISOR)**

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DATE: **(LALLAWMSANGI)**

ABBREVIATIONS

DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
ABTS	2'-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid
MHA	Mueller-Hinton Agar
ISP7	Tyrosine Agar
AIA	Actinomycetes Isolation Agar
SCA	Starch Casein Agar
LB	Luria- Bertani
A549	Lung cancer cell line
IC_{50}	Half maximal inhibitory concentration
HT29	Colon cancer cell line
MTCC	Microbial Type Culture Collection
ITCC	Indian Type Culture Collection
PDA	Potato Dextrose Agar
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
ROS	Reactive Oxygen Species
NaOCl	Sodium Hypochlorite
NH_3	Ammonia
CN	Cyanide
EDTA	Ethylenediaminetetraacetic Acid
PBS	Phosphate Buffered Solution
OD	Optical Density
PKS	Polyketide Synthase
NRPS	Non-Ribosomal Peptide Synthetase
PCR	Polymerase Chain Reaction
EA	Ethyl Acetate

DCM	Dichloromethane
DMSO	Dimethyl Sulfoxide
MDR	Multi-drug Resistant
TLC	Thin Layer Chromatography
MeOH	Methanol
Nm	Nanometer
mL	Milliliter
Mg	Milligram
Mg	Microgram
SD	Standard Deviation
SE	Standard Error
Bp	Base Pair
NCBI	National Center of Biotechnology Information
BLAST	Basic Local Alignment Search Tool
Mm	Millimeter
µL	Micro litre
mM	Millimolar

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CHAPTER 1

INTRODUCTION

Endophytes are microorganisms that are colonizing the inner tissues of plants either inter or intracellularly by spending a period of their life exclusively in the host plant without any observable indication of disease and without showing their visible existence (Petrini 1991; Hardoim *et al*. 2015). Endophytes include diverse collection of microbial communities such as fungi, bacteria and actinomycetes which asymptomatically colonize the internal tissues of plants at least once in the part of their life cycle (Rodriguez 2009). They can live inside the tissues of stems, roots, flowers, fruits and in many other tissues of plants where they are able to stimulate the growth of the host plants under undesirable conditions (Strobel *et al.,* 2003; Hagesawa *et al.,* 2006). A variety of relationships exist between endophytes and their host plant that ranges from symbiotic or mutualistic to antagonistic or slightly pathogenic relationships (Arnold *et al.,* 2007; Padhi *et al.,* 2013). The description of an endophyte does not include bacterial and mycorrhizal fungal association in the rhizosphere since bacteria and fungi exist inside the tissues (endosphere) of the host plant and set up a safe and harmless mutualistic association along with the host plant (Kusari and Spitellar 2012). This complex relationship among the endophytes and the host plant is believed to indirectly benefit the growth of plant even under stress due to the production of special biologically active compounds by endophytes termed as secondary metabolites, so as to prevent the growth or activity of plant pathogens (Fernandez, 2015). Endophytes are ubiquitous in nature and their existence has been reported from every plant examined so far (Kusari *et al*. 2012). The endosphere of the plant are likely to provide nutrition with less competitive and stable environment to endophytic population (Farrar et al. 2014). Plants exclusively limit the growth of endophytes, and therefore these endophytes utilize many mechanisms to progressively adapt to their living environment. In order to sustain stable symbiosis, endophytes produce numerous compounds that promote growth of plants and help them adapt better to the environment (Nair *et al.*, 2014). In other words,

endophytes must synthesize metabolites for the competition with occurring microorganisms, host and pathogens in order to colonize the host as well as for nutritional purpose (Palanichamy *et al.,* 2018). Endophytes are considered to colonize their host and build up the symbiotic relationship with the host plant ever since the existence of plants on the Earth and it might also possible that they would have exchanged their genetic makeup with the host that enabled protection to them from the defense mechanism of the host (Botella and Diez 2011; Higgins *et al*. 2014). Endophytes exist in a far more protected environment providing competitive edge over the other microorganisms residing in the rhizosphere as well as those residing in the phyllosphere (Backman and Sikora, 2008). In turn, endophytes can provide protection to the host against biotic stresses including attacks by fungal pathogens and herbivore insects either by the mechanism of antibiosis or by induced resistance (Hardoim *et al*. 2015). The microbiome in the endosphere plays a very crucial role within the system of host plants and its associated macro and microorganisms. They do so by performing niche-specific activities in order to improve the fitness of the host plant and in that process they are producing secondary metabolites for defense, signaling molecule, allellochemicals and biosynthetic precursors (Scherlach and Hertweck, 2017; Eckelmann *et al*., 2018). The bioactive properties of plants may also be due to the metabolites produced by their endophytic counterparts (Kaul *et al*., 2012; Kusari *et al.,* 2013; Santos *et al*. 2016). Endophytes promote host plant growth as well as help the host to adapt in unfavorable environments by providing resistance to diverse biotic and abiotic stresses such as salinity, extreme temperatures, and attack from plant pathogens and insects (Rodriguez, 2008; Ali *et al*. 2014). Endophytes comprises of plant-colonizing microorganisms in a mutualistic symbiosis relationship. They are found in most ecosystems plummeting biotic and abiotic stressors of plant crops by exciting immune responses and eliminate plant pathogens by niche competition. They participate in activating antioxidant activities as well as phenylpropanoid metabolism and provide defense, structural support, and survival molecules to the plants (Pandey *et al.,* 2018). Metabolomic studies showed that endophytic genes that codes for specific metabolites are involved in plant

growth promotion (PGP) by stimulating plant hormones production such as auxins and gibberellins and act as defensive agents against pathogens and insect pests while being ecologically friendly and environmentally safe (Ramos *et al.,* 2019). Endophytes are found in plants of most ecosystems and are of agricultural importance since they possess plant growth promoting ability that affects plant growth through direct or indirect mechanisms. The direct growth promotion includes the production of several secondary metabolites which directly affects the plant growth for example phytohormones or making the accessibility of certain nutrients to the plant from its environment like phosphorous and iron (Dinesh *et al*., 2015; Sharma *et al*., 2016). Whereas the indirect mechanism is mainly involved in the defense of host by the bacteria from phytopathogens by the production of antagonistic compounds or by inducing plant defense mechanisms against plant pathogens (Bhattacharyya and Jha, 2012). Antagonistic endophytes mainly targets pathogens by mechanisms such as mycoparasitism due to physical inter-hyphal interference, competition for nutrients and colonizing space, production of volatiles and nonvolatile metabolites or stimulation of host defense system (Ting *et al*. 2010: Katoch *et al*., 2017) Natural resources proved to be an excellent source for potentially discovering novel biologically active molecules and endophytes are known to produce a range of bioactive substance in response to biotic stress with antimicrobial, antiviral, anticancer, antimalarial and antioxidant activities and these metabolites has great potential in pharmaceutical and agriculture industries (Kaul et al. 2012; Chen et al. 2014; Mondal *et al*., 2019). Endophytes are reported to produce several natural products known as secondary metabolites such as alkaloids, polyketides, terpenoids, flavonoids, phenols, steroids, cardiac glycosides, quinols and peptides (Gunatilaka 2006; Higginbotham et al. 2013; Tejesvi et al. 2013). The production of bioactive molecules by endophytes is directly related to the independent evolution of these microorganisms, which may have incorporated genetic information from higher plants, allowing them to adapt better to plant host and perform some important functions such as protection from pathogens and insects (Pimentel *et al*., 2011). Bacterial endophytes have been shown to mediate de novo synthesis of novel

compounds and metabolites to prevent disease development in host plant system (Ryan et al., 2007). Endophytes have proven to be a rich source of novel natural compounds with a broad range of biological activities and a high level of structural diversity. Endophytes have many roles and applications including phytostimulation, pigment production, enzyme production, nutrient cycling and boremediation (Nair *et al.,* 2014). Endophytic microorganisms are known to influence plant physiology and development and are important in activities such as biocontrol, plant growth and development, symbiotic-mutualistic, commensalistic, trophobiotic as well as control of soil borne pathogens (Ek-Ramos *et al*., 2019). An endophytic community is complex and several factors such as plant-microbe and microbe-microbe interactions and environmental conditions may affect its structure (Ryan *et al.,* 2008).

Endophytes are gaining the attention of researchers increasingly which is due to the fact that, in addition to providing protection to the host; they also produce host specific bioactive natural products (Kusari *et al*. 2012; Padhi and Tayung, 2013). Considering the fact that pathogenic microorganisms are developing multidrug resistance (MDR), there is an urgent need for more effective and less toxic natural products. Endophytes carry out defense mechanism against pathogenic microorganisms by synthesizing a large number of antimicrobial compounds that are belonging to several structural classes of secondary metabolites (Yu *et al*., 2010). The discovery of novel antimicrobial metabolites from endophytes serve as an important alternative to overcoming the disturbingly increasing levels of drug resistance by plant and human pathogens as well as tackling the insufficient number of effective antibiotic drugs against diverse bacterial species (Song *et al*., 2008). Plants having known ethnobotany found in atypical climate and growing in unique habitat or in a biodiversity hotspot are considered as promising source of novel endophytes. Such rare or novel endophytes can act as a source of isolation of natural products with new chemistry (Kaul *et al*. 2012; Kusari *et al.* 2013). Apart from production of novel chemical substances, many endophytes possess natural capability for xenobiotic degradation or may act as vectors to introduce degradative traits (Siciliano *et al.,* 2001; Barac *et al*., 2004; Germaine *et al.,* 2004). The ability to

confer resistance to heavy metals or antimicrobials by some endophytes and ability to degrade organic compounds is believed to most likely arise from their exposure to diverse compounds in the plant or soil niche. This natural ability shown by endophytes to degrade these xenobiotics is being largely investigated with regard to improving phytoremediation (Porteous-Moore *et al.*, 2006; Ryan *et al.,* 2007). Recently, endophytes has been viewed as an attractive source of study for evaluation and elucidation due their potential in production of bioactive compounds using advanced biotechnological processes that mainly targets production of secondary metabolites by these organisms (Owen *et al*., 2004). Many bioactive compounds have been successfully obtained from endophytes and such rare or novel endophytes can act as a source of isolation of natural products with new chemistry (Kaul *et al*. 2012; Pratiwi *et al.,* 2018).

Dillenia pentagyna Roxb. (Family: Dilleniaceae) is a medium sized tree (20 meters), belonging to a family of evergreen shrubs, sub-shrubs, or climbers and are distributed throughout India. *Dillenia pentagyna* is commonly located in the northeastern regions and has many traditional medicinal uses (Dubey *et al*., 2009). *Dillenia* is a genus of about 100 species of flowering plants however, up to now only 8 of *Dillenia* species have been reported to be used traditionally for various medicinal purposes including cancer treatment (Dubey *et al*., 2009; Yazan and Armania, 2014). *Dillenia pentagyna* is one of the most important ethnomedicinal plant which is used to cure various ailments by local healers and its traditional uses ranges from region to region where they use different parts of the trees such as the bark, flowers, leaves and fruits (Sharma *et al.,* 2001; Dubey *et al*., 2009; Rai and Lalramnghinglova, 2010). *Dillenia pentagyna* is commonly used to treat various diseases such as stomach ulcer, hypertension, hemorrhoids, asthma, cancer, diabetes and dysentery (Sharma *et al*., 2001; Rai and Lalramnghinglova, 2010; Sawmliana, 2013). It also possesses pharmacological properties such as anticancer or cytotoxic activities (Rosangkima and Prasad 2004), antimicrobial activity and antifungal properties (Hague *at al*., 2008) as well as antioxidant properties (Smitha *et al*., 2012). Furthermore, a few compounds have been isolated from the stem bark of *Dillenia pentagyna* such as a triterpene glycoside, which

us named α-1-rhamnopyranosyl-3β-hydroxyl-lup-20(29)-en-28-oic acid by acid hydrolysis (Tiwari *et al*., 1980), flavanol (rhamnetin) and flavonone (naringenin and dihydroquercetin) , glycosides (Srivastava, 1981) and a diterpene, dipoloic acid diterpene (Smitha *et al*., 2012). However, to our knowledge there has been no work or study carried out for the investigation and exploration of diversity of endophytes and bioactive potential of endophytic bacteria associated with *Dillenia pentagyna* which has been attempted in the present study.

The present study aimed to isolate endophytic bacteria associated with the medicinal plant *Dillenia pentagyna* Roxb. and to check for bioactive activity and production of their secondary metabolites. The main objectives set for the present work are:

- Isolation and identification of endophytic bacteria associated with the bark, leaves and root tissues of Dillenia pentagyna Roxb.
- Screening for their antimicrobial potential against multidrug resistant (MDR) pathogens
- Detection of biosynthetic genes in selected isolates based on antimicrobial screening and determination of secondary metabolites using HPTLC

CHAPTER 2

REVIEW OF LITERATURE

Medicinal plants are used traditionally as remedies for the treatment of various diseases, including asthma, skin disorders, respiratory and urinary problems, gastrointestinal problems and hepatic and cardiovascular disease (Tian *et al*., 2014). Medicinal plants have been extensively used in most countries as a large integral part of their traditional herbal medicine and with the increasing demand for herbal drugs, natural health products, and secondary metabolites produced by medicinal plants; the use of medicinal plants is growing increasingly throughout the world (Cole *et al*., 2007; Chen *et al*., 2016). Medicinal plants synthesize a diverse array of biologically active compounds that are essential for them to survive and thrive in the natural environment (Bajguz 2007; Cushnie *et al*., 2014; Egamberdieva *et al.*, 2017). Medicinal plants, as source of remedies are broadly used as alternative therapeutic tools for the prevention and treatment of many diseases and are natural source of antioxidants and the bioactive compounds isolated from medicinal plants have shown antiviral activity, antimicrobial activity and show potential to inhibit cancer cell lines both *in vitro* and *in vivo* (Abidemi *et al*., 2015; Balasubramaniyan and Padma, 2013). Medicinal plants are well known as natural antioxidant agents and the recent studies have investigated that the antioxidant effect of plant products is mainly attributed to secondary metabolites like phenolic compounds such as flavonoids, phenolic acids, tannins, etc. (Nagavani *et al.,* 2010). Although a large number of medicinal plants have been well-studied with respect to their phytochemical constitutes and pharmacological properties, their microbiome and physiological interactions between host plant and associated microorganisms still remain poorly understood (Kobert *et al*., 2014). The plant associated microbiome consists of distinctive microbial communities that live in the roots, shoots as well as the endosphere (Beneduzi *et al*., 2012; Berg *et al*., 2014). Most of the microorganisms that inhabit plants play a major role in the plant's health and development although, they are sometimes neutral (Mendes *et al*., 2013). The endosphere of plants harbor several groups of microorganisms including fungi, bacteria and protozoa (Passari *et al.,*

2016). Nearly all vascular plants studied were found to harbor endophytic bacteria and/or fungi (Rodriquz *et al.,* 2009; Hardoim *et al.,* 2015). Apart from the disease causing microorganisms, the presence of other non-pathogenic microorganisms inside the plant system was first pronounced by De Bary, 1866, who detected the presence of microbial cells in the microscopically analyzed plant tissues (Gouda *et al*., 2016). These microorganisms are commonly speculated to contribute to the evolutionary fitness of their host by manufacturing a range of secondary metabolites, which provides resistance against diseases and promotes survival (Strobel *et al*., 2004; Passari *et al.*, 2015). Recently, due to the intimate interaction of endophytes with the host and due to the believe that the phytochemical constitutes of plants can be related either directly or indirectly to endophytic microbes and their interactions with host plants, endophytic microorganisms have been under increased investigation (Egamberdieva *et al.,* 2017).

Endophytes comprise a diverse assemblage of ubiquitous microorganisms residing in the tissues of plants for at least a part of their lifecycle without causing any overt symptoms (Petrini 1991; Bacon and White 2000). The potential explanation for the ubiquitous presence of endophytic microorganisms in plant tissues is the diversity of positive effects on plant growth and fitness that they have shown by stimulation of the host phenylpropanoid pathway or by producing several linked metabolites to the plant's metabolism (Ramos *et al.,* 2019).Due to extensive study, the endophytic communities have been classified into different subgroups such as obligate or facultative, associated in all types of plant species (Rosenblueth and Martinez-Romero, 2006). Hardoim *et al*., 2008 termed endophytes that depend on the metabolism of plant for survival, being spread amongst plants by the activity of different types of vector transmission, as obligate endophytes. Whereas, facultative endophytes are microorganisms that live outside the host during a certain stage of their life cycle and are mostly associated with plants, from its neighboring soil environment and environment (Abreu-Tarazi *et al*., 2010). It is believed that the bioactive compounds of plants are related either directly or indirectly to endophytic microbes and their interactions with host plants (Chandra, 2012 and Qi *et al*., 2012). The bioactive compounds produced by endophytes exclusive of those to their host plants, are very important to increase the

adaptability of both endophytes and their host plants, such as tolerance to biotic and abiotic stresses. In addition, these compounds can induce the production of a plethora of known and novel biologically active secondary metabolites (Zhang *et al*., 2006; Rodriquez *et al.,* 2009) that can be exploited and applied as important medicinal resources. The particular mechanisms by which endophytic microbes fill the various functions in plants are likely to differ depending on the microbe and plant (White *et al*., 2019)

Endophytes are mostly fungi or bacteria that colonize plant tissues and have adapted themselves to the intracellular parts of the plants without harming the host plant (Erjaee *et al*., 2019; Islam *et al.,* 2019). The adhesion or attachment of microbial cells to the surface of plants is considered to be the initial step of the colonization process. Microorganisms that occupy the surrounding area of the plant roots migrate towards the roots, using chemotactic factors and affinities for root exudates. Microbes then attach themselves to the root surface and this step is crucial in getting access to potential entry sites at lateral root emergence areas or other openings that are caused by wounds or physical injuries (Kandel *et al.,* 2017). The attachment of microorganisms to the root surface of plants is facilitated by the synthesis of exopolysaccharides (EPS) by bacterial cells and this is an important process in the early stages of endophytic colonization (Meneses *et al*. 2007). More than 300,000 unknown plant species around the world are thought to be the host of one or more endophytes (Erjaee *et al.,* 2019). For these organisms all or most of their life cycle is spent within their host and they exhibit complex interactions with their hosts, which involve mutualism, antagonism and rarely parasitism (Gouda *et al*., 2016). The endophytic population in a plant species is highly variable and depends on various components such as host species, host developmental stage, inoculum density and environmental condition (Dudeja and Giri, 2014). Endophytic bacteria have the competence to systematically inhabit plant tissues and maintain a symbiotic relationship with the host, which makes them highly efficient biocontrol agents and several reports have investigated bacterial endophytes as possible biocontrol agents against diverse pathogenic fungi (Bakker *et al.,* 2013; Mohamad *et al*., 2018). Endophytes are also known to influence the physiology of plant and their development, among which gram-positive bacteria are essential in promoting activities such as biocontrol, bioremediation, plant

growth, commensalistic, symbiotic-mutualistic, trophobiotic interactions, control of soil-borne pathogens and maintaining host-plant defense system against environmental stress (Ryan *et al*., 2008; Rho *et al*., 2017: Ramos *et al.,* 2019).

In recent years, insect pests have been severely endangering different plant species on a large scale. Although chemically synthesized pesticides would protect the plant quite effectively, frequent use of pesticide may lead to serious environmental pollution that threatens both animal and human health. Increasing awareness of environmental protection and food safety contribute to the elevating public attention towards a safer biological control methods. Since endophytes showed considerable biocontrol potential, the use of endophytic bacteria to control plant diseases has become an important and promising approach of biological control (Yu *et al.,* 2018). Endophytes are found in plants of most ecosystems and are of agricultural importance as they help improve crop yields by stimulating plant growth and immune response, excluding plant pathogens by niche competition as well as actively participate in phenylpropanoid metabolism and antioxidant activities (Pandey *et al.,* 2018). Modulation of seedling development by endophytes is likely the result of the evolution of plants in continuous symbiosis with microbes that colonize plant tissues and thus reliably participate in the development process (Verma *et al*., 2017). Bacteria associated with medicinal plants have rarely been explored with regard to antagonistic activity against plant pathogens (Bakker *et al*., 2013; Bhuvaneshwan *et al.,* 2013; Egamberdieva *et al*., 2017). Endophytic bacteria can be found in most plant species and can be recovered from roots, leaves, stems and a few from flowers, fruits and seeds (Lodewyckx *et al*., 2002). Microbial enzymes are regularly used in several industries, especially because they are economical, environment-friendly and they create no ethical concerns, and can be identified easily by screening microorganisms from various environmental conditions (Hoondal *et al.*, 2002; Dalvi *et al*., 2007). Endophytic bacteria have the ability to produce industrially important enzymes such as proteases, amylases, agarases, cellulases, and lipases (Cavaglieri *et al*., 2004)

Numerous studies have revealed that bacteria that live within plant tissues, collectively called as endophytic bacteria play a crucial role in the growth and fitness of a wide variety of plant species. These beneficial functions attributed to endophytic bacteria includes plant growth promotion by supplying nutrients (e.g., nitrogen fixation), protection against biotic (e.g., pathogens) and abiotic stresses (e.g., salinity and drought), detoxification of harmful compounds (e.g., NH_3 or CN) and the production of bioactive compounds (e.g., secondary metabolites and hormones) (Zhang *et al*., 2019). Endophytes can be described as chemical synthesizer in plants as they play an important role as a selection system for microorganisms to produce bioactive compounds with low toxicity towards higher organisms (Pimentel *et al*., 2010). Endophytes are reported to produce a number of biologically active metabolites in a single plant or microbe which served as an excellent source of drugs for treatment of various diseases and which have potential application in agriculture, food, medicine and cosmetics industries (Strobel and Daisy, 2003; Jalgaonwala *et al.,* 2011; Godstime *et al.,* 2014; Shukla *et al.,* 2014). Natural bioactive compounds produced by endophytes have been promising potential usefulness in terms of safety and human health concerns although there is still a significant demand of drug industry for synthetic products due to the reason that it is less time consuming as well as due to economic reasons (Strobel and Daisy, 2004: Pimentel *et al*., 2010). The secondary metabolites that are obtained from endophytes are categorized into functional groups such as, alkaloids, flavonoids, phenolic acids, steroids, saponins, tannins, chinons, terpenoids, benzopyranones, quinines, tertralones, xanthones, etc. (Gouda *et al*., 2016). These compounds can be extracted from the natural source by microbial production via fermentation or microbial transformation using innovative technological advancements that provides promising alternative for establishing an inexhaustible, cost-effective and renewable resource of high-value bioactive products and aroma compounds (Borges *et al.*, 2009).The biotransformation method has a large number of applications for instance, it has been extensively employed for the production of volatile compounds that possess not only sensory properties but other potential properties such as antimicrobial (vanillin), antifungal and antiviral (alkanolides), antioxidant (eugenol, vanilin), blood pressure regulating (2-[E]-hexena), somatic fat reducing (nootkatone) and

anti-inflammatory properties (1,8-cineole) and many others (Krings *et al.*, 2006; Berger *et al*., 2009). Several metabolites that are produced by microbial endophytes also have the potential to act as antimicrobial compounds against human, animals and plant pathogens. The antimicrobial effect against phytopathogens will have a positive effect on the host plant and may show a great potential for medical and veterinary treatments. These metabolites produced by microbial endophytes are of low molecular weights that inhibit the growth or kill phytopathogens, bacteria, fungi, viruses and protozoans that cause human and animal diseases (Jakubiec-Krzesniak *et al*., 2018; Tripathi *et al.,* 2018). The need for antimicrobial compounds from biological materials is inevitable for the food, agricultural and pharmaceutical industry. Therefore, the search for a safe, new and innovative bioactive compounds and alterative to synthetic drug is necessary and has led to the natural antimicrobial substances which are synthesized by microorganisms (Jeong *et al*., 2011; Erjaee *et al.,* 2019) In addition, some antibiotic producing microorganisms are recently found to be endophytes in different plant species (Eljounaidi *et al.,* 2016). It is believed that the widespread capacity of microbe to produce plant signal molecules such as nitric oxide, growth regulators such as auxins and ethylene could be co-evolutionary association of microbes and plants (Verma *et al.,* 2017).

Bioactive compounds from endophytes, such as Bacillus, Pseudomonas, and Rhizobium, are known to promote host plant growth, whereas endophytic bacteria can confer resistance to pathogen-induced diseases in the host plant (Islam *et al*., 2019) without causing any apparent disease symptoms by themselves (Braun and Hirsch 1992). However, considerable evidence has been accumulated supporting the beneficial role of endophytes in host plants in promoting plant growth (Verma *et al.,* 2001; Sziderics *et al*., 2007) improved nutrient acquisition from soil (Upson *et al.,* 2009) abiotic stress tolerance (Rodriguez *et al*., 2008), and enhanced production of plant defensive compounds by upregulating the gene expression of biochemical pathways involved in plant defense mechanisms (Gond *et al.,* 2015). Numerous studies have reported several mechanisms by which the host plant resistance against multiple plant pathogens is induced by inhabiting endophytes. They include competition against pathogens for nutrition and space (Bolwerk *et al*., 2005), induction of

host resistance genes (Waller *et al*., 2005), promotion of plant growth and physiology (Chaturvedi and Singh 2016), hyperparasitism, predation (Grosch *et al*., 2006; Dutta *et al*., 2014), and stimulation or production of natural biostatic and biocide compounds (Shankar *et al*., 1994). Among the many mechanisms, suppression of pathogen growth and fitness is one of the ways that endophytes significantly improve plant health (White *et al*., 2018). There are several mechanisms involving in the suppression of pathogens by endophytes which include direct antagonism by competing with pathogens for space and nutrients through production of antimicrobial metabolites and through induction of systematic resistance or increasing resistance in plants against pathogens via upregulation of host defense genes (Irizarry *et al*., 2017; Hardoim *et al*., 2015). There are studies which suggested that endophytes such as bacteria and fungi provide host plant defense against pathogens and other pests at the time of seed germination therefore known to last throughout the plant life (White *et al*., 2019). Bacterial endophytes such as *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* are producing bioactive compounds which have antifungal activity such as phenazine-1-carboxylic acid, 2,4-diacetylphloroglucinol, pyrrolnitrin, pyoleutirin and volatiles like hydrogen cyanide that have the ability to extensively inhibit the growth of pathogens (Ongena *et al*., 2008; Bastias *et al*., 2017). *Bacillus* species are one of the most important endophytic bacteria that act as control agents against diseases by the production of bioactive molecules that serve as potential inhibitors of plant pathogens. These bioactive compounds include molecules such as lipopeptides that induce leakage in fungal hyphal membranes and this mechanism reduces their virulence (Ongena *et al.*, 2008)

Endophytes have shown to produce bioactive compounds originally isolated from their host plants as well as unique and exclusive bioactive molecules that are evidently different from those of host plants and found to have different structural and molecular characteristics (Hughes *et al*., 2015). Endophytes are found to be producing significant amount of antioxidants which prevent oxidative damage to cellular components (Palanichamy *et al.,* 2018). Endophytes are capable of reducing oxidative stress in plants which are caused by environmental stresses such as the kind that produces reactive oxygen species (hydrogen peroxide, hydroxyl radicals, hydroperoxyl radicals and superoxides), thereby providing

oxidative stress tolerance in plants. Some endophytes produce stress tolerance to both biotic and abiotic stresses (Lata *et al*., 2018). Studies showed that endophytic bacteria at their early stage of colonization caused upregulated transcripts level of ROS-degrading genes including glutathione reductase and superoxide dismutase and this upregulation host ROS-degrading genes may further reduce oxidative damage to plants caused by pathogens (Lata *et al*., 2018; White *et al*., 2019).

Medicinal plants and their associated endophytes are an essential source of bioactive compounds and metabolites that contribute to more than 80% of the available natural drugs in the market (Singh and Dubey, 2013). Approximately half of the deaths worldwide are known to be caused by infectious diseases and parasitic diseases (Menpara and Chandra, 2013). Several researchers have reported that endophytic actinobacteria isolated from medicinal plants bearing ethnomedicinal properties can serve as potential candidates for the recovery of crucial bioactive natural products (Sharma and Puri 2007; Akshatha *et al.*2014). Although many advances have been made in the field of research and in contrast to today's world being the generation of nano to pico drugs, natural resources have been proven to be the best source for discovery of drugs. Endophytic microorganisms are a storehouse of biologically active, secondary metabolites that can serve as a source for antimalarial, antimicrobial, antidiabetic, antiarthritic and immunosuppressant activity (Jalgaonwala *et al*., 2014; Goldstime *et al*., 2014). Among the natural resources, medicinal plants are good sources of broad-spectrum bioactive compounds. To date, only a few plants have been studied for their endophytic bacterial diversity and potential to express bioactive secondary metabolites (Mondal *et al*., 2019). The discovery of novel antimicrobial secondary metabolites and bioactive compounds form different types of endophytic bacteria is an important alternative to overcome the increasing levels of antimicrobial resistance in pathogenic microorganisms (Goldstime *et al*., 2014). It is globally well known that immunocompromised individuals are at elevated risk for developing opportunistic microbial infections by different microbial pathogens. Furthermore, improper and frequent use of antibiotics, poor hygienic conditions and delay in diagnosis of the disease are among some of the important factors that play a role in the increasing drug resistance (Mishra *et al.,*

2107) Moreover, drug resistance is a recognized and increasing phenomenon that disease-causing microbial agents develop against pharmaceutical therapy (Housman et al., 2014). The urgent need for human diseases prevention and treatment, has promoted the discovery and development of novel and efficient therapeutic agents to which resistance has not been produced (Strobel and Daisy, 2003; Chinedum, 2005). Development and improvement of endophyte resources could bring us a variety of benefits, such as novel and effective bioactive compounds that cannot be synthesized by synthetic chemical reactions (Nair *et al*., 2014)

CHAPTER 3

MATERIALS AND METHODS:

3.1. Sample collection

Fresh Bark, Leaves and Roots of *Dillenia pentagyna* were collected from a forest in Aizawl District **(24˚01′N;92˚54′E),** Mizoram, Northeast India. Sample collection was done during the month of December, 2018. The leaves, barks and roots samples were randomly selected at a distance of a minimum of 100 m to avoid isolating identical microbiota of endophytes. A voucher specimen was prepared and preserved at the Department of Biotechnology, Mizoram University, and Tanhril. The collected tissues were kept in plastic bags and kept at 4˚C. The plant samples were processed within 24 hours of collection.

3.2. Surface sterilization and isolation of endophytic bacteria

The collected leaves, barks and roots samples were thoroughly washed in running tap water continuously for 10 min. The tissues of bark, root and leaf were cut into small pieces of $1cm^2$ and were surface sterilized in 75% ethanol for 1 min. This was followed by immersing tissues in 4% sodium hypochlorite for 3 min and was rinsed with sterile distilled water to get rid of the traces of sodium hypochlorite. Lastly the tissues were washed in 75% ethanol for 30 sec and then washed with sterile distilled water (Cannon and Simmons, 2002). The water obtained from the last wash was stores for checking the growth of epiphytic bacteria.

Fragments of the surface sterilized tissues were placed on 90 mm Petri dishes containing growth media such as Starch Casein Agar (SCA), Luria-Bertani Agar (LB), Tyrosine Agar (ISP7) and Actinomycetes Isolation Agar (AIA). The media were supplemented with antifungal compounds such as Nystatin and Cyclohexamide (60 µg/ml each) to suppress fungal growth on the media. The plates were incubated at 28ºC± 2ºC for 2-4 weeks (Passari *et al*., 2016). Pure cultures were obtained by repeated sub-culturing of emerging endophytic bacteria on respective media. The pure cultures obtained were then stored at 4˚C (Taechowisan *et al.,* 2003).

3.2.1. Validation of surface sterilization

After surface sterilization was performed, method of validation for surface sterilization was done. In this method sterilized tissues of barks, leaves and roots were taken and placed on nutritional media (ISP7 and LB) by pressing them, a technique called as tissue fingerprinting. Additionally, an aliquot of 0.1ml of distilled water that is obtained from the last wash of surface sterilization step was spread on the same media using L-shaped spreaders. The plates were incubated at 28±2˚C for 2-3 days and checked for growth of any remaining bacteria or fungi. (Passari *et al*., 2016)

3.3. Screening of isolates by antimicrobial activity

For preliminary screening of isolates, antimicrobial activity was tested against all the isolated pure cultures. Screening is done by preparation of broth cultures of all the isolates in Tryptone Yeast Extract Broth (ISP1) media. The cultures were incubated in an incubator shaker at 28˚C at 150 rpm. After 2-3 days of culture, cell-free supernatant was obtained by centrifugation of the broth cultures at 10,000 rpm for 10 min at 4˚C (Ebrahimipour *et al.,* 2013). The supernatant was collected and used for screening of antimicrobial activity. For screening, 11 pathogenic microorganisms which are multidrug resistant (MDR) pathogens were used. The test organisms were procured from American Type Culture Collection (ATCC) and maintained in Molecular microbiology and systematic Laboratory, Department of Biotechnology, Mizoram University. The test organisms used were *Pseudomonas aeruginosa* (ATCC-10145), *Staphylococcus aureus* (ATCC-BAA-44), *Escherichia coli* (ATCC-10536), *Candida albicans* (ATCC-64124), *Enterococcus faecalis* (ATCC-51575), *Salmonella typhimorium* (ATCC-51812), *Bacillus subtilis* (ATCC-11774), *Klepsiella pneumonia* (ATCC-BAA-2814), *Saccharomyces cerevisiae* (ATCC-2601), *Streptococcus pneumonia* (ATCC-10015) *and Micrococcus luteus* (ATCC-10240).

Antimicrobial assay was done using the agar well diffusion assay (Rios *et al*., 1987). In this method, nutritional media Mueller-Hinton Agar (MHA) was prepared and poured on media plates. Broth cultures of all the pathogens were prepared in respective media and 70µl of the

culture broth was taken at exponential stage of growth and then spread on MHA plates using L-shaped spreaders. Agar well was made by punching a hole with the help of sterile cork borer. In the agar well plates, extracts of different concentrations including negative control as well as positive control (standard antbiotic) was added. The plates were incubated at 28˚C for 24-48 hours and zone of inhibition was observed and compared against that of the control.

3.4. Preparation of microbial extract

Selected isolates were cultured in bulk on respective media at 28˚C for 2-3 days. The culture plates were cut and placed in conical flasks and soaked with 100% Methanol for 48 hours with repeated sonication and shaking in between. The soaked media was then filtered through Whatmann No.1 filter paper. The filtrate was further filtered twice and evaporated to dryness at 40˚C under reduced pressure using Rotary Evaporation System (BUCHI, Switzerland) to obtain crude bacterial extract. The extracts were then collected and stored at 4˚C until further use (Al-Bari *et al*., 2007).

3.5. *In vitro* antioxidant activity

The bacterial extracts having potent antimicrobial activities were selected for determination of their antioxidant activities using two methods DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2, 2'-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid) assays. Each experiment was conducted in 96 well microtiter plates and performed in triplicate.

3.5.1. DPPH radical scavenging assay

DPPH radical scavenging activity assay was performed as per Villano *et al*., 2007. A stock solution of each extract (5mg/ml) was prepared and it was diluted to concentrations ranging from 10-5000 µg/ml. An aliquot of 50 µl of each dilution was transferred to a 96-well microtiter plate (Tarsons, Kolkata, India). A solution of 0.1mM DPPH (2,2-diphenyl-1-picrylhydrazyl) in methanol was prepared freshly and 200 µl of

the solution was added to plate wells. After mixing thoroughly, the mixture was allowed to react then placed in dark room for 30 min. The absorbance of the mixture was read at 517 nm using Thermo Scan Go microplate reader (Thermo Scientific, USA). Extract without DPPH was used as blank, Ascorbic acid was used as positive control and Methanol as negative control. IC_{50} which represents the amount of antioxidant necessary to produce a 50% reduction of the DPPH was calculated with the calibration curve by linear regression. Results were expressed as a percentage of reduction of DPPH absorption compared to control.

3.5.2. ABTS decolorization assay

$ABTS^{+}$ radical scavenging capacity of the extract was measured with 96-well micotiter plate method (Re *et al.,* 1999). A stock solution of each extract (5mg/ml) was prepared and diluted to concentrations ranging from 10-5000 µg/ml. An aliquot of 150 µl of each dilution was transferred to 96 well microtiter plates. A solution of 7mM ABTS (2, 2'-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid) and 2.45 mM Potassium persulfate (1:1) was prepared in distilled water, incubated for 12-16 hours and diluted with methanol in such a way that an absorbance of 0.7 read at 734 nm was maintained, using Thermo Scan Go microplate reader (Thermo Scientific, USA). To each wells containing extracts, 150 µl of the solution mixture was added mixed thoroughly and then allowed to react in dark room for 7 min, after which absorbance was read at 734 nm. Extract without ABTS was used as blank, Ascorbic acid as positive control and Methanol as negative control

The percentage of DPPH and ABTS+ radical scavenging was calculated by using the equation:

$$\% \text{ Scavenging} = (A_{control} - A_{sample}) / A_{sample} \times 100$$

Where A_{sample} is the absorbance of the test sample and $A_{control}$ is the absorbance of the control.

IC_{50} values were calculated with the help of graph plotted as inhibition percentage against the concentration.

3.6. Antimicrobial activity of selected isolates

The bacterial extracts of the selected isolates were further tested for their antimicrobial activity. A culture plate consisting of nutritional media Mueller-Hinton agar (MHA) was prepared. The pathogens (70µl) were spread on the plates and incubated at 28-30˚C for 24 hours. Extract preparation was done by dissolving each extracts in 5% methanol at varying concentrations of 5,10,20 and 30 mg/ml each. The antimicrobial activity of the extracts was checked using the agar well diffusion assay (Rios *et al.,* 1988). The antimicrobial activity of the crude extracts was evaluated by measuring the diameter of the inhibition zone formed around the wells containing extracts. The mean of the triplicate readings were recorded.

3.7. Antifungal activity

The antifungal activity of all the pure endophytic isolates obtained were tested using dual culture *in vitro* assay (Khamna *et al*., 2008). The test organisms were obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India and Indian Type Culture Collection (ITCC), New Delhi, India. The 10 fungal pathogens that are used were *Aspergillus flavus* (MTCC-9064), *Fusarium graminearum* (ITCC-3437), *Fusarium oxysporum f.sp. Lycopersici* (ITCC-1322), *Fusarium lycopersicum* (MTCC-2480), *Fusarium udum* (MTCC-2755), *Fusarium proliferatum* (MTCC-286), *Fusarium oxysporum* (MTCC-1893), *Trychophyton mentagrophytes* (MTCC-8476), *Macrophomina phaseolina* (MTCC-7428), *Fusarium culmorum* (MTCC-2090). Antifungal test was done by placing 1cm^2 fungal plug on the centre of Potato Dextrose Agar (PDA) plates, each bacterial culture were streaked with a sterile loop on the sides of the plate 2.5 cm from the fungus and incubated at 28˚C for 72 hours, along with control plates without bacterial cultures (Petatán-Sagahón *et al.*, 2011). The percentage of inhibition was calculated using the formula:

C-T/C X 100

where,

C is the growth of fungal pathogens in control

T is the colony growth in dual culture. All experiments were carried out in triplicates

3.8. Cytotoxicity

Methanolic extracts of the endophytic bacterial isolates having significant antimicrobial activity were tested against lung cancer (A549) cell lines, colon cancer (HT29) and cervical cancer (HeLa) cell lines using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (Singh *et al*. 2016). The three test cell lines (A549, HT29 and HeLa) were acquired from National Centre for Cell Sciences (NCCS), Pune, India. Dulbecco's Modified Eagle's Medium (DMEM) amended with 10% inactivated Fetal Bovine Serum (FBS), streptomycin (100 µg/ml), penicillin (100 IU/ml) and amphotericin B (5 µg/ml) was employed to culture stock cells at 37°C in an humidified atmosphere of 5% CO_2 until confluency. The cells were dissociated with trypsin solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 well microtiter plates (Tarsons India Pvt. Ltd., India).

$1x10^4$ cells per 100µl of media per well were seeded in 96-well culture plates and incubated for 24 hr. The cells with 70–80% confluency were treated with the bacterial extracts in different concentrations (5, 10, 20, 40, 80, 100 µg mL-1) and incubated for 72 h. Post incubation, 20 µL of MTT (5mg/mL) solution was added to cells per well and the plate was moved to a cell incubator for another 4 h. Remaining MTT-formazan crystals were dissolved by adding 150 µL of DMSO and the absorbance was measured using a Spectramax M2 Microplate Reader (Molecular Diagnostic, Inc.) at a wavelength of 550

nm. Relative viability was calculated taking wells with non treated cells as 100% control. The results are expressed as mean values (±SD) of six repeats.

The percentage of cell viability was calculated using the formula:

Cell viability (%) = (OD sample – OD blank)/ (OD control – OD blank) x 100%

Where,

OD sample = absorbance of the samples,

OD blank= the absorbance of the blank (of the respective concentration solutions), and

OD control =the absorbance of the control wells

3.9. Molecular characterization and phylogenetic analysis of bacterial isolates

3.9.1. Genomic DNA extraction, amplification of 16S rRNA gene and sequencing

Bacterial universal primers- PA: 5′-AGA GTT TGATCC TGG CTC AG-3′as forward and PH: 5 ′ AAG GAG GTG ATC CAG CCG CA-3′ as reverse (Qin *et al*. 2009) was used for the amplification. The amplified products were quantified by agarose gel electrophoresis (1.5%) and analyzed using a Bio-rad Gel Doc XR+ system (Hercules, CA, USA). The PCR products were purified using Pure-link PCR Purification Kit (In-vitrogen), and was sequenced commercially at Chromegene Pvt. Ltd. India.

3.9.2. Phylogenetic analysis of the isolates

The sequences of the 16S rRNA gene were compared with the GenBank database using BlastN and the most similar match sequence was selected. The sequences were aligned with pair wise alignment using the program Clustal W packaged in the MEGA 6 software (Thompson *et al*., 1997). From this data, a phylogenetic tree was constructed using the maximum likelihood tree (Felsenstein, 1981). Bootstrap analysis was

performed with MEGA 6 using Kimura 2-parameter (K2) for gram-negative bacteria and gram-positive bacteria (Kimura M, 1980).

3.10. Detection of Biosynthetic genes (PKSII and NRPS)

The selected isolated endophytic bacteria were screened for the presence of polyketide synthase (PKSII) and nonribosomal peptide synthetase (NRPS) genes to evaluate their ability to synthesize polyketides and non-ribosomically synthesized oligopeptides. PCR amplification of PKSII was done using the degenerate primers: KS∞-5′-TSGCSTGCTTGGAYGCSATC-3′ and KSβ-5′-TGGAANCCGCCGAABCCTCT-3′ and amplification of NRPS was done using the primers A3F5′-GCSTACSYSATSTACACSTCSGG-3′ and A7R5′-SASGTCVCCSGTSGCGTAS-3′. (Passari *et al.,* 2016). The PCR reaction was carried out in a total volume of 25µl containing 1X reaction buffer, 4mM $MgCl_2$, 0.2mM dNTPs, 10 pmole of each primer and 2U of *Taq* polymerase along with 100 ng of template DNA. Thermal cycling was carried out in verity thermal cycler (Applied Biosystems, Singapore). The PCR conditions for PKSII were: initial denaturation at 94ºC for 5 mins followed by 35 cycles of denaturation at 95 ºC for 1 min, primer annealing for 1 min 30 sec at 58ºC followed by extension at 72 ºC for 2 and final extension at 72 ºC for 10 min. The PCR conditions for NRPS were: initial denaturation at 94ºC for 5 minsfollowed by 35 cycles of denaturation at 95 ºC for 1 min, primer annealing for 2 min at 59ºC followed by extension at 72 ºC for 4 and final extension at 72 ºC for 8 min and the amplified bands were visualized on 1.5% agarose gel Bio-rad Gel Doc XR+ system (Hercules, CA, USA).

3.11. Detection of secondary metabolites using HPTLC

The selected endophytic extract which was sort out after different tests and activities was prepared into fractions using three different solvents with varying polarities. The three different fractions obtained from different solvents i.e., Dichloromethane (DCM), Ethyl acetate (EA) and Methanol (MeOH) were analyzed by TLC and HPTLC. Different

mobile phases were tested for the separation of compounds by TLC. The only mobile phase that allowed us to visualize differences among the extracts for non polar fractions (for DCM and EA) includes Toulene:chloroform:ethanol (4:4:1 v/v/v) and for polar fractions (MeOH) includes Toulene:chloroform:ethanol (4:4:4 v/v/v) at 254 nm and 366 nm. The extract was further analysed using CAMAG HPTLC instrument equipped with win CAT software. A stock solution of all the fractions was prepared. TLC plates with 0.2 mm precoated silica gel 60F254 (Merck, Germany) was used and the sample was spotted using Linomat5 automated sample spotter (CAMEG) using 100µl of syringe (Hamilton, Switzerland). 5 µl of each sample was spotted with band width of 6 mm. The loading of the samples on the TLC plate was done by keeping space of 10 mm from the bottom and 10 mm from the side, and the space between two spots was kept 10 mm of the plate. The TLC plate was kept in a CAMAG twin-trough chamber (10 cm × 10 cm), which was pre-saturated with 10 ml of respective mobile phase for 20 min. The plate was developed upto 90 mm length. After development the dried plate was scanned at 254 nm and 366 nm. The image of this TLC plate was captured using TLC visualizer (CAMEG) under 254 nm and 366 nm. The developed plates were post-derivatized with Anisaldehyde-Sulphuric acid reagent. The TLC plate was heated at 80˚C for 15 min after dipping in Anisaldehyde-Sulphuric acid reagent then the plates were scanned by the CAMAG TLC Scanner III at a wavelength of 500 nm.

CHAPTER 4

RESULTS

4.1. Isolation of bacteria from the bark, leaf and root endospheric tissues of *Dillenia pentagyna*

In total, 96 bacterial endophytes were isolated from the barks, leaves and roots of *Dillenia pentagyna* Out of which 39 isolates were obtained from bark tissues, 37 isolates from root tissues and 20 isolates from leaf tissues. The colonization of bacterial endophytes was recorded highest in barks (48.62%) followed by roots (38.54%) and leaves (20.83) (**Figure1**). Among the nutritional media used, Starch Casein Agar (SCA) (n=42) media yields highest number of endophytic isolates followed by Luria Bertiani (LB) Agar (n=25). (**Figure 2**)

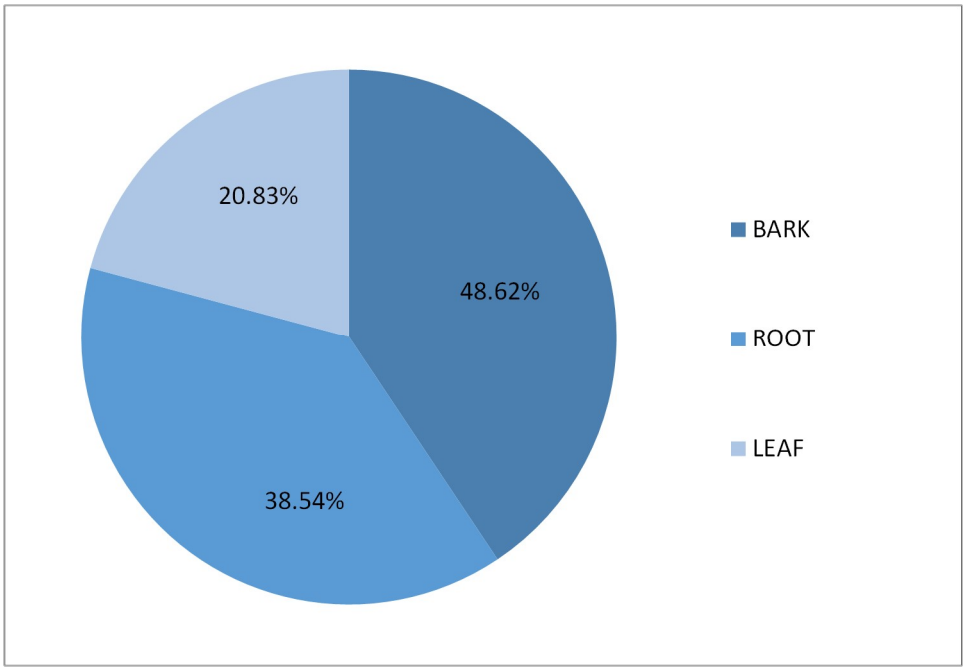


Figure 1: Pie chart showing the distribution of endophytic bacteria isolated from different tissues.

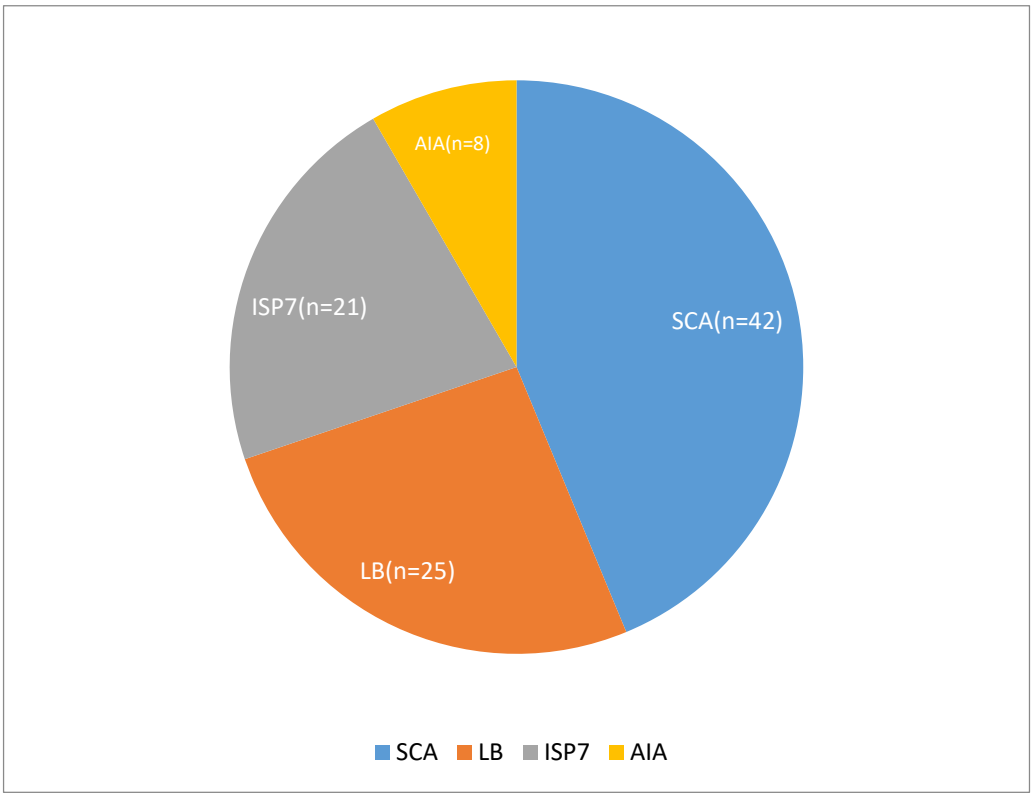


Figure 2: Pie chart showing the distribution of obtained bacterial isolates using different nutritional media.

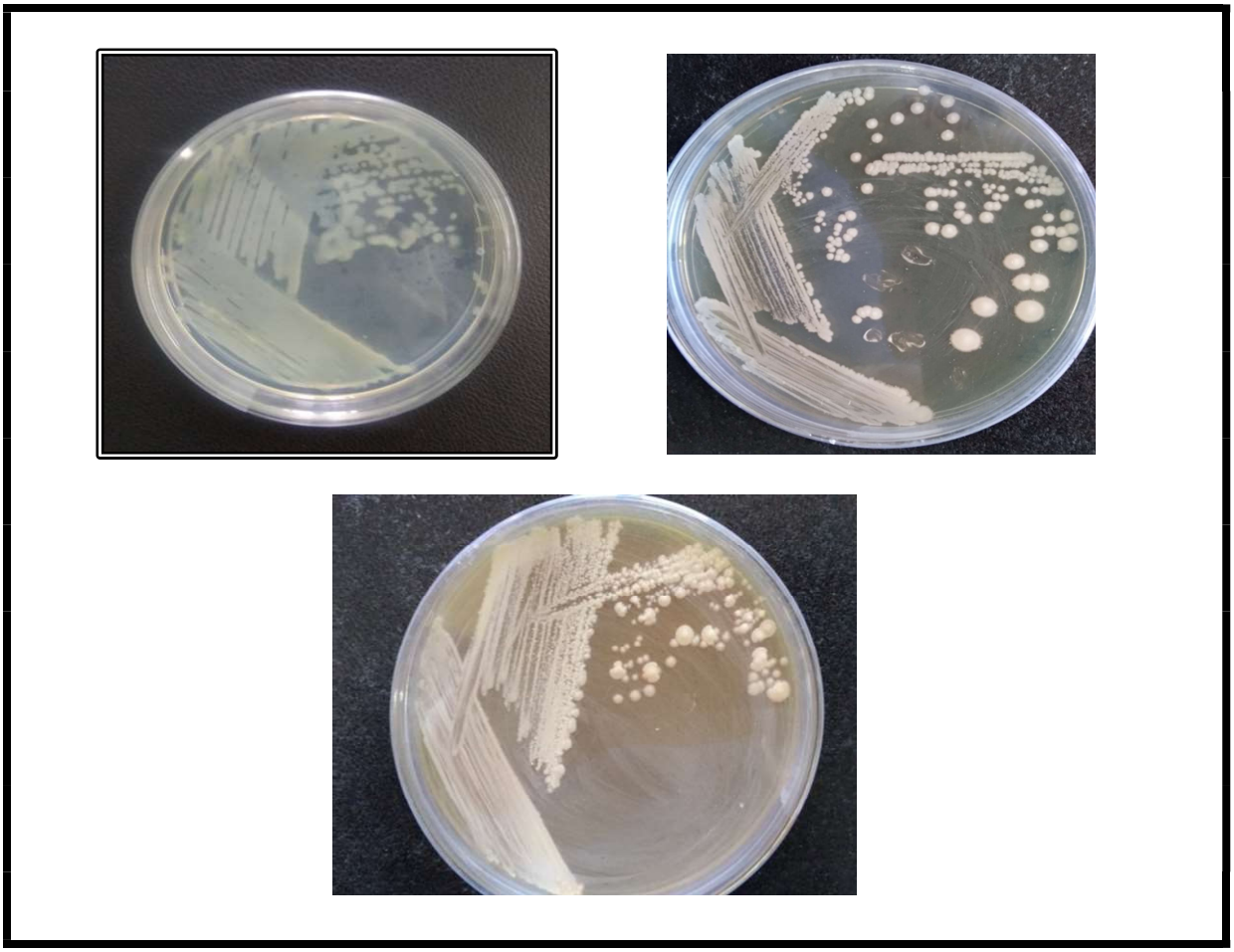


Figure 3: Pure culture isolates

4.2. Surface sterilization validation

After incubation of the plates obtained from the tissue fingerprint as well as the last wash, no growth was observed which showed successful surface sterilization of the tissues of bark, leaf and root of *Dillenia pentagyna* **(Figure 4)**

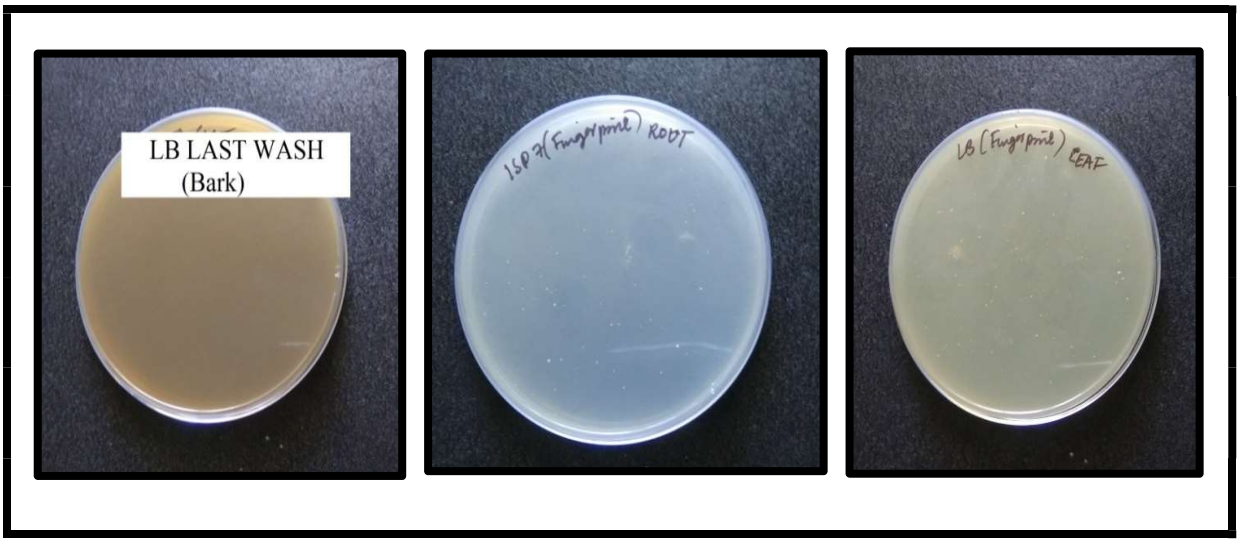


Figure 4: Validation of surface sterilization of tissue fingerprint and last wash (from left to right)

4.3. Antimicrobial activity

Antimicrobial activity test showed that out of the 96 bacterial endophytic broth cultures, 22 isolates showed activity against at least one of the tested pathogen

Table 1: Antimicrobial activity of broth cultures (-) Negative (+) Positive activity

Isolate no.	KP	SA	BS	EC	CA	ML	PA	EF	ST	SC	SP
BPSL6	-	+	-	-	-	-	-	-	-	-	-
BPSL7	+	-	-	-	-	-	-	-	-	-	-
BPSL8	-	-	+	-	-	+	-	+	-	-	-
BPSL11	-	-	-	-	-	+	-	-	-	-	-

BPSL17	+	-	+	-	-	+	-	-	-	-	-
BPSL18	-	-	+	+	-	+	-	-	-	-	-
BPSL21	+	-	+	+	-	+	-	-	-	-	-
BPSL26	-	-	-	-	+	-	-	-	-	-	-
BPSL29	-	+	+	+	-	+	-	-	-	-	-
BPSL38	-	-	-	-	-	+	-	-	-	-	-
BPSL44	-	+	+	+	-	+	-	-	-	-	-
BPSL45	+	-	+	-	-	+	-	-	-	-	-
BPSL48	-	+	+	+	+	+	-	-	-	-	-
BPSL53	-	-	-	-	-	+	-	-	-	-	-
BPSL56	-	-	-	-	-	+	-	-	-	+	-
BPSL85	-	-	-	-	-	+	-	-	-	-	-
BPSL97	+	-	-	-	-	+	-	-	-	+	-
BPSL98	-	-	-	-	-	+	-	-	-	-	-

BPSL105	-	+	-	-	-	+	-	-	-	+	-
BPSL107	-	+	-	-	-	-	-	-	-	+	-
BPSL108	+	-	-	-	-	+	-	-	-	-	-
BPSL109	-	-	-	-	-	+	-	-	-	+	-

(Pseudomonas aeruginosa (PA),Staphylococcus aureus (SA), *Escherichia coli* (EC), *Candida albicans* (CA), *Enterococcus faecalis* (EF), *Salmonella typhimorium* (ST), *Bacillus subtilis* (BS), *Klepsiella pneumonia* (KP), *Saccharomyces cerevisiae* (SC), *Streptococcus pneumonia* (SP) *and Micrococcus luteus* (ML)) (-) Negative (+) Positive activity

4.4. DPPH radical scavenging assay

A total of 22 extracts (methanolic) obtained from different selected isolates were used for DPPH assay. DPPH radical scavenging assay results showed that the methanolic extract BPSL8 showed a potent reducing capacity of DPPH measured at an IC50 value of **6.29**µg/ml **(Table 2)**. This activity shown by BPLS8 was found to be the best out of all the tested 22 endophytic extracts.

Table 2: DPPH activity from selected isolates

(Data presented in Mean ± Standard deviation from triplicate sample)

Sl.no	SAMPLE	IC_{50} (µg/ml) ± SD
1	BPSL6	131±0.13

2	BPSL7	20.38 ±0.54
3	BPSL8	**6.29±0.62**
4	BPSL11	226 ±0.34
5	BPSL17	11.01 ±0.56
6	BPSL18	91.02 ±0.52
7	BPSL21	1147±0.21
8	BPSL26	76.6±0.89
9	BPSL29	1231 ±0.20
10	BPSL38	72.4 ±0.18
11	BPSL44	55.59 ±0.63
12	BPSL45	269.8 ±0.71
13	BPSL49	383.6 ±0.53
14	BPSL53	14.5 ±0.74
15	BPSL56	14.28±0.38
16	BPSL85	432.6±0.40
17	BPSL97	238.2±0.51
18	BPSL98	230.1 ±0.69
19	BPSL105	62.1 ±0.31
20	BPSL107	20.04 ±0.62

21	BPSL108	12.9±0.54
22	BPSL109	12.51 ±0.47
Ascorbic Acid		**33.24±0.52**

4.5. ABTS Decolorization Assay

Out of the 22 tested endophytic extracts obtained from different selected isolates, the methanolic extract of BPSL97 was found to exhibit a potent reducing potential of ABTS radical cation with an IC_{50} value of 4.988µg/ml **(Table 3)**. ABTS activity of BPSL97 is considerably higher compared to the other tested extracts.

Table 3: ABTS activity of selected isolates

(Data presented in Mean ± Standard deviation from triplicate sample)

Sl.no	SAMPLE	IC_{50} (µg/ml) ± SD
1	BPSL6	90.5±0.21
2	BPSL7	26.56±0.2
3	BPSL8	15.4±0.35
4	BPSL11	374.2±0.7
5	BPSL17	33.05±0.13
6	BPSL18	38.14±0.11
7	BPSL21	152.9±0.62

8	BPSL26	126.4±0.87
9	BPSL29	71.4±0.54
10	BPSL38	24.69±0.09
11	BPSL44	59.31±0.38
12	BPSL45	2923±0.44
13	BPSL49	638.6±0.23
14	BPSL53	13.7±0.16
15	BPSL56	437.5±0.12
16	BPSL85	15.1±0.41
17	BPSL97	**4.988±0.75**
18	BPSL98	107.2±0.23
19	BPSL105	15.22±0.12
20	BPSL107	170±0.18
21	BPSL108	16.94±0.66
22	BPSL109	14.91±0.46
Ascorbic Acid		**14.65±0.92**

4.6. Antimicrobial activity of selected bacterial extracts

To evaluate antimicrobial activity, methanolic extracts prepared from selected 22 bacterial isolates were tested against 11 pathogens. Methanolic extract obtained from a total of 13 isolates out of 22 selected isolates showed antimicrobial activity by forming a zone of inhibition at the concentration of 30mg/ml (**Table 4; Figure 5**). Among the bacterial isolates extracts, isolate number BPSL85 showed the highest zone of inhibition (18±0.12 mm) which is followed by BPSL8 (16±0.28mm) against *Micrococcus luteus.* **(Table 5)**

Table 4: Antimicrobial activity of selected bacterial extracts

	BPSL6	BPSL7	BPSL8	BPSL11	BPSL17	BPSL21	BPSL29	BPSL45	BPSL49	BPSL53	BPSL56	BPSL85	BPSL108
Bacillus subtilis	+	-	+	-	+	+	-	+	+	-	-	+	+
Klepsiella pneumonia	+	+	-	-	+	+	+	-	-	-	-	+	-
Micrococcus Luteus	+	+	+	+	+	-	-	+	+	+	+	+	-

Table 5: Antimicrobial activity with zone of inhibition

	Zone of inhibition (mm±SE)												
	BPSL6	BPSL7	BPSL8	BPSL11	BPSL17	BPSL21	BPSL29	BPSL45	BPSL49	BPSL53	BPSL56	BPSL85	BPSL108
Bacillus Subtilis	10.05±0.26	0.0±0.0	12.5±0.12	0.0±0.08	6.2±0.26	2.2±0.3	0.0±0.0	3.6±0.1	1.5±0.2	0.0±0.0	0.0±0.0	4.2±0.18	5.5±0.28
Klepsiella Pneumoniae	9.66±0.76	8.5±0.28	0.0±0.0	0.0±0.0	7.2±0.16	3±0.24	2.5±0.28	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	8.2±0.26	0.0±0.0
Mirococcus Luteus	8±0.28	11±0.12	16±0.28	8±0.22	14.5±0.18	0.0±0.0	0.0±0.0	4.4±0.12	5±0.16	12.5±0.2	6±0.08	18±0.12	0.0±0.0

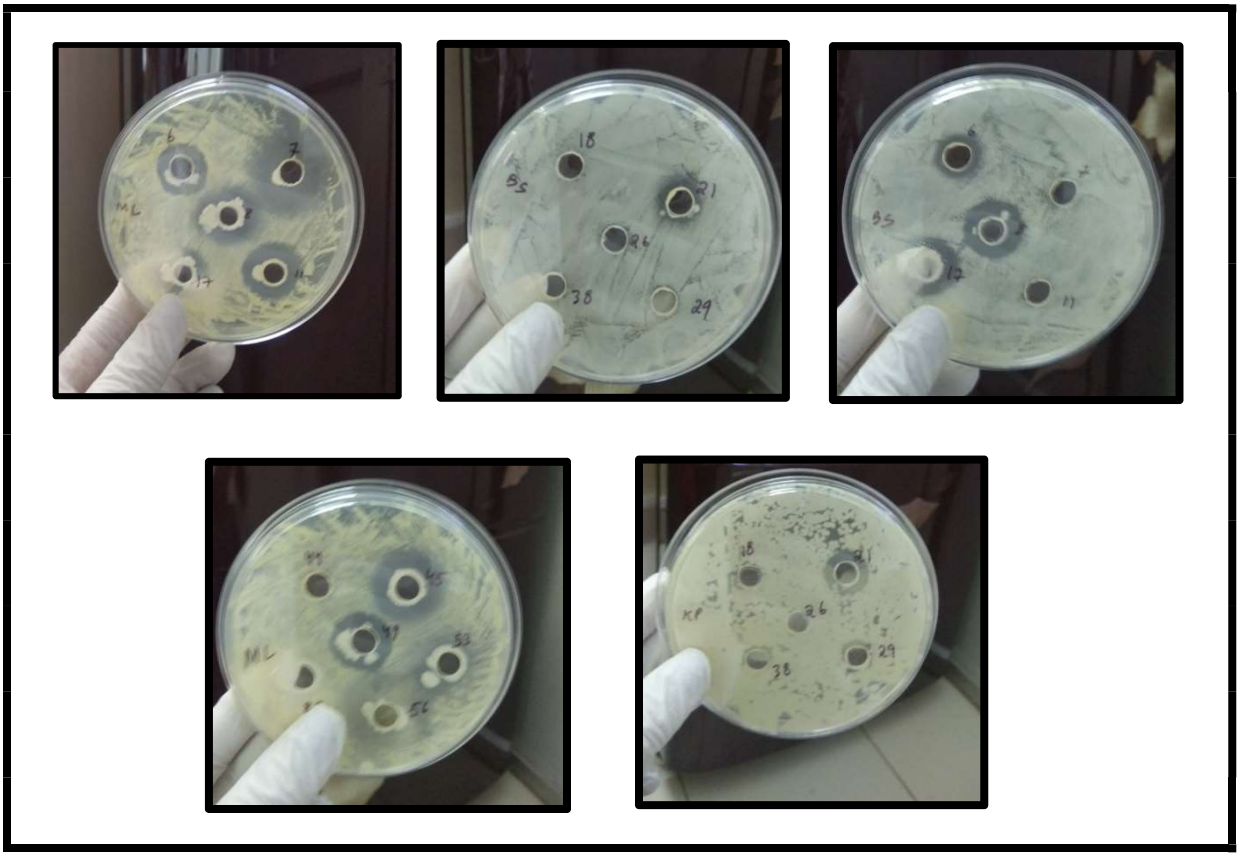


Figure 5: Image showing inhibition zone (Agar well diffusion method)

4.7. Antifungal Assay

To evaluate antagonistic property of bacterial endophytes, antifungal assay was performed for all the 96 pure culture isolates obtained. Screening was done in triplicates and a total of 19 endophytic bacterial cultures showed potential activity against 2 or more of the tested fungal pathogens (**Table 5**). Out of 19 potent isolates two isolate

number BPSL21 and BPSL16 showed highest inhibition against *F. culmorum* and *T.mentagrophytes* with inhibition percentage of 95% and 93.6% respectively (**Table 6;Figure 6**)

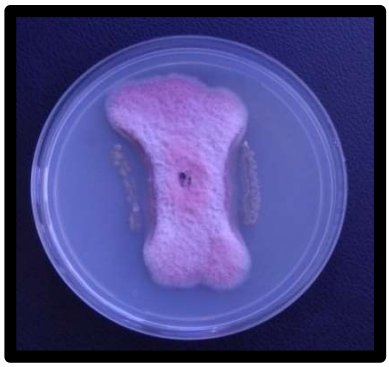
Table 6: Percentage of inhibition of fungal pathogens (%)

(Data presented in Mean ± Standard deviation from triplicate sample)

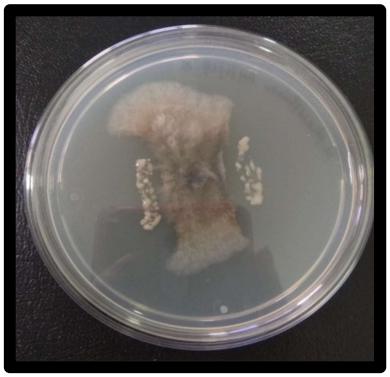
Endo Bacterial Isolate	Percentage of inhibition (%) ± Standard Deviation (SD)									
	F.lycopersicum (MTCC)	*F.oxysporum (MTCC)*	*F.culmorum (MTCC)*	*F.gramineaeum(ITCC)*	*A.flavus (MTCC)*	*F.udum (MTCC)*	*F.oxy* f.sp.*lyco (ITCC)*	*T.mentagrophytes (MTCC)*	*M.phaseolina (MTCC)*	*F.proliferatum (MTCC)*
BPSL8	50±0.7	-	91.6±0.3	36.6±0.4	-	46.7±0.3	50±0.2	91±0.1	**92.6±0.1**	66.6±0.3
BPSL10	58.3±0.4	50.1±0.1	90.6±0.2	60±0.2	-	50±0.5	58.3±0.1	89±0.2	91.6±0.2	58.3±0.4
BPSL15	50±0.2	66.7±0.3	88.3±0.1	61.6±0.2	-	61.7±0.8	48.3±0.3	**93.6±0.1**	91.6±0.1	66±0.9
BPSL16	46.6±0.1	58.3±0.6	90±0.4	53.3±0.1	-	53.3± 0.4	90±0.6	**92.5±0.3**	-	-
BPSL17	58.3±0.5	70±0.2	91.67±0.7	66.6±0.3	-	53.4±0.6	55±0.5	86.6±0.4	90±0.7	58±0.4
BPSL18	48.3±0.3	-	-	41,7±0.1	-	-	-	85±0.2		50±0.6
BPSL21	56.6±0.2	60±0.1	**95±0.6**	66.7±0.6	-	50±0.3	58.3±0.1	86±0.4	83.3±0.1	66.7±0.4
BPSL26	58.2±0.8	-	-	-	-	-	-	83.3±0.8	80±0.7	41.6±0.1
BPSL28	55±0.4	81.6±0.3	92.7±0.1	61.7±0.6	-	50±0.2	50±0.7	85±0.6	91±0.3	66.6±0.1
BPSL29	46.7±0.5	83.4±0.2	91.6±0.2	58.3±0.8	-	43.4±0.4	-	86±0.1	91.6±0.2	58.3±0.3

BPSL44	41.6±0.3	-	91.7±0.6	-	-	45±0.1	58.3±0.9	80±0.2	83.3±0.2	-
BPSL45	61.6±0.5	76.6±0.5	-	60±0.7	-	41.6±0.1	61.6±0.4	86±0.1	88.3±0.1	-
BPSL48	-	-	-	41.6±0.9	-	-	-	80±0.3	83.3±0.1	41.6±0.8
BPSL50	-	-	-	-	-	41.7±0.2	-	83±0.1	-	41.6±0.1
BPSL80	-	83.3±0.1	-	-	-	55±0.8	50±0.2	-	-	50±0.2
BPSL85	-	-	-	-	-	-	-	-	-	-
BPSL105	58.3±0.7	-	-	36.7±0.2	-	41.6±0.5	2.5±0.3	83.3±0.6	-	-
BPSL106	-	83.3±0.2	-	-	-	-	-	83.2±0.4	-	-
BPSL109	41.6±0.1	78.3±0.1	-	-	-	-	-	-	-	-

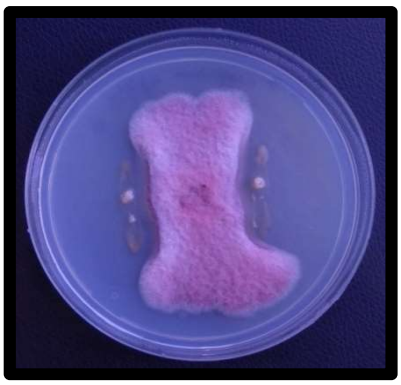
(*Fusarium lycopersicum, Fusarium oxysporum, Fusarium culmorum, Fusarium graminearium, Aspergillus flavus, Fusarium udum, Fusarium f. sp. lycopersicum, Trichophyton mentagrophytes, Macrophomina phaesolina, Fusarium proliferatum)*



T.mentagrophytes



M.phaesolina



F.Oxy f.sp.lyco

F.udum

F.lycopersicum

F.proliferatum

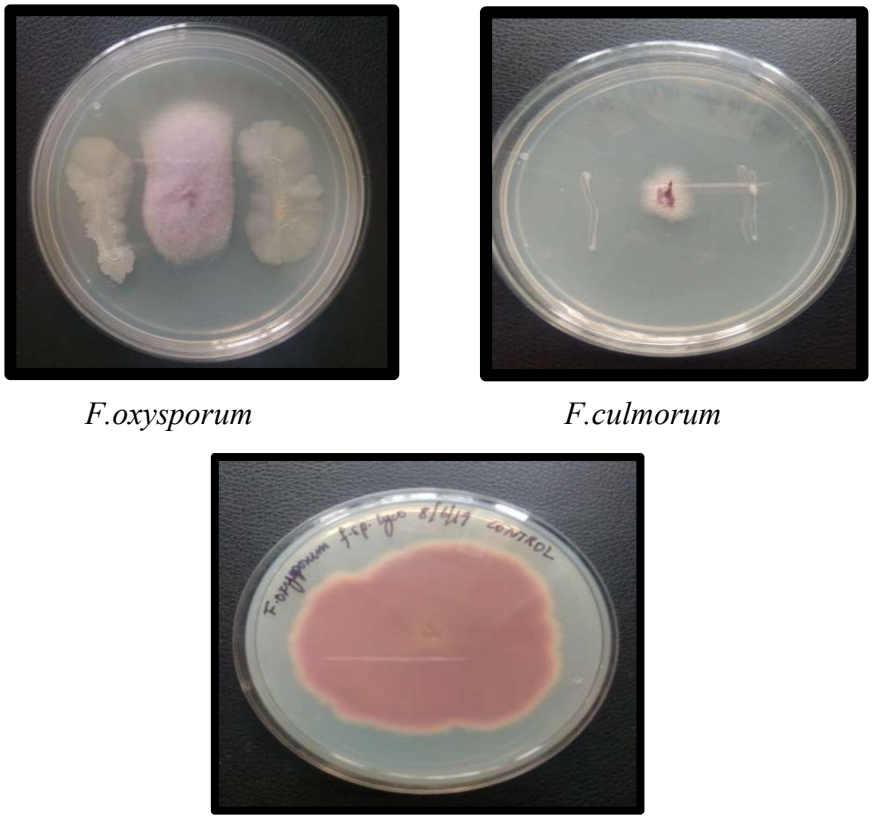


Figure 6: Antifungal pictures showing zone of inhibition by different fungal species (*T.mentagrophytes, M. phaesolina, F. oxy f. sp. lyco, F.udum, F. lycopersicum, F. proliferatum, F.oxysporum, F. culmorum* and Control)

4.8. Cytotoxicity

The best four isolates BPSL6, BPSL8, BPSL17 and BPSL85 were selected based on their antioxidant and antimicrobial activity. The four isolates were tested to evaluate their cytotoxic activity against three human cancer cell lines A549, HT 29 and HeLa. The results revealed that isolate number BPSL85 showed significant cytotoxicity against HT29 and HeLa with IC_{50} of 67.01 and 61.2 respectively **(Table 6; Figure 7)**

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Table 7: Cytotoxicity of endophytic bacterial extracts against human cancer cell lines

Cell lines	IC_{50} (µg/ml)±SE			
	BPSL6	BPSL8	BPSL17	BPSL85
A549	1073±0.7	125.8±0.8	106.3±1.8	609±1.61
HT29	443.7±2.4	323.2±1.5	322.2±1.9	**67.01±2.0**
HeLa	119±1.9	168.7±3.1	392±1.7	**61.2±1.6**

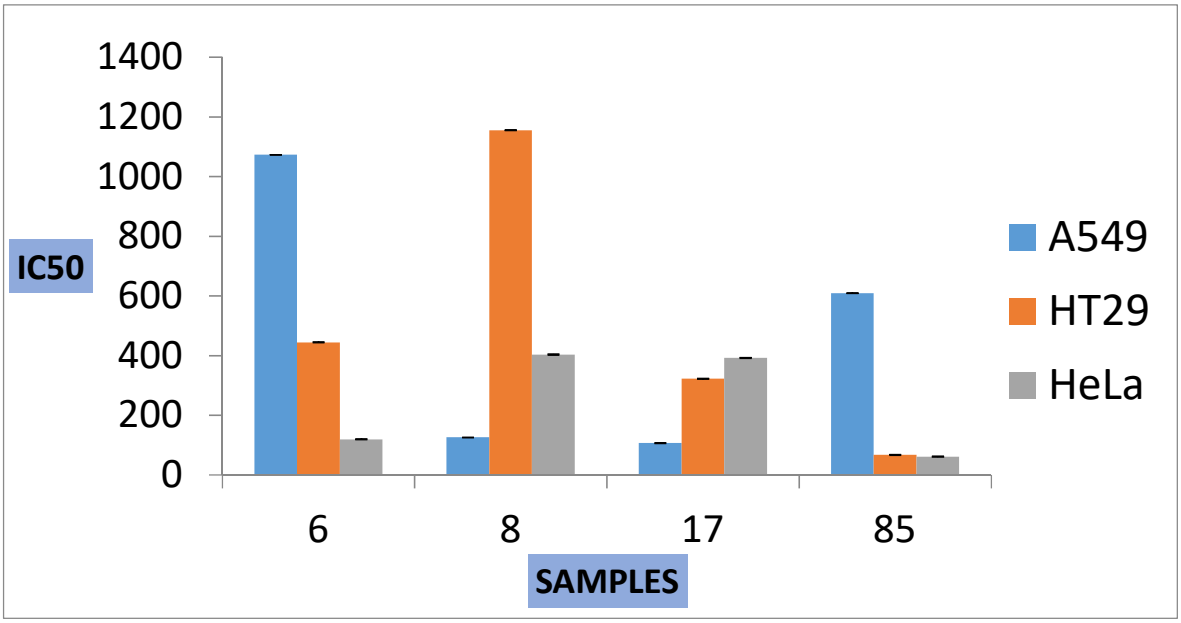

Figure 7: Graphical representation of IC_{50}±SE

4.9. Genomic DNA extraction, amplification of 16S rRNA gene and sequencing

Genomic DNA of the isolates organisms was extracted and the quality was checked on 0.8% agarose gel and by using spectrophotometer (**Figure 8**). Amplification of 16S rRNA gene was done using Applied Biosystems thermal cycler PCR. The PCR product was run on 1.2% agarose gel with low range DNA ruler plus (100 bp to 3 kb) as molecular markers. For all the isolates a single amplicon of 1500 bp was amplified. The amplified PCR product was commercially sequenced and the sequences were analyzed using NCBI BLAST (Basic Local Alignment Search Tool) and the analyzed sequences were submitted to NCBI Gen Bank (**Table 8**)

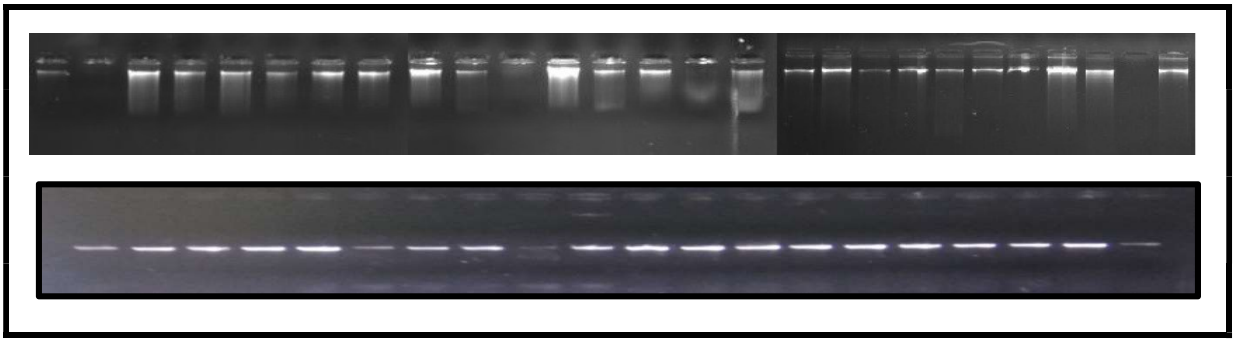

Figure8: Genomic DNA bands of the isolates under UV light, documented using a Bio-rad Gel Doc XR+ system

Table 8: Identification of the positive bacterial isolates based on 16S rRNA Gene sequences

Isolate no.	Gene Bank Accession no.	Closest species with Accession No.	Similarity	Identification
BPSL6	MT795822	*Burkholderia sp*. MK691482	99.58%	*Burkholderia sp*.
BPSL8	MT795803	*Bacillus pumilus* MK748178	100%	*Bacillus pumilus*
BPSL11	MT795811	*Bacillus thuringiensis* MT178209	99.67%	*Bacillus thuringiensis*
BPSL17	MT795814	*Bacillus subtilis* MT423973	100%	*Bacillus subtilis*

BPSL18	MT795806	*Lysinibacillus boronitolerans* MF111565	100%	*Lysinibacillus boronitolerans*
BPSL21	MT795819	*Bacillus toyonensis* MT052648	100%	*Bacillus toyonensis*
BPSL26	MT795804	*Bacillus sp.* KX817879	99.5%	*Bacillus sp*
BPSL29	MT795816	*Bacillus safensis* JX536689	99.86%	*Bacillus safensis*
BPSL38	MT795810	*Bacillus cereus* KF956599	99.51%	*Bacillus cereus*
BPSL44	MT795823	*Pseudomonas aeruginosa* MH727999	99.6%	*Pseudomonas aeruginosa*
BPSL45	MT795820	*Pseudomonas aeruginosa* MK713646	99.8%	*Pseudomonas aeruginosa*
BPSL48	MT795805	*Serratia sp*. MK397529	100%	*Serratia sp*
BPSL49	MT795809	*Bacillus sp.* MT472064	100%	*Bacillus sp.*
BPSL53	MT795812	*Bacillus altitudinis* MN543810	99.72%	*Bacillus altitudinis*
BPSL56	MT795824	*Burkholderia cenocepacia* MK615919	99.6%	*Burkholderia cenocepacia*
BPSL85	MT795813	*Brevibacterium sp*. MT433875	100%	*Brevibacterium sp*.
BPSL97	MT795815	*Bacillus aryabhattai* MT091981	100%	*Bacillus aryabhattai*
BPSL98	MT795808	*Bacillus tropicus* MT611943	99.6%	*Bacillus tropicus*

BPSL105	MT795817	*Bacillus altitudinis* MT071740	100%	*Bacillus altitudinis*
BPSL107	MT795818	*Bacillus cereus* MK743993	99.21%	*Bacillus cereus*
BPSL108	MT795807	*Bacillus velezensis* MT649755	100%	*Bacillus velezensis*
BPSL109	MT795821	*Alcaligenes faecalis* MH793406	100%	*Alcaligenes faecalis*

4.10. Phylogenetic Analysis

The relationship among the selected potential isolates (22) was carried out by aligning their 16S rRNA gene sequences along with the type strains retrieved from EZTaxon database.

The Gram positive bacteria were observed to be clubbed within a single clade belonging to the genera of *Bacillus, Lysinibacillus* and the lone actinobacteria *Brevibacterium*. Out of the total 22 selected 15 belongs to the family of Bacillaceae formed the major clade accounting for 68.18% of the total bacteria analyzed. The remaining Gram positive bacteria accounting for 4.5% belonged to Brevibacteriaceae family.

Among the Gram negative bacteria 13.6% belonged to the Class Gammaproteobacteria (3 isolates) and Betaproteobacteria (3 isolates) respectively which was the second biggest clade. *Pseudomonas* (Order: Pseusomonadales) and *Serratia* (Order: Enterobacterales) were the two genera of Gammaproteobacteria which were claded separately. Similarly, Alcaligene and Bulkhorderia formed the two genera of Betaproteobacteria which belonged to the same order of Bulkhorderiales.

The phylogenetic tree was constructed using Maximum likelihood method with Kimura 2 parameter to the lowest BIC values using Mega 6 with the estimated Transition/Transversion bias (R) value is 1.83 and 1.36 respectively. The tree with the

highest log likelihood (-1443.0171) is shown (Figure 9). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 40 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 289 positions in the final dataset.

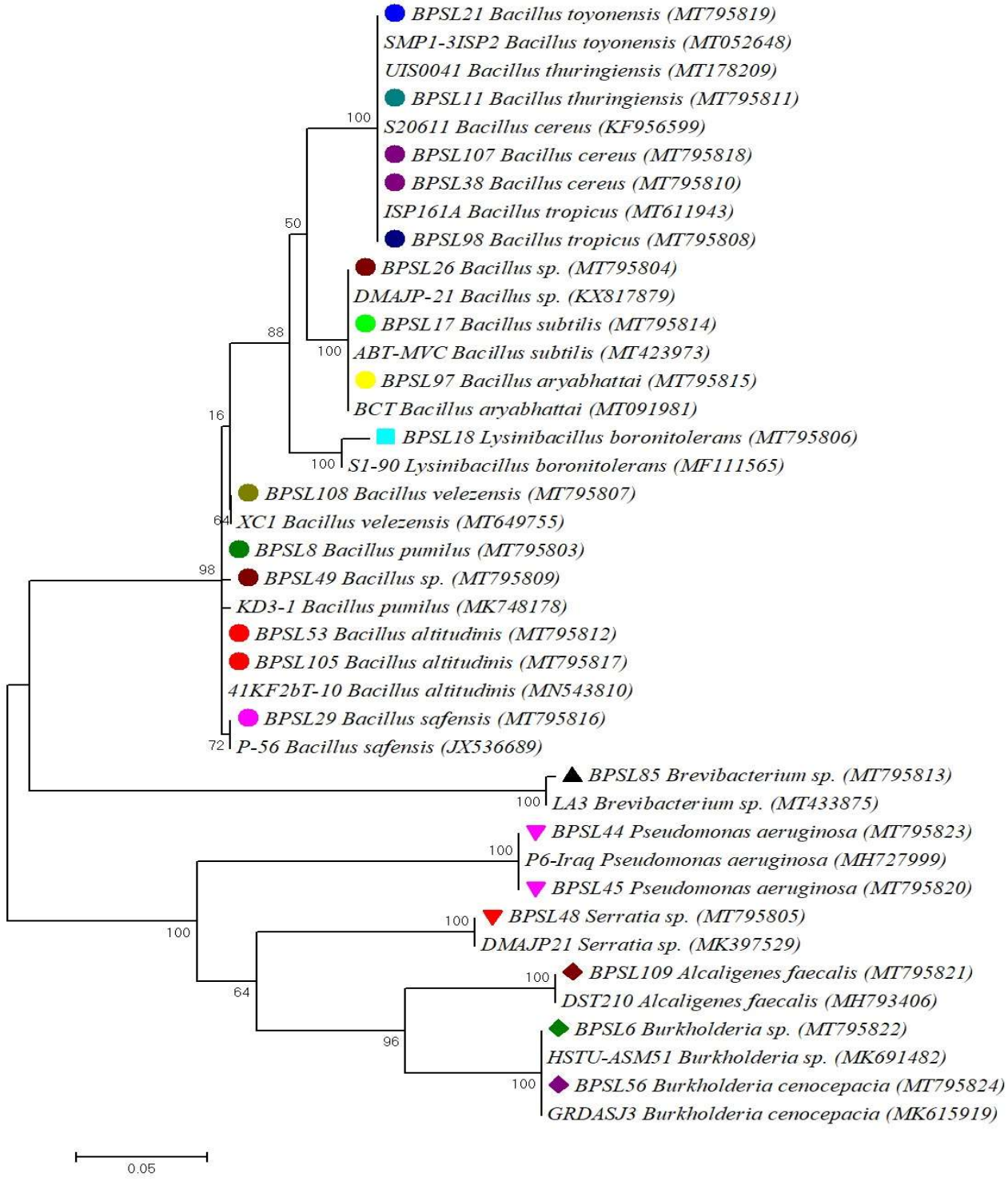

Figure 9: Molecular Phylogenetic analysis by Maximum Likelihood method using MEGA 6

4.11. Detection of PKSII and NRPS in selected strains

PCR amplification was done to detect PKSII and NRPS gene using specific primers in the samples selected based on antimicrobial activity. PCR amplification of expected size for PKSII was observed for BPSL108 when viewed in 1.5% Agarose gel. Whereas, NRPS candidate amplicons were detected in three isolates (BPSL17, BPSL85, BPSL108)

Table 9: Detection of biosynthetic genes using PCR amplification

	Biosynthetic genes	
Isolates	PKSII	NRPS
6	-	-
7	-	-
8	-	-
11	-	-
17	-	+
21	-	-
29	-	-
45	-	-
49	-	-
53	-	-
56	-	-

85	+	+
108	+	+

(+) positive (-) negative)

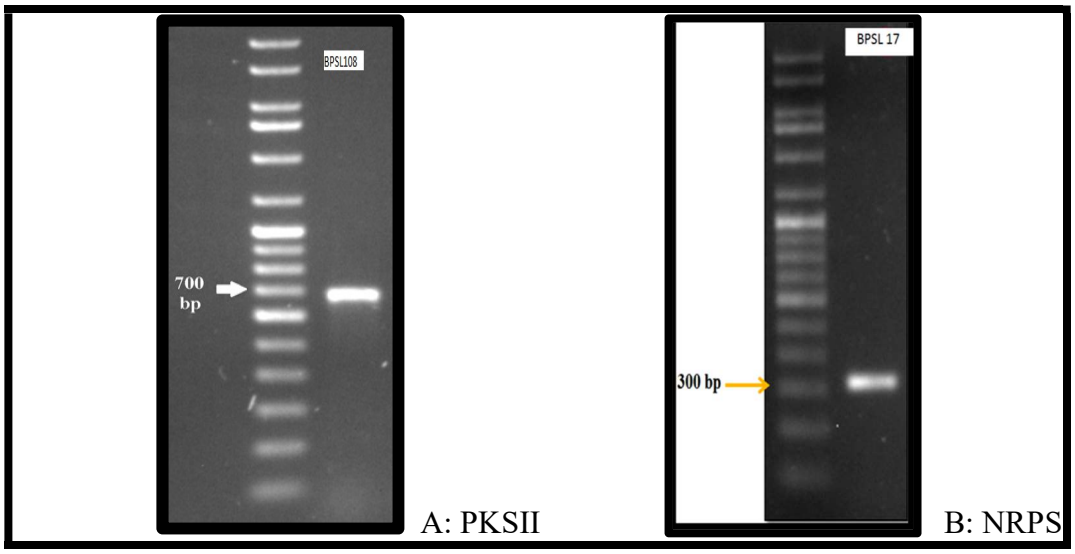

Figure 10: PCR amplification of PKSII (A) and NRPS gene (B)

4.12. HPTLC result for endophytic bacterial extracts

HPTLC method was developed for three different fractions i.e., Dichloromethane (DCM), Ethyl Acetate (EA) and Methanol (MeOH) of BPSL8. In this technique performed clear separation was achieved by using Toulene:chloroform: ethanol (4:4:1 v/v/v) for non polar fractions (DCM and EA) and Toulene: chloroform: ethanol (4:4:4 v/v/v) for polar fractions (MeOH). Post-derivatization with anisaldehyde-sulphuric acid reagent gave good results. Different color bands were observed which indicates the presence of phenols, sugars, steroids, and terpenes **(Figure 15)**

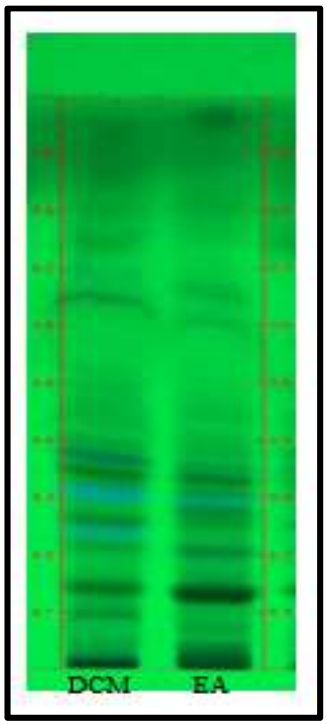

Figure 11: TLC plate of DCM and EA fractions visualized at 254 nm using CAMEG

Table 10: HPTLC profiling at 254nm for BPSL8 DCM and EA fractions

DCM				EA			
Peak No	R_f	Area	% Area	Peak No	R_f	Area	% Area
1	0.04	104.0 AU	0.23%	1	0.04	403.8 AU	0.72%
2	0.14	3293.7 AU	7.44%	2	0.10	1199.5 AU	2.15%
3	0.24	7650.1 AU	17.28%	3	0.22	16292.8 AU	29.17%
4	0.39	2594.5 AU	5.86%	4	0.38	5988.6 AU	10.72%
5	0.49	7215.3 AU	16.30%	5	0.48	5070.4 AU	9.08%

6	0.58	3816.9 AU	8.62%	6	0.52	4642.4 AU	8.31%
7	0.66	8734.5 AU	19.73%	7	0.58	4826.9 AU	8.64%
8	0.72	8593.8 AU	19.42%	89	0.64	7046.6 AU	12.62%
9	0.87	1569.7 AU	3.55%	10	0.68	2876.9 AU	5.15%
10	0.96	687.2 AU	1.55%	11	0.74	4375.8 AU	7.84%
				12	0.87	3123.5 AU	5.59%

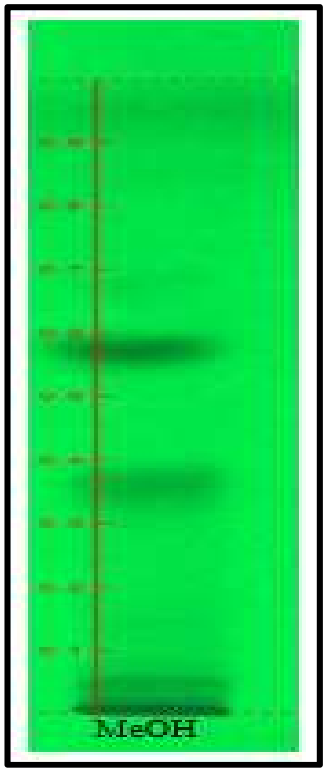

Figure 12: TLC plate of Methanol fraction visualized at 254 nm using CAMEG

Table 11: HPTLC profiling at 254nm for BPSL8 Methanol fraction

Methanol			
Peak No	R_f	Area	Area %
1	0.07	1045.0 AU	5.42%
2	0.10	129.7 AU	0.67%
3	0.16	789.2 AU	4.14%
4	0.39	8047.6 AU	41.73%
5	0.60	8030.6 AU	41.65%
6	0.65	221.9 AU	1.15%
7	0.68	359.1 AU	1.86%
8	0.71	650.9 AU	3.38%

Figure 13: TLC plate of DCM and EA fractions visualized at 366 nm using CAMEG

Table 12: HPTLC profiling at 366nm for BPSL8 DCM and EA fractions

DCM				EA			
Peak No	R_f	Area	Area %	Peak No	R_f	Area	Area %
1	0.14	184.6 AU	0.59%	1	0.21	344.9 AU	3.22%
2	0.45	9698.8 AU	30.79%	2	0.24	301.2 AU	2.81%
3	0.58	14661.5 AU	46.55%	3	0.37	2089.3 AU	19.51%
5	0.72	6487.0 AU	20.60%	4	0.56	4841.2 AU	45.21%
5	0.95	463.8 AU	1.47%	5	0.69	2501.1 AU	23.36%
				6	0.88	240.3 AU	2.24%
				7	0.93	391.0 AU	3.65%

Figure 14: TLC plate of Methanol fraction visualized at 366 nm using CAMEG

Table 13: HPTLC profiling at 366nm for BPSL8 Methanol fraction

Methanol			
Peak	R_f	Area	Area%
1	0.09	176.9 AU	27.86%
2	0.16	383.1 AU	60.35%
3	0.65	74.8 AU	11.79%
4	0.15	178.9 AU	7.81%

Figure15: Post derivatization of DCM, EA and Methanol fractions

CHAPTER 5

DISCUSSION

Endophytes are the microorganisms living in mutualistic association with plants by living inside the plant tissues without producing symptoms of disease. Medicinal plants have been used throughout history for their therapeutic properties, therefore, they are considered a good choice for endophytic investigations (Kaul *et al*., 2012; Ebrahimi *et al*., 2012) Plants are not sessile entities, but they live in close proximity with microorganisms such as those living in the rhizospheric and phyllospheric environment. However, plants develop a special and more complex relationship with microorganism living inside the plant tissues in their intercellular or intracellular spaces. The intricacy of this complex interaction can be embodied between enormously committed mutualism and saprophytism (Nain and Padmavathy, 2014). Endophytes have several indispensable functions that confer various benefits to their hosts. In this mutualistic cost-benefit interaction both endophyte and host are benefitted and none of the partners were detected to be harmed. The most commonly encountered endophytes are those which confer a number of benefits to the host in the uptake of nutrients (Islam *et al*., 2019). Endophytic bacteria protect the host plants by producing several classes of secondary metabolites to protect the host from biotic stresses such as attacks by fungal phytopathogens and insect pests. They also mitigate the harmful impact of abiotic stresses such as drought, salinity and high temperatures (Tripathi *et al.,* 2018).

In the age of multi drug resistant microorganisms, new locations or niches are urgently needed to be explored to discover new or potent source of bioactive metabolites. Endophytic bacteria living in the endosphere of plants are widely regarded as reservoir of bioactive secondary metabolite sand only a few studies have been undertaken on the diversity, distribution and bioactivity of endophytic bacteria harbored by plants (Kobert *et al*., 2014). Therefore the present study seeks to evaluate the bioactive potential of bacterial endophytes from the selected traditional medicinal plant of Mizoram, northeast

India and their direct or indirect correlation with the phytochemical constitutes and biological activity of the host plant.

Plant has long been a very important source of drug and screening has been done for many plants to see if they contain compounds with therapeutic activity. Therefore, it is vital to evaluate the antioxidant activity of the endophytes isolated from such plants (Rosy *et al*., 2010) The reactive oxygen species (ROS), like peroxides, superoxide, singlet oxygen, etc exert oxidative damaging effects by reacting with nearly every molecules found in living cells including protein, lipid, amino acids and DNA (Apel and Hirt, 2004). Compounds having antioxidant activity can reduce the level of these ROS to a normal (Ahmad *et al*., 2014). DPPH is a stable free radical which produces deep purple color in methanol solution and his can be reduced to purple colored solution in the presence of hydrogen donating antioxidants by the formation of yellow coloured diphenyl-picryl hydrazine (Sahoo *et al*., 2013). Our findings suggested that *Bacillus pumilu*s (BPSL8) is an antioxidant molecule- producing organism whose methanolic extracts showed a potent reducing capacity of DPPH with an IC_{50} value of 6.26µg/ml.

ABTS delocorization assay is based on the inhibition of the absorbance of radical cation, ABTS+ generated by potassium persulfate (a bluegreen chromogen) by antioxidants. In the presence of an antioxidant molecule, the colored radical is reduced to colorless solution (Sreejayan *et al*., 1996). Our findings showed that the methanolic extracts of *Bacillus aryabhattai* (BPSL97) showed the highest reduction potential of ABTS with an IC_{50} value of 4.988µg/ml which suggested that it contains a very potent antioxidant molecule or compounds.

Endophytes associated with medicinal plants are rich sources of secondary metabolites with antimicrobial activity, and they spend their whole life cycle within plant tissues without causing any infections or signs of disease (Bacon and White, 2000; Saikkonen *et a*l., 2004). In addition, it has also been documented that endophytes associated with medicinal plants may produce the same metabolites in vitro and within host plant tissue

(Kusari *et al.,* 2013; Dos Santos *et al*., 2016). In the present study, we analyzed the antimicrobial activity of all isolated bacteria from *Dillenia pentagyna* by making broth cultures from which the supernatant obtained by centrifugation was tested against 11multidrug resistant pathogenic microorganisms. Our findings showed that 22 isolates out of 96 isolates showed activity against at least one of the tested pathogens which can be attributed to the extracellular or intracellular bioactive molecules. Extract preparation was done for the 22 isolates that showed activity using the solvent Methanol to obtain crude extract which were then tested again for their antimicrobial activity against the same pathogens. Results showed that 13 bacterial extracts from selected isolates showed activity against three pathogens (*Bacillus subtilis, Klepsiella pneumonia, and Micrococcus luteus*). Out of the 13 isolates, two bacterial extracts of *Brevibacterium sp*. (BPSL85) and *Bacillus pumilis* (BPSL8) showed the highest zone of inhibition with 18±0.12 mm and 16±0.28 mm respectively against *Micrococcus luteus.* This suggested that the BPSL85 and BPSL8 are caplable of production of bioactive molecules having antimicrobial properties.

Antagonistic activity against fungal pathogens by endophytic bacteria increases the resistance towards different plant pathogens and thus have potential application in crop management (White *et al.,* 2019) Antifungal activity test was carried out for all bacterial isolates obtained from *Dillenia pentagyna* tissues. Our results showed that out of 96 isolates, 50% showed antifungal activity of at least one of the tested pathogens with 19 good isolates showing activity against two or more tested pathogens. From our results, *Bacillus toyonensis* (BPSL21) showed the highest percentage of inhibition with 95% against the tested pathogen *Fusarium culmorum,* while other isolates also showed very potent percentage of inhibition with 93% and 92% against *T.mentagrophytes* by isolate no.BPSL16 and BPSL15 respectively, including *Bacillus pumilis* (BPSL8) showing 92.6% inhibition against *M. phaesolina.* Our bacterial isolates were found to be very potent against fungal phytopathogens and these organisms could have an excellent agricultural uses

Cancer causes serious problems worldwide and plant based compounds are thought to be better curative compounds than synthetic compounds. Endophytes inhabiting plant tissues are potential source of various bioactive molecules and a well known anticancer drug Paclitaxol was isolated from endophytes (Newman *et al.,* 2007; Swarnalatha *et al*., 2016). In this study, four isolates BPSL6, BPSL8, BPSL17 and BPSL85 were selected and their extracts were tested for cytotoxic activity against three cancer cell lines, A549, HT29 and HeLa. The cancer cells were exposed to bacterial extracts in dose and time dependent manner to check the ability of extracts to inhibit the proliferation for 12-48 hours. Our results revealed that methanolic extracts of *Brevibacterium sp*. (BPSL85) showed significant cytotoxicity against HT29 with IC_{50} value of 67.01 and against HeLa with IC_{50} value of 61.2. This showed that *Brevibacterium sp.* extracts create an irreversible effect on the proliferation of cells and there was a considerable decrease in the number of cells due to induction of apoptosis in HT29 as well as HeLa cancer cell lines and can be considered that the extracts of BPSL85 possess anticancer activity

Twenty two bacterial isolates were identified using 16S rRNA gene sequencing. The bacterial strains showed diversities at the genus-species to- strain level. All the bacterial isolates belonging to both Gram positive and Gram negative was phylogenetically analyzed effectively using Maximum Likelihood Tree model. This model was successful in ensuring that both the Gram positive and Gram negative bacteria were claded separately with the Bacillus genera dominating among the selected isolates.

Endophytic bacteria provide benefits to host plants by producing a diverse class of secondary metabolites (natural products). Arrays of polyketide and non ribosomal peptides natural products are synthesized by specific classes of polyketide synthases (PKS I ,II and III) and non ribosomal peptide synthetases (NRPS) in host organisms. In the present study, we attempt to detect PKSII and NRPS gene responsible for expression of bioactive protein molecules in the selected twenty two bacterial isolates by PCR amplifications using specific primers. Our results detected PKSII bands in BPSL108 identified to be *Bacillus velezensis* and NRPS bands in BPSL17, BPSL85 and BPSL108

identified to be *Bacillus subtilis, Brevibacterium sp. and Bacillus velezensis* respectively.

Endophytes are reported to produce a number of bioactive metabolites in a single plant or microbe which served as an excellent source of drugs for treatment against various diseases (Gouda *et al.,* 2016). The present study focuses on detection of secondary metabolites from endophytic bacteria with potential applications in agriculture, medicine, food and cosmetics industries. High Performance Thin Layer Chromatography (HPTLC) method was used for detection of secondary metabolite from selected isolate BPSL8 based on different activities such as antioxidant, antimicrobial, antifungal as well as cytotoxicity. Different extracts obtained from Ethyl Acetate, Dichloromethane (DCM) and Methanol solvent of BPSL8 which was identified to be *Bacillus pumilus* were used for HPTLC method. Our results showed that different color bands were observed for *Bacillus pumilus* extracts which indicates the presence of phenols, sugars, steroids, and terpenes. From our results it can be said that these secondary metabolites may attribute to the antimicrobial, antioxidant and antifungal activity shown by *Bacillus pumilus* which can be furthermore purified and explored on a larger scale for further applications. Endophytes are a poorly investigated group of microorganisms capable of synthesizing bioactive compounds that can be used to combat numerous pathogens and can be a source for novel drug discovery.

While, still many questions remained unanswered with respect to the actual ecological roles of bacterial endophytes within the endosphere of the host plant, the increasing number of potent strains isolated from medicinal plants suggests that the plant endosphere is a unique niche which needs to be explored more in order to study their endophytes which are promising source for natural products.

CONCLUSION

In the present study, a total of ninety six bacterial isolates were isolated from *Dillenia pentagyna* Roxb. The Starch Casein Agar (SCA) was found to be the most suitable nutritional media for bacterial endophytes and it yields the highest number of isolates (n=42). The endophytic bacterial colonization was found to be the highest in the bark tissue with a percentage of 48.62%. All the isolates were investigated for their antimicrobial activity which indicated twenty two isolates showing activity against at least one of the tested pathogenic microorganisms. Among the twenty two isolates, methanolic bacterial extracts of thirteen isolates were further screened wih *Brevibacterium sp*. (BPSL85) showing the best antimicrobial activity followed by *Bacillus pumilus* (BPSL8). All the isolates were also tested for their antifungal activity which showed that 50% of the isolates exhibit antagonistic activity against one or more fungal pathogens and *Bacillus toyonensis* (BPSL21) was found to be the best isolate with highest percentage of inhibition. Antioxidant activity tested on the selected isolates showed that DPPH reduction capacity was highest for *Bacillus pumilus* (BPSL8) and ABTS radical cation reduction potential was highest for *Bacillus aryabhattai* (BPSL97). Cytotoxic activity test done for selected isolates against three human cancer cell lines suggested that *Brevibacterium sp*. (BPSL85) showed significant cytotoxicity against two of the three cell lines: HT29 and HeLa by inducing apoptosis. Out of the total isolates, twenty two selected isolates were identified based on 16S rRNA gene sequences. Detection of secondary metabolites was done by detection of polyketide synthase (PKSII) and non ribosomal peptide synthetase (NRPS) gene which is considered to be

responsible for synthesizing different classes of secondary metabolites in the selected strains. Further detection of secondary metabolites was done using High Performance Thin Layer Chromatography (HPTLC) whose results revealed that different color bands were observed for the Ethyl Acetate, DCM and Methanol fractions of *Bacillus pumilus* (BPSL8) extracts on TLC plate. This indicates the presence of phenols, sugars, steroids, and terpenes which are classes of secondary metabolites. From our study, we conclude that *Bacillus pumilus* (BPSL8) is the best isolate amongst all and showed significant results in all the activities testes. Detection of secondary metabolites in BPSL8 suggested that the antioxidant, antimicrobial and cytotoxic activity shown by the isolate is attributed to the presence of phenols and terpenes. Therefore, isolate BPSL8 identifies to be *Bacillus pumilus* can potentially be used to make bioformulation for antioxidant, antimicrobial and anticancer compounds and can be further studies with respect to their structural and chemical properties or exploited as an alternative for discovery of potent biological metabolites.

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Publications : 01

1. Leo, V. V. **Lallawmsangi**, Lalrokimi, and Singh, B. P. (2018). Microbes as Resource of Biomass, Bioenergy, and Biofuel. In: Singh, D. B., Gupta, V. K., Prabha, R. (Eds).Microbial Interventions in Agriculture and Environment: Research Trends, Prioritie sand Prospects, 1, 241-260. Springer, Cham. DOI: 10.1007/978-981-13-8391-5

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EXPLORATION AND CHARACTERIZATION OF ENDOPHYTIC BACTERIA ASSOCIATED WITH DILLENIA PENTAGYNA ROXB.

AN ABSTRACT SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY

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BACKGROUND AND AIM

Medicinal plants have been traditionally used and explored for a long period of time due to their medicinal properties used as remedies for treatment of various diseases. Although a large number of medicinal plants have been well-studied with respect to their phytochemical constitutes and pharmacological properties, their microbiome and physiological interactions between host plant and associated microorganisms still remain poorly understood. Recently, endophytic microorganisms have been under increased investigation due to the intimate interaction of endophytes with the host and the believe that the phytochemical constitutes of plants can be related either directly or indirectly to endophytic microbes and their interactions with host plants. Our study aims to isolate and identify endophytic bacteria from a medicinal plant *Dillenia pentagyna* and to evaluate the bioactive potential of the endophytes by checking their antimicrobial activities, antioxidant activities, antifungal properties, cytotoxic activities and production of secondary metabolites

METHODOLOGY

Endophytic bacteria were isolated from the surface-sterilized tissues of *Dillenia pentagyna* using different nutritional media and repeated sub-culturing to obtain pure culture isolates (Taechowisan *et al.,* 2003). All the pure culture obtained were screened for their antimicrobial activity against Multi-drug resistant pathogens (MDR) by preparing broth cultures of the isolates and testing their activity using the Agar well diffusion method using the nutritional media Mueller –Hinton Agar (Rios *et al*., 1987). Based on the antimicrobial properties, isolates were selected to twenty two isolates whose extracts were prepared using the solvent Methanol to obtain crude Methanolic extracts (Al-Bari *et al*., 2007). The methanolic extracts were tested again for their antimicrobial activity against the same pathogens using agar well diffusion assay. The methanolic extracts of twenty two isolates were also tested for their antioxidant activity to see their free radical reduction potential using two methods DPPH radical scavenging

assay and ABTS decolorization assay. DPPH assay was done as per the protocol given by Villano *et al*., 2007 and ABTS assay was done as per Re *et al.,* 1999. For all the pure culture isolates obtained from *Dillenia pentagyna,* antifungal activity was tested to check the antagonistic activity of endophytic bacterial isolates against fungal phytopathogens. Antagonistic activity was done by dual culture assay on Potato Dextrose Agar (PDA) where test was done by placing 1cm^2 fungal plug on the centre of PDA plates (Petatán-Sagahón *et al.*, 2011) and eleven fungal pathogens were used for this test (Khamna *et al*., 2008). To test the potential of selected bacterial isolates to induce apoptosis in human cancer cell lines, cytotoxicity test using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was performed (Singh et al. 2016). Cytotoxic activity test was done against three human cancer cell lines namely A549, HT29 and HeLa and percentage of cell viability was calculated. Molecular characterization was done by identifying bacterial isolates using 16S rRNA gene amplification using Polymerase Chain Reaction (PCR) (Qin *et al*. 2009). Phylogenetic analysis was done by construction of phylogenic tree based on the 16s rRNA sequences (Thompson *et al*., 1997). For detection of the responsible genes for production of bioactive molecules, PCR amplification of PKSII and NRPS gene was performed on the selected bacterial isolates using specific primers (Passari *et al.,* 2016). Detection of secondary metabolites of selected isolates was done using High Performance Thin Layer Chromatography (HPTLC) where three different fractions obtained from different solvents i.e., Dic.hloromethane (DCM), Ethyl acetate (EA) and Methanol (MeOH) were analyzed by TLC and HPTLC. TLC plate was captured using TLC visualizer (CAMEG) under 254 nm and 366 nm. The developed plates were post-derivatized with Anisaldehyde-Sulphuric acid reagent. The TLC plate was heated at 80˚C for 15 min after dipping in Anisaldehyde-Sulphuric acid reagent then the plates were scanned by the CAMAG TLC Scanner III at a wavelength of 500 nm.

RESULTS

In total, 96 bacterial endophytes were isolated from the barks, leaves and roots of *Dillenia pentagyna* Out of which 39 isolates were obtained from bark tissues, 37 isolates from root tissues and 20 isolates from leaf tissues. The colonization of bacterial endophytes was recorded highest in barks (48.62%) followed by roots (38.54%) and leaves (20.83). Among the nutritional media used, Starch Casein Agar (SCA) (n=42) media yields highest number of endophytic isolates followed by Luria Bertiani (LB) Agar (n=25). After incubation of the plates obtained from the tissue fingerprint as well as the last wash, no growth was observed which showed successful surface sterilization of the tissues of bark, leaf and root of *Dillenia pentagyna.*

Antimicrobial activity test showed that out of the 96 bacterial endophytic broth cultures, 22 isolates showed activity against at least one of the tested pathogen. A total of 22 extracts (methanolic) obtained from different selected isolates were used for DPPH assay. DPPH radical scavenging assay results showed that the methanolic extract BPSL8 showed a potent reducing capacity of DPPH measured at an IC50 value of **6.29**µg/ml. This activity shown by BPLS8 was found to be the best out of all the tested 22 endophytic extracts. Out of the 22 tested endophytic extracts obtained from different selected isolates, the methanolic extract of BPSL97 was found to exhibit a potent reducing potential of ABTS radical cation with an IC_{50} value of 4.988µg/ml. ABTS activity of BPSL97 is considerably higher compared to the other tested extracts.

To evaluate antimicrobial activity, methanolic extracts prepared from selected 22 bacterial isolates were tested against 11 pathogens. Methanolic extract obtained from a total of 13 isolates out of 22 selected isolates showed antimicrobial activity by forming a zone of inhibition at the concentration of 30mg/ml. Among the bacterial isolates extracts, isolate number BPSL85 showed the highest zone of inhibition (18±0.12 mm) which is followed by BPSL8 (16±0.28mm) against *Micrococcus luteus.* To evaluate antagonistic property of bacterial endophytes, antifungal assay was performed for all the 96 pure culture isolates obtained. Screening was done in triplicates and a total of 19 endophytic bacterial cultures showed potential activity against 2 or more of the tested fungal pathogens. Out of 19 potent

isolates two isolate number BPSL21 and BPSL16 showed highest inhibition against *F. culmorum* and *T.mentagrophytes* with inhibition percentage of 95% and 93.6% respectively The best four isolates BPSL6, BPSL8, BPSL17 and BPSL85 were selected based on their antioxidant and antimicrobial activity. The four isolates were tested to evaluate their cytotoxic activity against three human cancer cell lines A549, HT 29 and HeLa. The results revealed that isolate number BPSL85 showed significant cytotoxicity against HT29 and HeLa with IC_{50} of 67.01 and 61.2 respectively.

Genomic DNA of the isolates organisms was extracted and the quality was checked on 0.8% agarose gel and by using spectrophotometer. Amplification of 16S rRNA gene was done using Applied Biosystems thermal cycler PCR. The PCR product was run on 1.2% agarose gel with low range DNA ruler plus (100 bp to 3 kb) as molecular markers. For all the isolates a single amplicon of 1500 bp was amplified. The amplified PCR product was commercially sequenced and the sequences were analyzed using NCBI BLAST (Basic Local Alignment Search Tool) and the analyzed sequences were submitted to NCBI Gen Bank. The relationship among the selected potential isolates (22) was carried out by aligning their 16S rRNA gene sequences along with the type strains retrieved from EZTaxon database.

The Gram positive bacteria were observed to be clubbed within a single clade belonging to the genera of *Bacillus, Lysinibacillus* and the lone actinobacteria *Brevibacterium*. Out of the total 22 selected 15 belongs to the family of Bacillaceae formed the major clade accounting for 68.18% of the total bacteria analyzed. The remaining Gram positive bacteria accounting for 4.5% belonged to Brevibacteriaceae family. Among the Gram negative bacteria 13.6% belonged to the Class Gammaproteobacteria (3 isolates) and Betaproteobacteria (3 isolates) respectively which was the second biggest clade. *Pseudomonas* (Order: Pseusomonadales) and *Serratia* (Order: Enterobacterales) were the two genera of Gammaproteobacteria which were claded separately. Similarly, Alcaligene and Bulkhorderia formed the two genera of Betaproteobacteria which belonged to the same order of Bulkhorderiales. The phylogenetic tree was constructed

using Maximum likelihood method with Kimura 2 parameter to the lowest BIC values using Mega 6 with the estimated Transition/Transversion bias (R) value is 1.83 and 1.36 respectively. The tree with the highest log likelihood (-1443.0171) is shown (Figure 9). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 40 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 289 positions in the final dataset. PCR amplification was done to detect PKSII and NRPS gene using specific primers in the samples selected based on antimicrobial activity. PCR amplification of expected size for PKSII was observed for BPSL108 when viewed in 1.5% Agarose gel. Whereas, NRPS candidate amplicons were detected in three isolates (BPSL17, BPSL85, BPSL108)

HPTLC method was developed for three different fractions i.e., Dichloromethane (DCM), Ethyl Acetate (EA) and Methanol (MeOH) of BPSL8. In this technique performed clear separation was achieved by using Toulene:chloroform: ethanol (4:4:1 v/v/v) for non polar fractions (DCM and EA) and Toulene: chloroform: ethanol (4:4:4 v/v/v) for polar fractions (MeOH). Post-derivatization with anisaldehyde-sulphuric acid reagent gave good results. Different color bands were observed which indicates the presence of phenols, sugars, steroids, and terpenes

DISCUSSION AND CONCLUSION

Endophytic bacteria living in the endosphere of plants are widely regarded as reservoir of bioactive secondary metabolite sand only a few studies have been undertaken on the diversity, distribution and bioactivity of endophytic bacteria harbored by plants (Kobert *et al*., 2014). Therefore the present study seeks to evaluate the bioactive potential of bacterial endophytes from the selected traditional medicinal plant of Mizoram, northeast India and their direct or indirect correlation with the phytochemical constitutes and biological activity of the host plant. The reactive oxygen species (ROS) can exert oxidative damaging effects by reacting with nearly every molecules found in living cells and compounds having antioxidant activity can reduce the level of these ROS to normal. DPPH is a stable free radical that can be reduced by donation of hydrogen by antioxidants. Our findings suggested that *Bacillus pumilu*s (BPSL8) is an antioxidant producing organism whose methanolic extracts showed a potent reducing capacity of DPPH with an IC_{50} value of 6.26µg/ml. ABTS decolorization assay results showed that the methanolic extracts of *Bacillus aryabhattai* (BPSL97) showed the highest reduction potential of ABTS with an IC_{50} value of 4.988µg/ml which suggested that it contains a very potent antioxidant molecule or compounds.

Antimicrobial activity tested against our isolates showed that showed that thirteen bacterial extracts from selected isolates exhibit activity against three pathogens (*Bacillus subtilis, Klepsiella pneumonia, and Micrococcus luteus*) and out of them, two bacterial extracts of *Brevibacterium sp*. (BPSL85) and *Bacillus pumilis* (BPSL8) showed the highest zone of inhibition with 18±0.12 mm and 16±0.28 mm respectively against *Micrococcus luteus*. This suggested that the BPSL85 and BPSL8 are caplable of production of bioactive molecules having antimicrobial properties.

Antagonistic activity against fungal pathogens was also performed and from our findings, *Bacillus toyonensis* (BPSL21) showed the highest percentage of inhibition with 95% against the tested pathogen *Fusarium culmorum,* while other isolates also showed very potent percentage of inhibition with 93% and 92% against *T.mentagrophytes* by isolate no.BPSL16 and BPSL15 respectively, including *Bacillus pumilis* (BPSL8)

showing 92.6% inhibition against *M. phaesolina.* Our bacterial isolates were very potent against fungal phytopathogens and these organisms could have an excellent agricultural uses

Cytotoxicity test done against selected isolates revealed that methanolic extracts of *Brevibacterium sp*. (BPSL85) showed significant cytotoxicity against HT29 with IC_{50} value of 67.01 and against HeLa with IC_{50} value of 61.2. This showed that *Brevibacterium sp.* extracts create an irreversible effect on the proliferation of cells and there was a considerable decrease in the number of cells due to induction of apoptosis in HT29 as well as HeLa cancer cell lines and can be considered that the extracts of BPSL85 possess anticancer activity

For detection of secondary metabolites , detection of PKSII and NRPS gene responsible for expression of bioactive protein molecules was done and our results detected PKSII bands in BPSL108 identified to be *Bacillus velezensis* and NRPS bands in BPSL17, BPSL85 and BPSL108 identified to be *Bacillus subtilis, Brevibacterium sp. and Bacillus velezensis* respectively.

High Performance Thin Layer Chromatography (HPTLC) method was used for detection of secondary metabolite from a selected isolate BPL8 from where Ethyl Acetate, Dichloromethane and Methanolic extracts were prepared. Our results showed that different color bands were observed for *Bacillus pumilus* (BPSL8) extracts which indicates the presence of phenols, sugars, steroids, and terpenes.

Based on our study and results can be said that these secondary metabolites may attribute to the antimicrobial, antioxidant and antifungal activity shown by *Bacillus pumilus* which can be furthermore purified and studied. Therefore, isolate BPSL8 identifies to be *Bacillus pumilus* can potentially be used to make bioformulation for antioxidant, antimicrobial and anticancer compounds and can be further studies with

respect to their structural and chemical properties or exploited as an alternative for discovery of potent biological metabolites.

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