Comparison of putative phytochemicals in endophytic fungi of Frangipani (*Plumeria rubra*)

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Submitted By

Arshpreet Kaur

Roll no. 301401003

Under the supervision of

Dr. Sanjai Saxena

Professor

Department of Biotechnology

Thapar University

DEPARTMENT OF BIOTECHNOLOGY

Thapar University, Patiala, Punjab, 147004

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Certificate

This is to certify that the thesis entitled "**Comparison of Putative Phytochemicals in Endophytic fungi of Frangipani (*Plumeria rubra*)**" being submitted by **Ms. Arshpreet Kaur** (Roll No-**301401008**) in partial fulfillment of the requirements for the award of degree of master of science in biotechnology, Thapar University, Patiala is a bonafide work carried out under the esteemed supervision and conception of **Dr. Sanjai Saxena** and that no part of this thesis has been submitted for the award of any other degree.

15/7/16

Dr. Sanjai Saxena
Professor/Supervisor
DBT, Thapar University

Dr. Dinesh Goyal
Professor/ Head
DBT, Thapar University

Dr. S.S Bhatia
Dean, Academic Affairs
Thapar University, Patiala

Candidate's Declaration

I hereby declare that the work being presented in the thesis entitled "**Comparison of Putative Phytochemicals in Endophytic fungi of Frangipani (*Plumeria rubra*)**" in partial fulfilment of the requirements for the award of degree of Master of science, Department of Biotechnology, Thapar University, Patiala is my own laboratory work during the period of **January 2016 to June 2016**, under the conception and supervision of Dr. Sanjai Saxena, Professor, Department of Biotechnology, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree.

Patiala
Date: July 15th, 2016

Arshpreet Kaur
Roll No. 301401003

This is to certify that the above statement made by the candidate is correct and true to the best of our knowledge.

15/7/16

Dr. Sanjai Saxena
Professor / Supervisor

Department of Biotechnology
Thapar University, Patiala 147004

I feel grateful while dedicating this thesis to my parents...

Thank you mom and dad for being there in my thick and thin! Regards… ☺

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Arshpreet Kaur

CONTENTS

List of Tables

List of Figures

Abbreviations

S.No	Abbreviation	Full form
1.	**µg**	Micro Gram
2.	**µl**	Micro Liter
3.	**µm**	Micro Meter
4.	**AIDS**	Acquired Immuno Deficiency Syndrome
5.	**amu**	Atomic Mass Unit
6.	**cm**	Centi Meter
7.	**DNA**	Deoxyribose Nucleic Acid
8.	**EDTA**	Ethylene Diamine Tetra Acetic Acid
9.	**EtBr**	Ethyl Bromide
10.	**EtOH**	Ethanol
11.	**eV**	Electron Volt
12.	**GC-MS**	Gas Chromatography-Mass Spectrometry
13.	**GH**	Guest House
14.	**GLA**	Grass Leaf Agar
15.	**hCMV**	Human Cyto Megalo Virus
16.	**HIV**	Human Immuno Virus
17.	**HP**	Hewlett Packard
18.	**HPLC**	High Performance Liquid Chromatography
19.	**ITS**	Internal Transcribed Site

20.	**KB**	Kilo Bites
21.	**MeOH**	Methanol
22.	**ml**	Milli Litre
23.	**mm**	Milli Meter
24.	**MPCE**	Micro Nucleated Polychromatic Erythrocytes
25.	**PCR**	Polymerase Chain Reaction
26.	**PDA**	Potato Dextrose Agar
27.	**pH**	Potential of Hydrogen
28.	**PLA**	Pinus Leaf Agar
29.	**PR**	Passi Road
30.	**R&D**	Research and Development
31.	**Rf**	Retention Factor
32.	**RPM**	Rotation Per Minute
33.	**RT**	Reverse Transcription
34.	**SNA**	Synthetischer Nahrstoffarmer Agar
35.	**TAE**	Tris Acetate EDTA
36.	**TLC**	Thin Layer Chromatography
37.	**UV**	Ultra Violet
38.	**WA**	Water Agar

Executive Summary

Frangipani (*Plumaria* sp.) is commonly known ornamental and medicinal plant. The bio actives of this plant possess anticancer, antitumor, antifouling and cosmeceutical properties thus making it a prime subject of research. However, endophytic fungi, an endosymbiont are known to mimic the properties of host plant and produce similar bioactive compounds like that of host plant. Hence, the focus of the present study was to compare the bioactive constituents of endophytic fungi of Frangipani plant with its own phytochemicals.

In the present study, 33 endophytic fungi were isolated from leaves of Frangipani (*Plumaria rubra*) collected from different locations of Patiala. All the endophytic fungi were subjected to culture filtrate production followed by extraction with ethyl acetate. Subsequently, secondary metabolites were also isolated from leaves of frangipani plant so as to compare its bioactive compound profiling with crude EA fraction of endophytic fungi. The EA fractions of all endophytic fungi were compared with crude leaf fraction of Frangipani by using preparative thin layer chromatography (TLC). Out of 33 cultures, only #88FPLPR was showing three bands with identical R_f value to that of plant fraction. Further, crude EA fraction of #88FPLPR and plant fraction was analysed by HPLC. On comparing the HPLC chromatogram, two peaks were found to be similar in both fractions at same retention time of 2 min and 5.3 min. For further confirmation of presence of identical compounds were deduced from GC-MS analysis. In GC-MS data, 7 compounds of crude EA fraction of #88FPLPR were found to be similar to crude plant fraction which shows the presence of 21 compounds in total. The potential isolate was identified as *Nigrospora* sp through classical taxonomy while sequencing data of ITS region is awaited till now. Further speciation of #88FPLPR would be done after analyzing sequencing data. However, the results of the present study were providing preliminary data about the ability of endophyte to mimic the properties of host plant for validation of this hypothesis.

1. Introduction

Since ancient times, India has been one of the pioneers in the field of medicine, particularly, Unani, Ayurveda and Siddha. From past years, plants are the major source of biologically active compound. The major advantage of Ayurvedic medicines is their efficacy, minimum chances of side effects and low cost (Burkill, 1950).

Ornamental plants were not only grown for display purpose, but also grown for nutritive and medicinal purpose. The use of herbal compound for medicinal purpose had slowly increased in India, According to world health organisation (2010), only 20% of population from developing countries is able to afford medicine and other 80% rely on traditional medicine which are plant based (Kumar, 2005). Flowering shrubs like frangipani are now being exploited in the traditional medicine and their curative potentials were well documented.

Genus *Plumeria L* (Apocynaceae) was well known for tropical trees or shrubs which is cultivated as ornamental plants. *Plumeria* is commonly known as frangipani or temple tree. It is a member of family Apocynaceae. It was native species of the Bahamas and the Greater Antilles in Central America. In Thailand it is commonly pronounced as Lanthom, In India it is most famously called Champa. Various species of this genus were also known for their medicinal importance (Nandkarni, 1976). Their medicinal properties were often due to their latex which was frequently drastic and corrosive. *Plumeria accuminata*, *Plumeria alba*, *Plumeria rubra*, *Plumeria lancifolea*, *Plumeria drastic* and *Plumeria phagidenica* were some of the species with medicinal utility (Cowen, 2012).

Nature had been a prime source of a number of drugs for curing various diseases in Primates and animals (Dev, 1997). Many species belonging to *Plumeria* genus is medicinally important due to diverse activities such as diuretic, antipsychotic, antifouling (Coppen *et al* 1983), antitumor (Fujimoto, 1988), anticancer (Anderson, 1988), antibacterial and algicidal effects (Coppen, 1983). The iridoids plumieride isolated from *Plumeria* had possess antifertility and anticancer activities.

Plumeria sp. is used to cure pain, fever, diabetes, malaria, gastrointestinal ailments. Roots and leaves of *Plumeria sp.* is used to treat many worms, ulcers, leprosy, dysentery, tumors, skin and liver disease. Leaves of *Plumeria rubra* were used as an antidote, purgative and externally applied to treat headache. Leaves of Plumeria obtusa is very useful in skin disease and wounds. The latex and bark have purgative properties. Herbal paste of roots and barks were traditionally used to treat asthma, promote menstruation, reduce fever, and ease constipation since ancient times. The latex of *Plumeria*

is also used to irritation in sensitive skin. *Plumeria rubra* also had been reported to possess anticancer properties (Wong et al., 2011). Extracted bioactive compounds from *Plumeria sp* are reported for their anti-bacterial, molluscicidal and cytotoxic activities. The plant also contains sitosterol, isoplumericin, plumieride, amyrinacetate, scopotetin, iridoids, amyrins, plumieride coumerate, and plumieride coumerate glucoside the essential oil extracted from *Plumeria rubra* had the best antimicrobial property compare to other species of Plumeria.

Essential oil obtained from *Plumeria rubra* is used in cosmetic products and also used in aromatherapy like in scenting candles, perfumes, creams for pleasant smell. In the previous study on the fragrance of *Plumeria*, chemical compound and yield from water distillation also reported.

An endophytic fungus exists as an endo-symbiont with in the healthy parts of host plants without exerting any negative effects. They live inside the tissues of host organisms, that provide protection to them and in return endophytic fungi support their hosts by fighting with pathogens through the production of antimicrobial compounds. The world's billion dollar endophyte, *Pestalotiopsis microspora*, was discovered from Himalayan yew tree, *Taxus wallichiana*. This fungus produces anticancer drug – Paclitexel. Endophytes have potential benefits for humans as they are known to produce antidiabetic, anticancer, immunosuppressive compounds. Among different species of plants, a plethora of endophytes exist. Endophytic fungi benefit the host plant by rescuing them from Biotic and abiotic stress including Salt, Drought or Heat stress (Brink, 2010). Endophytic fungi are also known to increase plant more resistant against many potential pathogens by increase expression of some defence related gene (Gond, 2015).

Despite of immense research work about various applications of endophyte it is quite understood that the relationship between endophyte and host may be considered as a balance antagonism with both positive and negative effect on host depending on environmental conditions (Schulz, 1999).

The present project was focused on isolation of endophytic fungi from *Plumeria rubra* plant and comparison of secondary metabolite profile of host plant and endophytic fungi for identification of similar compounds.

2. Review of Literature

Frangipani (*Plumeria* sp) belongs to Apocynaceae family comprising of generally latex bearing plants and deciduous shrubs. It is native plant of Central America but now days it is found from South America to Mexico and also most abundant in India. About 155 Genera and 2000 species are extensively distributed in warmer areas around the globe (Krishnamurthi 1969). In India, various varieties of *Plumaria* are being cultivated as ornamental plants. It is 3-7 meter in height. It is well known for its attractiveness, fragrance and differently coloured species. Some species of *Plumeria* exhibit fruity fragrance, while others produce fragrance just like jasmine, peaches, or citrusy. They had long leathery, generally oblong shape. Leaves tend to fall in early winters because they were sensitive to cold climate.

Kingdom	Plantae
Sub-kingdom	Tracheobionta
Division	Spermatophyta
Class	Magnoliopsida – Dicotyledons
Sub-class	Asteridae
Order	Gentianales
Family	Apocynaceae
Genus	*Plumeria L.- Plumeria*
Species	*rubra*

Table 1 showing Taxonomical classification of Frangipani (*Plumeria* sp).

2.1 Chemical Constituents:

- Triterpenoids: These are widespread medicinally active compounds present in frangipani plant (*Plumaria* sp.). Till date 51 different compounds had been reported.
- Iridoids: they are the first medicinally compound which is isolated from the genus Plumeria, till date Twenty four iridoids had been reported.
- Fatty Acid Esters: Frangipani plant has also been reported to produce 4 fatty acid esters compounds.
- Alkaloids: Eleven alkaloids had been isolated from *Plumeria* species. Thereof, plumericidine and plumerianine exists in enantiomeric forms.

- Coumarins: The isolation of one coumarin from *Plumeria* species had been described.
- Cardenolides and Lignans were also isolated from *P. obtusa*.

2.2 Traditional Uses: Nature had been a prime source of number of medications for diagnosing various diseases in Humans and animals (Dev 1997). Many species of the genus *Plumeria* is medicinally important, as they were reported to exhibit wide array of activities such as diuretic, antipsychotic, antifouling (Coppen *et al* 1983), antitumor (Fujimoto 1988).

2.3 Pharmacological Application of *Plumeria*

2.3.1 Anticancer effect: Two Triterpenes namely plumeric acid and methyl plumerate were found to be completely inhibiting *Yoshida* sarcoma cells in vitro (Fujimoto 1988). Antitumor activity was also reported from plumericin, isoplumericin, allamcin, allamandin in brine shrimp (BS), 9KB, 3PS (Anderson 1988).

2.3.2 Antifertility and Embryo toxic Effects

Plumeria species are found to possess antifertility potential. The stem and root bark extract had been administered orally as menstrual inducers. There are reports claiming infertility activity in *Plumeria*, and they carry out the investigation on pregnant rats. The results revealed that EtOH extract was significantly active, but was poisonous at a dose of 1.52 g/kg. The DCM extract exhibited antifertility activity at 1.36 g/kg, and equivocal activity at 0.6 g/kg. The methanol extract exhibited antifertility activity due to presence of plumieride (Gunawardana 1997).

Plumieride treatment blocks the spermatogenesis without any severe side effect. However, motility and density of sperms were found to be reduced significantly. There was a fall in the number of Leydig cells and complete repression of fertility was observed in male albino rats (Gupta 2004). Plumieride was also found to cause statistically significant early post-implantation and foetal resorption (Gunarwardana 1997).

2.3.3Antimutagenic Property

Four compounds, A_1 (long hydrocarbon chain), C_1 (stigmast-7-en-3-ol), D_3 (lupeol carboxylic acid), and F_2 (ursolic acid) isolated from ethyl acetate extract of green leaves of *Plumeria*

exhibited antimutagenic activity. At a dosage of 2 mg isolates/25 g mouse, A_1 reduced the number of micro nucleated polychromatic erythrocytes (MPCE), induced by the mutagen mitomycin C, by 75%, C_1 by 80%, D_3 by 57%, and F_2 by 76%. (Guevara 1996) Results suggest that an aqueous extract of *Plumeria* leaves had an agonist and cholinergic action on α-adrenoceptors.

2.3.4 Antimicrobial Property

Rubinol, a phytochemical isolated from *Plumaria* was found to exhibit significant antimicrobial activity against *Bacillus anthracis*, *Pseudomonas aeruginosa*, *P. pseudomallie* and

Corynebacterium pseudodiphthericum (Akhtar 1994). Three new proaporphines, grandines A, B and C, were isolated from *Plumeria* in addition to phoebegrandine B and laurelliptine. The former four compounds were found to be active against Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram-negative bacteria (*Pseudomonas aeruginosa* and

Escherichia coli) (Almahy 2007). Three major iridoids; *viz*, plumieride, protoplumericine A and plumieride acid (=15-dimethylplumieride) were isolated from the bark and leaves of *Plumeria*. Protoplumericin A and plumieride acid were reported for the first time from this species. They displayed distinct activities against some pathogenic bacteria and fungi (Afifi 2006).

2.3.5 Cytotoxic Property

Various Iridoids such as fulvoplumierin, allamcin, plumericin, etc are found to be cytotoxic constituents of *Plumeria*. All of these compounds exhibited general cytotoxic activity on murine lymphocytic leukaemia (P-388) and a number of human cancer cell types (breast, colon, fibro sarcoma, lung, melanoma, KB) (Shehata 2002). Recent investigations showed that modified plumieride analoges had an enhanced cytotoxicity. Such modification consists of the replacement of the methyl ester functionally with alkyl amides of variable carbon-chain length and the conversion of the glucose moiety into di- and trisaccharide moieties (Dobhal 2004).

2.3.6 Antioxidant Property

When treatment was carried out in alloxan-induced hyperglycemic rats, a flavone glucoside isolated from *Plumeria* produced a significant reduction in the level of serum triglycerides. This

experiment demonstrated the antioxidant and hypolipidemic activity of the flavonoid. Further, antioxidant activity of the drug was confirmed through *in vitro* studies (Merina 2010).

2.3.7 Anti-Inflammatory and Analgesic Property

Lupeol acetate inhibited the edema formation by 35.9% at a dose of 200 mg/kg but no activity was observed at 100mg/kg (Miranda 2000).

2.3.8 HIV Treatment

Inhibition of human immunodeficiency virus reverse transcriptase was currently considered as a useful approach in the prophylaxis and intervention of acquired immunodeficiency syndrome (AIDS). The reverse transcriptase assay developed for the detection of the enzyme in virions; involving poly rA oligo dT and radio-labelled thymidine 5'-triphosphate (TTP), can be applied as a simple method for screening the human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT). Natural products had not been extensively explored as inhibitors of this enzyme. The iridoid fulvoplumierin was demonstrated to possess potent activity in the HIV-1 RT system (Tan 1991).

Many microorganisms had been developed resistance against the current drugs. So it is very essential to discover new drugs in order to provide aid and comfort in all aspects of human's health and life. For this it is very important to make drugs which had safe bioactive compounds which is not dangerous for human health. Endophytes constitute a large diversity of microbial adaptations that had developed in special and hidden environments and because of their huge diversity and particular habituation; they could provide a good area for research in the field of making new medicines and novel drug like molecules.

2.4 Endophytes as Store House of Bioactive Compound

Fungi were rich sources of many therapeutic agents. A number of substances of pharmaceutical properties had been isolated and identified from endophytic fungus. Endophytic fungus was also believed novel sources of bioactive compound and attempts were being made to isolate and identify bioactive compound from it. Endophytic fungi produce antibiotics as secondary

metabolite that had been isolated from endophytic fungi emphasizing their potent ecological role (Jalgaonwalal et al 2011)

2.4.1 Fungal Endophytic as a source of Anticancer Compound: Cancer is a disease in which the uncontrolled spread and growth of abnormal cells, which can result to death if not cured. It had been considered as major cause of death all over world. The anticancer drug was toxic to proliferating normal cells, possess numerous side effects and also not effective against many other forms of cancer. Due to this, the cure of cancer had been improved mainly on the diagnosis basis which allows earlier and more precise treatments. The diterpenoid "Taxol" had created more awareness and attraction than any other drug due to its distinct phase as compared to other anticancerous agents, which was produced from endophytic fungi '*Taxomyces andreanae'*. Taxol interrupting the cancer cell growth and spreading. Basically, Taxol was used for the treatment of advanced lung cancer, breast cancer and refractory ovarian cancer (Jalgaonwalal et al 2011).

Second important compound against anticancer was the alkaloid "Camptothecin" ($C_{20}H_{16}N_2O_4$) an antineoplastic agent which was isolated from the wood of plant named as *Camptotheca acuminate Decaisne*, found in China. The 10-hydroxycamptothecin and Camptothecin were two important forerunners for the synthesis of anticancer drugs, topotecan and irinotecan (Jalgaonwalal et al 2011).

2.4.2 Fungal Endophytes as a Source of Antioxidant Compounds: It was reported that endophytes which produced metabolites could be a possible cause of novel naturally produced antioxidants. Polysaccharides produced from plants and microorganisms which had been extensively studied and considered as natural antioxidant. Antioxidant metabolites were often obtained by endophytic fungi- Isopestacin and Pestacin, isolated from plant *Terminalia morobensis* and *Pestalotiopsis microspora* (Waller et al 2005).

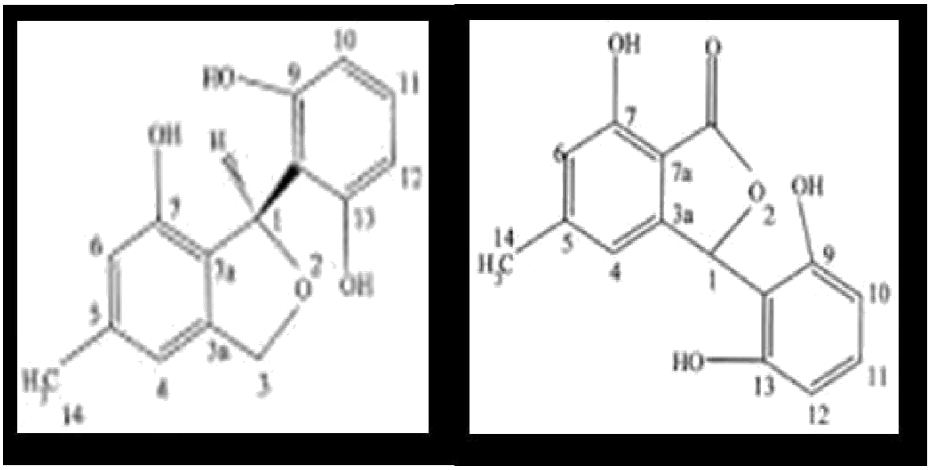

Figure 1 showing structure of bioactive compounds produced by endophytic fungi

2.4.3 Fungal Endophytes as a Source of Antiviral Compound

Many antiviral agents were investigated from endophytic fungi. Different compounds like cytonic A and B had been isolated from the endophytic fungus Cytonaema sp. These compounds were preventing human cytomegalovirus (hCMV) protease (Waller et al 2005).

2.4.4 Fungal Metabolites a Source of Antibacterial Compound

Metabolites having antibiotic activity was defined as low molecular-weight organic natural compound derived from microorganisms which were active at lower concentration against other microorganisms. Endophytic fungi were carried out a resistance mechanism to get better pathogenic invasion by producing secondary metabolites. Previously, it was reported that a large number of antimicrobial compounds isolated from endophytes, belonging to several structural classes like alkaloids, terpenoids, phenols, quinines, flavonoids and steroids (Guo et al 2000).

New antimicrobial metabolites from endophytes was an important cause to get increasing in the levels of drug resistance by human and plant pathogens, the inadequate number of effective antibiotics against different bacterial species, and new antimicrobial agents in development, due to the unfavourable returns on speculation (Liu 2008).

2.4.5 Fungal Endophytes as a source of Antifungal Compound

The function of fungal infection had increased during cancer chemotherapy, organ transplantation and allogenic bone marrow transplantation. Only a few numbers of antifungal

agents were now available for the treatment for various life-threatening fungal infections. Endophytes microorganisms live in internal tissues of plant species and represent as an abundant and symbiotic source of bioactive and chemically unique compounds with potential for desecration in a wide range of agricultural, medical and industrial field. In present different metabolites obtained from fungal endophyte with ability as antifungal agents (Schulzt 2002).

2.4.6 Fungal Endophytes as a source of Secondary metabolites

Endophytes that produce antibiotics had origins in ethnobotany, which utilizes the medicinal belief of common people. Microbial metabolite was useful agent for pharmaceuticals industry. The secondary metabolites and their role were explained shortly. Secondary metabolites may refer to compounds which were produced by organisms which was not necessary for the cell growth. Secondary metabolites were first recognized in 1873 and it may refer as a natural product.

Fungi were remarkable organism that produce a wide variety of natural products and are often called the secondary metabolites (Strobel 2004). Secondary metabolite was important for many metabolic interactions between their plant host and fungi host, such as signalling, regulation and defence of the symbiosis (Demain 2000).

2.5 Thin Layer Chromatography (T.L.C)

TLC [Thin Layer Chromatography] is an easy, rapid, and economical method that gives a fast respond as to how many components are in a mixture.TLC was performed out for different organic solvent extracts from the plant samples to separate the plant and Fungal constituents. TLC is also used to support the identity of a compound in a mixture when the Rf of a compound is compared with the Rf of a known compound (preferably both run on the same TLC plate). According to the principle of TLC the components will differ in solubility and in the strength of their adsorption to the adsorbent and will separate accordingly. TLC method was generally used by researchers to identify the compounds present in *Plumeria rubra.*

Thin layer chromatography had been performed by using solvent Methanol: Chloroform (3:2) and reported the presence of Iridoids, Fatty acid esters, Cardenoloid, Coumarins etc.

2.6 Gas Chromatography-Mass Spectroscopy (GC-MS):

Gas chromatography- Mass Spectroscopy (GC-MS) is a technique for analysis and quantification of volatile and semi volatile organic compound. In some reported data, for the analysis of compound GC-MS analysis was performed on an Hewlett Packard gas 6890 model (Agilent's Technologies, Network series) coupled to a mass detector HP 5971 model, equipped with library spectra Wiley (software 59943B) and capillary column HP-5 (30 m × 0.25 mm × 0.25 mm liquid phase). Oven temperature program of 290 to 350℃ at the rate of 5°C/min; carrier gas: helium 11.3 L/min; split mode of (5:1) and finally held for 30 min (Lawal *et al.*,2015).

In GC-MS experiments, 1 mg of extract and standard were transferred into glass vial and subjected to methylation with Diazomethane, with 100% yield. The samples were dissolved in Dichloromethane in concentrations of 1.4 mg/mL and injected for GC-MS analysis. The identification of compound methylated from extract was done with the use of Wiley and NBS peak matching library search system. Authentic standard of the compound and data reported in the literature were also used for further identification.

2.7 Identification of Endophytic fungi

2.7.1 Morphological identification

Morphological taxonomy has to be carried by microscopic study of the fungal structures such as mycelia, fruiting bodies; spores are very important features as fungal taxonomy is traditionally based on comparative morphological features (Zhang et al., 2008). Various optimization of growth conditions have been used in case of the fungi which are nonsporulating in the culture as, they cannot be identified by the conventional techniques otherwise so different media such as Potato dextrose agar (PDA), Water agar (WA), Grass leaf agar (GLA), Pinus leaf agar (PLA), (SNA) have been used in which the fungi sporulate (Guo *et al*., 2000)

2.7.2 Molecular technique

In contrast the molecular techniques exhibit more sensitivity and specificity for identifying microorganisms and can be used to classify the microbes on the diverse hierarchical taxonomic levels. Most of the endophytic fungi are being identified and detected by the

comparative analysis of ribosomal DNA sequences especially ITS region. The arbuscular mycorrhizal fungi is identified using the ITS (internal transcribed site) region. The 5.85 S ribosomal gene flanking region ITS 1 and ITS 4 have also been used extensively to identify the fungi.

3. Aim of the study

1. Isolation of endophytic fungi from *Plumeria rubra*.
2. Screening and identification of endophytic fungi producing host related metabolites.

4. Materials and Methods

4.1 Plant sample collection

The healthy parts (leaves and stem) of *Plumaria rubra* was collected from various locations of Patiala listed in table 2 during the month of February and April 2016. The collected samples were kept in sterilized zipper pouches and stored at 4˚C till further use.

S. No	Sampling site	Location
1.	Library Backyard	Thapar University, Patiala, Punjab
2	Garden area, Guest House	Thapar University, Patiala, Punjab
3	TIFAC-CORE	Thapar University, Patiala, Punjab
4	Auditorium	Thapar University, Patiala, Punjab
5	Passi Road	Patiala, Punjab

Table 2 showing sampling sites of plant collection

4.2 Isolation of Endophytic Fungi:

Collected plant parts (leaves and stem) was thoroughly cleaned with tap water for 15-20 min. The plant samples were surface sterilized by soaking in 1% sodium hypochlorite for 2 min followed by 70% ethanol and 30% ethanol for 1 min and 45 sec respectively. The sterilized sample was then allowed to surface dry under aseptic condition and further cut into small segments (2-3 mm) by using sterile blade. These small segments were inoculated into potato dextrose agar (PDA) plates and incubated at 28 ± 2˚C for 7- 10 days. Hyphal tips of fungi budding out of the segments were picked up using sterile fine needle and transferred onto fresh PDA plate. The actively growing pure cultures were further transferred on to PDA slants containing 10% glycerol for long term preservation (Ezra et al 2004).

4.3 Production of Culture Filtrate

Submerged fermentation of all isolated endophytic fungi was carried out in Potato Dextrose broth (PDB) medium. Briefly describing, 5mm mycelial plugs of 7day old culture was inoculated into 50 ml pre-sterilized PDB broth in Erlenmeyer flasks aseptically. Further, flasks were incubated at 120 rpm, 26℃ for 7-10 days. After 10 days, mycelium was separated from filtrate

through filtration using Whatman filter paper 4 followed by centrifugation at 10,000 rpm for 15 min to get cell free culture filtrate.

4.4 Liquid-Liquid Extraction

The cell free filtrate of each endophytic fungi was subjected to liquid-Liquid extraction by ethyl acetate. Ethyl acetate was added to culture filtrate in 2:1 ratio and was vigorously shaken for 10-15 min to recover maximum yield of bioactive residue in organic layer. This step was repeated thrice and aqueous and organic layer was separated. The organic phase was washed with sodium sulphate and solvent was evaporated to dryness to obtain crude fraction. The fractions so obtained were weighted, dissolved in methanol and stored at 4˚C.

4.5 Isolation of secondary metabolites from *Plumaria rubra* leaves

The healthy leaves of *P. rubra* were initially washed with double distilled water and air dried for 1 h at room temperature. Further, the leaves were grinded in a mixer to obtain fine powder. To 10g of this powder in a flask, 4 volumes of ethyl acetate was added followed by incubation at

130 rpm, 28˚C for 1 week (Siddiqui and Khan 1970). After the incubation is over, separate the organic layer by filtration and wash it with small amount of anhydrous sodium sulphate. Allow the organic solvent to evaporate so as to obtain crude fraction. The crude fraction so obtained was weighed, reconstituted in methanol and stored at 4˚C until use.

4.5 Optimization of TLC of crude plant extract and fungal extracts

The crude ethyl acetate (EA) fraction of each endophytic fungi was subjected to thin layer chromatography along with crude fraction of plant. The TLC plate of 0.5 mm thickness was prepared by layering silica gel (Merck) on to 20 x 15 x 5 mm clean glass plates and was activated by incubating at 100ºC for 2h prior to use. The sample was spotted on to activated TLC plate just 1 cm above the edge of plate with the help of capillary. Simultaneously, the TLC chamber was saturated with different solvent systems (Binary and tertiary) consisting of mixture of solvents of different polarities and ratios for 30 min. The TLC plate was kept in saturated TLC chamber in such a way that the sample spot is above the solvent level. When the solvent front reaches up to the desired level, the TLC plate was taken out and was air dried. The Chromatogram was developed by visualizing the TLC plate under UV light. Crude plant fraction

was used as standard for the comparison of R_f value. Retention factor (R_f) value of each band was obtained as the ratio of distance moved by solute to that of solvent (Nithya and muthumary 2010).

$$Rf = \frac{\text{Distance Traveled by the solute}}{\text{Distance traveled by the solvent}}$$

The crude EA fraction of endophytic fungi showing bands similar to that of crude plant fraction was selected for further characterization.

4.6 High Performance Liquid Chromatography

The crude fraction of selected endophytic fungi and plant was subjected to HPLC (Perkin Elmer-200 series pump). 50µg of crude fraction was dissolved in methanol and injected into the HPLC column. Gradient elution of orthophosphoric acid (0.1%) and acetonitrile ranging from 10% to 70% was used as mobile system with a flow rate of 1 ml/min. 20µl of each sample was injected into C18 (5 µm) reverse phase Discovery column (Sigma Aldrich) with 4.6 mm internal diameter x 150 mm Length. The peaks obtained in both chromatograms were used to ascertain identity of similar compounds in both fractions.

4.7 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

GC-MS analysis of the crude fraction was performed in a Hewlett Packard Gas Chromatography HP 6890 connected with Hewlett Packard 5973 Mass spectrometer system. The column used in HP-5MS capillary column with 30m height and 0.25mm internal diameter. The film thickness was 0.25µm. The temperature of the oven was programmed from 70-250˚C. For mass spectrometry, ion source was adjusted at 250˚C and 70 eV. Helium was used as the carrier gas with a flow rate of 1mL/min. Scanning range was set to 35 to 425 amu. The compounds suspended in methanol (HPLC grade) was injected into the GC/MS system with a run time of 30 min.

4.8 Identification of endophytic Fungi

Selected potential isolate, #88FPLPR was identified using classical and molecular taxonomy tools.

4.8.1 Classical taxonomy

The endophytic fungal isolate was examined under the microscope to identify its morphological and microscopic characters. The culture was grown on different media namely Potato Dextrose Agar (PDA), Water agar (WA), Grass leaf agar (GLA), Corn meal agar (CMA) and Synthetischer nahrstoffarmer agar (SNA). The glass slide was cleaned with alcohol and dried. A drop of water was placed on glass slide, upon which the mycelial mass taken from the tip of the colony using a fine needle was placed along and teased properly. It was then stained with Lactophenol cotton blue (Himedia). The slide was covered with 18×10 mm coverslip avoiding the formation of air bubble and mounted with DPX. The slide was microscopically observed at 100X, 400X, 1000X using Nikon binocular microscope. The fungi was identified based upon their spore structure and other morphological characteristics.

4.8.2 Molecular Taxonomy

Fungal Genomic DNA was isolated from 3-4 day old culture fully grown on PDA plates. 0.5-1 g of wet mycelium was crushed into very fine powder by using liquid nitrogen. To the crushed powder, 660-750 µl of the extraction buffer was added and was crushed again. The contents were shifted to a 1.5 ml micro centrifuge tube followed by addition of 10µl of β-mercaptoethanol and 4µl of Proteinase K. The contents were vortexed and incubated at 65˚C in water bath for 1 hour, they were mixed after every 15 minutes. After the incubation was over, the micro centrifuge tubes were centrifuged at 12,000 rpm for 15 min to remove cell debris. Further, 6 µl of RNase was added followed by incubation at 37˚C for half an hour. For the removal of protein, equal volume of Phenol: Chloroform (1:1) solution was added to each tube and mixed properly for 15 min and centrifuged at 12,000 rpm for 10 min, this step was repeated three times. Transfer the aqueous layer containing DNA to the fresh micro centrifuge tube carefully avoiding the inclusion of debris and other impurities along with it. Further, 20µl of 3M sodium acetate was added and the content of each micro centrifuge tube were top up with absolute ethanol and incubated at -4˚C overnight. Mix the contents by swiftly inverting the tubes to observe the white threads of precipitating DNA. On the next day, the micro centrifuge tubes were centrifuged at 12,000 rpm for 10 min and the pellet was rinsed with 70% ethanol followed by centrifugation at 12,000 rpm for 5 min. The DNA pellet was dried and further dissolved in 30µl of Tris EDTA buffer (pH=8). The qualitative estimation of the isolated DNA was done by Agarose Gel Electrophoresis.

4.8.2.1 Agarose Gel Electrophoresis

0.8% agarose gel containing ethidium bromide (Et Br) at the concentration 0.5 µg/ml was prepared 1X Tris Acetate EDTA (pH=8) and casted in the electrophoretic apparatus along with 8 well comb. The gel was allowed to solidify and the comb was carefully removed. Electrophoretic running buffer (1 X TAE) was put into the tank so that the gel is fully immersed into the buffer. The DNA samples were mixed with the 5 X loading dye. The sample were loaded into the wells and allowed to run at 60 volts for 1h. The gel was observed under UV transilluminator for presence of DNA. Gel imaging was carried out under UV light in Bio-Rad Gel Documentation System using Quantity-1-D analysis software.

For the quantitative analysis of DNA, the absorbance of DNA was taken at 260 and 280 nm. 50 µg/ml of DNA sample is equal to 1O.D. The concenteration of DNA was calculated by following formula–

$$\text{Concentration } (\mu g/ml) = O.D_{260nm} \times 50\mu g/ml \times \text{Dilution factor}$$

The purity of the DNA sample was analyzed by taking ratio of O.D. at 260 and 280 nm. If the value comes below 1.6, the DNA is contaminated with RNA. If it is between 1.6 – 1.8, the DNA sample is pure. If the value comes above 1.8, the DNA sample is contaminated by protein.

4.8.2.2 PCR Amplification

PCR is a rapid process for *in vitro* amplification of desired DNA sequence by using specific primer to produce a large amount of desired DNA fragment of sequence length. ITS1-5.8S-ITS2 rDNA sequence was amplified by utilizing ITS 1 (5' TCC GTA GGT GAA CCT GCG G 3')and ITS 4 (5' TCC TCC GCT TAT TGA TAT GC 3') primers synthesized by Xcelris Labs Pvt Ltd, Gujarat in a Thermocycler (Verti 96 well Thermal cycler, Applied Biosystems). Amplification was carried out in 25µl reaction mixture containing 1µl of extracted fungal DNA, 10µM of each primer (ITS1 and ITS4, 2.5mM of dNTP (Bangalore GeNei), 1.5 µ of Taq DNA Polymerase in 10 X Taq buffer (Table 3). The PCR cycling conditions consisted of initial denaturation at 96˚C for 5 min followed by 39 cycles for 95˚C for 45 sec, 60˚C for 45 sec, and 72˚C for 45 sec followed by final extension at 72˚C for 5 min. The PCR amplicon were observed using 1.5% agarose gel dissolved in 1X TAE buffer at 50V for 1.30 h. Gel imaging was performed under UV light in Bio-Rad Gel Documentation System using Quantity-1-D

analysis software. An approximate 550-600 bp PCR product sent for sequencing to Xcelris labs Pvt. Ltd, Ahmedabad, Gujarat**.**

S.No.	Reagents	Stock Concentration	Quantity	Final Concentration in 25 µl
1.	Autoclaved Double Distilled Water	-	15 µl	-
2.	Taq buffer	10 X	2.5 µl	1 X
3.	dNTPs	2.5 mM	2.0 µl	0.2 mM
4.	Primers	10 µM	2.0 µl	0.8 µM
5.	Taq DNA Polymerase	3 U/µl	0.5 µl	1 U
6.	Template DNA	65 ng/µl	1.0 µl	65 ng

Table 3 Different reagents used in PCR reaction mixture

5. Results and Discussion

5.1 Isolation of endophytic fungi

In the present work, 33 endophytic fungi were isolated form *Plumeria rubra*, regularly cultured on PDA plates and maintained at 28˚C. All the isolates were preserved on PDA-glycerol slants for long term storage. During the course of evolution, there is exchange of genetic and chemical information between endophyte and host plant thereby endophytes possess the potential to mimic the property of host plants and were capable of producing the host related bioactive compounds. Hence the present study deals with isolation of endophytic fungi from *Plumeria rubra* and further exploration and identification of host related bioactive compounds.

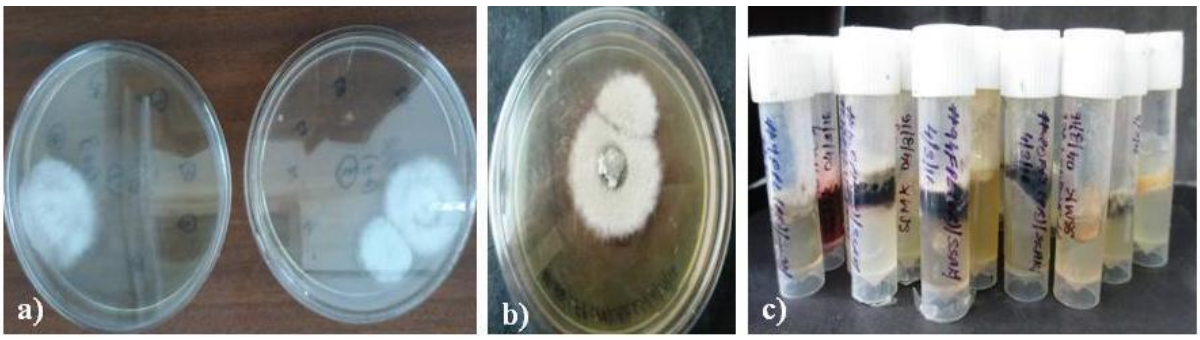

Figure 2 showing a) endophytic fungi emerged out from inoculated plant part b) Pure culture of endophytic fungal isolate c) PDA-glycerol stock of different endophytic fungi

All the 34 cultures were isolated from leaf of *Plumeria rubra* collected from different location. No visible growth of fungi seen on inoculated stem parts. 19 cultures were isolated from leaves collected from Passi Road, 7 cultures from Auditorium sample, 4 samples from Library Backyard and 5 samples from guest house garden area.

5.2 Production of culture filtrate

All 34 isolates under study were subjected to secondary metabolites production in PDB medium. Biomass production was considered as a parameter to infer the growth rate of fungal cultures. #44FPLTU showed the highest biomass production while the cultures with the lowest biomass production were #118FPLGHTU (Table 4). #118FPLGHTU gave maximum amount of culture filtrate while #29FPLPR gave the lowest amount of culture filtrate. Most of the cultures exhibited acidic pH but some of the cultures also showed basic pH.

S.No.	Culture Code	Plant Part	Place of Collection	Dry weight of Biomass (g)	Volume of filtrate (ml)	pH
1.	#2FPLPR	Leaves	Passi Road, Patiala	1.05	10.5	6.63
2.	#4FPLPR	Leaves	Passi Road, Patiala	1.01	10	5.58
3.	#7FPLPR	Leaves	Passi Road, Patiala	0.96	9	6.26
4.	#10FPLPR	Leaves	Passi Road, Patiala	1.13	9	6.87
5.	#13FPLPR	Leaves	Passi Road, Patiala	1.03	8	6.71
6.	#14FPLPR	Leaves	Passi Road, Patiala	1.22	10.5	6.96
7.	#19FPLPR	Leaves	Passi Road, Patiala	1.03	11.5	6.78
8.	#20FPLPR	Leaves	Passi Road, Patiala	1.14	9.5	6.72
9.	#23FPLGHTU	Leaves	Guest House, Thapar University, Patiala	0.20	6.5	8.06
10.	#25FPLPR	Leaves	Passi Road, Patiala	1.05	10.5	6.33
11.	#29FPLPR	Leaves	Passi Road, Patiala	1.19	2	6.83
12.	#31FPLLBTU	Leaves	Library, Thapar University, Patiala	0.22	8.5	7.68
13.	#39FPLPR	Leaves	Passi Road, Patiala	1.14	7	8.19
14.	#40FPLADTU	Leaves	Auditorium, Thapar University, Patiala	0.19	4	7.52
15.	#40FPLTUPR	Leaves	Passi Road, Patiala	1.25	14.5	5.31
16.	#44FPLADTU	Leaves	Auditorium, Thapar University, Patiala	0.25	3	7.34
17.	#44FPLGH TU	Leaves	Guest House, Thapar University, Patiala	0.15	10.5	7.07
18.	#47FPLPR	Leaves	Passi Road, Patiala	1.22	4	7.24
19.	#50FPLADTU	Leaves	Auditorium, Thapar University, Patiala	0.15	11	6.30

20.	#51FPLADTU	Leaves	Auditorium, Thapar University, Patiala	0.21	*5*	7.16
21.	#53FPLLBTU	Leaves	Library, Thapar University, Patiala	0.21	8.5	7.50
22.	#54FPLADTU	Leaves	Auditorium, Thapar University, Patiala	0.20	4.5	6.57
23.	#65FPLPR	Leaves	Passi Road, Patiala	1.02	9	6.41
24.	#76FPLLBTU	Leaves	Library, Thapar University, Patiala	0.21	10	7.50
25.	#77FPLLBTU	Leaves	Library, Thapar University, Patiala	0.18	8.5	6.62
26.	#80FPLPR	Leaves	Passi Road, Patiala	1.14	7	6.14
27.	#83FPLLBTU	Leaves	Library, Thapar University, Patiala	0.21	7	7.23
28.	#88FPLPR	Leaves	Passi Road, Patiala	1.06	12	5.90
29.	#90FPLPR	Leaves	Passi Road, Patiala	1.09	6	7.51
30.	#94FPLPR	Leaves	Passi Road, Patiala	1.06	6	6.88
31.	#103FPLGHTU	Leaves	Guest House, Thapar University, Patiala	0.23	9.5	8.20
32.	#104FPLPR	Leaves	Passi Road, Patiala	1.13	7	7.33
33.	#118FPLGHTU	Leaves	Guest House, Thapar University, Patiala	0.10	16.5	4.58

Table 4 showing biomass weight, pH and volume of filtrate of endophytic fungi isolated from *Plumeria rubra*

5.4 Solvent extraction

The cell free filtrates of all endophytic fungal isolates were subjected to solvent extraction by ethyl acetate. The obtained bioactive compound further was reconstituted in methanol. #118FPLGHTU (3.48mg) gave the highest yield of bioactive compound and was lowest in case of #54FPLADU (0.10 mg) (Table 5). The crude ethyl acetate residue of #88FPLPR produced crimson red colored compound while that of #77FPLLBTU produced yellowish and #23FPLGHTU produced pale yellow colored compound.

Culture Code	Yield of compound (mg)
#54FPLADTU	0.10
#76FPLLBTU	1.26
#78FPLADTU	1.32
#88FPLPR	1.24
#118FPLGHTU	3.48

Table 5 Yield of bioactive compounds recovered from different cultures under study

5.5 Isolation of secondary metabolite from plant leaves

The compound isolated from plant leaves were dark green in color (Figure 3) and the yield of the compound was 760 mg. The compound was dissolved in methanol and was further analyzed by preparative TLC. The crude fraction was emitting sweet fragrance.

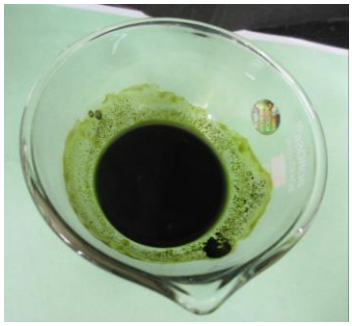

Figure 3 showing dark green colored crude plant fraction

5.6 Thin Layer Chromatography (TLC)

The crude EA fractions of all endophytic fungi were spotted along with crude plant fraction. Out of 34 endophytic fungi, #88FPLPR was showing three bands with same R_f value to that of plant fraction. The best separation of crude bioactive fraction of #88FPLPR was achieved in Hexane: Chloroform: Ethyl Acetate (6:4:50 µl) which gives 7 bands. The R_f value of these

7 bands was 0.829, 0.719, 0.634, 0.551, 0.468, 0.401 and 0.382. Out of these 7 bands, band 1, 3, 7 with Rf value 0.382, 0.468, 0.829 respectively was similar to that of crude plant fraction.

S. No	Solvent System	Ratio Used	Results
1.	Chloroform : Methanol	9:1	No separation
2.	Chloroform : Methanol	8:2	No separation
3.	Ethyl Acetate : Methanol	6:4	No separation
4.	Hexane : Ethyl Acetate	6:4	2 bands
5.	Hexane : Ethyl Acetate	8:2	1 band
6.	Hexane : Ethyl Acetate	1:1	No separation
7.	Hexane : Chloroform	6:4	2 bands
8.	Hexane : Chloroform : Methanol	3:2:50µl	No separation
9.	Hexane : Chloroform: Methanol	3:2:100µl	No separation
10.	Hexane : Chloroform : Ethyl Acetate	6:4:100µl	3 bands
11.	**Hexane:Chloroform: Ethyl Acetate**	**6:4:50µl**	**7 bands**

Table 6: Different mobile phase used for TLC of crude bioactive extract of #88FPLPR

5.7 HPLC analysis

The crude EA fraction of #88FPLPR and crude plant fraction was further analysed through HPLC. The peaks of crude plant fraction obtained at different retention time was compared with peak and retention time data of crude EA fraction of #88FPLPR. In Crude plant fraction, 8 minor peaks at retention time of 2, 3.2, 3.5, 3.7, 4.2, 4.7, 4.8, 7.3 min and three prominent peaks at retention time of 2.5 min, 3 min and 5.3 min was observed. However, In crude EA fraction of #88FPLPR, one major peak at 2 min and 5 small peaks at retention time of 2.4, 2.8, 5.0, 5.3 and 6.5 min was observed. On comparing the HPLC spectra of crude EA fraction and crude plant fraction, only two peaks at retention time of 2 min and 5.3 min was found to be common. Hence, further similarity between the compounds of crude plant fraction and EA fraction of #88FPLPR was deduced by GC-MS analysis.

5.8 GC-MS analysis

The crude EA fraction of #88FPLPR and plant fraction was further analysed by GC-MS carried out at SAIF, Panjab University, Chandigarh. The GC-MS spectra of crude plant

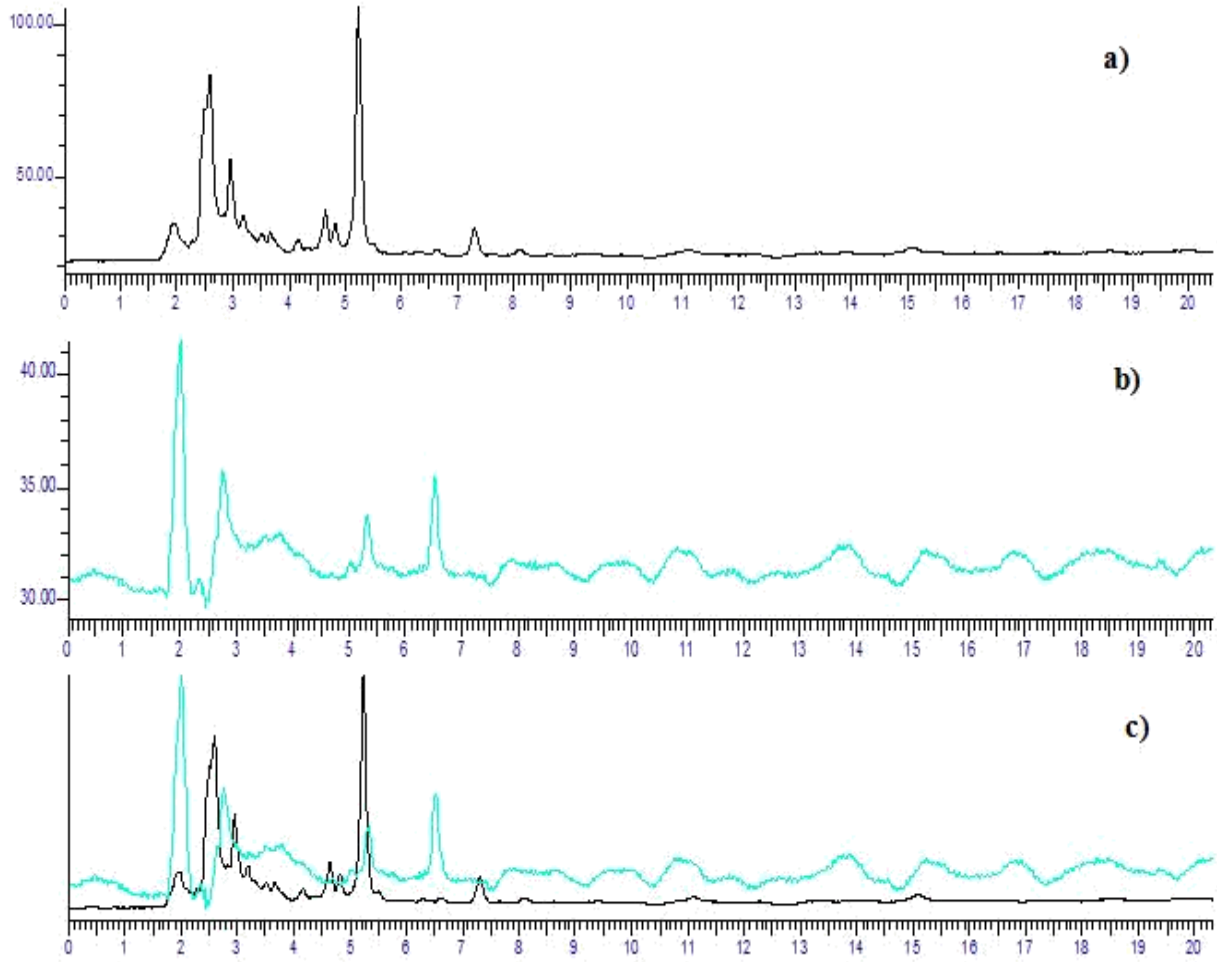

Figure 4 HPLC spectra of a) crude leaf extract of *Plumeria rubra* b) crude fungal extract of fungus c) combined spectra of leaf extract and fungal extract.

Name of Compound	RT (Plant fraction)	RT (#88FPLPREA fraction)	% Area (Plant)	Area% (#88FPLPR fraction
Cyclohexasiloxane, dodecamethyl-	13.69	13.70	5.30	2.72
Cycloheptasiloxane, tetradecamethyl	17.99	17.99	4.58	1.06
Hexadecanoic acid, methyl ester	27.31	27.30	7.66	12.23
9,12-Octadecadienoic acid (Z,Z)-, methyl ester	30.48	30.57	38.45	38.45
9-Octadecenoic acid (Z)-, methyl ester	30.59	30.61	25.66	36.23
Methyl stearate	31.10	31.07	3.40	3.87
9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)-	36.98	36.97	5.99	5.45

Table 7: GCMS result of purified plant compound and fungal compound

fraction exhibited presence of 21 different compounds while crude EA fraction was showing 10 different compounds. On comparing the GC-MS spectra, 7 compounds were found to be common between the crude EA fraction of #88FPLPR and crude plant fraction (Table 7).

5.9 Identification of endophytic fungi

5.9.1 Morphotaxonomy

The endophytes were grown on different medium such as PDA, CMA, SNA, Water Agar, GLA, and PLA so that they can be identified on the basis of their morphological and microscopic properties. Colony of #88FPLPR over PDA medium was white in color from front and dark brown in color from back, slow growing with smooth margins (Figure 5). Over Pine Leaf Agar (PLA) colony was grey from front and from reverse side it was black in color with no pigmentation or odour with smooth margins. While over GLA, colony exhibited brown colored pigmentation but colony was observed to be grey in color from front with rough margins. Its appearance was velvety to woolly. Further,morphological characteristics over different media is listed in Table 8.

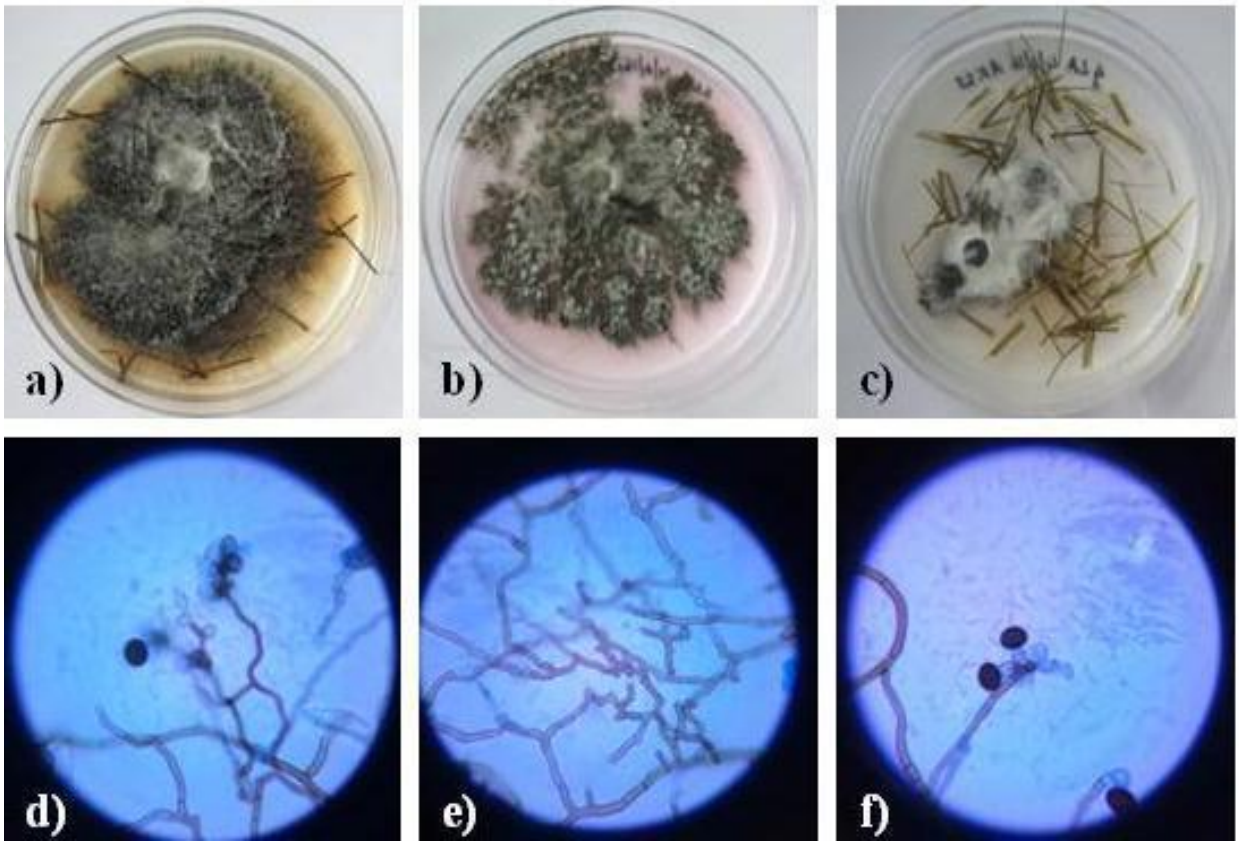

Figure 5 Colony morphology of #88FPLPR over different media a) PLA with brown pigment b) SNA, pink pigment c) GLA, d-f) oval shaped conidia observed

Character	GLA	PLA	SNA	WA	CMA
Diameter (mm)	33 mm	90 mm	90 mm	39 mm	50 mm
Colony color (Front)	White	Grey	Greyish white	Grey	Black
Colony color (Back)	Black	Black	Black	Grey	Black
Pigment formation	No Pigment	Brown	Pink	Pink	No Pigment
Margin	Smooth	Rough	Rough	Rough	Rough
Odour	No Odour	No Odour	No Odour	No Odour	No Odour

Table 8 Characteristics of Endophytic fungi on different medium

Conidia were single celled, oval shaped, black in color with smooth surface, hyaline sepate hyphae, pigmented conidiophores were observed. Conidiogenous cells were monoblastic and bear single conidium at the apex. Based on above morphological and microscopic characteristics, the potential isolate, #88FPLPR was tentatively identified as *Nigrospora* sp.

5.9.2 Molecular Identification:

The DNA was qualitatively estimated using agarose gel electrophoresis and the size of the genomic DNA deciphered by comparing its mobility in the gel with the 1kb DNA ladder (Stepup, Bangalore Genie) which ranges from 1kb to 10 kb. There was no RNA band seen, hence the RNase treatment was successful (Figure 6a). The concentration of DNA was estimated by taking the absorbance at 260 nm and the amount was 65ng/µl by using formula

$$\text{Concentration } (\mu g/ml) = \text{O.D}_{260nm} \times 50\ \mu g/ml \times \text{Dilution factor}$$

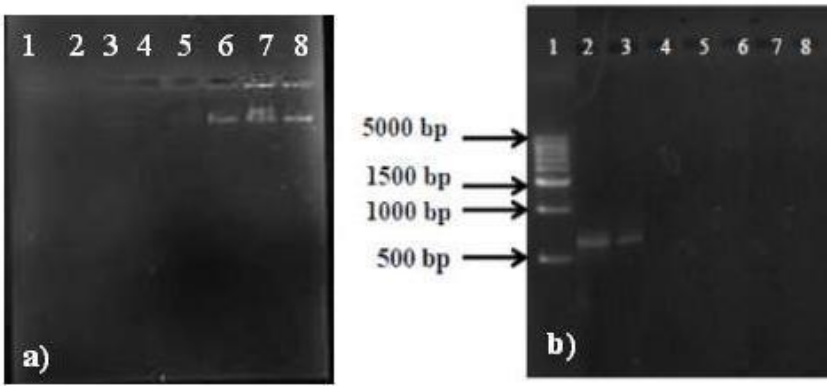

Figure 6 a) Genomic DNA isolation of #88FPLPR b) PCR amplicon of ITS region of #88FPLPR, lane 1: 500bp ladder; Lane 2-3: PCR amplicon of ITS region

The size of the PCR amplicon was found to be approximately 550 bp to 600 bp (Figure 6b). This size can be easily compared to the ITS region, which was amplified in order to characterize the fungi at molecular level.

Further, appropriate phylogenetic speciation of the endophytic fungi will be ascertained by analyzing the sequencing data.

6. Conclusion

From the current study, we conclude that the endophytic fungi, #88FPLPR isolated from Leaf of Frangipani (*Plumeria rubra)* have potential to mimic the properties of host plant. The results were confirmed through TLC, HPLC and GC-MS. In HPLC, crude plant fraction and EA fraction of #88FPLPR was showing two similar peaks but the similarity between the compounds were further confirmed by GC-MS. In GC-MS, 7 compounds were found to be identical in both plant and fungal fraction. The culture was further identified as *Nigrospora sp.* The species level identification of #88FPLPR will be deduced after getting sequencing data of ITS region. Further isolation and purification of identical compound is warranted to confirm this host plant relationship of genetic and chemical exchange of information.

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