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# AN EVALUATION OF POTENTIAL BIOACTIVE METABOLITES OF ENDOPHYTIC FUNGI ISOLATED FROM MEDICINAL PLANT

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## ABSTRACT

Medicinal plants and their endophytes are potent resources for discovery of novel bioactive metabolites. Medicinal plants harbor endophytic mycoflora. The endophytic fungi from plants were an important source for the production of various secondary metabolites, bioactive compounds which are useful in pharmacology, agriculture and in industries. Endophytic fungi are a group of fungi that colonize living and internal tissues of plants without causing any immediate, over negative effects. In our present research work, endophytic fungi were isolated from the leaves of medicinal plant *Pisonia grandis*. The frequency of the presence of endophytic fungi was studied based on seasonal variation. Eight endophytic fungi were isolated, of which three isolates were sporulating and was identified based on colony morphology, sporulating structures and taken for further studies. The ethyl acetate extract of the isolates were screened for phytochemical studies and also assayed for invitro antibacterial activity. Further, the fungal isolates were checked for their ability to produce extracellular enzymes by qualitative assay. The isolates were also screened for extracellular biosynthesis of silver nano particles for which UV-VIS absorption was recorded. FT-IR analysis was also performed to confirm the presence of various functional groups.

**KEYWORDS**: Medicinal plant, endophytic fungi, phytochemical, antibacterial, silver nano, FT-IR.

## INTRODUCTION

Endophytes are microbes (fungi/bacteria) that live within the plant tissues (leaves, stem, roots) without causing any noticeable symptoms of disease.[1] The most frequently isolated endophytes are fungi. The term "endophytic fungi" refers to an organism which lives within plant tissue by forming symbiotic relationship with host [2] and does not cause harmful effect to the plant species. The endophytic fungi involves in defense mechanism of host against pathogen. In symbiotic association, inhabited plants benefit by increasing resistance to herbivores grazing through the production of various alkaloids and hence can also be used as bioprotectants and biofumigants. The endophytic fungi from plants were an important source for the production of various secondary metabolites, bioactive compounds which is useful for pharmaceutical industries.[3]. Production of unique bioactive metabolites such as alkaloids, benzoquinones, flavanoids, phenols, stereoids, terpenoids, tetralones, xanthones etc have been identified from endophytic fungi.[4] Endophytes were also isolated from plants having insectidical, antimicrobial, antioxidant and antipyretic activities and can be used for pharmacological activities. Recently, certain isolates of endophytic fungi from Chinese herbs have been used as biocontrol agents for agricultural crops.[5&6] Medicinal plants are reported to harbor endophytes which in turn provide protection to their host from infection agents and also provide adaptability to survive in adverse environmental condition. It is therefore important to determine the endophytic fungi diversity of medicinal plants.[7] The endophyte will provide a form of systematic, natural resistance that means farmers will not have to spray crops routinely in a bid to avert diseases. Essentially the plant will be protected by organisms that remain active with in the plant through the entire season. By using the endophytic fungi as bio fumigation, the endophyte suppresses the soil borne pests and pathogens by releasing more natural biocides. In the United States, an endophyte isolated from Cinnamon tree, demonstrated a broad biocidal activity against a wide range of destructive pathogens and is being commercially developed as a mycofumigant – placed into the soil prior to planting to disinfect soils. These endophytic fungi are much needed and useful for present world.[8]

In the present study, the leaves of *Pisonia grandis*, an important medicinal plant, was used for the isolation of

endophytic fungi and to evaluate their potential bioactive metabolites. The plant *Pisonia grandis* (synonym: *Pisonia alba*, *Pisonia morindifolia*) commonly known as Leechikottai kerai in Tamil. The plant *Pisonia grandis* R.Br belonging to the family Nyctaginaceae, is an evergreen glabrous garden tree. It is native of Hawai island also neutralized throught India.[9] The leaves are ovate-oblong, 15-25cm long, 5-7 cm wide usually unequal obtuse at the base and cute apex.[10] Plants are antidiabetic, anti inflammatory, wound healing, diuretic, analgesic, filariasis, dysentry and rhuematic disorders. In the alternative system of medicine, *Pisonia grandis* leaves are used as analgesic, anti-inflammatory, diuretic and hypoglycemic agent. Leaves are used as carminative and anti inflammatory, diuretic. Root is considered purgative.[11] Hence, the objective of our present study is to isolate endophytic fungi from the leaves of medicinal plant *Pisonia grandis* and to screen the potential metabolites by screening their antimicrobial activity, production of extra cellular enzymes, synthesis of silver nano particles and FT-IR analysis.

## MATERIALS AND METHODS

**Collection of Plant Material:** The leaves of medicinal plant, *Pisonia grandis* was collected from in and around Chennai. The healthy leaves were used for the isolation of endophytes at different seasons such as in the rainy season (November) and late summer (July) to study the seasonal variations of endophytic fungi.

**Isolation and Identification of Endophytic Fungi**

The healthy leaves were collected and washed under running tap water. The endophytic fungi from the leaves were isolated using standard procedure.[12] The number of individual fungal colonies were counted and each fungal colony was isolated and sub cultured separately to get the pure culture. The morphology of surface texture, pigmentation, and spores at the hyphal tips, were used to identify the different endophytic fungi at genus level using standard manual.[13] The endophytic fungi isolated from the host plant collected at two different seasons were analyzed based on Colonization frequency and Percentage of endophytic infection rate (EIR).[14]

Colonization Frequency**:-** (CF%)

$$CF\% = \frac{\text{No. of species isolated}}{\text{No. of segments screened}} \times 100.$$

Endophytic infection Rates : - (EIR %)

$$EIR\% = \frac{\text{No.of infected segments}}{\text{Total no.of segments screened}} \times 100$$

**Fermentation and extraction:** A 500 ml Erlenmeyer flask containing 200 ml of potato dextrose broth (PDB) was inoculated with 2-3 agar plugs containing mycelia. It was taken from the culture of each endophytic fungal isolate purified on PDA and incubated at room temperature for 15days. The fungal culture was filtered with cheese cloth to remove mycelium and filtrate separately. Both Culture filtrate and mycelia were extracted with ethyl acetate (1:1 ratio) three times. Then the organic phase was evaporated to dryness and finally Culture filtrate extract (CFE) and Culture mycelial extract (CME) were stored for further use.

**Preliminary Qualitative Phytochemical Screening:**

The ethyl acetate extract of the endophytes were checked for the presence of the following secondary metabolites such as Alkaloids, Terpenoids, Phenols, Carbohydrates, proteins& amino acids, Saponins gum/mucilage, by standard procedure.[15]

**Antimicrobial activity:** Different concentrations (50µg & 100µg) of both culture filtrate extract (CFE) and culture mycelial extract (CME) were assayed against bacteria such as *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus.* Assay was carried out using well diffusion method using standard procedure. Muller Hinton Media was used. Gentamycin (10mg) were used as positive control. The plates were incubated at 37$^{o}$C. Zone of inhibition around the well was observed after 24hrs. Triplicates were maintained for all the samples.

**Extra cellular enzyme assay:** Extra cellular enzymes (amylase, cellulase, laccase, lipase & Protease) assay were carried out with standard procedure to investigate the production of enzymes by the endophytic fungi. It was assessed by digestion of (or) dissolved suspended substrate in agar plates after inoculation with 3mm mycelia plugs and incubation for 3-5 days at 37$^{o}$C. The diameter of the clear zone was used as a measurement of the amount of enzyme production.[16]

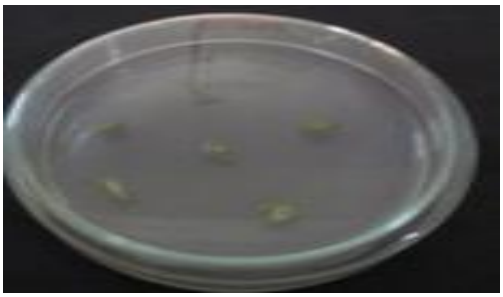
**Extra cellular synthesis & characterization of silver nano particles from endophytes:** For the synthesis of silver nano particles, the fungal isolates were grown in 250 ml flask containing 100 ml Potato dextrose broth (PDB) at room temperature for 72 hours and then the biomass was harvested and filtered through Whattman filter paper No.1. The fungal mat was washed with distilled water to remove media component and suspended in 100 ml distilled water for 48 hours. After 48 hours of incubation, the cell filtrate was separated by filtration. The fungal cell filtrate was then collected and it was challenged with the AgNO3 salt (final conc. 1mM).[17] After 24 hours of incubation, the formation of silver nano particles was screened by visual observation of colour that changes from pale white to brown. Then it was further confirmed by subjecting the reaction mixture to UV – Visible spectrophotometer analysis. The spectrum was scanned at the resolution of 1 nm, between 200 – 800 nm for each sample.

**Fourier Transform Infrared Spectrophotometric analysis:** Spectrum FT-IR system (Shimadzu, IR Affinity 1, Japan), equipped with a DLATGS detector with a mirror speed of 2.8mm/sec. scan range: from 400-

4000cm$^{-1}$ with a resolution of 4cm$^{-1}$ was used for the FT-IR study. The ethyl acetate extract of the endophytes were prepared. The extract was evaporated by flash evaporator and it was mixed with a KBr salt, using a mortar and pestle and compressed into a thin pellet. Infrared spectra were recorded on KBr pellet on a Shimadzu FTIR spectrometer 4000 - 500cm$^{-1}$.

## RESULTS AND DISCUSSION

**Isolation and identification of endophytes:** A total of eight endophytic fungi have been isolated from the leaves of medicinal plant *Pisonia grandis* Three isolates (37%) were sporulating and were identified as *Aspergillus niger, Aspergillus fumigatus & Aspergillus japonicus* and non sporulation forms accounted for (63%) of the endophytic population. Hence, further work was carried out only with identified endophytes. (Plate: 1a & 1b). Medicinal plants are reported to harbor endophytes, which in turn provide protection to their host from infectious agents and they are unexpected producers of metabolites useful in pharmaceutical and agricultural industries. Occurrence of sterile mycelium as endophytic fungi is not unusual.[18] The fungal communities live inside the healthy tissue of medicinal plants which increases the absorption of soil nutrients and also change in nutrient cycle.[19] Recent studies have revealed the ubiquity of these fungi, with an estimate of 1 million species of endophytic fungi residing in plants.[20]



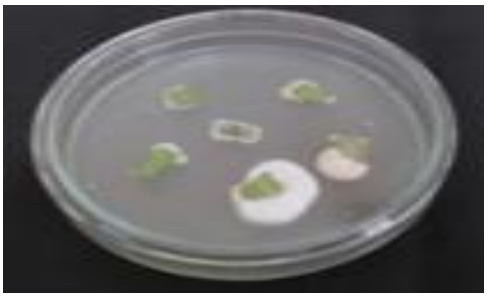


**Plate 1a- inoculation of leaves for isolation of endophytes Plate 1b- Emergence of endophytic fungi.**

The endophytic fungal isolate *Aspergillus niger (PaAn)* showed the following characteristic features: Colonies spread rapidly with mycelium white to dark brown to black conidial heads globose, radiate; conidiophores aroused from the substratum varying several millimeters long, smooth; philades conidia small, globose, rough.

The endophytic fungal isolate *Aspergillus fumigatus (PaAf)* showed the following characteristic features: Growth spreading, dark smoky green becoming darker, less velvety; conidial heads columnar varying length. conidiophores smooth,short, vesicles flask shaped, often greenish; philades borne directly on vesicles, closely packed. Conidia small, globose, smooth.

The endophytic fungal isolate *Aspergillus japonicus (PaAj)* showed the following characteristic features: Colonies purple brown, not very deep, phialides directly borne on the vesicles, closely packed, often appearing short, light brown in colour, conidia globose, distinctly spinulose,.

The leaves collected during rainy season (November) showed a higher percentage of colonization frequency compared to the leaves collected during late summer (July). Endophytic infection rate was higher in leaves collected during November month (Fig.1). The distribution of endophytes in a host may be influenced by the environmental factors, (Temperature, edaphic factors, extreme climatic condition and quality of air) nutrients supplied by the host plant, age of the host plant or tissues and chemicals present in the host plant.[21] Endophytic population varies from plants to plants and from species to species. Within the same species it not only varied from region to region but also differs with change in climatic conditions of the same region. Temporal changes in relative frequency of total endophytic fungi were studied in the matured leaves of teak (*Tectona grandis L.)* and rain tree (*Samanea saman* Merr.) which showed greater number of genera and species, with higher colonization frequency, when compared to the young leaves. During rainy season the occurrence of endophytes in leaves were found to be increased.[22]

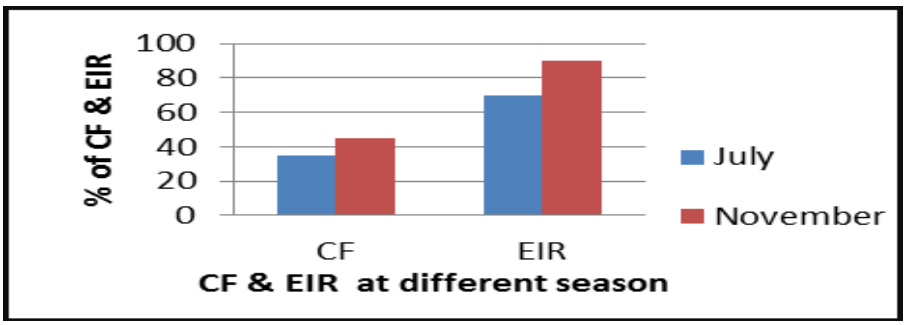



**Fig.1: Colonization frequency and Endophytic infection rate of *Pisonia grandis* at different season.**

**ANTIBACTERIAL ACTIVITY**

The results of inhibitory action of various concentrations (50µg, 100µg) of both culture filtrate extract (CFE) and culture mycelial extract (CME) of both endophytes were tested against bacteria (*Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus).*

The culture filtrate extract of *Aspergillus niger* at 100µg showed maximum inhibition against *Staphylococcus aureus* (12±0.3mm) and minimum inhibition against *Bacillus subtilis* (8±0.2mm). (Fig.2) The culture mycelial extract of *Aspergillus niger* at 100µg showed maximum inhibition against *Pseudomonas aeruginosa* (14±0.4mm) and minimum inhibition against *Bacillus subtilis* (7±0.2mm). (Fig.3)

The culture filtrate extract of *Aspergillus fumigatus* at 100µg showed maximum inhibition against *Bacillus subtilis* (13±0.1mm) and minimum inhibition against *E.coli* (7±0.2mm). (Fig.2) The culture mycelial extract of *Aspergillus fumigatus* at 100µg showed maximum inhibition against *Staphylococcus aureus* (10±0.3mm) and minimum inhibition against *E.coli* (8±0.2mm). (Fig. 3).

The culture filtrate extract of *Aspergillus japonicus* at 100µg showed maximum inhibition against *Pseudomonas aeruginosa* (12±0.1mm) and minimum inhibition against *E.coli* (7±0.2mm). (Fig.2) The culture mycelial extract of *Aspergillus japonicus* at 100µg showed maximum inhibition against *E.coli* (11±0.3mm) and minimum inhibition against *Staphylococcus aureus* (9±0.2mm). (Fig. 3).

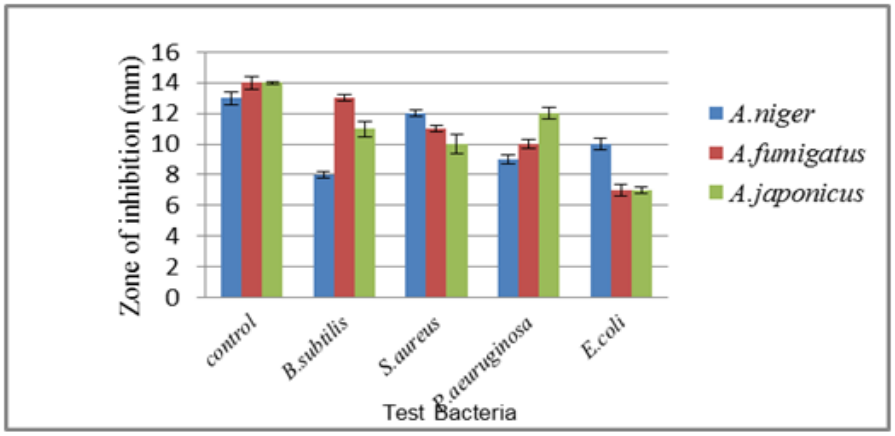



**Fig.2: Antibacterial activity of different endophytic fungal extracts (culture filtrate extract at 100µg) against Bacterial pathogens.**

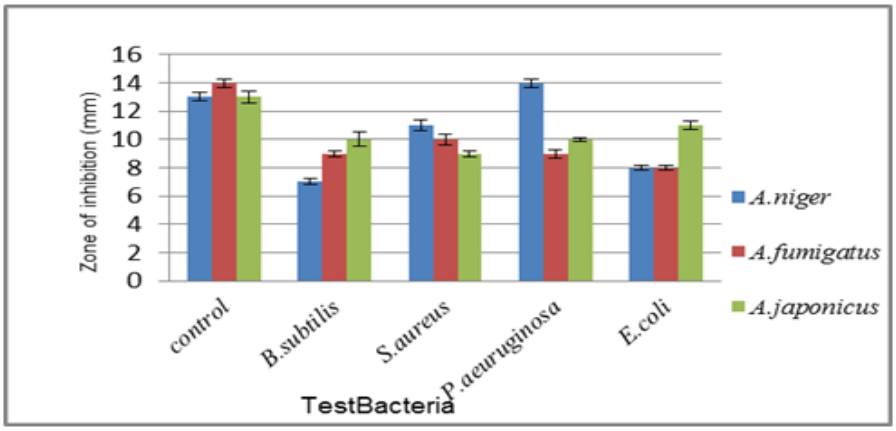



**Fig.3: Antibacterial activity of different endophytic fungal extracts (culture mycelial extract at 100µg) against Bacterial pathogens.**

Endophytic fungi have proved to be an important source for bioactive and antimicrobial compounds. They are rich source of functional metabolites such as alkaloids, amines, amides, indole derivatives, steroids, terpenoids, flavonoids, phenols, aliphatic compounds.[23] Antimicrobial metabolites produced by the fermentation of endophytic fungi have many advantages, including sustainable use, large-scale industrial production and quality control. From the results, it was observed that all the isolates exhibited different strengths of anti microbial activities. The differential susceptibility was attributed to the culture condition, extraction procedure and test strain used for antimicrobial analysis.[24] Our results indicate that the CFE and CME of *A.niger, A.fumigatus and A.japonicus* have a broad antimicrobial spectrum and strong toxicity of bio active components. The broad

antimicrobial spectrum was found to be variable in CFE and CME. Variation between CFE and CME may be due to the production of extra and intracellular metabolites. Similar finding were observed in the reports of antimicrobial activity of *Aspergillus sp*. isolated from *Justicia adathoda*. [25]

**Enzyme assay:** Endophytic fungi *A.niger* showed the presence of enzymes namely amylase and cellulase. *A.fumigatus and A.japonicus* showed the presence of amylase and cellulase respectively. (Plate 2a & 2b). Laccase, protease and lipase activity was found to be absent in all three endophytic isolates.

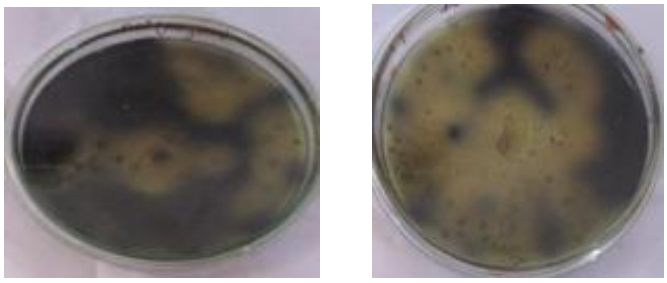


**Plate 2a- Enzyme amylase produced by *A.niger, A.fumigatus***

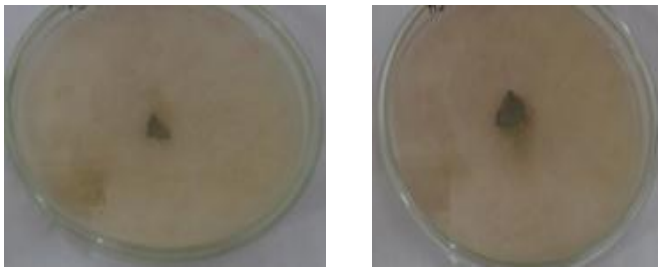


**Plate 2b- Enzymes cellulase produced by *A.niger & A.japonicus***

Fungal endophytes are gaining importance because of the production of enzymes. These fungal enzymes are often more stable (temperature ranges and diverse pH) than enzymes derived from plants and animals. It is useful in agriculture, industries (food processing, production of beverages, textiles and leather industry) and in human health.[26] Presence of amylase activity of the fungal isolates may have industrial and agricultural application with wide spectrum of biological property that can function at diverse pH and temperature ranges. The extra cellular degradative enzyme cellulase observed in fungal isolate may find its application in paper industry. The absence of laccase activity may be attributed to the endophytic nature of these fungi, since an active enzyme may damage the host plant.

**Synthesis of Silver nano particles by fungal Endophytes:**

Biological method is environment friendly and economically feasible way to synthesize silver nano particles.[27]. Fungi can produce large amount of nano particles as compared to bacteria because they can secrete larger amounts of protein which directly translate to higher productivity of nano particles. Their efficacy in the production of enzymes on a large scale, the ease of handling biomass makes it a model system for nano particle synthesis.[28] Silver nano particles are synthesized from endophytic fungi and they possess antimicrobial activity. Our results have revealed the rapid colour change of dark brown only in two fungal cell filtrates (after 24hrs) with addition of silver nitrate solution. *A.japonicus* showed no colour change. The appearance of colour change indicates the synthesis of silver nano particles. The formation of silver nano particles in fungal cell filtrate was further characterized by using UV- VIS Spectrophotometer. The reaction mixture of *A.niger* showed peak at 433 nm and *A.fumigatus* showed sharp peak at 422nm. (Fig: 4 & 5).

The characteristic brown colour arises due to the excitation of surface Plasmon vibrations in silver nano particles.[28] The mechanism of silver nano particles production by fungi is due to trapping of Ag+ ions at the surface of the fungal cells and the subsequent reduction of the silver ions by the enzyme present in the fungal system. Silver nano particles are used to solve the problem of emerging pathogens including multi-drug resistant bacteria. Silver nano particles are more effective because of the high surface area to volume ratio so that a large proportion of silver nano particles is in direct contact with their environment.[29] Silver nano particles are used in bio – labeling, biosensoration filters, nano dressing, textile fabrics beneficial for burnt patients, for surgical masks, in tissue conditioner etc., thus, silver nano particles are the ideal nano particles for the development of novel antimicrobial product and these are said to be antimicrobials of new generations.

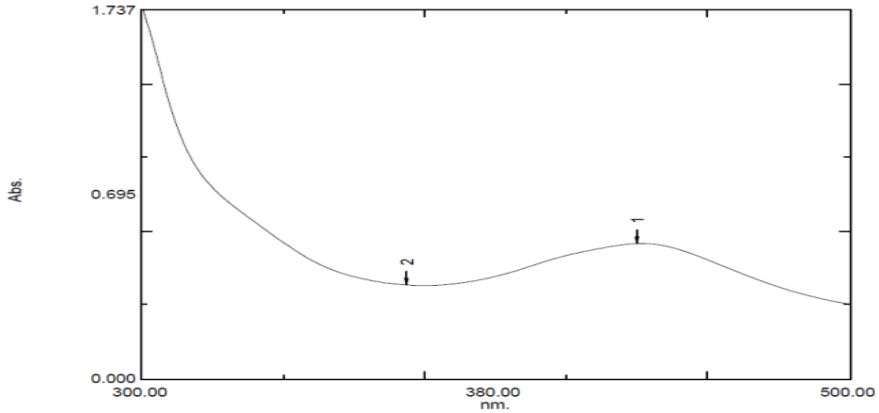



**Fig. 4: UV Vis Spectra of silver nano particles by *Aspergillus niger* showing peak at 433nm**

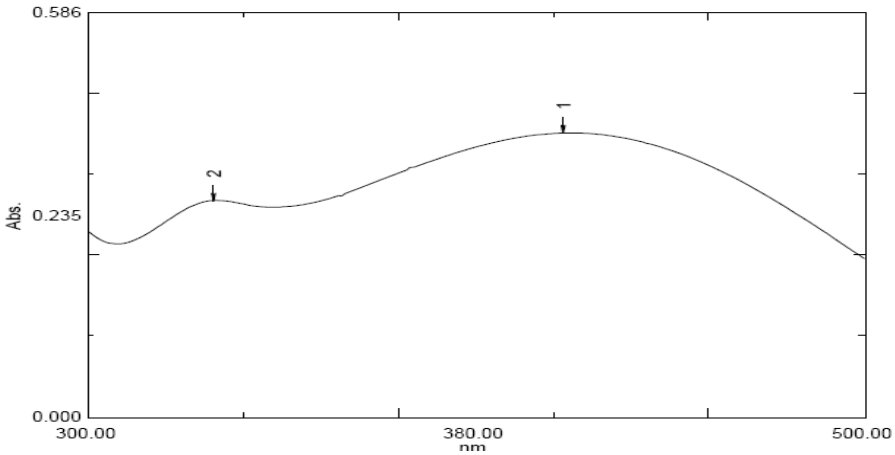



**Fig. 5: - - UV Vis Spectra of silver nano particles by *Aspergillus fumigatus* showing peak at 422nm**

**Phytochemical analysis**

The results of qualitative phytochemical analysis indicates that the ethyl acetate extract of endophytes contain alkaloids, carbohydrates, proteins, phenolic compounds and tannins. (Table:1)

**Table: 1 Phytochemical test for endophytes.**

| S.no | Phytochemical tests | *A.niger* | *A.fumigatus* | *A.japonicus* |
|---|---|---|---|---|
| 1 | **Alkaloid test** | | | |
| | Mayer's test | + | + | - |
| | Wagner's test | + | - | + |
| | Dragendroff's test | - | - | + |
| 2 | Terpenoid (Salkowski test) | - | - | - |
| 3 | **Phenolic compounds and Tannins** | | | |
| | Ferric chloride test | + | - | + |
| | Lead acetate test | - | + | + |
| | Alkaline reagent test | - | - | - |
| 4 | **Carbohydrates test** | | | |
| | Fehling's test | + | - | + |
| | Barfoed's test | - | + | - |
| | Benedict's test | - | + | - |
| 5 | **Protein and Amino acid tests** | | | |
| | Million's test | + | - | - |
| | Biuret test | - | + | - |
| | Ninhydrin test | - | + | + |
| 6 | Saponin Test | - | - | _ |
| 7 | **Fixed oils and fats** | | | |
| | Spot test | - | - | - |
| 8 | Gum and mucilage | - | - | - |

Endophytes are rich source of bioactive metabolites which have important potencies in medicine, agriculture and industries. The production of secondary metabolites from endophytes is associated with environmental factors. The endophytic interaction with its hosts may favour the synthesis of secondary metabolites.[30] They are rich source of functional metabolites such as alkaloids, amines, terpenoids, steroids, flavonoids, phenol compounds, quinines, isocoumarin derivatives, peptides etc**.** The presence of phytochemicals within the endophytes can be a potential source for medicinal and industrial use and it is considered to be an indicator that they can be potential source of precursors in the development of synthetic drugs.[31] There is an increasing effort to characterize and identify the endophytic fungi isolated from medicinal plants. Many studies have revealed that the medicinal properties of plants may be related to the presence of bioactive compound found in endophytic fungi hosted by these plants.[32] So, in our study the presence of various phytochemicals in the endophytic fungi may be attributed to that phytochemicals of the medicinal plants / vice versa.

**FT-IR Analysis**

(Fig. 6,7 & 8) FT-IR spectra of culture filtrate extract of fungal isolates indicates the presence of alkylhalides, carboxylic acids, aliphatic amines, alkanes, primary amines, esters, aromatics, nitro compounds, and aldehydes.

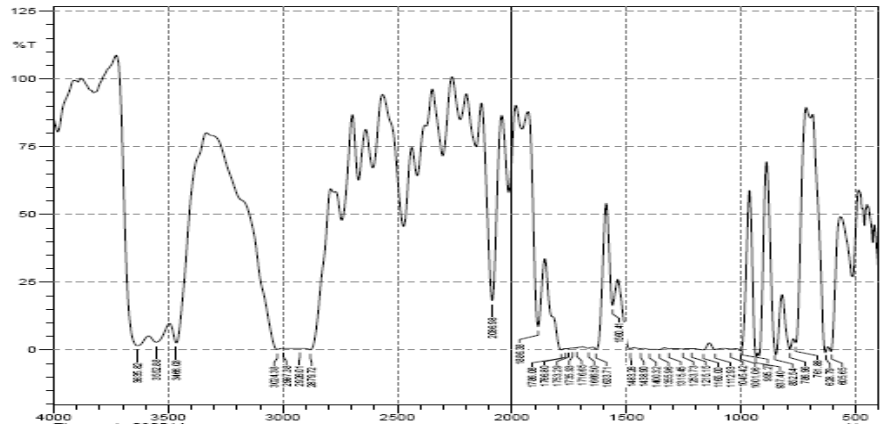



**Fig. 6:- FT IR spectrum of culture filtrate extract of *A.niger***

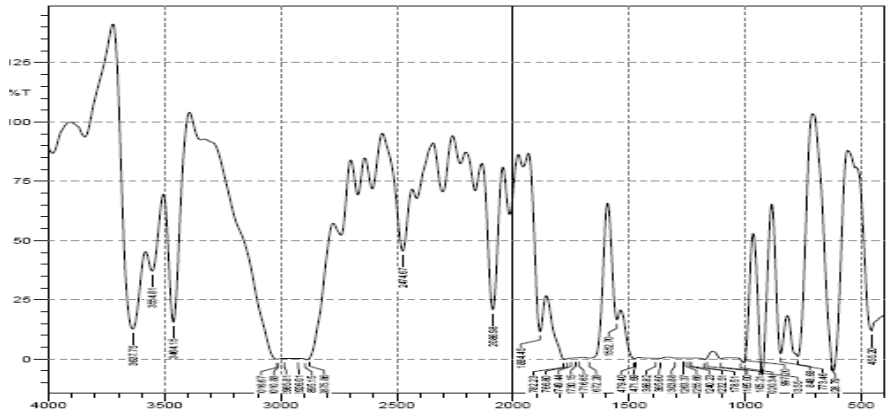



**Fig.7:- FTIR spectrum of culture filtrate extract of *A.fumigatus***

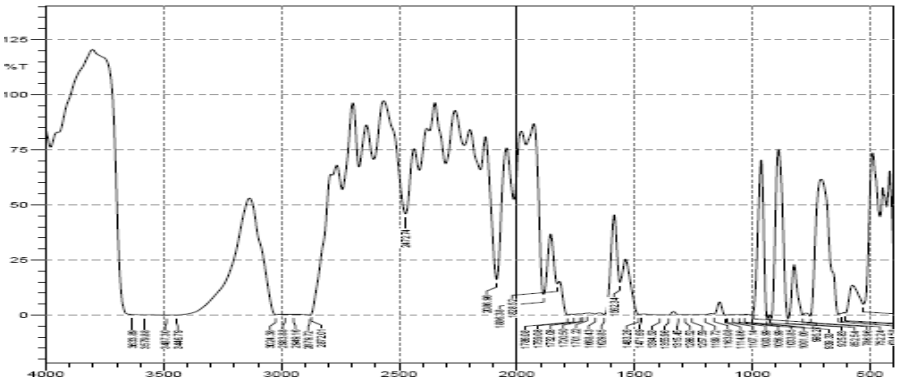



**Fig.8:- FTIR spectrum of culture filtrate extract of *A.japonicus***

FT-IR analysis of these endophytic isolates revealed the distribution of functional group within the organic fractions. The presence of various functional groups may be attributed to the existence of variety of potential phytochemicals.[33]

## CONCLUSION

In conclusion, the present study reveals that the endophytic fungi isolated from the medicinal plant *Pisonia grandis* showed the presence of potent antimicrobial activity, capable of producing enzymes, synthesizing silver nano particles and revealed the production of various phytochemicals. Thus endophytes from *Pisonia grandis* are remarkable natural source of biologically active metabolites. Further investigation will focus on the structure elucidation of bioactive compounds and its effective application strategies and the influence of environmental factors when used in different field conditions.

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