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Isolation and Identification of Endophytic Fungi from Tree Bark Showing Potent Antioxidant Activity

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Abstract
Endophytes are mutualistic harbors inside the living tissues of healthy plants without causing any symptoms of infection. These are important source of bioactive molecules, which have broad range of biological activities. An endophytic fungus isolated from bark of *Tamarindus indica*, *Ficus benghlensis* and *Mangifera indica*. The fungus was identified as *Aspergillus spp.1*, *Cladosporium* spp. and *Aspergillus* spp.2 based on morphological characterization. The ethyl acetate cell free extract was qualitatively studied by using TLC, which showed Rf values for phenol standard 0.45, and 0.45,0.47,0.42, for samples A1, C and A2 respectively, and for flavonoids standard was 0.82,for one sample i.e. *Aspergillus spp.1* showed Rf value 0.85.Total phenol content by Folin-catetu method, among three extract highest concentration ,98.58 µg/mg,other two showed 83.90 and 70.70 µg/mg.Total flavonoids by AlCl3 method, the total flavonoids content in *Aspergillus spp.1* was found to be 90.10 µg/mg. protein. All three extracts showed DPPH radical scavenging and H_2O_2 Scavenging property. *Aspergillus spp. 2 (Mangifera indica)* showed maximum % inhibition as compared to Ascorbic acid, *Aspergillus spp*. 1from (*Tamarindus indica)* and *Cladosporium spp.* from *(Ficus benghalensis).* The present study suggests that endophytic fungi associated with tree bark are potential agent as antioxidant activity.

Keywords
Endophytic fungi, antioxidant, *Cladosporium sphaerospermum, Aspergillus chevellie*, *Aspergillus rubur,*phytochemicals.

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INTRODUCTION:
It is seen that endophytic microbial population can easily adapt their physiology in order to establish themselves in the plant host tissue. These physiological changes result in production of useful biologically active metabolites which could bbe exploited for human use. They produce a wide range of compounds useful for plants for their growth, protection to environmental conditions, and sustainability in favor of a good dwelling place within the host. They protect plants from herbivore by producing certain compounds which will prevent

animals from further grazing on the same plant and sometimes act as biocontrol agent, increases nutrient uptake ability of plant.

Endophytic fungi area poorly investigated group of microorganisms that represent an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of pharmaceutical and industrial areas. Some plants and their associated endophytes were found to provide identical natural compounds.

Endophytic fungi of inner bark of *Prosposis cineraria* have been investigated 32 species belonging to 21 genera were isolated. The colonization frequency of the endophytic fungi was reportable as sixty-two. 55%. Fungus composition included thirteen.6% class Zygomycetes five.6% ascomycetes, 72.8% hyphomycetes, four-dimensional coelomycetes and four-dimensional sterile fungi are found**. (Praveen Gehlot,2008).**

Establishment of methodology for the isolation and characterization of a completely unique endophytic fungus from the inner bark of healthful plant *Nothapodytes foetida*, that created camptothecin in Sabouraud broth (SB) under shake flask conditions. Camptothecin and its connected compounds are at the moment obtained by extraction from intact plants, however fungal endopytes is also another supply of production**. (Touseef Amna; 2012).**

A novel phenolic compound, 4-(2,4,7-trioxa-bicyclo [4.1.0] heptan-3-yl) phenol was isolated from *Pestalotiopsis mangiferae*, an endophytic fungus associated with *Mangifera indica* Linn. The structure of the compound was elucidated on the basis of comprehensive spectral analysis (UV, IR, 1H-, 13C- and 2D-NMR, as well as HRESI–MS). Compound shows potent antibacterial and antifungal activity against Bacillus subtilis, *Klebsiella pneumoniae*, Escherichia coli, *Micrococcus luteus*, *Pseudomonas aeruginosa* and *Candida albicans.* **(Kamalraj Subban;2013)**

The aim of the study is to analyze the antioxidant properties of fungal cell free extract, also to evaluate the phytochemical properties of endophytic fungi from tree bark. The qualitative screening of chemicals was performed.

MATERIALS AND METHODS:

2.1 Collection of samples:

Tree bark was selected for isolation of endophytic fungi. Three tree *Tamarindus indica,Ficus benghalesis,and Mangifera indica* were selected. The part slightly above the soil of bark was selected to collect the sample. The sample was collected from Trimbkeshwar road, district Nashik.

2.2 Isolation of endophytic isolates:

Collected bark samples were first washed with tap water to remove all dust and soil from the surface. Cut bark into small square pieces. After, washed bark sample is washed with sterile distilled water. Dried overnight. Dried samples next day surface sterilized with first sterile 0.01 % $Hgcl_2$ solution and 70% ethanol for 30 seconds and 1 min. respectively. Later washed with sterile distilled water. Surface sterilized and washed bark teased again into small pieces. Then such treated and small teased pieces of bark samples were inoculated in Potato Dextrose Broth supplemented with chloramphenicol (100 µg/ml) antibiotic to avoid bacterial contamination. Incubated the flask at 28°C for 21 days on shaker.

2.3 Identification of isolates: Identification of these fungal strains was done by using on the basis of their cultural and microscopic properties these fungi show different characteristics. Identification based on Phenotypic Characters has been processed and the report generated using morphological characters.

2.4 Production of secondary metabolites

Production of secondary metabolites was carried out by using the protocol described by **(Deeksha Sharma *et. al.*, 2016)** with some modifications. All the endophytic cultures were inoculated in 50 ml of Potato Dextrose Broth (PDB) and incubated at 28°C for 20 days on rotary shaker at 120 rpm. After incubation this 50 ml broth with the grown endophytes were transferred to 350 ml of PDB and incubated at 28°C for 30 days on rotary shaker at 120 rpm. After the incubation period is completed the broth with the grown cultures were filtered using Whatmann No 1 filter paper and the broth was used for extraction of secondary metabolites.

2.5 Extraction of secondary metabolites:

The filtered broth was subjected to extraction using ethyl acetate as extraction solvent. In the filtrate, equal volume of ethyl acetate was added and kept overnight. This mixture of ethyl acetate and filtrate was separated using the separating funnel. The organic layer was collected and was allowed to dry. The dried extract was scrapped out and stored for further use in phytochemical compound screening, estimation total phenolic content and total flavonoids Content. **(Jia P. Marcellano *et. al.*, 2017).**

2.6 Qualitative analysis of Endophytic extract

2.6.1 Phytochemical screening

Test for flavonoid (Shinoda test)

A qualitative test was performed by adding Mg chips and few drops of concentrate

HCl to the extract. Appearance of reddish color indicate the presence of flavonoids. **(Sahiba Sumbal *et. al.*, 2012)**

Test for coumarin (NaOH test)

To the 200 µl of plant extract 300µl of 10% NaOH solution was added, formation of yellow colour indicates the presence of coumarin **(Majid *et. al.*, 2015).**

Test for Alkaloids (Wagner's reagent test)

100 µl of Wagner's reagent was added to the extract, formation of reddish brown ppt indicates the presence of alkaloids **(Sasikumar R. *et. al.*, 2014).**

Test for phenols (Lead acetate test)

10% lead acetate was added in the plant extract, development of pale yellow colour indicates the presence of phenols **(Usman *et. al.*, 2009)**.

Test for Tannins ($K_2Cr_2O_7$ test)

To the 500 µl of extract, few drops of $K_2Cr_2O_7$ solution was added. Formation of red `precipitate indicates presence of tannins **(Sasikumar R. *et. al.*, 2014)**.

Test for Amino acids (Ninhydrin test)

To 100 µl of the extract, few drops of 5% ninhydrin solution were added. It was then boiled in water bath for 5 minutes. The appearance of bluish purple colour indicates the presence of amino acids **(Priya S E. *et. al.*, 2015)**.

2.6.2 Thin Layer Chromatography for Phenols

Thin layer chromatography of the crude extract was carried out. A thin strip of thin-layer chromatography silica plate was impregnated with the fine drop of extract and Gallic acid as standard at marked places and allowed to air dry. The plate was developed in a chromatography chamber using a solvent system consisting of chloroform: ethyl-acetate: formic acid in a ratio 5:4:1. For visualization, plates were sprayed with 2% FeCl3 in ethanol as spraying reagent. **(Radomir V. Malbasa *et. al.*, 2014).**

2.6.3 Thin Layer Chromatography for Flavonoids

Thin layer chromatography of the crude extract was carried out. A thin strip of thin-layer chromatography silica plate was impregnated with the fine drop of extract and Quercetin as standarde at marked places and allowed to air dry. The plate was developed in a chromatography chamber using a solvent system consisting of Methanol: Chloroform: Hexane in a ratio 7:2:1. After the successful development, the plate was examined under the ultraviolet chamber for the presence of any spots. **(Vipin Nagda *et. al.*, 2017)**

2.6.4 Quantitative analysis of endophytic extracts

2.6.4.1 Determination of total phenolic contents

2.6.4.2 Determination of total flavonoids content

Total flavonoid content was measured with the aluminum chloride assay. 1 ml of sample was mixed with 4 ml of distilled water and 0.3 ml of sodium nitrite solution (5% w/v) and was allowed to stand for 5 minutes. 0.3 ml of aluminum chloride solution (10%) was added to the sample mix and after 1 minute, 0.2 ml of 1 M NaOH was added. The volume was made up to 10 ml with distilled water and mixed well. Tubes were observed for development of yellow color. The absorbance was measured at 510 nm in spectrophotometer. Varying concentrations of quercetin (10-100 µg/ml) were used for preparing the standard curve. The standard curve was plotted using the absorbance values obtained for quercetin. The total flavonoid content was calculated from standard curve and the result was expressed as µg quercetin equivalent per mg dry weight of crude fungal extract. **(Vipin Nagda *et. al.*, 2017)**

2.6.5 Radical scavenging activity

2.6.5.1 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay:

Various concentrations of crude fungal extracts (20-100 µg/ml, 1.75 ml) were mixed with methanolic solution of DPPH radicals (0.1 mM 0.25 ml). The mixture was shaken vigorously and left to stand in the dark for 30 minutes. The reduction in the DPPH radical concentration was determined by measuring the absorbance at 517 nm. Methanol was taken as blank and DPPH solution without the extracts was taken as a control. The percentage of inhibition of DPPH free radical activity was calculated using the equation. **(Vipin Nagda *et. al.*, 2017)**

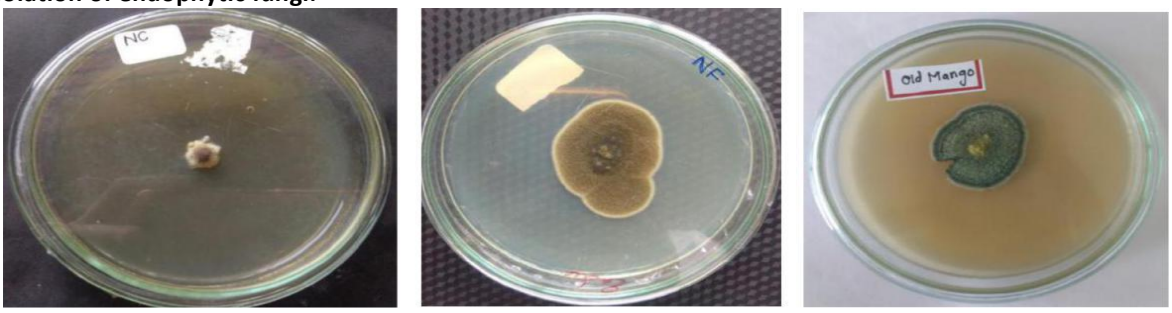
Percent Inhibition = (Ac-As)/Ac*100

Ac = Absorbance of control, and

As = Absorbance of solution containing sample extracts.

RESULTS:

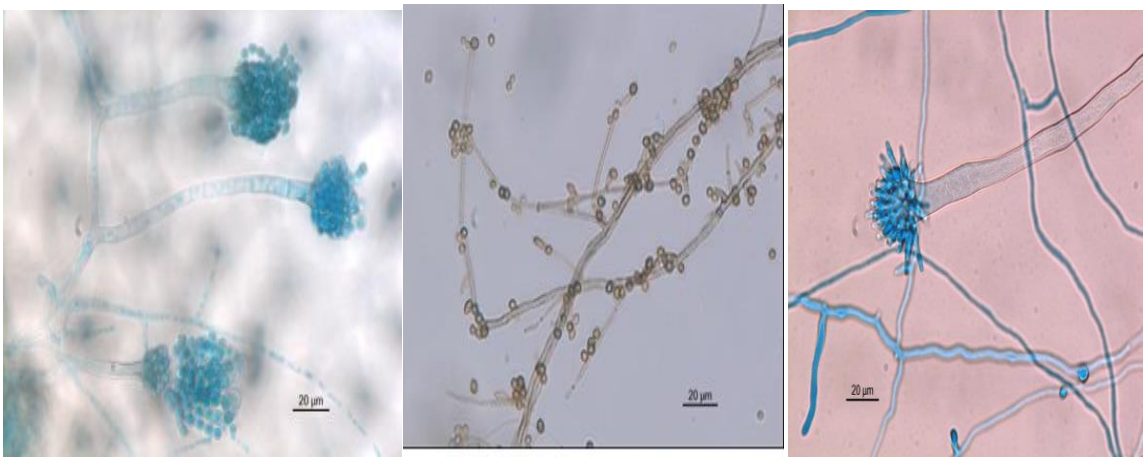
Isolation of endophytic fungi:



A **B** **C**

Photoplate no.1: A: ***Aspergillus spp*** from *Tamarindus indica* **B: *Cladosporium spp.***fro *Ficus benghalesis.* **C**: ***Aspergillus spp***.from *Mangifera indica*.

Identification:



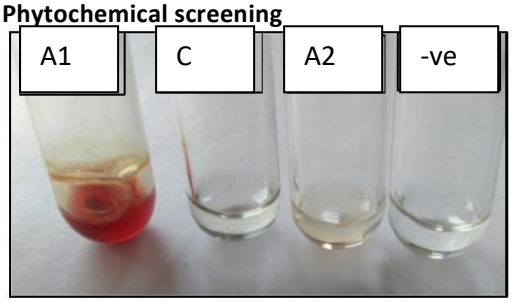
A B C

Optimization:

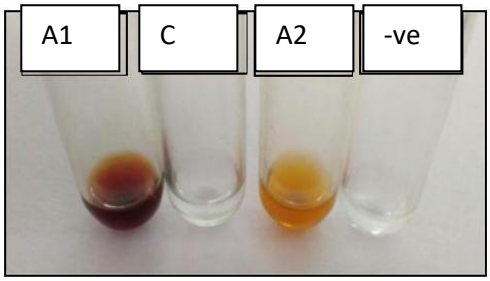
The optimized condition for the isolate from bark of Ficus tree has shown highest wet biomass weight in PDB at 28 °C, pH 6, at shaking conditions for 14 days. For isolate from bark of mango tree has shown highest biomass weight after 21 days, in PSB medium at pH 5, 28°C.and Isolate from Tamarindus tree bark has shown highest wet weight at 28°C, pH 6 after 28 days in PDB medium.

Quantitative analysis of Endophytic extract

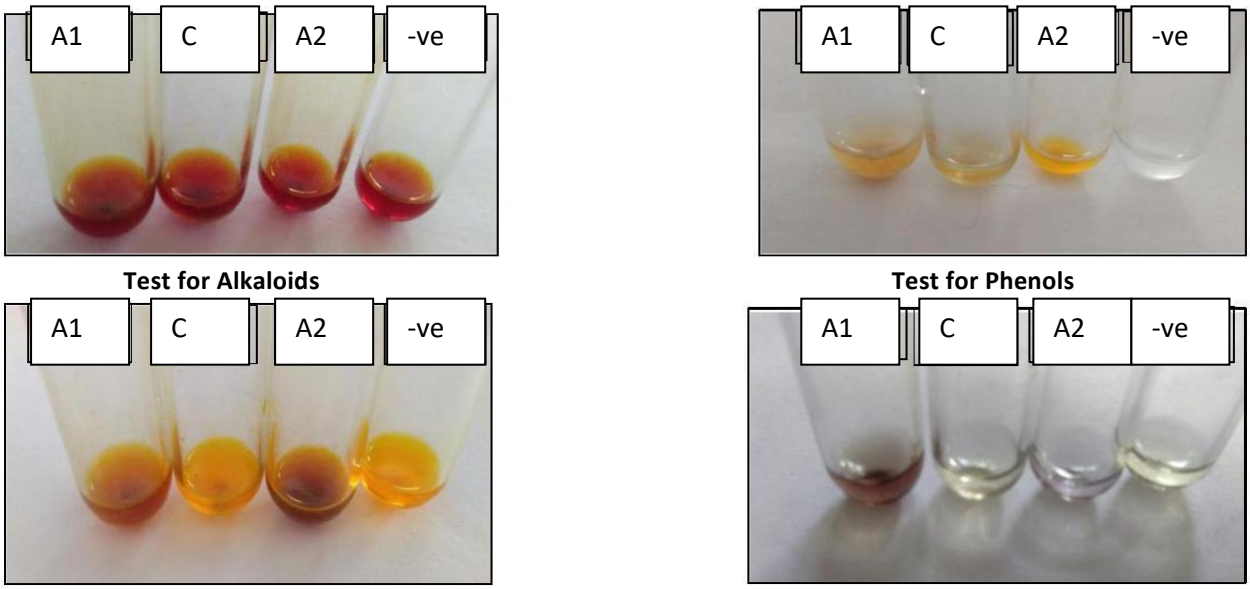
Phytochemical screening



Test for Flavonoid



Test for Coumarins



Photoplate No.3: Phytochemical screening of endophytic extract from A1-*Aspergillus spp* 1(*Tamarindus indica*), C- *Cladosporium spp. (Ficus benghalensis)*, A2- *Aspergillus spp.* 2 (*Mangifera indica*), -ve - Negative control.

Table No 1: Phytochemical screening of endophytic extract.

Test	A1	C	A2
Flavonoids	+++	-	-
Coumarins	+++	-	++
Alkaloids	-	-	_
Phenols	+++	+	++
Tannins	++	-	+++
Amino acids	+++	-	+

Phytochemical screening showed that the extract from *Cladosporium spp.* showed positive results only for test of phenols. Alkaloids showed negative test for all the three extracts, while flavonoids test showed positive result only for *Aspergillus spp*. 1.

Thin layer chromatography for Phenols and Flavonoids:

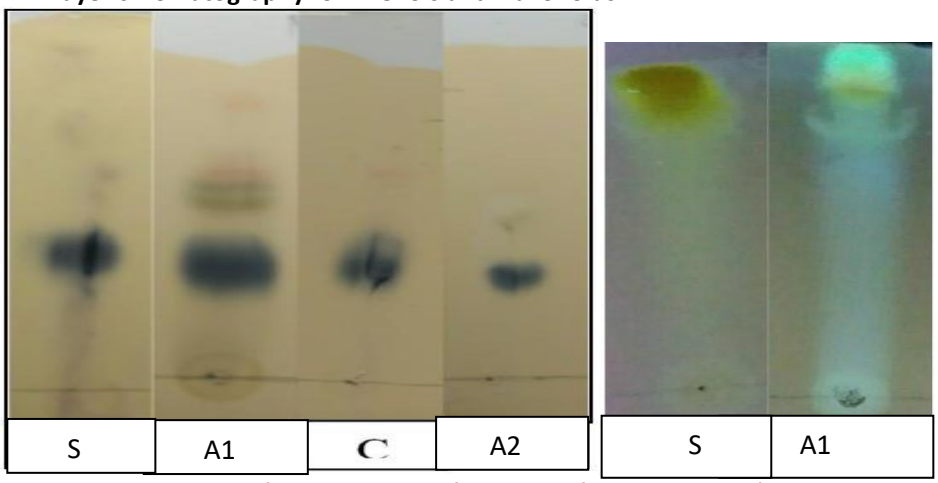


Photo Plate No: 4.TLC for Phenols and flavonoid of crude extract from A1-*Aspergillus spp.* 1 *(Tamarindus indica),* C- *Cladosporium spp. (Ficus benghalensis),* A2- *Aspergillus spp.* 2 (*Mangifera indica*), S- Standard.

Table No 2: Rf values of Phenol

Extract	RF values
Standard	0.45
A1	0.45

Table No 3: Rf values of Flavomoids

Extract	RF value
S	0.82
A1	0.83

Quantitative analysis of Endophytic extract

Total Phenol content by Folin Ciocalteau's method

Table No 4: Concentration of phenol content in endophytic fungi.

Extract	Total Phenol content in µg/mg of extracts
A1	98.58
C	70.70
A2	83.90

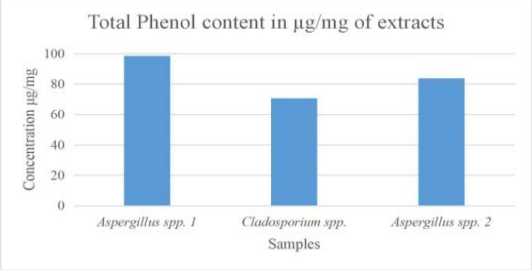


Fig.1 Concentration of phenol content in endophytic fungi.

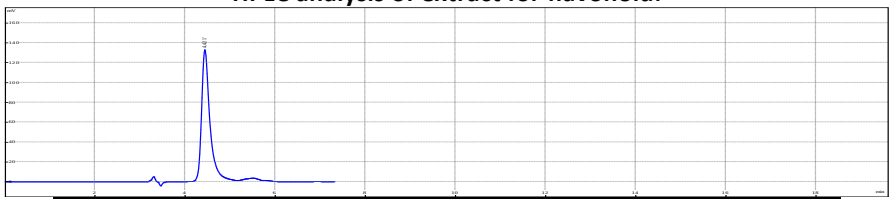
The assay for the estimation of total phenolic content using Folin Ciocalteau reagent showed that the *Aspergillus spp.* 1 *(Tamarindus indica)* produce 98.58 µg gallic acid equivalent per mg dry weight of crude fungal extract which is more than total phenolic content produced by *Cladosporium spp. (Ficus benghalensis)* and *Aspergillus spp.* 2 *(Mangifera indica).*

Table No 5: Concentration of total flavonoid content.

Extract	Total flavonoid content in µg/mg of extracts
A1	90.10

Flavonoids are important phytochemical which are responsible for several activities. The assay for the total flavonoid content was determined by AlCl3 method. The Total flavonoid content in *Aspergillus spp.* 1 (*Tamarindus indica)* was found to be 90.10 µg quercetin equivalent per mg dry weight of crude fungal extract.

HPLC analysis of extract for flavonoid:



Rank	Time	Area	Resolution	T.plate.No.	Asymmetry
1	4.421	2153581	0.00	3840	1.34

Photoplate no.5 Standard Qurcetin HPLC spectra.

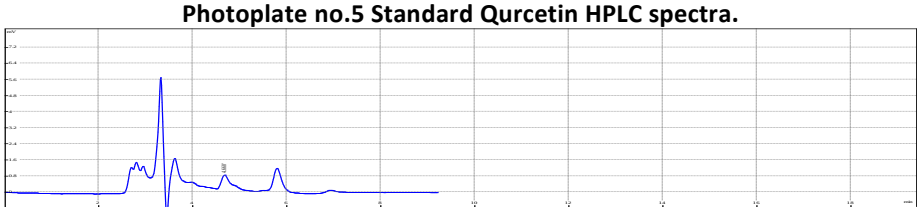


Photo plate No.6 *Aspergillus spp.*1 extract flavonoid HPLC spectra Radical scavenging activity:

DPPH activity:

Rank	Time	Area	Resolution	T. plate. No.	Asymmetry
1	4.660	57509	0.00	473	1.59

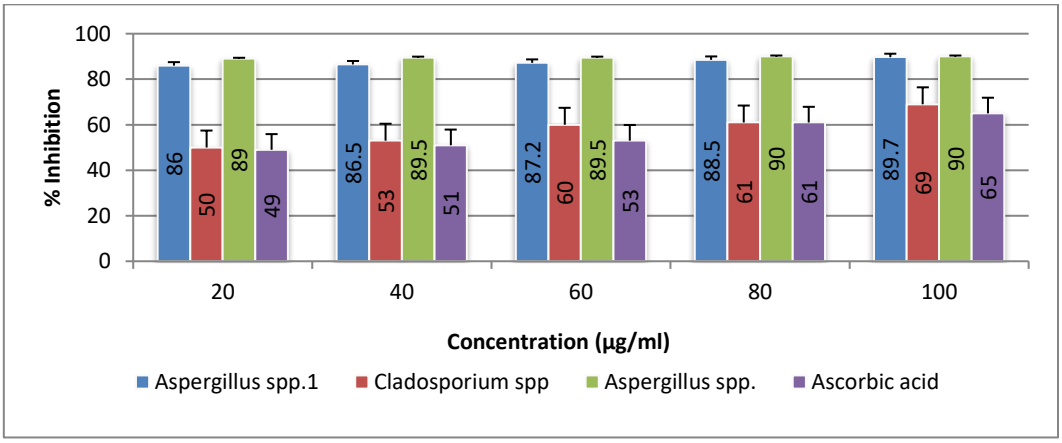


Fig.2: Radical Scavenging activity using DPPH

DPPH scavenging activity shown by all three isolate cell free extract.

Discussion:

The endophytes have created bigger interest within the use reservoir of natural bioactive compounds that they (host) made. The presence of phytochemical within endophytes is often potential supply for healthful and industrial use. The presence of phytochemicals within the endophytes are often potential supply of precursors within the development of healthcare product. Endophytic fungi are reported ubiquitously from each mad every higher plant, which has been investigated for their antimicrobial complement. The importance of compounds bearing antioxidant activity lays in the fact that they're extremely effective against harm caused by reactive O species (ROSs) and oxygen-derived free radicals, that contribute to a spread of pathological effects, as an example, DNA damages, carcinogenesis, and cellular degeneration. Antioxidants have been considered promising therapy for prevention and treatment of ROS-linked diseases as cancer, cardiovascular disease, atherosclerosis, hypertension, ischemia/reperfusion injury, diabetes mellitus, neurodegenerative diseases (Alzheimer and Parkinson diseases), rheumatoid arthritis, and ageing. (**Salini D;2017**).

The main objective of this study was to isolate, characterize, identify, endophytic fungi from three selected different tree bark i.e. *Mangifera indica, Tamarindus indica, Ficus benghalensis* and to study the bioactive compound production from them. Characterization and identification is performed by microscopic investigation. Endophytes are the microorganisms which have great potential as source for new bioactive compounds. Total three fungal isolates were obtained from three different plants i.e. *Tamarindus indica, Ficus benghalensis, Mangifera indica* which were screened for production of different bioactive compounds like phytochemicals. The present study contributed for phytochemical screening which is used to determine the presence of chemical components which is perspective source of medicinal and industrial use. To study the bioactive compounds i.e. for chemical analysis fungal crude extracts were used. **(Sharma *et. al.*, 2016).** The bioactive compounds that impart biologically active nature to the plant were screened and results ensured the presence of flavonoid and phenol. The presence of flavonoids confirmed by TLC (ethyl acetate fungal crude extract) showed the spots with solvent system Methanol: chloroform: hexane (7:2:1) with RF value **(Vipin Nagada *et. al.*, 2017)** and HPLC analysis.

The active metabolites contain chemical groups like phenols, flavonoids. From the isolated endophytes from coniferous plant *Cupressus torulosa* D. Don showed production of phenols and flavonoids from ethyl acetate extract (Sharma et. al., 2016). The isolates from tree bark showed various results like all three isolates produced phenols while none of them produced alkaloids. *Aspergillus spp.* 1 (*Tamarindus indica*) showed highest production of flavonoids, coumarins, amino acids and tannins were produced highest by *Aspergillus spp*. 2 (*Mangifera indica*), while *Cladosporium spp.* (*Ficus benghalensis*) only produced phenol. The ability of an endophyte to produce some metabolites but not others has been described by where different endophytes in a plant may produce different secondary metabolites hence play different functions in the plant and that the total number of metabolites in a plant extract may be a contribution of all the endophytes that live on the plant. The production and quality of bioactive compounds from endophytic fungi depend upon natural conditions of the association and therefore

the nature of the artificial medium used (**Strobel and flower et. al., 2003**).

Ethyl acetate extraction is most potential method of isolating fungal secondary metabolites. Ethyl acetates an extraction solvent selectively extracts low molecular weight phenolic compounds and high molecular weight polyphenol. (**Mariana Recco,2011**) Phenolic contents were the major constituents of the endophytes and possessing antioxidant activities. The ethanolic extract of an endophyte *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus terreus*, isolated from the medicinal plant *Achillea millefolium* exhibited strong antioxidant capacity due to the presence of "phenolic" and "flavonoid" constituents. Phenolic constitute the main class of natural antioxidants and possess a wide range of therapeutic uses that are usually quantified employing Folin's reagent Antioxidant activities of the endophytic fungal cultures were correlated with their total phenolic contents, indicates that phenolic compounds are the main constituents of the endophytes that are responsible for possessing the antioxidant activity. They endophytic fungi secrete some important bioactive components like alkaloids, flavonoids, phenolic acids, quinones, steroids, terpenoids which are directly correlated with antioxidant activities Presence of phytochemicals like saponins and phenolic possess strong antimicrobial and antioxidant activity the antioxidant activity was comparable with standard ascorbic acid. The results of this study demonstrate that the endophytic fungal have medicinal uses and indicate a promising potential for the development of an antioxidant agents. These selective endophytic fungi by *in vitro* results appear as interesting and promising and may be effective as potential sources of novel antioxidant drugs. (Kumaresan S and *et.al*; 2015)

CONCLUSION:

In this study, the three temperate region growing trees particularly tree bark has been studied for its endophytic assemblage and its crude extract were screened for antioxidant constituents like phenols, tannin, flavonoid, etc. The in vitro antioxidant potential is evaluated by reducing power assay and H_2O_2 scavenging activity. The three fungal species namely *Aspergillus spp.1*, *Cladosporium spp.* and *Aspergillus spp.2* were isolated from the bark of *Tamarindus indica*,*Ficus benghlensis* and *Mangifera indica* respectively.

It is evident that all the endophytic fungi studied here possess in vitro inhibitor activities. Biochemical screening of them revealed the presence of phenols, flavonoids, tannins, but their quantities varied, this may be due to the biochemical, genetical and physiological conditions of the fungus. Bioactive compound found in endophytic fungi await a major break-through for a variety of medical applications. Further, possible growth of endophytic fungi and easier downstream process of the fungus compounds provide quantitative and qualitative development of therapy agents particularly antioxidants could also be tried.

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