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Screening of entophytic pigmented fungi for Phytochemical and Antioxidant activity

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ABSTRACT

The present investigation was done to screen the endophytic fungi from the medicinal tree (*Azadirachtaindica*) for its antioxidant activity. About five fungal isolates were collected and cultured. Among the five isolates only two were taken for the study due to its coloured pigmented morphology. The two pigmented fungi were identified as *Fusarium*spand*Aspergillus sp*. Methanolic extract of the isolates were taken and dried. The dry solid residue was re- dissolved in methanol and the crude extract was evaluated for their antioxidant property. The methanolic extracts were qualitatively and quantitatively analysed for protein, flavanoids, and phenol content. From the results it was found that the endophytic fungi played an important role in the search of biological compounds and might also represents an alternative source for the therapeutic agents. The potential of these fungi is of great interest and warrants further investigation.

Keywords: Endophytic fungi, Phytochemicals screening, Total Antioxidant capacity.

INTRODUCTION

Biotechnology emphasises in the use of cellular systems for the development of processes and products holding economic and social relevance to humankind. The fungi are of great biotechnological interest in the fermentative processes that culminate in the production of secondary metabolites [9]. Antioxidants serve as the defensive factor against free radicals in the body. The term antioxidant originally was used to refer specifically to a chemical that prevent the consumption of oxygen. In a biological system, an antioxidant can be defined as ‘‘any substance that when present at low concentrations compared to that of an oxidizable substrate would significantly delay or prevent oxidation of that substrate’’ [3]. The theory called ‘‘catalyst poisoning’’ in oxidative reactors, and this was well before the free radical theory of per oxidation had been proposed. The term antioxidant broadly denotes any substance that prevents oxidation by bio molecules, either directly by scavenging the reactive oxygen species or indirectly, by up regulating the antioxidant defense or DNA repair system.Hence, there has been an increased interest in the food industry and in preventive medicine in the development of ‘Natural antioxidants’ from natural sources[15].

*Azadirachtaindica*is an indigenous medicinal plant in India and Africa, commonly known as Neem. Based on the recent claims that the metabolites secreted from the endophytes recovered from medicinal plants play a key role as therapeutics, extracts has been scientifically investigated for anti-microbial, antipyretic, anti-inflammatory effects and against malaria and cancer [5, 16].Endophytic are microbes that colonize the living internal tissues of plants without causing any immediate overt negative effects [4]. Endophytic fungi had been previously isolated from the various parts of the plants such as leaves, stem, roots and bark regions.

Antioxidants in fungi:

A number of plants and mushrooms (fruiting body) are commonly known to produce antioxidants but there are few reports on lower fungi. These include *Penicilliumroquefortii,*

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Aspergilluscandidus, Mortierella, Emericellafalconensis, *Acremonium*, *Colletotrichumgloeosporioides*, *Mycelia sterilia*, *Antrodiacamphorata*, *Chaetomium*sp., *Cladosporium*sp., *Torula*sp., *Phoma*sp and so on. Recently, fungi have emerged as the new sources of antioxidants in the form of their secondary metabolites [13,1]. Fungi are remarkably a diverse group including approximately 1.5 million species, which can potentially provide a wide variety of metabolites such as alkaloids, benzoquinones, flavanoids, phenols, steroids, terpenoids, tetralones, and xanthones[2]. They demonstrate variety of bioactivities along with antioxidant properties and function as varied as their structure. They are exploited in medicine and industry and considered to be potential sources of new therapeutic agents. A lot more fungi still needs to be explored as the production, downstream processing of actual bioactive phytochemicals from them is quite tougher as compared to plants.

MATERIALS AND METHODS

Isolation of Endophytic Fungi

Healthy endophytic fungi were collected from Neem tree in Bharathiar University campus. The samples were processed within 24 hours following collection. The collected sample was washed thoroughly with tap water. Surface sterilization was done by immersing the sample in 70% ethanol for 30 min, 0.5% sodium hypochlorite for 30 sec and 70% ethanol for 2 min, rinsed with sterile water and dried on sterile filter paper. The outer tissue was removed and inner tissue of 0.5 cm was placed on sabouraud's Dextrose agar plates supplemented with 100 mg/l streptomycin. The plates were incubated for 4-5 days at room temperature and 5 colonies were observed .The pure culture was maintained in Sabouraud's dextrose agar medium. Among the 5 isolated fungi only two fungi were taken for the study due to its colouredpigmentation.

Culture and morphological characterization of fungi

The fungi were identified on the basis of their morphological and culture characteristics. Fungi were grown on specified media at specified culture condition .The selected fungi were plugged in the Sabouraud dextrose agar (SDA) media with sterile condition and incubated for one week for the growth of spores on plate. And the fungal morphology was viewed under the microscope by using the lactophenol cotton blue stain. The pure culture was maintained in SDA medium. Among the 5 isolated fungi only two fungi were taken for the study due to its pigmentation.

Cultivation of the fungal isolate

Preparation of seed culture:

The endophytic fungi grown on SDA plates were taken(0.5 cm diameter) and inoculated onto 100 ml of Sabouraud dextrose broth (SDB) and incubated at room temperature under shaking condition for 24 hours for uniform dispersal of the spores. After incubation 10 ml of the broth was transferred onto another flask containing 90 ml of sterile SDB medium. The flask was kept for 7-14 days without any disturbance for fungal mycelia mat formation.

Harvesting and preparation of Methanolic crude extract of fungal biomass:

Once the medium is fully covered with fungal biomass it was harvested by filtering the culture through cheese cloth. The filtered biomass was washed with sterile water and dried. The biomass was extracted with methanol using mortar and pestle, centrifuged at 5000 rpm for 10 min and the supernatant was collected and the solvent was evaporated. The dry solid residue was re dissolved in methanol and the crude extract was evaluated for their antioxidant property

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DETERMINATION OF BIOACTIVE COMPONENTS

PHYTOCHEMICAL SCREENING:

Preliminary Phytochemicals screening of the crude extracts of the fungal mycelium were carried out by qualitative methods using standard protocols.

QUANTITATIVE ANALYSIS

Estimation of total Phenolic content

Total phenolic contents of the extracts were determined by Folin-Ciocalteau method [8]. An aliquot of the extract was mixed with 5 ml of Folin-Ciocalteau reagent (previously diluted with water 1:10 v/v) and 4 ml (7.5g/L) of sodium carbonate. The tubes were allowed to stand for 15min and the total phenols were determined by measuring the absorption in a UV/VIS Spectrophotometer (ELICO) at 765nm. Total phenol content was expressed in terms of Gallic acid equivalent in milligram per gram of material**.**

Estimation of total Flavanoid content

Total flavanoid content was determined by [11]Ordonezmethod. A volume of 0.5 ml of 2% Aluminium chlorideethanol solution was added to 0.5 ml of sample solution. After one hour at room temperature, the absorbance was measured at 420nm. A yellow colour indicated the presence of flavanoids. Flavanoid content was expressed as mg Quercetin equivalents in 1 g of the material**.**

Estimation of Protein

Protein was determined by Lowry's method [7]. Varying Concentrations of 0.2 to 1.0 ml of working standard solution was taken. 0.1 ml of the sample was taken. The volumes in all the tubes were made up to 1.0 ml with distilled water. A volume of 5.0 ml of alkaline copper reagent was added to each tube. It was mixed well and incubated at room temperature for 10 min. After Incubation, 0.5 ml of Folin-Ciocalteau reagent was added and mixed well and it was incubated at room temperature for 30 minutes. A reagent blank was also prepared. After 30 minutes the blue colour developed were read at 660 nm. The results were expressed as mg/g.

Evaluation of antioxidant activity

DPPH radical scavenging assay

The effect of fungal methanolic extracts on DPPH radical was determined using the method of Szabo*et al.* (2007). Different concentrations of the extracts (10 - 100 µg/ml) were prepared and subjected to antioxidant tests. To 1 ml of each of the extracts, 5 ml of 0.1mM methanol solution of DPPH was added, vortexes, followed by incubation at 27ºC for 20 min. The control was prepared without any extract and absorbance of the sample was measured at 517 nm using UV/VIS Spectrophotometer (ELICO) using methanol to set 0.The ability to scavenge DPPH radical was calculated by the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{[(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})]}{(\text{Abs}_{\text{control}})} \times 100$$

Reductive ability

Total reducing power was determined as described by Oyaizu (1986).1 ml of sample solution at different concentrations were mixed with 2.5 ml of phosphate buffer (0.2 mol/l, pH 6.6) and 2.5 ml of potassium ferricyanide (1%). The mixture was incubated at 50ºC for 20 min 2.5 ml of trichloroacetic

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acid (TCA,10%) was added to the mixture and centrifuged at 3000g for 10min. The supernatant (5 ml) was mixed with 1 ml of ferric chloride (0.1%), and the absorbance was measured at 700 nm in a Spectrophotometer. Increased absorbance of the reaction mixture indicated increased reducing power.

Super oxide anion scavenging activity

Measurement of super oxide anion scavenging activity of the fungal methanolicextracts were based on the method described by (Liu *et al.,*1997). Super oxide radicals were generated in PMS-NADH systems by oxidation of NADH and assayed by the reduction of nitrobluetetrazolium (NBT). 3 ml of sample solutions at different concentrations were mixed with 1 ml of NBT (156µM) and 1 ml of NADH (468 µM). The reaction was initiated by adding 0.1ml of phenazinemethosulphate (PMS) solution (60µM) to the mixture. The reaction was incubated at 25ºC for 5 min, and the absorbance at 560 nm was measured against blank. Decreased absorbance of the reaction mixture indicates increased super oxide anion scavenging activity. The percentage inhibition of super oxide anion generation was calculated using the following formula:

$$\text{Inhibition of super oxide generation (\%)} = \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \times 100.$$

Scavenging activity against Nitric oxide

The nitric oxide scavenging activity was done by following the method of Sreejayan*et al*.(1997). Nitric oxide interacts with oxygen to produce stable products, nitrite and nitrate. Scavengers of nitric oxide compete with oxygen, leading to a reduced production of nitrite; the concentration of nitrite in aqueous solution was assayed spectrophotometrically by using the Greiss reagent, with which nitrite reacts to give a stable product absorbing at 546 nm. Nitric oxide radicals were generated from sodium nitroprusside solution at physiologicalpH. Sodium nitroprusside (1 ml of 10 Mm) was mixed with 1 ml various concentrations of sample extracts in phosphate buffer (pH 7.4). The mixture was incubated at 25 °C for 150 min. To 1 ml of the incubated solution, 1 ml of greiss reagent (1% sulphanilamide, 2% ortho phosphoric acid and 0.1% napthyl ethylene diaminedihydrochloride) was added. Absorbance was read at 546 nm and percentage inhibition was calculated using the formula:

$$\text{Inhibition \%} = \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \times 100$$

Estimation of Total antioxidant capacity

Total antioxidant was determined by phosphomolybdenum method followed by[(12)].Samples and standard (1ml) was mixed with 2 ml of reagent solution [ammonium molybdate (4mM),sodium phosphate (28mM) and sulphuric acid(0.6M)]. All the reaction mixtures were incubated at 95ºC for 90 min. The absorbance was measured at 695nm. Total antioxidant activity was expressed as the number of equivalent of ascorbic acid (mg AA/g).

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RESULTS

Isolation of microorganisms

The endophyticfungi were identified on the basis of their morphological and culture characteristics. Purified culture of the fungi was grown on SDA media for 7-14 days for the growth of spores. And the fungal morphology were observed and shown in Fig 1 and 2. The fungal isolates were identified as *Fusariumsp*and *Aspergillussp.*

Figure.1 Fungal morphology of*Fusariumsp*

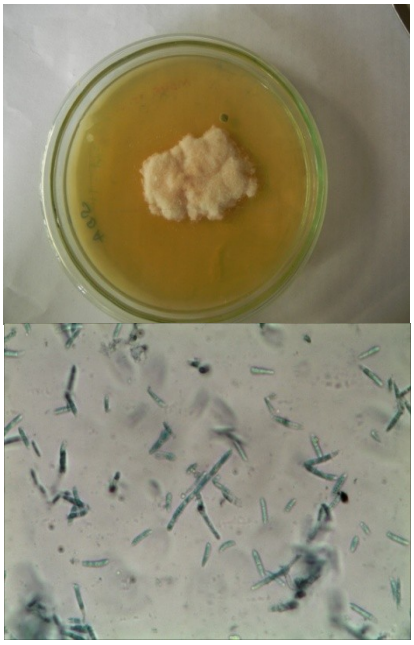
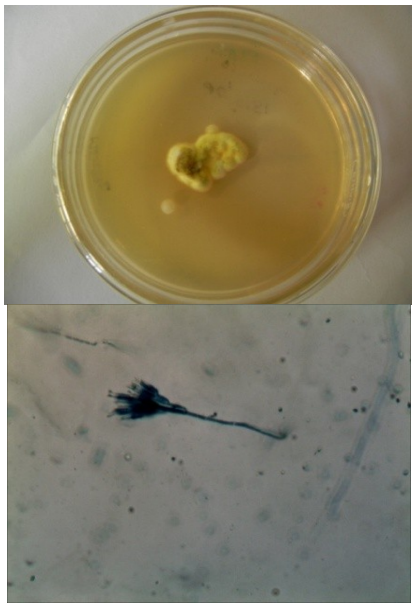


Figure. 2 Fungal morphology of *Aspergillus sp*



Harvesting of bio mass from fungal mycelia mat

The endophytic fungi of the *A.flavus* and *F.oxysporum* were inoculated onto 100 ml of Sabouraud dextrose broth (SDB) was kept for 7-14 days without any disturbance for fungal mycelia mat formation. Once the medium is fully covered with fungal biomass it was harvested by filtering the culture through cheese cloth. The filtered biomass was washed with sterile water and dried. The biomass was extracted with methanol using mortar and pestle, centrifuged at 5000 rpm for 10 min and the supernatant was collected and the solvent was evaporated. The dry solid residue was re dissolved in methanol and the crude extract was evaluated for their antioxidant property.

QUALITATIVE ANALYSIS:

By preliminary Phytochemical screening six different chemical compounds (Alkaloids, Glycosides, Flavanoids, Saponins,Tannins and carbohydrates) were tested for their presence or absence in the methanolic extract of the fungal mycelium was carried out and the results were given in Table 1. Thus out of (2x6=12) tests for the presence or absence of the above compounds, 4 tests gave positive results and the remaining

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8gave negative results. The 2 positive results showed the presence of Flavanoids and carbohydrates. Alkaloids, Glycosides, saponins and tannins did not show any positive result.

Table.1 showing the results of Preliminary phytochemicals screening

Phytochemicals	*Fusarium Sp.*	*Aspergillus sp.*
Alkaloids	-	-
Glycosides	-	-
Flavanoids	++	++
Saponins	-	-
Tannins	-	-
Carbohydrates	++	++

QUANTITATIVE ANALYSIS

Quantitative analysis for Phenol, Flavanoids, and Protein for antioxidant activity:

The content of total phenolics, flavanoids and protein was shown in Table 2. In this current study the content of total phenolics was equal to 0.7 and 2.3 mg GAE/g of fungal biomass of *Fusarium sp.*and *Aspergillus sp.*respectively. The total flavanoids content was measured by aluminium colorimetric method and was found to be 1.2 and 2 mg QE/g of fungal biomass. The methanolicextract of *Fusariumsp* has 2.9µg/ml of protein whereas Aspergillus*sp* contains 3.6µg/ml. When compared with the *Fusariumsp* the *Aspergillussp* contains high amount of protein in the methanolic extract.

Table.2 showing the results of total Phenol, Protein and Flavanoids contents

Serial no	Sample	Phenol(µg/ml)	Protein(µg/ml)	Flavanoids(µg/ml)
1	*Fusariumsp*	**0.7**	**2.9**	**1.2**
2	*Aspergillussp*	**2.3**	**3.6**	**2**

EVALUATION OF ANTIOXIDANT ACTIVITY:

DPPH radical scavenging assay:

DPPH assay is one of the most widely used method for screening antioxidant activity of natural product (Nanjo, 1996). DPPH is stable, nitrogen centered free radical which produces violet colour in methanol solution. It was reduced to a yellow coloured product, diphenylpicryl hydrazine, with the addition of the

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fraction in a concentration dependent manner. The reduction in the number of DPPH molecules can be correlated with the number of available hydroxyl groups (Nithya*et al., 2011).* Scavenging of DPPH represents the free radical reducing activity of antioxidants based on a once- electron reduction. Scavenging DPPH free radical determines the free radical scavenging capacity or antioxidant potential (AOD) of the sample, which shows its effectiveness prevention interception and repair mechanisms against injury in a biological system (Lee *et al*.,2001). The fungal methanolic extracts of the *F.oxysporum* exhibited 60% and *A.flavus*showed 80% DPPH scavenging activity. DPPH radical activity showed the better results in *A.flavus*.

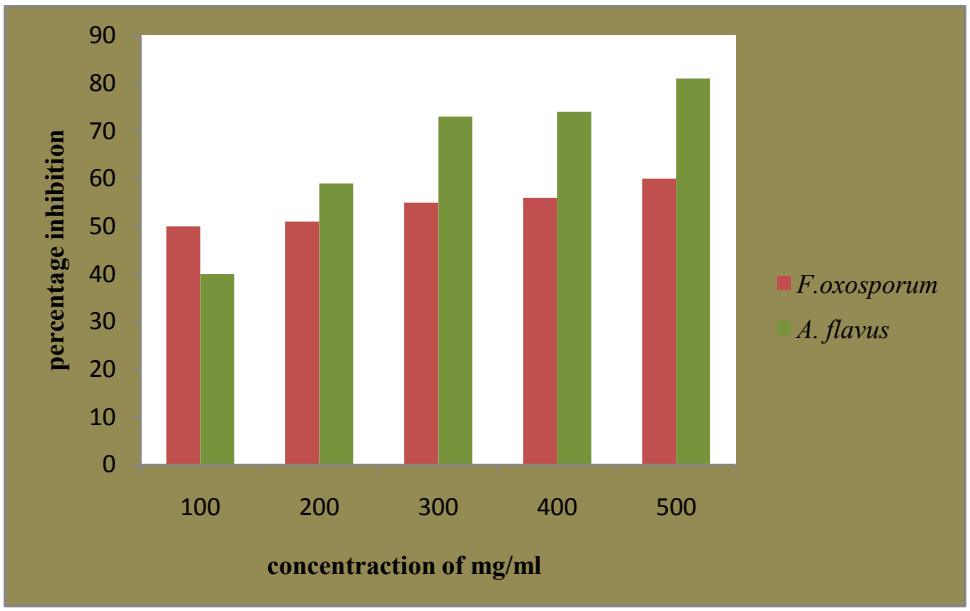

Figure 3 DPPH scavenging radical activity for *F.oxosporum&A.flavus*

Reductive ability

For the measurement of the reducing ability, the $fe^{3+}$to $^{-}fe^{2+}$ transformation was investigated in the presence of methanolic extract. The reducing capacity of the compound may serve as a significant indicator of it potential antioxidant activity. However of antioxidants has been assigned to various mechanisms such prevention of chain initiation, binding of transition- metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging. Similar to the antioxidant acitivity, the reducing power of methonolic extract of the fungal isolates (*F.oxysporum* and *A.flavus*) increased with increasing dosage. The result showed that methanolic extract consists of hydrophilic poly phenolic compounds that cause the greater reducing power. As the OD value increases the reducing capacity also increases which is shown in the figure 2.

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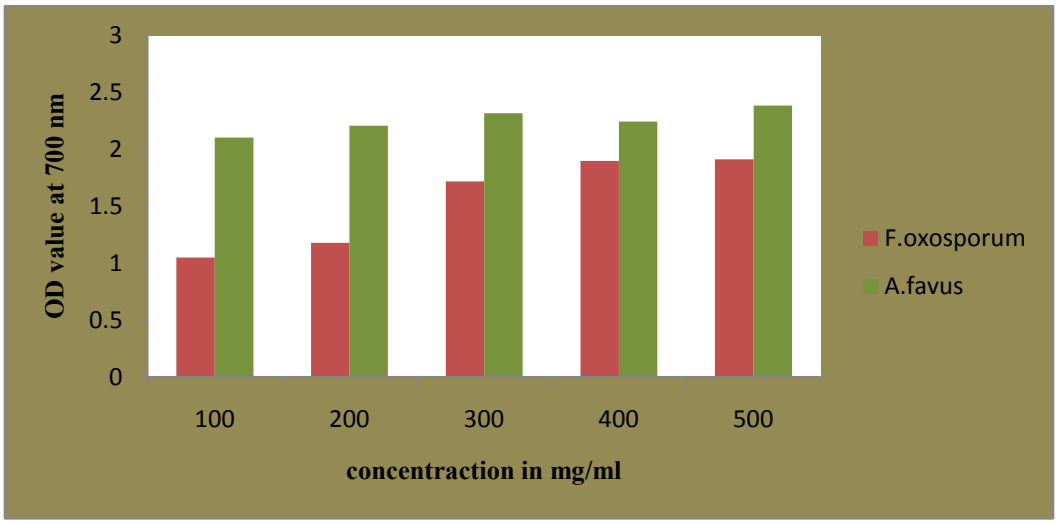

Figure.4. Comparison of reducing power for *F.oxysporum&A.flavus*

Super oxide anion scavenging activity

It is well known that superoxide anions damage biomolecules directly by forming H_2O_2, OH, peroxynitrite singlet oxygen during aging and pathological event such as ischemic reperinfusion injury (yen *et al.,*1994). The superoxide anion radical scavenging activity of methonolic extracts of*F.oxysporum and A.flavus* assayed by PMS-NADH system is given in figure.3 The superoxide scavenging activity of fungal extracts increased markedly with increase in concentration. Thus higher inhibitory effects of the methonolic extracts on superoxide anion formation noted here possibly renders them as a promising antioxidants on futher analysis, the *A.flavus*shows better superoxide anion scavenging activity than the *F.oxysporum*. which is shown in the figure 3.Superoxide is universally generated from organic compounds, protein and cells during metabolism and other normal biochemical function. Superoxide is harmful because it reduces iron – III to iron II. Super oxide can give rise to strong oxidant such as singlet oxygen and interact with the other compounds such as nitric oxide radical or hydrogen peroxide give rise to the hydroxyl radical and nitrogen dioxide(Vimala*et al .,*2003).

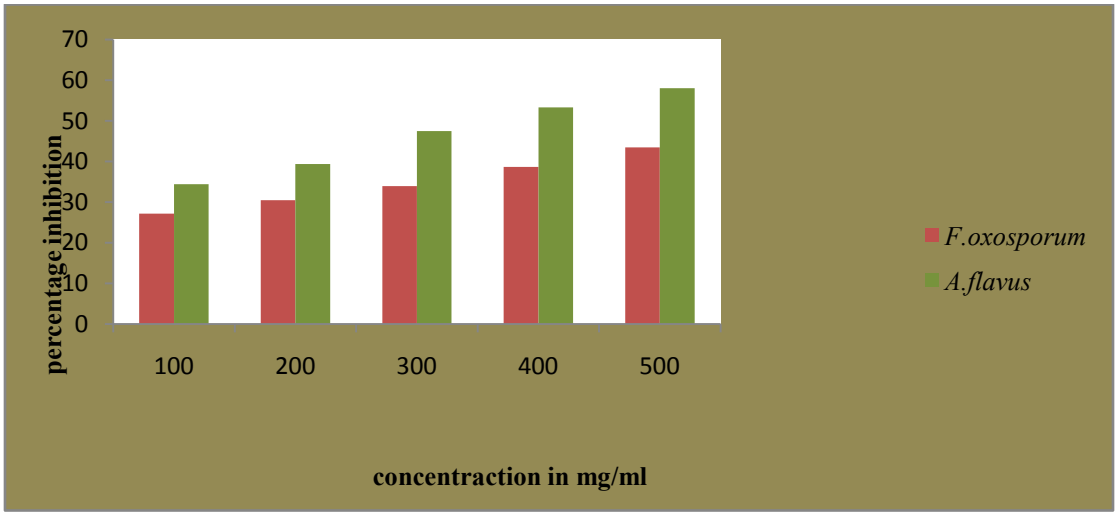

Figure.5. super oxide radical scavenging assay for *F.oxysporum&A.flavus*

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Nitric oxide scavenging activity

Nitric oxide (NO) is a potent pleiotropic mediator of physiological process such as smooth muscle relaxant, neuronal signalling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical which plays many roles as a effectors molecules in diverse biological systems including neuronal messenger, vasodilation and anti microbial activities (Miller *et al*., 1993). Although nitric oxide and superoxide radicals are involved in host defence, over production of these two radicals contributed to the pathogenesis of some inflammatory diseases (Guo*et al*.,1999). More over in the pathological condition, nitric oxide reacts with super oxide anion and form potentially cytotoxic molecules, peroxynitrite. Nitic oxide inhibitors have been shown to have beneficial effects on some aspect of inflammatory and tissue damage seen in inflammatory diaseases. The methanolic extract of *F.oxysporum* and *A.flavus* inhibit nitric oxide in a dose dependent manner. The result indicated that the methonolic extract of *Fusariumsp* (49%/500µg) and *A.flavus* (92%/500µg) has better nitric oxide scavenging activity which is shown in figure 4.

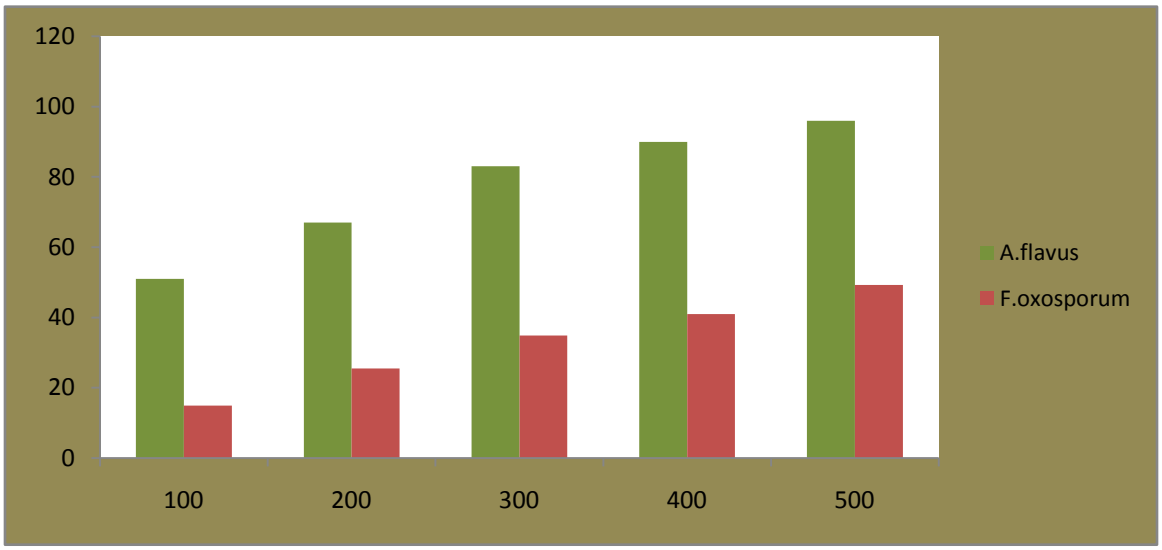

Figure.6.comparision of *F.oxsosporum&A.flavus*in nitric oxide radical scavenging assay

Similar work was done in the Invitro antioxidant of *Cyperusrotundus* in crude extract 42%/500µg/ml. Our result shows the better results in the nitric oxide radical scavenging assay.

Total antioxidant activity

The antioxidant activities of methanolic extract of both fungi along with the standard Ascorbic acid were shown in Fig 3. Among the two fungi *Aspergillus sp.*shows25mg AA/g exhibited higher radical scavenging activity when compared with *Fusarium*19mg AA/g. However relatively lower than that of standard Ascorbic acid

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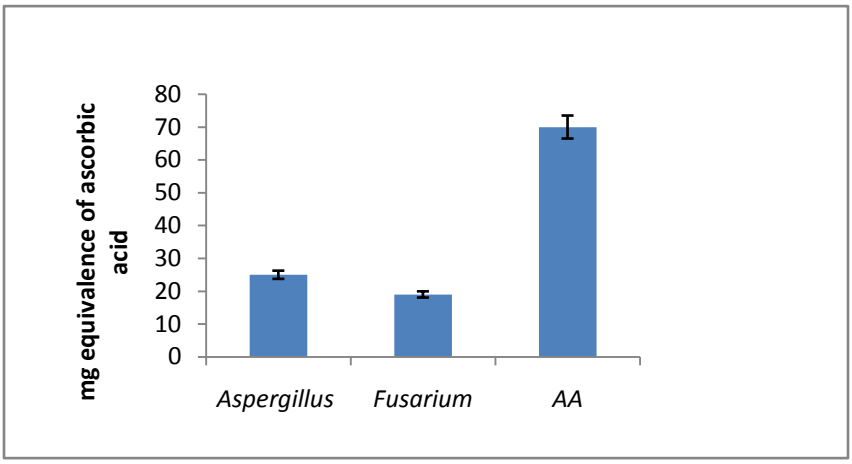
Figure.7. Total antioxidant activities



AA- Ascorbic acid

DISCUSSION

Endophytic fungi are one of the most unexplored and diverse group of organisms having symbiotic associations with higher life forms and that are belived to be associated with the production of pharmaceutical products [17]. In this present study, the endophytic fungi isolates from Neem tree were identified as *Fusarium* sp. and *Aspergillus* sp. Those organisms were grown and harvested, methanolic extract were prepared for screening of qualitative and quantitative analysis for total antioxidant activity in them. The total phenolic content of the fermentation extract of the endophytic fungus *Alternaria alternate* isolated from *Coffeaarabica L*. was 3.44µg GAE/mg[6].Flavanoids compound seems to have an important role in stabilizing lipid oxidation, associated with antioxidant activity. Endophytic fungus *Phyllostictas*p, isolated from *Guazumatomentosa*was reported with 1µg/g of flavanoidsin the extract[14].Our results showedhigher values than the above. Methanolic extract of *Aspergillus* sp. possess highest antioxidant activity when compared with *Fusarium*sp. however it is less than standard ascorbic acid.

CONCLUSION:

The endophytic fungi play an important role in the search for bioactive compounds and might also represents an alternative source for the production of the therapeutic agents. The potential of these fungi is of great interest and warrants further investigation. From this study it is concluded that the endophytic fungi from medicinal plants possess pharmacological properties like their host, results in isolation of novel bioactive compounds from them. It is anticipated to separate and characterization of compounds.

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