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Evaluation of *In-vitro* antioxidant and cytotoxic activities of endophytic fungi isolated from the medicinal plant *Madhuca neriifolia*

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Abstract

Endophytic fungi *Alternaria alternata* (*A. alternata*) and *Trichoderma asperellum* (*T. asperellum*), isolated from the medicinal plant *Madhuca neriifolia,* were used to evaluate their antioxidant and cytotoxic activities on the human lung cancer cell line A549. Ethyl acetate (EtOAc) was used for the extraction. DPPH, ABTS and FRAP assays were performed to evaluate their antioxidant activity. Ascorbic acid and Quercetin were used as a standard to compare the antioxidant potentiality of these fungi. Ascorbic acid shows 90% inhibition activities in 200ug/ml with an IC_{50} value of 41.89 µg/ml. in DPPH assay. In ABTS assay, Ascorbic acid shows 89.56% inhibition in 2000 µg/ml with an IC_{50} value of 577.82µg/mL. In FRAP assay, Quercetin shows 99.89% inhibition in 2000µg/ml with an IC_{50} value of 185.151 µg/mL. Compared to these two standards, in the DPPH assay, *A. alternata* and *T. asperellum, at the concentration of* 200 µg/ml, showed the inhibition activities of 33.26% with IC_{50} value of 413.923 µg/ml and 34.04% with IC_{50} value of 389.791 µg/ml respectively. Similarly, in ABTS assay *A. alternata* and *T. asperellum, at the concentration of 2000* µg/ml, showed the inhibition activities of 34.67% with IC_{50} value of 2853.1µg/ml and 31.57% with IC_{50} value of 3097 µg/ml respectively. In the FRAP assay, *A. alternata* and *T. asperellum,* at the concentration of 2000 µg/ml, showed the inhibition activities of 82.72% µg/ml with IC_{50} value of 585.38 µg/ml and 83.16% with IC_{50} value of 576.79 µg/ml respectively. Both these fungi exhibited good to moderate level of antioxidant activities in these three assays.

In the cytotoxicity assay, *A. alternata* and *T. asperellum,* at the concentration of 100µg/ml, showed an average cells viability of 49.72% with IC_{50} value of 92.356µg/ml and 57.22% with an IC_{50} value of

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110.314µg/ml respectively. Among these two fungi, *A. alternata* showed more stronger toxicity than *T. asperellum* on the cancer cell line-A549. Therefore, it may be concluded that since *A. alternata* is found to be more potent, it can be a better candidate for further exploration against human cancer cell lines.

Key words : *Alternaria alternata*, *Trichoderma asperellum,* Antioxidant, MTT, Human lung cancer cell line-A549, *Madhuca neriifolia*.

Free radicals are one of the major causes of cancer among humans. These are the molecules produced as a by-product during cellular metabolism[22] with an unpaired electron[1]. It induces oxidative damage to the biomolecules such as carbohydrates, proteins, nucleic acids and lipids because of their highly reactive and unstable nature[13]. These oxidative damages of the biomolecules not only create cancer but also cause ischemia and reperfusion injury, diabetes mellitus, neurodegenerative diseases, rheumatoid arthritis, obstructive sleep apnea, senescence *etc*[7].

Endogenous antioxidant defence and repair mechanism of human body are not enough to protect vital biomolecules from free radicals, but the human body may require supplements of natural compounds for protection. Therefore, it is necessary to explore new and more effective antioxidants from natural resources to protect the biomolecules from being damaged. Endophytes growing on medicinal plants do possess the ability to produce natural compounds having anti-oxidant abilities. Hence, traditionally used medicinal plant *Madhuca neriifolia* from South West of the Western Ghats of Karnataka, India has been selected. This plant is not widely studied before for its endophytic fungi and their medicinal values. Two endophytic fungi *A. alternata* and *T. asperellum* have been isolated and their morphology and molecular analysis have been carried out earlier. In the present study, an attempt has been made to explore the antioxidant and cytotoxic ability of *A. alternata* and *T. asperellum* against human lung cancer cell line-A549.

Isolation of endophytic fungi :

A. alternata and *T. asperellum* were isolated from the twigs of the medicinal plant *Madhuca neriifolia*. External tissues of the plant parts were exposed to 70% ethanol (v/v) for 5 sec. followed by 0.01% mercuric chloride ($HgCl_2$) for 30 sec. After 10 sec, samples were rinsed with sterilized distilled water three times and dried with blotting paper, and then the samples were cut into 1 cm in length with the help of a sterilized blade and placed in a sterilized PDA media (antibiotic streptomycin 150mg/l) aseptically.

Fermentation and extraction of endophytic fungi :

Three to five mycelial segments from both the fungi (5mm) from the plate were inoculated into different conical flasks of 500ml containing 250ml of Potato Dextrose Broth

(PDB) incubated for 8-10 days in a shaker at 180 -200 rpm. After ten days of incubation, the PDB cell cultures were filtered with the help of muslin cloth to remove the mycelial mate. The cell-free cultures were extracted thrice with an equal volume of ethyl acetate (EtOAc) in a separating funnel. The EtOAc phase was collected and dried to get the concentrated form of the sample.

Antioxidant assays :

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

The DPPH radical scavenging activity was performed as described by Chang *et al*[6]. Total five different concentrations such as 12.5µg/ml, 20µg/ml, 50µg/ml, 100µg/ml and 200µg/ml were taken from the stock solution of 10mg/ml. In each concentration, 1.48ml DPPH(0.1mM) solution was added. These mixtures were incubated at room temperature in the dark condition for 20 minutes. Later, the absorbance of the mixtures was read at 517nm. Ascorbic acid was used as a control. The percentage of scavenging activity was calculated by using the following equation;

$$\text{Percentage of scavenging activity} = \frac{control - test}{control} X100$$

The IC_{50} value is calculated by using ED50 plus V1.0 Software.

Ferric reducing power activity :

The reducing power of the extract was determined as described by Yen and Duh[24]. Total five different concentrations such as 125µg/ml, 250µg/ml, 500µg/ml, 1000µg/ml and 2000µg/ml were taken from a stock concentration of 10mg/ml. For each concentration, 2.5ml of phosphate buffer (200mM) (pH 6.6) and 2.5ml of 1% potassium ferric cyanide were added and boiled for 20 minutes at 50°C. Quercetin (10mg/mL DMSO) was used as the standard to compare the scavenging activity of the samples. After incubation of the samples, 2.5 ml of 10% TCA were added to this mixtures and centrifuge at 650g for 10 minutes. The top 5ml layer was mixed with 5ml of distilled water. Then, 1ml of 0.1% ferric chloride was added to it. Later, the absorbance was read at 700nm. The percentage of scavenging activity was calculated by the following formula;

$$\text{Percentage of inhibition} = \frac{Control - Test}{Control} X100$$

2, 2'azinobis 3 ethyl benzothiazoline 6-sulfonic acid (ABTS) :

The ABTS assay was determined as described by Bunea *et al*[5]. Two solutions, 20mM, 2, 2'azinobis 3 ethyl benzothiazoline 6-sulfonic acid (ABTS) and 17mM Potassium persulfate, were prepared. A 0.3ml potassium persulfate was added into 50ml of ABTS solution. After mixing these two solutions, it was kept at room temperature overnight in dark condition. Different concentrations such as 125µg/ml, 250µg/ml, 500µg/ml, 1000µg/ml, and 2000µg/ml were prepared. In each mixture, 0.16ml of ABTS was added. After 20 minutes, the absorbance was measured at 734nm. Ascorbic acid (10mg/mL) was used as the standard to compare the scavenging activity of the samples. The percentage of inhibition activity was calculated by the following formula:

$$\text{Percentage of inhibition} = \frac{Control - Test}{Control} X100$$

The IC_{50} value is calculated by using ED50 plus V1.0 Software.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay:

The selected crude extracts were used for anticancer activity against the human lung cancer cell line A549 through MTT assay according to the methods of Laura *et al*[16]. The cell line-A549 was initially procured from National Centre for Cell Sciences (NCCS), Pune, India, and maintained through Dulbecco's modified Eagles medium, DMEM (Sigma Aldrich, USA). A 100µl cell suspension at a density of $5x10^3$ cells/well was seeded in 96 tissue culture plate (Microtiter plate). Different concentrations of the samples such as 100µg, 50µg, 25µg, 12.5µg, and 6.25µg in 500µl of DMEM were prepared and added to each well and incubated for 24h at 37ºC in a humidified 5% CO_2 incubator. After 24h of incubation, 30µl of MTT solution was added to all test and cell control wells. The plates were gently shaken and incubated at 37ºC in a humidified 5% CO_2 incubator for 4 hours. After the incubation, the supernatant was removed. A 100µl of MTT solubilization solution Dimethyl sulfoxide (DMSO) was added to the wells to solubilize the formazan crystals. Later, the absorbance values were measured by using a microplate reader at the wavelength of 540 nm. The viability of cells was evaluated by direct observation of cells by an Inverted phase contrast microscope and the percentage of growth inhibition was calculated using following formula :

$$\text{Percentage of cells viability} = \frac{\text{Mean OD Samples x } 100}{\text{Mean OD of a control group}}$$

Antioxidants are among the most crucial natural components which can fight against reactive oxidant species. In the present study attempts have been made to evaluate the crude extracts of *A. alternata* and *T. asperellum*, isolated from the traditional medicinal plant *M. neriifolia* for their antioxidant potentialities. For this purpose, DPPH, FRAP and ABTS assays were used.

Though *A. alternata* and *T. asperellum* are morphologically and molecularly different endophytic fungi, surprisingly, in the DPPH assay, both these fungi showed almost similar levels of inhibition activities, that is 33.16% with an IC_{50} value of 389.791 µg/ml by *A. alternata* and 34.04% with IC_{50} value of 413.923 µg/ml by *T. asperellum* at the concentration of 200 ug/ml. The scavenging activity of these fungi at different concentrations is shown in Fig: 1.

In the ABTS assay, *A. alternata* at the concentration of 2000µg/ml exhibited an inhibition activity of 34.63%; with an IC_{50} value of 2853.1µg/ml, whereas *T. asperellum* showed 31.57% inhibition activity (2000 µg/ml) with 3097 µg/ml of IC_{50} (Fig: 2). Similarly, in the FRAP assay, *A. alternata* and *T. asperellum* at the concentration of 2000µg/ml; showed inhibition activities of 82.72% (IC_{50} value is 585.3817µg/ml) and 83.16 % (IC_{50} value is 576.79 µg/ml), respectively (Fig: 3). However, the efficiency of inhibition observed in FRAP assay seems to be significantly higher than the DPPH and ABTS assays.

To compare the antioxidant potential of these extracts, two different standards, viz, Ascorbic acid and Quercetin, were selected.

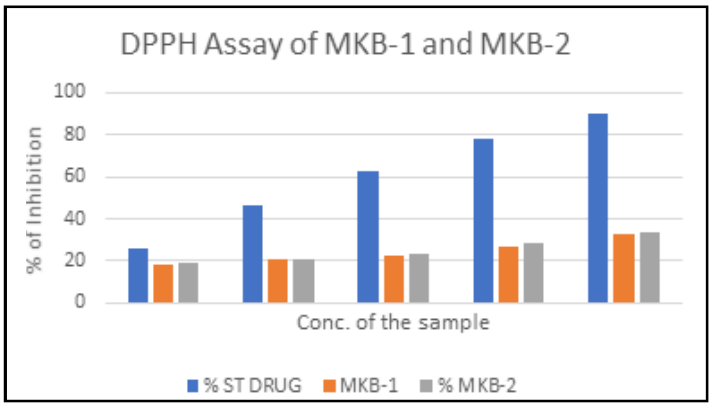


Fig: 1 DPPH Assay

Note: MKB1-*A. alternaria,* MKB2- *T. asperellum.*

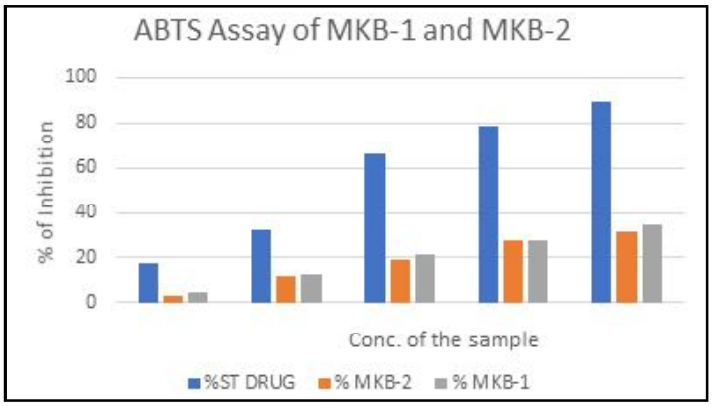


Fig: 2 ABTS Assay

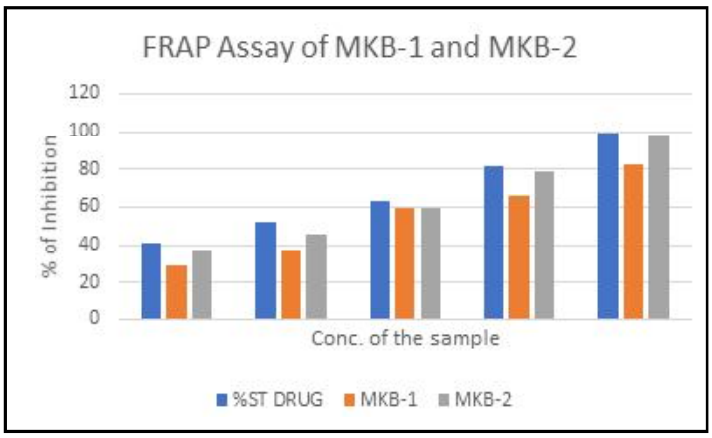


Fig: 3 FRAP Assay

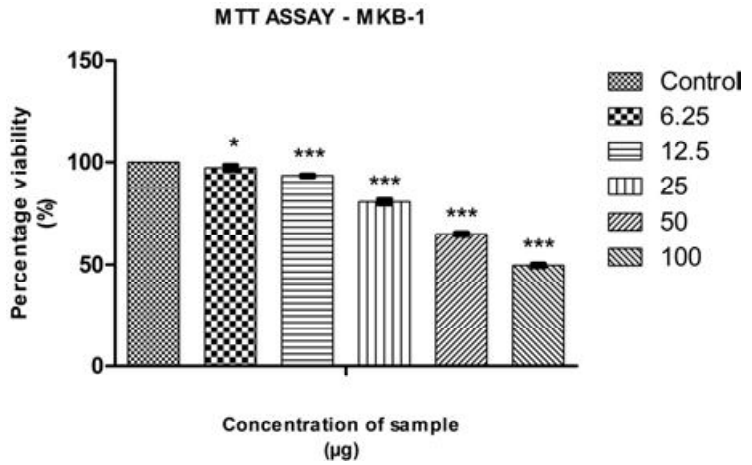


Fig: 4(a) MTT Assay of the sample MKB-1

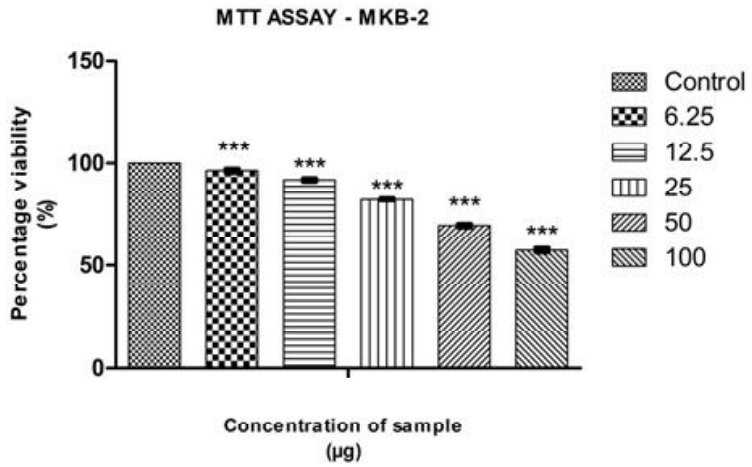
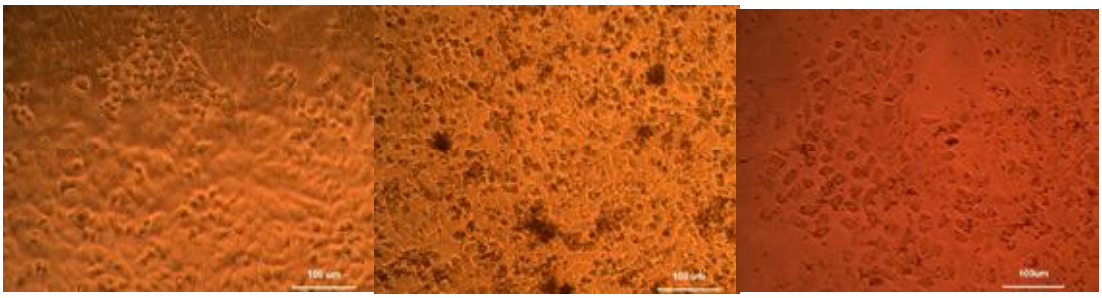


Fig: 4(b) MTT Assay of the sample MKB-2



Control (A549) Treated with *A. alternata* Treated with *T. asperellum*

Fig: 5 Treated and untreated cells morphology of A549 cell line

It is interesting to notice that when the concentrations of the extracts were increased, the inhibition ability also increased. However, this trend could not be seen with decreasing concentrations. Compared to the standard drugs, both these fungi showed good to moderate antioxidant activities in the three assays, that is, 83.16 % to 33.16 % inhibitions. The expression of antioxidant properties could be due to the presence of various phytocompounds such as Alkaloids, phenols, flavonoids, terpenoids, and tannins.

Several earlier reports also substantiate the antioxidant potential of many endophytic fungi. Among them, *A. alternata isolated from Coffea arabica L. performed moderate antioxidant activity.* Rasha *et al.,*[18] have reported the antioxidant activities of EtOAc crude extract of *A. alternata* isolated from *Ziziphus spina-christi* plant species. Kyeongwon *et al.,*[15] have shown antioxidant activities from different *Trichoderma* strains in the marine environment, they are, *T. afroharzianum, T. asperelloides, T. asperellum, T. atroviride, T. bissettii, T. capillare, T. citrinoviride, T. gamsii, T. guizhouense, T. hamatum, T. longibrachiatum, T. orientalis, T. paraviridescens, T. pyramidale, T. songyi, T. subviride, T. virens, Trichoderma sp. Similarly,* Harish *et al.,*[10] found antioxidant potential in the EtOAc crude extract of *A. alternata* isolated from *Picrorhiza kurroa* plant species. Similar reports are available for the exhibition of the antioxidant potential of *Alternaria sp.* of fungi isolated from various plant species[9,11,20,21].

The present results indicated that both the fungi have the potential to fight against free radicals. Hence, they were studied further to evaluate their cytotoxicity against cancer cell line A549. In cytotoxic analysis, it is observed that compared to the untreated cells, *A. alternata* kills more than 50% of cancer cells at 100 µg/ml concentration, whereas *T. asperellum* kills less than 50% at the same concentration (Fig-4). It is also observed that when the concentration of the samples increases, the toxicity also increases. This may be due to the presence of potential natural compounds present in the extracts (Fig. 5).

Several reports suggested the cytotoxicity of endophytic fungi. Gebarowska *et al.,*[8] have reported that *A. alternata* (*Solanum nigrum*) extracts cytotoxic effects on different cancer lines such as A549, THP1, MCF7 and HeLa. Similarly, Atef *et al.,*[2] observed similar results from two species of the *Trichoderma* genus that is *T. harzianum* and *T. asperellum,* on cancer cell lines viz, HeLa and MCF-7 cells. The crude extract of *Aspergillus terreus is toxic to* cancer cell line A549[12(b)]. The crude extracts of *Neofusicoccum* sp., *Phomopsis* sp., *Leptosphaerulina sp.,* and *Penicillium sp.* possess cytotoxic potential on the A549 and NCI-H460 cancer cell lines[23]. Sheeba *et al.,*[19] *showed cytotoxic activity by* the crude extracts of *Trichoderma viride* on the HeLa cancer cell line.

In the light of the previous reports, the present studies suggest that the endophytic fungi *A. alternata* and *T. asperellum* isolated from the plant *Madhuca neriifolia* do possess antioxidant abilities. Further, these fungi have exhibited moderate levels of cytotoxic effects against human lung cancer cell A549. The

expression of cytotoxic ability could be attributed to the presence of antioxidant potential, which can be correlated to the availability of a combination of phytochemicals in the crude extracts. Furthermore, detailed studies related to the characterization of lead compounds from these extracts and subsequent cytotoxic analysis may substantiate the results of the present study. However, the present results clearly indicate that the compounds isolated from the endophytes might become potential candidates to be used in combination with other drugs in cancer therapy.

Antioxidant and cytotoxic analyses are very crucial pharmacological tests to identify the effectiveness of natural compounds against free radicals and cancer cells respectively. In the present study, it is found that *A. alternata* and *T. asperellum* exhibits good to moderate levels of antioxidant activities. On the other hand, in terms of cytotoxic activity, *A. alternata* shows more potential than *T. asperellum* against human lung cancer cell line A549. *A. alternata* exhibits tremendous potential as a cytotoxic agent. The results pave the wat for a clear understanding of the mechanism of action of compounds responsible for exerting beneficial properties. Further, the extracts or their isolated compounds may be considered for use in therapeutic regimes in combination with standard drugs against cancers.

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