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ANTI-OXIDANT ACTIVITY OF SECONDARY METABOLITES PRODUCED BY *FUSARIUM OXYSPORUM*: AN ENDOPHYTIC FUNGUS ISOLATED FROM AN ENDANGERED PLANT *MAPPIA FOETIDA*

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Abstract:

Endophytes are the excellent source for the production of bioactive natural compounds. Endophytic fungi were isolated from the endangered medicinal plant Mappia foetida. Fusarium oxysporum has been cultured and identified. Qualitative assay of the bioactive natural compounds was investigated by using standard protocol. The extract was screened for possible antioxidant activities by free radical scavenging activity (DPPH). Hence this study showed promising DPPH (1, 1-diphenyl-2-picrylhydrazyl) scavenging activity for F. oxysporum. The endophytic fungal leaf extract of F. oxysporum showed highest antioxidant activity around 74.46% which has scavenging effect on DPPH assay.

Keywords: *Fungal endophytes, Bioactive natural compounds, DPPH (1, 1-diphenyl-2-picrylhydrazyl) scavenging activity.*

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INTRODUCTION:

Fungal and bacterial endophytes refer to the microorganisms which are colonising the intercellular and intracellular parts of healthy plant tissues at a specific time, whose presence is asymptomatic and unobtrusive [1]. Majority of plant species are considered to be host at least one type of endophyte [2]. Endophytes form a symbiotic relationship with their plant host. It is believed that in many cases the microbes function as the biological defence for the plant against foreign phytopathogens. The protection mechanism of the endophytes is exerted directly, by releasing metabolites to attack any antagonists or lyse affected cells, and indirectly, by either inducing host defence mechanisms or promoting growth. The functional metabolites produced by endophytes include alkaloids, terpenoids, steroids, quinones, isocoumarin derivatives, flavonoids, phenols and phenolic acids and peptides. Some species produce novel antimicrobial agents (e.g., Cryptocandin from *Cryptosporiopsis quercina*), others produce potent anti-cancer compounds (e.g., Taxol from *Taxomyces andreanae*), and yet others produce compounds that can be utilized industrially, such as enzymes and solvents [3]. Endophytes can produce the same or similar secondary metabolites as their host. Bioactive compounds which are co-produced by the plants as well as their associated endophytes include the anticancer drug camptothecin [4], the anticancer drug lead compound podophyllotoxin, and the natural insecticide azadirachtin [5]. There are several mechanisms proposed for the simultaneous production of these biological compounds. In some cases, such as that of gibberellin, the biosynthetic mechanism of the same compound evolves independently in plants and their microbial counterparts [6]. On the other hand, horizontal gene transfers between the plant host and its endophytes have long been hypothesised, although so far this process has only been shown to occur between microbial endophytes [7]. It has been strongly suggested, however, that interactions between endophytes and their respective plant host contributes to the co-production of these bioactive molecules [8].

The antioxidant compounds play an important role as a health protecting factor. There is some scientific evidence suggests that antioxidants reduce the risk for chronic diseases. The main characteristics of an antioxidant are its ability to trap free radicals. These free radicals may oxidize nucleic acids, proteins, lipids or DNA and can initiate degenerative disease. During a chemical reaction (oxidation), one reactant loses an electron and is called oxidant or free radical [9], while the other gains an electron. In living organisms' oxygen in unstable form is the most common free radical, this is called Reactive oxygen species (ROS) [10] and is generated during various metabolic activities.

Our daily diet contains vegetables, fruits, tea, wine etc., which possess compounds rich in anti-oxidative properties (Institute of Food Research Report, 1997). Potential sources of natural antioxidant have been searched in different types of plant materials such as vegetables, fruits, leaves, oilseeds, cereal crops, tree barks, roots, spices and herbs [11]. Plant sourced food antioxidants like vitamin C, Vitamin E, carotenes, phenolic acids, phytea and phytoestrogens have been recognized as having the potential to reduce disease risk. Several antioxidants from plant that aid in antioxidant defense system, protecting plants against damage caused by active O_2 formed due to exposure to ultraviolet radiation. These reduce the free radical formation as well as oxidative stress and reduce the possibility of cardiovascular disease [12]. A number of plants such as rosemary and sage belonging to Labiatae family have provided effective antioxidative extracts, used for the protection of oils, fats and salad dressings [13]. Several compounds such as phenolic diterpenoids- camosol, rosmanol, camosoic acid, etc. obtained from several aromatic plants possess strong antioxidant properties.

Natural antioxidants have an important role in the prevention of many age-related diseases and promotion of health. Among natural antioxidants from plants, flavonoids and other phenolic compounds are potent antioxidants and chelating agents. Antioxidant can be defined as any substance that delays or inhibits oxidative damage to a target molecule [14]. The main characteristic of an antioxidant is its ability to trap free radicals. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases [15]. Medicinal plants are considered as good antioxidant since ancient times.

MATERIALS AND METHODS:

Study area and Collection of plant material:

The study area is situated at Western Ghats of Mookambika Wild life Sanctuary in Shivamogga district of Karnataka. The average annual rainfall of 5000 – 8000mm. The region falls in 13°30'9.1"N and 75° 5' .18"E with elevation ranges 640 meters above Mean Sea Level (MSL) and the forest is composed with rich endemic flora. These forests are classified as tropical wet evergreen forests of the *Dipterocarpus indicus - Humboldt brunonis - Poeciloneuron indicum* type.

Freshly collected plant material is washed thoroughly under running tap water followed by sterile distilled water to remove the adhered debris. Twigs and leaves were surface sterilized under aseptic condition in sequential steps by immersing in mercuric chloride (1mg/1ml) for 10 min and 70 % ethanol for another min followed by washing finally with distilled water.

Inoculation of implants:
After successive surface sterilization of twigs and leaves of *M. foetida* were aseptically cut into small pieces (0.5-0.5cm^2) and placed 5-6 pieces on each of the solidified sterile Potato Dextrose Agar (PDA) media. The inoculated plant implants were incubated till the growth of distinguishable fungal endophytes.

Identification of endophytic fungi:
For the identification of endophytic fungal isolates, slides were prepared from cultures and were stained with lacto phenol cotton blue and examined with a bright-field and phase contrast microscope. Identification was based on morphological characteristics such as growth pattern, hyphae, the colour of the colony and medium, surface texture, margin character, aerial mycelium, mechanism of spore production and conidia characteristics using standard identification manuals [16,17]. The identified fungi sub cultured in PDA slants for further use and stored in refrigerated conditions.

Quantitative determination of secondary metabolites:
Alkaloids: The extraction is made with ethanol by solvent distillation process. Then the extract is purified by various methods. For qualitative detection crude extract is used [18].

Wagner's Test:
For 3ml of fungal crude extract from the endophytes, 1ml of Wagner's solution is added.

Flavonoids: Plant extract of 2ml is added to the test tube containing 1ml of dilute ammonia and mixed well with 1ml of concentrated sulphuric acid. Formation of yellow color indicates presence of flavonoids [19].

Steroids: 1ml of fungal crude extract from the endophytes is dissolved using 1ml of chloroform and 2-3ml of acetic anhydride. 1-2 drops of concentrated sulphuric acid is added. Upper layer turns red and concentrated sulphuric acid layer shows yellow with green fluorescence. Therefore, it indicates presence of steroids [20].

Terpenoids: 2ml of fungal crude extract from the endophytes + 2ml of chloroform is added. 3ml of concentrated sulphuric acid is added to form a layer. If reddish brown color appears, it indicates presence of terpenoids [21].

Tannins: Ferric chloride test: Fungal crude extract from the endophytes is mixed with 1% $FeCl_3$ solution which produces green or brownish green, blue color [22].

Saponins: 1ml of fungal crude extract from the endophytes is mixed using 20ml distilled water and kept on stirring for 15min. If foam forms, indicates the presence of saponins [23].

Triterpenoids- Salkowaski test: 2ml of fungal crude extract from the endophytes is mixed with 5 drops of conc. Sulphuric acid. If greenish blue color appears, it indicates the presence of triterpenoids [24].

Carbohydrate- Benedict's test: Benedict's reagent is mixed with 2ml of fungal crude extract from the endophytes and kept for boiling in water bath and observed for reddish brown precipitate which indicates the presence of carbohydrates [24].

Antioxidant activity assay of fungal crude extracts:
The free radical scavenging activities of extract were measured by using 1,1-diphenyl-2-picryl-hydrazyl (DPPH) [25]. Extract concentration around 0.1 mg/ml in 4 ml of methanol was mixed with 1 ml of methanol solution containing DPPH radicals of 0.2 mM. The mixture was shaken vigorously and allowed to stand for 30 min in the dark chamber. The absorbance was measured at 517 nm against a blank. IC50 value was obtained by interpolation from linear regression analysis. Butylated hydroxy toluene (BHT) was used as standard for comparison studies. The capacity of radical scavenging activity was calculated using the following equation:

The antioxidant activity of the bacterial isolates is due to their redox properties, which play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides [26, 27]. The antioxidant ability of the isolates maybe used to treat the human body for oxidative damages caused by free radicals and active oxygen [28].

DPPH scavenging effect (%) = $[\{A_0 - A_1\} A_0] \times 100$
Whereas A_0 is the absorbance of the control reaction and A_1 is the absorbance of the presence of the sample. DPPH activity was correlated with standard TBHQ.

RESULTS AND DISCUSSION:

Isolation and Identification of endophytic fungi

Plant samples were used to isolate many endophytic fungal endophytes which are highly used for the medicinal properties. The isolated fungal endophyte was identified based on morphological characters using Barnett and Hunter, 1972. Isolated fungal strains were cultivated on potato dextrose agar (PDA) media (Fig 1).

Fig 1: Endophytic fungi- *F. oxysporum* inoculated on PDA media

Qualitative detection of secondary metabolites

F. oxysporum isolated from both leaf and twig of *M. foetida* plant, were found to be able to produce all the functional metabolites (table 1). Although all the isolates showed more or less efficient (as observed from the intensity of color) for the production of alkaloids, flavonoids, tannins, saponins, sterols, Cardiac glycosides, terpenoids and triterpenoids (Fig 2).

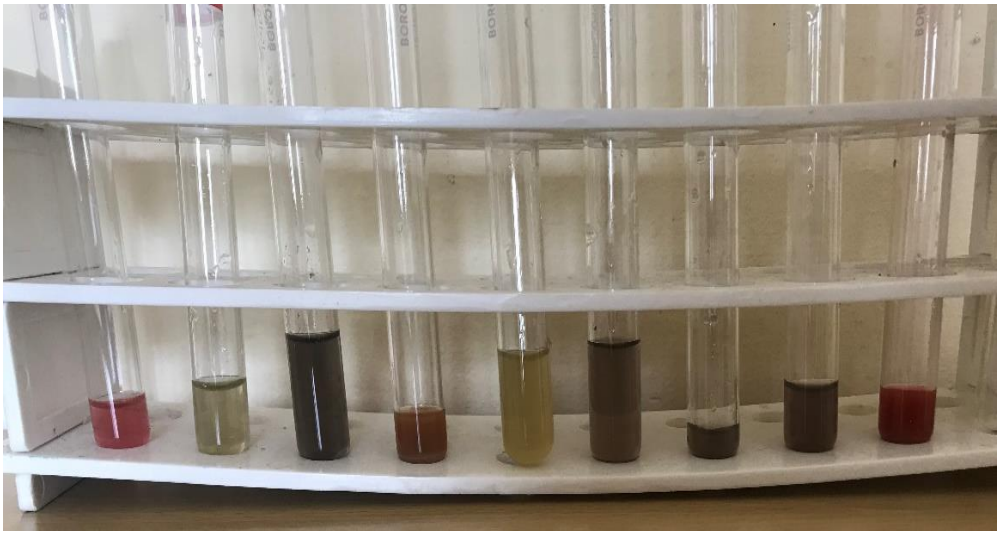

Fig 2: Isolation and identification of secondary metabolites from endophytic fungal species

Table 1: Qualitative detection of secondary metabolites from endophytic fungal species

Secondary Metabolites	Endophytic Fungi- *F. oxysporum*	
	Leaf	*Twig*
Alkaloids	+	+
Flavonoids	+	+
Steroids	+	+
Terpenoids	+	+
Tannins	+	-
Saponins	-	-
Triterpenoids	-	+
Carbohydrates	-	-

Antioxidant activity:

DPPH radical scavenging assay

The DPPH assay considered to be an basic and widely used for an antioxidant activity. It is considered as an most accurate screening method used to evaluate antioxidant activity for different extracts. The antioxidant activity of ethanol extract of an endophytic fungal extract measured by the ability of scavenging DPPH free radicals, was compared with standard BHT (Butylated Hydroxyl Toluene). It was observed that leaf *F. oxysporum* extract had higher scavenging activity followed by twig endophytes *F. oxysporum*. At a concentration of 0.1mg/ml, the scavenging activity of ethanol extract of leaf *F. oxysporum* and twig *F. oxysporum* reached to 74.46% and 49.98% respectively. Hence the study proves to be that the endophytic extracts have the proton donating ability and could serve as free radical inhibitors or scavenging.

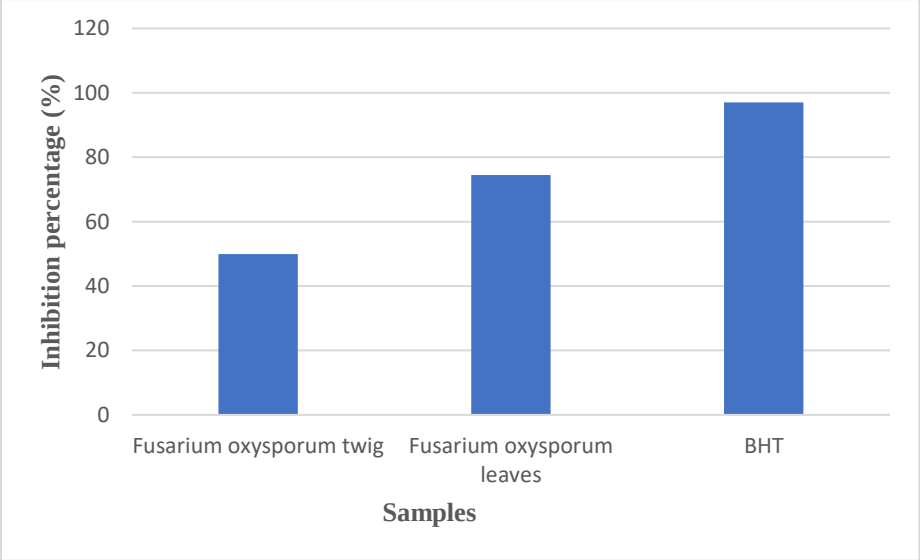

Fig 3: **DPPH scavenging activities of endophytic fungi *F. oxysporum***

DISCUSSION:

Endophytes have recently generated significant interest in the microbial chemistry community due to their immense potential to contribute to the discovery of new bioactive compounds. It has been suggested that the close biological association between endophytes and their plant host results in the production of a greater number and diversity of biological molecules compared to epiphytes or soil-related microbes [29]. Moreover, the symbiotic nature of this relationship indicates that endophytic bioactive compounds are likely to possess reduced cell toxicity, as these chemicals do not kill the eukaryotic host system. This study highlights that the endophytes are considered to be a potential source for the production of bioactive products [30]. The primary phytochemical analysis shows the presence of many secondary metabolites. Antioxidants are tremendously important

substances which possess the ability to protect the body from damage caused by free radical induced oxidative stress. The antioxidant potential of *M. foetida* methanol extracts were investigated in the search for new bioactive compounds from natural resources. It became clear that *M. foetida* leaves, present the highest antioxidant activity compared with twigs for DPPH scavenging activity.

The present study concludes, the discovery of phytochemical composition from the medicinal plants leads for the discovery of therapeutic agents. This identified compound which may be used to develop biopharmaceuticals against infectious discovery with antioxidant source in future. Plant based products are being used for medicinal and therapeutic since the dawn of the history. The use of plants as medicines has involved in the isolation of active compounds. Isolation and characterization of pharmacologically active compounds from medicinal plants in continues even today. Medicinal plants have a wide range of bioactive molecules and also have a lot of promising antimicrobial properties such as antidiabetic, anticancer agents and also process immunosuppressive compounds. Therefore, the bacterial endophytes can be utilized as the potential bioresources for the secondary metabolites other than host plant.

Even today, the most of the rural people are still depending on the local traditional medicines or drugs for their health issues because of the poor economic status. Traditional and Ayurveda medicine are cheaper and it is very much safe when compare to allopathy. Hence, in India medicinal plants and the extracts of plants are still being in use for various ailments.

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