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Taxol producing mangrove endophytic fungi *Fusarium oxysporum* from *Rhizophora annamalayana*

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ABSTRACT

Objective: To find out the anticancer properties of Taxol (paclitaxel) isolated from mangrove endophytic fungi. **Methods:** An endophytic fungus *Fusarium oxysporum* was isolated from *Rhizophora annamalayana*, a mangrove plant and analysis for Taxol production. The fungus was identified based on morphology and spore characteristics. The secondary metabolites Taxol were extracted with ethyl acetate. Taxol extracted was characterized by chromatographic and spectrometric analysis. **Results:** Thin layer chromatography plate shows violet red and IR spectrum values were conformed as group of terpenoid functional groups. The HPLC analysis showed the higher yield of Taxol 172.3 μg/L from potato dextrose liquid medium. **Conclusion:** The bioprospecting of entophytic fungus *F. oxysporum* isolated from mangrove is discussed and may serve as a potential material for the production of Taxol for anticancer treatment.

1. Introduction

Taxol, also known as paclitaxel, a chemical substance of tetracyclic diterpene lactam was first isolated from the bark, roots and branches of western yew, *Taxus brevifolia*[1]. Traditional methods of extracting Taxol from the bark of *Taxus* species are inefficient and environmentally costly. For example, while 1 kg of Taxol treats just five hundred patients; the production of 1 kg of Taxol requires 10 tons of bark or 300 trees. For this reason, seeking new ways of obtaining Taxol is the key to protecting this limited resource and reducing the cost of drug therapy. With this end goal in mind, scientists all over the world are researching areas related to Taxol production including chemical synthesis, plant tissue cell culture microbial fermentation[2-5]. In particularly microbial fermentation has demonstrated that the isolation and identification of Taxol-producing endophytic fungi is a new and feasible approach to the production of Taxol[6]. Presently, the development and utilization of Taxol producing fungi have made significant progress worldwide[7,8].

Extensive research such as searching for paclitaxel-producing endophytic fungi from *Taxus* species as well as from other related plant species, microbial fermentation processes and genetic engineering for improving paclitaxel production has been developed, and much progress has been achieved during the past two decades. In the case *Taxomyces andrenae*, *Pestalotiopsis microspora*, *Alternaria* sp., *Fusarium lateritium*, *F. solani*, *F. mairie* and *Periconia* sp. were screened to have the ability to produce paclitaxel and its derivatives[9-16]. Almost there is no report from the marine endophytic fungi for Taxol production. To date, mangrove endophytic fungi have produced novel bioactive compounds. The practical applications of mangrove endophytic fungi are multiple, as potential biocontrol agents, sources of novel metabolites for therapeutics, plant protection and other industrial applications. In general, the production of secondary metabolites that are potentially useful for pharmaceutical and agricultural applications is widespread among mangrove endophytic fungi[17,18]. Hence the present study was taken up to endophytic fungi of *F. oxysporum* was isolated from the mangrove leaves of *R. annamalayana*.

2. Materials and methods

2.1. Isolation and identification of endophytic fungi

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The fungus used in this study is one of the twenty six endophytic fungi isolated from mangrove leaves were collected from Vellar estuary and stored in sterile polythene bags and brought to the laboratory. After collection the leaves were processed separately within 2 h. To kill the unwanted phylloplane fungal propagules adhering to the surface of the cuticle of the leaves, they were surface sterilized. The leaves were washed thoroughly in running tap water and small pieces of approximately 0.5 cm diameter were cut with the help of flame - sterilized knife or blade. Then the leaf discs were surface sterilized by immersion in 70% ethanol for 5 seconds, followed by 4% sodium choloride for 90 seconds and then rinsed in sterile filter paper. The surface sterilized leaf segments were evenly spaced in Petri dishes (90 cm diameter) containing potato dextrose agar (PDA) medium (amended with chloramphenicol 150 mg/L). The surface sterilized leaf segments were evenly spaced in PDA medium (amended with chloramphenicol 150 mg/L). The Petri plates were incubated at 26 ℃ for four weeks. The Petri plates were monitored every day for the growth of endophytic fungal colonies from the leaf segments. The hypal tips, which grew out from leaf segments were isolated and sub cultured onto PDA. The endophytes were distinguished from one another by their cultural characteristics (growth rate, surface texture and margin, and hyphal pigmentation). *F. oxysporum* (RAEN-017) was screened for Taxol production.

2.2. Growth of fungi in liquid media

The test fungus *F. oxysporum* was grown in 2 liter standard flasks containing 500 mL of Potato Dextrose Broth. After 3 weeks of culture at 26 °C, the culture fluid was passed through four layers of cheese cloth to remove solids and extracted with organic solvent.

2.3. Detecting compounds from fermentation broths

After an appropriate incubation time, the entire culture (500 mL) was passed through four layers of cheesecloth to remove mycelia. To the culture filtrate 0.25 g sodium carbonate was added with frequent shaking in order to reduce the amount of fatty acids that may contaminate the culture. Then the culture filtrate was extracted with two equal volumes of solvent ethyl acetate for Taxol production. The organic phase was collected and the solvent was then removed by evaporation under reduced pressure at 35 ℃ using rotary vacuum evaporator. Crude extract were analyses by chromatographic separation and spectroscopy.

2.4. Chromatographic analysis

The crude ethyl acetate extract was loaded onto thin layer chromatogram sheet. The extract-loaded chromatogram was then developed with Saturated benzene-methanol mixture (solvents A: Chloroform+Hexane; 8:2, solvent B: Dichloromethane+Methanol 8:7 and solvent C: Ethyl acetate/2-propanol) Taxol was detected with the 1 % (w/v) vanillin/sulfuric acid reagent after gentle heating. It appeared as a bluish spot fading to dark grey after 24 h. The spots on developed chromatogram were scraped and centrifuged 2 000 r/min at 15 min. The supernatant were collected than analysis in UV absorption at 200 to 600 nm, and the fungal sample was analyzed in Fourier Transform Infra Red spectroscopic analysis (Avatar 330 FT-IR).To further presence of Taxol in crude extract was analysed by High performance liquid chromatography (HPLC) using C18 column. The mobile phase was methanol/acetonitrile/water (25:35:40) at 1.0 mL/min. The sample and the mobile phase were filtered through a 0.2 μm-pore-size PVDF filter before placing them on the column.

3. Results

3.1. Morphogical characteris of *F. oxysporum*

F. oxysporum is a hypomycetes fungus which was isolated from *Rhizophora annamalayana* (Figure 1). Based on the morphology of the fungal colony, mycelia as well as the characteristics of the conidia, the endophytic fungus was identified as *F. oxysporum*. The fungus produces three types of spores: microconidia, macroconidia and chlamydospores. Microconidia are borne on simple phialides arising laterally and are abundant, oval-ellipsoid, straight to curved, (5-12) mm×(2.2-3.5) mm, and nonseptate. Macroconidia, sparse to abundant, are borne on branched conidiophores or on the

Figure 1. Showing a *Rhizospora annamalaiyana* and 1b.endophtyic fungi growing in culture plate.

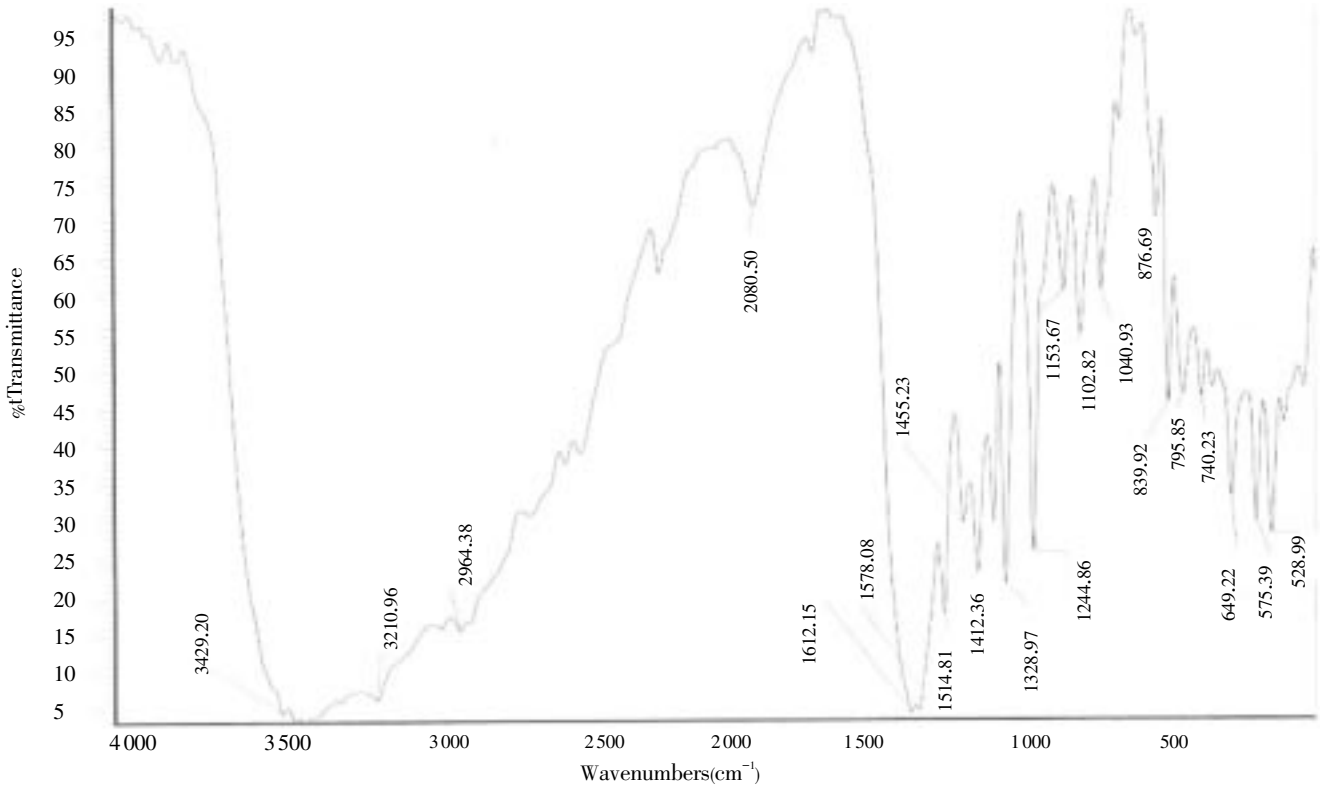

Figure 4. IR spectrum values for *F. oxysporum*.

surface of sporodochia and are thin walled, three- to five-septate, fusoid-subulate and pointed at both ends, have pedicellate base. Three-septate conidia measure (27-46) mm×(3-5) mm while five-septate conidia measure (35-60) mm×(3-5) mm. Three-septate spores are more common. Chlamydospores, both smooth and rough walled, are abundant and form terminally or on an intercalary basis the morphological characteristic of the fungus show in Figure 2.

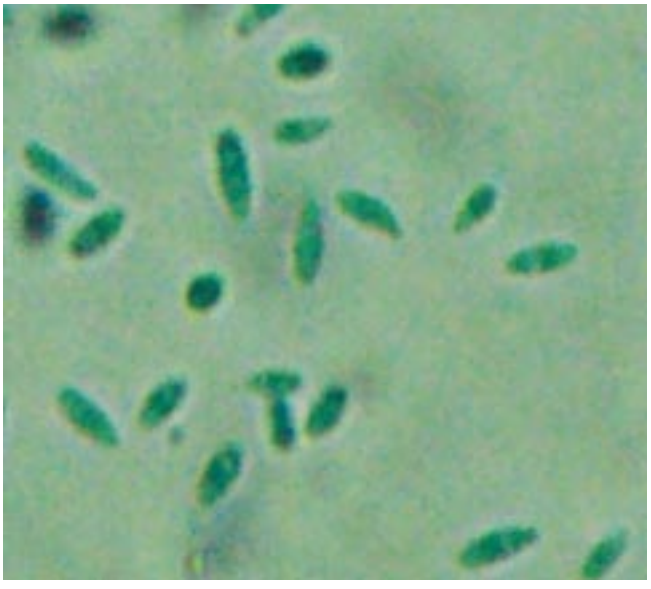

Figure 2. Microscopic observation of *F.oxysporum*.

3.2. Taxol extract and analysis

The culture filtrate was harvested and extracted with ethyl acetate. The crude extract was subjected to chromatographic analysis. Compound having chromatographic properties comparable to Taxol in solvent systems A and C, and giving a color reaction with the vanillin/sulfuric acid reagent, was consistently isolated from *F. oxysporum*. The presence of Taxol in the fungal samples was confirmed by TLC showing the color blue to gray with the vanillin sulfuric acid reagent. The active principle on development thin layer chromatogram appeared as dark band under UV illuminator at 273 nm (Figure 3). It was obtain on the TLC plate at region mobile phase I shows violet red which was conformed as group of terpenoid.

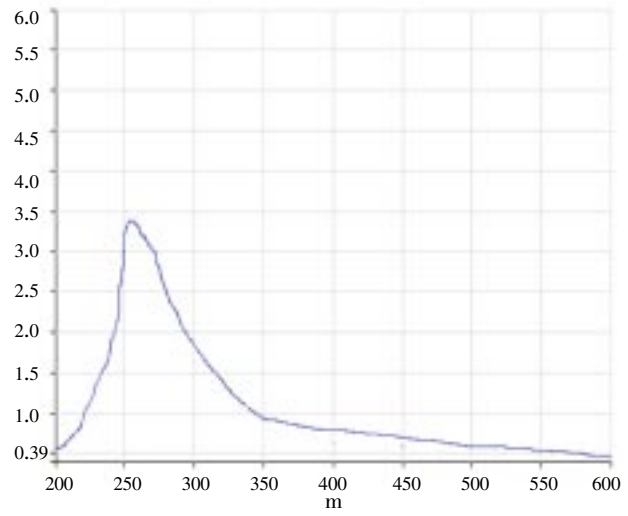

Figure 3. UV absorption spectrum of fungal Taxol.

Table1.
Summarizes the tentative assignments of the prominent peaks in the IR spectrum of the compound (Taxol).

Assignment	wave numbers (cm^{-1})
μOH, μNH	3429
μC=C	2964
Aromatic CH	795,649

The presence of broad peak at 3429 cm^{-1} in the IR spectrum of these compound are attributed to μOH and μNH modes respectively the C=C stretching vibration of the aromatic ring appeared at 2964 cm^{-1}. the peaks at 795 and 649 cm^{-1} are attributed to aromatic C-H out of plane bending vibration shown Table 1. The sharp peaks at 1612 cm^{-1} are COO-group vibration (Figure 4). HPLC analysis was performed to confirmation the presence of fungal Taxol. The fungal extract isolated from *F. oxysporum* gave a peak with a retention time shown in Figure 5. The amount of Taxol produced by *F. oxysporum* was found to be 172.3 μg/L.

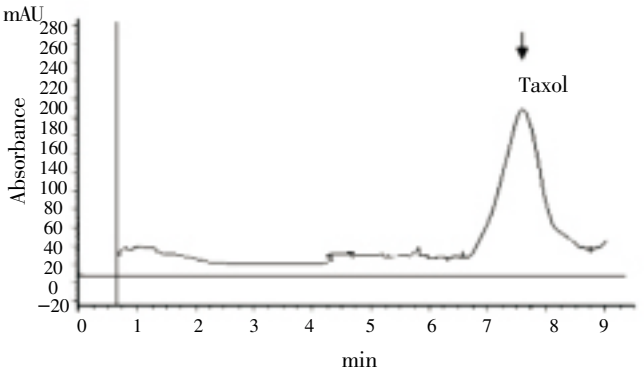

Figure 5. Extracted Taxol from *F. oxysporum* was purified by HPLC.

4. Discussion

Endophytes may contribute to their host plant by producing a plethora of substances that provide protection and ultimately survival value to the plant. The plant materials used in separation of endophytic fungi include various tissues and organs of yew trees, such as roots, stems, leaves and fruits. Endophytic fungi from coelomycetous *viz.*, *Chaetomell raphigera, Colletotrichum falcatum, Fusicoccum* sp. and *Pestalotiopsis neglecta* isolated from medicinal plants were screened for production of Taxol[19-20]. In additional, filamentous fungus *Aspergillus niger* isolated from *Taxus cuspidate* proficient to production of Taxol[21]. Up to now, Taxol production from endophytic fungi reported by only medicinal plants[22-24].

Recently, mangrove endophytic fungi has attracted many researches due to its importance in ecology[25,26]. Endophytic fungal association with mangrove plants confers protection from adverse environmental conditions and allows them to successfully compete with saprobic fungi decomposing senescent parts[27]. Although, the mangroves endophytic fungi has gained more metabolites with unique and novel structures have been isolated. Secondary metabolites production from mangrove endophytic fungi progressively (*i.e.*) alternation derivative, of alternariol 4-methyl-10-acethyl ester was isolated from mangrove endophytic fungus No.2240[28]. Moreover, isocoumarin exhibited cytotoxicity against Hep-2 and HepG2 cells isolated from mangrove endophytic fungus[29]. New xanthone derivative Compound exhibited cytotoxicity against KB and KBV200 cells with IC_{50} values greater than 50 _g/mL, isolated from endophytic fungus strain no 1850[30]. Anticancer activity of 14 anthracenedione derivatives separated from the secondary metabolites of the mangrove endophytic fungi *Halorosellinia* sp. and *Guignardia* sp. that inhibited potently the growth of KB and KBV200 cells[31].

However, several bioactive compounds are discovered from marine endophytic fungi and also five anthraquinones were elucidated by the spectroscopic methods from marine endophytic fungus *F. proliferatum*[32-34]. Enniating G, a new compound with a structure of cyclohexapeptide, was also isolated from the culture broth of mangrove fungus *Fusarium* sp. which was collected from Thailand, displayed antitumour activity to Heps 7402 with ED_{50} value of 12 μg/mL[35,36].

Previously studies evidenced that *F. mairei*, *Fusarium* sp. were capable to produce a Taxol by Chakravarthi *et al*. Cheng *et al*[16,37]. But there is no report from *F. oxysporum* especially mangrove endophytic fungi for Taxol from production. Based on these findings and literature survey, present study evaluated the endophytic fungal *F. oxysporum* extracts isolated from mangrove plants produced Taxol by TLC plate results and IR spectrum values shows functional groups conformed of terpenoid presents in fungal Taxol. and also confirmed by HPLC.

Detection of Taxol in fungal broth is TLC, UV, MS[38,29]. Totally, 109 endophytic fungi were isolated from yew trees, among them 28 isolates produces conformed through high-performance liquid chromatography mass spectrometry (HPLC-MS) analysis[22]. Most probably UV, TLC and HPLC analysis will be conform the incidence of Taxol[30,31].

In future study is supposed to be analysis on the Taxol produced endophytic fungus was quantified by cytotoxic activity towards human cancer cells and its strain provides an excellent opportunity for large scale production of Taxol.

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