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Paclitaxel and Its Chemo-preventive Role in the Management of Small Lung Cancer

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ABSTRACT

Based on a quantitative analysis using HPLC and UV, *Alternaria alternata* has been identified as a strong taxol producer. Taxol's biosynthetic efficiency has been checked using PCR detection.The development of taxol by *A. alternata* was investigated by inoculating the fungus on various media.The best medium for extracting the highest volume of taxol was found to be potato dextrose broth (PDB). After 21 days of incubation at 23°C with a pH of 8.5 and shaking (120 rpm) (250 g/L), the substance taxol was formed to perfection. HPLC was used to effectively purify the *Alternaria alternata* taxol. The taxol from *Alternaria alternata* displayed promising anticancer properties. The capacity of anticancer drugs to eradicate cancer cells may be used to determine their efficacy. As a result, the inhibitory effect of taxol on A549 lung adenocarcinoma cells was assessed by culturing the cells with different concentrations of the compound for 24 hours (MTT assay).

INTRODUCTION

Cancer is a disease in which the cell's natural division mechanisms, which are regulated by its genetic material (DNA), are disrupted.Viruses, chemical carcinogens, chromosomal rearrangement, tumour suppressor genes, and spontaneous transformation have all been linked to cancer (Anwar *et al.,* 2020). Cancer claimed the lives of 8.9 million people worldwide in 2017, according to the World Health Organization.This figure is projected to increase to 12.0 million by 2030. One of three factors can lead to cancer:a poor diet, a genetic predisposition, or environmental exposure. In the case of lung cancer, a bad diet is responsible for at least 35% of all cancers worldwide, with the addition of alcohol and cigarettes increasing the figure to 60%. A hereditary predisposition to cancer accounts for around 20% of cancer cases, leaving the majority of cancers to be caused by a number of environmental carcinogens (Rehm *et al.,* 2020).

Chemoprevention, which is intended to interact with the initiation, promotion, or progression stages of carcinogenesis, is an important method for cancer control. Taxol has recently been recognized as having high therapeutic importance, as it prevents or suppresses the growth of lung cancer while also improving abnormal lung function (Yadav *et al.,* 2020). It has been well developed and approved as a highly effective chemotherapeutic agent for a range of tumors by the Food and Drug Administration (FDA) since 1992 (Pillai, 2014). Taxol destroys tumour cells by increasing microtubule assembly and inhibiting depolymerization. This substance is the first anticancer drug worth a billion dollars in the world. It was used to treat cancers of the breast, lungs, and ovaries, as well as other human tissue proliferating diseases (Sykłowska-Baranek *et al.,* 2021).

Taxol (Paclitaxel) is a plant secondary metabolite discovered in the bark of the yew tree. Actually, most clinically used taxol is made using natural precursors from distinct plant tissues in a semi-synthesis process. Endophytic fungi provide the source of taxol, which is used in cancer chemotherapy and scientific research. Taxol was isolated from the plant palm tree and strawberry through *Alternaria* sp. in vitro. The taxol yield in discovered fungi is currently too low to be commercially useful, limiting large-scale taxol production through industrial fermentation (Nair and Padmavathy, 2014; Hamzah *et al.,* 2020). The method of separation and selection of taxol-producing fungi was previously studied by many scientists (Nair and Padmavathy, 2014; Hamzah *et al.,* 2020).The isolation of endophytic fungus from plant materials was the first step. Following that, taxol-producing fungi were identified among the endophytic fungi that had been isolated. Finally, spectroscopic methods were used to isolate and classify bioactive substances from fungal cultures.The main distinction between endophytic fungus and plant material was thought to be a straightforward process, but detecting taxol-producing endophytic fungus was time-consuming and costly (Nair and Padmavathy, 2014; Hamzah *et al.,* 2020).

The main goals of this study were to create an effective procedure for screening taxol-producing fungi from a variety of endophytic fungi, with the ultimate goal of reducing workloads. To investigate a simple and efficient protocol for screening taxol-producing endophytic fungi, unique PCR primers were used to screen a variety of isolates. This research laid the groundwork for the discovery of new molecular markers for taxol-producing fungi screening as well as the study of the A549 cell line.

MATERIALS AND METHODS

Chosen Host Plants for the Study and Samples Collection:

The analysis of the occurrence and distribution of fungal endophytes requires the collection of host plants from suitable sites and the acquisition of fresh plant material. Palm tree branches and strawberry fruits were selected for plant samples. Five leaf samples were obtained at random from both plants at Benha city, Qaliobya Gavernorate, Egypt, with an average temperature of 29°C. A polythene bag was used to collect the samples.

Media for Culture:

The potato infusion (boiled filtrate of macerated potatoes) was mixed with dextrose and agar to make the media. The pH was balanced, and the media was sterilised by autoclaving at 121°C for 15 minutes at 15 lbs. pressure. After cooling, the media was aseptically poured into sterile Petri dishes to allow for solidification.

Samples and Cultures Preparation:

The chosen leaf samples were washed thoroughly with tap water and surface sterilized with 75% ethanol (60 sec) and 1:3 dilution of water and NaCl (5

min). The sterilized leaf samples were cut into small segments (0.5– 0.6 cm^2) and transferred to Petri dishes containing PDA supplemented with Chloramphenicol. The plates were incubated at room temperature (27°C) for about 5-7 days. Beginning on day seven, regular observations of endophytic fungus growth were made. PDA slants were used to keep the isolates' pure cultures (Elavarasi *et al*., 2012).

Macroscopic and Microscopic Identification of Fungal Cultures:

The obtained fungi were identified macroscopically and microscopically according to their morphological features. Pure cultures of the obtained fungi were periodically examined macroscopically for the morphology of the fungal culture including colony features (color and texture), and microscopically by the mechanism of spore production and their characteristics (shape and the arrangement) using the standard mycological manuals (Nag Raj, 1993).

PCR Amplification:

The obtained fungi were also identified genetically using the PCR technique to confirm the morphological identification. ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (TCCTCCGCTTATTGATATGC-3') were used to amplify the ITS area in PCR. In a BioRad thermocycler, the PCR reaction was carried out (S 1000TM). An initial denaturation stage of 5 minutes at 94°C was followed by 40 cycles of one minute denaturation at 94°C, primer annealing for 45 seconds at 53°C, and primer extension for 90 seconds at 72°C, with an initial denaturation of 5 minutes at 94°C and a final extension of 10 minutes at 72°C. The reaction was carried out in a 25-liter container with 2 litres (20 ng/ml) of DNA templates, 12.5 litres of master mix, and 10 pmol of each primer.

Extraction of Taxol:

Endophytic fungi were grown in 1-liter Erlenmeyer flasks with 300 ml M1D medium and 1g soytone per litre.The culture fluid was passed through four layers of cheesecloth to extract solids after three weeks of culture at 23°C. To reduce the contamination of taxol by fatty acids, 0.25 mg Na_2CO_3 was applied to the culture filtrate when shaking. The organic phase was dried at reduced pressure at 35°C using a rotary vacuum evaporator after the culture filtrate was extracted with dichloromethane, one ml dichloromethane was used to remove the dry solid residue (Liu *et al.,* 2009).

Screening of Fungi for the Production of Taxol:

Alternaria alternata was one of the fungi tested for the development of taxol. *Alternaria alternata* was found to produce taxol, which was first detected by HPLC-UV. The presence of taxol in the extracts was determined by HPLC analysis. The samples' retention time was similar to that of the genuine taxol (Yang *et al.,* 2018).

Cell Line and Culture:

The National Cancer Institute provided cancer cell lines for purchase.To dilute out the DMSO, the cells were thawed in a water bath at 37°C for one to two minutes before being applied to 10 ml of pre-warmed (37°C) antibiotic-free RPMI 1640 (sigma) growth medium containing 10% Foetal Calf Serum (FCS) (Sigma). The cells were centrifuged at 1500 rpm for 5 minutes at 25°C to produce a cell pellet with the supernatant being discarded. The pellet was suspended in 10 mL of pre-warmed total growth medium and placed in a cell culture dish. The cells were incubated at 37°C in a humified CO2 (5%) incubator until the monolayer was subconfluent (Rose *et al.,* 1994).

RESULTS

Hyphomycetes Identification:

Alternaria alternate:

Colonies are thin, velvety almost black with aerial growth consisting almost entirely of spore chains. The reverse side of the colonies was dark

brown. Colorless hyphae, smooth conidiophores, 50 µm long, 3–6 µm thick in size arise singly or in groups often branched, straight and flexuous (Fig.1).

ITS Amplification:

PCR amplification was also used to identify the organisms down to the species level. On agarose gel electrophoresis, the molecular size of rDNA was observed in the range of 500 to 1500 bp, and the amplified products were sequenced (Fig.2).

After sequencing the phylogenetic tree was constructed using MEGA 6.0 software**.** Phylogenetic analysis for the isolate revealed the identification of fungus level of *Alternaria alternata* (Fig. 3).

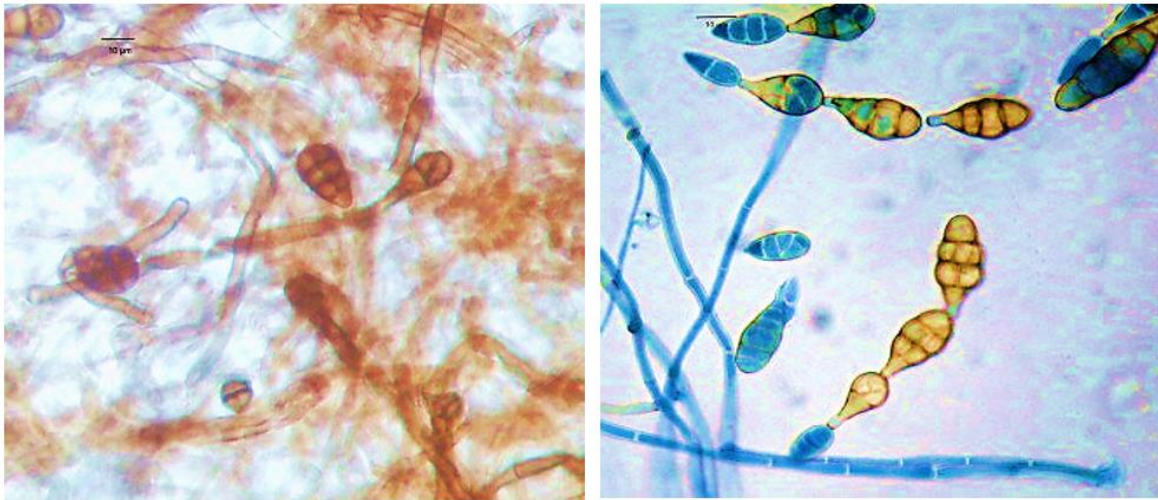

Fig. 1: Microscopic identification of *Alternaria alternata*

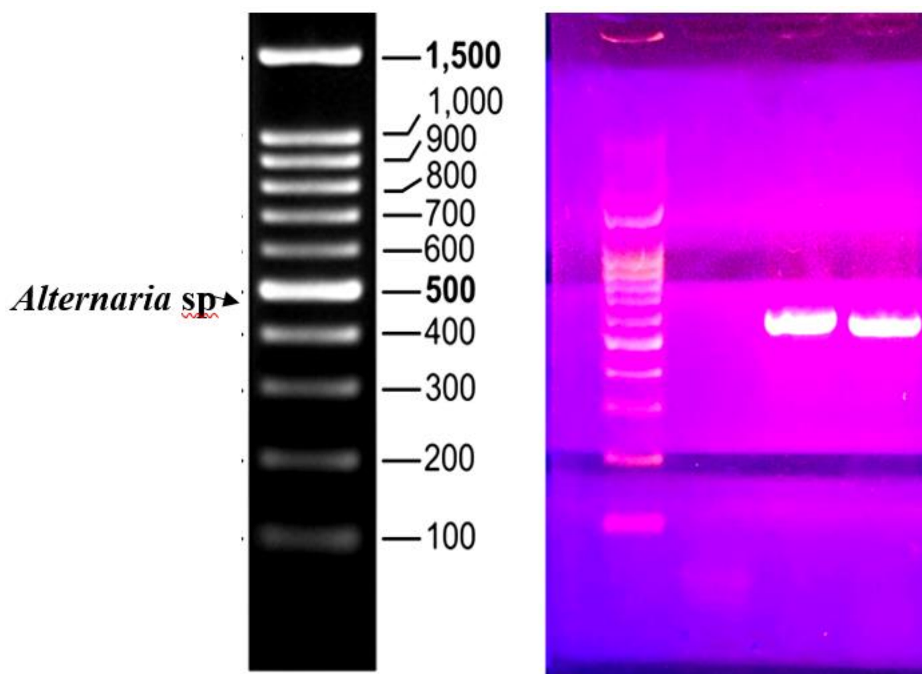

Fig. 2: PCR amplification of *Alternaria alternata*

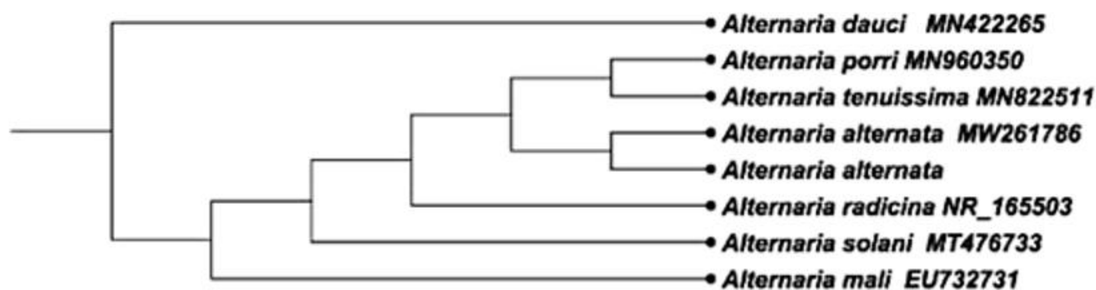

Fig. 3: The phylogenetic tree of *Alternaria alternata*.

Ultra Violet (UV) Spectrum:

UV spectrum was used to confirm the development of fungal Taxol. The absorption spectra of fungus and authentic Taxol were identical, with a characteristic peak at 220 and 273 nm, respectively (Fig. 4).

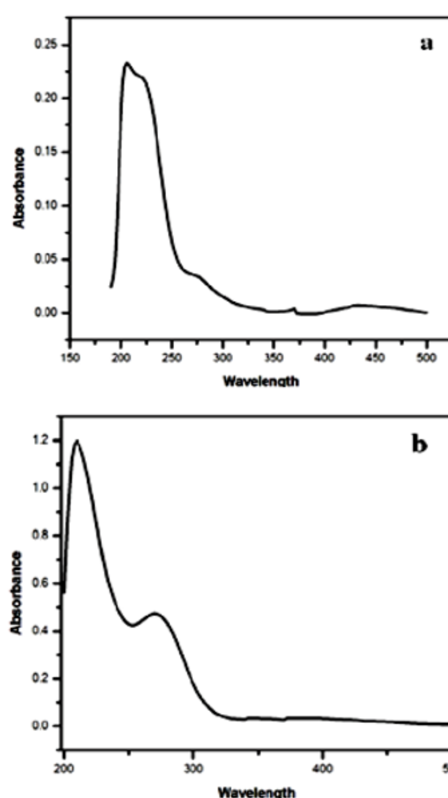

Fig. 4: Ultra Violet spectrum of authentic Taxol (a) and fungal Taxol (b).

High-Performance Liquid Chromatography (HPLC):

The fungal extract was subjected to High-Performance Liquid Chromatography to confirm the presence and quantification of taxol. The fungal sample produced a peak with a retention time that was similar to that of genuine taxol. The amount of taxol released by the fungus was measured and found to be 104.5 mg/L (Fig. 5).

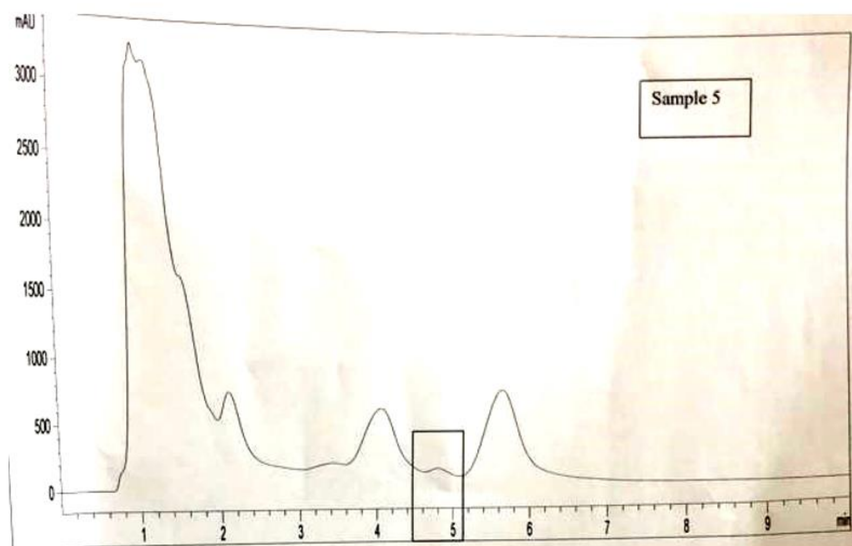

Fig. 5: HPLC analysis of fungal taxol from *Alternaria alternata*.

***In-vitro* Studies on Anticancer Activity of Fungal Taxol Against Lung Cancer Cell Line A549**:

Table 1 shows that taxol cytotoxicity in A549 cells in both fungi was dose-dependent.

A 50% reduction in cell viability was observed when cells were treated with 5.6 mg/L of taxol for 24 hours (58.95 in A549), Table (2) and Fig. (6).

Table 1: Cell viability of A549 cancer cell line treated with different concentrations of fungal taxol isolated from the *Alternaria alternata*.

Viability %	Taxol Conc (mg/L)
100	0
90.4	0.4
82.8	0.7
73.4	1.4
66.5	2.8
63.9	5.6
59.3	11.2

Table 2: IC_{50} calculation along with Hill Coeff results

Minimum	0.369576 +/- 0.2999 (81.15%)
Maximum	23.1322 +/- 20.16 (87.13%)
IC_{50}	58.9582 +/- 6.303 (10.69%)
Hill Coeff.	15.9415 +/- 6.845 (42.94%)

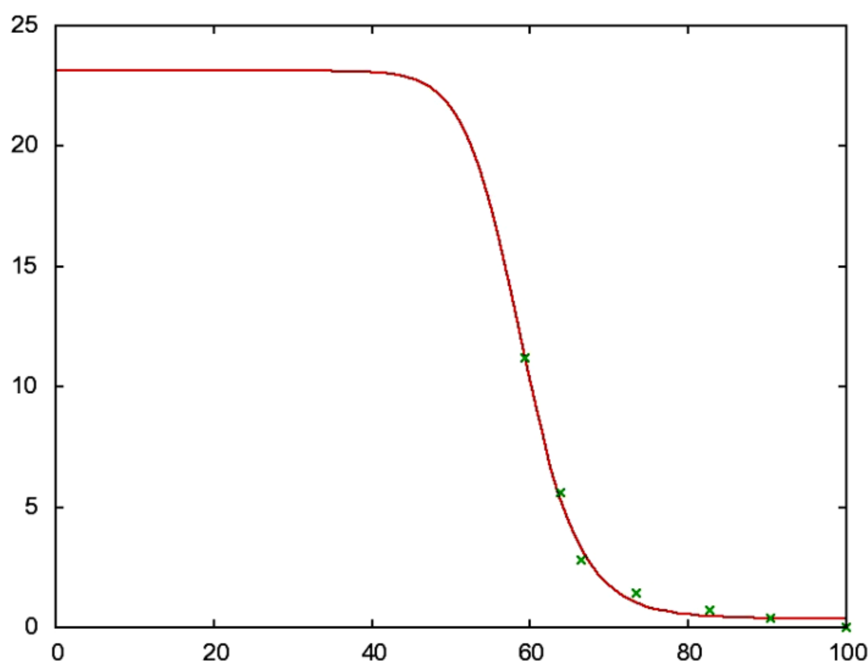

Fig. 6: Fitting curve of calculation of IC_{50} *in Alternaria alternata.*

DISCUSSION

Endophytic fungi are increasingly being recognized as sources of novel bioactive compounds and secondary metabolites that can aid biological regulation (Kumar *et al*., 2014). As a result, using endophytic fungi opens up new biotechnological possibilities, necessitating the isolation, cultivation, and bioprospecting of these species (Sharma *et al*., 2016). In light of the above, the current research aims to isolate and molecularly identify endophytic fungi from plants such as *Alternaria* spp. in order to extract, screen, and characterise fungal taxol from these isolated fungi. The therapeutic effectiveness of fungal taxol against lung cancer was also studied. The endophytic fungus can be found in a variety of plant hosts throughout the country.The chosen plants, palm branches and strawberries fruits from various families, and the fungus isolated from these plants, together with sterile morphotypes, form a wide range of Hyphomycetes members (Moloinyane, 2018).

Many plants have sterile forms that have been isolated as endophytes. In most endophytic research studies, sterile mycelium is contained, according to Vardhana *et al*., (2017). Endophytes were roughly classified into *Alternaria* sp. in our analysis based on their culture morphology and spore sequence. Identifying fungal endophytes diversity down to species level without a molecular analysis, according to previous studies, is difficult. Traditional approaches of identification and differentiation into the genera are difficult due to the highly variable morphological characteristics and natural phenotypic versatility of individual isolates (Nerva *et al*., 2019).

We ran into the same problem in this analysis, where we couldn't identify individual isolates at the species level using the conventional method. As a result, all the isolates were subjected to PCR analysis in order to amplify the ITS area, and individual phylogenetic trees were built to name the organism up to species level (Forsman *et al*., 1994). The organisms were identified to species level using PCR amplification of the ITS structure. *Alternaria alternata* was established as the fungus' species name through phylogenetic analysis of the isolate (Wirsel *et al*., 2001).

The isolate was also examined for the existence of taxol output using chromatographic and spectroscopic methods. The presence of fungal taxol in the extract was confirmed by chromatographic tests (HPLC). To evaluate the pattern of taxol assignments, spectral and analytical scrutiny, such as UV, was used to validate fungal taxol. The absorbance value of other compounds other than taxol may be messed up in UV, resulting in taxol weight values that are higher than the actual value. The absorbance value for test fungi (220–270 nm) was associated with the authentic taxol in UV (270 nm) (Allison, 2020).

The cytotoxic and anticancer effects of fungal taxol have only been identified in a few studies (El-Sayed *et al*., 2020). As a result, the current study was planned to determine the therapeutic efficacy of fungal taxol against lung cancer in both in vitro and in vivo. The MTT assay was used to determine the cytotoxic activity of fungal taxol in this sample, which showed that fungal taxol had a 24-hour effect on cell viability in the A549 cell line. The cell viability of control cells was consistently found to be between 85 and 90%. The cell viability was significantly reduced at concentrations of fungal taxol ranging from 0.4 to 0.7 mg/L. Just 50% of cells were viable after 24 hours of treatment with fungal taxol at a concentration of 6.0 mg/L. The IC50 of fungal taxol was determined to be 58.9582 +/- 6.303. (10.69 percent).

Conclusion:

The proven results for the anti-cancerous role of fungal taxol can be inferred in detailed studies like in *vivo* assays in the future. Altogether, the study reveals a novel method for the identification of taxol produced from *A. alternata* species. Henceforth, the chosen fungus possesses several secondary metabolites which can find application in industrial and therapeutic arenas as well as compounds for drug targets.

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