

Fusarium tricinctum, An Endophytic Fungus Exhibits Cell Growth Inhibition and Antioxidant Activity

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Abstract An endophytic fungus (strain T6) isolated from *Taxus baccata* was studied for its effect on the growth of human breast cancer cell line (MCF-7), human cervical cancer cell line (HeLa) and peripheral blood mononuclear cells (PBMCs) as well as for its antioxidant activity. Based on morphological characters and internal transcribed spacer (ITS) sequence analysis, this fungus (strain T6) was identified as *Fusarium tricinctum*. This fungus has shown inhibition in the growth of the MCF-7 and HeLa cancer cell lines. IC₅₀ values of the fungal extract were 225 ± 26 and 220 ± 18 µg ml⁻¹ for MCF-7 and HeLa cell lines, respectively. Further, *F. tricinctum* showed inhibition in the proliferation of concanavalin A stimulated PBMCs indicating its immunosuppressive potential (IC₅₀ value 110 ± 44 µg ml⁻¹). Tumour necrosis factor (TNF)-α production in concanavalin A stimulated PBMCs and MCF-7 were found to be inhibited which indicates that the antiproliferative effect may be associated with TNF-α. Free radical scavenging results revealed that this fungus also exhibited antioxidant activity (IC₅₀ value 482 ± 9 µg ml⁻¹). Present study results suggested that *F. tricinctum* has the potential to be used for therapeutic purposes because of its antiproliferative and antioxidant potential.

Keywords *Taxus baccata* · Cancer cell lines · Tumor necrosis factor-α · Antioxidant

Introduction

Endophytic fungi are reported to colonize internal plant tissues without causing any adverse effect to the host [1] and are known to protect the host against biotic and abiotic stresses [2, 3]. Endophytes are known to secrete secondary metabolites with potential use for therapeutic purposes [4, 5]. Screening of these diverse fungi for their ability to produce important bioactive molecules is a promising approach to obtain rare drugs. Endophytic fungi are also known to produce complex bioactive molecules produced by the host and this was proved with the isolation of an endophytic fungus from *Taxus brevifolia* capable of producing taxol (anticancer compound) [6]. Since then, extensive research on isolation of endophytic fungi producing important bioactive compounds has been taken up by many research groups [7–9].

Taxus baccata L. subsp. *wallichiana* (Zucc.) Pilger (Himalayan Yew) is the only species of *Taxus* reported from the temperate Himalayas at an altitude of 1800–3300 m amsl [10]. It is a very slow growing, ever-green conifer that has been reported to grow in sub-tropical climates. Some of the endophytic fungi isolated from *T. baccata* growing in this region are reported to produce taxol [11]. Many *Fusarium* spp., are reported as endophytes and often isolated from different forest plants. These endophytic species are well known to produce bioactive secondary metabolites with varied activities such as antibacterial [11] and anticancer [12, 13]. In the present investigation, an endophytic fungus *F. tricinctum* was isolated from the bark of the Himalayan yew and assessed

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for its effect on the growth of human breast cancer cell line (MCF-7), human cervical cancer cell line (HeLa) and peripheral blood mononuclear cells as well as for its antioxidant activity.

Materials and Methods

Isolation of Endophytic Fungi

Endophytic fungi were isolated from the bark samples of *T. baccata* L. subsp. *wallichiana* (Zucc.) Pilger collected from Bhadrehah (district Doda, India) according to the method described earlier [11]. The plates were incubated at 25 ± 2 °C for 5–10 days and were regularly observed for the growth of endophytic fungi. Each fungal culture was checked for its purity and subcultured to fresh potato dextrose agar (PDA) (HiMedia Laboratories Ltd. Mumbai, India) medium by the hyphal tip method [6]. In the present study, one of the endophytic fungal isolates (strain T6) was selected for its antiproliferative and antioxidant activities based on preliminary investigations.

Identification of Endophytic Fungus Strain T6

The fungal strain T6 was characterized based on its morphological characteristics such as colony, spores and the reproductive structures [14]. Molecular characterization was performed by ITS sequence analysis. For this purpose, mycelia harvested from actively growing strain T6 were ground into a fine powder in liquid nitrogen. Genomic DNA was extracted by CTAB method [15]. Quality and quantity of DNA was checked with Nanodrop 1000 spectrophotometer (Thermo Scientific, USA) and samples were stored at -20 °C until use. The ITS region of nrDNA was amplified with the universal primers ITS1 and ITS4 [16] using Thermal Cycler (Applied Biosystems, California, USA). The PCR programme for amplification of the ITS region was used as described earlier [17]. The PCR amplified fragments were gel purified using Qiaquick columns (Qiagen) as per the manufacturer's instructions and sequenced. The ITS sequence of strain T6 was deposited in the GenBank under the accession numbers KT779291. This ITS sequence was compared to those available in the GenBank database using the BLASTn algorithm. Alignment of the sequences was constructed using MAFFT ver. 7.0 [18] and edited with BioEdit 5.0.6 [19]. The phylogenetic tree was reconstructed using maximum parsimony method and the Kimura two-parameter distance calculation by MEGA5 software [20]. The bootstrap was 1000 replications to assess the reliable level to the nodes of the tree.

Fungal Extract Preparation

The mycelial mats (5.0 mm diam) of 7–10 days were inoculated into potato dextrose broth (100 ml) and incubated at 25 ± 2 °C on a shaker for 5 days and resulting culture was used as the seed culture. Seed culture (15 ml; 3 % v/v) was transferred into 500 ml of potato dextrose broth and incubated at 25 ± 2 °C for 21 days in dark as a stationary culture. The culture was then filtered through four layers of cheesecloth to remove the mycelia. The harvested mycelia were dried overnight at 35–40 °C and extracted with ethyl acetate for 12 h. The culture broth was also extracted with three equal volumes of ethyl acetate; the fractions were collected and combined, and the solvent was then evaporated under reduced pressure at 35 °C. The residue was re-dissolved in dimethyl sulfoxide (DMSO) and used for the assay.

Cell Growth Inhibition Assay

Human breast cancer cell lines (MCF-7) and human cervical cancer cell lines (HeLa) were procured from National Centre for Cell Science (NCCS), Pune, India. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, USA), containing 10 % (v/v) foetal bovine serum (Gibco), 100 IU ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, and 2.5 µg ml⁻¹ amphotericin. Cells were maintained in a humidified incubator with 5 % CO₂ at 37 °C. The effect of fungal extract on the growth of cancer cell lines was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) assay [21]. Cells were trypsinised and seeded at a density of $\sim 2 \times 10^4$ cells per well in 96 well cell culture plate and incubated overnight. After 16 h, fungal extracts were added in varying final concentrations (50, 100, 150, 200, 250, 300 and 350 µg ml⁻¹) to the wells. After 72 h of incubation, 20 µl of MTT reagent (Sigma USA, 5 mg ml⁻¹) was added to each well and again incubated for 4 h. The formazan crystals formed were solubilized in 100 µl DMSO (Merck, Germany). Finally, the absorbance of each well was recorded at 570 nm, taking 630 nm as the reference wavelength, using the microplate reader (Tecan infinite, Austria). Paclitaxel was used as a positive control at the concentration of 20 µg ml⁻¹. Percentage of inhibition was calculated as:

$$\left(\frac{\text{Mean OD of untreated cell} - \text{Mean OD of treated cell}}{\text{Mean OD of untreated cell}} \right) \times 100.$$

Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood (5 ml) which was drawn from healthy volunteers with their consent prior to the study. Blood was carefully layered onto the ficoll (Sigma, USA) and centrifuged at $400\times g$ for 30 min at room temperature. After centrifugation, top plasma layer was carefully removed with a pipette and collected opaque interface containing PBMCs in a clean conical centrifuge tube. The cells were washed by adding PBS and centrifuged at $250\times g$ for 10 min.

Lymphocyte Proliferation Assay

PBMCs were seeded at a density of 1×10^5 cells per well and then fungal extracts were added in varying final concentrations. After 2 h, concanavalin A ($5 \mu\text{g ml}^{-1}$), a mitogen was added in order to stimulate the PBMCs. After 72 h of incubation, MTT assay was carried out to measure the cell proliferation. MTT assay and calculation of inhibition in mitogen-induced proliferation were done as described in the case of a cell growth inhibition assay.

TNF- α Measurement

In order to evaluate the production of TNF- α , MCF-7 and concanavalin A stimulated PBMCs were incubated with varying concentrations of fungal extract for 72 h. After incubation, supernatants were collected and the secretion of TNF- α was measured using ELISA kit (Preprotech, USA) as per manufacturer's instruction. Inhibition in TNF- α production was calculated in the same manner as mentioned in the cell growth inhibition assay.

Antioxidant Assay

In order to know the antioxidant potential of fungal extract, 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was performed. Fungal extract (50 μl) with varying concentration was mixed with 150 μl of DPPH (100 μM) in methanol, added in wells of a 96-well microtiter plate. Ascorbic acid ($100 \mu\text{g ml}^{-1}$; 50 μl) was used as positive control. The plate was incubated in dark for 45 min, after which the absorbance of the solution was measured at 517 nm in ELISA microtitre plate (Tecan infinite, Austria). Free radical scavenging activity was expressed as the inhibition percentage calculated using formula,

$$\text{Free radical scavenging activity} = \left(\frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \right) \times 100.$$

Statistical Analysis

All assays were carried out in triplicates. Data were analysed using analysis of variance (ANOVA) and the means were compared by using Tukey's test at $p < 0.05$.

Results

Isolation and Identification of Endophytic Fungus Strain T6

A total of 25 endophytic fungi were isolated from the bark of *T. baccata*. One of the fungal isolates (strain T6) was selected for this investigation. The strain T6 grew well on PDA media at 28 °C in 7 days. The morphological traits of strain T6 were: pink coloured centre surrounded by white coloured margins, cottony appearance, nearly round margins and broken edges. Microscopic observations indicated the presence of tubular, thick walled, septate hyphae, simple or branched and sickle-shaped macroconidia and oval microconidia. Microconidia are sparse, one-celled, smooth, ovoid, non-septate, present solitary and measure 1.6–2.6 μm in length and 1–1.5 μm in width. The apical cells were elongated, blunt to conical, and basal cells were blunt to non-notched. Based on the morphology of the fungal mycelia and characteristics of the spores, the endophytic fungus T6 seemed to be *Fusarium* sp. ITS sequence analysis of T6 showed 100 % similarity with *Fusarium tricinctum* strain A1 (JF273496). Phylogenetic analysis also clustered T6 with *Fusarium tricinctum* species (Supplementary Fig. 1).

Cell Growth Inhibition Against Cancer Cell Lines

Inhibition in cell growth was observed in both human breast cancer cell line (MCF-7) and human cervical cancer cell line (HeLa). The cytotoxic effect significantly increased with increase in the concentration of the fungal extract (Fig. 1). The IC_{50} value for extract was found to be 225 ± 26 and $220 \pm 18 \mu\text{g ml}^{-1}$ for MCF-7 and HeLa cell lines, respectively. Paclitaxel, ($20 \mu\text{g ml}^{-1}$) an anti-cancer drug was used as a positive control which showed $93 \pm 5 \%$ inhibition in this experiment. The inhibition level of the crude fungal extract was comparable with paclitaxel ($20 \mu\text{g ml}^{-1}$) when the concentration of the extract was above $250 \mu\text{g ml}^{-1}$ (Fig. 1).

Anti-proliferative Activity Against Peripheral Blood Mononuclear Cells

Fusarium tricinctum extract was also looked for their effect on the proliferation of mitogenic (concanavalin A) stimu-

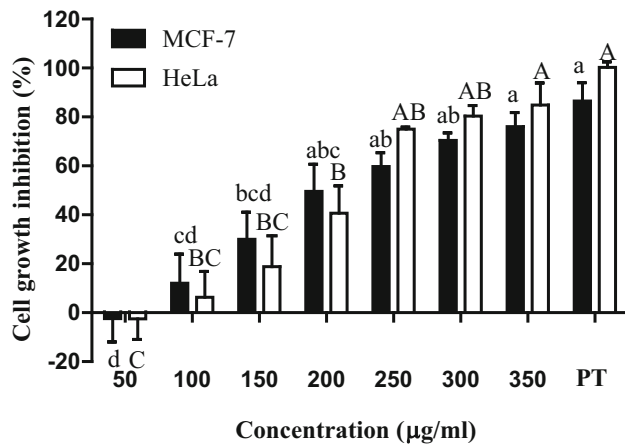


Fig. 1 Effect of *Fusarium tricinctum* extract on the growth of human breast cancer (MCF-7) and human cervical (HeLa) cancer cell lines. Means followed by the same letter are not significant at $p < 0.05$. PT represents paclitaxel ($20 \mu\text{g ml}^{-1}$) as a positive control

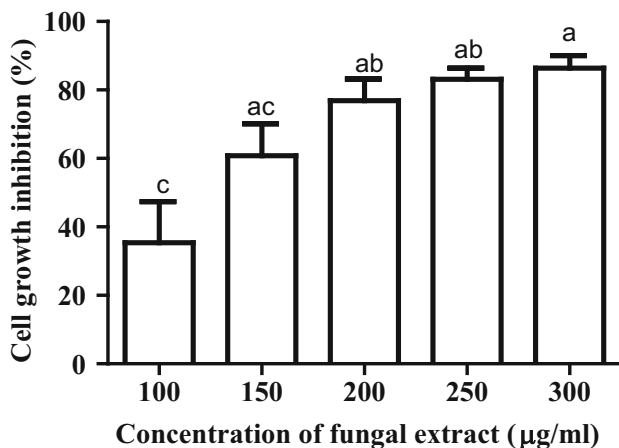


Fig. 2 Immunosuppressive effect of *Fusarium tricinctum* extract on concanavalin A stimulated peripheral blood mononuclear cells (PBMCs). Means followed by the same letter are not significant at $p < 0.05$

lated human PBMCs. PBMCs have not shown proliferating response against the mitogen concanavalin A in the presence of fungal extracts indicating its immunosuppressive activity. With the increase in the concentration of the fungal extract, the immunosuppressive effect became significantly more pronounced in PBMCs (Fig. 2). The IC_{50} value for extract was found to be $110 \pm 44 \mu\text{g ml}^{-1}$.

TNF- α Production

TNF- α is an inflammatory cytokine which plays an important role in cancer progression and metastases. Hence, effect of the fungal extract on TNF- α production in MCF-7 was estimated using ELISA. Interestingly,

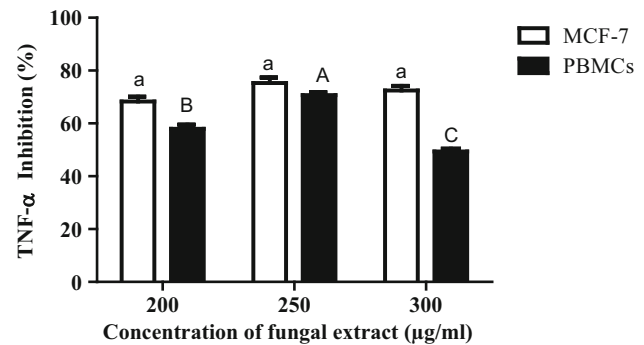


Fig. 3 Effect of *Fusarium tricinctum* extract on TNF- α production in MCF-7 and concanavalin A stimulated peripheral blood mononuclear cells (PBMCs). Means followed by the same letter are not significant at $p < 0.05$

inhibition in TNF- α production was observed in MCF-7 cells treated with fungal extract which show that cytotoxic effect may be mediated by inhibition of this cytokine production (Fig. 3). Similarly, TNF- α production was found to be inhibited in concanavalin A stimulated PBMCs. Inhibition in cytokines production against MCF-7 and concanavalin A stimulated PBMCs was observed in different concentrations of extract tested in this study, but the inhibition effect appears independent of concentrations.

Antioxidant Activity

Free radical scavenging activity was performed to evaluate the antioxidant activity of *F. tricinctum*. It was observed that the scavenging activity significantly increased with increase in the concentration of fungal extract (Fig. 4). The IC_{50} value of scavenging activity of extract was found to be $482 \pm 9 \mu\text{g ml}^{-1}$. Ascorbic acid was used as positive control and where it shows $80 \pm 2 \%$ antioxidant activity. The scavenging activity of the extract was comparable with ascorbic acid ($100 \mu\text{g ml}^{-1}$) when the concentration of the extract was 1 mg ml^{-1} .

Discussion

Endophytic fungi have been studied as a source of anti-cancer agents ever since the million dollar drug Taxol was isolated from the endophytic fungus *Taxomyces andreanae* [22]. Two human cancer cell lines were used in this study for the investigation of the antiproliferative effect of *F. tricinctum* extracts and the results clearly showed cell growth inhibition (cytotoxicity) against both cancer cell lines. Similar results were reported from *F. oxysporum* which showed cytotoxicity in different human cancer cell lines [23]. Puri et al. [9] reported the cytotoxic activity of

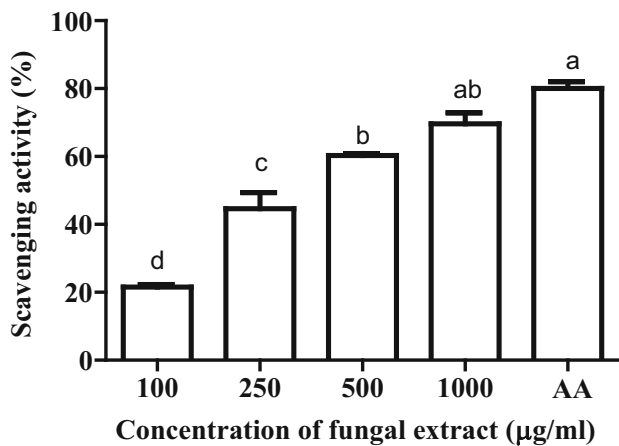


Fig. 4 Antioxidant effect of *Fusarium tricinctum* extracts based on free radical scavenging activity. Means followed by the same letter are not significant at $p < 0.05$. AA represents ascorbic acid ($100 \mu\text{g ml}^{-1}$) as positive control

endophytic fungus *Entrophospora infrequens* inhabiting *Nothapodytes foetida* against different human cancer cell lines and found activities comparable to the camptothecin.

It has been reported that several anticancer drugs have an impact on immune system and possess immunosuppressive properties [24]. Immunosuppressive compound such as subglutinol-A and subglutinol-B were isolated from the fungal endophyte *F. subglutinans* inhabiting *Tripterygium wilfordii* [25]. The present study revealed that PBMCs did not show proliferating response against the mitogen concanavalin A after addition of fungal extract indicating immunosuppressive effect. These findings were inline with the reported anti-lymphocyte proliferative activities against phytohemagglutinin (PHA) stimulated PBMCs by endophytic fungi from *Tripterygium wilfordii* [26]. The immunosuppressive effect was further supported by the fact that there is inhibition in the production of inflammatory cytokines TNF- α in stimulated PBMCs culture. TNF- α , a cytokine which mediates the inflammatory response and is also known to influence cancer growth [27]. The present study revealed that *F. tricinctum* extract inhibits the TNF- α production in breast cancer cell lines (MCF-7), which indicate that cytotoxic effect may be mediated by TNF- α inhibition.

Antioxidants protect the cells from the damages caused by free radicals. Free radical mediated reactions are associated with various diseases which include alzheimer's disease, diabetes, cardiovascular disorder and cancer [28]. In the present study, the extract of *F. tricinctum* showed antioxidant activity which increased with extract concentration indicating the possibility of exploring this fungus as a source of antioxidant agent. Different fungal compounds with antioxidant activity were isolated from various

endophytic fungi from different sources [29, 30] including phenolics showing potent antioxidant activity [31].

In conclusion, the extracts of *F. tricinctum* exhibited strong cell growth inhibition and antioxidant properties. Further studies focussing on purification and characterization of bioactive compound responsible for these activities may help in isolating some new compounds of pharmaceutical importance.

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