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Biological evaluation and preliminary screening of endophytes of *Taxus fauna* for taxol production

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Abstract

Endophytic fungi of *Taxus* species, being considered as economically most promising source for taxol production, prompted the current study for screening of endophytic fungi associated with *Taxus fuana* of Pakistan. Two endophytic fungi *Epicoccum sp.*NFW1 and *Mucorhemalis*NFW6 were explored for their biological activities and taxol production by utilizing bioassays, TLC, RP-HPLC and LC-ESI-MS analyses. Crude extract of NFW1 showed significant antimicrobial activity with zone of inhibition ranging from 11.8 - 23.1 mm against different test strains. It also expressed cytotoxicity in brine shrimp lethality assay with an IC_{50} value 10µg/mL. NFW6 expressed moderate antimicrobial activity and high antioxidant potential (89.2%) with IC_{50} 51.2 µg/mL in DPPH assay. RP-HPLC confirmed the presence of taxol in NFW1 at a concentration 280.7 µg/g of dry mycelial mass. It was further validated by LC-ESI-MS analysis. These findings reveal that *Epicoccum* sp.NFW1 should be studied for the isolation and characterization of taxol and related metabolites.

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Introduction

Taxol (Paclitaxel), a diterpenoid compound, is the world's first billion dollar drug approved for the treatment of cancer and other human tissue proliferating diseases. It can curb angiogenesis by enhancing the assembly of microtubules and inhibiting their depolymerisation (Wani *et al.,* 1971). It was originally obtained from the bark of Taxus (yew) plant. Its concentration in the bark is very low and isolating it from trees posed environmental threats (Xiong *et al.,* 2013; Roberts, 2007). This prompted the search for alternative sources for taxol production (Mukherjee *et al.,* 2002; Soliman and Raizada, 2013). One such approach is to exploit the symbiotic micro flora such as endophytes of these valuable plants.

An endophyte is a microorganism, which spends the whole or part of its life cycle in the healthy tissues of the host plant (Tan and Zou, 2001). Endophytes are emerging source of novel biochemical entities (Kumar *et al.,* 2013). Their role in drug discovery is tremendously heightened since the isolation of taxol from *Taxomycesandreanae*, a fungal endophyte of *Taxus brevifolia*(Stierle *et al.,* 2013; Backman and Sikora, 2008). Since then many scientists reported isolation and identification of taxol and various bioactive compounds from endophytic organisms (Strobel *et al.,* 2004; Khan *et al.,* 2010). These compounds could be employed as antibiotics, antioxidants, anticancer and immuno-suppressants (Pimentel *et al.,* 2011).

Endophytic fungi are prioritized for taxol production. Typical, taxol concentration is 0.01-0.02 % of the dry weight of bark, while in the initial studies its yield from endophytic fungi varied from 24ng/L to 70 ng/L(Sterlie*et al.,*1993). Gangadevi and Muthumary (2008) reported concentrations higher (163.4 µg/L) as compared to previous studies. The appreciable taxol yield along with short fermentation time and high growth rate of fungi makes it economical to continue the investigation on endophytes for production of such rare compounds (Gangadevi*et al.,* 2008; Kumaran*et al.,* 2010).

In present study two endophytic fungi (NFW1 and NFW6) isolated from *Taxus fauna* Nan Li & R.R. Mill (Shah *et al.,* 2008) of Himalayan region of Pakistan were investigated for their biological potential. Crude organic extract of NFW1 and NFW6 was screened in different bioassays. Potential of these isolates for taxol production was evaluated by thin layer chromatography and high performance liquid chromatography (HPLC) and further validated by liquid chromatography mass spectrometry (LC-ESI-MS).

Materials and methods

Sample Collection

Two endophytic fungi NFW1 and NFW6 were obtained from Microbiology Research Lab (MRL) Department of Microbiology, Quaid-i-Azam University, Islamabad. The strains were identified at molecular level as *Epicoccum* sp. NFW1 (Genbank accession no JX402049.1) and *Mucorhemalis* NFW6 (GenBank accession no JX845511.1) (unpublished data). Isolates were maintained on potato dextrose agar (PDA, Oxoid) for further studies.

Fermentation and extraction of metabolites

Fungal cultures were grown in 1L Erlenmeyer flasks containing 400 mL modified taxol media TM (Xu *et al.,* 2006). Flasks were run on a shaker incubator at 25 °C and 150 rpm for 21 days.

The composition of the TM medium (g/L distilled H_2O) was:sucrose, 40; phenylalanine, 0.01; peptone, 0.5; yeast extract, 0.8; $(NH_4)_2SO_4$,3.0; $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 2.0;NaCl, 0.6; sodium acetate, 0.5; and sodium benzoate, 0.1.

Extraction of the secondary metabolites was carried out as reported by Xu *et al.,* (2006) after slight modifications. Fungal culture was blended three times with ethyl acetate. Organic layers were collected and reduced to dryness under vacuum to obtain crude ethyl acetate extract labelled NFW1E (0.140g) and NFW6E (0.150g).

Bioassays

Antibacterial assay

Antibacterial assay was performed by using the disc diffusion method against *Bascillussubtilis*(ATCC 6633), *Salmonella Setubal* (ATCC 19196)and *Pseudomonaspicketii* (ATCC 49129) (Haq *et al.,* 2012). Cultures were obtained from MRL, Quaid-i-Azam University, Islamabad. Suspensions of test organisms were prepared in sterile physiological saline (0.9% NaCl) in accordance with Mac-Farland's turbidity standard [0.5x10^6colony forming unit (CFU) per mL]. Bacterial lawn was prepared by using sterilized cotton swabs. Pre-sterilized discs of 8 mm in diameter were loaded with crude ethyl acetate extract dissolved in dimethylsulfoxide (DMSO) at conc. of 8 mg/mL (each disc contained 200 µg crude extract).The loaded discs were placed on the bacterial lawn and sample was allowed to diffuse for 5 minutes. All plates were incubated at 37 °C for 24 hrs. Antibacterial activity was determined as diameter of zone of inhibition in millimetre (mm). DMSO served as negative and cefotaxime(100 µg/disc) as positive control. Assay was performed in triplicate.

Antifungal assay

Antifungal assay was performed by agar well diffusion assay as reported by Kanan and Al- Najar (2008) against *Fusariumsolani, Mucor sp.* and*Alternaria alternate*. Test cultures were obtained from First Fungal Culture Bank of Pakistan, University of the Punjab, Lahore. An aliquot of 100 µL spore suspension (1x10^8spores/mL) of each test isolate was spread evenly on the surface of PDA plates by using sterile glass rod. Wells were made at appropriate distance using sterilized borer of 8 mm diameter. 100 µL of crude ethyl acetate extract dissolved in DMSO,wasdispensed carefully in the respective wells (each well contained 0.4 mg crude extract). Fluconazole (100 µg/well) was used as positive control and solvent DMSO as negative control. Plates were incubated at 28 °C± 1°C and antifungal activity was expressed as diameter of zone of inhibition, measured after 72 hr. The assay was performed in triplicate.

Brine shrimp assay for cytotoxic activity

Brine shrimp cytotoxicity assay was performed using Brine shrimp (*Artemiasalina*) larvae (Haq*et al.,* 2012). Larvae were hatched in artificial seawater at 37°C.The crude extracts (20 mg) were dissolved in 2 mL of methanol to make stock solution. From this stock solution 5, 50 and 500 µL was poured separately in; 20 mL vials (3vials/concentration) to attain final concentration at 10, 100, 1000 µg/mL respectively. The vials were kept open over night with continuous air flow to evaporate the solvent. Then 3mL of artificial sea water was poured in each vial along with 10 mature brine shrimp larvae using pasture pipette. Finally volume of sea water in each vial was increased up to marked level of 5mL. The vials were kept under illumination for 24 hr, and survived nauplii were counted macroscopically. IC_{50} value of the extracts was calculated by probit analysis using finny software.

DPPH assay for antioxidant activity

The free radical scavenging assay was performed using 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) as described by Haq *et al.,* (2012). Test samples were dissolved in 100% DMSO. To the 96-well microtiter plate, 95 µl DPPH solution (316 µM in methanol) and 5 µL of test solution was added. The plate was incubated at 37°C for 1 hr after thorough mixing. Absorbance was measured at 515 nm using micro plate reader (DAD Agilent 8453). The highest final concentration of the test sample was 200µg/mL. The samples which showed more than 70% scavenging activity were further processed to determine IC_{50}. Ascorbic acid (IC_{50} = 35.6 µM) and pure DMSO were used as positive and negative control respectively. Assay was performed in triplicate. The following formula was used to calculate percentage scavenging activity and IC_{50} value was calculated by table curve software.

Scavenging effect (%) = [1-As/Ac] x100

Where "Ac" means Absorbance of control and "As" means Absorbance of test sample.

Chromatographic identification of Taxol

Thin layer chromatography

Comparative thin layer chromatographic analysis was carried out on 0.25mm (20cm x 20cm) aluminium coated silica gel plates (Merck, Germany) and developed in solvent system A chloroform/methanol (7:1, v/v) with authentic taxol (Sigma) as control. Compounds were detected by their UV absorbance at 254nm and 366nm. Presence of taxol was detected by staining with 10% phosphomolybidic acid and Dragendorff's reagent and gentle heating. The area of the plate containing putative taxol was marked and scrapped off carefully. Sample was purified using another TLC in solvent system B (chloroform/acetonitrile 7:3 v/v).

Reverse phase high performance liquid chromatography (RP-HPLC) and Liquid chromatography mass spectrometry (LCMS)

The presence of taxol in the fungal samples was further confirmed by performing HPLC (Agilent 1200) on a C18 column (250×4.6 mm 5µm particle size, supelco, USA),coupled with diode array detector. Crude extracts were dissolved in methanol (1mg/mL) and filtered through 0.2µM filter paper. A binary gradient system consisting of A (10%methanol in water) and B (100% methanol) was used. Program started with 90% A and 10% B at 0 min till it linearly increased to 100% B in 30min. 20µL of the sample was injected at flow rate of 1.0min/mL and detected at 228 nm (Pandi*et al.,* 2011). Taxol was quantified by comparing the peak area of the sample and the standard taxol and calculated as follow:

$$\text{Conc. of taxol} = \frac{\text{Total sample area x dilution of standard x purity of standard x 100}}{\text{Total standard area x dilution of sample}}$$

Total standard area x dilution of sample

Liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) was performed on the crude samples in positive ionization mode using an LC system (Agilent eclipse HP 1100) coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass). Samples were dissolved in methanol (1mg/mL) and injected into reverse phase C-18 column at flow rate of 20µl/min. A binary gradient solvent system A (0.1% formic acid in water) and B (0.1%vmn formic acid in acetonitrile) was used for elution and data analysed in Mass-Lnyx 4.0 software.

Results and discussion

Antibacterial and Antifungal activities of the crude extracts

Crude extract of NFW1 and NFW6 was active against test bacterial and fungal strains as shown in Table 1.

Table 1. Antbacterial and antifungal activity of the crude extracts of endophytic fungi of *Taxus fauna*.

Samples/Controls	Antibacterial assay, Zone of inhibition (mm)		
	B. subtilis	*S. Setubal*	*P. pickettii*
NFW1	23.1 ± 1.4	22.7 ± 1.1	11.8 ± 0.8
NFW6	19.6 ± 1.2	10.2 ± 0.1	9.0 ± 0.3
Ceftx.	20.1 ± 1.2	16.2±0.2	15.5 ± 1.1
DMSO	-	-	-
Samples/Controls	Antifungal assay, Zone of inhibition (mm)		
	Mucor sp.	*F. solani*	*A. alternate*
NFW1	10.2 ± 1.1	9.6 ± 0.2	-
NFW6	11.0 ± 1.2	10.5 ± 0.1	-
Flu.	17.1± 2.1	±	16.2±1.2
DMSO	-	-	-

Data shows the mean of three independent replicates, ± = Standard deviation, - = No activity, Flu= fluconazole, Ceftx= Ceftaxime,

Both isolates showed significant antibacterial activity. NFW1 was most active against *B. subtilis*and formed zone of inhibition 23.1mm. This is because genus *Epicoccum* produces chemically diverse classes of bioactive metabolites like epicoccin, epicorazines and epicoccamide (Fávaro *et al.,* 2012; Musetti *et al.,* 2007; Wright *et al.,* 2003). NFW6 was most active against*B. subtilis* and formed zone of inhibition

19.6mm. Zhang *et al.,* (2012) reported antimicrobial activities of *Mucor sp. SPS-11* against*E. coli, S. aureus* and *R. cerealis, Mucor* and *Penicillium*.NFW1 and NFW6 expressed moderate antifungal activities against *Mucor sp.* and*F. solani* as shown in Table 1. None of the samples inhibited growth of *A. alternata*.These findings are correlated with previous reports by different research groups (Qadri *et al.,* 2014; Bhimba *et al.,* 2012). Almost 19 genera of Taxus plant associated endophytic fungi have been reported for taxol production and antifungal activity (Tayung and Jha, 2010).

Table 2. Brine shrimp lethality (cytotoxicity) and DPPH free radical scavenging activity of crude extract.

Endophytic strain	Cytotoxicity Assay					DPPH Assay	
	% Survival				IC_{50} (µg/mL)	% scavenging at 200 µg/mL	IC_{50} (µg/mL)
	1000 µg/mL	100 µg/mL	10 µg/mL	1 µg/mL			
NFW1	0.0	10	50	100	10.00	5.00	-
NFW6	98.00	100	100	100	-	89.2	51.2

Brine shrimp assay for cytotoxic activity

Crude extract of NFW1 displayed promising lethality with 0% survival of larvae at 1000 µg/mL. IC_{50} value was found to be 10 µg/mL. NFW6 did not exhibit cytotoxicity at all concentrations tested (Table 2).Taxol was used as positive control with IC_{50}value 1.2 µg/mL. Endophytes may attain cytotoxicity from their host plant (Hazalin *et al.,* 2009).

DPPH assay for antioxidant activity

In DPPH assay, only NFW6 showed significant antioxidant activity. Crude extract exhibited free radical scavenging potential of 89.2% and IC_{50} value 51.2 µg/mL (Table 2). Many reports describe antioxidant potential of endophytic fungi (Liu *et al.,* 2007).

TLC analysis

Fungal endophytes were screened for taxol production by chromatographic analysis. On TLC plates, crude extract showed properties identical with that of Taxol (Fig. 1a). Spot pattern and R*f* values of the sample when visualized under UV 254nm showed that they may contain paclitaxel or its analogues. Samples gave color identical with taxol when sprayed with 10% phosphomolybidic acid however; showed varying stain with Dragendorff's reagent (Fig. 1b, 1c). It was observed that there are more relevant compounds in NFW1 sample as compared to NFW6.Pandi and coworkers studied that taxol showed UV illumination at 254 nm while appeared as blue-graycolor when stained with vanillin/sulfuric acid reagent (Pandi *et al.,* 2011). Some studies report Dragendorff's negative taxol derivatives which support our results (Cardellina, 1991; Pandi *et al.,* 2011).

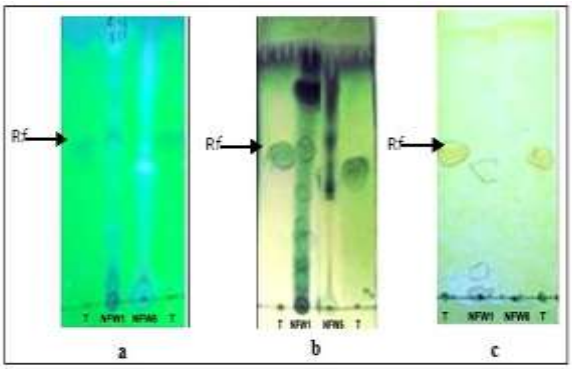

Fig. 1. TLC plates showing the detection of different compounds in crude extracts of NFW1 and NFW6 as compared to standard taxol (T) (a) Short-range UV light, (b) 10% phosphomolibdic acid reagent (sprayed and heated), (c) Dragendorff's reagent.

RP-HPLC analysis

The qualitative and quantitative screening for the presence of taxol and related moieties in crude extract was done by using RP-HPLC. The standard taxol was eluted after 26 minutes at set UV range. When injected into HPLC, crude extract obtained from NFW1 showed overlapping peak distribution at similar retention time, indicating the presence of taxol and related moieties (Fig. 2a, 2b). The final concentration of taxol in the mycelia extract of NFW1 was calculated using standard curve and found to be

280.7 µg/g of dry mass. Previous studies report 60 µg/L to 163.4 µg/L of Taxol production from endophytic fungi (Gangdevi *et al.,* 2008; Strobel *et al.,*1996).

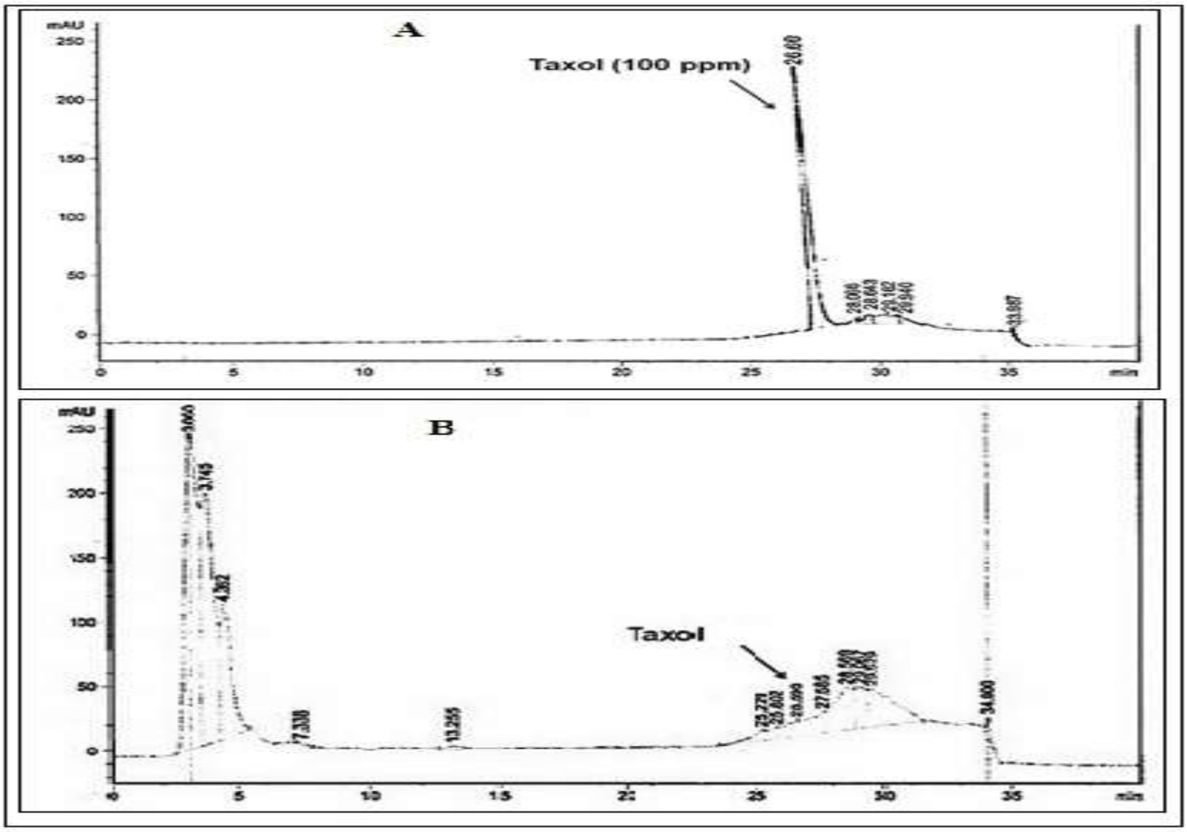

Fig. 2. HPLC analysis of the (A) authentic taxol (retention time 26.6±.0.1) and crude extract of NFW1 (B) indicating overlapping peaks (retention time 26.5±0.1min – 28.9±0.1).

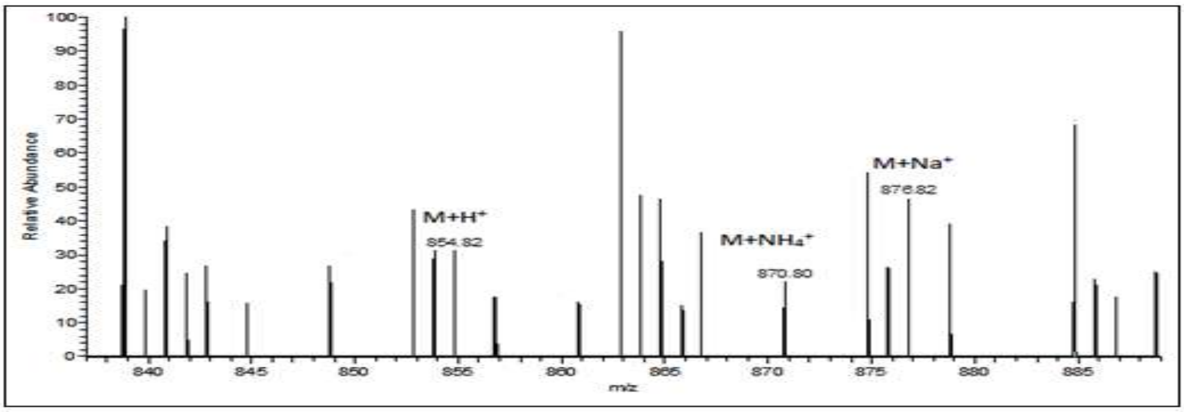

Fig. 3. Extracted LC-ESI-MS spectrum of NFW1 showing characteristic taxol peaks at molecular ions m/z 854.82 $(M+H)^+$, 870.80 $(M+NH_4)^+$ and 876.82 $(M+Na)^+$

LC-ESI-MS analysis

LC-ESI-MS analysis provided another convincing evidence for the presence of taxol in our sample. Authentic taxol yields peaks at 854nm $(M+H)^+$, 870nm $(M+NH_4)^+$ and 876nm $(M+Na)^+$ (Strobel *et al.,* 1996; Wang *et al.,* 2000). Similar peaks were observed in crude extract of NFW1 (Fig. 3). NFW6 isolate did not show the presence of relevant peaks in HPLC or MS analysis. Our findings show that *Epicoccum* sp. NFW1 should be prioritized for the isolation and biochemical characterization of taxol and related secondary metabolites.

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