INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 8, No. 2, p. 82-94, 2016

RESEARCH PAPER OPEN ACCESS

Extracellular enzymatic activity, phytochemical analysisand *in vitro* biological assessment of endophytic fungi isolated from *Taxus fauna*

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Key words: *Taxus fauna, Plectaniamilleri, Trichoderma asperellum*T77, Endophytic fungi, Extracellular enzymes.

http://dx.doi.org/10.12692/ijb/8.2.82-94 Article published on February 20, 2016

Abstract

The present study was aimed to investigate biological activities and phytochemical profiling of endophytic fungi isolated from *Taxus fauna*. A total of two endophytic strains*Plectaniamilleri*(NFL1) and*Trichoderma asperellum* T77 (NFL2) were selected and 8 samples were prepared by solid state fermentation on four different media (PDA, SDA, TM, Rice). Phytochemical profiling was conducted by analyzing total phenolic and total flavonoid contents colorimetricallyand the samples were biologically evaluated by employing antimicrobial, antileishmanialand several multimode antioxidant assays. The maximum phenolicand flavonoid contents was observed in NFL1-PDA (3.12 ± 0.10 µg GAE/mgE)(1.40±0.05 µg QE/mgE) and NFL2-PDA (3.403 ± 0.10 µg GAE/mgE) (2.6 ± 0.10 µg QE/mgE) extracts respectively. Maximum antioxidant and reducing power potential was displayed by NFL1-PDA, NFL2-PDA (25.4±0.58, 27.94±0.50 µg AAE/mg E) and NFL1-TM, NFL2-SDA (15.4 ± 0.35, 17.41 ± 0.30 µg AAE/mg E) respectively.Highest DPPH radical scavenging activity was displayed by NFL1-PDA (79.2±2.4) and NFL2-PDA (77.5±18) extracts. Significant antibacterial activity was also observed by NFL1-PDA and NFL2-PDA against all the tested bacterial strains. Remarkable antileishmanial activity was also manifested by NFL1-PDA extract (IC50 1.5 µg/mL).*P milleri* (NFL1) was found positive for the production of amylase, pectinase laccase and lipase while *T. asperellum*(NFL2) tested positive for laccase and amylase.The results of presentstudy show that endophytic fungi associated with *Taxus fauna*are potential source of antimicrobial and antioxidant agents and could be exploited for the production of industrially important enzymes.

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Introduction

Microorganisms have alwaysplayed their role in serving mankind by producing infinite number of enzymes andpharmaceutically important bioactive secondary metabolites(Craggand Newman, 2013; Tan *et al.,* 2001; Strobel *et al.,* 2004; Gunatilaka*et al.,* 2006). Microbial habitats are highly diverse and range from amazing deep oceans to hot thermal vents. One of such uniquebiological niche is the intracellular space between cells of higher plants that supports the growth of microorganisms known as endophytes(Strobel *et al.,* 2004). Endophytes are ubiquitous microorganisms present in virtually all plants on earth from the arctic to the tropics (Strobel and Daisy 2003).Plants area reservoir ofcountlessendophytes (Stone *et al.,* 2000) includinga variety of bacteria, fungi and actinomycetes. The most frequently encountered group of endophytes is fungal endophytes and the number of secondary metabolites produced by them is incomparable to any of their counterpart (Arnold and Engelbrecht2007; Zang*et al.,* 2006).

Fungal endophyteshave the ability to produce pharmacologically important natural productse.g. anticancer paclitaxol produced by an endophyte *Taxomycesandreanae* (Stierle*et al.,* 1993), antileishmanial metabolitesPalmarumycin CP17 and Palmarumycin CP18 by *Edenia* sp. (Martìnez-Luis *et al.,* 2008), antibacterial compound e.gnodulisporins by*Nodulisporium* (Dai *et al.,* 2009), novel wide-spectrum antibiotics, kakadumycins, isolated from *Streptomycetes*(Castillo *et al.,* 2003), and antifungal agent ambuic acid from *Pestalotiopsismicrospora* (Li*et al.,* 2001).

These metabolites belong to diverse chemical classes such as alkaloids, terpenoids, flavonoids, phenols, phenolic acids and steroids (Debbab*et al.,* 2011).Given the large no of interesting chemical scaffolds being discovered, the potential of these unique organisms is still largely untapped (Puri*et al.,* 2006)and needs to be investigated for developingtherapeutic agents and agrochemicals which are highly effective, less toxic and have minor environmental impact (Tenguria*et al.,* 2011).

Endophytes harboring *Taxus* plant around the world have been reported to produce several bioactive metabolites. Northern areas of Pakistan have rich biodiversity of medicinal plants including *Taxus* species and few studies have been done on fungal endophytes colonizing *Taxus* plant from this region.So, this study focuses on the phytochemical analysis along with the evaluation of biological activities of selected endophytic fungiisolated from *Taxus fauna* of Himalayan region of Pakistan. As the biosynthesis of secondary metabolites is regulated by nutritional parameters and manipulations of these have shown significantinfluence on the diversity of secondary metabolites so, different solid state mediaare used in this study to investigate the diverse metabolites produced by these fungi (Shang *et al.,* 2012; Kossuga*et al.,* 2012).This research may also acts as a foundation for isolation ofuseful and new natural products.

Materials and methods

A total of 2 strains of endophytic fungi were obtained from Microbiology Research Lab(MRL) of Quaid-i-Azam University, Islamabad.The strains were previously isolated from leaves of *Taxus fauna* and identified at the molecular level as *Plectaniamilleri*(NFL1) [GenBank: KC812764.1], *Trichoderma asperellum*T77 (NFL2) [GenBank: JX838791.1](Fatima, 2013).The strains were maintained on PDA slants at 4℃.

Fermentation and Extraction of Secondary Metabolites

Solid state fermentation of the endophytic fungi was carried out on four media PDA (Oxoid), Sabouraud Dextrose Agar(SDA, Oxoid), Rice and Modified Taxol media (TM). The composition of the TM medium (g/L distilled H_2O) was: sucrose-40g, peptone-0.5g, yeast extract-0.8g, $(NH_4)_2SO_4$-3.0g, $MgSO_4.7H_2O$- 0.5g, KH_2PO_4- 2.0g, NaCl-0.6g, phenylalanine-0.01g, sodium acetate-0.5g and sodium benzoate-0.1g. For preparation of Rice medium, 20g of rice were soaked overnight in flasks containing 30ml of distilled water.

After autoclaving,mycelial agar plug (0.5×0.5 cm^2) from fresh plate of fungal isolates was transferred in the center of plates and flasks and incubated at 25 °C for three weeks.After the incubation period, all the fungal cultures were extracted with ethyl acetate (HPLC grade, SIGMA-Aldrich). Fungal mycelial mass, along with the agar, was sectioned with sterile scalpel and extracted three times with ethyl acetate, followed by overnight incubation under shaking conditions. The organic layers were pooled and concentrated to dryness under vacuum. Similarly, rice cultures were soaked in ethyl acetate overnight prior to pooling and concentration of the organic layers (Smedsgaard1997).Then the crude extract obtained was weighed and used for initial biological screening.

Phytochemical Screening

Determination of Total Phenolic Contents (TPC)

Folin-Ciocalteau (FC) method was used to determine the total phenolic content by using standard protocol described by Clarke *et al.*(2013). Briefly, stock solutions (4 mg/mL) of the extracts were prepared in DMSO and an aliquot of 20 µl was transferred to each well of 96 well plate followed by addition of 90 µL of Folin–Ciocalteu reagent.After 5 min, 90 µl of Na_2CO_3 (7.5% w/v in H_2O) was added and incubated for 1 hr. Absorbance of the samples was recorded at 650 nm (Bioteck, USA). DMSO and gallic acid were used as blank and control, respectively. A calibration curve (y = 0.0135x + 0.0846, R^2 = 0.986) was obtained in parallel under the same operating conditions using gallic acid as standard (6.25-50 µg/mL). The amount of total phenolics was determined as µg gallic acid equivalent per mg extract (µg GAE/mg E).

Determination of Total Flavonoid Content (TFC)

Total flavonoid content of the crude ethyl acetate extracts was determined by ammonium chloride method (Haq*et al.,* 2012). Crude ethyl acetate extracts (20 ul) were mixed with the solution of aluminum chloride (10% w/v) and 10 µL of potassium acetate (1 M) solutions. It was followed by addition of distill water to attain volume of 200ul. The entire mixture was incubated at room temperature for 30 min.After the incubation period, absorbance was measured by using microplate reader (Bioteck, USA Microplate reader ELX 800). The calibration curve (y = 0.0269x + 0.00765, R^2 = 0.998) was drawn by using quercetin as standard at 2.5 to 40 µg/mL and the flavonoid content were established in µg quercetin equivalent per mg extract (µg QE/mg E).

TLC Screening

Thin layer chromatography was performed using coated silica gel plates0.25mm (20cm x 20cm) (Merck, Germany) anddeveloped in solvent system Hexane: Chloroform (1:1) (HPLC grade, SIGMA-Aldrich) in case of NFL1, while in pure Chloroform in NFL2. TLC chromatograms were visualized under UV light(254nm and 366nm).

Biological Screening

Determination of Antioxidant Activity

a) Total antioxidant capacity (TAC)

Total antioxidant activity of extracts was evaluated by phosphomolybdenum complex formationmethod described by Baydar*et al.* (2007) with slight modification. Samples of crude extract (100µl each at concentration of 4 mg/mL) were mixed with 900 µL of reagent solutions(0.6 M sulfuric acid, 4 mM ammonium molybdate and 28 mM sodium phosphate). The reaction mixtures were incubated in water bath at 95°C for 90 minutes. Samples were cooled at room temperature and absorbance was measured at 695 nm by using microplate reader. DMSO was used as negative control. For calibration curve, ascorbic acid was used as positive control at 6.25-100 µg/ml. The resultant TAC was expressed as µg ascorbic acid equivalent per mg extract (µg AAE/mg E).

b) Reducing power assay

The reduction potential of the extracts was investigated in accordance with the method described previously byClarke *et al.* (2013). Stock solutions of 4mg/ml was prepared and 100 µL of each sample was mixed with 200 µL of phosphate buffer (0.2 M, pH 6.6) and 250 µL of 1% w/v potassium ferricyanide. The mixture was incubated at 50°C for 30 min. It was followed by addition of 200 µL of 10% w/v

trichloroacetic acid to acidify the reaction. The resultant mixtures were then centrifuged at 3000 rpm for 10 min. Supernatant (150 µL) was mixed with 50 µL of 0.1% w/v ferric chloride solution and optical density was measured at 700 nm. Ascorbic acid was used as positive control and results were expressed as µg ascorbic acid equivalent per mg extract (µg AAE/mg E). Assay was performed as triplicate analysis.

c) DPPH free radical scavenging assay

Free radical scavenging activity of the crude extracts was determined by using 2,2-diphenyl-1-picryl hydrazyl reagent as described by Clarke *et al.* (2013). Briefly, 190 µL of DPPH (9.2mg/100ml methanol) was mixed with 10 µL of test extract (4 mg/mL stock solution). Test samples were evaluated at final concentration of 200 µg/mL.The reaction mixture was incubated in dark for 1 hr and the optical density was measured at 515 nm using microplate reader. Lower absorbance of reaction mixture indicated higher free radical scavenging activity.Ascorbic acid was maintained as positive standard while DMSO served as negative control.Free radical scavenging activity was expressed as percentage and measured using the following formula.

$$\% \text{ Scavenging Activity} = \frac{\text{Absorbance of control} - \text{Absorbance of the sample}}{\text{Absorbance of control}} \times 100$$

Antimicrobial Screening

AntibacterialActivity

The antibacterial activity of extracts was evaluated by agar well diffusion method (Yadav *et al.,* 2010). The strains were refreshed on nutrient broth and adjusted to turbidity standadrd of MacFarland 0.5 $BaSO_4$. Culture broth containing 10^4 CFU/mL was swabbed to make the bacterial lawn. Using the sterile cork borer of 7mm, wells were made in the plates,followed by addition of 100 µl (4mg/ml) of the samples in respective wells.Roxithromycin and Cefixime-USP at a concentration of 20 µg/well and DMSO were included as positive and negative controls, respectively. After 24 hr incubation period, clear zone of growth inhibition were measured in millimetre (mm) by using microscale. Three replicates of each test extracts were examined and the mean values were recorded.

Test Microorganisms

Activity of extracts was tested against two Gram positive (*Staphylococcus aureus* ATCC# 6538 and *Staphylococcus epidermidis* ATCC# 12228) and seven Gram negative strains *Klebsiella* sp.(clinical isolate), *Acinetobacter* sp. (clinical isolate), *Bordetella bronchiseptica* ATCC# 4617, *Pseudomonas aeruginosa* ATCC# 9027, *Salmonella typhimurium* ATCC# 14028 and *Enterobacter aerogenes* ATCC# 13048.All the cultures were obtained from MRL, Quaid-i- Azam University, Islamabad.

Antileishmanial Activity

In vitro antileishmanial activity was performed with *Leishmaniatropica* khw23 strain by protocol described previously by Shah*et al.*(2014). The *Leishmaniatropica* promastigote was cultured in M199 media supplemented with 10% fetal bovine serum at 24°C. Stock solution of each extract was prepared in DMSO (10 mg/mL) and serial drug dilutions were prepared with M199 medium covering a range of 10 points from 0.2-100 µg/mL. Log phase parasite (100µl) with 1×10^6 promastigotes were seeded in 96-well plates. The plates were incubated in a shaker incubator at 27°C for 72 hrs.Amphotericin-B was taken as positive control while DMSO as negative control. After incubation, the plates were inspected under an inverted microscope to check sterility and growth of controls.A total of 20 µl was taken from each dilution, put on improved neubar counting chamber and live parasites were counted under microscope. Assay was run in triplicate and a mean of values was calculated. IC_{50} values of compounds withantileishmanial activity were calculated by Prism software.

Enzymes Assays

Extracellular enzymeproduction from endophytic fungi was investigated by dissolving substrates in agar plates and inoculating with 8 mm fungal mycelia

plugs. After incubation period of 3-5 days at 25°C, the amount of enzyme produced was evaluated by measuring the diameter of the clear zone around the fungal colony(Amirita*et al.,* 2012).

Amylase

Amylase production was measured using Glucose yeast peptone agar medium with 0.2% starch.The composition of GYP agar medium (g/L distilled H_2O) was glucose-1g, yeast extract-0.1g, peptone-0.5gand agar-16 g.After inoculation and incubation for 5 days, the plates wereflooded with 1% iodine in 2% potassium iodide.The plates showing formation of clear zone around fungal colony were considered positive for amylase.

Cellulase

Cellulase production was measure using GYP agar medium supplemented with 0.5% Carboxy-methylcellulose (CMC). The plates were inoculated and kept for incubation. After the incubation period, the plates were flooded with 0.2% aqueous Congo red solution and destained with 1M NaCl for 15minutes. Cellulase activity was accessed by appearance of yellow areas around the fungal colony in an otherwise red medium.

Laccase

For analyzing the laccase activity, GYP agar medium augmented with 0.005% 1-naptholpH 6, was prepared. After inoculation followed by incubation, the plates were observed visually. Plates positive for laccase activity showed a change in color of media, from clear to blue due to oxidation of 1-napthol.

Lipase

Lipase activity was checked by growing the fungi on Peptone Agar medium supplemented with1% separately sterilized Tween-20.The composition of peptone agar medium (g/L distilled H_2O) was peptone-10g, NaCl-5g, $CaCl_2$ $2H_2O$-0.1g, agar- 16g at pH 6.0.At the end of the incubation period, positive lipase activity was observed by visible precipitates around the colony due to the formation of calcium salts of the lauric acid liberated by the enzyme.

Protease

GYP agar medium amended with 0.4% gelatin (pH 6.0) was used to access protease activity. Gelatin solution in water was sterilized separately and added to GYP medium. Afterincubation period, protease activity was indicated by clear zones around the colony. Then, plates were flooded with saturated aqueous ammonium sulphate, which resulted in formation of precipitate; this made the agar opaque and enhanced the clear zone around the fungal colony.

Pectinolytic activity

Pectinolytic activity was determined by growing fungi on Pectin Agar medium (g/L; Pectin-5g, yeast extract-1g, agar- 15g pH 5.0). After incubation period, the plates were flooded with 1% aqueous solution of hexadecyltrimethylammonoium bromide. Pectinolytic activity was seen in the form of clear zones around fungal colony.

Tyrosinase activity

Tyrosinase activity of endophytic fungi was checked by growing them on GYP agar medium. After the incubation period,a mixture of 0.11% p-cresol and 0.05 % glycine was overlaid on the surface of the fungal colony. Appearance of reddish brown color around the colony, after 24 hours, indicatestyrosinase activity.

Results and discussions

Phytochemical analysis

Total Phenolic and flavonoid Content

Phenolic content was estimated by the addition of Folin-Ciocalteu reagent. This yellow acidic solution containscomplex polymeric ions formed from phosphomolybdic and phosphotungsticheteropoly acids. These reagents oxidizedphenolates and resulted in the production of complex molybdenum-tungsten blue which can be detected spectrophotometrically and calculated as GAE (Ishtiaq *et al.,* 2014). The equivalents of TPC and TFC are calculated on the basis of standard regression lines for gallic acid (y = 0.0103x + 0.1875; R2 = 0.9978) and quercitin (y= 0.00028x + 0.497; R2 = 0.998). The total phenolic

content of selected endophyticfungi is presented in Fig. 1. NFL1 extract on PDA medium showed maximum quantity of TPC (3.176 ± 0.10 µg GAE/mgE)among all the four extract, followed by TM>Rice>SDAextracts (Fig. 1).In case of NFL2 extracts,PDAmedium extract exhibited maximum quantity (3.403± 0.10 µg GAE/mgE), followed by TM>SDA>Rice extracts.

The total flavonoid content was determined by aluminum chloride method. TFC of all extracts was determinedin terms of mg quercetin equivalent per gram extracts and presented in Fig. 1.Among all the extracts of NFL1, flavonoids were found to be rich in PDA medium extract (1.45±0.05 µg QE/mgE) followed by TM >Rice>SDA extracts. While in case of NFL2, the flavonoid content was seen in following order PDA extract (2.7±0.10 µg QE/mgE)>TM> SDA >Rice extract (Fig. 1). A positive correlation was found between the phenolic and flavonoid contents suggesting that theantioxidant potential of phenols might be attributed to the presence of flavonoids.

Table 1. Antibacterial activities of crude extracts of endophytic fungi of *Taxus fauna* in terms of zone of inhibition (ZOI).

Samples	Culture Media	Zone of inhibition (mm)							
		E.aerogenes	*Pseudomonas*	*Klebsiella*	*Acinetobacter*	*S. typhi*	S.epidermidis	*S.aureus*	B.bronchi-septica
NFL1	PDA	17±0.4	13±0.9	12±1.2	16.1±0.92	10±1.1	21±0.87	10±1.1	-
	SDA	-	8±0.8	-	-	-	14±1.0	-	7±0.95
	TM	9±1.3	-	10±0.21	-	-	-	-	-
	Rice	11±0.8	-	-	10±1.2	17±0.6	-	-	13±0.76
NFL2	PDA	15±1.1	10±1	13.4±1.34	-	12±0.7	9.8±0.45	13±0.68	-
	SDA	-	9±0.87	-	-	13±0.9	13±0.69	-	-
	TM	11±0.87	-	10±0.93	-	15±0.2	12±0.84	-	11±0.29
	Rice	10±0.35	-	9±0.58	15±0.64	10±1.3	15±1.4	11±1.2	-
Standard	Ceftx	26±1.4	20.4±1.3	19.3±0.98	24.4±0.81	25±1.1	18.6±1.5	24.6±1.3	24±1
	Roxi	25.7±1.0	21.5±1.5	21.1±0.88	23.2±0.80	24±1.4	20.1±1.4	23.5±1.1	26±0.98

Values (mean ± SD) are average of three samples of each fungal extract, analyzed individually in triplicate (n = 1x3).- = No activity in antibacterial assay or not active, Ceftx= Ceftaxime, Roxi=Roxithromycin.

TLC Screening

TLC separation of EtOACcrude extracts of NFL1 and NFL2 showed variable secondary metabolic profile in all samples. The obtained pattern might be attributed to the presence of lipophilic, sterols andterpenoids compounds in addition to different classes of compounds (Fig. 2).

Antioxidant Activities

a) Total antioxidant capacity

The total antioxidant activity was evaluated by phosphomolybdenum method which is based on the reduction of Mo (VI) to Mo (V) by the sample and the subsequent formation of green phosphate Mo (V) complex at acidic pH. Electron transfer occurs in this assay which depends upon the structure of the antioxidant (Djeridane A. *et al.,* 2006). In our study, premier antioxidant capacities were found in PDA medium extract of NFL1 (25.702±0.58µg AAE/mg E) followed by Rice>TM >SDA extracts (Figure 3). Among NFL2 extracts (Fig. 3), the highest TAC was found in PDA extract (28.02±0.50 µg AAE/mg E) followed by TM > Rice >SDA extracts.High antioxidant and reducing power activity of sample are attributed to high amounts of phenolic compounds. Phenolic compounds disrupt chain oxidation reactions by donation of a hydrogen atom or chelating metals (Viuda-Martos*et al.,* 2010).

In our study, positive correlation was seen between the antioxidant and total phenolic contents of the extracts and it was linear with an excellent correlation coefficient, R^2 of 0.9669 (NFL1) and 0.7499 (NFL2) respectively.

Table 2. Antileishmanial activity of crude extracts of endophytic fungi of *Taxus fauna*.

No.	Samples	Culture Media	IC_{50} (µg/ml)
1	NFL1	PDA	1.5±1.1
2		SDA	15.8±0.5
3		TM	8.6±1.2
4		Rice	26±1.5
5		PDA	30.2±1.4
6	NFL2	SDA	50.6±0.7
7		TM	>100
8		Rice	40.8±2.4
9	Standard	Amphotericin B*	0.02±

Table 3. Production of extracellular enzyme by endophytic fungi of *Taxus fauna*.

Fungal strain	Amylase	Pectinase	Cellulase	Laccase	Lipase	Protease	Tyrosinase
NFL1	+	+	-	+	+	-	-
NFL2	+	-	-	+	-	-	-

Total Reducing Power Assay

In total reducing power assay, iron (Fe^{+3}) in ferric chloride is converted to ferrous (Fe^{+2}) by antioxidant compounds which results in change of color of the test solution from yellow to green.Higher the reducing power of the sample, greater will be the intensity of green color (Gordon 1990).

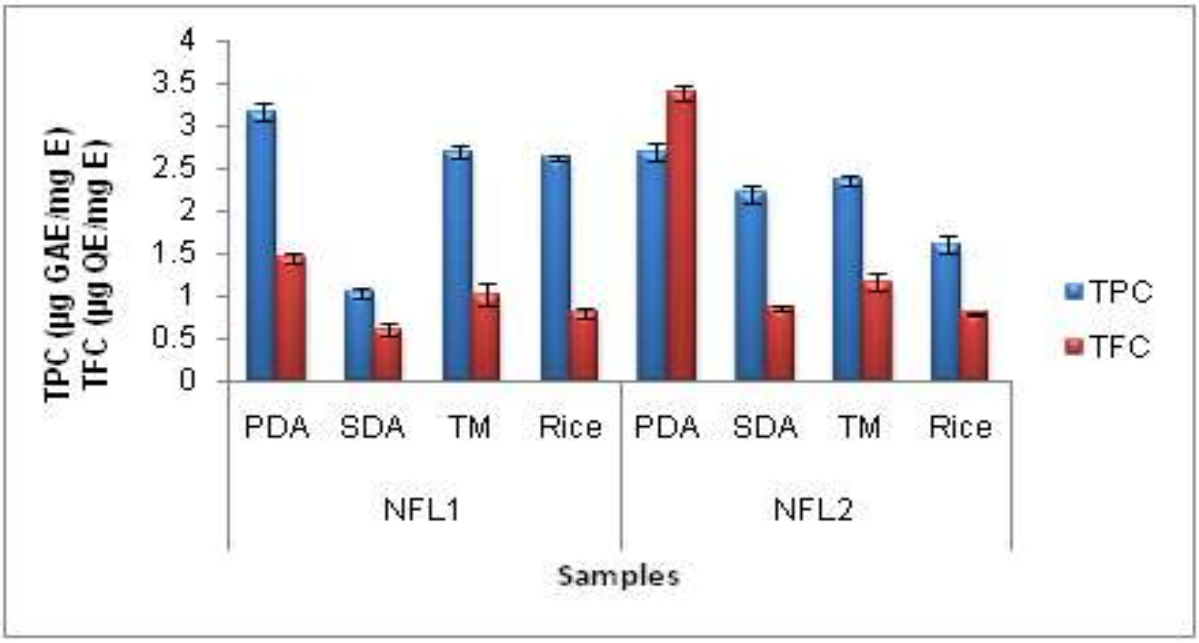

Fig. 1. Total phenolic content (µg GAE/mg E), Total flavonoid content (µg QE/mg E),determination in crude extracts of endophytic fungi. Values are presented as mean ± Standard error from triplicate investigation.

The reducing power of extracts has been shown in Fig. 3. In the present study, the highest reducing power in NFL1 extracts was achieved in the TM extract (15.4±0.35µg AAE/mg E) followed by PDA > SDA > Rice extracts. Total reducing power of the NFL2 extracts was found to decrease in order SDA (17.41± 0.30 µg AAE/mg E) followed by PDA > Rice >TM extracts (Figure 3).

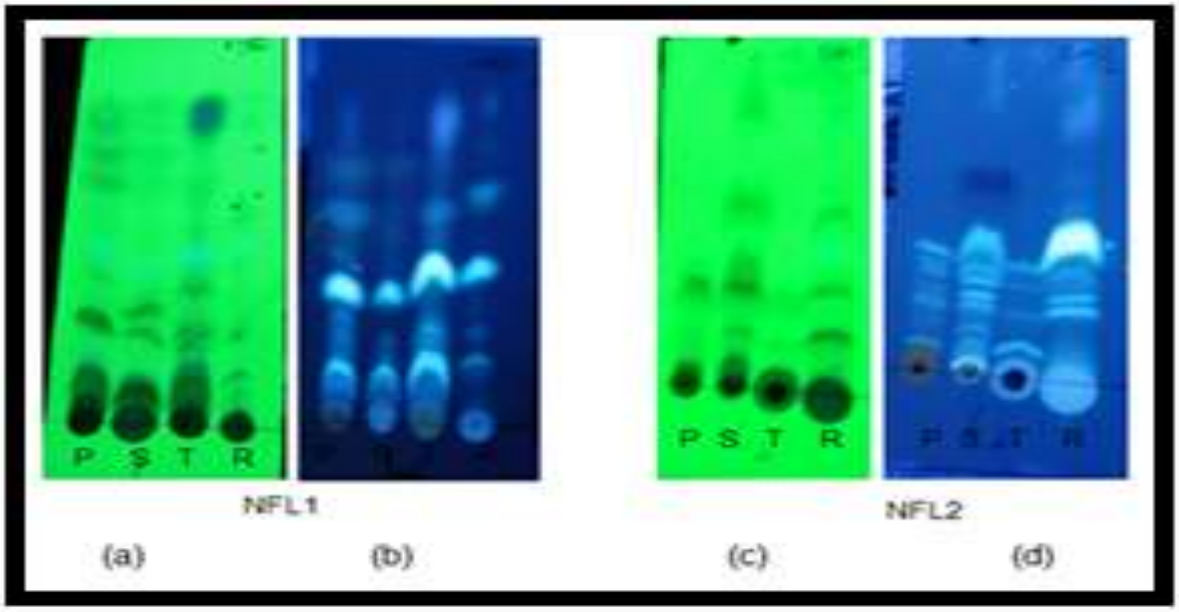

Fig. 2. TLC plates showing the detection of different compounds in crude extracts of NFL1 and NFL2 in order P=PDA, S=SDA, T=TM, R=Rice (a,b) Short (254nm) and Long UV (365nm) lightThin layer chromatogram of NFL1(c,d)Short and Long UV light Thin layer chromatogram of NFL2.

DPPH Radical Scavenging Activity

DPPH free radical scavenging potential is based on the ability of antioxidants to donate hydrogen which reacts with the DPPH radical. When a solution of DPPH is mixed with a sample that can either donate a hydrogen atom or transfer electron to DPPH, it neutralizes the free radical character and reduced form of DPPH (non-radical) is formed which results in loss of the violet color (Schreiner *et al.,* 2009). NFL1extract on PDA medium showed highest DPPH scavenging activity (79.2 ± 2.4 µg/mL) while the others followed the trend SDA > TM >Riceextracts. In case of NFL2 extract, remarkable DPPH scavenging activity was again observed by PDA extract of NFL2 (77.5±3.51µg/mL) and the others followed the trend TM> SDA>Rice (Fig.4).

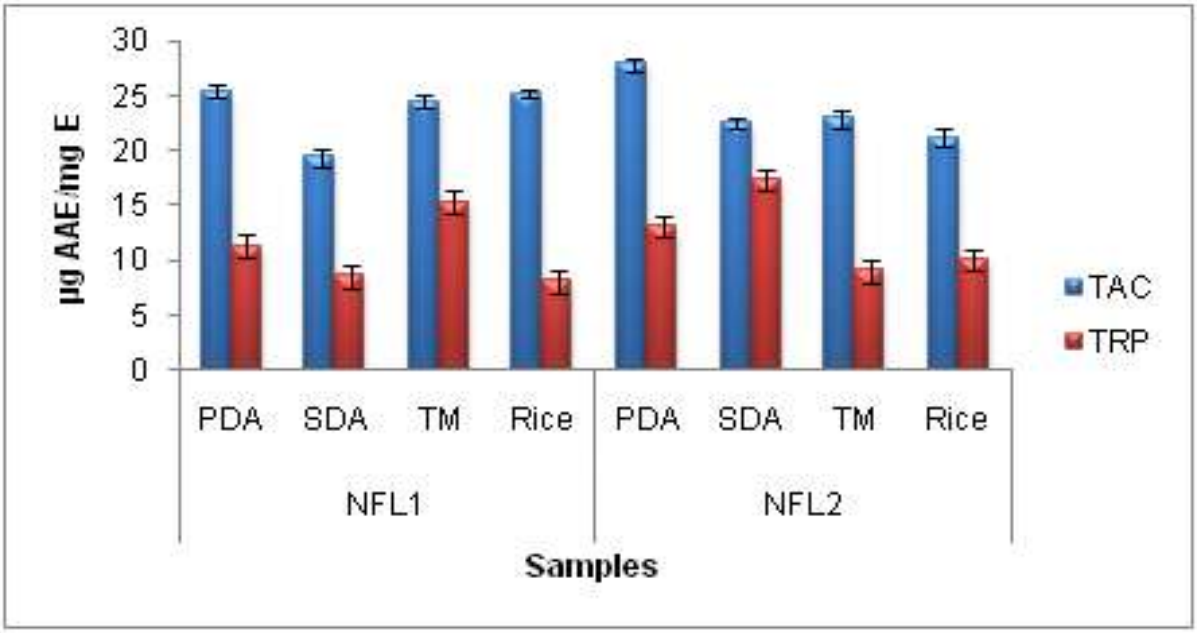

Fig. 3. Total antioxidant capacity (µg AAE/mg E), Reducing power (µg AAE/mg E) determination in crude extracts of endophytic fungi. Values are presented as mean ± Standard error from triplicate investigation.

Antimicrobial Screening

Antibacterial Activity

Endophytes harboring the Taxus plant produce several antimicrobial metabolites belonging to diverse structural classes i.e. alkaloid, flavonoid, terpenoids, phenols and steroids (Wang *et al.,* 2000; Tan and Zou 2001). Crude extracts of NFL1 and NFL2 showed significant activity againstall the tested bacterial strains as shown in Table 1. Among all the four tested extractsof NFL1, PDA medium extractshowed activity against various bacterial strains andshowed strong inhibition against *S.epidermidis*forming a zone of inhibitionof20mm.NFL2 expressed similar behavior against *E. aerogenes* when cultured in PDA medium by forming clear zone of inhibition of 16 mm.Among all the tested extracts, the PDA medium extract of NFL1 and NFL2showed activity against several bacterial strains including gram positive and gram negative bacteria. Severaldiverse bioactive secondary metaboliteshave also been characterized from *Trichoderma* spp. i.e.Harzianolide, T39butenolide and harzianopyridone(Vinale *et al.,* 2006; Howell 1998).

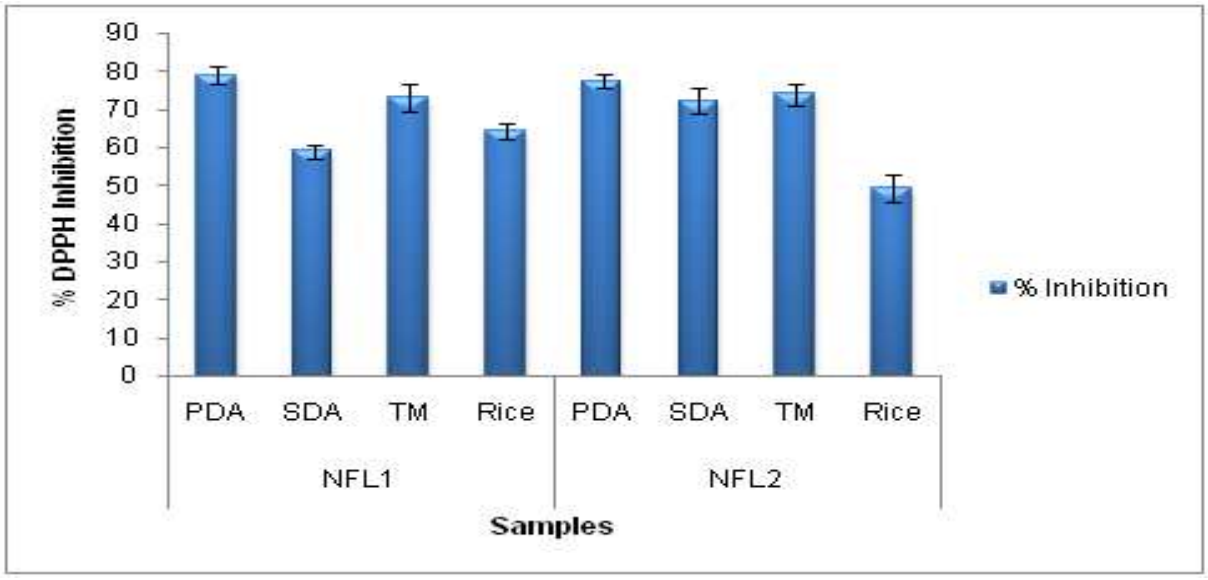

Fig. 4. % radical scavenging activity (DPPH) determination in crude extracts of endophytic fungi. Values are presented as mean ± Standard error from triplicate investigation.

Antileishmanial Activity

NFL1 PDA media extract, showed notable antileishmanial potential with IC_{50} 1.5±1.1µg/mL, followed by the NFL1 on TM extract (IC_{50}8.6±1.2µg/mL). The remaining extracts also showed sufficient potential in the order Rice>SDA extracts(Table 2).In the case of NFL2, highest activity was observed PDA extract (IC_{50}30.2±1.4µg/mL) followed by the Rice>SDA>TM extracts. Thus far, only a few natural fungal-derived compounds with antileishmanial activity have been described (Luque-Ortega *et al.,* 2010).Development of resistance to the first line drugs and alarming increase in number of cases of leishmaniasis has led to the increased demand of new therapies for the treatment of this disease (Al-Harmini*et al.,* 2011).The biosynthetic potential of the strains showed sound influence of cultivation media by displaying variations in their activities in different media extracts. In the present study, it is suggested that starch based media PDA because of its simple compositionshowed the ability to produce diverse bioactive secondary metabolites from fungi.

Enzymatic Activity

Enzymeshaving industrial or agricultural applications are gaining attention apart from pharmaceutically important compounds from endophytes (Liu and Yan, 2010).Like amylase is used infood and detergent industry(De Souza and De Oliveira, 2010), cellulase in

paper industry (Eriksson, 1993), pectinase in fruit and textile industry (Kashyap*et al.,* 2001), lipase in leather and dairy industry (Sharma *et al.,* 2001), while the laccase and tyrosinaseenzyme are involved in lignin degradation (Bucher *et al.,* 2004) andproteases have pharmaceutical applications in diabetes (Ladenburger*et al.,* 1997).

The results of enzymatic potential of endophytic fungi are represented in Table 3. In the present study,*P. milleri* (NFL1) showed positive results for enzymes i.e. amylase, pectinase, laccase and lipase while *T. asperellum*(NFL2) showed positive results for enzymes amylase and laccase. None of the tested strains showed extracellular enzyme production for protease and tyrosinase.The patterns of substrate utilization and the enzymes produced by endophytic fungi will help to establish functional role of endophytic fungi (Carroll and Petrini, 1983).

Acknowledgments

Authors are thankful to Higher Education Commission of Pakistan for financial support.

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