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Research Article

**BIOLOGICAL SCREENING OF ENDOPHYTIC FUNGI
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University Multan, Pakistan.³ Riphah Institute of Pharmaceutical Sciences, Lahore, Riphah
International Univeristy Islamabad, Pakistan.⁴ Department of pharmacy, University of Florida, Gainesville, USA.**Abstract:**

The purpose of this study was to screen and evaluate the physiochemical and biological potential of three endophytic fungi isolated from the medicinal plant Nerium oleander (Apocynaceae). Trichoderma, Penicillium and Taloromyces species, isolated from Nerium oleander, were subjected to antioxidant activity by DPPH method, total antioxidant potential by phosphomolybdic acid method and total phenolic contents by Folin's Ciocaltue method, antibacterial, antifungal and anti-leishmanial assay. All the extracts showed significant antifungal, antioxidant and antileishminial potential. The antioxidant nature of the extracts was concentration dependent. Phytochemical analysis showed the presence of various secondary metabolites including flavonoids.

Key words: *Endophyte, Biological Screening, Antioxidant, Antileishmaniasis, Antifungal***Corresponding Author:****Tariq Ismail,**

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INTRODUCTION:

Plant and plant derived materials have been used for treatment preventing and cure of disease. Epidemiologic data showed reduced risk of cancer with high consumption of vegetables and fruits [1]. Plant are not only good source of chemicals but there reside some other species like endophytes which by down streaming or up streaming start producing chemicals of different origin. [2]. It is time of need to develop/ discover/ investigate highly effective, less toxic and less environmental pollutant chemical from natural or synthetic sources [3].

Nerium oleander is perennial herb of apocynaceae family, indigenous to Indian subcontinent but present up to tropical area and Mediterranean belt. It is present from Nepal to Baluchistan province of Pakistan, Afghanistan and in gardens throughout India and Pakistan [4]. In folk medicine leaves are used in skin disease like leprosy, alopecia veneral disease and cardiotoxic [4]. Compound isolated from *Nerium species* are gitoxigenin, oleanderol, neriantin, deacetyloleanderin, neriifolin), rutin, adynerin, dambonitol, ; strospeside, class odorosides triterpene, steroidal cardenolide, uzarigenin, , triterpenoidal saponins, oleanderin, and odoroside H in flowers [5]. Endophytes are present intracellularly inside plant with symbiotic relationship [6]. Endophytes are omnipresent in living world [7]. Fungal endophytes are most commonly associated with plants and they produce unmatched numbers of secondary metabolites [8] [9]. Endophytes produce many different kinds and classes of chemical compound like saponin, alkaloid benzophenones phenolic acids and others. [10]. These secondary compounds produced by endophytes are antiviral, anticancer, anticancer antidiabetics and immunosuppressant in nature [11]. Paclitaxol [6], Palmarumycin CP17 and Cp18 [12], gnodulisporins [13] , kakadamycin [14] and ambuic acid [15] are few examples of secondary metabolites which were isolated from endophytic fungi. Due to large number of effective and novel scaffold discovered from endophytic fungi, with diverse areas of activity but this source is still untapped [16]. The bioactive compounds produced by plant endophytes can be explored for their medicinal values. [17]. The possibility of discovering exciting possibilities in field of endophyte exploration still exist as it is most untapped area of scientific research. [18]. In this study initial screenings, made up of total phenolic content, antibacterial, antifungal antioxidant, Brine shrimp assay and anti-leishmanial assay of secondary metabolites of ethyl acetate extract of endophytes isolated from endophytes of *Nerium oleander* was investigated.

EXPERIMENTAL:**Endophyte materials**

A total of three different strains were used which were previously isolated from leaves of *Nerium oleander* and identified at molecular level as *Penicillium polonicum* NL1, *Talaromyces purpureogenus* NL2 and *Trichoderma atroviride* NL3. These strains were maintained on PDA slants at 4C.

Phytochemical Screening**Total Phenolic Contents**

Total phenolic contents were determined by Folin-ciocaltau method [19]. Extracts in concentration of 4mg/ml dissolved into DMSO were transferred into 96 well plate in quantity of 20ul each. 90ul of Folin-Ciocaltau reagents was added into each well followed by 90ul of NaCo3 7.5% w/v after five minutes. The whole mixtures were incubated for one hour. Absorbance of samples was recorded at UV wavelength of 650nm. A calibration curve was obtained using gallic acid as positive standard. The total phenolic content was determined as ug gallic acid equivalent per mg extract.

Total Flavonoid Contents

Ammonium chloride method [20] was used to determine the flavonoid contents of crude ethyl acetate extracts. 20ul crude extracts, ethyl acetate extracts, with equal amount of 10% w/v aluminum chloride solution were mixed with 10 ul of 1M solution of potassium acetate. Volume was made up to 200ul with distilled water. After 30-minute incubation at room temperature absorbance was at 650nm using microplate reader (Biotech, USA Microplate reader Elx800). The calibration curve was drawn using Quercetin as standard from 2.5ug/ml to 40ug/ml. Flavonoid contents was determined using ug quercetin equivalent per mg extract.

Biological Screening**DPPH free radical scavenging assay**

DPPH(2,2-diphenyl-1-picryl hydrazyl) free radical scavenging assay was done in accordance with [21]. Test extracts (4mg/ml) in 10ul quantity was mixed with 190ul DPPH (9.2mg/ml methanol), 1hour dark incubation and OD was measured at 515nm using plate micro reader using DMSO as negative and Ascorbic acid as positive standard. Absorbance in low values indicated higher scavenging activity.

Antibacterial Activity and Antifungal Assay

Agar well diffusion method was used to evaluate the antibacterial activity[22]. The turbidity of strains was adjusted according to MacFarland 0.5 BaSO4 standards after refreshing of culture in nutrient agar. Bacterial lawn was made by swabbing broth having colony concentration in 10⁴CFU/mL. Sterile cork

borer (7mm) was used for making wells in plates. These well were filled with 100ul (4mg/ml) of samples and Cefixime USP, Roxithromycin USP and DMSO as positive and as negative control respectively. Microscale was used for measuring clear zone of inhibition in millimeter. Test was repeated in triplicate and mean values were determined.

Test Microorganisms

Antimicrobial activity of extracts was determined against two bacterial and three fungal strains i.e., *P. aeruginosa*, *S. aureus*, *A. flavus*, *A.niger*, *F.solani* and *Candida albican* All test cultures were obtained from microbiological research laboratory, Department of Microbiology, Quaid-i-Azam University Islamabad.

Antileishmanial Activity

Leishmania tropica khw23 strains were commonly used for the in vitro anti-leishmanial activity[23]. *L. tropica* promastigote was grown in M199 culture augmented with 10% fetal bovine serum at room temperature. Extract stock solution (10mg/ml) was prepared in DMSO. Ten serial dilution of drug were prepared with M199 in 0.2ug/ml to 100ug/ml. Log phase 1X10⁶ promastigotes, seeded into 96 well plates, were incubated with 27C for 72 in shaker incubator. Each dilution (20ul) was put on neubar counting chamber of inverted microscope and number of live parasites were counted. Triplicate reading was taken with amphotericin and DMSO as positive and negative control. IC₅₀ values were determined with Graph pad prism.

Brine shrimp lethality potential

Brine shrimp lethality test was performed according to the procedure developed by [24]. The test was conducted in 96 well plates with each extract (1000, 100 and 10 µg/ml) with final volume of 5ml. Four replicates were used for treatments and control. The control was solvent of dissolution for extracts. Approximately 10, 12 h old brine shrimp hatchlings were transferred to each well using 9 in disposable pipette. Brine shrimp lethality test was based on

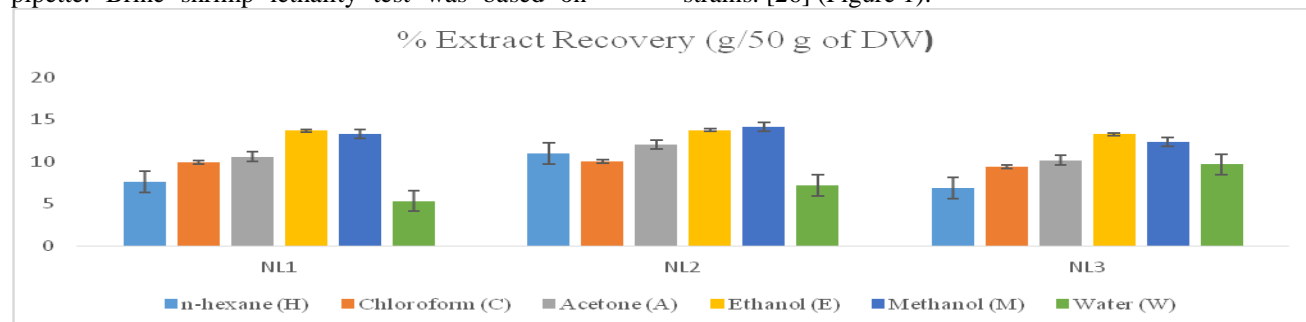
exposure of 10 *Artemia* nauplii to different concentration of extracts and determining toxicity by counting the number of dead nauplii. The toxicity was determined after 12 hrs, 24 hrs and 48 hrs of extracts exposure. Percentage of deaths was determined by counting number of survivors. Nauplii was considered dead if they showed no movement internally or externally for many seconds of observation.

Protein kinase inhibition potential

The protein kinase inhibition assay was performed by observing hyphae formation in purified isolates of *Streptomyces* 85E strain [25]. Bacterial lawn was allowed to develop by spreading mycelia fragments of *streptomyces* on sterile agar plates containing mineral ISP4 medium. About 5 µl of each extract (20 mg/ml of DMSO) was loaded onto sterile 6 mm filter paper discs. The impregnated paper discs with a final concentration of 100 µg/disc were applied directly on the surface of the agar plates seeded with *Streptomyces* 85E. Surfactin (sporulation inhibitor) and DMSO infused discs were included as positive and negative control respectively. The plates were then incubated at 30°C for 72-96 hours (time required for hyphae formation in *Streptomyces* 85E) and the results were interpreted as bald zone of inhibition around samples and controls infused discs.

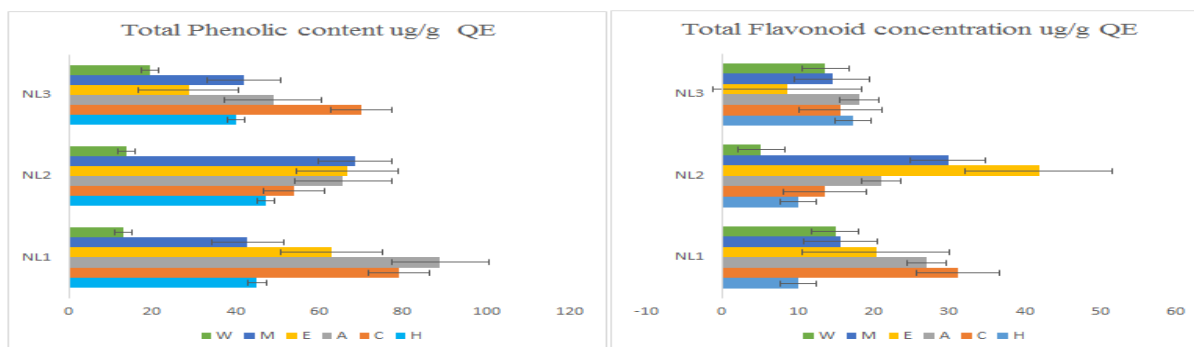
RESULTS AND DISCUSSION:

Clinical, pharmacological and chemical investigations of natural sources, mainly from plants were the basis of most of the early medicines such as aspirin, digitoxin, morphine, quinine and pilocarpine [22]. In current study phytochemical and biological potential of three strain isolated from a selected medicinal plant were determined. Mercuration technique was used for the determination of percentage of the extract recovery from endophytic fungus. Significant amount of extract yield was obtained in case of NL1 (*Penicillium polonicum*) with Methanol solvent in comparison of all of three strains. [26] (Figure 1).



*DW = Dry weight, Values (mean ± SD) are average of three samples of each endophyte, analyzed individually in triplicate

Fig 1: Percent recovery and respective solvents used for extraction



QE is Quercetin equivalent.

Fig 2: Total phenolic and flavonoid contents in ug/g QE

Total phenolic and flavonoid contents:

As in medicinal plants, the phenolic and polyphenolic compounds like flavonoids, phenolic acids and tannins play a significant role in antioxidant potential of plant antioxidant activity so is the case of endophytes. In combating oxidative stress due to environmental toxins these antioxidants play an important role by neutralizing free radicals [27]. Folin-Ciocalteu (F-C) reagent based assay results showed that C, A and E extracts of NL1 showed significant amount of phenolic contents (Figure 2S). Some extracts of NL2 also showed significant amount of phenolic contents with highest value of 63.65 and 63.94 GAE/mg for M and W sample while only sample C of NL3 showed moderately significant result with value of 65.02 μg GAE/mg. These findings are comparable with previous reports about antioxidant potential of extract of *Albizia* leaves because of presence of phenolic contents (Islam *et al.*, 2013) (Figure 2). It was reported earlier that methanol was an efficient solvent for extraction of

low molecular weight polyphenols whereas aqueous acetone, ethanol, ethyl acetate are good for extraction of high molecular weight flavones. [28]

DPPH Free Radical Scavengers Assay:

DPPH assay was used to determine the free radical scavenging activity while table curve software was used to obtain percent scavenging and IC_{50} values. The range of scavenging activity showed between 85 to 92%. NL2 ethanol samples showed highest value of percentage scavenging of 91% among all endophytic samples with IC_{50} value of 26.47 $\mu\text{g}/\text{ml}$, while NL3 sample showed highest value of percentage scavenging of 89.67% (Table 1). Ahmed and his coworkers reports about antioxidant potential of different compounds isolated from endophytic fungus can be correlated with these findings.[29]. A positive correlation was observed between the phenolic and flavonoid contents suggesting that the antioxidant potential of phenols might be attributed to the presence of flavonoids [30].

Table 1: DPPH free radical scavenging assay

Samples	NL1		NL2		NL3	
	% scav.	IC_{50}	% scav.	IC_{50}	% scav.	IC_{50}
H	27.33 \pm 1.25	>200	50.67 \pm 0.47	197.8	8 \pm 0.82	>200
C	21.67 \pm 0.47	>200	5 \pm 2.16	>200	20 \pm 0.82	>200
A	85.67 \pm 1.25	19.52	28 \pm 0.82	>200	24 \pm 0.82	>200
E	89.67 \pm 1.25	18.65	91 \pm 0.82	26.47	21.33 \pm 0.47	>200
M	27.33 \pm 1.25	>200	87 \pm 0.82	57.42	12.67 \pm 0.47	>200
W	16.33 \pm 2.49	>200	88 \pm 0.82	29.58	17 \pm 1.63	>200

% scav. = Percent scavenging at 200 $\mu\text{g}/\text{ml}$, Values (mean \pm SD) are average of three samples of each endophyte, analyzed individually in triplicate

Antimicrobial and Antifungal Assay:

Significant results were observed against *P. aeruginosa* with highest value of 36mm of M extract of NL3. Results against *S. aureus* were not highly significant (Table 2). The antimicrobial potential of NL1 to NL3 were closely related to the findings reported in literature. Our results were correlated with previous reports that Nerium oleander endophytes species showed anti-bacterial activities [31] [32]. In case of antifungal assay significant

results were obtained against *C. albican*, *A. flavus* and *A.niger* while samples didn't show noticeable results against *F.solani* (Table 3). Maximum zone of inhibition of 13.97 ± 0.05 mm was observed with acetone extract of leaves against *A. flavus*. While in case of NL2 samples highest zone of inhibition of 13.23 ± 0.21 mm was observed with Ethanol sample. These results are comparable with previous reports about antifungal activities of *Albizia* species [33] [34].

Table 2: Antibacterial assay

Samples	<i>S. aureus</i>			<i>P. aeruginosa</i>		
	NL1	NL2	NL3	NL1	NL2	NL3
H	---	---	9.46 ± 0.04	---	---	30 ± 0.06
C	---	1 ± 0.02	10.81 ± 0.34	---	---	33 ± 0.05
A	---	2 ± 0.1	10.14 ± 0.23	9 ± 0.1	10 ± 0.12	39 ± 0.45
E	---	2 ± 0.02	8.78 ± 0.01	8 ± 0.02	20 ± 0.12	42 ± 0.56
M	---	31 ± 0.75	10.14 ± 0.12	1 ± 0	27 ± 0.07	36 ± 0.26
W	---	---	7.43 ± 0.04	11 ± 0.05	11 ± 0.15	30 ± 0.28

Values (mean \pm SD) are average of three samples of each endophyte, analyzed individually in triplicate, sample concentration= 100 μ g per well

Table 3: Antifungal assay

Samples	NL1				NL2				NL3			
	<i>A. niger</i>	<i>A. flavus</i>	<i>C. albican</i>	<i>F. solani</i>	<i>A. niger</i>	<i>A. flavus</i>	<i>C. albican</i>	<i>F. solani</i>	<i>A. niger</i>	<i>A. flavus</i>	<i>C. albican</i>	<i>F. solani</i>
H	11 ± 10.87	7.98 ± 0.02	7.68 ± 7.85	7.98 ± 0.07	10.74 ± 0.36	----	8.93 ± 0.25	8.99 ± 0.02	10 ± 0.08	7.97 ± 0.05	8.78 ± 0.31	7.8 ± 0.25
C	8.87 ± 8.98	9.93 ± 0.05	8.93 ± 8.9	7.91 ± 0.12	10.89 ± 0.16	7.92 ± 0.06	8.88 ± 0.1	9 ± 0.08	8.93 ± 0.17	10.97 ± 0.05	9.99 ± 0.09	8.49 ± 0.19
A	8.99 ± 8.56	13.97 ± 0.05	7.68 ± 7.85	7.89 ± 0.16	----	7.87 ± 0.15	8.97 ± 0.08	8.98 ± 0.17	9.92 ± 0.08	8.96 ± 0.06	7.96 ± 0.06	7.94 ± 0.04
E	9.29 ± 9.99	10.96 ± 0.04	8.93 ± 8.9	8.99 ± 0.08	9.01 ± 0.07	7.99 ± 0.03		8.88 ± 0.16	9.9 ± 0.16	6.91 ± 0.08	7.68 ± 7.85	7.7 ± 0.15
M	8.5 ± 8.43	7.95 ± 0.04	7.68 ± 7.85	7.98 ± 0.07	8.86 ± 0.2	----		8 ± 0.08	9.93 ± 0.17	----	8.93 ± 8.9	----
W	10 ± 9.87	----	8.93 ± 8.9	7.95 ± 0.04	----	6.99 ± 0.02		7.98 ± 0.02	9.88 ± 0.13	8.95 ± 0.04	7.85 ± 0.05	7.85 ± 0.05

Values (mean \pm SD) are average of three samples of each endophytet, analyzed individually in triplicate. Sample concentration= 400 μ g per disc.

Table 4: Brine shrimp lethality assay

Samples	NL1					NL2					NL3				
	200 µg/ml	100 µg/ml	50 µg/ml	25 µg/ml	LD50	200 µg/ml	100 µg/ml	50 µg/ml	25 µg/ml	LD50	200 µg/ml	100 µg/ml	50 µg/ml	25 µg/ml	LD50
H	100.00	93.33	70.00	46.67	27.92	90.00	83.33	73.33	60.00	15.78	100.00	70.00	63.33	46.67	<25
C	100.00	70.00	56.67	50.00	24.67	100.00	86.67	70.00	63.33	6.29	100.00	100.00	76.67	63.33	<25
A	100.00	56.67	50.00	26.67	67.00	90.00	83.33	66.67	53.33	22.56	100.00	100.00	100.00	86.67	<25
E	90.00	76.67	63.33	20.00	39.67	100.00	86.67	70.00	60.00	14.24	100.00	100.00	100.00	90.00	<25
M	100.00	86.67	73.33	30.00	33.03	80.00	63.33	46.67	30.00	57.43	100.00	100.00	86.67	73.33	<25
W	90.00	76.67	63.33	53.33	19.28	90.00	66.67	46.67	30.00	56.51	100.00	90.00	83.33	66.67	<25

Brine shrimp lethality potential:

In case of brine shrimp lethality assay most of the NL1 extracts showed more potent effects against *A. salina* larvae with LD₅₀ values ranging between 25 to 37.38 µg/ml. In case of NL2 samples the lowest LD₅₀ (<25 µg/ml) was shown by most of the samples while NL3 sample showed moderately significant effects (Table 4). These reports are correlated with previous reports of *Albizia* plant for brine shrimp cytotoxicity [35].

Values (mean ± SD) are average of three samples of endophyte, analyzed individually in triplicate

Protein Kinase Inhibition Potential:

In the recent years, it is needed that to investigate inhibitors of protein kinases from natural products because kinases play major role in regulatory mechanisms of cells especially cell. Therefore inhibition of protein kinases has emerged as a promising target for cancer treatment[25] and whole cell *Streptomyces* assay is considered a simple assay to determine cytotoxic potential of samples. Maximum zone of inhibition was shown by M extract

of NL3 with value of 28.01 ± 0.28 mm followed by E extract (21.87 ± 0.13 mm). Three of NL1 samples H, C and A and some bark samples also showed significant activity. This is first report of protein kinase inhibition study. These results suggest that a moderately polar extraction solvent would be suitable for the extraction of bioactive metabolites that may serve as a promising source of kinase inhibiting compounds (Figure 3).

Anti-leishmanial potential

Leishmania is a parasite which cause leishmaniasis whose prevalence is 0.7 million (700,000) to 1.2 million (1,200,000) [36] Results indicated that extracts of NL1 were highly effective against leishmaniasis. Among these extracts water W, Methanol M and Acetone A samples showed highest % mortality i.e., 88% mortality. While some low polar extracts of leaves also showed significant results. Our findings suggest that both of these plants could be a potential source of antileishmanial compounds (Figure 3).

**Fig 3: Protein kinase & Anti-leishmanial potential**

Values (mean ± SD) are average of three samples of each endophyte, analyzed individually in triplicate. Sample concentration=50ug/ml

CONCLUSION:

It is concluded from current study that different solvents and their combinations for the purpose to explore pharmacological potential of endophytes could be a suitable strategy for extraction of secondary metabolites, which may be downstream from plant to fungus or upstream from fungus to plant. As culturing of fungus and extraction of fungal metabolite is comparatively easy than plants, they may use for identification and isolation of new and unknown compounds with minimum environmental effects. It is also concluded that these endophytes i.e., *Penicillium polonicum*, *Talormyces purpureogenus* and *Trichoderma viridae* possess antileishmanial and enzyme inhibition activity. Antileishmanial, antioxidant, antimicrobial and anticancer lead compounds can be isolated from these fungi through bioactivity guided isolation. Further *in vivo* studies will also provide ample information about medicinal potential of selected plants.

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