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Original Article

EVALUATION OF IN VITRO ANTIMICROBIAL POTENTIAL OF ENDOPHYTIC FUNGI ISOLATED FROM EUGENIA JAMBOLANA LAM

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ABSTRACT

Objective: In this study ethyl acetate crude extract of 22 endophytic fungi associated with *Eugenia jambolana* were investigated for their antimicrobial potential against Human pathogenic ATCC reference strains of bacteria and fungi.

Methods: Antimicrobial potential of endophytes was evaluated by Agar well diffusion method and MIC values were calculated by 96 multi-well micro titer plates against (ATCC) strains of human pathogenic gram negative bacteria viz. *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginos*a, *Klebsiella pneumonia, Salmonella typhi*, *Shigella flexneri, Serratia marcescens* and gram positive bacteria *Staphylococcus aureus*, *Enterococcus faecalis*. Reference fungal strains were *Candida albicans* and *A. niger*.

Results: 68% fungi showed significant antibacterial and antifungal activities. Isolated endophytes *Aspergillus terreus, Aspergillus species and Aspergillus tubingensis* showed a wide range of activity against microbes while *Curlvularia lunata* was least active. *Escherichia coli, Serratia marcescens* and *Salmonella typhi* were resistant against all fungal extracts. MIC range of endophytic fungal extract varied from 1.87mg/ml to 7.50mg/ml.

Conclusion: The results showed that endophytic fungi isolates of *Eugenia jambolana* with antimicrobial potential could be outstanding source of novel bioactive compounds.

Keywords: Endophytic fungi, *Eugenia jambolana*, *Syzygium cumini,* In vitro, Antimicrobial activity, MIC.

INTRODUCTION

Overuse of antibiotics and increased resistance to drugs in microorganisms are becoming a serious global concern [1]. It leads to urgent search for new sources of antibiotics that are cheap, effective, non-toxic, and have little environmental impact. Moreover, the frequency of advent infectious diseases has increased worldwide accompanied with an increasing extent of environmental degradation, spoilage of land, industry sewage and poisonous gases [2].

Microorganisms are highly diverse source of bioactive natural products. Endophytic microbes are fungi and bacteria that colonize internal tissues of living plants without causing any harm to its host [3]. All higher plants are hosts to one or more endophytic microbes capable of producing similar secondary metabolites as its host plant [4, 5].

Bioactive natural compounds isolated from fungal endophytes have been playing a promising role in the search for novel drugs [6] and becoming an inspiring source for researchers due to their enormous structure diversity and complexity.

Eugenia jambolana Lam. (Syn. *Syzygium cumini*) commonly known as Jamun or black plum belongs to family Myrtaceae having potent medicinal and pharmaceutical applications. Entire plant such as seed, fruit, leaves flower, bark used in folk medicine in countries where Jamun is reported to grow [7]. The various parts of plant possess a range of medicinal properties such as antimicrobial, antiviral, anti-inflammatory, anti-genotoxic, anti-ulcerogenic, cardio protective, anti-allergic, anticancer, chemo preventive, radio protective, antioxidant, hepatoprotective, anti-diarrheal, hypoglycemic and antidiabetic effects [8].The present study was conducted to investigate in vitro antimicrobial potential of endophytic fungi associated with *E. jambolana* against pathogenic microbes.

MATERIALS AND METHODS

Endophytic strains

The 22 endophytic fungal strains were isolated from different tissues (leaf, petiole and stem) of *E. jambolana* following protocol of Fisher *et al.* [9] were selected for present study. All fungal strains were molecularly identified and the sequences were deposited in NCBI (Data not yet published).

Preparation of Fungal Extracts

The endophytes were mass cultured on Potato Dextrose Broth (PDB) media for 10 days at 27°C on a shaker at 160 rpm. The mycelium were filtered and dried. The dried powdered materials were then extracted with organic solvent ethyl acetate (1:10) by using cold percolation for 48-72 h. The obtained extract was then filtered by using Whatman No. 1 filter paper and then concentrated under vacuum at 40°C by using a rotary evaporator.

Assay for antimicrobial testing

Preparation of inoculums

Bacterial strains were grown on nutrient agar plates and fungal strains were grown on Sabouraud Dextrose Agar plates. Bacterial inoculums were prepared from overnight grown cultures (24 h) in peptone water and the turbidity was adjusted equivalent to 0.5 McFarland units (approximately 10^8 CFU/ml for bacteria and fungi inoculums turbidity was equivalent to 10^5 or 10^6 CFU/ml). The microorganisms were inoculated into peptone water and incubated at 35± 2°C for 4 h. Streptomycin was taken as positive control (10µg) for antibacterial activity and ketoconazole (10µg) for antifungal activity. The antimicrobial activity of endophytes was evaluated by Agar well diffusion method [10]. Inoculums were spread over the surface of agar plates with a sterile glass spreader. 4 wells were made at equal distance using sterile cork borer. To test

the antimicrobial activity all extracts were made a final concentration of 100 mg/ml. 10µl, 20µl, 30µl, 40µl of extract was poured to each well and plates were incubated for a period of 24 h at 37°C in incubator for bacteria and at 30°C for 24 to 48 h in B.O.D incubator for fungi. Each experiment was done in triplicate and mean values were taken. Diameter (mm) of the clear inhibitory zone formed around the well was measured.

Reference Strains

Human pathogenic ATCC reference strains of bacteria and fungi were used to screen antimicrobial activity. Two fungal strain of *Candida albicans* (ATCC10231) and *A. niger* (ATCC160404) and nine bacterial strains of *Escherichia coli* (ATCC25922), *Proteus mirabilis* (ATCC43071), *Klebsiella pneumoniae* (ATCC700603), *Pseudomonas aeruginosa* (ATCC 27853), *Shigella flexneri* (ATCC12022), *Serratia marcescens* (ATCC 27137), *Salmonella typhi* (ATCC 13311), *Enterococcus faecalis (*ATCC29212) and *Staphylococcus aureus (ATCC259323)* were used.

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. The MIC values of extracts were determined based on a micro broth dilution method in 96 multi-well micro titer plates with slight modifications [11]. A volume of 100 µl of test materials in 10% (v/v) DMSO (usually a stock concentration 100 mg/ml for crude extracts) added into the first row of the plate. 50µl of nutrient broth and 50µl of normal saline were added to each well of plate. Serial dilutions were performed using a multichannel pipette such that each well had total 100µl of the test material in serially descending concentrations.10 µl of resazurin indicator solution (prepared by dissolving a 270mg tablet in 40ml of sterile distilled water) was added in each well. Finally 10µl of bacterial suspension was added to each well to achieve a concentration of 5 x 10^6 CFU/ml. Each plate had a column with streptomycin as positive control.

The plates were prepared in triplicate and placed in an incubator set at 37°C for 18 to 24 h. Any color changes from purple to pink or to colorless indicate growth of microbes. The lowest concentration at which no color change occurred was taken as the MIC value of extract.

RESULTS

In the present study 22 fungal crude ethyl acetate extracts were tested for antimicrobial activity by agar well diffusion assay. Out of 22 endophytic fungi 15 (68%) showed significant antimicrobial activities. The diameter of inhibition zones ranging from 10 to 31mm for bacteria and from 8 to 13mm for fungal strains. Data has been listed in table 1 as mean ± SD. Remaining extracts showed a narrow spectrum of activity or showed no activity. Maximum inhibition zone (31mm) was shown by extract of *A. niger* against *Klebsiella pneumoniae*. Similarly extracts of *Coprinopsis cinerea* and *A. terreus* showed 25mm and 17mm zone of inhibition against *Enterococcus faecalis* and *Proteus mirabilis* respectively.

Aspergillus terreus showed a wide range of activity against 6 bacterial and 2 fungal test strains. The fungal isolates, *Aspergillus species and Aspergillus tubingensis* both showed activity against 5 bacterial and 2 fungal strains*. Curlvularia lunata* was least active, showing activity against only 1 bacterial and 1 fungal strain. *Escherichia coli, Serratia marcescens* and *Salmonella typhi* were resistant against all fungal extracts*. A. niger and Klebsiella pneumoniae* was found most susceptible microbes inhibited by 13 fungal extracts. MIC ranges of tested microbes have been shown in table 2. Outcomes of the study shows that MIC range of fungal extract varies from 1.87mg/ml to 7.50mg/ml. *Aspergillus terreus and A. niger* fungal extracts have least MIC range (1.87mg/ml) against *Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Shigella flexneri* which means these possess the most significant antimicrobial activity. All fungal extracts have same MIC range (3.75mg/ml) against reference strain of *A. niger.*

DISCUSSION

Many studies have been done on the investigation of antimicrobial activity of endophytic fungi isolated from various medicinal plants. No reports are available on antimicrobial activities of the endophytic fungi isolated from *E. jambolana.* It is a well known plant which possesses diverse health and industrial benefits. The outcomes of our study indicate that endophytic fungi have significant antimicrobial potential which supports the views of Schulz *et al.* [12]. Ethyl acetate extraction is most efficient method for obtainment of fungal secondary metabolites [13]. Results of our study correlate with some of the previous studies. 19 Endophytes isolated from Amazonian toxic plants *Palicourea longiflora* and *Strychnos cogens* showed antimicrobial activity against at least one of the pathogenic microorganisms tested: *Bacillus* sp., *B. subtilis, S. aureus, E. coli, Candida albicans, Trichoderma sp*., and *Aspergillus flavus.* [14]. Endophytic fungi isolated from *Salvadora oleoides Decne* showed potent antimicrobial activity against *S. typhi, E. coli, K. pneumoniae* and *Aspergillus niger* [15].

Endophytic fungi from medicinal plant *Simphytum officinale* inhibit growth of *S. aureus* [16]. In our study none of the fungal extract showed the activity against *Escherichia coli, Serratia marcescens* and *Salmonella typhi.* It is in agreement with earlier studies on endophytes from Chilean native gymnosperms [17]. In our study *Aspergillus terreus* is the most potent endophyte followed by *Aspergillus species* and *Aspergillus tubingensis* respectively.

A previous study by Rao *et al* [18] showed antibacterial activities of *Aspergillus terreus* against *S. aureus, Enterococcus faecalis, B. subtilis, Pseudomonas aeruginosa* and *E. coli*. *Aspergillus* genera are a major contributor of antimicrobial compound of fungal origin [19]. Extracts of *A. niger, Coprinopsis cinerea* and *A. terreus* produced zone of inhibition greater than that of control against *Klebsiella pneumoniae, Enterococcus faecalis* and *Proteus mirabilis* respectively.

Klebsiella pneumoniae and *Proteus mirabilis* is *gram negative* while *Enterococcus faecalis* is gram positive one. Gram-negative bacteria are more resistant to antibiotics so they are more difficult to control. This is an indication for isolation of strong antibacterial compounds from these fungi. Previous study has been done on isolation of antibacterial compound phomodione from endophytic fungi *Phoma* strain, from *Saurauias caberrinae* plant [20] and compounds with cytotoxic and antifungal potential are isolated from *Periconia atropurpurea*, an endophyte from plant *Xylopia aromatica* [21].

CONCLUSION

Finding of our study indicates that endophytic fungi associated with *E. jambolana* are a rich source of natural antimicrobial compounds. Antimicrobial compound isolation from endophytic fungi can meet the ever growing need of antimicrobial novel compounds and help in conservation of medicinal plants.

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CONFLICT OF INTEREST

There is no conflict of interest between authors**.**

Table 1: Antimicrobial activity of endophytic fungi isolated from *Eugenia jambolana.*

Fungal isolates	Diameter of Inhibition zone (mm±SD)										
	CA	**AN**	**PM**	**KP**	**PA**	**SF**	**EF**	**SA**	**EC**	**SM**	**ST**
Fusarium sp.	-	12.83±0.76	14.66±0.57	-	15.83±0.76	-	13.00±0.57	19.16±0.76	-	-	-
Coprinopsis cinerea	8.16±0.76	-	12.83±0.76	20.66±0.76	-	-	25.00±0.57	-	-	-	-
A. flavus	11.00±0.57	-	-	13.33±0.57	-	12.33±0.57	-	13.00±0.57	-	-	-
Unknown fungus	13.00±0.57	12.66±0.57	13.16±0.76	13.00±0.57	10.00±1.0	-	-	-	-	-	-
Isaria tenuipes	10.83±0.76	11.33±0.57	-	10.00±1.0	11.33±0.57	10.5±0.5	-	-	-	-	-
Aspergillus sp.	12.16±0.76	12.50±0.57	-	15.00±1.0	19.0±1.0	13.00±0.5	15.5±0.57	18.5±0.57	-	-	-
A. niger	12.50±0.57	13.00±1.0	-	31.50±0.57	13.16±0.28	12.33±0.57	13.33±0.57	-	-	-	-
A. tubingensis	13.00±1.00	11.5±0.57	15.00±0.57	16.0±0.57	-	20.33±0.57	15.83±0.76	11.5±0.57	-	-	-
Curlvularia lunata	-	10.0±1.00	-	12.66±0.57	-	-	-	-	-	-	-
S. racemosum	9.00±1.00	9.50±0.57	-	13.66±0.57	-	-	-	-	-	-	-
Choanephora sp.	8.66±0.57	10.66±0.57	-	11.00±1.0	-	-	-	-	-	-	-
Chaetonium sp.	13.83±0.76	9.00±0.57	-	13.83±0.76	-	-	-	-	-	-	-
Trichoderma longibrachiatum	12.00±0.57	12.83±0.76	-	-	13.0±1.00	-	13.0±0.50	-	-	-	-
A. japonicus	-	9.5±0.57	-	12.0±0.57	-	12.83±0.76	10.0±1.00	10.0±0.57	-	-	-
A. terreus	12.66±0.57	13.0±0.57	17.0±1.00	16.0±0.57	14.5±0.50	14.0±0.57	13.02±0.76	17.5±0.57	-	-	-
Control	18.0±1.00	23.0±0.57	14.0±1.00	23.0±0.57	26.0±1.00	23.5±0.57	24.0±0.57	25.5±0.57	12.0±1.0	13.0±1.0	18.0±1.0

Fungal strains = CA-C*andida albicans* and AN- *A. niger,* Bacterial strains = EC- *Escherichia coli*, PM- *Proteus mirabilis*, KP- *Klebsiella pneumoniae*, PA-*Pseudomonas aeruginosa*, SF- *Shigella flexneri*, SM- *Serratia marcescens*, ST-*Salmonella typhi,* EF- *Enterococcus faecalis* and SA- *Staphylococcus aureus* . Control for fungal strains= Ketoconazole (10µg). Control for bacterial strains= Streptomycin (10µg).

Table 2: Minimum Inhibitory Concentration (MIC in mg/ml) of Various Fungal Extracts.

Fungal isolates	CA	AN	PM	KP	PA	SF	EF	SA
Fusarium sp.	-	3.75	5.0	-	5.0	-	5.0	5.0
Coprinopsis cinerea	5.0	3.75	5.0	3.75	-	-	5.0	-
A. flavus	5.0	3.75	-	3.75	-	5.0	-	5.0
Unknown fungus	5.0	3.75	5.0	3.75	5.0	-	-	-
Isaria tenuipes	5.0	3.75	-	3.75	5.0	5.0	-	-
Aspergillus sp.	5.0	3.75	-	3.75	5.0	7.5	5.0	5.0
A. niger	3.75	3.75	-	1.87	1.87	1.87	3.75	-
A. tubingensis	3.75	3.75	3.75	1.87	-	3.75	3.75	3.75
Curlvularia lunata	-	3.75	-	3.75	-	-	-	-
S. racemosum	7.5	3.75	-	3.75	-	-	-	-
Choanephora sp.	7.5	3.75	-	3.75	-	-	-	-
Chaetonium sp.	7.5	3.75	-	3.75	-	-	-	-
Trichoderma longibrachiatum	-	3.75	-	3.75	5.0	-	5.0	-
A. japonicus	-	3.75	-	-	-	5.0	5.0	5.0
A. terreus	3.75	3.75	3.75	1.87	1.87	1.87	3.75	3.75

Fungal strains = CA-*Candida albicans*, AN-*A. niger.* Bacterial strains =PM- *Proteus mirabilis*, KP- *Klebsiella pneumoniae*, PA-*Pseudomonas aeruginosa*, SF- *Shigella flexneri*, EF- *Enterococcus* faecalis and SA-*Staphylococcus aureus.*

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