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Phytochemical Analysis and Antibacterial Activity of Endophytic Fungi Isolated from *Basella rubra* L. - A Medicinal Plant

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Now-a-days endophytic fungi serve as a source of antimicrobial compounds. In the present study a total of 21 endophytic fungi were isolated from *Basella rubra* L. a medicinal plant and screened for their antibacterial potential and for the presence phytochemical constituents. The results of the preliminary screening of antibacterial activity showed that out of 21 endophytic fungi *Chaetomium indicum*, *Cladosporium sphaerospermum*, *Curvularia lunata* and *Curvularia pallescens* showed inhibition to all the test pathogens with a maximum zone of inhibition of 20 mm and a minimum of 11 mm. Likewise, crude ethyl acetate extract of these four endophytic fungi, the crude extracts of *Chaetomium indicum* offered 21, 21, 28 and 35 mm of inhibition zone against *B. subtilis*, *E. coli*, *S. typhi* and *Staph. aureus*, respectively followed by *Curvularia lunata*, *Chaetomium indicum* and *Aspergillus flavus extracts*. The zone of inhibition obtained in the secondary screening can be attributed to the antimicrobial potency of the antibiotic streptomycin used in the present study.

Key words: Antibacterial activity, Endophytic fungi, *Basella rubra,* Medicinal plant.

Most, if not all, plants studied in natural ecosystems are infested by fungi that cause no disease symptoms. These fungi are called endophytes, in contrast to parasites, which lead to disease and reduce the fitness of their host plants. There are reports that endophytes can become parasites under certain conditions and vice versa[1]. Hence, host microbe interactions can range from mutualism through commensalism to parasitism in a continuous manner[2,3]. Endophytes are considered to be plant mutualist's because they receive nutrition and protection from the host plant while the host plant may benefit from enhanced competitive abilities and increased resistance to herbivores, pathogens, and various abiotic stresses[4]. But some fungal endophytes may become plant pathogens, depending on the developmental stage of host and fungus, environmental factors, and host defense responses[1].

Role of natural products in the field of medicine, industry, and agriculture has increased. In recent past most of the drugs currently in use have been isolated from plants and more recently attention has turned to endophytic microorganisms as they exhibit immense potential for new bioactive compounds[5]. Endophytes are regarded as a source of natural bioactive products

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and some of these are capable of synthesizing bioactive compounds that can be used for defense against human pathogens and also been proven useful for novel drug discovery[6-8]. These endophytes may also benefit the host plant by producing bioactive substances to enhance plant growth and competitiveness of the host in nature[8].

A high proportion of endophytic fungi (80%) produce biologically active compounds in tests for antibacterial, fungicidal and herbicidal activities[9]. The continued development of new antimicrobial compounds is important to overcome the difficulties related to the treatment of infections caused by resistant pathogens[10]. Thus, endophytic fungi have emerged as an alternative source for the production of new antimicrobial agents. Owing to their great importance of secondary metabolite production by endophytic fungi, the study was undertaken to isolate endophytic fungi from a medicinal plant *Basella rubra* L. and to evaluate their phytochemical constituents along with their antibacterial potential.

MATERIALS AND METHODS

Collection of plant material

Healthy plants of *Basella rubra* were collected from Mysore region, Karnataka. For sampling selection, the plant was randomly collected from different sites in the same vicinity for the study. The healthy stems and leaves of *B. rubra* were collected and brought to the laboratory and processed immediately to reduce the risk of contamination and subjected for isolation of endophytic fungi.

Isolation and identification of endophytic fungi

The collected plant samples were washed thoroughly 2-3 times in running tap water to remove the dust and debris present on the surface of explant, followed by repeated washing in distilled water. After proper washing, stem and leaf samples were cut into small pieces (1 to 2 cm length) under aseptic condition using sterile scalpel. Endophytic fungi were isolated following standardized and modified method[11]. The efficiency of surface sterilization procedure was ascertained for every segment of tissue following the imprint method. About 08 to 10 stem segments were placed on Petri plates containing 20 ml of potato dextrose agar (PDA) medium supplemented with antibiotic chloramphenicol to avoid the emergence of endophytic bacteria and incubated at 25±2° C for 15 days. Endophytic fungal colonies emerging from their host were picked with sterile fine tip needle and sub cultured on to Petri plates containing PDA devoid of antibiotic to obtain pure cultures. The fungi were identified based on their morphological, conidial and cultural characters[12]. All the fungal isolates were maintained in test tubes and Petri plates on PDA media.

Evaluation of antibacterial activity

Test organisms

All the isolated endophytic fungal isolates were screened for antibacterial activity. The indicator bacteria included both Gram-positive (*Staphylococcus aureus* MTCC 7443 and *Bacillus subtilis* MTCC 121) and Gram-negative (*Escherichia coli* MTCC 7410 and *Salmonella typhi* MTCC 733) bacteria obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India and used throughout the study. All the microbial cultures were adjusted to 0.5 McFarland standards, which is visually comparable to a microbial suspension of approximately $1.5x10^8$ cfu/ ml.

Preliminary screening

The preliminary screening of antibacterial activity was done by following the agar plug method[13]. To evaluate antibacterial activity, the nutrient agar (NA) medium was poured into Petri plates and inoculated with 100 µl of the bacterial suspension ($1.5x10^8$ cfu/ ml) and spread uniformly by using a sterile cotton bud on the medium. Mycelial discs (6 mm) of each endophytic fungal isolate (15 day-old) grown on PDA were obtained from actively growing margins isolates using a sterile cork borer and placed on the surface of the NA medium previously seeded with test organisms[14]. PDA media (6 mm discs) devoid of any fungal colony served as negative control. The plates were sealed using Para film and incubated at 37° C for 24 h. After incubation, antibacterial activity was confirmed by the visualization and measurement of inhibition zones. The average of three repeated trials was taken to evaluate the antibacterial activity.

Fermentation and extraction of secondary metabolites

The endophytic fungal isolates which offered antibacterial activity in primary screening were subjected to fermentation. Each endophytic fungus (5 -10 discs) was picked from actively growing margins and were fermented in 1000 ml Erlenmeyer flasks containing 500 ml of PDB for 21 days at 25 ± 2° C under static conditions devoid of antibiotic. After incubation, the culture broth was filtered through double layer sterile muslin cloth to harvest mycelium. The culture filtrate was extracted with ethyl acetate (500 ml x 3) followed by evaporation of the solvent using flash evaporator.

Secondary screening

The fungal isolates with relatively broader antibacterial spectrum or stronger activities shown in preliminary assay were selected for secondary assay. The secondary antibacterial screening was done by following disc diffusion method[15]. The test bacteria ($1.5x10^8$ cfu/ ml) were seeded onto the surface of NA media and uniformly spread using sterile cotton bud. Each sterile disc (6 mm) were loaded with 50 µl of fungal extract (concentration 1 mg/ disc) and 50 µl of ethyl acetate and equidistantly placed on NA plates. Streptomycin discs were also used as standard. The plates were sealed using Para film and incubated at 37° C for 24 h. After incubation, antibacterial activity was confirmed by the visualization and measurement of inhibition zones. The average of three repeated trials was taken to evaluate the antibacterial activity.

Phytochemical Screening

The crude ethyl acetate extracts of endophytic fungi of *B. rubra* plant which exhibited stronger antibacterial activities in preliminary assay were subjected to qualitative phytochemical screening for identification of various classes of active chemical constituents like alkaloids, carbohydrates, proteins, aminoacids, phytosterols, phenolic compounds, flavonoids and anthraquinones using the standard methods[16-17].

Statistical Analysis

Data from three replicates were analyzed for each experiment and analysis of variance (ANOVA) using SPSS Inc. 16.0. Significant effects of treatments were determined by F values ($P \leq 0.05$). Treatment means were separated by Tukey's Honestly Significant Differences (HSD) test.

RESULTS

Isolation and identification of endophytic fungi

A total of 21 endophytic fungi were isolated from a total of 50 stem and 50 leaf segments of *B. rubra* (Table 1). The overall colonization frequency of endophytic fungi from stem and leaves was found to be 21% (stem- 18% and leaves- 24%). The isolated endophytic fungi were classified into 9 different taxa of *Alternaria* sp., *Aspergillus* sp., *Chaetomium* sp., *Cercospora* sp., *Cladosporium* sp., *Corynespora* sp., *Curvularia* sp., *Nigrospora* sp. and *Monilia* sp. Among the isolated fungi *Curvularia* sp. was the dominant fungus showing 28.57% of colonization frequency, followed by *Cladosporium sp.* with colonization frequency of 23.8%. *Aspergillus* sp., *Cercospora* sp. and *Chaetomium indicum* with colonization frequency of 9.52%, *Corynespora* sp., *Alternaria* sp., *Nigrospora* sp. and *Monilia* sp.with colonization frequency of 4.76% (Table 2).

Evaluation of Antibacterial activity

Preliminary screening

All the endophytic fungi isolated were screened for their antibacterial activity against four test bacteria as mentioned above. Out of 24 isolates six isolates were able to inhibit the test bacteria. The results revealed that all the endophytic fungi offered varied degree of inhibition against the test pathogens. Among the tested endophytic fungi, *Chaetomium indicum*, *Cladosporium*

Table 1. Endophytic fungi isolated from stem and leaf segments of *B. rubra*

Parts used	No. of samples	No. of fungi isolated	Frequency of colonization (%)
Stem	50	09	18%
Leaves	50	12	24%
Total	100	21	21%

sphaerospermum, *Curvularia lunata* and *Curvularia pallescens* showed inhibition to all the test pathogens with a maximum zone of inhibition of 20 mm and a minimum of 11 against test pathogens, while all the other endophytic fungi except *Cladosporium* sp. and *Cercospora* sp. showed no inhibition to test pathogens (Table 3).

Secondary screening

The crude ethyl acetate extracts of selected endophytic fungi were further subjected for their potential to inhibit test bacterial pathogens. Among the tested endophytic fungi, crude extracts of *Chaetomium indicum* offered maximum inhibition of 21, 21, 28 and 35 mm of inhibition zone against *B. subtilis*, *E. coli*, *S. typhi* and *Staph. aureus*, respectively followed by *Curvularia lunata*, *C. pallescens* and *Aspergillus flavus* (Fig. 1). The results of the study can be attributed to the antimicrobial potency of the antibiotic streptomycin used in the study (Table 4).

Phytochemical screening

Phytochemical analysis was carried out on the isolated endophytic fungal extracts to

Table 2. Frequency of endophytic fungi isolated from the stem and leaf of *B. rubra*

Endophytic fungi	No. of isolates	Colonization rate (%) Stem	Leaves	Dominant fungi (%)
Alternaria alternata	01	-	01	8.33
Aspergillus flavus	02	-	02	16.66
Chaetomium indicum	02	02	-	22.22
Cercospora sp.	02	-	02	16.66
Cladosporium sphaerospermum	01	01	-	11.11
Cladosporium sp.	04	-	04	33.33
Corynespora sp.	01	01	-	11.11
Curvularia sp.	03	03	-	33.33
Curvularia lunata	02	-	02	16.66
Curvularia pallescens	01	-	01	8.33
Monilia sp.	01	01	-	11.11
Nigrospora sp.	01	01	-	11.11

Table 3. Preliminary antibacterial screening of endophytic fungi (inhibition zone in mm)

Endophytic fungi	Zone of inhibition (mm) *Bacillus subtilis*	*Escherichia coli*	*Salmonella typhi*	*Staphylococcus aureus*
Alternaria alternata	0.0 ± 0.0^{f}	0.0 ± 0.0^{d}	0.0 ± 0.0^{f}	0.0 ± 0.0^{e}
Aspergillus flavus	11 ± 0.1^{e}	15 ± 0.2^{c}	15 ± 0.2^{d}	13 ± 0.3^{d}
Chaetomium indicum	12 ± 0.2^{d}	20 ± 0.3^{b}	17 ± 0.4^{c}	15 ± 0.2^{c}
Cercospora sp.	15 ± 0.3^{c}	0.0 ± 0.0^{d}	0.0 ± 0.0^{f}	25 ± 0.4^{b}
Cladosporium sphaerospermum	0.0 ± 0.0^{f}	0.0 ± 0.0^{d}	0.0 ± 0.0^{f}	0.0 ± 0.0^{e}
Cladosporium sp.	20 ± 0.2^{b}	0.0 ± 0.0^{d}	18 ± 0.1^{b}	0.0 ± 0.0^{e}
Corynespora sp.	0.0 ± 0.0^{f}	0.0 ± 0.0^{d}	0.0 ± 0.0^{f}	0.0 ± 0.0^{e}
Curvularia sp.	0.0 ± 0.0^{f}	0.0 ± 0.0^{d}	0.0 ± 0.0^{f}	0.0 ± 0.0^{e}
Curvularia lunata	11 ± 0.4^{e}	15 ± 0.1^{c}	15 ± 0.3^{d}	13 ± 0.4^{d}
Curvularia pallescens	11 ± 0.3^{e}	15 ± 0.2^{c}	11 ± 0.2^{e}	15 ± 0.1^{c}
Nigrospora sp.	0.0 ± 0.0^{f}	0.0 ± 0.0^{d}	0.0 ± 0.0^{f}	0.0 ± 0.0^{e}
Monilia sp.	0.0 ± 0.0^{f}	0.0 ± 0.0^{d}	0.0 ± 0.0^{f}	0.0 ± 0.0^{e}
Positive control	30 ± 0.2^{a}	30 ± 0.0^{a}	30 ± 0.1^{a}	30 ± 0.2^{a}
Negative control	0.0 ± 0.0^{f}	0.0 ± 0.0^{d}	0.0 ± 0.0^{f}	0.0 ± 0.0^{e}

Values are means of three independent replicates. ± indicate standard error. Means followed by the same letter(s) within the same column are not significantly different according to Tukey's HSD.

determine the presence of phytochemical components. In the current study, phytochemical analysis of ethyl acetate extracts of all the selected endophytic fungi showed the presence of carbohydrates and phytosterols, while alkaloids were only present in the crude extracts of *Curvularia lunata* and *Curvularia pallescens*, while the other phytochemical constituents tested in the present study were absent in all the extracts (Table 5).

DISCUSSION

Herbs and herbal products are known to have antibacterial potential[18]. Herbal treatments become very popular because it is easily available, cheaper and less toxic than the synthetic drugs. Plants are often colonized by many fungi that do not cause any disease symptoms. Some fungi are organ specific and colonization frequency varies with environmental conditions[19]. The colonization of the endophytic fungi is ubiquitous yet selective in nature. This selective colonization of the endophyte may lead to the production of special compounds within the host plant[20]. Numerous studies have been conducted with the crude extracts of endophytic fungi from various plants, screening antimicrobial activity as well as for the discovery of new antimicrobial compounds[21]. The search for antimicrobial compounds from natural sources has received much attention and efforts have been put in to identify compounds that can act as suitable antimicrobials agent to replace synthetic ones.

In the present investigation, endophytic fungi were isolated from leaf and stem segments of *B. rubra* and a total of 21 endophytic fungi belonging to eight different genera were isolated. This low rate of colonization may be attributed to the secretion of the phyto-chemicals, since they

Table 4. Secondary antibacterial screening (inhibition zone in mm)

Endophytic fungi	Zone of inhibition (mm)			
	Bacillus subtilis	*Escherichia coli*	*Salmonella typhi*	*Staphylococcus aureus*
Aspergillus flavus	15.3±0.3^{e}	17.2±0.2^{c}	15.1±0.2^{d}	22.2±0.3^{c}
Chaetomium indicum	21.2±0.2^{c}	21.1±0.4^{b}	28.2± 0.4^{b}	35.4±0.4^{a}
Curvularia lunata	28.4±0.2^{b}	15.2±0.2^{d}	25.4±0.5^{c}	18.2±0.2^{e}
Curvularia pallescens	16.2±0.1^{d}	13.1±0.3^{e}	25.1±0.2^{c}	18.6±0.3^{d}
Streptomycin	30.2±0.2^{a}	30.4±0.1^{a}	31.0±0.1^{a}	30.2±0.2^{b}
Negative control	0.0^{f}	0.0^{f}	0.0^{e}	0.0^{f}

Values are means of three independent replicates. ± indicate standard error. Means followed by the same letter(s) within the same column are not significantly different according to Tukey's HSD

Table 5. Phytochemical analysis of ethyl acetate extracts of selected endophytic fungi

Phytochemical analysis	Endophytic Fungi			
	Aspergillus flavus	*Chaetomium indicum*	*Curvularia lunata*	*Curvularia pallescens*
Alkoloids	--	-	++	++
Carbohydrates	++	++	++	++
Proteins	--	--	--	--
Amino acid	--	--	--	--
Phytosterols	++	++	++	++
Phenolic compounds	--	--	--	--
Flavonoids	--	--	--	--
Anthraquinines	--	--	--	--

Note: + Presence, - absence

contain certain antifungal and antibacterial components[22]. Further, all the isolated endophytic fungi were subjected to antibacterial activity against both Gram positive and Gram negative bacteria. The preliminary screening results of antibacterial activity showed that, among the endophytic fungi tested, *Aspergillus flavus*, *Chaetomium indicum*, *Curvularia lunata* and *Curvularia parescens* showed antibacterial activity against the test pathogens. Likewise, the isolated endophytic fungi when subjected for preliminary screening and fermentation assay, the isolates were found to produce antimicrobial metabolites against bacteria and fungi[23].

The endophytic fungi which showed antibacterial activity in preliminary screening were subjected for fermentation assay and further the crude extracts were obtained through ethyl acetate extraction. The crude extracts from the culture of endophytic fungi grown aerobically in PDA medium displayed anti-bacterial activity. The ethyl acetate crude extracts of all the four endophytic isolates were effective against all the bacterial strains tested in the present study. These results might be

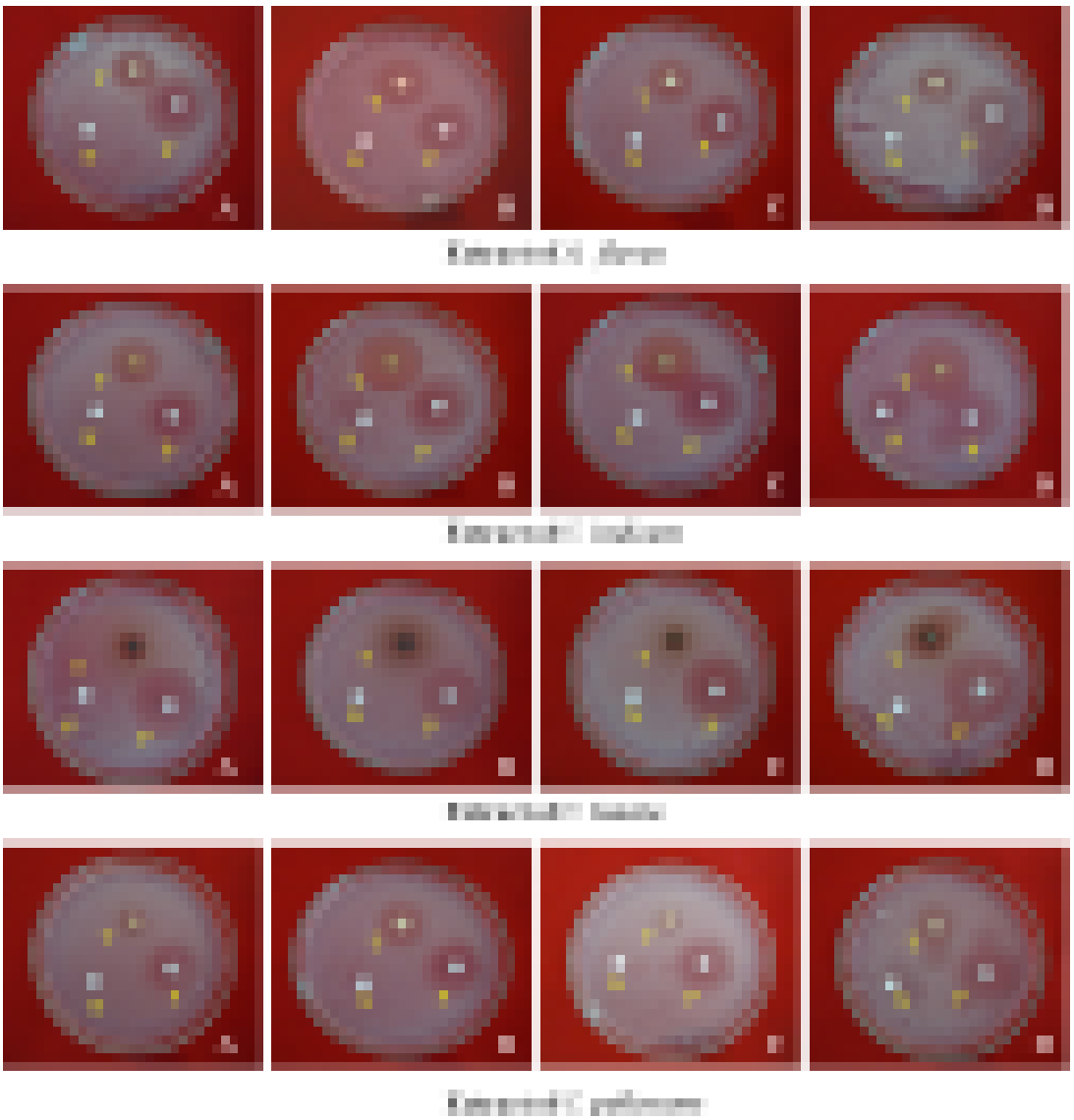

Fig. 1. Antibacterial activity of selected endophytic fungi against test pathogens by disc diffusion method. A: *B. subtilis*; B: *Staph. aureus*; C: *E. coli*; D: S. *typhi*; N: Negative control; P: Positive control; T: Treatment

attributed either to the antimicrobial potency of the extract or to the high concentration of unidentified active principle in the extracts. Similarly, crude extracts of endophytic fungi yielded more potent compounds once they had undergone some purification[24].

Similarly, the ethyl acetate extract of endophytic fungi were also evaluated for its phytochemical constituents using standard procedures[16-17]. The results of the study revealed the presence of alkaloids, carbohydrates and phytosterols which are known to be biologically active and therefore aid the antimicrobial activities of *B. rubra*. Similar results were also observed with *Penicillium* sp. isolated from *Centella asiatica*[25]. Likewise, the phytochemical screening of crude extracts of the endophytic fungi isolated from *Kigelia africana*, revealed the existence of a diverse group of secondary metabolites, which also resembled those in the host plant extracts[26]. Similarly, ethyl acetate extracts of endophytic fungi *C. gloeosporioides* isolated from *Plumeria acuminata* and *P. obtusifolia* revealed the presence of alkaloids, flavonoids, steroids, phenol and phenolic compounds[27]. The phytochemical analysis of endophytes isolated from *Ginkgo biloba* also showed a varying number of secondary metabolites in the ethyl acetate extract[28]. Hence from the present findings it can be stated that, the endophytic fungal crude extracts from *B. rubra* have a wide spectrum of antibacterial activity. Further studies are needed to identify the active metabolites using analytical chemistry and to discover more about the symbiotic role of these fungi in *B. rubra* in order to understand the benefits that these endophytes confer on this medicinal plant.

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