Journal of Applied and Natural Science
10 (4): 1286-1290 (2018)
ISSN : 0974-9411 (Print), 2231-5209 (Online)
journals.ansfoundation.org

Antibacterial activity of endophytic fungi from some medicinal plants of Biligirirangana hill, India

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Article Info
DOI:10.31018/jans.v10i4.1923
Received: October 20, 2018
Revised: November 20, 2018
Accepted: November 28, 2018

How to Cite
Sushma, K.S. *et al*. (2018). Antibacterial activity of endophytic fungi from some medicinal plants of Biligirirangana hill, India. *Journal of Applied and Natural Science*, 10(4): 1286-1290

Abstract
Twenty fungal endophytes were isolated from different medicinal plants of Biligirirangana Hill, Chamarajanagar dist. Phytochemical analysis of ethyl acetate extract showed positive results for tanins, flavonoids, steroids, terpenoids, glycosides, saponins and alkaloids except saponin and flavonoid tests. The crude extracts of the fungal endophytes were tested against two gram positive and two gramnegetive bacteria for its antibacterial activity. The highest zone of inhibition was produced by *Fusarium sps* (AB9)35mm. All the crude extracts were found to be effective against *Escherichia coli, Bacillus subtilis* and *Staphylococcus aureus* rather in *Pseudomonas aerogenosa.* Among them the highest inhibition zone was produced in *Penicillium* sp (AB11) 24mm, *Cladosporium sp*(AB3) 21mm, and *Aspergillus sp*(AB12) 30mm. Therefore, endophytic fungi can be a good source to inhibit the growth of harmful pathogens.

Keywords: Antibacterial activity, Crude extracts, Endophytic fungi, Phytochemical analysis

INTRODUCTION

Plants consists of endophytes which colonize inter or intracellular tissue of the host plant and spend whole or part of its life cycle without causing any apparent symptoms (Tan and Zhou, 2001). Endophyic fungi are the group of unexplored organism of bioactive potential compounds (Palem *et al*., 2016). All plant species harbor endophytes in order to protect host against pathogens, environmental factors, nutritional status, as well as the developmental stages of the host and endophyte (Schulz and Boyle 2006). Endophytes constitutes large microbial diversity which are the sources of secondary metabolites helps in the production of novel bioactive molecules. After the discovery of (Taxol) paclitaxel an anticancer drug was isolated from *Taxomyces adrenae* (Stierle *et al*., 1993). Endophytic fungi exhibit properties such as anticancer, anti-fungal, anti-bacterial (Wiyakrutta *et al*., 2004) anti-diabetic andimmuno-suppressant compound (Strobel and Daisy, 2003). The endophytic fungi produce constituents such as alkaloids, steroids, tanins, flavonoids and phenolic compounds (Hill, 1952). The present study investigates the phytochemical analysis and antibacterial activity against human pathogen.

MATERIALS AND METHODS

Collection of samples: Medicinal plants (Table 1) were collected from BR Hills, Chamarajanagar district, hill range situated in south eastern part of Karnataka, lies in the coordinates of 77°–77°16'E, 11°47'– 12°9'N, covering an area of 540 sq km (Madegowda 2009). Plants were identified based on morphological characteristics (Bremer *et al*., 1998). Fresh healthy plants samples were collected in sterile polythene bags and brought to the laboratory within 24 hrs.

Isolation of endophytic fungi: The collected plants samples were surface sterilizedwith some modification (Barmet, 1992). Plant material was first washed under running tap water to remove

dirt, washed with distilled water then sequentially rinsing the plant material with 70% ethanol for 30 sec followed by 0.5% sodium hypochloritefor 2–3 min, and rinse with 70% ethanol for 2 min, and finally with distilled water. The plant materials were cut into small segments aseptically and dried using sterile filter papers. The dried segments were placed in petri dishes containing Sabaraud dextrose agar (SDA) media and incubated at 27°C for 4–6 days. After incubation period, the visual growth for each fungus was observed. Each colony of the fungal isolates were then transferred separately to obtain pure culture and maintained at 4°C for further use.

Identification of endophytic fungi: The morphological study of fungal colony were observed bygrowing the fungi on SDA media and incubating for 7 days and was noted by observing back and front views of the plates macroscopically (Barnett and Hunter, 1972). The unknown fungal colony was identified microscopically by studying their cultural characteristics, mycelium andspore formation. Slides were prepared by wet mount method using lacto phenol cotton blue stain and observed under binocular microscope at 40x and 100x magnifications (Schulz *et al*, 1993).

Preparation of crude extracts: Preparation of crude extracts was followed by the procedure of Subramanian (1983).Theisolates of endophytic fungi were inoculated into 250 ml Erlenmeyer flasks containing 100 mL sabaruads dextrose broth and incubated at room temperature for 21 days ina rotary shaker. After incubation, the broth culture was filtered to separate mycelia and the filtrate. To the filtrate equal volume of ethyl acetate was added, mixed well for 10 minutes and kept till the two clear immiscible layers were formed. The upper layer of ethyl acetate containing the extracted compounds was separated using separating funnel. The mycelium was grinded in pestle and mortar using ethyl acetate as solvent and filtered using cheese cloth. Both mycelia and culture filtrate extracts were pooled together and evaporated to dryness in hot air oven of 100°C and stored at 4°C to be used for further analysis.

Tests against human pathogens: The antibacterial potentiality of the isolated endophytic fungi was tested against four human pathogenic bacteria two Gram positive *Bacillus subtilis* and *Staphylococcus aureus* and two Gram negative bacteria *Escherichia coli* and *Pseudomonas aerogenosa* collected from Microbial Type Culture Collection (MTCC), Chandigarh and maintained in our laboratory.

Agar well diffusion method: Agar well diffusion test was performed according to(Lin *et al*, 2002) by using sterilized Mueller Hinton Agar (pH 7.3 ± 0.1). These plates were swabbed by bacterial cultures and wells were prepared with a sterile cork borer of diameter 6mm in a distance of 15mm from each well. Different concentrations (30µl, 50µl and 100µl) of the fungal extracts were dispensed aseptically included with their respective controls using a micropipette and incubated at 37° C for 24 hours. The diameters of the zone of inhibition (ZOI) were measured with a ruler (mm).

Phytochemical property: The endophytic fungal crude extracts were subjected to qualitative chemical tests to determine phytochemical constituents carried out on the aqueous extract using standard procedures to identify compounds (Collee *et al*, 1996), (Harborne, 1973), (Bandoni *et al*, 1976).

Tanins: About 1 ml of the samplewas taken and few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black coloration.

Flavornoids: 5 ml of dilute ammonia solution were added to a portion of the aqueous filtrate of each plant extract followed by addition of concentrated H_2SO_4. A yellow coloration observed in each extract indicated the presence of flavonoids.

Steriods: Two ml of acetic anhydride was added to 1ml of sample with 2 mlH_2SO_4. The colour changed from violet to blue or green in samples indicating the presence of steroids.

Terpenoids: Five ml of each extract was mixed in 2 ml of chloroform, and 3ml of concentrated H_2SO_4 was carefully added to form a layer. A reddish brown coloration of the inter face was formed to show positive results for the presence of terpenoids.

Glycosides: Five ml of each extracts was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This wasunderlayed with 1 ml of concentrated sulphuric acid. A violet ring may appear below the brown ring indicates positive result.

Saponin: 2 ml of sample was mixed with 3 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion.

Alkaloids: To the sample a drop of iodine was added, the appearance of reddish brown indicates positive result for alkaloids.

RESULTS AND DISCUSSION

The ethyl acetate crude extract of isolated fungal endophytes from the few selected medicinal plants tested against two gram positive and two gram negative pathogenic bacteria for antibacterial activity are given in Table 1 while the zone of inhibitions measured in mmare mentioned in Table 2. All the crude extracts were found to be effective against *Escherichia coli, Bacillus subtilis* and *Staphylococcus aureus* and not in *Pseudomonas aerogenosa.* Among them *Penicillium sp, Cladosporium sp, Aspergillus sp.* and *Fusarium sp.*showed the highest zone of inhibition of 24 mm, 21 mm 30 mm and 35 mm. AB12 isolate

Table 1. List of endophytic fungi isolated and identified from few selected medicinal plants collected from BR Hills.

Sl. No.	Code no.	Identified isolates	Isolated from medicinal plant
1.	AB1	*Cladosporium sp*	*Tylophoraindica*
2.	AB2	*Fusarium oxysporum*	*Eucalyptus nilgirans*
3.	AB3	*Cladosporium cladosporoids*	*Rauvolfiatetraphyla*
4.	AB4	*Alternaria alternate*	*Citrus sinensis*
5.	AB5	*Colletotricum sp.*	*Tylophoraindica*
6.	AB6	*Gliocladium sp.*	*Emblica officinalis*
7.	AB7	*Penicillium notatum*	*Gymnemasylvestri*
8.	AB8	*Penicillium sp.*	*Azadirachtaindica*
9.	AB9	*Fusarium sp.*	*Eucalyptus nigirans*
10.	AB10	*Alternaria sp.*	*Murrayakoiengii*
11.	AB11	*Penicillium sp*	*Fever plant*
12.	AB12	*Aspergillus repens*	*Citrus medica*
13.	AB13	*Phomaherbarum*	*Cycleapeltata*
14.	AB14	*Phyllostictasp*	*Asclepiascurassavica*
15.	AB15	*Penicillumitalicum*	*Catharanthesrosea*
16.	AB16	*Alternaria sp*	*Eryngium foetidum*
17.	AB17	*Bipolaris maydis*	*Ocimumtenuliformis*
18.	AB18	*Mycelia*	*Tinosporacordifolia*
19.	AB19	*Leptospherulinaarachidicola*	*Tinosporacordifolia*
20.	AB20	*Colletotrichum sp.*	*Ricinus communis*

Table 2. Antibacterial activity of the endophytic fungal isolates against four human pathogens.

Sl.No.	Code no. of fungal isolates	Zone of Inhibition measured in (mm)			
		Escherichia coli	***Bacillus subtilis***	***Staphylococcus aureus***	***Pseudomonas aeruginosa***
1.	AB1	15	15	20	-
2.	AB2	16	14	20	-
3.	AB3	30	14	21	-
4.	AB4	12	17	18	-
5.	AB5	15	15	19	-
6.	AB6	15	18	18	-
7.	AB7	16	18	17	-
8.	AB8	23	24	23	-
9.	AB9	17	14	35	-
10.	AB10	12	16	17	-
11.	AB11	24	22	22	12
12.	AB12	30	30	30	25
13.	AB13	22	22	21	-
14.	AB14	-	20	23	-
15.	AB15	16	16	10	-
16.	AB16	16	16	14	-
17.	AB17	18	22	17	-
18.	AB18	16	24	15	-
19.	AB19	30	30	-	-
20.	AB20	-	-	30	-

could inhibit all these four bacteria. 50µl and 100µl of the extracts produced maximum zone of inhibition in Gram positive bacteria *S. aureus* (30mm) and *B. subtilis*(30mm) and Gram-negative bacteria *P. aerogenosa*(25mm) and *E. coli*(30mm) which showed antibacterial activity. AB8 isolate showed inhibition zone in *S. aureus* (23mm), *B. subtilis* (24mm), *E. coli* (23mm) and no zone was formed in *P. aerogenosa*. AB10 isolate formed the minimum zone of inhibition (Table 2). The antibacterial activity of fungal samples resistant to pathogenic bacteria further screened for phytochemical constituents'analysis of the crude extracts for the presence of tanins, flavonoids, steroids, terpenoids, glycosides, saponins and alkaloids showed positive results except for saponin and flavonoid tests (Table 3).

Antibacterial activities for endophytic fungi were found to be effective against Gram positive bacteria *S. aureus*, *B. subtilis* and Gram-negative bacteria *P. aerogenosa*, *E. coli*. The result showed that fungal crude ethyl acetate extract inhibited gram-positive than gram-negative bacteria. The endophyte could inhibit the growth of Gram positive bacteria *Staphylococcus aureus* and *Bacillus subtilis* to a greater degree than Gram negative bacteria *Escherichia coli* (Chareprasert *et al*, 2006). The antimicrobial activity of the fungal isolates have

Table 3. Phytochemical analysis of the fungal crude extracts for the presence of compounds.

Sample	Tanin	Flavanoid	Steroid	Terpenoid	Glycosides	Saponin	Alkaloid
AB1	+	-	+	+	-	-	+
AB2	+	-	+	+	+	-	+
AB3	+	-	+	+	+	-	+
AB4	+	-	-	-	+	-	+
AB5	+	-	+	-	-	-	+
AB6	+	-	-	+	-	-	-
AB7	+	-	+	+	+	-	+
AB8	+	-	+	+	+	-	+
AB9	-	-	-	+	-	-	+
AB10	-	-	-	-	-	-	-
AB11	+	-	+	+	+	-	+
AB12	+	-	+	+	+	-	+
AB13	+	-	+	+	+	-	+
AB14	-	-	-	+	-	-	+
AB15	-	-	+	+	-	-	-
AB16	-	-	-	+	+	-	+
AB17	+	-	-	-	+	-	+
AB18	+	-	-	+	-	-	+
AB19	-	-	+	-	-	-	+
AB20	+	-	+	+	-	-	+

+ Present, - Absent

shown good inhibition *Gliocladium sp* (80%) followed by *Penicillium adametzi*(73.3%) and *P. chrysogenum* (71.1%) (Vinu and Jayashankara, 2011). Antibacterial activity of endophytic fungi was observed against selected test organisms. The number of extracts of sea grasses *Cymodoceaserrulata*, *Halophila ovalis* and *Zosteracapensis* indicated that sea grasses are good source of antimicrobial-producing endophytic fungiwith some extracts also possess antibacterial activity(Kumar *et al*, 2008).

The active metabolites contain chemical groups such as phenols, steroids, flavonoids, quinines, terpenoids, xantones, peptides, cytocatalasins, alkaloids, aliphatic compounds, and phenyl propanoids (Ladoh-Yemed *et al*., 2015) In the current study, phytochemical analysis of ethyl acetate extracts of most endophtic fungi extract showed the presence of alkaloids, steroids, tannin and glycoside. A study for (Ramesha and Srinivas, 2014) revealed the presence of alkaloids, flavonoids, steroids, phenol and phenolic compounds in crude extracts of endophytic fungi isolated from *Plumeria acuminata* L. and *Plumeria obtusifolia* L. Our results are in accordance with previous reports wherein the endophytes have shown the presence of different phytochemicals have antimicrobial activities. In addition, the use of endophytes as potential factories for the production of secondary metabolites might revolutionize agricultural, pharmaceutical and biotechnological research in the near future (Suresh *et al*., 2013)

Conclusion

The present study represents the antibacterial activity of endophytic fungi isolated from medicinal plants, most of isolates had inhibited active ranged between 12 -30 mm against tested strains. Compounds from tested plants can be exploited to commercial values provided in vivo assessment of the compounds is studied in future and could be a potential source of bioactive compounds to explore for the development of new drugs for human diseases and phytopathogens.

ACKNOWLEDGEMENTS

The authors are thankful to Mangalore University for providing the fellowships and all the facilities required to carry out this work.

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