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Preliminary screening of endophytic fungi from *Enicostemma axillare* (Lam.) Raynal. for antimicrobial activity

Ujwala S. Deepake, Yamilee Das, Shital Algunde and G. Gyananath*

School of Life Sciences, Swami Ramanand Teerth Marathwada University, Nanded-431606, Maharashtra, India.

Abstract

Enicostemma axillare (Lam.) Raynal. belonging to Gentianaceae family is a perennial herb found throughout India and is reported to possess medicinal properties. Considering the importance of this plant, a study was conducted to determine the colonization frequency of endophytic microbes and to evaluate the antimicrobial activity of crude extracts of fungal endophytes from *Enicostemma axillare.* The endophytic fungi from different parts of the plant (leaves, stem and roots) were isolated, pure cultures were raised and identified based on the morphology and characteristics of fungal spores. The highest colonization frequency of bacteria and actinomycetes was observed in leaves (100 %). However, colonization frequency of endophytic fungi was around 92 % higher in stem. The endophytic fungi that displayed broad spectrum antimicrobial activity include *Aspergillus flavus*, *Penicillium sp*., *Eurotium sp., Sartorya sp.* and *Phomopsis sp.* Our preliminary results indicate that crude extracts of endophytic fungi of *Enicostemma axillare* (Lam.) Raynal. may possess some antimicrobial compounds.

Keywords: Endophytic fungi, crude extracts, antimicrobial activity, *Enicostemma axillare.*

INTRODUCTION

Endophytic microbes and fungi have received considerable attention because of their antimicrobial properties and also as biopesticides [1]. A vast majority of plants have been recognized as a repository of fungal endophytes with novel metabolites of pharmaceutical importance [2-4]. *Enicostemma axillare* (Lam.) Raynal. belonging to Gentianaceae family is a medicinal plant and widely distributed in South America, Africa, and Asia. It is a perennial herb found throughout India and common in coastal areas. The parts of this plant are extremely bitter due to presence of amrogentian [5]. A survey of Ayurvedic literature showed that the fresh juice of leaves has been used as a bitter tonic to control arthritis, in typhoid fever and as cooling agent. The plant is used in folk medicine to treat diabetes mellitus, rheumatism, abdominal ulcers, hernia, swelling, itching and insect poisoning. It has anti-inflammatory, hypoglycemic, anticancer, antioxidant , antibacterial , and hepato-protective activity [6-11]. The lack of scientific data regarding fungal endophytes of this plant led us to determine colonization frequency of endophytic microbes, isolate and evaluate antimicrobial activity of endophytic fungal extracts against various pathogenic microorganisms.

MATERIALS AND METHODS

Nutrient agar, starch casein agar, potato dextrose agar, nystatin, cycloheximide, penicillin and streptomycin were purchased from HiMedia Pvt. Ltd., Mumbai. Ethyl acetate, diethyl ether, dichloromethane and dimethylsulfoxide were purchased from Sd. Fine Chemicals, Mumbai.. The microbial cultures were obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India.

Collection of Plant Materials

Healthy leaves, roots and stems of *Enicostemma axillare* (Lam.) Raynal. (Gentianaceae) were collected from Swami Ramanand Teerth Mararthwada University Campus, Nanded, Maharashtra, India in January 2011 (Figure 1). They were placed in sterile plastic bags and stored in a refrigerator prior to isolation of endophytic fungi.

Isolation of endophytic fungi and determination of colonization frequency of endophytic microbes

The colonization frequency of endophytic microbes and fungi was determined from surface sterilized plant segment according to the methods previously described with slight modification [12-13]. The surface sterilized segments were placed in petridish containing potato dextrose agar supplemented with penicillin (100 µg/ml) and streptomycin (100 µg/ml) for fungi, Starch casein agar supplemented with nystatin (50 µg/ml) and cycloheximide (50 µg/ml) for actinomycetes and Nutrient agar supplemented with nystatin (50 µg/ml) for bacteria. The plates were sealed, and incubated for 5 days (bacteria), 3 weeks (actinomycetes) and 5 weeks (fungi) at 30 ºC. The colonization frequency was determined as reported earlier [14]. Subsequently, endophytic fungi were isolated and pure cultures were maintained on potato dextrose agar slants at 4°C.

Extraction and determination of total yield of endophytic fungal extracts

Extraction was carried out as per the procedures described previously [15]. In brief, the crude extracts of fermentation broth of endophytic fungi were prepared in ethyl acetate, diethyl ether,

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*Corresponding Author

G. Gyananath
School of Life Sciences, S. R. T. M. University, Nanded, India.

Tel: +919850486910
Email: gyananath52@gmail.com

dichloromethane and water, evaporated to dryness and total yield of extract (mg) was determined by gravimetric method. The crude extracts were then dissolved in 3 ml of dimethyl sulphoxide for antimicrobial assay.

Test organisms & their growth conditions

Total 12 test microorganisms were selected for the study and among them eight were bacterial species and four were non-filamentous fungi. The pathogenic bacteria (*Escherichia coli* MTCC *2939, Bacillus subtilis* MTCC *1789, Proteus vulgaris* MTCC 1771*, Staphylococcus aureus* MTCC 96*, Pseudomonas morgani* MTCC 2487*, Bacillus sp. tc 09* (HQ844242)*, Bacillus pumilus strain P 012* (HQ844240) and *Bacterium P 07* (HQ844241)) were inoculated in nutrient broth & non-filamentous fungus viz *Candida albicans* (MTCC 3017), *Candida blankii* (MTCC 1442)*, Candida parapsilosis* (MTCC 1965) and *Cryptococcus neoformans* in yeast potato dextrose broth. Incubated for 24 to 48 hours with shaking & also prepared broth without test organism, this served as diluent. After incubation period, the optical density was taken at 625 nm. The optical density was adjusted to get 0.082 to 0.13 reading by adding diluent. This 0.5 Mcfarland standard of test organism was used for antimicrobial assay.

Antimicrobial activity of fungal extracts by agar well diffusion method

The endophytic fungal extracts were tested for antimicrobial activity by agar well diffusion method [16]. The ethyl acetate, diethyl ether, dichloromethane, dimethyl sulphoxide and sterile potato dextrose broth were used as control. The zone of inhibition was measured in mm.

Identification of endophytic fungi

The fungi were identified on the basis of colony characteristics, morphology and arrangements of spores on fungal hyphae by using slide culture technique [17]. These slides were observed for the morphology and arrangements of spores on fungal hyphae under light microscope [18].

RESULTS

Colonisation frequency (CF) of endophytic microorganisms

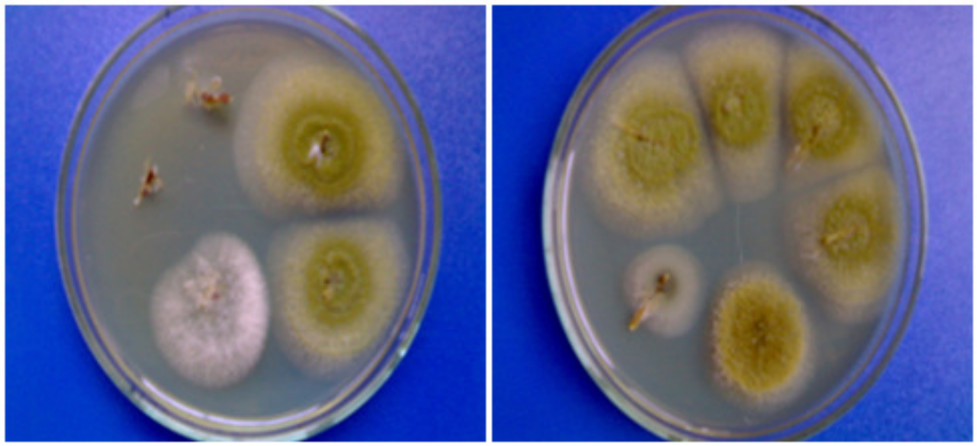
Table-1 illustrates the percent colonization frequency of endophytic microbes in leaf, stem and root of *Enicostemma axillare* (Lam.) Raynal. It is apparent from the data that the colonization frequency of endophytic bacteria and actinomycetes was observed to be more in the leaves (100 %). However, the highest colonization frequency of endophytic fungi was observed in stem (91.67 ± 14.43 %) when compared to that of leaves (87.87 ± 21.05 %) and root (38.27 ± 11.5 %) (Figure 2).

Table 1. Colonization frequency of endophytic microorganisms in *Enicostemma axillare*

Sr.No.	Plant Part	Colonization Frequency (%) of Endophytic microorganisms		
		Bacteria	Actinomycetes	Fungi
1.	Leaf	100 ± 0.00	100 ± 0.00	87.87 ± 21.05
2.	Stem	96.67 ± 5.77	96.67 ± 5.77	91.67 ± 14.43
3.	Root	100 ± 0.00	90.77 ± 15.99	38.27 ± 11.5

Values are mean ±SD of three replicates.

Fig 1. *Enicostemma axillare* (Lam.) Raynal. growing in the grassland region.



(a) (b)

Fig 2. Stem (a) and root (b) segments of *Enicostemma axillare* (Lam.) Raynal. colonised by endophytic fungi.

Total yield of endophytic fungal crude extracts

Table 2 depicts the amount of endophytic fungal extracts in different solvents. The fermentation broth of endophytic fungi were extracted in the various solvents viz ethyl acetate, diethyl ether, dichloromethane and water (aqueous extract). The high amount of all the fungal extracts was found in water which ranges from 122.7 mg to 691.2 mg, followed by diethyl ether (from 6.4 mg to 50.1 mg), ethyl acetate (from 5.5 mg to 92.8 mg) and dichloromethane extract (from 4.6 mg to 50.2 mg).

Table 2. Total yield of crude extracts of endophytic fungi in various solvents

Sr. No.	Fungal Isolate	Total yield (mg)			
		Ethyl acetate	Diethyl ether	Dichloromethane	Aqueous extract
1.	F-1	5.5± 0.061	37 ± 0.924	25.1 ± 0.264	323.1 ± 7.716
2.	F-2	22.8 ± 0.883	25.8 ± 0.968	12.6 ± 0.837	540.8 ± 8.01
3.	F-3	16.9 ± 0.792	11.4 ± 0.064	10.4 ± 0.374	546.5 ± 6.243
4.	F-4	13.4 ± 0.693	11.4 ± 0.069	4.8 ± 0.177	585.6 ± 6.246
5.	F-5	36 ± 0.979	21.17 ± 1.056	7 ± 0.292	436.3 ± 9.669
6.	F-6	37.5 ± 0.747	33.5 ± 1.565	25.9 ± 0.109	534.3 ± 12.02
7.	F-7	28.8 ± 0.51	15.3 ± 0.627	17.3 ± 0.162	122.7 ± 3.57
8.	F-8	23.39 ± 0.183	50.1 ± 0.615	22.35 ± 0.946	509.4 ± 2.549
9.	F-9	28.1 ± 0.102	10 ± 0.379	45.9 ± 0.648	302 ± 7.068
10.	F-10	13.5 ± 0.588	11.6 ± 0.173	4.6 ± 0.053	585.5 ± 9.109
11.	F-11	25.5 ± 0.176	21.7 ± 1.039	24.8 ± 0.094	691.2 ± 3.315
12.	F-12	92.8 ± 1.196	10.3 ± 0.429	6.9 ± 0.542	546.4 ± 5.525
13.	F-13	36.1 ± 0.672	21.18 ± 0.148	7.1 ± 0.509	436.1 ± 2.576
14.	F-14	10.1 ± 0.253	6.4 ± 0.193	50.2 ± 1.43	323.5 ± 6.256
15.	F-15	27.1 ± 0.126	41.7 ± 1.748	24.7 ± 0.257	691.1 ± 15.26

Values are mean±SD of three replicates.

Antimicrobial activity of crude extracts of endophytic fungi

The endophytic fungal extracts were tested against *Escherichia coli, Bacillus subtilis, Proteus vulgaris, Staphylococcus aureus, Pseudomonas morgani, Bacillus sp. tc 09, Bacillus pumilus strain P 012* and *Bacterium P 07.* Among them *Bacillus sp. tc 09, Bacillus pumilus strain P 012* and *Bacterium P 07* have been isolated from oral saliva of patients suffering from tooth caries and periodontitis. Endophytic fungal extracts were also tested against *Candida albicans, Candida blankii, Candida parapsilosis and Cryptococcus neoformans.* From the data it is observed that fungal extracts were inhibitory against one or more tested organisms.

Figure 3 depicts antimicrobial activity of 15 fungal extracts prepared in ethyl acetate against test microorganisms. The maximum broad spectrum antimicrobial activity was shown by F-6 isolate followed by F-7, F-11 and F-3. The maximum zone of inhibition was displayed by F-3 and F-6 isolate against *Bacillus subtilis* (11 mm) followed by F-11 against *Staphylococcus aureus* (10 mm). In contrast, isolates F-7, F-11 and F-13 was found to have potent antifungal activity against *Candida albicans* (6 mm).

In case of diethyl ether extracts, the isolates F-5, F-4, F-6, F-2 and F-11 exhibited broad spectrum activity. Isolate F-4 inhibited *Staphylococcus aureus* (10 mm) and *Proteus vulgaris* (9 mm) remarkably. The growth of *Bacillus subtilis* was effectively inhibited by isolate F-2 and F-6 (9 mm). Moreover, isolates F-6, F-11 and F-14 displayed antifungal activity against *Candida albicans* (8 mm) (Figure 4).

Figure 5 illustrates antimicrobial activity of endophytic fungal extracts prepared in dichloromethane against test microorganisms. The broad spectrum antimicrobial activity was displayed by isolates F-6, F-7, F-11, F-4, F-5 and F-3. Especially, F-5 and F-6 isolates showed maximum zone of inhibition (11 mm) against *Bacillus subtilis.* In addition, F-9 isolate exhibited potential to inhibit fungus *Candida albicans* (9 mm).

Figure 6 shows antimicrobial activity of aqueous extracts of endophytic fungal isolates. The isolates that possessed broad

spectrum activity include F-5, F-4, F-6, F-3, F-14 and F-7 in descending order. The maximum zone of inhibition was shown by F-3, F-4 against *Proteus vulgaris* and by F-6 against *Bacillus subtilis* (12 mm). However, F-11 isolate exhibited maximum zone of inhibition (10 mm) against fungus *Candida albicans.*

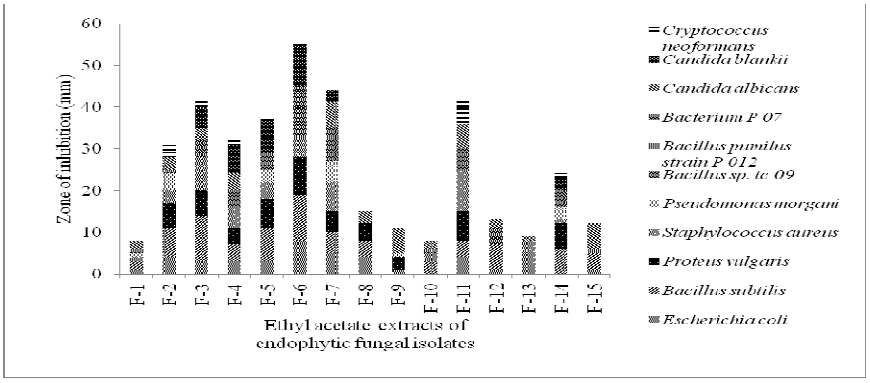


Fig 3. The antimicrobial activity of ethyl acetate extracts of endophytic fungi

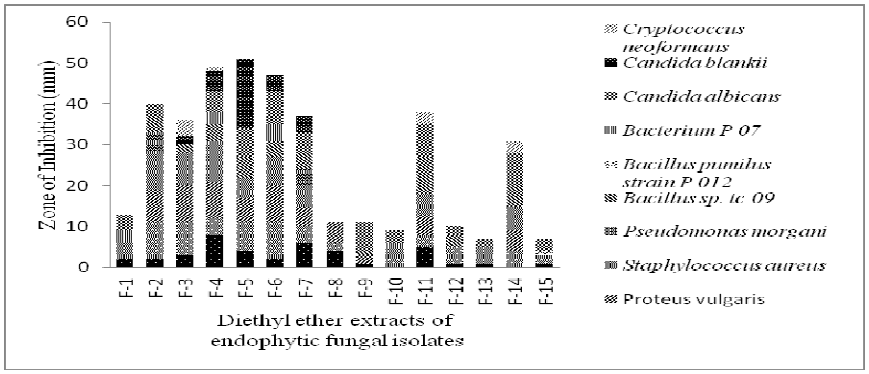


Fig 4.The antimicrobial activity of diethyl ether extracts of endophytic fungi

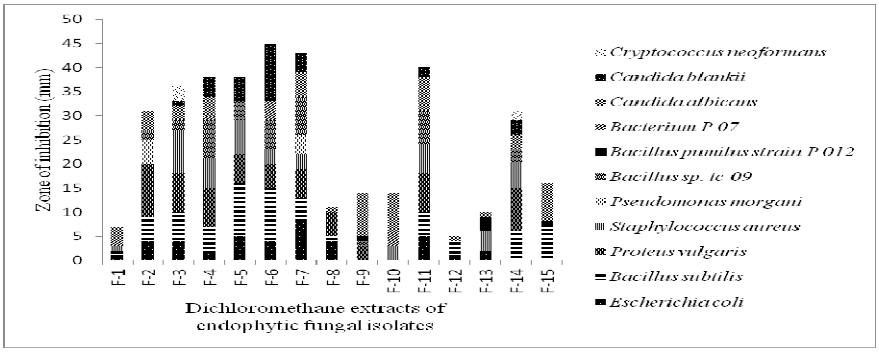


Fig 5. The antimicrobial activity of dichloromethane extracts of endophytic fungi

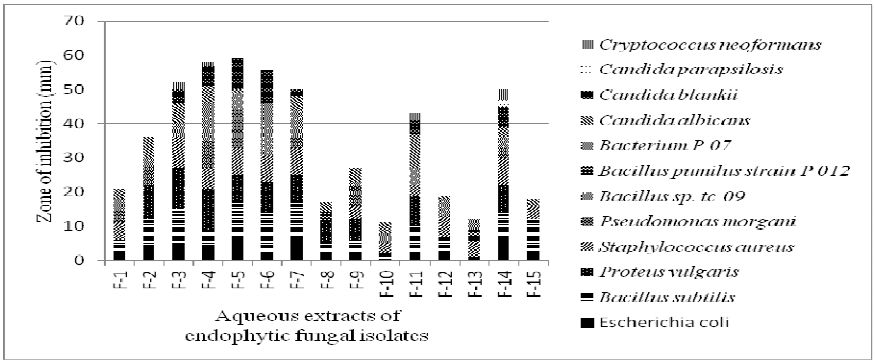


Fig 6. The antimicrobial activity of aqueous extracts of endophytic fungal isolates.

Endophytic fungal community of *Enicostemma axillare* (Lam.) Raynal.

The identified endophytic fungi isolated from various part of medicinal plant are given in Table-3. Majority of endophytic fungi belongs to *Aspergillus* sp. (F-6, F-7, F-8, F-9, F-10, F-12, F-13, F-15). In addition, *Penicillium sp.* (F-2, F-3, F-4), *Cladosporium sp.* (F-1), *Eurotium sp.* (F-5), *Phomopsis sp.* (F-14) and one unidentified fungus (F-11) were also observed (Figure 7).

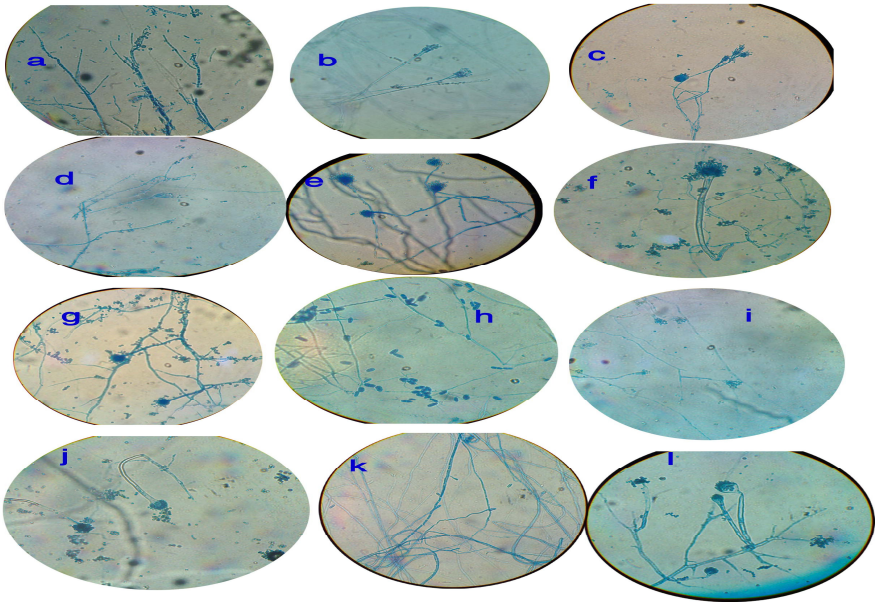


Fig 7. Light microscopic (400X) observation of endophytic fungi
(a): F-1- *Cladosporium sp.* (b): F-2- *Penicillium sp.* (c): F-3- *Penicillium sp.* (d): F-4- *Penicillium sp.* (e): F-5- *Eurotium sp.* (f): F-6- *Aspergillus flavus*
(g): F-7- *Aspergillus sp.* (h): F-11- *Unidentified fungus* (i): F-12- *Aspergillus sp.* (j): F-13- *Sartorya sp.* (k): F-14- *Phomopsis sp.* (l): F-15- *Aspergillus fumigatus.*

Table 3. Endophytic fungi isolated from various parts of *Enicostemma axillare* (Lam.) Raynal.

Sr. No.	Endophytic Fungal Isolate	Source	Identification
1.	F-1	Root	*Cladosporium sp.*
2.	F-2	Root	*Penicillium sp.*
3.	F-3	Root	*Penicillium sp.*
4.	F-4	Root	*Penicillium sp.*
5.	F-5	Stem	*Eurotium sp.*
6.	F-6	Stem	*Aspergillus flavus*
7.	F-7	Leaves	*Aspergillus sp.*
8.	F-8	Root	*Aspergillus sp.*
9.	F-9	Leaves	*Aspergillus sp.*
10.	F-10	Leaves	*Aspergillus sp.*
11.	F-11	Root	*Unidentified fungus*
12.	F-12	Stem	*Aspergillus sp.*
13.	F-13	Stem	*Sartorya sp.*
14.	F-14	Root	*Phomopsis sp.*
15.	F-15	Stem	*Aspergillus fumigatus*

DISCUSSION

Fungi have been widely investigated as a source of bioactive compounds. A well-known example is taxol derived from the plant, which is reported for its anticancer properties [19]. In India, the extracts from many types of local plants are used in traditional manner for treatments of various ailments [20]. Previous studies demonstrated that crude extracts from culture broth of endophytic microorganisms displayed antibacterial, antifungal, antiviral, anti-inflammatory and anti-tumor activity [21]. Therefore, the use of endophytic fungi as antimicrobials opens up new areas for biotechnological exploitations.

It is apparent from the data that the colonization frequency of endophytic bacteria and actinomycetes was observed to be more in the leaves. However, the highest colonization frequency of endophytic fungi was observed in stem of *Enicostemma axillare* (Lam.) Raynal. as compared to that of leaves and root. The results indicated that the colonization of plant tissues by endophytic fungi

occurs in a manner similar to those of plant pathogens and mycorrhizae [22]. These observations are in conformity with the earlier report on *Withania somnifera* (L.) Dunal [23], though minor variations exist.

Our studies with crude extracts of 15 endophytic fungi prepared in four different solvents namely ethyl acetate, diethyl ether, dichloromethane and water revealed that the majority of aqueous extracts showed antibacterial activity against *Staphylococcus aureus, Proteus vulgaris* and *Bacillus subtilis.* This may be attributed to the high concentration of active chemical constituents in the aqueous extracts. The dichloromethane extracts showed maximum antimicrobial activity against *Candida albicans* and *Proteus vulgaris.* The results are in conformity with previous study of Yi et al. [24] in that broad-spectrum antimicrobial activity of ethyl acetate extracts of epi- and endophytic fungal strains from the local seaweeds, sea grass, sponge, and other invertebrates have been reported. Similarly, the ethyl acetate extracts of F-11 and F-13 isolates showed maximum antibacterial activity against *Staphylococcus aureus.* Interestingly, antifungal activity was found against *Candida blankii* due to diethyl ether extracts of F-5 endophytic fungal isolate. These findings further substantiate that endophytic fungi have shown significant and positive biological activities. Screening of other endophytic fungal extracts showed low antimicrobial activity in the bioassay, suggesting that they may show greater antimicrobial activities once they are purified [25]. The differential sensitivity of crude extracts of endophytic fungi isolated from *Enicostemma axillare* (Lam.) Raynal. can be used to explore novel bioactive molecule with a high activity against human pathogenic microorganisms.

The data on the diversity of fungal endophyte community associated with *Enicostemma axillare* (Lam.) Raynal. is scanty. Therefore, the results obtained in the study are compared with the endophytes from other hosts from tropical regions. Endophytic fungal diversity has been predicted initially in the leaves of many angiosperms of tropics [26-27]. The majority of reported endophytes are from leaves and twigs of tropical forest trees [14, 28-29] and few reports are also available on the isolation and diversity of endophyte from *Azadirachta indica* A. Juss. and *Catharanthus roseus* (L.) G.DON. [30-31]. In the present study, among the endophytic fungi the most dominant being *Aspergillus* that was observed in various parts of *Enicostemma axillare* (Lam.) Raynal. Previously, this fungus was reported as common endophyte in other plant species like *Withania somnifera*, *Calotropis procera, Tripterygium wilfordii*, *Calotropis gigantea, Azardirachta indica* A. Juss. and *Melia azardarch* L. [23, 32-36]. Other endophytic fungi include *Cladosporium sp., Penicillium sp., Eurotium sp., Sartorya sp.* and *Phomopsis sp.* These fungal isolates belong to anamorphic group and one unidentified fungus was found in the roots of *Enicostemma axillare* (Lam.) Raynal.

In conclusion, the crude extracts of endophytic fungi obtained from *Enicostemma axillare* (Lam.) Raynal. may serve as a potential source for exploring novel bioactive metabolites with a broad spectrum antimicrobial activity against human pathogenic microorganisms.

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