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Antimicrobial potential of endophytes isolated and characterized from aerial and non aerial parts of *Murraya koenigii* L.

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ABSTRACT

Endophytes are the microbes that live within the host plant tissues without causing any visible disease symptoms. Depending on their nutritional requirements they can live as biotrophic parasites or saprotrophs. They also represent a huge reservoir of microbes that are explored very poorly. The present study was done first ever in India to investigate the biodiversity of endophytic bacteria and fungi in parts of the plant, Murraya koenigii. The results have shown that the both bacterial and fungal isolates were diverse both in morphology and characteristics. In the present investigation, the results were found to be very surprising and interesting. Total of 08 bacterial and 11 fungal endophytes were isolated from the plant parts. It was found that the inner core of leaves, stems and roots of Murraya koenigii L. were found to have the presence of bacterial and fungal endophytes as observed on LB and PDA plates. Different colonial growth was observed in the petriplates while some were obtained as mixed cultures. The pure cultures were maintained separately for further use. In case of bacterial endophytes as observed on LB agar plates. 3 isolates were obtained from stem, 3 were obtained from leaves and 2 were obtained from roots. In case of fungal endophytes as observed on PDA plates, 6 isolates were obtained from stem, 4 were obtained from leaves and single isolate was obtained from roots. It was found that bacterial endophytic fractions were most effective against the pathogens and drug resistant strains in comparison to fungal endophytic fractions. The results showed that bacterial endophytic fractions of stems viz. S1, S2 and S3 possessed significant antimicrobial activity against E. coli NCIM 2065, Lactobacillus plantarum NCIM 2083, Micrococcus luteus ATCC 9341, Salmonella abony NCIM 2257 while no activity was observed against Methicillin resistant strains of S. aureus and any of the fungal cultures, Candida albicans NCIM 3471; Aspergillus niger NCIM 1196. Amongst bacterial endophytic fractions of leaves, L1 and L3 showed no antibacterial activity against any of the bacterial pathogens while showed potency against fungal cultures studied. Only L2 bacterial endophytic fraction showed potency against all the bacterial and fungal pathogens studied except Lactobacillus plantarum NCIM 2083. The L2 bacterial endophytic fraction also showed antibacterial activity against one of the Methicillin resistant strain of S. aureus. Bacterial endophytic fractions of roots viz. R1 and R2 showed potency against all the bacterial and fungal cultures except E. coli NCIM 2065. No antimicrobial potential of any of the fungal endophytic fraction of stem viz. S1-S6 against any of the test organism was revealed. Although S3 fraction showed slight antibacterial activity against one of the Methicillin resistant strain of S. aureus. Simultaneously fungal endophytic fraction of leaves viz. L1, L3 and L4 also showed no activity against any of the test organism. L4 fraction showed significant antifungal activity against Candida albicans NCIM 3471. The endophytic fraction of leaves, L2 showed significant activity against one of the Methicillin resistant strain of S. aureus, E. coli NCIM 2065 and Lactobacillus plantarum NCIM 2083. The isolated bacterial endophytes were screened for gram staining and different biochemical tests. The results confirmed that out of 08 isolated cultures of bacterial endophytes, 05 were gram positive, non-motile, non-spore forming bacilli and 03 were gram negative, motile, non spore forming bacilli. Gram positive colonies showed positive sugar (Glucose, sucrose, fructose, rhamnose, mannose) fermentation test, catalase test, amylase test, urease test, esterase test and methyl red test while Gelatin liquefication test, Indole test, Vogues Prosker test, Citrate utilization test, H_2S production tests were found to be negative. Gram negative colonies showed positive sugar (Glucose, sucrose, lactose, cellobiose) fermentation test, Mac Conkey growth, Vogues Prosker test, Citrate utilization, Indole and acetate utilization test. The data of the tests were evaluated by the previous studies which confirm gram positive bacilli as Bacillus megaterium while gram negative bacilli were nominated as Enterobacter cloacae. The fungal endophytes isolates were classified by colony and hyphal characters as stained by lactophenol. Cultures were deposited at National Centre of Fungal Taxonomy (NCFT), New Delhi. The results confirmed that amongst, 11 fungal endophytes isolates, 05 were found to be Aspergillus niger, 02 were Aspergillus flavus, 02 were Candida albicans, 01 was Phoma hedericola and 01 was Penicillium sublateritium.

Key words: Endophytes, identification, *Murraya koenigii*, aerial and non aerial parts, antimicrobial activity.

INTRODUCTION

Endophytes are those microorganisms that inhabit interior of plants especially leaves, stems, roots shows no apparent harm to host [1]. Almost all classes of vascular plants and grasses examined to date are found to host endophytic organisms [2]. Different groups of organisms such as fungi, bacteria, actinomycetes and mycoplasma are reported as endophytes of plants [3]. The endophyte may present in a metabolically hostile environment and continuously encountering host defense chemicals [4]. Endophytic fungi from medicinal plants could be a rich source of functional metabolites [5-7]. Endophyte plant association could be could also be subjugated to stimulate the production of secondary metabolites by host plant. Plants growing in adverse habitats have to be screened for isolation of endophytes and their metabolites [8-10]. Endophytes from angiosperms and gymnosperms have been studied for novel secondary metabolites. The leaves of *Murraya koenigii* are used extensively for seasoning and flavouring dishes. Curry leaf is exported as curry leaf and as curry leaf oil from India. The oil extracted from the leaves and extracts of different parts of the plant possessed pharmacological activities [11]. In the present investigation, an attempt was made for isolation, identification and screening of bacterial and fungal endophytes from aerial and non aerial parts of *Murraya koenigii* (Curry leaf). This study was conducted for the first time for identification and screening of all available bacterial and fungal endophytes from the aerial and non aerial parts of the plant.

MATERIALS AND METHODS

All the chemicals and reagents used in the experiments were procured from C.D.H and Ranchem. Glass wares used were of Borosil. The media and broth used for microbial culture was procured from Hi-Media Ltd., Mumbai.

Collection and Identification of the plant parts

The plant, *Murraya koenigii* L. (Rutaceae) and its parts viz. leaves stem and roots were collected from local garden and was taxonomically identified by some Taxonomists/Botanists in the form of herbarium. The plant parts collected was stored as herbarium and deposited in ITLS, Dehradun for future reference **(Figure 1)**.

Figure 1: Collection of parts for surface sterilization of *Murraya koenigii* L.

Surface sterilization of plant tissues and Isolation of endophytes

The tissues of the plant were washed in running tap water followed by soaking in 70% alcohol for few seconds; further in 0.5-3.5% sodium hypochlorite for 1-2 minutes followed by rinsing in sterile double distilled water before placing it on a LB medium for isolation of endophytic bacteria [12]. Some isolates require months or more time in culture before they sporulate. The LB plates were incubated for about 10 days for observation of any growth of bacterial endophytes. For isolation of fungal endophytes surface sterilization of tissue requires 70% ethanol for 1-3 minutes, aqueous sodium hypochloride (4% available chlorine) for 3-5 minutes, again rinsed with 70% ethanol for 2-10 seconds and finally rinsed with double distilled water and dried in Laminar air flow [13] also addition of 50mg/l chloramphenicol was done within PDA medium to suppress bacterial growth [14]. Sterile knife blade was required to remove outer tissues from sample and to excise inner tissues [15]. The PDA plates were kept for about 6 days for observation of growth of any fungal endophyte. All the plates were incubated at 28ºC to promote the growth of endophytes and were regularly monitored for any microbial growth. On observing the microbial growth,

subculturing was done. Each endophytic culture was checked for purity and transferred to freshly prepared PDA plate. Appropriate controls were also maintained in which no plant tissues were inoculated.

Maintenance of Endophytes for Identification and Future Use

The purified endophytic isolates were transferred separately to LB/PDA slants and broths depending on the case for bacterial and fungal endophytes respectively and accessioned accordingly depending upon the plant parts from which they were isolated. Finally all the purified endophytes were maintained at 4ºC till further used. Different biochemical tests were done for identification of bacterial and fungal endophytes. The bacterial isolates were tested for their morphological and biochemical characteristics (catalase enzyme activity). A Gram stain was performed to determine the characteristics of the cell wall, cell shape and the arrangement of cells. The morphology of the endophytic bacterial strains was observed on slides under a microscope. For staining, 15 µL of a bacterial culture that was grown in nutrient broth overnight at room temperature with shaking at 150 rpm will be heat-fixed onto a slide and then stained. To test the strain for catalase enzyme activity, 15 µL of the culture will be placed on a slide and 15 µL H_2O_2 (30%) was added. The catalase-positive strains were characterized by the intense production of bubbles. Furthermore, the fungal isolates were characterized using a micro cultivation technique on slides after first being cultivated on corn meal agar (CMA) for seven days at 30°C. The slides were then stained with lactophenol. The structures were observed using a photomicroscope. The samples were then compared to other samples reported in the literature.

Production of Secondary metabolites

LB broth and Potato Dextrose broth were prepared and autoclaved. Endophytic bacterial and fungal cultures were inoculated in the medium separately within the flasks. Flasks were then incubated at 28°C for 14 days in shaker. After incubation, extraction was done with different solvents (Chloroform and Ethyl acetate). First the endophytic bacterial and fungal grown in the media was removed by filtering the medium using filter paper. Then the solvents were added to the media in the ratio 1:1 in separating funnel and left for 15 minutes. The organic phase was collected and kept for drying at 37°C. The dry weight of the extract was determined.

Determination of antimicrobial activity of Endophytic fractions

Culture Media

For antibacterial test, Nutrient agar/broth and Sabouraud's dextrose agar/broth of Hi Media Pvt. Bombay, India was used for antifungal test.

Inoculum

The pathogenic bacterial cultures were inoculated into Nutrient broth and incubated at 37 ^{0}C for 18 h and suspension was checked to provide approximately, 10^5 CFU/ml. The same procedure was done for fungal pathogens and there strains will be inoculated into Sabouraud's dextrose broth but the fungal broth cultures were incubated at 48-72 h.

Microorganisms used

Pure cultures of various pathogenic bacterial and fungal strains, *E. coli* NCIM 2065, *Lactobacillus plantarum* NCIM 2083, *Micrococcus luteus* ATCC 9341, *Salmonella abony* NCIM 2257, *Candida albicans* NCIM 3471, *Aspergillus niger* NCIM 1196 and Methicillin resistant strains of *Staphylococcus aureus* (MRSA) isolated from clinical specimens viz. pus and blood of infected patients were procured with authentication for the study. The standard bacterial and fungal cultures used for the study were procured from Roorkee Research & Analytical Labs Pvt. Ltd., Roorkee (U.K), India and MRSA strains were procured from Shooloni University, H.P., India.

Determination of diameter of zone of inhibition by well diffusion method

The agar well diffusion method [16] was modified. Nutrient agar medium (NAM) was used for growth of pathogenic bacterial cultures. The culture medium was inoculated with the bacterial pathogen separately suspended in nutrient broth. Sabouraud's dextrose agar/broth was used for growth of pathogenic fungal cultures. The culture medium was inoculated with the fungus separately suspended in Sabouraud's dextrose broth. A total of 8 mm diameter wells were punched into the agar and filled with separate endophytic fractions and solvent blanks. Standard antibiotic (Erythromycin, 1 mg/ml) was simultaneously used as the positive control. The plates were incubated at 37 ^{0}C for 18 h. The antibacterial activity was evaluated by measuring the diameter of zone of inhibition observed. For assaying, antifungal activity of endophytic fractions, Sabouraud's dextrose agar/ broth medium plates was used. The same procedure as that for determination of antibacterial property was adopted and then after the diameter of zone of inhibition was observed after 48-72 h. Fucanazole (1mg/ml) was used as standard for determination of antifungal activity. The procedure for assaying antibacterial and antifungal activity was performed in triplicates to confirm the average readings of diameter of zone of inhibition observed for each of the test organism.

RESULTS AND DISCUSSION

Isolation of Endophytes

In the present investigation, the results were found to be very surprising and interesting. Total of 08 bacterial and 11 fungal endophytes were isolated from the plant parts. It was found that the inner core of leaves, stems and roots of *Murraya koenigii* L. were found to have the presence of bacterial and fungal endophytes as observed on LB and PDA plates. The results shown in **Figure 2** indicates the initial inoculum of plant parts on LB and PDA plates comparing with that of the growth of endophytes after incubation.

Different colonial growth was observed in the petriplates while some were obtained as mixed cultures. The pure cultures were maintained separately for further use. In case of bacterial endophytes as observed on LB agar plates. 3 isolates were obtained from stem, 3 were obtained from leaves and 2 were obtained from roots. In case of fungal endophytes as observed on PDA plates, 6 isolates were obtained from stem, 4 were obtained from leaves and single isolate was obtained from roots. The results are shown in **Figure 3.**

(A) Initial inoculum of surface sterilized roots, stem and leaves of the plant

(B) Growth of bacterial and fungal endophytes from inoculum of roots, stem and leaves on LB and PDA plates

Figure 2: Growth of endophytes from exposed inner parts of surface sterilized leaves, stems and roots of *Murraya koenigii* L.

Antimicrobial activity of bacterial and fungal endophytic fractions

It was found that bacterial endophytic fractions were most effective against the pathogens and drug resistant strains in comparison to fungal endophytic fractions.

Figure 3: Growth of fungal endophytes on PDA from exposed inner parts of surface sterilized leaves, stems and roots of *Murraya koenigii* L.

Bacterial endophytic fractions

The results showed that bacterial endophytic fractions of stems viz. S1, S2 and S3 possessed significant antimicrobial activity against *E. coli* NCIM 2065, *Lactobacillus plantarum* NCIM 2083, *Micrococcus luteus* ATCC 9341, *Salmonella abony* NCIM 2257 while no activity was observed against Methicillin resistant strains of *S. aureus* and any of the fungal cultures, *Candida albicans* NCIM 3471; *Aspergillus niger* NCIM 1196. Amongst bacterial endophytic fractions of leaves, L1 and L3 showed no antibacterial activity against any of the bacterial pathogens while showed potency against fungal cultures studied. Only L2 bacterial endophytic fraction showed potency against all the bacterial and fungal pathogens studied except *Lactobacillus plantarum* NCIM 2083. The L2 bacterial endophytic fraction also showed antibacterial activity against one of the Methicillin resistant strain of *S. aureus*. Bacterial endophytic fractions of roots viz. R1 and R2 showed potency against all the bacterial and fungal cultures except *E. coli* NCIM 2065. The results are shown in **Table 1** and **Figure 4 (a & b).**

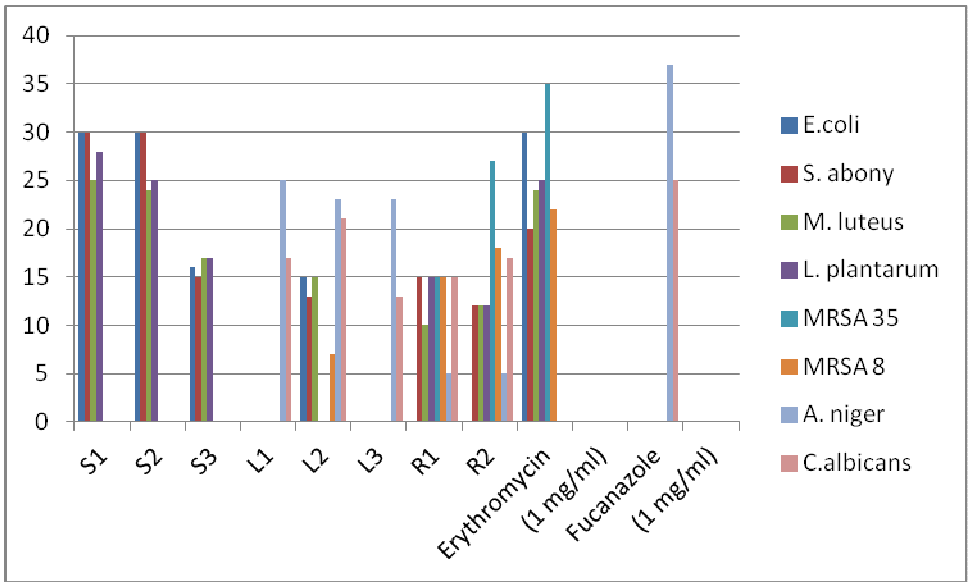

Figure 4 (a): Graphical representation of Antimicrobial activity of bacterial endophytic fractions

Figure 4 (b): Antimicrobial activity of bacterial endophytic fractions against animal pathogens

Table 1: Antimicrobial activity of bacterial endophytic fractions

Bacterial Endophytic fractions	Diameter of zone of inhibition (mm)							
	Pathogens studied							
	ECO	**SA**	**ML**	**LP**	**MRSA 35**	**MRSA 8**	**AN**	**CA**
S1	30.0	30.0	25.0	28.0	NA	NA	NA	NA
S2	30.0	30.0	24.0	25.0	NA	NA	NA	NA
S3	16.0	15.0	17.0	17.0	NA	NA	NA	NA
L1	NA	NA	NA	NA	NA	NA	25.0	17.0
L2	15.0	13.0	15.0	NA	NA	07.0	23.0	21.0
L3	NA	NA	NA	NA	NA	NA	23.0	13.0
R1	NA	15.0	10.0	15.0	15.0	15.0	05.0	15.0
R2	NA	12.0	12.0	12.0	27.0	18.0	05.0	17.0
Erythromycin (1 mg/ml)	30.0	20.0	24.0	25.0	35.0	22.0	NT	NT
Fucanazole (1 mg/ml)	NT	NT	NT	NT	NT	NT	37.0	25.0

**NA, No Activity; NT, Not Tested*

**ECO- E. Coli; SA- Salmonella abony; ML- Micrococcus luteus; LP- Lactobacillus plantarum; MRSA 35- Methicillin resistant Staphylococcus aureus (isolated from pus); MRSA 8- Methicillin resistant Staphylococcus aureus (isolated from blood); AN- Aspergillus niger; CA- Candida albicans*

Fungal endophytic fractions

No antimicrobial potential of any of the fungal endophytic fraction of stem viz. S1-S6 against any of the test organism was revealed. The S3 fraction showed slight antibacterial activity against one of the methicillin resistant strain of *S. aureus*. Simultaneously fungal endophytic fraction of leaves viz. L1, L3 and L4 also showed no activity against any of the test organism. L4 fraction showed significant antifungal activity against *Candida albicans* NCIM 3471. The endophytic fraction of leaves, L2 showed significant activity against one of the Methicillin resistant strain of *S. aureus*, *E. coli* NCIM 2065 and *Lactobacillus plantarum* NCIM 2083. The results are shown in **Table 2** and **Figure 5 (a & b).**

Table 2: Antimicrobial activity of fungal endophytic fractions

Fungal Endophytic fractions	Diameter of zone of inhibition (mm) Pathogens studied							
	ECO	***SA***	***ML***	***LP***	***MRSA 35***	***MRSA 8***	***AN***	***CA***
S1	NA	NA	NA	NA	NA	NA	NA	NA
S2	NA	NA	NA	NA	NA	NA	NA	NA
S3	NA	NA	NA	NA	13.0	NA	NA	NA
S4	NA	NA	NA	NA	NA	NA	NA	NA
S5	NA	NA	NA	NA	NA	NA	NA	NA
S6	NA	NA	NA	NA	NA	NA	NA	NA
L1	NA	NA	NA	NA	NA	NA	NA	NA
L2	22.0	NA	NA	20.0	21.0	NA	NA	NA
L3	NA	NA	NA	NA	NA	NA	NA	NA
L4	NA	NA	NA	NA	NA	NA	NA	24.0
R1	NA	NA	NA	NA	NA	NA	NA	NA
Erythromycin (1 mg/ml)	30.0	20.0	24.0	25.0	35.0	22.0	NT	NT
Fucanazole (1 mg/ml)	NT	NT	NT	NT	NT	NT	37.0	25.0

**NA, No Activity; NT, Not Tested*

**ECO- E. Coli; SA- Salmonella abony; ML- Micrococcus luteus; LP- Lactobacillus plantarum; MRSA 35- Methicillin resistant Staphylococcus aureus (isolated from pus); MRSA 8- Methicillin resistant Staphylococcus aureus (isolated from blood); AN- Aspergillus niger; CA- Candida albicans*

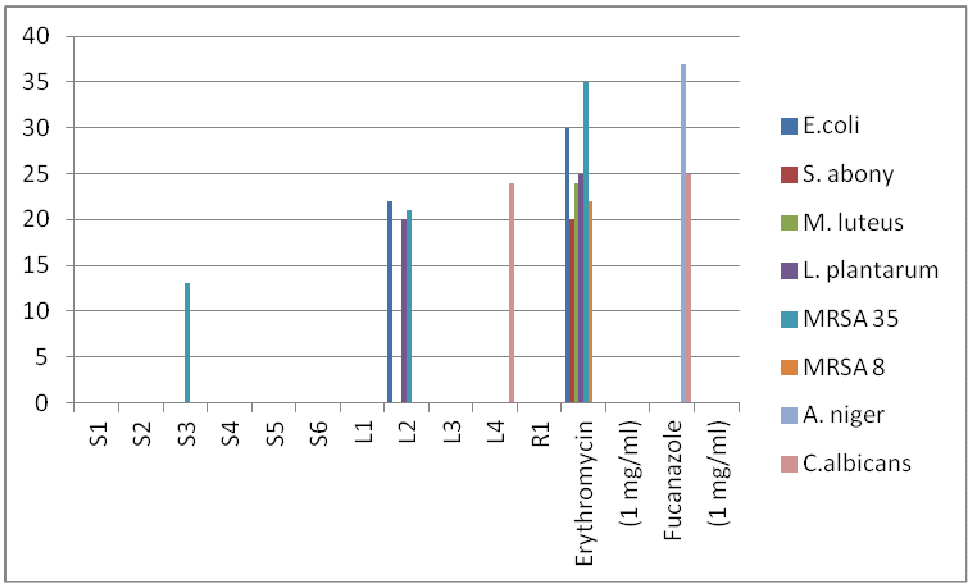

Figure 5 (a): Graphical representation of fungal endophytic fractions against animal pathogens

Figure 5 (b): Antimicrobial activity of fungal endophytic fractions against animal pathogens

Identification of morphology of bacterial and fungal endophytes

Bacterial endophytes

The isolated bacterial endophytes were screened for gram staining and different biochemical tests. The results confirmed that out of 08 isolated cultures of bacterial endophytes, 05 were gram positive, non-motile, non-spore forming bacilli and 03 were gram negative, motile, non spore forming bacilli.

Gram positive colonies showed positive sugar (Glucose, sucrose, fructose, rhamnose, mannose) fermentation test, catalase test, amylase test, urease test, esterase test and methyl red test while Gelatin liquefication test, Indole test, Vogues Prosker test, Citrate utilization test, H_2S production tests were found to be negative. Gram negative colonies

showed positive sugar (Glucose, sucrose, lactose, cellobiose) fermentation test, Mac Conkey growth, Vogues Prosker test, Citrate utilization, Indole and acetate utilization test. The data of the tests were evaluated by the previous studies which confirm gram positive bacilli as *Bacillus megaterium* while gram negative bacilli were nominated as *Enterobacter cloacae*. The results are shown in **Table 3 (a & b).**

Table 3 (a): Morphology and Biochemical Characteristics of Gram positive Bacterial endophytes

Isolate	Gram Staining/Shape/Motility/Spore formation	Sugar Fermentation Test	Amylase test	Indole test	Methyl red test	VP test	H_2S production	Citrate test
S1	Gram positive, Bacilli, non-motile, spore forming	+	+	_	+	-	_	-
S2	Gram positive, Bacilli, Non motile, Non spore forming	+	+	_	+	-	_	-
L1	Gram positive, Bacilli, Non motile, Non spore forming	+	+	_	+	-	_	-
L2	Gram positive, Bacilli, Non motile, Non spore forming	+	+	_	+	-	-	-
R2	Gram positive, Bacilli, Non motile, Non spore forming	+	+	_	+	-	-	-

+, present; -, absent

Table 3 (b): Morphology and Biochemical Characteristics of Gram negative Bacterial endophytes

Isolate	Gram Staining/Shape/Motility/Spore formation	Sugar Fermentation Test	Mac-Conkey growth	Indole test	Methyl red test	VP test	Acetate test	Citrate test
S3	Gram negative, Bacilli, motile, Non spore forming	+	+	+	_	+	+	+
L3	Gram negative, Bacilli, motile, Non spore forming	+	+	+	_	+	+	+
R1	Gram negative, Bacilli, motile, Non spore forming	+	+	+	_	+	+	+

+, present; -, absent

Fungal endophytes

The fungal endophytes isolates were classified by colony and hyphal characters as stained by lactophenol. Cultures were deposited at National Centre of Fungal Taxonomy, New Delhi. The results confirmed that amongst, 11 fungal endophytes isolates, 05 were found to be *Aspergillus niger*, 02 were *Aspergillus flavus*, 02 were *Candida albicans*, 01 was *Phoma hedericola* and 01 was *Penicillium sublateritium*. The results are shown in **Table 4**.

Table 4: Identification of fungal endophytic isolates

Isolate	Fungal Endophytes
S1	*Aspergillus niger*
S2	*Aspergillus niger*
S3	*Aspergillus niger*
S4	*Aspergillus flavus*
S5	*Aspergillus flavus*
S6	*Aspergillus niger*
L1	*Aspergillus niger*
L2	*Candida albicans*
L3	*Candida albicans*
L4	*Penicillium sublateritium*
R1	*Phoma hedericola*

Endophytic fungi are one of the most unexplored and diverse group of organisms that make symbiotic associations with higher life forms and may produce beneficial substances for host [17]. Fungi have been widely investigated as a source of bioactive compounds. Endophytic organisms have received considerable attention after they were found to protect their host against insect pests, pathogens and even domestic herbivorous. However, only a few plants have been studied for their endophyte biodiversity and their potential to produce bioactive compounds. Recently studies have been carried out about the endophytic biodiversity, taxonomy, reproduction, host ecology and their effect on host [18-20]. Endophytes are now considered as an outstanding source of bioactive natural products, because they occupy unique biological niches as they grow in so many unusual environments. Endophytic fungi from medicinal plants can therefore be used for the development of drugs. The endophytic flora, both numbers and types, differ in their host and depends on host geographical position [21-23]. *Murraya koenigii* L. is a well known medicinal plant and its different parts are reported to have antimicrobial, antioxidant, anti-inflammatory and anticancer activities.

The medicinal properties of the plant could be attributed to their endophytic fungi. Therefore, the present work was initiated to find out endophytic flora associated with in this widely used medicinal plant.

CONCLUSION

The results of the present study suggest that, endophytes residing in the medicinal plants are the potential bioresource of secondary metabolites. The diversity of these endophytes is variable and still unexplored. The medicinal plants thus are the bioreactors of natural molecules and diverse useful microbes as well. These endophytes can be isolated and screened for secondary metabolites which can be potent pharmacological molecules. Furthermore the exploitation of the endangered plant species can be stopped by using such endophytes for production of pharmacologically active molecules.

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