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ANTIMICROBIAL PROPERTIES OF ENDOPHYTIC FUNGI ISOLATED FROM *CYNODON DACTYLON AND MORINGA OLIEFERA*

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ABSTRACT

Fungal endophytes were isolated from selected *Cynodon dactylon* and *Moringa oliefera* species, on MRBA media. The findings show that the isolated endophytic species were identified as *Curvularia sp*, *Penicillium oxalicum*, *Basidiomycete sp*, *Fusarium graminareum*, *Aspergillus sp1*, *Aspergillus sp2*, *Helminthosporium* (*Drescheleria haolodes*), *Alternaria brasicola*. Further these fungi (mycelium and growth broth) were used to extract secondary metabolites. The extracts were used to test their antimicrobial potency against medical pathogens such as *Escherichia coli, β-Strepto pneumococcus, Staphylococcus albus, Kleibsiella pneumoniae and Pseudomonas aeruginosa*. They were further tested with plant pathogens such as *Erwinia caratovora, Xanthomonas campestris, Xanthomonas punicara*. Significant findings emerged as the antimicrobial potency was estimated by measuring the zone of inhibition of each of these extracts with the chosen pathogens on the petriplate. The outcome of the exercise undertaken proved successful for furthering the scope and standardizing of the endophytic secondary metabolites as an alternative to the conventional use of antibiotics.

Key Words: Endophytes, Secondary metabolites, Antimicrobials, Pathogen.

INTRODUCTION

Fungi and bacteria live asymptomatically within the plant tissue without instigating seemingly impairment to the host tissue. Endophytes are found in almost all species of plants and hence permeating. Among them, the most frequently encountered endophytes are the fungal isolates. Fungi may be endophyte, epiphyte or latent pathogen (Bacon *et al.,* 1993). Endophytes remain within the host tissue but with exclusion where the fruiting structures may arise through the surface of the plant tissue. A wide variety of endophytic fungi can be observed as colonizing a leaf within the few weeks of its emergence, these colonies remain asymptomatic and the plant tissue remains unaffected and functional. Several fungi have been isolated from the tissues of aquatic and terrestrial plants and red and brown algae (Carroll, 1978). These endophytic fungi are found to habitat in all parts of the plants especially the leaves which are colonized with the variety of fungi (Dreyfuss and Petrini, 1984)

Among these, the most common endophytic fungi are anarmorphic members of Ascomycota. Phylogenetic evidence suggests that some endophytes have evolved from pathogens. The mechanism of host tissue recognition and colonization within the tissue is thought to be common in the closely related fungi. (Fisher and Petrini, 1994)

It has been observed that there is a complex relationship between the endophytic fungus and the host. In one instance, Boursnell an investigator, showed that in

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the plant *Helianthemum chamaecistus* the endophytes passes through the seed from one generation to another, and through the seedling it tends to spread throughout the plant entering new tissue. It is thought that the endophytes are transmitted horizontally where in the plant is colonized by fungal propagules that come to from the environment. Propagule of some endophytic fungi can be observed in the body of insect pest of the host (Petrini, 1999). It is thought that the dispersion of endophytic fungi occurs from host to host through insects in a complex manner perhaps through aerial dispersion of the fungal spores involving wind or vector. Fungal organisms are thought to have certain potential to release their spores in air. The sporulation is seen only after senescence of the host tissue.

An endophytic fungus tends to avert colonization of the pathogenic organisms in the host plant. The wide colonization of an endophyte in plant tissue creates a barrier effect which prevents an attack of the pathogens to the host. It has been understood that metabolites produced by fungal endophytes inhibit the growth of competitors including pathogens. Certain endophytic fungi may produce metabolites with a hermo-protective role for the plant growing in volcanic area. The endophytic infected plant could withstand the environmental stress and also from certain predation (Schardl, 1997). Endophytes such as Mycorrhiza benefits the host plants by increasing the absorption of the soil nutrients like phosphorous and inorganic elements. They also enable in the fixation of atmospheric nitrogen thus empowering the plants to endure several years without addition of nitrogen. The roots of plants infected with the endophytes show enhancement of the water acquisition and water holding capacity in the soil. Endophytes also increase the water flow through plants by producing water-soluble compounds like alkaloids that increase the osmotic flow through the roots. The plant in return provide protective environment to the endophytic fungus where the nutrients are made readily available. The loss of plant nutrients decreases the rapid colonization of the fungal endophytes indicating the importance of the host tissue in regulating the colonization of fungus (Sussman and Halvorson, 1966)

Endophytic fungi are significant for their role in the production of anticancer, anti-diabetic, insecticidal and immunosuppressive compounds. Due to their tremendous role in making plant to adapt to the stress conditions and because of the production of secondary metabolites with the pharmaceutical significance, the study of endophytic fungus occupy an important part in fugal biology (Meijer and Leuchtmann,1999) Therefore endophytic fungi are effective in the survival or overcoming drought situations and preventing pathogenic entry. The secondary metabolites produced by the fungal endophytes are attractive to the pharmaceutical companies for the production of novel drugs. There are a number of secondary metabolites that have been discovered from endophytic fungi some of them are alkaloids, steroids, terpenoids, peptides, polyketones, flavoniods, quinols and phenols.

To name a few, world's foremost anticancer drug paclitexel, commonly called taxol was discovered from a fungus *Pestalotiopsis microspora*, isolated from yew tree *Taxus wallichiana* in the Himalayan region. Taxol is an anti-fungal compound. Strobel and colleagues identified a potent fungal organism which was known to produce Oocydin A which provided the plant with the requisite protection from the pathogenic microbes (Strobel, 2003). The earliest work on the endophytic fungus was by Freeman in the year 1904. Freeman isolated the endophytic fungi from an annual grass Persian darnel. Thus endophytic fungus has significance role both in protecting plant (Strobel, 1999) and in the production of the secondary metabolites which as a drug has medicinal value and agricultural value to control the harmful pathogens.

Our aim was to isolate and identify certain important endophytic fungi on selected plants species such as *Moringa oleifera* and *Cynodon dactylon* which are ecologically and industrially important to human welfare. The rich nutrients of the selected plants provides good environment for the luxuriant growth and helps in the maximum colonization of the endophytic fungi

Moringa oleifera

The plant species is commonly called as drumstick belonging to the family *Moringaceae.* This mainly habitats in semi-arid tropical and subtropical areas. Drumstick pods and leaves are of great value as sources of carotene, calcium, phosphorous and vitamin.

Cynodon dactylon

The plant species is commonly called as Durva and belongs to the family *Poaceae*. The Sanskrit word durva literally means that which is cut or eaten by the animals. It is perennial creeping grass belonging to the family *poaceae* and the leaves vary in length from 1-10cm, 0.5-1cm broad. *Cynodon dactylon* contains proteins, carbohydrates, mineral constituents, oxides of magnesium, phosphorous, calcium, sodium and potassium. It contains carotene vitamin C, cartone, palmitic acid, triterpenoides, alkaloid and sergonovine.

The chosen plants were screened for endophytic fungi, identified and the isolates maintained. Extraction of the crude secondary metabolites from the isolated endophytic fungus by rotary evaporation technique and to maintain the extract. Testing of the antimicrobial effect of the crude extract obtained from the endophytes on the selected important medical pathogens and plant pathogens.

MATERIALS AND METHODS

Sampling site

Selected plants were collected from a nursery located at Electronic City, Bangalore.

Microbial cultures

The selected test organisms of medical importance;*Escherichia coli, β-Strepto pneumococcus, Staphylococcus albus, Kleibsiella pneumoniae* and *Pseudomonas aeruginosa* were procured with permission from Manipal Acunova, Bangalore. Selected plant pathogens of agricultural importance *Erwinia caratovora*, *Xanthomonas campestris*, *Xanthomonas punicara* were procured with permission from Gandhi Krishi Vignana Kendra (GKVK), Bangalore.

Collection of plant parts

Healthy plant species were selected and samples of leaves, stem, and roots were collected from field. They were stored in the polythene bags to prevent moisture loss and were transported to laboratory within 12hrs and stored at 4°C

Isolation of endophytic fungi

Samples collected were washed thoroughly with sterile water before processing. Plant material was surface sterilized by immersing them in 70% ethanol for 3minutes and 0.5% sodium hypochlorite for 1min and rinsed thoroughly with sterile distilled water. Excess water was dried under laminar airflow chamber. Sterile scalpel was used to remove the outer tissue and the samples were cut approximately to 1cm in length and they were placed on Martin Rose Bengal Agar (MRBA) media containing antibiotic streptomycin which is meant to suppress bacterial growth. The cut end of the material was made to contact the media. After 7 days hypha growing from the plant material was transferred to other MRBA plates and were incubated for nearly 10days and the purity of the cultures were checked periodically. The fungal isolates were identified based on their morphological and reproductive character with the help of identification manual (Barnett and Hunter, 1972)

Fungal cultivation and extraction of metabolites

Blocks of actively growing fungal cultures were cultivated on Potato Dextrose Agar (Himedia) in 250ml conical flask containing 100ml of media. The flasks were incubated at 28℃ in the shaker incubator at 100 rpm for 3 weeks. After incubation, cultures were filtered to isolate mycelia and the ferment broth separately. The mycelia were then extracted with 100ml of methanol overnight. The broth was extracted with 150ml of ethyl acetate using separating funnel. The extract was collected thrice. Both methanol extract and ethyl acetate extract were filtered, combined and evaporated to dryness by rotary evaporator. The crude extract obtained was finally dissolved in 3ml of methanol for antimicrobial assay

Antimicrobial assay

In aseptic condition 50ul of the crude extract was added using micropipette to a sterile paper disc (5mm diameter, Whatman). The paper was air dried and placed on the surface of the Muller Hinton medium (Himedia) seeded with the test organism. The plates were incubated at 37°C for 16hrs and the zone of inhibition measured. The results of an average of 3 repeated paper discs were used to evaluate activity. Ampicillin (dose) the broad spectrum antibacterial agent was used as positive control and methanol was used as negative and blank control

RESULTS

Table 1. Identified endophytic fungi isolated from *Moringa oleifera* and *Cynodon dactylon*

Endophytic fungi isolated from *Moringa oleifera and Cynodon dactylon*
Curvulariasp
Pencilliumoxalicum
Basidiomycetessp
Fusariumgraminareum
Aspergillus spp1
Aspergillus spp2
Helminthosporium (Drescheleriahaolodes)
Alternariabrasicola

Fig. 1. Human pathogens inhibited by secondary metabolites(sample.A) extracted from Endophytic fungi isolated from *Muringa oleifera* and *Cynodon dactylon*

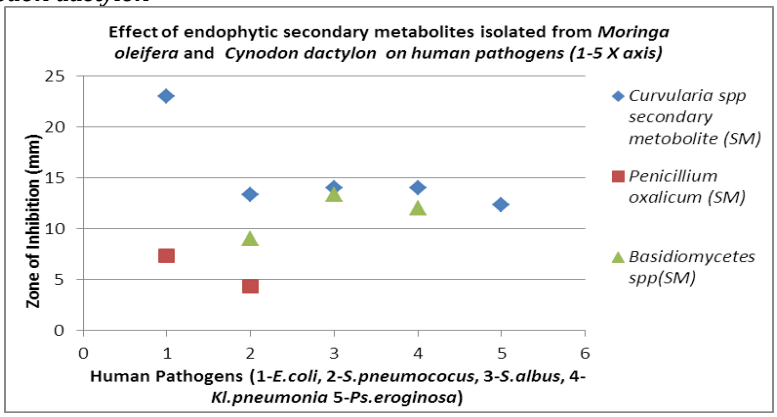


Fig. 2. Human pathogens inhibited by secondary metabolites(sample.B) extracted from Endophytic fungi isolated from *Muringa oleifera* and *Cynodon dactylon*

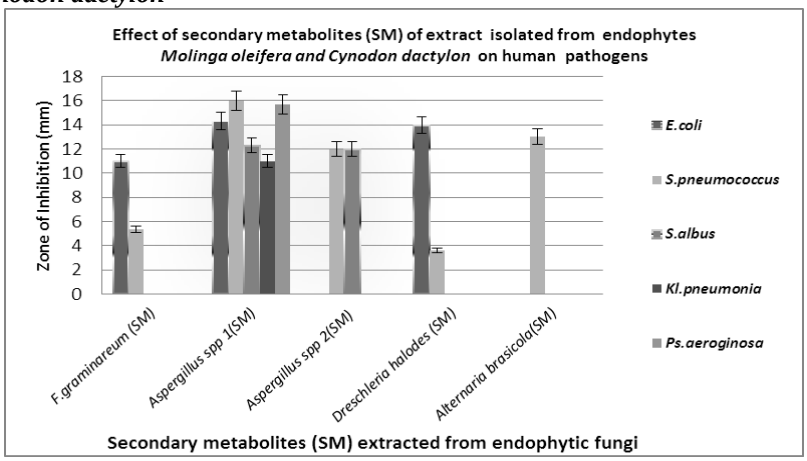


Fig. 3. Plant pathogens inhibited by secondary metabolites(sample.A) extracted from endophyticfungi isolated from *Muringa oleifera*and *Cynodon dactylon*

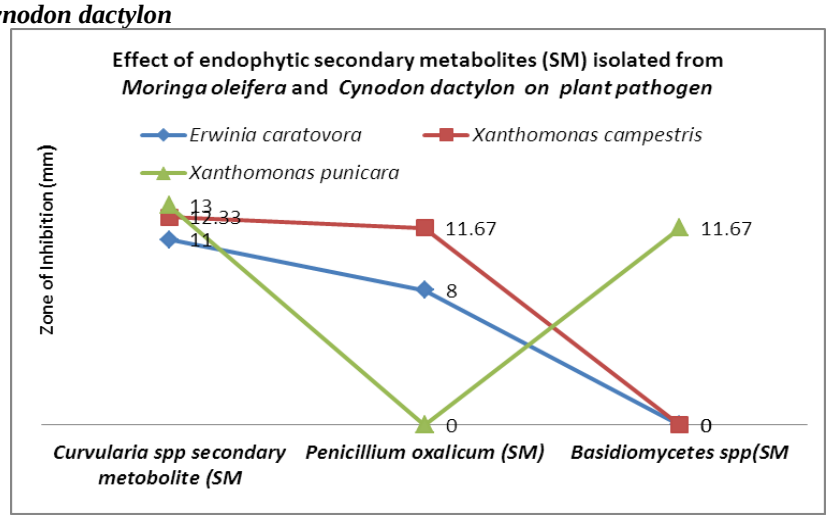


Fig. 4. Plant pathogens inhibited by secondary metabolites (sample.B) extracted from endophytic fungi isolated from *Muringa oleifera* and *Cynodon dactylon*

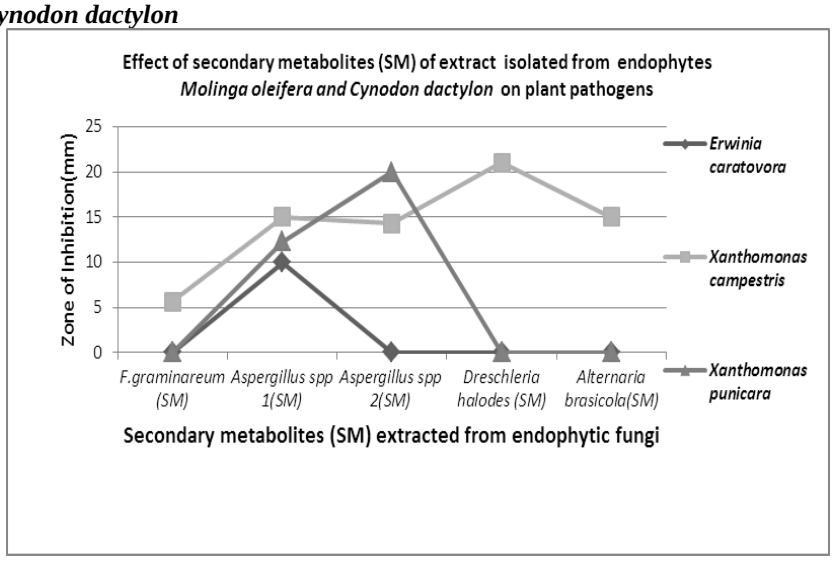


Fig. 5. Inhibition zone to β-*S.pneumococci* Secondary metabolite (S.M)extract of *P.oxalicum*

Fig. 6. Inhibition zone toβ-*S pneumococci* by S.M of Basidiomycetes

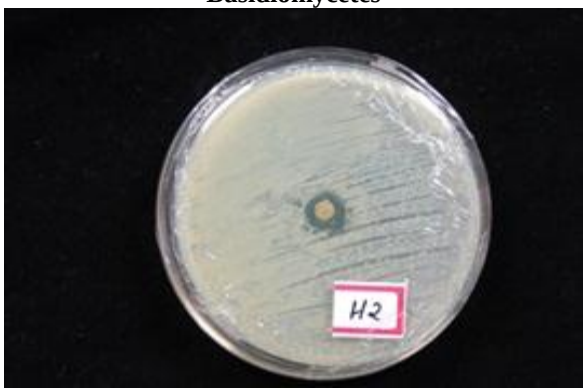


Fig. 7. Inhibition zone to *S. albus* by S.M of *Helminthosporium sp*

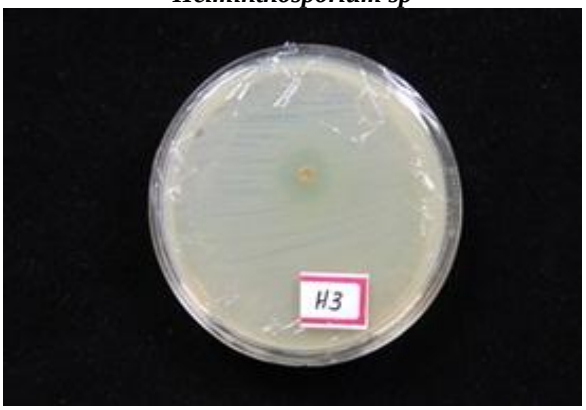


Fig. 8. Inhibition zone to *Kl. pneumonia* by S.M of *Alternaria brasicola*

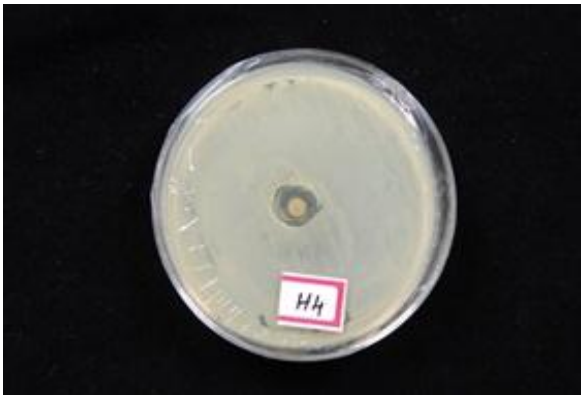


Fig. 9. Inhibition zone to *X.punicara* by S.M of *F.graminareum*

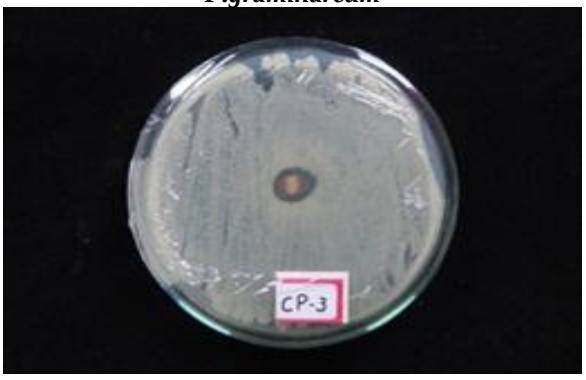
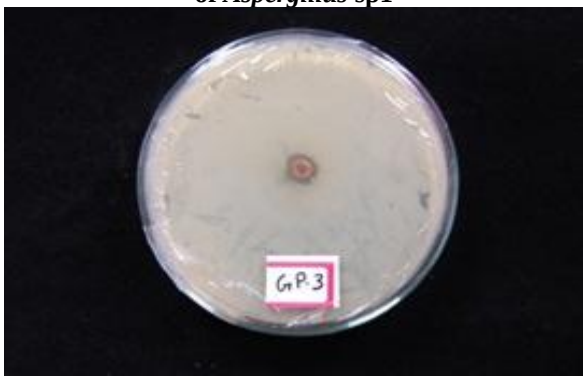


Fig. 10. Inhibition zone to *Xanthomonas punicara* by S.M of *Aspergillus* sp1



DISCUSSION

Fungal endophytes were isolated from *Cynodon dactylon* and *Moringa oliefera* species, on MRBA media and were identified as *Curvularia* sp, *Penicillium oxalicum*,Basidiomycetes sp, *Fusarium graminareum*, *Aspergillus* sp1, *Aspergillus* sp2, *Helminthosporium* (*Drescheleria haolodes*), and *Alternaria brasicola*, these findings are recorded in Table.1. These fungi whose mycelium and broth were used to extract secondary metabolites were tested for their antimicrobial potentcy against medical pathogens namely *Escherichia coli*, β-*Strepto pneumococcus*, *Staphylococcus albus*, *Kleibsiella pneumoniae* and *Pseudomonas aeruginosa* they were they were also analyzed for their antimicrobial potency against plant pathogens such as *Erwinia caratovora*, *Xanthomonas campestris*, *Xanthomonas punicara*.

The antimicrobial properties of secondary metabolites were analyzed according to the disc diffusion method, a standardized protocol from Indian pharmacopeia. Broad spectrum antibiotic Ampicillin was used as a positive control. Sterile whatmann filter paper disc with methanol were used as negative control. The secondary metabolites obtained from the isolated fugal endophytes were tested for its antimicrobial activity by disc diffusion method and the zone of inhibition was measured and recorded in mm and plotted as in Fig.1-Fig.4

The secondary metabolites(SM)obtained from *Curvularia* had shown inhibition against *E.coli* (23mm), β-S*treptococcus pneumococcus* (13mm), *Staphylococcus albus* (14.00mm), *Klebsiella pneumonia* (13.00mm), *Pseudomonas aerogenosa* (12.33mm). The extract from *Penicillium oxalicum* showed inhibition against *E.coli* (7.33mm), β-*Streptococcus pyogenes* (4.33mm). The metabolites obtained from Basidiomycetes showed inhibition against β-*Streptococcus pyogenes* (9.00mm) and *Stapyloccocus albus* (13mm), *Klebsiella pneumonia* (12.00mm).

From Fig.1 the antimicrobial potency was recorded the highest for the secondary metabolite (SM) of *Curvularia* with *E.coli* followed by SM of *Curvularia* with β-S*treptococcus pneumococcus, Staphylococcus albus, Klebsiella pneumonia* and *Pseudomonas aerogenosa*.
SM of Basidiomycetes had been observed to have a good antimicrobial potency and least antimicrobial effect in Fig.1.was observed by secondary metabolites of *Penicillium oxalicum*

In Fig.2 secondary metabolites of *Aspergillus* sp1 and *Aspergillus* sp2 had been observed to have maximum antimicrobial potency on all followed closely by *Alternaria brasicola* on *Pneumococcus* and *Helminthosporium (Drescheleria haolodes*) on *Kleibsiella pneumonia*, least SM effect was observed for extracts of *Fusarium graminareum*

From Fig.3 *Curvularia* sp had shown maximum effect of antimicrobial potency of secondary metabolite on all the three plant pathogens *Erwinia caratovora, Xanthomonas campestris, Xanthomonas punicara.* Followed by secondary metabolites of *Penicillium oxalicum*Lastly the secondary metabolites of Basidiomycetes sp although a fairly good inhibition was observed for *Xanthomonas punicara.*

In Fig.4 all most all the extracts of secondary metabolites of *Fusarium graminareum*, *Aspergillus* sp1, *Aspergillus* sp2, *Helminthosporium* (*Drescheleria haolodes*), and *Alternaria brasicola*, were observed to have antimicrobial potency on *Xanthomonas campestris, followed by Aspergillus sp2* showing maximum antimicrobial effect on *Xanthomonas punicara and Aspergillus* sp1on *Erwinia caratovora.* The zones of inhibition of these antimicrobial effects caused by extracts of secondary metabolites obtained from the endophytes, can be observed from Fig.5-Fig.10

CONCLUSIONS

In the study undertaken, it has been demonstrated that, crude metabolites extracts of fungal endophytes isolated from the two medicinal plants had shown considerable anti-microbial activity against a panel of human and plant pathogenic micro-organisms. Out of the fungal endophytes, *Cuvularia* sp, *Aspergillus* sp1, *Aspergillus* sp2 and *Helminthosporium* (*Drescheleria haolodes*) had demonstrated maximum antimicrobial potency. The extract was observed to be significantly effective against both Gram-positive and Gram–negative pathogen, and fungal pathogens, this showed the broad spectrum nature of the secondary metabolite. The variation in the effectiveness of the extract against different micro-organisms may perhaps depend upon the chemical composition of the extracts and membrane permeability of the microbes, for these biomolecules extracted as secondary metabolites and their characteristic metabolism. Our studies continue to consolidate the antimicrobial potency of the secondary metabolites of the endophytes isolated.

Plant extracts continue to serve as a viable source of drug for the world population and several plant based drugs are already in extensive clinical use. Of late several species of plants have been used widely for the treatment of various diseases due to their antimicrobial property. The plants which harbour these endophytic fungi known to produce secondary metabolites are considered highly significant, as drug based compounds used for various clinical and agricultural trails. It is conceptualized that the secondary metabolites produced by plants are identical to the secondary metabolites produced by fungi present in the tissues of the plant hence these endophytic fungi are considered to have highly potential in producing novel metabolites for exploration in medicine.

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