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Original Article

ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF TWO ENDOPHYTIC FUNGI ISOLATED FROM *MELASTOMA MALABATHRICUM* L. LEAVES

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ABSTRACT

Objective: The objectives of this study were to isolate endophytic fungi from *Melastoma malabathricum* L. leaves and to study their antimicrobial and antioxidant potentials.

Methods: Disc diffusion method and Agar well diffusion method was carried out for studying the antibacterial and antifungal range of the isolated endophytes MMF5 and MMF9. DPPH (2, 2-diphenyl-2-picrilhydrazyl) reduction assay was followed for the study of antioxidant activities of the isolates. CFU counting method was followed for determining the MICs of the isolates against two selected bacteria and to know the mode of action.

Results: Two endophytic fungi have been isolated which were identified as species of *Penicillium* by studying their morphology under compound light microscope. Both were found to show great antibacterial activities against Gram-negative as well as Gram-positive bacteria. The Minimum Inhibitory Concentration (MIC) value of the ethyl acetate extracts of MMF5 and MMF9 were found to be 150µg/ml and 100µg/ml respectively for *Staphylococcus aureus* and *Escherichia coli* with the bacteriostatic mode of action. They also exhibited good antifungal activities against animal as well as plant pathogenic fungi. IC_{50} value of MMF5 and MMF9 in an antioxidant assay using stable DPPH radical was found to be 52.38µg/ml and 24.44µg/ml respectively in comparison to the control ascorbic acid having the value 8.5µg/ml.

Conclusion: From this study, it can be said that due to having good antimicrobial and antioxidant activities, the strains can be used as prospective source in the medicinal industry for the drug development.

Keywords: *Melastoma malabathricum*, Antimicrobial, Antioxidant, Indigenous plants, Minimum Inhibitory Concentration, Bacteriostatic

INTRODUCTION

The concept of 'endophytes' was first introduced by De Bary in the year of 1866. Endophytes are the microorganisms that colonize the internal tissues of plant parts like a leaf, petiole, internodes, flower, etc of many plant groups especially angiosperms and gymnosperms [1]. They live in the plant tissues either symbiotically or detrimentally or neutrally making their own ecological niche. Mainly three groups of microorganisms are reported as endophytes till date viz. fungi, bacteria, and actinomycetes [2]. The earliest and most isolated endophytes belong from the fungal kingdom. Till now many species of several fungal genera like *Colletotrichum*, *Curvularia*, *Fusarium*, *Phoma* etc. are reported [3-6]. The second major groups of endophytes are from the bacterial group. Some examples of bacterial endophytes are the species of *Pseudomonas*, *Bacillus*, *Enterobacter*, etc [7-10]. Generally, endophytes are beneficial microorganisms because they produce many secondary metabolites that protect the plants from the attack of pathogenic microorganisms. They are also helpful for the host plants as they are involved in the mineralization process that added nutritive value to the host plant. Many times they aid in the plant metabolism by synthesizing the precursor molecules required for the plant metabolisms. Endophytes are also clinically important in recent days because they can produce many bioactive compounds that are found in the crude drugs. *Taxomyces andreanae* is an endophyte that generally lives in the inner side of the bark of a gymnosperm named *Taxus brevifolia* [11] is reported to produce the anticancerous drug 'Taxol'. So if an endophyte having known functions is isolated from its natural source, the production of the compounds responsible for the particular functions can be made in large scale in the laboratory and it would also be a time-saving procedure. Several endophytes from several plants are also reported having excellent antimicrobial as well as antioxidant activities [12]. So, endophytes are very much useful for both the plants as well as animals including human being. *Melastoma malabathricum* belonging to the family Melastomataceae is largely used among the many ethnic groups of Tripura in several medical problems. Number of ethnic people live in this area [13]. It is also used by the people outside of India like China, Malaysia, and Indonesia. Ethnic people or tribes learn and gather huge knowledge about the plants which are used ethnically for curing many diseases [14]. Several plant parts of it are used ethnopharmacologically like leaves, barks, seeds, roots, etc. It is used by the ethnic people for the treatment of dysentery, cuts and wound, diarrhea, stomachache, etc [15]. From the previous reports, it was found that the dried leaf extracts of *Melastoma malabathricum* contain several phytochemical constituents like triterpenes, saponins, flavonoids, steroids and tannins. Alkaloids were not reported. Leaves of *Melastoma malabathricum* extracted with methanol are reported to have good antioxidant activities [16]. In this study, the leaves of *Melastoma malabathricum* were collected from Tripura and its antimicrobial and antioxidant activities were measured *in vitro*.

MATERIALS AND METHODS

Collection and selection of plant materials

In our previous study, it has been found that the leaf extract of *Melastoma malabathricum* (Melastomataceae) which is an indigenous plant of Tripua, showed excellent antimicrobial as well as antioxidant activities [17]. So, in this study, the leaves of that particular plant were collected from Tripua and the endophytic organisms of the plant were in concern of study. Fresh leaves were washed in running tap water for 2-3 times followed by sterile water twice and were kept in 4 ˚C for the further studies.

Isolation of the endophytes

Fresh, dust free uninfected leaves of *Melastoma malabathricum* were cut into various sizes with sterilized blades and scissor under laminar air flow. After this, the pieces of laminas were surface sterilized with 4% sodium hypochloride (NaOCl) for 5-7 min. They were again washed gently with the sterile water thrice for removing the excess NaOCl from the surfaces of the leaf pieces. Finally, they

were treated with 70% ethyl alcohol for 4-5 seconds for removing any further contamination. After the completion of surface sterilization, the samples were kept in a sterile Petri plate for mild drying. Next, the leaf pieces were transferred to the MEA plates containing Malt Extracts (2%) with 2% agar-agar. The medium was mixed with streptomycin (50 mg/ml) for avoiding any bacterial contamination. After inoculation, the plates were incubated at 28C until the appearance of fungal growth. Single colonies of fungi coming out from the leaf pieces were picked up. Based on their morphological variations they were labeled with a strain number and checked for antimicrobial potential from their extracts.

Identification of the endophytes

Two strains viz, MMF5 and MMF9 were found potent against a spectrum of microbes. So they were identified based on their morphological features observed under a compound light microscope. The strains were grown on MEA Petri plate for 3-4 d till the appearance of prominent mycelial growth. From each strain, few mycelia were taken on clean slides and stained with cotton blue followed by lactophenol. The mycelia were separated by little stirring with the aid of two sterile needles and were finally mounted by a cover slip. Finally, the morphology of MMF5 and MMF9 were observed under a compound light microscope both under low power (10 X) and high power (40 X) magnifications.

Antibacterial activity of the endophytic isolates

Antibacterial efficiencies of the isolated endophytic fungi were measured against several pathogenic bacteria such as *Escherichia coli* MTCC1667, *Salmonella typhimurium* MTCC98, *Pseudomonas aeruginosa* MTCC741, *Staphylococcus aureus* MTCC96; *Bacillus subtilis* MTCC121, *Listeria monocytogenes* MTCC657. The strains were procured from Microbial Type Culture Collection (MTCC), IMTech, Chandigarh. The antibacterial assay of the endophytes was checked by the disc diffusion method [18]. The antibacterial activities of the endophytes were checked in every seven days for one month time period, i.e., on the 7th, 14th, 21st and 28th day after inoculation of endophytes in ME broths. Pathogenic bacterial suspensions (1.6×10^8) were evenly inoculated on Nutrient Agar (NA) plates using a sterile swab. Paper discs (6 mm in diameter) of the Whatman No. 1 filter paper were impregnated with the Cell Free Supernatant (CFS) of liquid cultures of endophytic isolates and were placed over the bacterial mat in the NA plates. Ciprofloxacin (10 µg/ml) and Dimethyl Sulfoxide (DMSO) were used as positive and negative control respectively. After the incubation of the plates at 37˚C for 24 h it was found that zones of inhibition were developed against the sensitive organisms.

MIC of the ethyl acetate extracts of the isolates

MICs of the CFS of the isolates were studied against *Staphylococcus aureus* and *Escherichia coli* i.e., one Gram-positive and one Gram-negative bacteria respectively. The ethyl acetate extracts of the CFSs were prepared by extracting the CFSs in 40% ethyl acetate. From the dried ethyl acetate extracts of the isolates different concentrations viz., 25, 50, 100, 150 and 200µg/ml were made using DMSO as a solvent. The different concentrations of ethyl acetate extracts were added to the different test tubes of NBs followed by the addition of fixed volume of bacterial culture to each and allowed for the incubation at 37ºC for overnight. After the incubation, 100 µl of bacterial cultures were withdrawn and colony forming units (CFUs) were measured following plate dilution method.

Study of mode of action

Time killed study was performed for determining the mode of action of the ethyl acetate extracts of MMF5 and MMF9 against *Escherichia coli* and *Staphylococcus aureus* respectively. In each experiment two sets were run simultaneously, i.e., one control set and the other is the treatment set. The growing cultures of *Escherichia coli* and *Staphylococcus aureus* were treated with the ethyl acetate extracts of MMF5 and MMF9. The doses of the extracts used in this study were their respective MIC values. To determine the mode of action of the extracts, the calculation was done by counting the colony of the pathogenic bacteria in both controls and treated sets in successive ten hours [19].

Antifungal activity of the isolates

The antifungal activities of the two endophytic fungal isolates were checked against two plant pathogenic fungi viz. *Helminthosporium oryzae* MTCC351, *Alternaria alternata* VBAV007, and two animal pathogenic fungi viz. *Candida albicans* MTCC1644, *Aspergillus parasiticus* MTCC2796. The pathogenic fungal strains were procured either from MTCC, IMTech, Chandigarh or were taken from our laboratory stock. Agar well diffusion method was followed for checking the antifungal efficiency of both MMF5 and MMF9 [20]. Both the endophytes were inoculated in ME broths for their growth. Like the antibacterial assay here also the activity was tested in every 7 d for one month time period. Pathogenic fungi were also grown in ME broths. At every 7th day after the inoculation of endophytes, 100 µl of pathogenic fungal cultures were spread on ME Agar Petri dishes. Then 50 µl of CFS of MMF5 and MMF9 were poured in the wells which were formed by cork borer. The petriplates were then incubated at 28ºC for 3-4 d.

Antioxidant activity

The ethyl acetate extracts of MMF5 and MMF9 were subjected for the antioxidant assay. The study was performed using the stable DPPH radical [21]. The ethyl acetate extracts were dissolved in methanol (0.01 gm in 1 ml of methanol) for making the stock solutions. A solution of DPPH (0.004%) was also prepared for this assay. Different concentrations of ethyl acetate extracts ranging from 5 to 150µg/ml were made from the stock solutions. The concentrations were made by mixing of a specific volume of endophyte stock solutions and a fixed volume of DPPH stock solution. These reaction mixtures were subjected to incubation for half an hour in dark condition. Finally, using spectrophotometer the optical densities were measured at 517 nm. In this experiment methanol with DPPH solution (0.004%) was the blank set. The standard used in this experiment was the ascorbic acid of same concentrations. After the recording of the optical densities, the Percentage of Inhibition (POI) of the test samples were calculated and finally the IC_{50} values of the ethyl acetate extracts of MMF5 and MMF9 were calculated. The formula used in this study for calculating of Percentage of Inhibition is given below:

POI of DPPH activity (%) = [(a-b)/a] ×100 where, a = O. D. of the blank set and b = O. D. of the sample set.

RESULTS AND DISCUSSION

Antibacterial activity of the endophytic isolates

A total of 18 endophytic fungal strains were isolated from the leaves of *Melastoma malabathricum* (table 1). But among them only two i.e., MMF5 and MMF9 showed good antibacterial activity by producing a zone of inhibition of significant size. They were also able to kill both Gram-negative as well as Gram-positive pathogenic bacteria. The length of the zones of inhibition developed by MMF5 and MMF9 were noted (fig. 1-2). Test bacteria were sensitive to Ciprofloxacin and resistant to DMSO. Both MMF5 and MMF9 exert their maximum activity against the Gram-positive bacteria, i.e., *Staphylococcus aureus, Bacillus subtilis* and *Listeria monocytogenes*. Their activity was moderate against *Salmonella typhimurium* and *Escherichia coli*. *Pseudomonas aeruginosa* was the most resistant bacteria among the bacteria used. It was sensitive to the positive control, but the endophytes had little effect on it even in the 21st and 28th day of the experiment.

Table 1: Shows the list of endophytes isolated from leaves of *Melastoma malabathricum*

Leaf No.	Endophytic fungal strain
Leaf1	MMF1, MMF2, MMF3
Leaf2	MMF4, MMF5, MMF6, MMF7, MMF8
Leaf3	MMF9, MMF10, MMF11,MMF12, MMF13,MMF14
Leaf4	MMF15,MMF16, MMF17, MMF18

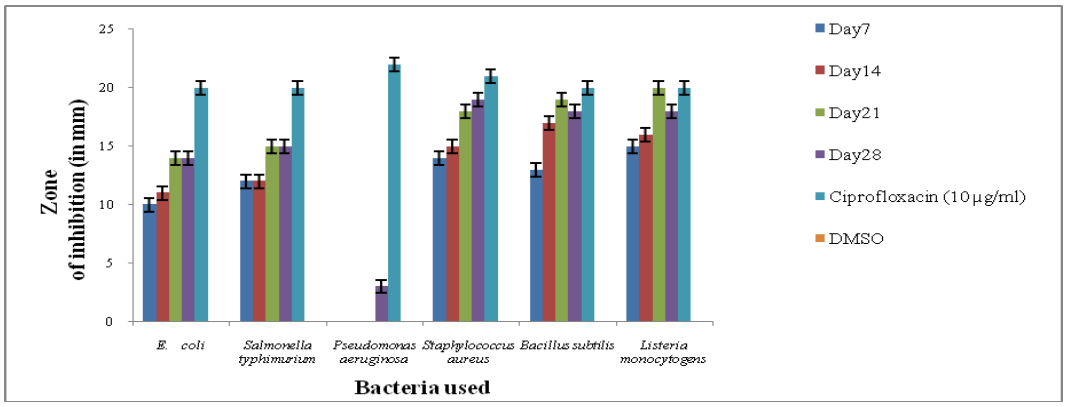

Fig. 1: Shows day wise Antibacterial activity of MMF5

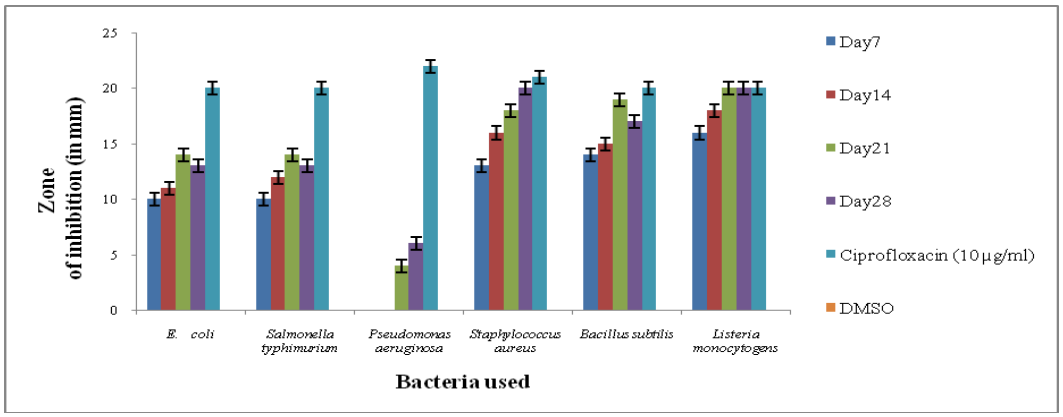

Fig. 2: Shows day wise Antibacterial activity of MMF9

MIC of the ethyl acetate extracts of the isolates

The MICs of the ethyl acetate extracts of MMF5 and MMF9 were determined by counting the CFU against *Staphylococcus aureus* and *Escherichia coli*. The MIC values of MMF5 and MMF9 were 150µg/ml and 100µg/ml respectively against both the bacteria indicates the superiority of MMF9 over MMF5 in killing both Gram-negative as well as Gram-positive bacteria (table 2-3).

Table 2: Shows MICs of MMF5 against *Staphylococcus aureus* and *Escherichia coli*

	CFU/ml	
Concentration (µg/ml)	***Staphylococcus aureus***	***Escherichia col***
Control	1.8×10^{9}	2.4×10^{9}
25	1.7×10^{8}	2.0×10^{8}
50	1.1×10^{8}	1.8×10^{8}
100	1.0×10^{8}	3.2×10^{7}
150	**1.0×10^{6}**	**2.3×10^{5}**
200	1.5×10^{4}	3.1×10^{3}

Table 3: Shows MICs of MMF9 against *Staphylococcus aureus* and *Escherichia coli*

	CFU/ml	
Concentration (µg/ml)	***Staphylococcus aureus***	***Escherichia coli***
Control	2.0×10^{9}	4.0×10^{9}
25	3.8×10^{8}	3.2×10^{8}
50	2.4×10^{8}	1.6×10^{8}
100	**1.6×10^{6}**	**1.1×10^{6}**
150	1.4×10^{4}	2.4×10^{4}
200	2.2×10^{3}	2.1×10^{3}

Study of mode of action

By counting the Colony Forming Units (CFU) in both control and treatment set at every hour the pattern of the growth curve of the bacteria used were found, and the mode of action of the ethyl acetate extracts were determined. Ethyl acetate extracts of MMF5 and MMF9 exhibited a bacteriostatic mode of action against the bacteria used in this experiment (fig. 3A-3D). Antibacterial activities were not bactericidal as no sharp declines in the growth of bacteria in the treatment sets were found.

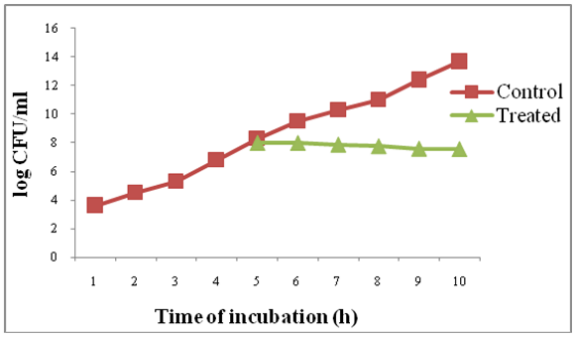

Fig. 3A: Shows the mode of action of MMF5 against *Staphylococcus aureus*

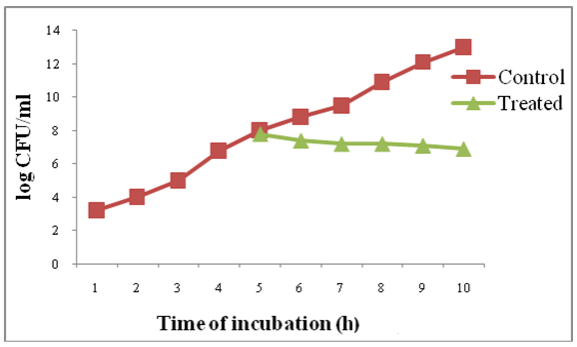

Fig. 3B: Shows the mode of action of MMF5 against *Escherichia coli*

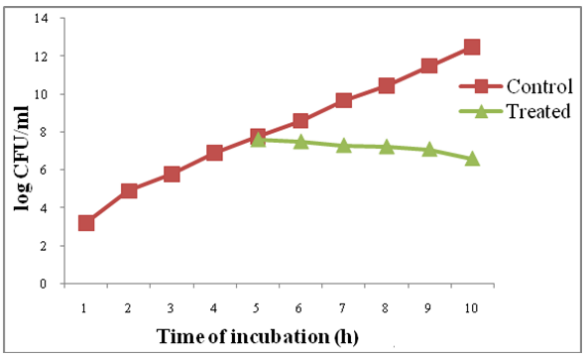

Fig. 3C: Shows the mode of action of MMF9 against *Staphylococcus aureus*

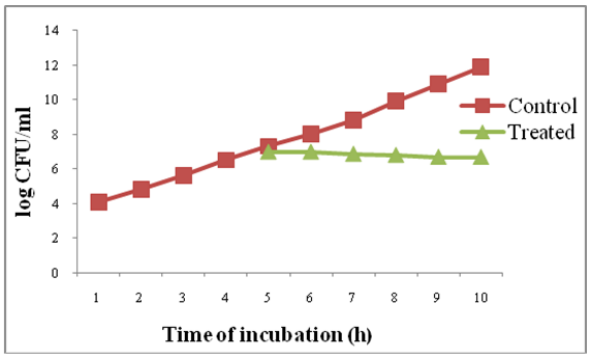

Fig. 3D: Shows the mode of action of MMF9 against *Escherichia coli*

Antifungal activity of the isolates

Clear zones of inhibition were developed around the well in the ME plates against the sensitive fungi. Both MMF5 and MMF9 were found to exhibit antifungal activity against all the four pathogenic fungi used. Both the endophytes were more effective against the plant pathogenic fungi, i.e., *Helminthosporium oryzae* and *Alternaria alternata* than the animal pathogenic fungi, i.e., *Candida albicans* and *Aspergillus parasiticus*. The antifungal activity of the endophytes are compared and presented by measuring the zones of inhibition (in mm) produced against the test fungi (fig. 4A-4B).

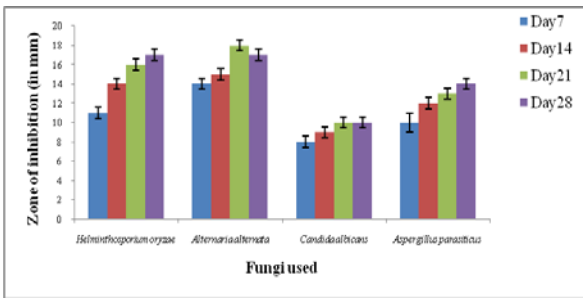

Fig. 4A: Shows day wise Antifungal activity of MMF5

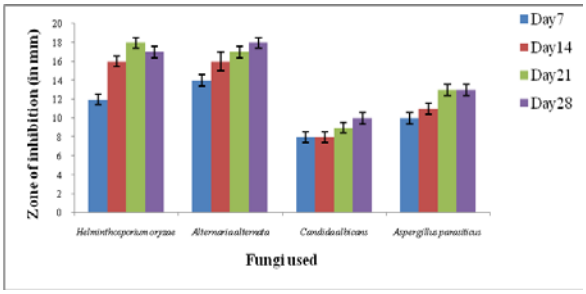

Fig. 4B: Shows day wise Antifungal activity of MMF9

Antioxidant activity

In the antioxidant assay, the ethyl acetate extracts of two isolates MMF5 and MMF9 showed good antioxidant potentials with IC_{50} values of 52.38µg/ml and 24.44µg/ml respectively. The IC_{50} value of ascorbic acid was recorded as 8.5µg/ml (fig. 5).

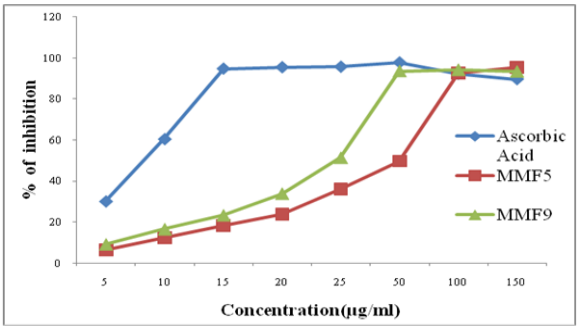

Fig. 5: Shows antioxidant activities of MMF5 and MMF9

Identification of MMF5 and MMF9

Based on the morphological characteristics both MMF5 and MMF9 were identified as species of *Penicillium*. Both MMF5 and MMF9 were found to be with conidiophores with metulae at their apex that divides further to form the sterigmata. The sterigmata bear exogenously produced conidia in long chains. Conidia were globose in shape. Terminal portion of the conidiophores along with its branches and sterigmata developed broom shaped structures. (fig. 6A-6B).

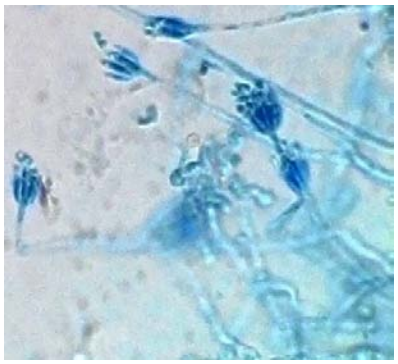

Fig. 6A: Shows the microscopic view of MMF5 under compound light microscope

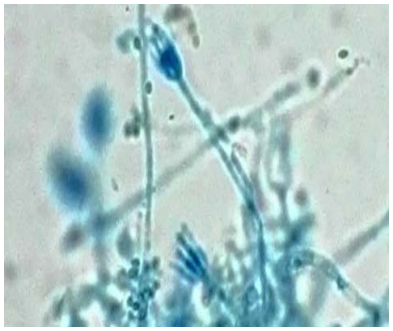

Fig. 6B: Shows the microscopic view of MMF9 under compound light microscope

CONCLUSION

In the antibacterial study among the 18 endophyte isolates, MMF5 and MMF9 were found to have better activities against both Gram-negative as well as Gram-positive bacteria used in the assay and both were further used in antifungal as well as antioxidant study. Both the endophytes exhibited good antifungal activities against the plant pathogenic fungi like *Alternaria alternate*, *Helminthosporium oryzae* as well as the human pathogenic fungi like *Candida albicans*, *Aspergillus parasiticus*. *Alternaria alternata* and *Helminthosporium oryzae* cause leaf spot in several plants and brown spot of rice respectively. *Candida albicans* is the causal agent of oral and vaginal candidiasis; *Aspergillus parasiticus* is the pathogen responsible for aspergillosis. Thus MMF5 and MMF9 have a great prospect in the medicinal industry for having the ability to kill such broad spectrum of pathogens those cause diseases in plants and humans. They were also found to show good antioxidation potential because of having low IC_{50} value. The antimicrobial and antioxidant potentials of both the endophytic fungal strains added value towards their applicability.

CONFLICT OF INTERESTS

Declare none

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CONFLICT OF INTERESTS

Declare none

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