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EVALUATION OF ANTIMICROBIAL POTENTIAL OF ENDOPHYTIC FRACTIONS OF MENTHA PIPERITA AGAINST PATHOGENIC MICROBES

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ABSTRACT

Endophytic microorganisms asymptomatically colonize healthy plant tissues and may be related to the plant's resistance to attack by pathogens or even to the synthesis of secondary metabolites. The present study was aimed at isolating and characterizing endophytic strains from the leaves, stems and roots (aerial and non aerial parts) of *Mentha piperita* (Lamiaceae). Coarse and fine leaves, stems and root fragments after surface sterilization with internal procedures were collected to isolate endophytes. After 10 days of incubation, the colonization rate (CR) of the fragments and the endophytic were purified and maintained in culture medium. The bacteria were partially characterized using Gram staining and fungi were identified by distinguishing between reproductive structures using a micro-culture technique. The endophytic CR was more significant in coarse root fragments. In roots, the percentage of bacteria was higher than the percentage of fungi while in leaves and stems, the percentage of fungi was higher in comparison to bacteria. Gram-positive bacilli (MPGPB) and gram positive coco-bacilli (MPGPCB) were accounted for the majority of bacterial isolates. Amongst all the fungal isolates, the majority had sporulating mycelium, which mainly consisted of fungi from the genus *Penicillium*, *Fusarium*, *Trichoderma* and *Papulaspora*. The antimicrobial activity of dominant endophytes were checked after preparation of solvent broth culture against test bacterial cultures viz. *Bacillus subtilis*, *Bacillus licheniformis*, *Micrococcus luteus* and *Pseudomonas aeruginosa*. The results were found to be very significant and surprising as all these endophytic solvent fractions showed potent antimicrobial activity against the test organisms.

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INTRODUCTION

Natural products and their derivatives represent more than 50% of the drugs in clinical use in the world (Cowan, 1999). One of the paramount reasons for pursuing natural products chemistry resides in the actual or potential pharmacological activity to be found in alkaloids, terpenoids, coumarins, flavanoids, lignans, glycosides etc. Since the advent of antibiotics in the 1950's, the use of plant derivatives as a source of antimicrobials has been virtually non-existent (Cowan, 1999). Plants are biologically and chemically diverse resources. The number of higher plant species (angiosperm and gymnosperm) on this planet is estimated at 250,000 with lower level at 215,000 and upper level as high as 500,000. Of these only about 6% have been screened for biological activity and 15% have been evaluated for phyto-constituents. It is estimated that only 5000 plant species have been studied for medicinal use with a large chunk, at least 80% remaining to be investigated, a greater proportion of which are tropical forest

plants. 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 (Hirch and Braun, 1992). It is believed that plants which are able to survive in harsh environment, plants that are used for special purpose such as herbal medicine and plants which show an unusual longevity contains endophytes which produces novel bioactive compounds (Strobel *et al*., 1999). Endophytes include a variety of bacteria, fungi and actinomycetes. Cultivable endophytic colonizing microbes can be isolated from wild and agricultural crop plants (Fisher *et al*., 1992; Adams and Kloepper, 2002; Monica and Esperanza, 2006). Antimicrobial potential of the endophytes isolated from Murraya koenigii was determined (Shawl *et al*., 2015). In the present investigation, the endophytes were isolated on LB and PDA medium for determination of the nature of endophytes. These were further identified by gram staining in form of type of bacteria and lactophenol cotton blue staining for determination of spores or other microscopic arrangements in type of fungi.

MATERIALS AND METHODS

Surface sterilization of plant tissues and Isolation of endophytes

Further the tissues of the plant were soaked in 70% alcohol for few seconds or in 0.5-3.5% sodium hypochlorite for 1-2 minutes followed by rinses in sterile double distilled water before placing it on a LB medium for isolation of endophytic bacteria (Kharwar *et al*., 2009). For isolation of fungal endophytes surface sterilization of tissue requires 70% ethanol for 1-3 minutes, aqueous sodium hypochlorite (4% available chlorine) for 3-5 minutes again rinse with 70% ethanol 2-10 seconds and final rinse with double distilled water and drying in laminar air flow. Sterile knife blade was required to remove outer tissues from sample and to excise inner tissues. The PDA plates were kept for about 5-6 days for observation of growth of any fungal endophytes. All the plates were incubated at 28ºC to promote the growth of endophytes and were regularly monitored for any microbial growth (Maheshwari, 2006). On observing the microbial growth, sub-culturing was done. Each endophytic culture were checked for purity and transferred to freshly prepared PDA plate. Appropriate controls were also be maintained in which no plant tissues were inoculated. The bacterial and fungal endophytes isolated were identified.

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 have been 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). Gram stains were 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 is grown in nutrient broth overnight at room temperature with shaking at 150 rpm were heat-fixed onto a slide and then stained. The fungal slides if isolated were stained with lactophenol. The structures were observed using a photomicroscope. The samples were then compared to other samples reported in the literature (Schilmiller *et al*., 2008; Sutton, 1980; Carmichael *et al*., 1980).

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 10-14 days in shaker. After incubation, extraction was done with different solvents (Chloroform, Ethyl acetate). The organic phase were collected and kept for drying at 37°C. The dry weight of the extract was determined.

Determination of Antimicrobial activity

Culture Media

For antibacterial test, Soyabean Casein Digest agar/broth was used.

Inoculum

The bacteria viz. *Bacillus subtilis*, *Bacillus licheniformis*, *Micrococcus luteus* and *Pseudomonas aeruginosa* were inoculated into Soyabean Casein Digest broth and incubated at 37 ^{0}C for 18 h and suspension were checked to provide approximately, 10^5 CFU/ml.

Determination of diameter of zone of inhibition by well diffusion method

The agar well diffusion method was modified (Perez *et al*., 1993). Soyabean Casein Digest agar medium (SCDM) was used for bacterial cultures. The culture medium was inoculated with the bacteria separately suspended in nutrient broth. A total of 8 mm diameter wells were punched into the agar and filled with secondary metabolites at 200 mg/ml. 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. The procedure for assaying antibacterial activity was performed in triplicates to confirm the readings of diameter of zone of inhibition observed for each of the test organism.

RESULTS AND DISCUSSION

In the present investigation, after the surface sterilization of leaves, stems and roots of the plant, different bacterial and fungal endophytes from *Mentha piperita* were isolated on Luria-Bertani (LB) and Potato dextrose agar (PDA) medium respectively. In roots, the percentage of bacteria was higher than the percentage of fungi while in leaves and stems, the percentage of fungi was higher in comparison to bacteria **(**Figure 1; Table 1; Figure 2; Figure 3). Gram-positive bacilli (MPGPB) and gram positive coco-bacilli (MPGPCB) were accounted for the majority of bacterial isolates.

Figure 1: Inoculation of surface sterilized plant parts (leaves; stems and roots) of Mentha piperita on LB and PDA media

Table 1: Percent endophytic cultures isolated from aerial and non aerial parts of *Mentha piperita*

Parts of *Mentha piperita*	Percent endophytes isolated	
	Bacterial	Fungal
Leaves	10	95
Stems	20	80
Roots	85	20

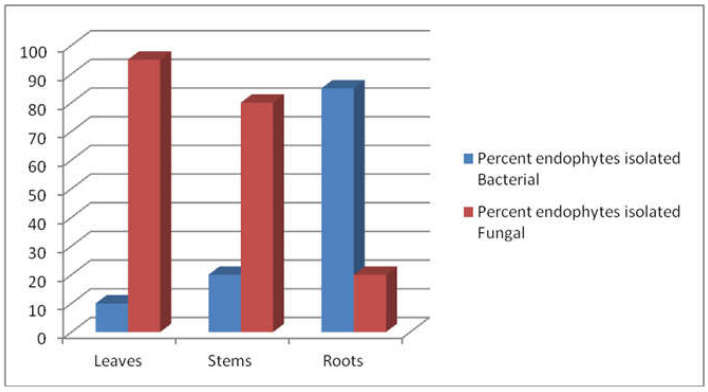

Figure 2: Graphical representation in percentage of endophytic cultures isolated from aerial and non aerial parts of *Mentha piperita*

Figure 3. Isolation of bacterial and fungal endophytes on LB and PDA medium

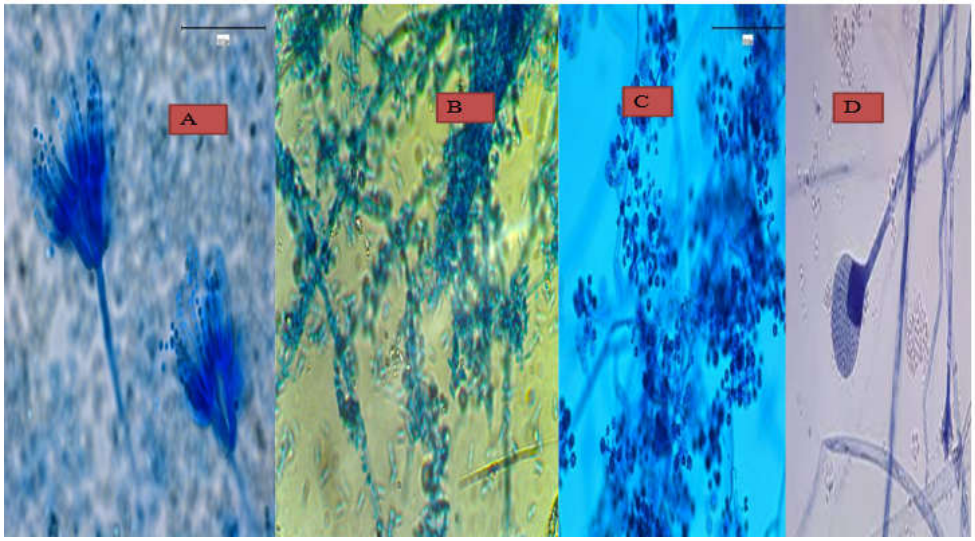

Figure 4. Microscopic identification of fungal endophytes (A) *Penicillium chrysogenum*; (B) *Fusarium oxysporum*; (C) *Trichoderma viride*; (D) *Papulospora* sp

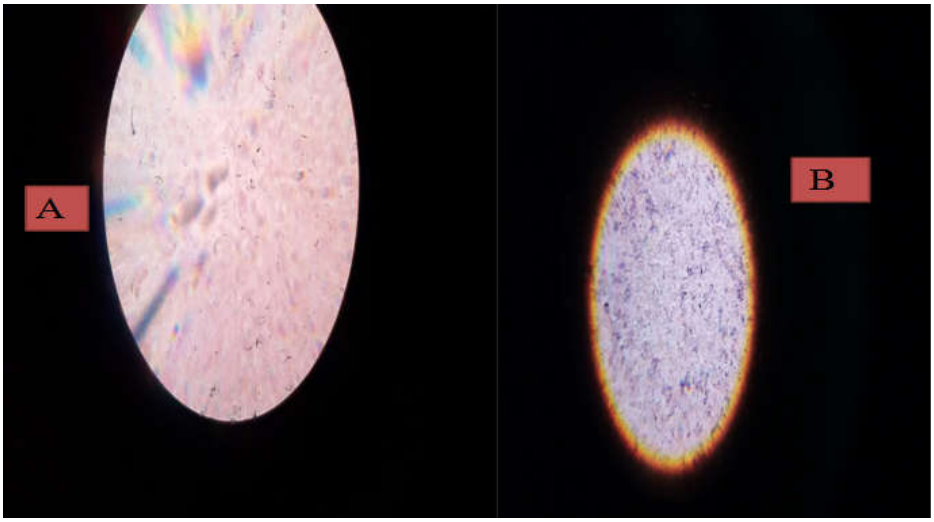

Figure 5. Microscopic identification of bacterial endophytes (A) Gram positive cocco-bacilli (MPGPCB); (B) Gram positive bacilli (MPGPB)

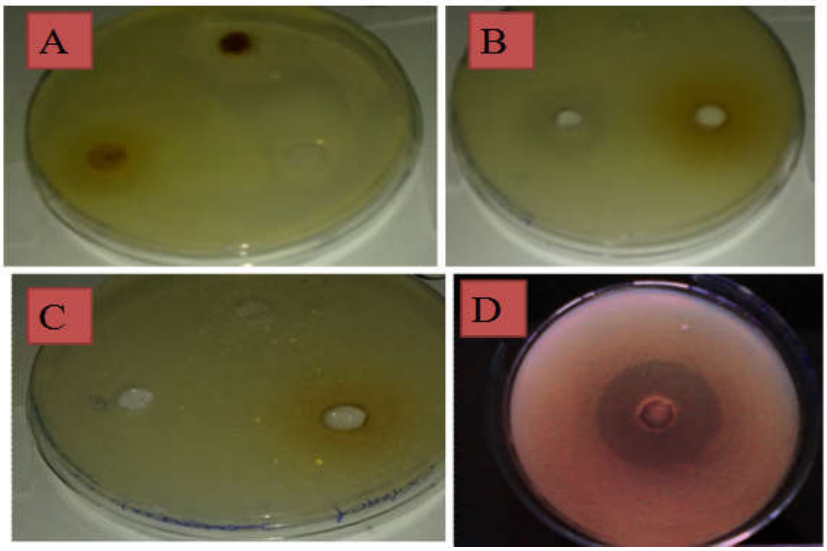

Figure 6. Antimicrobial activity of different fractions against (A) *Pseudomonas aeruginosa*; (B) *Bacillus subtilis*; (C) *Micrococcus luteus*; (D) *Bacillus licheniformis*

Amongst all the fungal isolates, the majority had sporulating mycelium, which mainly consisted of fungi from the genus *Penicillium*, *Fusarium*, *Trichoderma* and *Papulaspora* (Figure 4; Figure 5). The antimicrobial activity of dominant endophytes were checked after preparation of solvent broth culture against test bacterial cultures viz. *Bacillus subtilis*, *Bacillus licheniformis*, *Micrococcus luteus* and *Pseudomonas aeruginosa*. The results were found to be very significant and surprising as all these endophytic solvent fractions showed potent antimicrobial activity against the test organisms (Figure 6). Previous study performed by our group on isolation of endophytes from aerial parts of *Mentha piperita* reported no pure culture isolation of fungal endophytes while bacterial endophytes were isolated as bacilli, cocci and cocco-bacilli (Kumar & Mathur, 2017).

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