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Purification and Characterization of Secondary Metabolites from Bacterial Endophytes Isolated from *Mentha piperita*

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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. 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. In the present investigation, the putative endophytes were isolated from leaves and stems of *Mentha piperita* (peppermint) plant. A total of 4 different bacterial endophytes were isolated from leaves which were marked as L1, L2, L3 and L4 while only 2 different types of bacterial endophytic cultures were isolated from stems of the plant which were marked as S1 and S2. These bacterial endophytes were isolated and maintained in pure form on LB medium. No recording of fungal endophytes was observed on PDA medium plates. These bacterial cultures were further progressed for the production of secondary metabolites. The secondary metabolites were extracted using ethyl acetate as solvent. Further, secondary metabolites were dried to obtain the crude extract and were purified using column chromatography, and identified via TLC, GC-MS and FT-IR spectra. These metabolites were characterized as terpenes viz. cinnamaldehyde, cinnamyl alcohol and eugenol as recorded by GC-MS spectra.
Key words : Bacterial endophytes, Mentha piperita, leaves, stems, secondary metabolites.

Introduction

Natural products and their derivatives represent more than 50% of the drugs in clinical use in the world.[1] 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. Antimicrobial plant extracts have been recognized as a future source of new antimicrobials in the event of the current downturn in the pace at which these are being derived from micro-organisms.[2] The public is also becoming more aware of problems with over prescription and misuse of traditional antibiotics. 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. [3] 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 endophyte which produces novel bioactive compounds.[4] Endophytes include a variety of bacteria, fungi and actinomycetes. Cultivable endophytic

colonizing microbes can be isolated from wild and agricultural crop plants.[5-7] Little is known about endophytes, but several researches revealed that the endophytes are the source of secondary metabolites as that of its host plant. It has been speculated that the beneficial effects in the host plant are the combined effect of endophytic activities. *Mentha piperita;* peppermint belong to the family, Labiatae/Lamiaceae is a species of mint native to much of Europe and southwest Asia, though it's exact natural range is uncertain due to extensive early cultivation. It grows in wet soils. It is an invasive species in the Great Lakes region where it was first sighted in 1843. It is an herbaceous rhizomatous perennial plant growing 30–100 cm tall, with variably hairless to hairy stems and foliage, and a wide-spreading fleshy underground rhizome.[8, 9] The oil extracted from the leaves of the plant is having pharmacological activity.[10] The present investigation is focused upon the isolation and identification of bacterial endophytes from the aerial parts (viz. leaves and stems) of *Mentha piperita* plant.

Experimental

Collection and Identification of the plant parts

The aerial parts of *Mentha piperita* L. viz. leaves and stem were collected and were taxonomically identified by some Taxonomists/Botanists in the form of herbarium.

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.[11] Some isolates require months or more time in culture before they sporulate. Even stop sporulating after they have been transferred several times. The LB plates were incubated for about 7-10 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 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, also added 50mg/l chloramphenicol within PDA medium to suppress bacterial growth.[12] 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. 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 will also be 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 have been isolated. Finally all the purified endophytes were maintained at 4ºC till further used.

Production of Secondary metabolites

LB broth was prepared and autoclaved. Endophytic bacterial cultures were inoculated in the broth medium separately within the flasks. Flasks were then incubated at 32°C for 36 h in incubator shaker. Further, the broth culture was centrifuged to produce the supernatant/filtrate. The extraction of the supernatant/filtrate with different solvents (Chloroform: Ethyl acetate) in 1:1 ratio and left for 15-30 minutes. The organic phase were collected and kept for drying at 37°C.

Phytochemical screening of the extract

The portions of the dry extract were subjected to the phytochemical screening using the method adopted.[13, 14] Phytochemical screening was performed to test for alkaloids, saponin, tannins, flavanoids, steroids, sugars and cardiac glycosides.[15, 16].

Test for alkaloids

The 0.5 g of the dried extract was dissolved in 5 ml of 1% HCl and were kept in water bath for about 2 minutes. 1ml of the filtrate was treated with Dragendroff's reagent Turbidity or precipitation was taken as indicator for the presence of alkaloids.

Test for Tannins

About 0.5 g of the dried extract was dissolved in 10 ml of boiling water and was filtered. Few ml of 6% FeCl3 was added to the filtrate. Deep green colour appeared confirmed the presence of Tannins.

Test for Flavanoids

About 0.2 gm of the dried extract was dissolved in methanol and heated for some time. A chip of Magnesium metal was introduced followed by the addition of few drops of conc. HCl. Appearance of red or orange color was taken as an indicator of the flavanoids.

Test for Saponin

About 0.5 g of the dried extract was stirred with water in the test tube. Frothing persists on warming was taken as an evidence for the presence of saponin.

Test for Steroids

Salkowaski method was adopted for the detection of steroids. About 0.5 g of the dried extract was dissolved in 3 ml of chloroform and filtered. To the filtrate, conc. H_2SO_4 was added to form a lower layer. Reddish brown color was taken as positive for the presence of steroids ring. [17]

Test for Cardiac glycoside

About 0.5 g of the dried extract was dissolved in 2 ml of glacial acetic acid containing 1 drop of 1% $FeCl_3$. This was under laid with conc. H_2SO_4. A brown ring obtained at the interphase indicates the presence of deoxy sugar. A violet ring appeared below the ring while in the acetic acid layer a greenish ring appeared just above ring and gradually spread throughout this layer.

Test for reducing Sugars

1ml each of Fehling's solutions, I and II was added to 2 ml of the aqueous solution of the extract. The mixture was heated in a boiling water bath for about 2-5 minutes. The production of a brick red precipitate indicated the presence of reducing sugars.

Purification of Secondary metabolites via chromatographic and spectroscopic techniques

Conventional preparative TLC

Silica gel G used for thin layer chromatography (TLC) was activated in hot air oven at 110°C for one hour.

Preparation of thin layer plates and loading of sample

A quantity of the finely divided absorbing agent silica gel G was prepared by the absorbent with twice the weight of distilled water and the mixture were made homogeneous by vigorous shaking for 5 minutes, then it was applied to the glass plate in a thin and uniform layer by using a Stahl–type applicator or by means of a spreading device. The thickness of the applied layer was maintained at 2 mm to 4 mm for leaves fraction and the plates were activated by being dried in a hot air oven, usually for 24 hours at 60°C. The plates were developed in the solvent, Ethyl acetate: methanol: water (EMW) in a ratio of 40:5:4 to separate the polar compounds. Another solvent system was used for separation of intermediate polar/acidic compounds were, Chloroform: ethyl acetate: formic acid (CEF) in the ratio of 5:4:1. Chromatograms were sprayed with vanillin-sulphuric acid to view the vanillin-active compounds. At least 90 % of the plate was covered only the exposed part was sprayed with the detection system. The active fractions/ pure compounds were scraped from the silica

gel plate and eluted from the silica gel with ethanol. The active compounds were filtered through Millipore filters (0.45 µm and 0.22 µm) to remove the silica gel and this yielded more of compound(s) fraction.

Gas Chromatography–Mass Spectrometry (GC/MS) analysis

GC/MS analysis of the potent volatile constituents present in the secondary metabolite crude extract was performed using a Perkin Elmer GC Claurus 500 system and Gas Chromatograph interfaced to a Mass Spectrometer (GC/MS) equipped with Elite-1 fused silica capillary column (30 m × 0.25 mm) composed of 100% Dimethyl poly siloxane) from Perkin Co., Germany. For GC/MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1 ml/minute and an injection volume of 2 µl was employed (split ratio of 10:1). Injector temperature 250°C; Ion-source temperature 280°C. The oven temperature was programmed from 110°C (isothermal for 2 minutes), with an increase of 10°C/min, to 200°C, then 5°C/min to 280°C, ending with a 9 minutes isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 seconds and fragments from 45 to 450 Da. Total GC running time was 36 minutes. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Software adopted to handle mass spectra and chromatograms was Turbo Mass Ver 5.2.0

Fourier Transform Infrared (FT-IR) studies

The IR spectrum of crude extract of secondary metabolite was recorded using a computerized FT-IR spectrometer (Perkin Co., Germany) in the range of 4000–400 cm^{-1} by the KBr pellet technique.

Results and Discussions

The present investigation illustrates the isolation and identification of putative endophytes from *Mentha piperita* (peppermint) plant. A total of 4 different bacterial endophytes were isolated from leaves which were marked as L1, L2, L3 and L4 while only 2 different types of bacterial endophytic cultures were isolated from stems of the plant which were marked as S1 and S2. These bacterial endophytes were isolated and maintained in pure form on LB medium. No recording of fungal endophytes was observed on PDA medium plates. The bacterial endophytes were inoculated in LB broth flasks separately for production of secondary metabolites. The crude extract of the secondary metabolites were processed for phytochemical screening. The extracts were found positive of alkaloids, flavanoids, tannins, saponin, steroids and reducing sugars while glycosides were found to be absent **(Table 1)**. The secondary metabolites extracted were further chromatographed in TLC and GC-MS. The metabolites isolated from bacterial endophytes viz. L1, L2, L3, L4, S1 an]d S2 were found to have similar Rf values (i.e 0.65) as recorded by TLC chromatogram. This confirms that, the crude secondary metabolites of each of the bacterial endophytes were of similar composition and nature. The results are shown in **Figure 1**. These metabolites were found to be in the form of terpenes viz. cinnamaldehyde, cinnamyl alcohol and eugenol as recorded by GC-MS spectra. The results are shown in **Figure 2**. The presence of some of the secondary metabolites reported by the earlier workers from the leaves such as eudesmanoids[18], isoflavone glycosides[19] and essential oils may be the cause of the antibacterial activity of this plant. It is evident from the literature that, the phenols[20], tannins[21, 22], terpenoids[23], flavonoids and flavonoid glycosides[24] are active against a wide range of microbes. Essential oils of plants have a very varied chemical composition, including over 60 different kinds of volatile molecules which can be extracted by distillation and belong to two main groupings, terpenes and aromatic products. Besides contributing to the scent of plants, many of these compounds present interesting bioactive properties, ranging from antibiotic to antitumor effects[25]. The ability to produce such volatile antibiotics has stimulated to investigate several endophytic strains for their possible use in the so-called myco-fumigation of foodstuffs.[26-28] Additional prospects for biotechnological applications reside in their use as flavoring agents in the food industry[29], and even as biofuels.[30] Previous studies reported the endophytic cultures isolation from *Murraya koenigii*[31].

Table 1: Phytochemical screening of the crude extract (s) of secondary metabolites

Secondary metabolites extract of *Mentha piperita*	Phytochemical constituents					
	Alkaloids	**Flavanoids**	**Tannins**	**Steroids**	**Saponin**	**Glycosides**
Leaves	+	+	+	+ +	+	_
Stem	+	+	+	+ +	+	_

*+, present; ++, dominant; -, absent

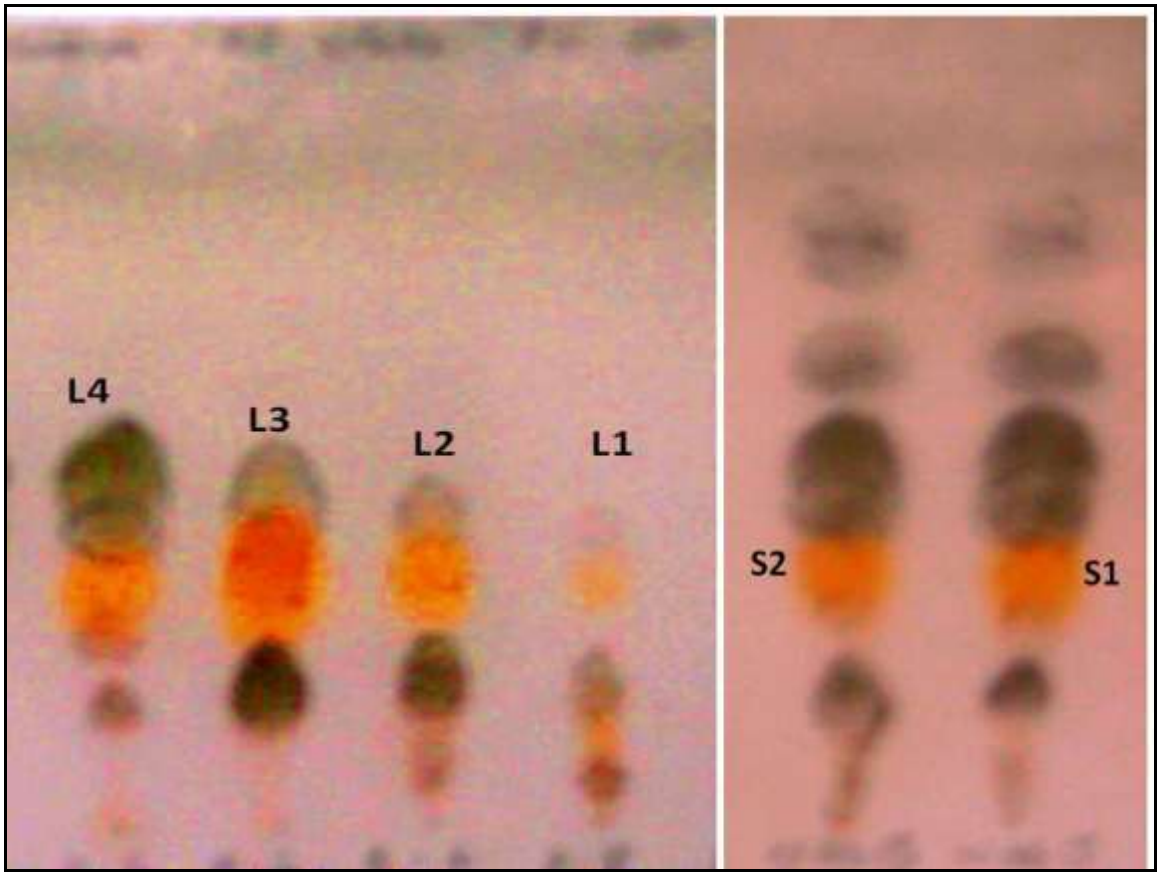

Figure 1: Separation of crude secondary metabolites via TLC (Rf value: 0.65) obtained from bacterial endophytes isolated from *Mentha piperita*

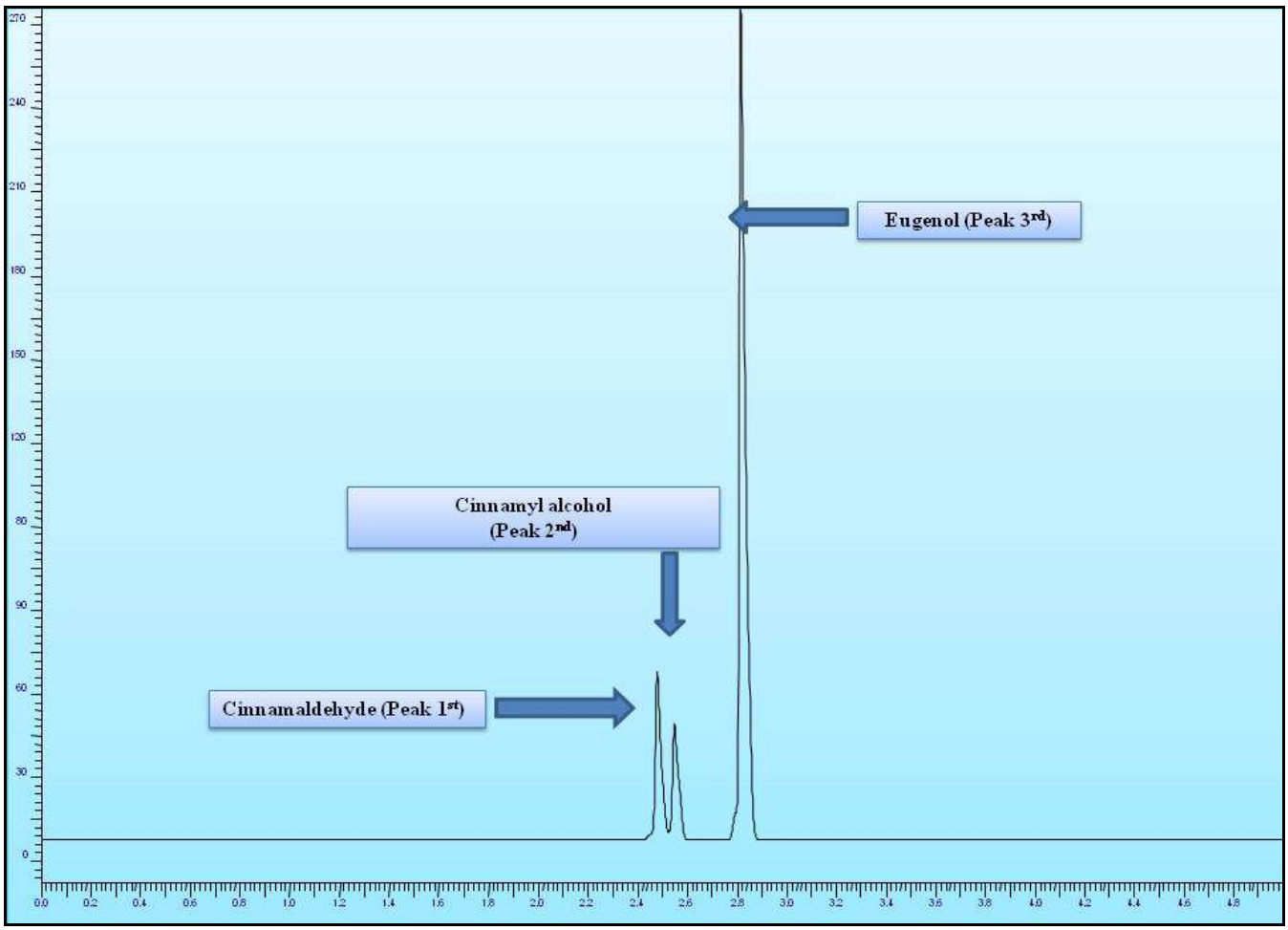

Figure 2: GC-MS spectra of terpenes (secondary metabolites) purified from *Mentha piperita*

Conclusions

The present study suggests that, endophytes can be utilized as the potential bioresource for secondary metabolites other than host plant. Significant molecules having unique pharmacological activities can thus be isolated, identified and screened for different pharmacological activities. New acquisitions in these fields will be fundamental in order to exploit microbial strains for a large-scale production of plant-derived drugs in controlled fermentative processes.

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