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Metabolic Profiling of Endophytic *Bacillus subtilis* US2 Isolated from *Rosmarinus officinalis* Leaves with Potential Antimicrobial Activity

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Abstract: Twelve isolates of endophytic bacteria from *Rosmarinus officinalis* (Rosemary) have been screened for their potential in preventing growth of pathogens (*Bacillus subtilis, Staphylococcus epidermidis, Serratia marcescens, Proteus vulgaris, Bacillus cereus, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumonia, Fusarium oxysporum, Acremonium solani, Aspergillus flavus* and *Penicillium griseofulvum*) in addition to non-pathogenic *Aspergillus columnaris* and *Aspergillus ochraceus*. Endophytic bacterial isolates displayed considerable antimicrobial activity. The mean zones of inhibition produced by the different bacterial endophytes fluctuated between one and 49 mm. The most active endophyte inhibited growth of *Staphylococcus epidermidis* (28.67mm), *Serratia marcescens* (48.67mm) and *Bacillus cereus* (10mm). 16S rRNA sequence has been deposited in the NCBI GenBank database under accession number: MH093646 (*Bacillus subtilis*). Metabolic profiling analysis by GC-MS revealed that five compounds were major constituents of the crude extract obtained from endophytic *Bacillus subtilis* MH093646; (Cyclo-hexanone / 2-butoxy-Ethanol / Acetic acid, butyl ester / Propanoic acid, ethyl ester and 2-Butoxyethyl acetate). Twelve chemical constituents were identified from the crude extract of *Rosmarinus officinalis*. Alpha-Pinene / Camphene / 3-Carene / p-Cymene / D-Limonene / Bornyl acetate and Caryophyllene compounds had antimicrobial properties. Data indicated that antimicrobial activity of *Rosmarinus officinalis* is not related to the activity of the endophytic bacteria.
Keywords: Endophyte, *Rosmarinus officinalis*, *Bacillus subtilis*, 16s rRNA, Antimicrobial activity and GC-MS.

1 Introduction

Rosmarinus officinalis (Rosemary) is a common household plant grown in many parts of the world. Rosemary oil is an effective antibacterial agent which can control many food micro-organisms such as *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli* O157:H7, *Shigella dysenteria*, *Bacillus cereus* and *Staphylococcus aureus* [1]. It can also inhibit the activity of food spoilage bacteria and yeast strains [2]. Rosemary plant is cultivated for its aromatic oil [3]. It is used for flavouring food, a beverage drink, as well as in cosmetics. Rosmarinic acid have a therapeutic potential in treatment or prevention of bronchial asthma, spasmogenic disorders, peptic ulcer, inflammatory diseases, hepatotoxicity, atherosclerosis, ischaemic heart disease, cataract, cancer and poor sperm motility [4].

Endophytes are an endosymbiotic group of microorganisms – often bacteria or fungi – that colonize the inter-and intracellular locations or in the vascular tissues of plants [5]. Endophytes reside inside the living plant tissues for their whole life or at least part of their life without causing any obvious disease symptoms to the host [6] and after attaining residence in the host tissues, the endophytes are known to produce a diverse range of natural products which could be used as a source of drugs. Thus the natural products from endophytes have a great potential in pharmaceutical, agrochemical and biotechnology industries [7]. Endophytes are treated as a subdivision of the population of the rhizospheric microbes [8]. The entrance of endophytes into the host plant cells is primarily happen through the roots and the aerial parts of hosts, such as leaves, flowers, stems and cotyledons [9]. Endophytes are localized at the point of entry and then spread to the entire host plant body [10]. The population of endophytes in a plant species is highly

variable and depends on various components, such as host species, host developmental stage, inoculum density and environmental condition [11]. Many studies have emphasized endophytes from medicinal plants and their application in different areas [12]. Recently, new endophytic bioactive metabolites, possessing a wide variety of biological activities as antibiotic, antiviral, anticancer, anti-inflammatory, antioxidant, etc., have been identified [13].

The endophytic bacteria can produce the similar secondary metabolites to the host plant and also has antibacterial activity to the pathogenic bacteria [14]. They have been isolated from a range of plant types which are mainly crop plants such as rice [15], potato [**16**], carrot [17], tomato [18] and citrus [19]. The endophytic bacteria are known to increase resistance of host plants to pathogens [**20**]. Several endophytic bacteria have been reported to produce vast range of natural products like phytohormones, compounds of low molecular weight, enzymes, siderophores, and antibiotics [20, 21]. They are ubiquitous in nature and exhibit complex interactions with their hosts, which involve mutualism, antagonism and rarely parasitism [22]. Ecomycins, Pseudomycins, Munumbicins, Kakadumycins are some examples of the novel antibiotics produced by endophytic bacteria. It is well known that until now most of the antibiotics have been derived from soil bacteria. Now the endophytic bacteria presents itself as a promising alternative potential source of new antibiotics [23].

The present study was made as an attempt to explore the antimicrobial activity of the bacterial endophytes of *Rosmarinus officinalis* and a selected isolate with a promising antimicrobial activity is identified and examined for its metabolic profiling.

2 Materials and Methods

2.1 Collection of Plant Samples

Leaves of *Rosmarinus officinalis* medicinal plant were collected from five different sites (Baja Village Area "BVA", Karaman Island "KI", El-tel Alawsat Area "EAA", Sohag University Staff Club "SUSC" and Sohag University Campus "SUC") at Sohag city. Three samples of *Rosmarinus officinalis* were collected per site. The collected samples were stored in separate plastic bags at 4°C in an ice box until isolation could commence [13].

2.2 Isolation of Endophytic Bacteria

Collected samples were washed in running tap water followed by 70% ethanol, 2% Sodium hypochlorite and deionized water respectively and air dried under a laminar flow hood for surface sterilization **[**24**]**. Under aseptic conditions the surface-sterilized segments were cut into about 1×1×0.5 cm (length × width × thickness) pieces then placed on nutrient agar (NA) plates. Plating was done in triplicates and incubated at 37°C for 48 hours. After attaining visible growth, all the isolated colonies were subcultured in nutrient agar (28 g/L) plates and stored at 4°C **[**25**].**

To confirm that the plant surfaces were effectively decontaminated, one ml aliquots of the sterile distilled water that was used in the final rinse of surface sterilization procedures were plated onto nutrient agar medium **[26]** and incubated at 37ºC for 48 hours. Bacterial growth was observed after 48 hours. Also, surface sterilized segments were rolled on nutrient agar plates, incubated at 37ºC for 48 hours and checked for possible microbial growth **[**10**].**

Colonization rate and isolation rate were calculated using the following equations [27]:

$$\textbf{Colonization rate} = \frac{\text{Total number of samples yielding} \geq \text{isolate}}{\text{Total number of samples in that trial}} \times 100$$

$$\textbf{Colonization rate} = \frac{\text{Total number of isolates yielding in a given trial}}{\text{Total number of samples in that trial}} \times 100$$

2.3 Determination of Antimicrobial Activity of Endophytes

Test organisms

Bacterial pathogens used were *Bacillus subtilis, Staphylococcus epidermidis, Serratia marcescens*, *Proteus vulgaris*, *Bacillus cereus, Staphylococcus aureus, Pseudomonas aeruginosa* and *Klebsiella pneumonia* which were provided by Bacteriology lab., Botany Department, Faculty of Science, Assuit University.

Fungal pathogens, *Fusarium oxysporum*, *Acremonium solani, Aspergillus flavus* and *Penicillium griseofulvum* in addition to non-pathogenic *Aspergillus columnaris* and *Aspergillus ochraceus* which were provided by Physiology of fungi lab., Botany and Microbiology Department, Faculty of Science, Sohag University.

Culture conditions

Endophytic bacteria were grown in 250 ml Erlenmeyer flasks containing 100 ml of sterilized nutrient broth and incubated for 48 hrs at 37ºC in shaker (BTC; E7CN, Taiwan) at 125 rpm/min. After the incubation period, culture media were centrifuged at 10,000 rpm for 15 min in centrifuge (Heraeus; Biofuge 15, Germany) and the supernatant was collected by filtration. This is used as the starting material for antimicrobial activity assays **[**28**].**

Antimicrobial Activity

The antimicrobial activity against the test microorganisms was detected using agar well diffusion method **[**29**].**

Bacterial pathogens were grown on nutrient agar medium while fungi were grown on potato dextrose agar medium. Agar wells were prepared using a sterilized stainless steel cork borer. Each well was loaded with 100 µl of the supernatant obtained after centrifugation and filtration, cultures incubated for 48 hrs at 37°C to observe the zones of inhibition. Clear zones were recorded in mm after subtracting the diameter of the well (6 mm) prepared using cork borer.
The most bioactive endophytic bacterium as well as the host (*Rosmarinus officinalis*) were selected for further investigations.

2.4 Identification of Endophytic Bacterial Isolates

The most bioactive endophytic bacterium was tentatively identified according to Bergey's manual of Systematic Bacteriology **[**30**]** by morphological, biochemical, physiologically characterization. Identification was confirmed by molecular identification with 16S rRNA gene sequencing [31, 32**]** in Macrogen Inc., Seoul, Korea.

2.5 Preparation of Crude Extracts

2.5.1 Bacterial Crude Extracts

Bacillus subtilis MH093646 inoculated into 100 ml of sterile nutrient broth and incubated at 37± 2°C for 24 h with continuous shaking at 200 rpm/min (BTC; E7CN, Taiwan). Twenty ml of grown culture was transferred into 1000 mL of sterile nutrient broth and incubated at 37±2°C for 5 days under continuous shaking at 200 rpm/min. The culture broth was sonicated on a sonicating water bath (Bandelin, Sonorex Digitec, DT 156 BH) for 30 min to break the cells and was extracted with ethyl acetate in a separating funnel by shaking vigorously for 10 minutes. The mixture was allowed to settle until the appearance of two distinct layers, the upper solvent layer was separated from the lower aqueous layer and the extraction process was repeated three times **[33].** The solvent (ethyl acetate) was evaporated on a rotary evaporator (Heidolph Hei-VAP Platinum 2 Rotary Evaporator) and the powder crude extract obtained was dissolved in hexane (10 mg/10 ml).

2.5.2 Rosmarinus Officinalis Crude Extract

Five grams of freshly collected leaves from *Rosmarinus officinalis* were harvested and extracted three times with ten ml of hexane. The hexane extract was evaporated under reduced pressure **[**34**]**. The powder crude extract obtained was dissolved in hexane (10 mg/10 ml).

2.6 Gas Chromatography and Mass Spectrometry (GC–MS)

Natural products of endophytic *Bacillus subtilis* MH093646 and extract of *Rosmarinus officinalis* were analyzed using gas chromatography coupled with mass spectrometry (GC/MSD) in Sohag Company for Drinking water and Sanitation based on peak area percentage, retention time, molecular formula then the molecular weight. GC-MS was performed on Agilent 5975 GC/MSD system. A DB-5 MS UI stainless steel capillary column 30m _ 0.25 mm (1.0µm film thickness).The column temperature was initially held at 35 °C for 1.0 min, and then programmed to 200 °C at rate of 25 °C / minute with holding time 1.0 minute finally programmed to 280 °C at rate of 10 °C / minute with holding time 2.0 minute.

Mass unit conditions were as follows:

Ion source 230 °C, ionization energy 70 eV and electron current 1435 mA. Helium was used as the carrier gas at 1.0 ml per minute.The injection temperature was 200 °C. The WILEY data base was used for identification of GC/MS peaks and linear retention indices were compared with the published data [35].

3 Results and Discussion

3.1 Isolation of Endophytes

Present study was carried out to isolate and characterize endophytic bacteria for antimicrobial activities. In this study, the growth of endophytic bacteria was observed after 24 hrs on the nutrient agar plates, isolated, grown and subsequently pure cultures were maintained on nutrient agar slants at 4°C. No bacterial or fungal growth was recorded on the used media, indicating the effectiveness of surface sterilization. Through these results, the stages of the sterilization process can be considered sufficient to dispose of epiphytes and that the obtained isolates can be considered endophytes for the medicinal plant selected in this study. The major key to succeed in isolating and studying endophytes is to ensure the sterility of the plant surface [10]. The diversity of isolated endophytic bacteria was also largely dependent on the isolation methods [**36**]. The results shown in **table (1)** indicated that twelve isolates were isolated from the *Rosmarinus officinalis* leaves with isolation rate 48% and colonization rate 32%. Previously numerous reports studied diversity of endophytic bacteria, and fungi in medicinal plants [37].

Table 1. Counts of bacterial endophytes, isolation and colonization rates recovered from leaves of *Rosmarinus officinalis.*

Plant name	Number of isolates	Isolation rate (%)	Colonization rate (%)
Rosmarinus officinalis	12	48	32

3.2 Antimicrobial Activity

In the present study, endophytic bacteria isolated from *Rosmarinus officinalis* showed considerable antimicrobial activity. After the incubation time, clear zones were observed against tested bacteria and fungi and were recorded in millimeters. The mean zones of inhibition produced by the different bacterial endophytes fluctuated between one and 49 mm (**Table 2 and figure 1**).

Nine isolates (75%) out of twelve endophytic bacteria isolated from leaves of *Rosmarinus officinalis* plant could display antibacterial activity inhibiting at least two of the bacterial pathogens while three isolates (25%) could display antifungal activity inhibiting at least one of the tested fungi. Bacterial endophyte number (6) showed promising antibacterial activity against *Staphylococcus epidermidis* (28.67mm), *Serratia marcescens* (48.67mm) and *Bacillus cereus* (10mm).The most bioactive endophytic bacterium (6) and its harboring *Rosmarinus officinalis* were selected for further investigations.

Bacterial endophytes have been recognized as repository of novel secondary metabolites for potential therapeutic use [**38**]. Further, **Strobel and Daisy (2003)** necessitated that medicinal and endemic plants should use for endophytic studies as they are expected to harbor rare and interesting endophytes with novel bioactive metabolites [13]. The discovery of novel antimicrobial metabolites from endophytes is an important alternative to overcome the increasing levels of drug resistance by plant and human pathogens [39]. The production of bioactive substances by endophytes is directly related to the independent evolution of these microorganisms, which may have incorporated genetic information from higher plants, allowing them to better adapt to plant host and carry out some functions such as protection from pathogens, insects, and grazing animals [40]. Endophytes are chemical synthesizer inside plants [41], in other words, they play a role as a selection system for microbes to produce bioactive substances with low toxicity toward higher organisms [40].

Chao *et al.* (2013) reported broad antifungal metabolites from endophytic *Bacillus amyloliquefaciens* isolated from healthy *Cinnamomum camphora* leaves [42]. Similarly, **Yuan *et al.* (2012)** reported anti-fungal activity of *Bacillus amyloliquefaciens* isolated from Chinese medicinal plant, *Ginkgo biloba* [43]. **Susilowati *et al.* (2015)** find that *B. subtilis* strain, bacterial symbionts of brown algae *Sargassum* sp., shows clear zone diameter 3.9 mm to MRSA [44]. **Sulistiyani *et al.* (2015)** also finds 1 of 9 isolated symbionts bacteria in seagrass *Enhalus* sp. that belongs to *Bacillus* sp., has antibacterial activity against bacteria of Multi Drugs Resistant Tuberculosis (MDR-TB) came from their bioactive crude extracts [45]. Related research also conducted by **Susilowati *et al.* (2015)** which has isolated brown algae bacteria symbionts that are *B. subtilis* and it has an ability to obstruct pathogenic bacteria like MRSA and *Staphylococcus epidermidis* [44]. Thus, endophytes can be a good source for the industrial production of antibiotics.

3.3 Identification of the most Active Endophyte

The most active endophytic bacterium no. (6) isolated from *Rosmarinus officinalis* was tentatively identified based on morphological characteristics and various biochemical tests according to Bergey's Manual of Systematic Bacteriology (1984). The bacterial endophyte number (6) isolated from *Rosmarinus officinalis* was gram positive, spore forming bacilli and based on the results obtained in **table (3)** the isolate was identified as *Bacillus* sp. Occurrence of both gram-positive and gram-negative endophytic bacteria has been reported from large diverse terrestrial and aquatic plants [46]. Bacteria of the genus *Bacillus* are dominant rhizospheric bacteria but have also been reported as endophytes in several plant species [47].

Further identification at the species level was carried out by 16s rRNA gene analysis. The endophytic bacterial DNA was isolated and the 16S rDNA sequence was amplified using the universal primers and sequenced. The 16S rDNA sequence thus obtained was compared with the non-redundant BLAST database in order to acquire the sequences that displayed maximum similarity. All the sequences reported by BLAST program revealed that the 16S rDNA sequence of the selected endophytic bacterial species (6) isolated from *Rosmarinus officinalis* showed a very high percentage of similarity (99%) with the sequences of *Bacillus subtilis*, with a reasonably high score and e-value being zero.

The phylogenetic relationship between the homologous sequences obtained by BLAST program using sequence data from gene bank for strains that showed high percentage of similarities with our strains was constructed using a freely available alignment program, CLUSTALW employing the neighbor-joining algorithm. This relationship is important to identify the species related to the endophyte. The evolutionary relationship is depicted in the form of a dendrogram (**Figure 2**) that shows a clear rooted evolution. All the sequences are shown to be derived from a common ancestor who later diverged into two different clusters that grouped the various strains of *Bacillus* sp.

Based on the results obtained and correlating with the results from morphological and biochemical tests, the endophytic bacterium no. (6) isolated from *Rosmarinus officinalis* is identified and designated as *Bacillus subtilis* US2. The 16S rRNA sequence of selected endophytic bacterium reported in this article has been deposited in the GenBank database under accession number: MH093646 (*Bacillus subtilis* US2).

Previously numerous reports studied diversity of endophytic bacteria in medicinal plants [37]. The

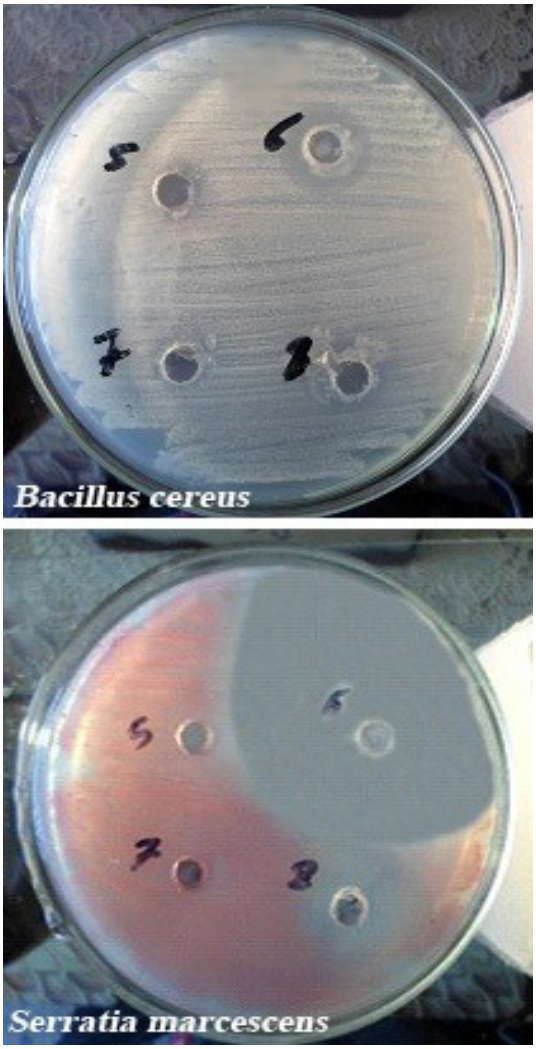


Fig. 1: Antibacterial activity of bacterial endophytes (5, 6, 7 and 8) isolated from *Rosmarinus officinalis* against (*Bacillus cereus* and *serratia marcescens*).

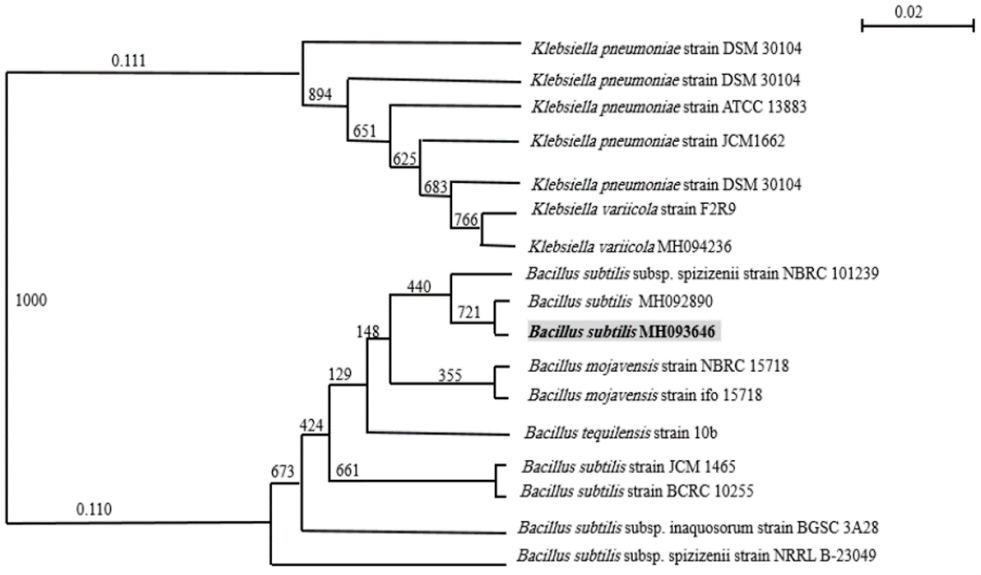


Fig. 2: Phylogenetic tree depicting the relation between the 16s rDNA sequence of the endophytic *Bacillus subtilis* MH093646 with its possible homologous sequences accessed from the GeneBank.

endophytic bacterial community isolated from *Plectranthus tenuiflorus* included *Paenibacillus* sp., *B. megaterium*, and *Pseudomonas* sp. has been previously characterized as a Korean ginseng root endophytes [48]. *Paenibacillus* has also been found as an endophyte in different woody plants like pine, coffee, and poplar [49]. *Acinetobacter, Bacillus, Pseudomonas* have been identified as an endphytes in *Echinacea* medicinal plant [50], while *Bacillus pumilus, B. subtilis, B. megaterium, Pseudomonas mendocina* were isolated as an Endophyte from the root of Medicinal Plant *Chlorophytum borivilianum* Safed musli [51]. *B. licheniformis* has been identified in *Jacaranda decurrens* plant [52].

Table 2. Antimicrobial activities of bacterial endophytes recovered from Rosmarinus officinalis against tested bacteria and fungi.

Isolate No. / Tested organisms	Zone of inhibition (mm)											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Bacillus subtilis	0	1.00±0	0	0	1.00±0	0	1.00±0	0	0	0	0	1.00±0
S. epidermidis	5.00± 1.00	5.33± 1.52	5.33± 0.57	6.00± 1.00	4.33± 0.57	28.67± 3.51	9.66± 1.15	24.33± 1.52	6.00± 1.00	1.00±0	5.33± 1.15	2.66± 0.57
Serratia marcescens	1.00± 0	0	1.00±0	0	0	48.67± 1.53	0	18.66± 1.52	5.33± 0.57	4.00± 1.00	4.33± 0.57	0
Proteus vulgaris	0	1.00±0	0	0	0	0	0	0	0	0	0	0
Bacillus cereus	1.00± 0	6.66± 1.52	1.00±0	5.66± 1.15	0	10.00± 1.73	0	0	6.33± 1.52	0	0	1.00±0
S. aureus	6.33± 1.52	0	0	1.00±0	16.66± 1.52	0	13.66± 1.52	0	0	0	0	0
P. aeruginosa	1.00± 0	1.00±0	0	0	0	0	0	0	0	1.00±0	0	0
Klebsiella pneumonia	0	0	0	0	0	0	0	0	0	0	0	0
Fusarium oxysporum	0	0	0	1.67± 0.58	0	0	0	0	0	0	0	0
Acremonium solani	0	0	0	0	0	0	0	0	0	0	0	0
Aspergillus flavus	0	0	0	4.33± 0.58	0	0	0	0	0	0	0	4.33± 1.53
Penicillium griseofulvum	0	0	0	0	0	0	0	0	0	0	0	0
Aspergillus columnaris	0	0	0	3.00± 1.00	0	0	0	0	0	0	0	0
Aspergillus ochraceus	0	0	0	0	0	0	0	0	0	0	2.67± 1.15	6.67± 1.15

Values are means of three replicates ± standard deviation (SD)

Table 3: Biochemical activities of selected bacterial endophyte (6) isolated from *Rosmarinus officinalis* leaves.

Test	Isolate (6)
1-Gram staining	+
2-Bacterial shape	Bacilli
3-Spore staining	spore forming
4-Starch hydrolysis	+
5-Growth at 65ºc	+
6-Reduction of nitrate	+
7-Acid and gas from glucose	-
8-Growth on 7% NaCl	+
9-V-P reaction	+
10-MR test	-
11-Catalase test	-
12-Oxidase test	-
13-Peroxidase test	-
14-Urease test	-
15-Citrate test	+
16-Amylase test	+
17-Protease test	-
18-Esterase test	-
19-O-F test	Facultative anaerobe
20-Gelatin hydrolysis	+
21-Pigment	-

3.4 Gas Chromatography Mass Spectrometry (GC-MS) Analysis of Crude Extracts

3.4.1 GC-MS analysis of Endophytic Bacillus Subtilis MH093646 Crude Extract

In chemical screening performed by GC-MS, an impressive diversity of the chemical constituents of the crude extract obtained from endophytic *Bacillus subtilis* MH093646 was observed. The crude extract showed a number of peaks in its chromatogram at different retention times with different abundance values. The GC-MS chromatogram of endophytic *Bacillus subtilis* MH093646 isolated from *Rosmarinus officinalis* exhibited most exciting diversity of the chemical constituents in its crude extract which showed 26 compounds were identified (**Table 4, figure 3**). Five compounds were major constituents (Cyclo-hexanone / 2-butoxy-Ethanol/ Acetic acid, butyl ester/ Propanoic acid, ethyl ester and 2-Butoxyethyl acetate) that gave five distinct peaks at retention times of 6.08, 6.02, 5.19, 4.26 and 7.32 min respectively with the highest abundance values.

Antimicrobial properties were detected in Cyclo-hexanone / 2-butoxy-Ethanol and Acetic acid, butyl ester (**figure 4**) and were considered as bioactive compounds because of their potential against the clinical pathogens according. Substituted cyclohexanones have long been served as potential bioactive compounds as well as starting materials for the synthesis of natural products and their derivatives and have potent pharmacological activity in the treatment of a broad spectrum of medical conditions [53, 54]. 2-Butoxyethanol is usually found in disinfectant formulations and was found biocidal at progressively lower concentrations down to 1 to 2% [55, 56]. Butyl acetate is an organic solvent commonly used in cosmetics. Cosmetic products containing more than 5% butyl acetate deny microorganisms the physical and chemical requirements for growth [57]. The tert-butyl acetate of 2-endo-hydroxy-1,8-cineole showed the highest antimicrobial and bactericidal activities against all kinds of the test bacteria [58].

3.4.2 GC-MS Analysis of Rosmarinus Officinalis Crude Extract

Twelve compounds identified in crude extract of *Rosmarinus officinalis* were shown in **table (5)** and **figure (5)**. Previous literature indicated that Alpha.-Pinene [59, 60] / Camphene [61, 62] / 3-Carene [63, 64] / p-Cymene [65, 66] / D-Limonene [67, 68, 69] / Bornyl acetate [70, 71, 72] and Caryophyllene compounds [73, 74] had antimicrobial properties. Despite the economic interest and broad popular medicinal usage of *Rosmarinus officinalis* there are very few reports on the chemical composition of this plant.

Data indicated that antimicrobial activity of the secondary metabolites produced by *Rosmarinus officinalis* is not related to the activity of the ones produced by the

Table 4. Active compounds identified from *Bacillus subtilis* MH093646 crude extract isolated from *Rosmarinus officinalis* by GC-MS.

No.	Compound	Retention time/min	Molecular Weight	Molecular. formula
1	Benzene	3.901	78.05	C_6H_6
2	Ethyl ester propanoic acid	4.262	102.07	$C_5H_{10}O_2$
3	Isobutyl acetate	4.845	116.08	$C_6H_{12}O_2$
4	Toluene	4.914	92.06	C_7H_8
5	Butyl ester acetic acid	5.189	116.08	$C_6H_{12}O_2$
6	3-methyl-butanoic acid	5.280	102.07	$C_5H_{10}O_2$
7	3-methyl acetate-1-Butanol	5.738	130.10	$C_7H_{14}O_2$
8	Ethyl-benzene	5.767	106.08	C_8H_{10}
9	p-Xylene	5.830	106.08	C_8H_{10}
10	2-butoxy-Ethanol	6.024	118.10	$C_6H_{14}O_2$
11	Cyclo-hexanone	6.081	98.07	$C_6H_{10}O$
12	4-methyl-Pentanoic acid	6.167	116.08	$C_6H_{12}O_2$
13	3,7-dimethyl-1-Octene	6.767	140.16	$C_{10}H_{20}$
14	Propyl-benzene (1-ethyl-2- methyl-benzene)	6.848	120.09	C_9H_{12}
15	o-Cymene	7.003	134.11	$C_{10}H_{14}$
16	2-Butoxyethyl acetate	7.323	160.11	$C_8H_{16}O_3$
17	(Z)-2-Dodecene	8.107	168.19	$C_{12}H_{24}$
18	2-Piperidinone	8.239	99.07	C_5H_9NO
19	Naphthalene	8.467	128.06	$C_{10}H_8$
20	Hydrocinnamic acid	9.297	150.07	$C_9H_{10}O_2$
21	1-methyl-Naphthalene	9.412	142.08	$C_{11}H_{10}$
22	(E)-3-Tetradecene	9.778	196.22	$C_{14}H_{28}$
23	2,4-bis (1,1-dimethylethyl)-phenol	10.985	206.17	$C_{14}H_{22}O$
24	(E)-3-Tetradecene	11.729	196.22	$C_{14}H_{28}$
25	1-Nonadecene	13.783	266.30	$C_{19}H_{38}$
26	Di-isooctyl phthalate	18.143	390.28	$C_{24}H_{38}O_4$

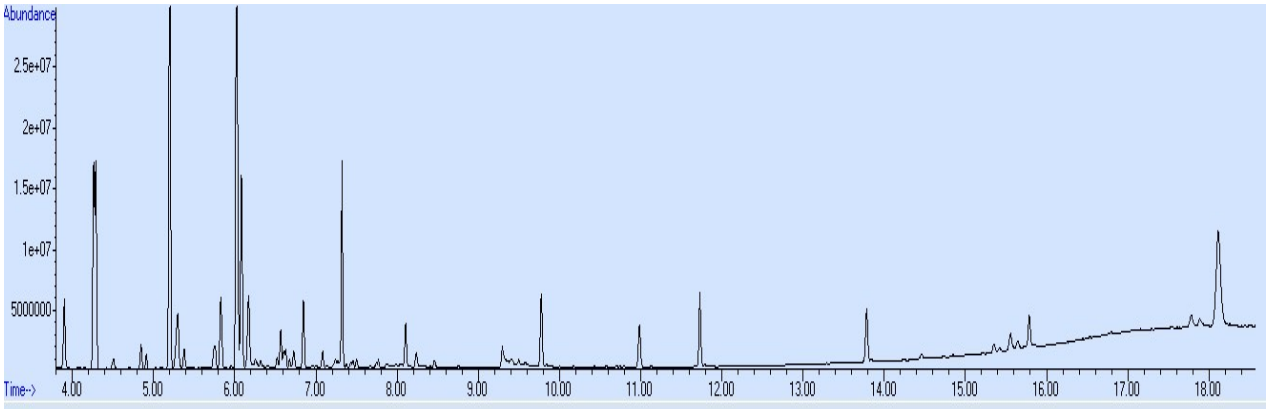
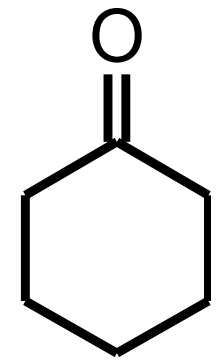
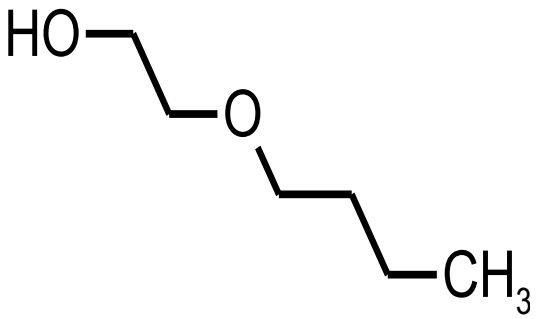


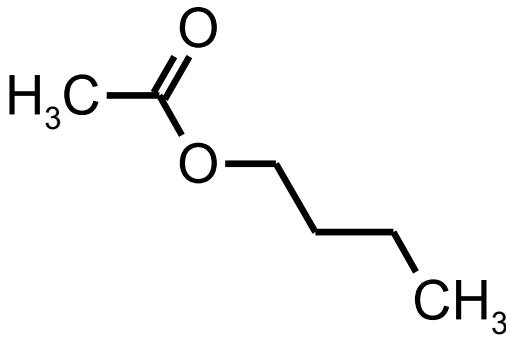
Fig. 3: GC-MS chromatogram of *Bacillus subtilis* MH093646 crude extract isolated from *Rosmarinus officinalis.*



a) Cyclohexanone



a) 2-Butoxyethanol



c) Butyl ester acetic acid

Fig. 4: Major active compounds identified in crude extract of Bacillus subtilis MH093646 (a, b and c) by GC-MS.

endophytic bacteria as there was no relationship or similarity between the previous compounds and others identified in crude extract of *Bacillus subtilis* MH093646 isolated from *Rosmarinus officinalis*. These results are in agreement with **Glienke *et al.* 2012** that suggested the presence of phenolic and anthraquinone compounds at the crude extract of pepper-tree leaves and the chemical analysis of the compounds with antimicrobial activity extracted from the endophytes of the pepper-tree leaves [75].

However, results disagree with **Pachkore *et al.* 2011** reported that antimicrobial activity of the endophytic bacteria may be attributed to host derived metabolites [76]. It was suggested that endophytic bacteria could produce the same bioactive metabolites that also produced by the plant itself [77].

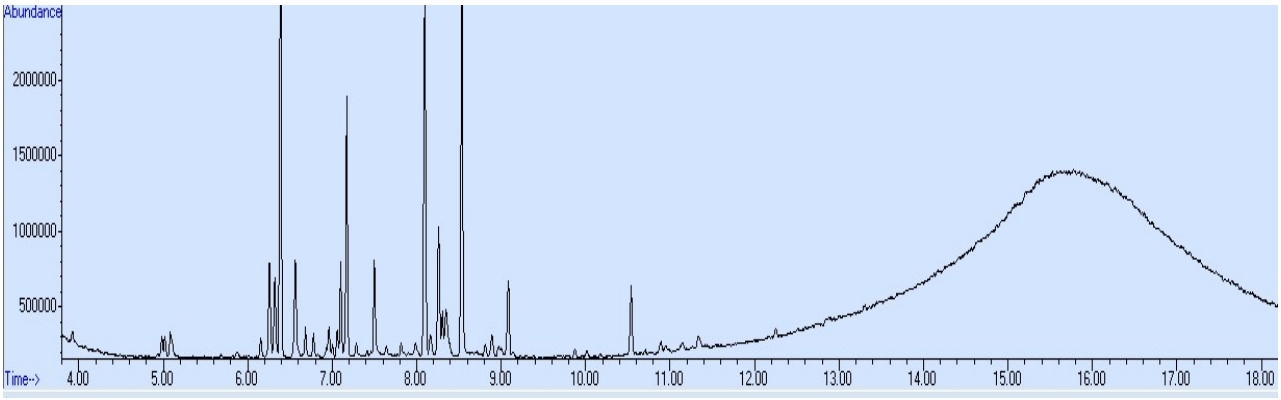


Fig. 5: GC-MS chromatogram of *Rosmarinus officinalis* crude extract.

Table 5: Active compounds identified in *Rosmarinus officinalis* crude extract by GC-MS.

No.	Compound	Retention time/min	Molecular Weight	Molecular formula
1	Alpha-Pinene	6.390	136.13	$C_{10}H_{16}$
2	Camphene	6.562	136.13	$C_{10}H_{16}$
3	3-Carene	6.962	136.13	$C_{10}H_{16}$
4	p-Cymene	7.054	134.11	$C_{10}H_{14}$
5	D-Limonene	7.100	136.13	$C_{10}H_{16}$
6	Eucalyptol	7.174	154.14	$C_{10}H_{18}O$
7	3,7-dimethyl-1,6-Octadien-3-ol	7.500	154.14	$C_{10}H_{18}O$
8	1,7,7- trimethyl-, (1S)-bicyclo[2.2.1]heptan-2-one (+)-2-Bornanone)	8.084	152.12	$C_{10}H_{16}O$
9	1,7,7-trimethyl-, (1S-endo)-bicyclo[2.2.1]heptan-2-ol	8.256	154.14	$C_{10}H_{18}O$
10	4,6,6- trimethyl-, (1S)-bicyclo[3.1.1]hept-3-en-2-one	8.530	150.10	$C_{10}H_{14}O$
11	1,7,7-trimethyl-, acetate, (1S-endo)-bicyclo[2.2.1]heptan-2-ol (Bornyl acetate)	9.085	196.15	$C_{12}H_{20}O_2$
12	Caryophyllene	10.539	204.19	$C_{15}H_{24}$

4 Conclusions

Rosmarinus officinalis is a potential source for various bioactive endophytic bacteria. Endophytes have proven to be rich sources of novel natural compounds with a wide-spectrum of biological activities and a high level of structural diversity. One promising endophyte isolated form *Rosmarinus officinalis* identified as *Bacillus subtilis* MH093646 by 16S rRNA showed a significant antibacterial activity against pathogenic bacteria by producing Cyclo-hexanone, 2-butoxy-Ethanol and Acetic acid, butyl ester as bioactive compounds. Data indicated that antimicrobial activity of the secondary metabolites produced by *Rosmarinus officinalis* is not related to the activity of the ones produced by the endophytic bacteria. Detailed investigations on endophytic bacteria of medicinal plants are needed to prove their further potential that will lead to the discovery of numerous high value metabolites.

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