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فعالیت بازدارنده برخی از باکتری‌های اندوفیت برگ *Satureja khuzestanica* در برابر باکتری‌های بیماری‌زای گیاهی

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چکیده

مقدمه: میکروب‌هایی که درون بافت‌های گیاهی قرار دارند، به نام اندوفیت شناخته می‌شوند. این میکروب‌ها مجموعه‌ای از ترکیبات را تولید می کنند که قابلیت استفاده در پزشکی و کشاورزی مدرن را دارند. هدف از این مطالعه جداسازی، غربالگری و شناسایی باکتری‌های اندوفیت با فعالیت ضدمیکروبی علیه باکتری‌های پاتوژن گیاهی بود. گیاهان دارویی مانند *Satureja khuzestanica* به‌دلیل داشتن برخی متابولیت‌های ثانویه در طب سنتی استفاده می‌شوند؛ اما اطلاعات مربوط به اندوفیت‌های باکتریایی طبیعی آن محدود است.

مواد و روش‌ها: در مطالعه حاضر، ۲۷ سویه اندوفیت از *S. khuzestanica* جداسازی شد. براساس توالی‌یابی ژن *16S rRNA*، سویه‌های باکتریایی جداشده با بیشترین فعالیت در برابر باکتری‌های بیماری‌زای گیاهی متعلق به جنس‌های *Bacillus*، *Streptomyces* و *Pseudomonas* بودند. متابولیت‌های ثانویه زیست‌فعال این باکتری‌های اندوفیت با استفاده از اتیل استات استخراج شدند و سپس آنالیز کروماتوگرافی گازی - طیف‌سنجی جرمی (GC-MS) در شرایط استاندارد انجام شد. حداقل غلظت مهاری (MIC) برای پنج گونه باکتری با روش رقت میکروبراث تعیین شد.

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نتایج: تجزیه و تحلیل داده‌ها نشان دادند تفاوت معنی‌داری برای فعالیت ضدمیکروبی ثبت شد که حداقل غلظت بازداری آن از ۰/۳۱۲ میلی‌گرم بر میلی‌لیتر تا ۲/۵ میلی‌گرم بر میلی‌لیتر بود. حداقل غلظت باکتری‌کشی ۰/۶۲۵ میلی‌گرم بر میلی‌لیتر تا ۱۰ میلی‌گرم بر میلی‌لیتر بود.

بحث و نتیجه‌گیری: ترکیبات اصلی در تجزیه و تحلیل GC-MS باکتری‌های اندوفیت در این مطالعه ۲،۴--di-tert-butylphenol، beta-d-glucopyranose، hexadecane، tetradecane، eicosane و dibutyl phthalate بودند. این مطالعه برای نخستین‌بار اندوفیت‌های باکتریایی *S. khuzestanica* با فعالیت ضدمیکروبی علیه فیتوپاتوژن‌های باکتریایی را گزارش می‌دهد. یافته‌های ما بینش جدیدی را دربارهٔ فعالیت‌های ضدمیکروبی باکتری‌های اندوفیت طبیعی *S. khuzestanica* ارائه می‌دهد.

واژه‌های کلیدی: *باسیلوس*، *استرپتومیسز*، *سودوموناس*، کروماتوگرافی

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The Inhibitory Activity of Some Endophytic Bacteria from *Satureja Khuzestanica* Leaves against Phythopathogenic Bacteria

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Abstract

Introduction: Microbes that reside inside the plant tissues are known as endophytes. These microbes produce an array of compounds that have the potential to be used in modern medicine and agriculture. The aim of this study was to isolate, screen, and identify endophytic bacteria that have antimicrobial activity toward phytopathogenic bacteria. Aromatic plants such *as Satureja khuzestanica* are used in traditional medicine due to their secondary metabolites but information regarding their naturally occurring bacterial endophytes is limited.

Materials and Methods: In the present study, 27 strains of endophytes were isolated from *S. khuzestanica*. Based on the sequencing of the 16S rRNA gene, isolated bacterial strains with the highest activity against phytopathogenic bacteria belong to *Bacillus*, *Streptomyces*, and *Pseudomonas* genera. The bioactive secondary metabolites of these endophyte bacteria were extracted using ethyl acetate, followed by Gas Chromatography-Mass Spectrometry (GC-MS) analysis under standard conditions. The minimum inhibitory concentration (MIC) values were determined for five species of bacteria via the microbroth dilution technique. The crude extracts of the five selected bacterial endophytes indicated an antimicrobial activity against five phytopathogenic species.

Results: Data analysis showed significant differences in antimicrobial activity that ranged from 0.312 mg/ml to 2. 5 mg/ml. The minimum bactericidal concentrations ranged from 0.625 mg/ml to 10 mg/ml.

Discussion and Conclusion: The major compounds in GC-MS analysis of endophyte bacteria in this study were 2,4-di-tert-butylphenol, beta-d-glucopyranose, hexadecane, tetradecane, eicosane, and dibutyl phthalate. This study reports for the first-time bacterial endophytes of *S. khuzestanica*, with antimicrobial activity against bacterial phypathogens.

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Our findings provide new insights into the antimicrobial activities of natural bacteria endophytes from *S. khuzestanica.*

Key words: *Bacillus*, *Streptomyces*, *Pseudomonas*, Chromatography

Introduction

How did medicinal herbs usage start-from nature or planting is unknown. However, historically, in the great civilizations of the world, medicinal plants have been used as the main agent in the healing and treatment of pain. In the late eighteenth and early nineteenth centuries, scientific studies on medicinal plants were expanded and medicinal plants were used as scientifically important medicinal agents (1). Iran is one of the centers of diversity of *Satureja* species. The genus *Satureja* belongs to the Lamiaceae family and the Nepetoideae subfamily. *S. khuzestanica* grows naturally in the western and northwestern parts of Iran (2, 3). *S. khuzistanica* and *S. reshingeri* are valuable herbs used in pharmaceutical and food industries due to their pharmacological and biological properties (4). Recently a wide range of biological activities of *S. khuzestanica* have been reported such as antibacterial (5, 6, 7, 8), antifungal (9, 10), antiparasitic (11, 12), antioxidant (13, 14, 15, 16, 17, 18), and anti-inflammatory (7, 15, 19, 20, 21,) properties. Bacterial endophytes are obligate symbiotic microorganisms, which live in apparently healthy internal plant tissues, without causing disease (22). Endophytic bacteria can be found in most plant species and can be recovered from various parts of the plant including roots, leaves, stems, and a few from flowers, fruits, and seeds (23). Endophytic microorganisms affect plant physiology through activities such as biocontrol roles, plant growth regulation, bioremediation, symbiotic-mutualistic, commensalistic, and trophobiotic interactions, control of pathogens, and support of host plants (24). Endophytes help to improve crop yields, through an immune response and stimulating plant growth, excluding plant pathogens through niche competition, and antioxidant activities (25). They have the potential to produce a variety of secondary metabolites with applications in the agriculture and pharmaceutical sectors (23, 24, 26).

Several secondary metabolites produced by bacterial endophytes act as antimicrobial agents against human, animal, and plant pathogens. Whereas the antimicrobial effect against phytopathogens will have a positive effect on the host plant, the efficacies of endophyte metabolites may show great clinical potential for medical and livestock treatments. Indeed, antibiotics are low-molecular-weight products made by microbes that inhibit the growth or kill phytopathogens agents such as bacteria, fungi, viruses, and protozoans (27, 28). Many important antibiotics are produced by endophytes in different plant species (29). However, only a few of all the plants existing on earth have ever been studied regarding their bacterial endophytic pool (30), increasing the probability to find new and beneficial endophytes with the potential to be applied in biotechnology. The microbiome of medicinal plants is extremely important because of increasing evidence on the spectrum of bioactive metabolites related to the activity of associated bacterial endophytes (31). Plant diseases caused by bacterial pathogens pose major constraints in crop production and cause significant annual damage worldwide (32). New and emerging bacterial disease problems and established problems in new geographical regions grab the headlines (32). Many bacterial pathogens predominantly colonize internal locations within plants that are inaccessible to most spray-applied chemical and biological

pesticides that target plant surfaces (33). Management strategies for plant bacterial diseases require great knowledge of the environmental conditions in order to identify the most suitable time for targeting the pathogen populations and to determine when the acute host tissues are sensitive to infection. The major pathogens of plants are parasitic plants, fungi, viruses, nematodes, and bacteria. (34). The most important bacterial ones belong to the genera of *Agrobacterium*, *Ralstonia*, *Pectobacterium*, *Xylella*, *Erwinia*, *Xanthomonas*, *Pseudomonas*, and *Dickeya*. An integrated management approach, including the use of plant host resistance, chemical intervention and biological controls, and cultural practices for inoculum reduction, typically represents the best strategy for effective and stable disease management

Materials and Methods

Plant Material/Samples Collection: *S. khuzestanica* leaves samples were collected from three different areas including Lorestan, Khuzestan, and Ilam provinces located in the south and southwest of Iran. Plant leaves were cut and placed separately in polythene bags to avoid moisture losses and stored at 4^{o}C for further investigation.

Isolation of Endophytic Bacteria: Briefly, leaves were washed under tap water to remove dust, and disinfected by sequential immersion in 70% ethanol for 5 minutes or sodium hypochloride for 20 minutes. Finally, and disinfected leaves were washed three times in sterilized distilled water and soaked in 10% NaHCO3 solution to disrupt the plant tissues and inhibit the growth of fungi (35). A gram of each leave sample was homogenized in potassium phosphate buffer (pH 7.0) followed by serial dilutions preparation. For isolation of endophytic bacterial strains, a loopful of homogenate samples was stork on modified *Tryptic soy agar* (TSA) and nutrient agar (NA) in three replications and incubated at 28 ºC for three days. Bacterial single colonies were selected based on their phenotypic characteristics such as colony morphology, color, and growth rate. They were kept in sterilized distilled water at 4 °C for further investigation.

Determination of Antibacterial Activity: Purified endophytic bacterial strains were grown on Tryptic Soy Broth (TSB) medium and their antibacterial activity against the phytopathogens *R. solanacearum*, *P. carotovorum*, and *C. michiganensis*, was determined in a randomized design in three replicates, and inhibition zone data were recorded and analyzed (24). Analysis of the variance of data was done based on a randomized complete design with three replications. The resulting data were analyzed using SAS software, and a comparison of means was done through Duncan's multiple range test.

Identification of Efficient Endophytic Bacteria: Phenotypic features of the efficient bacterial strains were determined based on standard bacteriological methods followed by 16S rRNA amplification and sequencing. For the extraction of bacterial genomic DNA, 50 mg of the freshly grown colonies was transferred into a 1.5 ml Eppendorf tube, and 480 µl TE buffer and 20 µl lysozyme solution (2 mg/ml) were added. The bacterial suspension was mixed and placed in a shaker incubator for 1 h, treated with 50 µl SDS solution 20 % and 5 µl Proteinase K solution, and kept in a bain-marie at 55 °C for 1 h. The bacterial DNA was extracted twice with phenol-chloroform-isoamyl alcohol, followed by precipitation with 80 µl sodium acetate (3 mol/l, pH 5) and 800 µl absolute ethanol. The DNA precipitate was centrifuged at 12000 rpm for 10 min, washed with ethanol 70 %, and air-dried. The extracted DNA was resuspended in 40 µl sterilized distilled water and stored at -20°C for further use (36). For the amplification of 16S rRNA

genes, the extracted DNA was subjected to a polymerase chain reaction (PCR) using 16SF- (AGAGTTTGATCCTGGCTCAG) and 16SR- (GGTTACCTTGTTACGACTT) primers (37). The PCR program for 35 cycles was as follows: DNA denaturation at 94 °C for 5 minutes, annealing for 30 seconds at 53 °C, and extension for 60 seconds at 72 °C. The PCR products were purified with a PCR purification kit (Macherey-Nagel, Germany) and subjected to sequencing (Bioneer Korea Co.). The obtained sequences were analyzed using BLAST software in the GenBank database NCBI.

Preparation of Cell-free Supernatant from Efficient Bacterial Strains: Selected bacterial strains were cultured in 250 ml Erlenmeyer flasks containing 100 ml of standard medium (TSB+ 0.1 g of $MgSO_4$ $7H_20$, 0.1 g of KC1, 0.05 g of KH_2PO_4, 0.05 g of K_2HPO_4, 0.2 g of $CaC1_2$ $2H_20$, 0.4 g of yeast extract, 0.4 g of malt extract, 0.2 mL of trace elements, pH 7.2), incubated at 27 °C for 3 days in a shaker incubator at 160 rpm. A total of one-liter volume of culture broth was centrifuged at 8000 rpm at 4 °C for 15 min and was filtered through Whatman no.1 filter paper. The spent culture broth was aseptically transferred into 250 ml flasks, and an equal volume of 1:1 (v/v) of ethyl acetate was added. This mixture was shaken vigorously for 30 min and was kept stationary for another 15 min to phase separation. The solvent was dried in a rotary evaporator under a vacuum to obtain the crude metabolite (38). The remaining residues were dried in a vacuum desiccator, re-dissolved in a small volume of ethyl acetate (1mg/ml), and stored at -20 °C for further use (39). A total of 2 µl of the ethyl acetate extract from each bacterial strain was used for GC/MS analysis. New unknown metabolites from bacterial crud extract were characterized using GC-MS [SHIMADZU QP2010] instrument (GC column oven temperature 35 °C, injector temperature 250 °C at split mode ratio 100 with a flow rate 1.25 ml/min). The MS with ion source temperature 200 °C, interface temperature 250 °C, scan range 45-450 m/z, event time 0.3 sec, solvent cut time 1 min, MS start time: 1 min, MS end time 50 min, ionization EI (-70ev) was employed for metabolites characterization.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Determination: The minimum inhibitory concentration of crude extracts of bacterial endophytes was determined according to Andrews' method (40) with some modifications. Briefly, the extracted solutions were prepared by dissolving 0.02 g/ ml in dimethyl sulfoxide (DMSO) and diluted to a final concentration of 20 mg/ ml in nutrient broth medium followed by preparation of serial dilution including 10 mg/ ml, 5 mg/ ml, 2.5 mg/ ml, 1.25 mg/ ml, 0.625 mg/ ml, and 0.312 mg/ ml in the same medium. Using McFarland standard number 0.5 (10^8 × 1.58 CFU/ ml) as a reference, a volume of 50 µl suspension of each sample was inoculated in 15 ml of nutrient broth medium and incubated for 24 hours at 28 °C. The 96-well microtiter plates were employed, and a volume of 100 µl of phytopathogenic bacterial strain suspension was added horizontally followed by adding 100 µl of prepared diluted crude extract at different concentrations vertically from top to bottom. The negative controls were 100 µl DMSO and 100 µl nutrient broth medium, and the positive control was 1 mg/ ml streptomycin (Sigma-Aldrich Switzerland). Three replicates were employed for each sample and plates were incubated for 24 hours at 28 °C. MIC values (lowest concentration of each extract without visible growth) were determined visually.

Identification of Bioactive Metabolites: The volatile and semi-volatile secondary metabolites from the crude extracts of bacterial strains were analyzed by GC–MS

(SHIMADZU QP2010, 41 Osama A. A. Mohamad). Accordingly, for GC-MS analysis, a Shimadzu model GC 2010 Plus (Kyoto, Japan) gas chromatograph coupled with a Shimadzu Quadruple-MS model QP2010 SE mass spectrometer was used. Secondary metabolites were separated on a 30 m × 0.22 mm i.d. fused-silica capillary column coated with 0.25 μm film of BP-5 (Shimadzu), and a splitless injector with a 1 mm internal diameter glass liner. The Shimadzu Quadruple-MS model QP2010 SE mass spectrometer was used to identify volatile and semi-volatile secondary metabolites from the crude extract of bacterial strains. Also, an HP-5MS fused silica capillary column was applied (Hewlett-Packard, 30 m × 0.25 mm i.d. 0.25 μm film, cross-linked to 5% phenyl methyl siloxane stationary phase). The entire system was checked using ChemStation software (Hewlett- Packard, version A.01.01). Electron impact mass spectra were recorded at 70 eV, and ultra-high pure (99.999%) HAE gas was used as the carrier gas at a flow rate of 1 ml. min^{-1}. The injection volume was found to be 1 μl, and all injections were done in a split-less mode. The injector and detector temperature settings were 250 °C and 280 °C, respectively. The column oven temperature was set initially at 35 °C for 5 min, then raised to 300 °C (ramp: 4°C/min) and held for 20 min. The database of the National Institute of Standards and Technology (NIST) was applied to interpret the mass spectrum of GC-MS with more than 62000 patterns. Using the obtained data from NIST05 (National Institute of Standards and Technology, US) and WILEY 8 libraries, the existing bioactive compounds in bacterial crude extracts were identified through comparison to mass spectra. Finally, the molecular weights and structural metabolites formula of the tested bacterial metabolites were determined.

Results and Discussion

Isolation and Determination of the Antibacterial Activity of Endophytic Bacterial Strains: Based on the different cultural conditions on the TSA medium, 27 endophytic bacteria were isolated from *S. khuzestanica*. All endophytic strains were screened for their antibacterial activity toward five plant pathogens. Based on the inhibitory activity of endophytic bacterial strains against the different species of plant pathogenic bacteria, the most bioactive five strains were selected as the representative (Table 1). They showed significant antibacterial activity against plant pathogenic bacteria strains with the diameter of inhibition zones as follows: 24 mm for *P. carotovorum*, 21 mm for *R. solanacearum*, 20 mm for *B. nigrifluens*, 17 mm for *C. michiganensis*, and 19 mm for *E. amylovora*. All values are the mean of three repetitions of independent experiments. The comparison of the average interaction effect of endophytic bacteria and pathogenic bacteria is presented in Table 2.

Table 1- Antagonistic Activity of Endophytic Bacterial Strains Isolated from Satureja khuzestanica against some Plant Pathogenic Bacteria

	B.megaterium	*P.fluorescens*	*P. gessardii*	*P. azotoformans*	*P. oryzihabitans*	Streptomycin
R. Solanacearum	21	19	9	15	16	23
C. michiganensis	17	14	7	16	17	20
P. carotovorum	11	20	13	12	24	28
B.nigrifluens	9	20	10	12	20	30
E. amylovora	9	16	8	18	19	27

*numbers show inhibition zone in mm

Table 2- Comparison of the average inhibitory effect of the endophytic bacterial strains against plant pathogenic bacteria *in vitro*

Inhibition Zone	Endophytic Bacteria	Plant Pathogenic Bacteria
21 ± 1.73 ^{de}	*B.megaterium*	*R. Solanacearum*
19 ± 1 ^{efg}	*P.fluorescens*	
9 ± 1 ^{nop}	*P. gessardii*	
15 ± 2 ^{hijk}	*P. azotoformans*	
16 ± 1.73 ^{ghi}	*P. oryzihabitans*	
23 ± 2 ^{cd}	Streptomycin	
17 ± 2.65 ^{fghi}	*B.megaterium*	*C. michiganensi*
14 ± 1 ^{ijkl}	*P.fluorescens*	
7 ± 1 ^{p}	*P. gessardii*	
16 ± 3 ^{ghij}	*P. azotoformans*	
17 ± 1 ^{fghi}	*P. oryzihabitans*	
20 ± 1 ^{def}	Streptomycin	
11 ± 1 ^{lmno}	*B.megaterium*	*P. carotovorum*
20 ± 1 ^{def}	*P.fluorescens*	
13 ± 1.41 ^{jklm}	*P. gessardii*	
12 ± 1 ^{klmn}	*P. azotoformans*	
24 ± 3.61 ^{c}	*P. oryzihabitans*	
28 ± 2.65 ^{ab}	Streptomycin	
9 ± 1 ^{nop}	*B.megaterium*	*B.nigrifluens*
20 ± 2.45 ^{def}	*P.fluorescens*	
10 ± 0.82 ^{mnop}	*P. gessardii*	
12 ± 1.41 ^{klmn}	*P. azotoformans*	
20 ± 0.82 ^{def}	*P. oryzihabitans*	
30 ± 1.41 ^{a}	Streptomycin	
9 ± 0.82 ^{nop}	*B.megaterium*	*E. amylovora*
16 ± 0.82 ^{ghij}	*P.fluorescens*	
8 ± 0.82 ^{op}	*P. gessardii*	
18 ± 0.82 ^{efgh}	*P. azotoformans*	
19 ± 0.82 ^{efg}	*P. oryzihabitans*	
27 ± 0.82 ^{b}	Streptomycin	

Different letters between rows indicate a significant difference at the 1% probability level

Representative bacterial strains (MON 01, MON 02, MON 05, MON 06, and MON 07) were identified based on their 16S rRNA gene sequences. They showed 99% homology with 16S rRNA encoding gene sequences of verified species and they submitted to the GenBank database with allocated accession numbers (Table 3).

Bacteriostatic and Bactericidal Activity of the Bacterial Crude Extract: Metabolites from endophytic bacterial representative strains showed bacteriostatic and bactericidal activity against three Gram-negative and one Gram-positive phytopathogenic bacteria. Ethyl acetate extracts showed bacteriostatic effects with MIC ranging from 0.312 to 5 mg/ ml and bactericidal activity ranging from 0.625 to 20 mg/ ml (Table 4, 5).

Table 3- NCBI 16S rRNA Genes of Endophytic Bacterial Strains Isolated from *Satureja khuzestanica* Accession Number

Bacterial name	GenBank accession number
Bacillus(Priestia) megaterium strain MON 02	OL342346
Pseudomonas fluorescens strain MON 06	OL342347
Pseudomonas gessardii strain MON 07	OL342348
Pseudomonas azotoformans strain MON 05	OL342349
Pseudomonas oryzihabitans strain MON 01	OL342350

Table 4- Minimum Inhibitory Concentrations of Crude Extracts of Secondary Metabolites of Bacterial Endophytes

isolated from *Satureja khuzestanica*

	*B. megaterium*MIC (mg/ml)	*P. Fluorescens* MIC (mg/ml)	*P. gessardii* MIC (mg/ml)	*P. azotoformans* MIC (mg/ml)	*P. oryzihabitans*MIC (mg/ml)	Streptomycin MIC (mg/ml)
R. Solanacearum	0.625	0.625	1.25	1.25	2.5	0.312
C. Michiganensis	0.625	5	2.5	1.25	1.25	0.312
P. carotovorum	1.25	0.625	1.25	1.25	0.312	0.312
B. nigrifluens	1.25	0.312	1.25	2.5	0.312	0.156
E. amylovora	2.5	2.5	2.5	0.625	0.625	0.156

Table 5- Minimum Bactericidal concentrations of crude extracts of secondary metabolites of bacterial endophytes isolated from *Satureja khuzestanica* of bacterial endophytes isolated from *Satureja khuzestanica*

	B. megaterium MBC (mg/ml)	*P. fluorescens* MBC (mg/ml)	*P. gessardii* MBC (mg/ml)	*P. azotoformans* MBC (mg/ml)	*P. oryzihabitans* MBC (mg/ml)	Streptomycin MBC (mg/ml)
R. solanacearum	2.5	1.25	2.5	5	5	0.625
C. michiganensis	1.25	10	5	2.5	5	0.625
P. carotovorum	5	1.25	5	5	0.625	1.25
B. nigrifluens	2.5	1.25	2.5	5	0.625	0.625
E. amylovora	5	5	5	1.25	1.25	0.625

Detection of Bioactive Metabolites by GC-MS Analysis: Spectra of GC-MS from representative endophytic bacterial strains isolated from *Satureja khuzestanica* were interpreted by the database of the National Institute of Standards and Technology (NIST) with 62000 patterns. The chromatogram of bacterial metabolites was identified according to the peak area, retention time, and effect. Characterization metabolites from tested endophytic bacterial strains isolated from *S. khuzestanica* revealed that they have the capacity to produce bioactive agents.

Among the 27 bacterial strains isolated from *S. khuzestanica* on the TSA medium, the most active representative was selected for identification based on the 16S rRNA gene sequences. The strain *MON 02* was identified as *Bacillus (Priestia) megaterium* by 99% similarity to *B. megaterium* which was submitted in the GenBank under accession number OL342346. Its major constituent with 67.34 % area was Prodox (2, 4-Di-tert-butylphenol) at the retention time of 20.578, which is a common natural product that exhibits potent toxicity against almost all testing microbes, including the producing species (Table 6). The 2, 4-DTBP is found in 16 species of bacteria in 10 families, such as nitrogen-fixing cyanobacteria and Gram-positive bacteria such *Bacillus*. Bioactivities of 2, 4-DTBP as antibacterial activities, antiviral activities, antifungal activities, pesticides (Nematicidal activities), antioxidant activities, and anti-inflammatory activities are reported in the literature.

Table 6- GC-MS Analysis of *Bacillus* (*Priestia*) *Megaterium* Crude Extract Isolated from *Satureja khuzestanica*

Retention time (min)	area%	metabolite	efficacy
20.578	67.34	prodox (2,4-di-tert-butylphenol)	antibacterial, antiviral, antifungal, nematicide, antioxidant, anti-inflammatory
21.476	10.59	hexadecane	antimicrobial and antioxidant
22.006	4.89	2,5-cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-	-
22.695	6.87	eicosane	antibacterial
24.853	4.70	isobutyl phthalate	antibacterial
25.910	5.61	dibutyl phthalate	antibacterial antitumor anticancer herbicide
	100		

Bacillus is a Gram-positive bacterium that has been used industrially for decades and its product portfolio is continuously growing. Its metabolites include enzymes such as α-amylases, β-amylases, penicillin G acylase, xylanase, hydrolases, and cytochrome monooxygenase. Furthermore, it has been shown that *Bacillus* spp. has well biocontrol properties by promoting plant growth and reducing plant diseases caused by both plant-pathogenic fungi and bacteria (42). These properties are mostly related to their secondary metabolite profiles (43).

The strain *MON 06* was identified as *Pseudomonas fluorescens* by 99.66 % similarity to *P. fluorescens*, which was submitted to the GenBank under accession number OL342347. One of its major constituents was Dibutyl phthalate (DBP) at the retention time of 13.820 (table 7).

Dibutyl phthalate (DBP) is a bioactive ester produced by actinomycetes (44, 45), fungi (46), algae (47), and also higher plants (48). It acts as an antitumor and anticancer compound (49), and herbicide (50). Also, the cytotoxic activity of DBP against tumor cell lines has been evaluated by Mabrouk et al. (2008). The other identified metabolites from *P. fluorescens* were beta-D-Glucopyranose (anti-bacterial and antioxidant) (51), Cyclo-Leu-Pro-diketopiperazine (antifungal) (52), and Staflex.

Table 7- GC-MS Analysis of *Pseudomonas fluorescens* Crude Extracts Isolated from *Satureja khuzestanica*

retention time (min)	area%	metabolite	efficacy
11.774	18.16	beta-d-glucopyranose	anti-bacterial/ antioxidant
13.719	10.49	l-phe-d-pro lactam	anticancer
13.820	7.22	kodaflex (dibutyl phthalate)	antibacterial antitumor anticancer herbicide
14.344	1.45	staflex DBP	antibacterial
14.758	11.35	Cyclo (leucyloprolyl)	antibacterial
14.872	16.15	staflex DBP	antibacterial
14.950	28.54	cyclo-leu-pro-diketoiperazine	antifungal
15.059	5.09	cyclo-(leu-pro)	antifungal
15.125	1.56	pyrrolo(1,2-a) pyrazine-1,4-dione, hexahydrate	antifungal
	100		

Strain *MON 07* was identified as *P. gessardii* which was submitted to the GenBank under accession number OL342348. Its major metabolites were 2, 4-di-tert-butylphenol (40.44% area), trans-2-isopropyl-trans-5-methyl-1-hexene (17.34 area), tetradecane (12.08), hexadecane (11.56), and l-phe-d-pro lactam (7.78), (Table 8).

2,4-Di-tert-butylphenol is a common natural product that exhibits potent toxicity against almost all testing organisms, including bacteria and fungi species (53). The antimicrobial efficacy of dibutyl phthalate (DBP) has been reported from *Streptomyces* (54). Also, this compound shows strong activity against Gram-positive and Gram-negative bacteria, as well as unicellular and filamentous fungi (45).

Table 8- GC-MS Analysis of *Pseudomonas gessardii* Crude Extracts Isolated from *Satureja khuzestanica*

retention time (min)	area%	metabolite	efficacy
15.722	17.34	trans-2-isopropyl-trans-5-methyl-1-hexene	antifungal
18.781	12.08	tetradecane	antibacterial
20.556	40.44	2,4-di-tert-butylphenol	antibacterial, antiviral, antifungal, nematicide, antioxidant, anti-inflammatory

21.453	11.56	hexadecane	antimicrobial and antioxidant
23.838	5.42	eicosane	antibacterial
24.780	7.78	l-phe-d-pro lactam	anticancer
25.889	5.38	dibutyl phthalate	antibacterial antitumor anticancer herbicide
	100		

Strain *MON05* was identified as *P. azotoformans*. It showed 99.66 % similarity to *P. azotoformans* strain D95_SO which was submitted to the GenBank under accession number OL342349. It is reported that *P. azotoformans* was used as an effective biocontrol bacterium against *Colletotrichum orbiculare* on cucumber (55). The findings of scholars indicated that *P. azotoformans* has efficacy on drought stress alleviation in wheat plants through various biochemical mechanisms. The *P. azotoformans* isolated from soil samples in China appeared to have strong inhibitory activities against *Fusarium fujikuroi*, a serious rice fungal pathogen. The identified metabolites from *P. azotoformans* include tetradecane (22.76 % area), 2,4-di-tert-butylphenol (22.44 % area), eicosane (10.40 % area), dodecane (10.88 % area), dibutyl phthalate, heptadecane, behenyl chloride, hexadecane, and isomenthone (Table 9).

Table 9- GC-MS Analysis of *Pseudomonas Azotoformans* Crude Extracts Isolated from *Satureja khuzestanica*

retention time (min)	area%	metabolite	efficacy
15.367	1.68	Iso-menthone	antibacterial, antiviral, antifungal
15.762	10.80	dodecane	antibacterial, antifungal
18.841	22.76	tetradecane	antibacterial
20.183	2.88	hexadecane	antimicrobial antioxidant
20.590	22.44	2,4-di-tert-butylphenol	antibacterial, antiviral, antifungal nematicide, antioxidant, anti-inflammatory
21.513	21.04	hexadecane	antimicrobial antioxidant
22.015	2.38	behenyl chloride	antibacterial
22.704	2.71	heptadecane	anti-inflammatory antineoplastic, antibacterial, antifungal
23.883	10.40	eicosane	antibacterial
25.911	2.90	dibutyl phthalate	antibacterial antitumor anticancer herbicide
	100		

Rahbar et al (2012) found the good antimicrobial activity of tetradecane, hexadecanoic acid, and pentadecane against seven Gram-positive and Gram-negative bacteria (*Bacillus subtilis, Enterococcus faecalis, S. aureus, Staphylococcus epidermidis, Escherichia coli, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae*), as well as three fungi (*Candida albicans, Saccharomyces cerevisiae* and *Aspergillus niger*) (56). Also, Hexadecane is reported for antibacterial and antioxidant activities.

Bacterial strain *MON 01* was identified as *P. oryzihabitans* and showed 99 % similarity to *P. oryzihabitans strain ER34* which was submitted to the GenBank under accession number OL342350. In a study by Kleopatra Leontidou, *P. oryzihabitans*as was isolated as plant growth-promoting rhizobacteria from halophytes and drought-tolerant plants.

Endophytic *P. oryzihabitans* was isolated from soybean grown in soil treated with glyphosate. The bacterium *P. oryzihabitans* was the most potent antagonistic bacteria which reduced disease incidence and severity compared to the untreated control (168-2016). So far, it has shown promising results as a potential biocontrol agent against plant parasitic nematodes (s0038). In the present study, metabolites from P. *oryzihabitans* were phenol,2-methyl-5- (1- methylethyl) or carvacrol (26.47 % area), 2,4-di-tert-butylphenol (26.06 % area), hexadecane (13.08 % area), and tetradecane (11.74 % area) (table10).

Table 10- GC-MS Analysis of *Pseudomonas Oryzihabitans* Crude Extracts Isolated from *Satureja khuzestanica*

retention time(min)	area%	metabolite	efficacy
15.748	7.12	n-nanodecane	antibacterial antifungal
16.054	3.29	beta.fenchyl alcohol	antibiofilm and antihyphal
17.761	26.74	phenol, 2-methyl-5-(1-methylethyl) [carvacrol}	antibacterial, antiviral, antifungal
18.818	11.74	tetradecane	antibacterial
20.178	2.05	hexadecane	antimicrobial antioxidant
20.593	26.05	2,4-di-tert-butylphenol	antibacterial, antiviral, antifungal, nematicidal, antioxidand, anti-inflammatory
21.496	13.08	hexadecane	antimicrobial antioxidant
23.870	6.58	eicosane	antibacterial
25.905	1.77	dibutyl phthalate	antibacterial antitumor anticancer herbicide
26.012	1.84	eicosane	antibacterial
	100		

All aspects of the interaction between microbes and plants are not fully understood. Nevertheless, more documents show that plant-associated microorganisms provide substantial benefits to agriculture and the environment. In brief, in this study, for the first time, bacterial endophytes were isolated from *S. khuzestanica* and their crude extracts showed notable inhibitory activities against tested phytopathogenic bacteria. The antibacterial analysis result of *S. khuzestanica* endophytic bacteria showed the potential use of these bacteria for the isolation of pure bioactive metabolites and the possibility of making new drugs. Further studies need to be carried out to isolate and identify pure active metabolites produced by *S. khuzestanica* endophytic bacteria.

The authors have no conflicts of interest to declare.

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