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Antibacterial effect and chemical composition of *Satureja bachtiarica*

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Abstract
In this study antimicrobial activity of methanolic extract and essential oil of *Satureja bachtiarica* were evaluated by "disk diffusion method" on *Pseudomonas syringae* pv. *syringae, Rhizobium radiobacter, Ralstonia solanacearum, Xanthomonas axonopodis* pv. *citri, Bacillis subtilis, Pectobacterium cartovorum* and *Pectobacterium atrosepticum*. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determined by using a serial dilution method. The lowest inhibitory consistency of MIC and MBC values of *Satureja bachtiarica* essential oil and methanolic extract was observe on *Bacillus subtillis.* Chemical composition of essential oil and methanolic extract were determined by GC-MS and HPLC respectively. The major components of essential oil were: Carvacrol 53.94, γ- terpinene13.08, Tymole 11.16, P-symene 6.54, E- caryophylene2.16, Bornole1.2, Linalool2.49, α- terpnene. HPLC analysis of methanolic extract showed ten type of compound: Carcacrol (461.48mlgr/lit), Quercetin (75.80mlgr/lit) Eugenol (60.61mlgr/lit) Hesperetin (24.29mlgr/lit), Hesperedin(13.75mlgr/lit), Rutin (13.23mlgr/lit), Catechin (9.721mlgr/lit), Vanillin(1.01mlgr/lit), Caffeic acid (0.0812ml gr/lit), P-coumaric acid (2mlgr/lit), that are present in varying amount. Our result indicated that *Satureja bachtiarica* essential oil showed high antibacterial activity against all selected bacteria whereas, methanolic extract showed antibacterial activity against *Xanthomona saxonopodis* pv. *citri, Bacillis subtilis, Ralstonia solanacearum, Pseudomonas syringae* pv. *syringae*. In general, essential oil and methanolic extract have strong antimicrobial activity against these pathogens.

Keywords: *Satureja bachtiarica*, essential oil, methanolic extract, antibacterial activity

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Introduction

During the last decade, natural products with antimicrobial effect are investigated. In order to eliminate use of synthetic antibiotics which cause the resistance of microorganisms and several side effect (Heidari *et al*., 2013).The genus *Satureja* sp. (Lamiacea) constituents about 200 species of herbs and shrubs (Cantino, 1992). *Satureja* genus also showed antimicrobial and antioxidant activity (Serrano *et al*., 2011). *Satureja bachtiarica* is a well-known aromatic plant which is usually used as flavoring agents in meat and as a traditional medicinal herb in Iran. *Satureja* genus also showed antimicrobial and antioxidant activity (Serrano *et al*., 2011). Because of the strong phenolic character of its essential oil, *Satureja* is reminiscent of the taste and fragrance of commercial oregano and thymole oil (Escudero *et al*., 1985). At the present study, antimicrobial potency of *Satureja bachtiarica* essential oilsandmethanolic extract were determined by implementing disk diffusion method and broth microdilution technique against seven plants pathogenic bacteria for the first time. In addition, chemical composition of essential oil and methanolic extract of *Satureja bachtiarica*were recognized. We have evaluated essential oil and methanolic extract composition and antibacterial property of *Satureja bachtiarica*an attempt to contribute to use of these as alternative products for controlling plant pathogenic bacteria.

Materials and methods

Collection of plant material:

Satureja bachtiarica (P930300) was collected from Kohenjanthat is one area of Fars province of Iran, from March to April 2015. The collected plant material air-dried in darkness at room temperature (25℃) for two weeks. Taxonomic identification of plant was confirmed by third author.

Extraction of essential oil

The air dried part of *Satureja bachtiarica* were subjected tohydro-distillation using a Clevenger-type apparatus for 3hours. The oil dehydraitedby anhydrous sodium solfate. The extracted oil was stored at 4° until analysis (Dehghanzadeh *et al.*, 2012).

Chemical composition

Essential oil was analyzed by Helwet-packard GC/ MS (model 6890) operating at 70eV ionization energy equipped with a HP-5MS capillary column phenyl methylesiloxans (30m x 0.25mm 0.25µm film thickness) with Helium as the carrier gas and a split ratio of 1:20. The retention indices for all the components were determined according to the van Den Dool & Kratz method (16) using n-alkanes as standard. The compounds were identified by comparison of retention indices HP-5MS with those reported in the literature and by comparison of their mass spectra with the wiley and mass finder3 libraries or with the published mass spectra (Adams, 2001).

Preparation of crude extracts

Both stems and leaves were air dried at room temperature (25℃) to constant weights. The dried plant materials were separately ground to powders. Hundred grams of leaves and stem powdered were soaked in 1000 ml of methanol separately for 48 hrs. Extract was filtered using a Whatman No 1 filter paper.

Each filtrate was concentrated to dryness under reduced pressure at 40°C using a rotary evaporator to yield of crude extract of *Satureja bachtiarica* (Taylor *et al*., 1996)

Polyphenol Extraction

For extraction of polyphenol from plant material, the procedure of (Justesen *et al.*, 1998) has been used. Powdered sample of plant, were extracted with maceration method and was used methanol/acetic acid mixture (85:15 v/v), with the ratio of raw materials to methanol solution of 1:10, then was left at freezer temperature (-18 ºC) for 24h and was extracted in an ultrasonic bath for 15 min. After that centrifuged at 10000 rpm for 20 min in 0 ºC and mix, supernatants was collected , and n-Hexane was added on mass (1:1 v/v), after that it was centrifuged at 10000 rpm for 10 min in 0 ºC. Fallow by, solution was Filtered through 0.2 µm pore size membrane filters and was stored in darkness in a freezer at -18 ºC until analysis.

HPLC analysis

HPLC analysis was carried out on an Agilent 1200 series, equipped with a zarbox eclipse xdb-c18 column (10cmx 5 µmi.d, x 150 mm film thickness, RP), and photodioidedarry detector (PDA) elution was monitored at 280 and 230 nm. Gradient elution was selected to achieve maximum separation and sensitivity. The elution was performed by varying the proportion of solvent a (formic acid 1% in deionized water). To solvent B (Methanol (v/v) as follows: Methanol: formic acid 1% (10:90), at 0 min; Methanol: formic acid 1% (25:75), at 10 min, methanol: formic acid 1% 60:40) at 20 min, and finally, Methanol: formic acid 1% (70:30), at 30 min. The total running time was 30 min. The column temperature was 30 ºC. The injection volume was 20µL and it was done automatically using auto Samper (Najafian & Rowshan, 2013). Chemical analyzing detect total phenol content (Caffeic acid, Carvacrol, Catechin, Chloregenic acid, Coumarin, Ellagic acid, Eugenol, Gallic acid, Hesperetin, Coumaricacid, Qercetin, Rutin, Sinapicacid, Trans-ferulic acid, Vanillin) only ten type recognized they were Carcacrol, Quercetin Eugenol) Hesperetin, Hesperedin, Rutin, Catechin Vanillin, Caffeic acid P-coumaric acid.

Microorganism

A board of organisms comprising 6 Gram negative bacteria: *Pseudomonas syringae* pv. *syringae, Rhizobium radiobacter, Ralstonia solanacearum, Xanthomonas axonopodis* pv. *citri, Pectobacterium atrosepticum, pectobacterium cartovorum,* and one gram positivebacreria*: Bacillus subtilis,* were selected to test *Satureja bachtiarica* extract and essential oil ability to inhibit the growth by disk diffusion method. All strains were isolated from plant disease and rejuvenated in nutrient agar and sub cultured as needed. All strains were provided from subculturing local isolations (Garrity, 2006). For bioassay experiments, suspension of approximately 10^8 cells/ml in sterile normal saline was obtained.

Antibacterial activity

The paper disc diffusion method was used to screen the antibacterial activity of plant extracts or essential oil and performed by using nutrient agar (NA). The experiment was carried out according to the National Committee for Clinical Laboratory Standards Guidelines (Watts, 2008). Test Bacteria were grown in NA.

Bacterial concentration were measured spectrophotometrically (10^8CFU/ml, OD_{600}=0/1). One hundred microlitres of bacterial suspension were swabbed uniformly on surface of NA and the inoculums were allowed to dry for 5 minutes. Sterilized filter paper discs (Whatman, 6 mm in diameter) were placed on the surface of the NA and soaked with 20 µl of a solution of each plant extracts (1000,800.6000,400 µl/disc for methanolic extracts and 100 ,80,60,40 µl/disc for essential oil. DMSO was used as a negative control. Plats were then incubated at 37 °C for 24 h in the inverted position. The diameters (mm) of the inhibition zones were measured.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

A broth micro dilution susceptibility assay was performed using National Committee for Clinical Laboratory Standards Guidelines methods for the determination of the MIC (NCCLS 2001). The 96-well plates were prepared by dispensing into each well 100 µl of Mueller Hinton broth (NB), 100 µl of the plant extract or essential oil and 100 µl of the inoculum. Two- fold serial dilution was prepared with nutrient broth to make the concentrations of 100,50,25,12.5,6.25,3.125,1.562,0.781µl. Negative control containing 100 µl of plant extract or essential oil and 100 µl NB without inoculum were included on each microplate. The contents of the wells were mixed and the micro-plates were incubated at 37 °C for 24 h. The MIC was defined as the lowest concentration of the compounds to inhibit the growth of microorganisms. The experiment was carried out in triplicate. MBC was determined by subculturing the 5 µl of test dilution from each well on to a nutrient agar plates and incubating further at 37 °C for 24 h. The complete absence of growth at applied concentration was considered as the minimum bactericidal concentration

Statistical analysis

All the assays were carried out in triplicates. The experimental results were expressed as mean±error. The data were analyzed using one way analysis of variance (ANOVA) using SPSS.

Result

Based on the results, this plant essential oil showed high antibacterial activity against all selected bacteria. Also methanolic extract showed antibacterial activity against, *Pseudomonas syringae, Xanthomonas axonopodis* pv *citri, Bacillus subtillis, Ralstonia solanacearum.* The maximum antibacterial activity of essential oil was observe on, *Rhizobium radiobacter, Ralstonia solanacearum*in 100 concentration also minimum antibacterial activity of this plant essential oil was observe against *Xanthomonas axonopodis* pv. *citri* in 40 concentration(Table 1). The maximum antibacterial activity of methanolic extract was observe against *Xanthomonas axonopodis* pv. *citri* inconcentration of 1000 and the minimum antibacterial activity of methanolic extract was observe on *Ralstonia solanacearum* in 400 concentration (Table 2).

In our study there were significantly different at 1% level of Duncan test between all assays. The lowest inhibitory consistency of MIC and MBC values of *Satureja bachtiarica* essential oil and methanolic extract was observe on *Bacillus subtillis.*

But other bacterial strains have been inhibited on higher consistency (Table3). In our study a total of 54 chemicals were identified by GC- MS according for 99.99 of the oil given in the (Table5). The major components of essential oil were: Carvacrol 53.94%, γ- terpinene 13.08%, Tymole11.16%, P-simene 6.54%, E-Caryophylene 2.16%, Bornole1.2%, Linalool 2.49%.

Total phenol content of plant extract was revealed in table4, only Carcacrol (461.48mlgr/lit), Quercetin (75.80mlgr/lit) Eugenol (60.61mlgr/lit) Hesperetin (24.29mlgr/lit), Hesperedin (13.75mlgr/lit), Rutin (13.23mlgr/lit), Catechin (9.721mlgr/lit), Vanillin(1.01mlgr/lit), Caffeic acid (0.0812ml gr/lit),P-coumaric acid(2mlgr/lit).

Table 1. Means of diameter inhibition zone (mm) of essential oil of *Satureja bachtiarica* against bacterial growth

Bacterial strain	Inhibition zone	Concentration of essential oil
Xanthomonasaxonopodis*pv.*citri	17.50±0.5 m	100
	15.16±0.28n	80
	14.00±00.00o	60
	10.66±0.57p	40
Bacillus subtilis	46.00±1.73b	100
	34.66±0.57hi	80
	31.66±0.57j	60
	29.33±1.15k	40
Pectobacteriumatrosceptium	43.33±1.15d	100
	38.33±0.57f	80
	35.50±0.50gh	600
	32.66±0.57j	40
Rhizobium radiobacter	48.16±0.28a	100
	44.66±0.57c	80
	40.33±0.57e	60
	37±0.28f	40
Ralstoniasolanacearum	47.66±0.57a	100
	43.00±1.00d	80
	40.66±0.57e	60
	37.66±0.57f	40
Pectobacteriumcartovorum	42.16±0.28d	100
	38.00±1.00f	80
	36.33±0.57g	60
	34.00±00.00i	40
Pseudomonas syringae* pv. *syringae	19.33±0.57 l	100
	18.16±0.28m	80
	15.83±0.76n	60
	13.83±0.76o	40

Not: Significant at p≤0/01

Note: in each column, mean with the same letters are not significantly different at 1% level of Duncan test.

Table 2. Means of diameter inhibition zone (mm) of methanolic extract of *Satureja bachtiarica* against bacterial growth

Bacterial strain	Inhibition zone	Concentraition of methanolic extract
Xanthomonas axonopodis* pv. *citri	23/33±0/57a	1000
	21/33±0/57b	800
	00±00c	600
	18/16±0/28d	400
Bacillus subtilis	21/66±0/57b	1000
	20/00±00c	800
	18/16±0/28d	600
	13/83±0/76fg	400
Pectobacterium atrosceptium	00±00 l	1000
	00±00 l	800
	00±00 l	600
	00±00 l	400
Rhizobium radiobacter	00±00 l	1000
	00±00 l	800
	00±00 l	600
	00±00 l	400
Ralstoniasolanacearum	12/16±0/28h	1000
	11/33±0/28i	800
	9/83±0/76j	600
	8/00±00k	400
Pectobacterium cartovorum	00±00 l	1000
	00±00 l	800
	00±00 l	600
	00±00 l	400
Pseudomonas syringae* pv. *syringae	15/16±0/28e	1000
	14/16±0/28f	800
	13/16±0/28g	600
	9/83±0/76j	400

Not: Significant at p≤0/01

Note: in each column, mean with the same letters are not significantly different at 1% level of Duncan test.

Table 3. MIC and MBC values of methanolic extract and essential oil of *Satureja bachtiarica*

Bacteria	Essential oil		Methanolic extract	
	MIC(µl)	MIC(µl)	MIC(µl)	MIC(µl)
Pectobacterium cartovorum	12.5	-	-	-
Xanthomonas axonopodis* pv. *citri	12.5	25	25	50
Rhizobium radiobacter	25	50	-	-
Ralstoniasolanacearum	12.5	-	25	-
Pseudomonas syringae* pv. *syringae	12.5	-	50	-
Pectobacterium atrosepticum	12.5	-	-	-
Bacillus subtilis	3.125	6.25	12.5	25

Table 4. Chemical compound of *Satureja bachtiarica*methanolicextractwas analyzed by HPLC

Phenolic compounds	Amount (mg/lit)	Retention time (min)
Caffeic acid	0.812696	11.2
Carvacrol	461.488	28.4
Catechin	9.721979	8.2
Chloregenic acid	-	10.2
Coumarin	-	17.4
Ellagic acid	-	19.039
Eugenol	60.61145	23.7
Gallic acid	-	3.2
Hesperedin	13.70446	18.5
Hesperetin	24.29836	22.5
p-coumaric acid	≈ 2	15.9
Quercetin	75.80777	21.6
Rutin	13.23855	12.4
Sinapic acid	-	16.6
Trans-ferulic acid	-	16.3
Vanillin	1.012617	13.5

Discussion

Antimicrobial activity of *Satureja bachtiarica* agains, *Rhizobium radiobacter, Ralstonia solnacearum Xanthomona saxonopodis* pv. *citri, Pectobacterium cartovorum, Pseudomonas syringae, Pectobacterium atrosepticum ,Bacills subtillis* was represented in this study for the first time. In our study the extract was not inhibited selected bacteria at concentrations of (100, 80, 60, 40) compare with essential oil so we have been increased concentrations of the extract at (1000, 8000, 6000, 4000).Most of the bacterial species showed the fairy high degree of sensitivity to the methanolic extract and essential oil. The best activity of essential oil was observed against *Rhizobium radiobacter*, *Ralstoniasolnacearuma*. Methanolic extract showed the best action against *Xanthomonas axonopodis* pv. *citri.*

The antibacterial activity of the essential oil and methanolic extract of this plant has already been reported against different pathogen except against plant pathogenic bacteria. The ethanolic extract of this plant showed antimicrobial activity against *Escherchia coli, Staphylococcus aureus,* but aqua extract did not show antimicrobial activity against these bacteria (Heidari *et al.*, 2013). Also (Ahanjan *et al.*, 2011) reported that this plant essential oil was suitable plant drug against human pathogenic bacteria. Total phenol content of plant extract was reveald in table 4: Carcacrol (461.48mlgr/lit), was the major compound. Our results indicate that it was the first report compound in this plant. Also Carvacrol 53.94% was the highest percent in this plant essential oil. A phytochemical screening result of this plant was in accordance with the results previously obtained. (Mohammadpour *et al.*, 2012) reported that phenolic compounds (37.4%), Thymol (22.6%) and P-cymene (19.3%) were the main components in *Satureja bachtiarica*essential oil. In another study Thymol (44.5%), γ-terpinene (23.9%), P-cymene (7.3%), β-caryophyllene (5.3%) and Borneol (4.2%) were the

Table 5. Essential oil compound of *Satureja bachtiarica*was analyzed by GC-MS

Compound(P930300)	RI	% of compound
Tricyclene	921	0.018
α-Thujene	924	0.876
α-Pinene	931	0.573
Camphene	946	0.459
Sabinene	970	0.053
ß-Pinene	974	0.145
Myrcene	988	1.186
3-Octanol	993	0.018
α-Phellandrene	1003	0.214
δ-3-Carene	1009	0.047
α-Terpinene	1015	2.16
p-Cymene	1023	6.548
Limonene	1025	0.184
ß-Phellandrene	1026	0.202
1,8-Cineole	1029	0.025
(Z)-ß-Ocimene	1034	0.1
(E)-ß-Ocimene	1044	0.153
γ-Terpinene	1058	13.084
cis-Sabinene hydrate	1064	0.321
Terpinolene	1086	0.133
Linalool	1098	2.493
n-Nonanal	1102	0.038
Borneol	1163	2.018
Terpinene-4-ol	1174	0.476
α-Terpineol	1187	0.034
cis-Dihydrocarvone	1199	0.03
trans-Dihydrocarvone	1207	0.022
Thymol	1290	11.166
Carvacrol	1299	53.941
δ-Elemene	1334	0.017
Thymol acetate	1352	0.086
Carvacrol acetate	1370	0.4
α-Gurjunene	1406	0.03
(E)-Caryophyllene	1416	2.164
Aromadendrene	1435	0.029
α-Humulene	1449	0.142
allo-Aromadendrene	1456	0.016
Germacrene D	1477	0.006
Viridiflorene	1491	0.028
Bicyclogermacrene	1492	0.094
ß-Bisabolene	1505	0.01
γ-Cadinene	1510	0.007
δ-Cadinene	1519	0.019
Spathulenol	1573	0.079
Caryophyllene oxide	1578	0.155

major compound Sefidkon & Ahmadi **(**2000). In our study the numbers of major components in Satureja bachtiarica essential oil were higher than previous report in this plant. Also the main components observed in the essential oil of this plant were almost similar to those obtained by other studies. As we seen the percent of some major component like: P-cymene, Thymole, Linalool, Carvacrol, Borneole

in this study were different with other reported. Soil nutrient level, temperature regime, relative humidity, irradiance and photoperiod may play a specific role in the composition of the oil (Chauhan *et al.*, 2011). The plants are a reserve of biologically active substance. Essential oil and methanolic extract can be a significant source of a great diversity of chemical species equipped with antimicrobial capacity. In general, the antimicrobial activity of the tested essential oil and methanolic extract can be related to the contribution major compound such as Thymol & Carvacrol in essential oil or methanolic extract, because these compound due to the ability to penetrate cell membrane and rapid exit inter-cellular compounds have antibacterial properties (Dehghanzadeh *et al.*, 2012).

Conclusion

In general there are many difficulties and deficiencies to control plant pathogenic bacteria. Also *Satureja bachtiarica* extract and essential oil in *in vitro* have considerable antimicrobial ability over the studied strains. So using extract and essential oil of this plant cause more effective to control these pathogenic. But applying extracts instead of essential oil is more cost effective and easier to apply and can be a proper substitute for agricultural toxin.

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چکیده

امروزه آلودگی محیط زیست و تجمع مواد سمی در آن یکی از مسئله‌های بسیار خطرناک برای سلامتی بشر و موجودات زنده میباشد. همینطور پدیده مقاوت به آنتی بیوتیک ها و پیدایش سویه های مقاوم یکی از مشکلاتی است که کشاورزان با آن روبرو می‌باشند. استفاده از ترکیبات طبیعی یکی از راهکارهای موثر برای این مشکلات میباشد. در این تحقیق اثر ضد میکروبی اسانس وعصاره گیاه *Satureja bachtiarica* به روش انتشار دیسک روی پاتوژن های *Pseudomonas syringae pv. Syringae, Rhizobium radiobacter , Ralstonia solanacearum , Xanthomonas axonopodis pv. citri, Bacillis subtilis, Pectobacterium cartovorum, Pectobacterium atrospeticum.* مورد مطالعه قرار گرفت.همچنین جت محاسبه حداقل غلظت بازدارندگی (MIC) و حداقل غلظت گشندگی (MBC) از روش میکروبراث دایلوشن استفاده گردید. براساس نتایج حاصله بهترین غلظت بازدارندگی و کشندگی اسانس و عصاره این گیاه روی باکتری *Bacillus subtillis* میباشد.همچنین ترکیبات شیمیایی اسانس و عصاره این گیاه به ترتیب با روشهای GC-MS وHPLC جداسازی گردید. مهمترین ترکیبات شیمیایی اسانس شامل: Carvacrol 53.94, γ- terpinene13,08, Tymole11.16, P-symene 6.54, E-caryophylene2.16, Bornole1.2, Linalool2.49, α- terpnene. و مهمترین ترکیبات شیمیایی عصاره شامل: Carcacrol (461.48mlgr/lit), Quercetin (75.80mlgr/lit) Eugenol (60.61mlgr/lit) Hesperetin (24.29mlgr/lit), Hesperedin(13.75mlgr/lit), Rutin (13.23mlgr/lit), Catechin (9.721mlgr/lit), Vanillin(1.01mlgr/lit), Caffeic acid (0.0812ml gr/lit), P-coumaric acid(2mlgr/lit), میباشد. به طور کلی اسانس گیاه *Satureja bachtiarica* خاصیت آنتی میکروبی مناسبی روی تمامی میکرو ارگانیسم ها دارا میباشد. اگرچه عصاره این گیاه تنها روی پاتوژن های *Xanthomonas axonopodis pv. citri, Bacillis subtilis , Ralstonia solanacearum, Pseudomonas syringae pv.syringae* اثر ضد میکروبی نشان داده است.

واژه‌های کلیدی: مرزه بختیاری ,اثر ضد میکروبی ,اسانس, عصاره

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