Original Article

Bull Emerg Trauma 2018;6(4):355-362.

Bulletin of Emergency And Trauma

Antimicrobial Survey of Local Herbal Drugs against *Acinetobacter baumannii* Isolated from Patients Admitted to a Level-I Trauma Center

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Received: May 13, 2018
Revised: July 22, 2018
Accepted: July 29, 2018

ABSTRACT

Objective: To determine the antimicrobial activity and entity of several local herbal plants against *Acintobacters* isolated from trauma patients admitted to a Level-I trauma center.
Methods: The antibacterial activities of the *Satureja bachtiarica* oil and some selected Iranian medicinal plants (*Artemisia sieberi* and *Tanacetum dumosum* belonging to the Asteraceae/Compositae; *Salvia mirzayanii* and *Mentha mozaffarianii* belonging to the Lamiaceae/Labiatae) were assayed on *A. baumannii* by microdilution and agar disc diffusion methods. Having obtained the acceptable antibacterial data, the shade-dried aerial parts of the plants were extracted by hydrodistillation method using Clevenger apparatus according to European pharmacopeia for 3 h. The analysis of *S. bachtiarica* essential oil accompanied by other herbal drug oils were performed by using GC/FID and GC/MS methods.
Results: Outcomes revealed that the *S. bachtiarica* essential oil exhibited the potent antibacterial capability against *Acinetobacter* strains in comparison with Colistin, as a positive control. For *S. bachtiarica*, the growth inhibition zone and minimum inhibitory concentration (MIC) values were 21 mm and 0.5 mg/ml, while, for Colistin, the data were in order: 8 mm and 0.016 mg/ml. Consequently, GC/MS outcomes demonstrated that the major components of the essence were carvacrol (48.6%), followed by *p*-Cymene (16.6%), γ-terpinene (6.9%) and linalool (5.3%).
Conclusion: Based on the considerable inhibitory activity against nosocomial infections by essential oil of *S. bachtiarica*, it could be considered as the suitable candidate in the food industry and pharmaceutical uses.

Keywords: Trauma; *Acintobacter baumannii*; Antibacterial activity; *Satureja bachtiarica*; Carvacrol.

Please cite this paper as:
Abdolrahimzadeh H, Bolandparvaz S, Abbasi HR, Dehghankhalili M, Paydar S, Dehghanian AR, Hafez Ghoran S, Asadollahi M, Zare M. Antimicrobial Survey of Local Herbal Drugs against *Acinetobacter baumannii* Isolated from Patients Admitted to a Level-I Trauma Center. *Bull Emerg Trauma*. 2018;6(4):355-362. doi: 10.29252/beat-060414.

Introduction

Trauma is a cellular disruption caused by an environmental energy that is beyond the body's resilience. Trauma remains the most common cause of death for all individuals between the ages of 1 and 44 years and is the third most common cause of death regardless of age. It is also the number one cause of years of productive life lost. Death after trauma has a trimodal distribution. Acute deaths (less than 24 hours) usually result from uncontrolled conditions, but infections and multiple organ dysfunction syndrome, which often arise from infection, are responsible for a significant proportion of late deaths. Indeed, infection is responsible for most deaths in patients who survive longer than 48 hours after trauma. Trauma infections can be divided into the injury and nosocomial infections. Most post traumatic infections are polymicrobial, involving a mixture of aerobic and anaerobic organisms. In one series, 37-45% of all trauma patients experienced infectious complications during their initial hospitalization. In that same study, 80% of all trauma patients who were in ICU at least 7 days met the criteria for SIRS [1]. Humans have evolved mechanisms to avoid infection despite presence of bacteria in our environment. Under normal circumstances there is a balance between our microbial, intact environmental barriers, and host defenses. Traumatic injury disrupts this balance and significantly increases the probability of developing an infection [1].

Acinetobacter baumannii is an aerobic Gram-negative bacillus which is non-fermentative, catalase-positive and oxidase-negative [2]. Considering the resistance to multiple antimicrobial agents along with the wide spreading nosocomial infections, *A. baumannii* has been evolved from being an underestimated microorganism to one of the most important factors causing the various infections at hospital environments, mainly in immunocompromised patients over the last 30 years [3-6]. Surprisingly, the intrinsic resistance of *Acinetobacters* could be related to not only the genetic materials including plasmids, transposons and integrons but also the ability to survive on anhydrous surfaces for the long interval periods [3,7-9]. Multidrug-resistant *A. baumannii* strains are frequently found to be responsible for epidemics of nosocomial infections, such as respiratory, bloodstream, urinary tract, skin, and soft tissue infections [10].

According to ethnopharmacological survey in Iranian folk medicine, we found some medical plants that used to prevent post-operative infection rate [11,12]. Therefore, we evaluated the antibacterial activity of some of these medical plants against *Acinetobacters* infection. The main purpose of this *in vitro* study was to evaluate the antibacterial activity of volatile oils obtained from some medicinal plants belonging to the Asteraceae/Compositae (*Artemisia sieberi* and *Tanacetum dumosum*) and Lamiaceae (*Salvia mirzayanii*, *Mentha mozaffarianii* and *Satureja bachtiarica*) on *Acintobacters* isolated from Trauma patients in Shahid Rajaee Hospital of Shiraz.

Materials and Methods

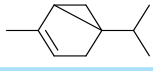
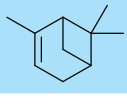
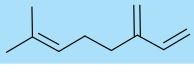
Plant Material

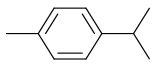
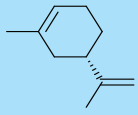
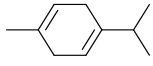
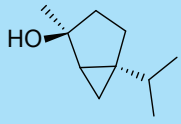
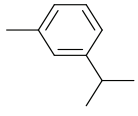
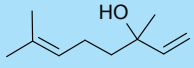
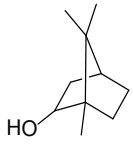
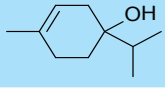
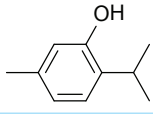
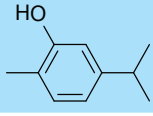
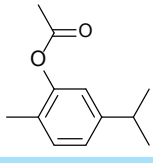
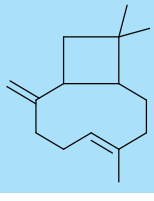
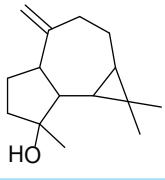
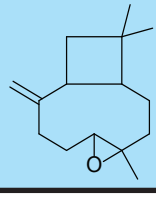
The aerial parts of the plants were collected from South and Southwest of Iran in March until July 2017 in the flowering stage of the plants. The plants were identified by regional floras and authors with floristic and taxonomic references [13], and voucher specimens were deposited at the herbarium of Medicinal and Natural Products Chemistry Research Center, Shiraz University of Medical Sciences, Shiraz, Iran. The shade-dried aerial parts of the plants were extracted by hydrodistillation for 3 h, giving relatively high yield oils in all collections (Table 1). The oils were dried over anhydrous sodium sulfate and kept at -4ºC.

Gas Chromatography Analysis

Gas Chromatography was performed on a Varian CP-3800 chromatograph, with a FID and a HP-5 column (30 m×0.25 mm i.d., 0.25 µm film thickness). The oven temperature was programmed from 60°C to 240°C at 5°C/min and finally kept 10 min at this temperature. The carrier gas was

Table 1. Chemical constituents (%) of the essential oil of *S. bakhtiarica* analyzed by GC-FID

No.	Compounds	Structures	*S. bakhtiarica* (%)	RI
1	α-Thujene		1.3±0.04	932
2	α-Pinene		1.14±0.08	938
3	Camphene		0.46±0.05	953
4	β-Myrcene		1.71±0.07	993

5	p-Cymene		16.55±1.4	1025
6	Sylvestrene		0.78±00	1031
7	γ-Terpinene		6.86±1	1064
8	cis-Sabinene hydrate		0.47±00	1072
9	m-Cymene		0.3±0.00	1087
10	Linalool		5.25±0.08	1099
11	Borneol		1.85±1	1168
12	Terpinen-4-ol		1.98±0.09	1180
13	Thymol		0.77±00	1291
14	Carvacrol		48.57±2.5	1301
15	Carvacrol acetate		1.96±02	1372
16	β-Caryophyllene		2.08±0.4	1422
17	Spathulenol		0.56±0.02	1550
18	Caryophyllene oxide		1.96±0.4	1589

*The values are the means of three different FID area percentage±SE; **The major compounds with more than 1.0% area concentration were formatted in bold font.

N_2 with a flow rate of 0.9 mL/min. Injector and detector temperature were set at 250°C. The injection volume was 0.4 µl for the volatile oils and also *n*-alkanes for calculation of the retention indices was separately injected.

Gas Chromatography-Mass Spectroscopy Analyses

The GC-MS was carried out on an Agilent 7890N chromatograph, coupled to an Agilent 5975C mass spectrometer (Agilent Technologies, USA), operating at 70 eV ionization energy, 0.5 s/scan and the mass range: 35-400, equipped with a HP-5 MS capillary column (30 m×0.25 mm, film thickness 0.25 µm). Helium was used as the carrier gas at the constant flow of 0.9 ml/min and split ratio of 1:50. The oven temperature was 60°C rising to 250°C at a rate of 5°C /min, then held at 250°C for 10 min; transfer line temperature was set at 250°C.

Identification of Compounds

Retention indices (RI) for all constituents were calculated according to Van den Dool approach [14], using *n*-alkanes (C_6–C_{22}) as standards and the essential oils on a HP-5 column under the same chromatographic conditions. The identification of the components was made based on comparison of their mass spectra with those of the internal computer reference mass spectra libraries (Wiley 7.0 and Nist), as well as by comparison of their retention indices with data mentioned in the previous literatures [15].

Antibacterial Activity

The antimicrobial activity of the obtained essential oils was tested by using the disc-diffusion method on Muller Hinton Agar (MHA) [16] using determination of inhibition zones, and determining the minimal inhibitory concentration (MIC) using the macro dilution broth susceptibility assay as recommended by NCCLS [17].

Study Population and Sampling

All trauma patients admitted in Shahid Rajaee Hospital, the unique center of trauma in Shiraz, affiliated to Shiraz University of Medical Sciences (SUMS) hospital with single of sepsis and positive blood culture for *Acintobacter baumannii*. The hospital is a referral center of trauma for Fars Province situated in Southern part of Iran.

Microorganisms and Media

Disc Diffusion Assay

Muller Hinton agar (MHA) was autoclaved at 121°C for 20 min. The plates (8-cm diameter) were prepared with 10 ml agar inoculated with 1 ml of each bacterial suspension. Microorganisms were cultured for 16 to 24 h at 37°C and prepared to turbidity equivalent to McFarland Standard No. 0.5. Sterile paper discs (6 mm in diameter) were impregnated with 20 µl of dilutions of known essential oil concentrations (5 µg/disc) and incubated at 37°C for 24 h. The essential oils were dissolved in dimethyl sulfoxid (DMSO, 15 µl) before the test for antimicrobial activity. Discs (6 mm diameter) of Colistin (10 µg) was used as positive controls. Bacterial growth inhibition was determined as the diameter of the inhibition zones around the discs (mm). The growth inhibition diameter was an average of three measurements, taken at three different directions. All tests were performed in triplicate.

Minimum Inhibitory Concentration (MIC) Using Muller Hinton Broth Micro-Dilution (MHB)

The minimal inhibitory concentration (MIC) values were evaluated using the broth serial dilution method according to standard methods [18]. The MIC was defined as the lowest concentration of the compound to inhibit the growth 50% of microorganisms. Bacterial strains were cultured overnight at 37 °C in Muller Hinton Broth (MHB, Oxoid). Stock solution of the essential oil was prepared in 33.3% (v/v) dimethyl sulfoxide (DMSO). Dilution series, using MHB, were prepared from 0.062-8 mg/ml. After incubation at 37 °C for 24 h, the microorganism growth inhibition was evaluated by measuring absorbance at 600 nm, and prepared to turbidity equivalent to McFarland Standard No. 0.5 using a spectrophotometer. An aliquot of the samples (5 µl) was added to 95 µl of fresh media followed by 100 µl of the bacterial suspension (OD=0.1 at 600 nm) in a 96-well plate. The plates were incubated at 37ºC for 24 h in a shaking incubator. Colistin was used as positive control in each assay. DMSO solution was used as a negative control. Control tubes were incubated under the same condition. Antibacterial activity was detected using a colorimetric method by adding 10 µl of 0.5% INT solution in water in each well at the end of the incubation period for further 30 min. Experiments were performed in triplicate but at three different times.

Results

In terms of measuring the antibacterial activity using disc diffusion method, the significant zones of inhibition around the discs were noted in Tables 2 and 3. Our examinations investigated the antimicrobial activities of medicinal plants belonging to the Asteraceae (*Artemisia sieberi* and *Tanacetum dumosum*) and Lamiaceae (*Salvia mirzayanii*, *Mentha mozaffarianii* and *Satureja bachtiarica*) families. Careful results on examined bacterial strains revealed that the essential oil of *Satureja bachtiarica* Bunge assayed by both Disk Diffusion and Minimum Inhibitory Concentration (MIC) methods was the most potent one against all of *Acintobacters* (Figures 1 and 2). Our findings showed that the aerial part of *S. bachtiarica* essential oil had high ability against Gram-negative *Acintobacter* strains. The essential oil seems to be active against all bacterial strains used (MIC values of 0.5 mg/ml). Hence, the most active concentration was 0.5 mg/ml

Table 2. Antimicrobial potential (MICa) of essential oil from *S. bachtiarica*, against 6 *Acintobacters* isolated from individual Trauma patients in Shahid rajaei Hospital of Shiraz, Iran as determined by nutrient-broth micro-dilution bioassay.

	Acintobacter					
Plant oil	**From Ttauma patient 1**	**From Ttauma patient 2**	**From Ttauma patient 3**	**From Ttauma patient 4**	**From Ttauma patient 5**	**From Ttauma patient 6**
Satureja bachtiarica	0.5	0.5	0.5	0.5	0.5	0.5
Colistin	0.016	0.016	0.016	0.016	0.016	0.016

*Minimum inhibitory concentration (MIC) the plant extracts in the bacterial suspension in the nutrient broth media (mg/ ml) determined in three replicates

Table 3. Disk diffusion results of five medical plants against 6 *Acintobacters* isolated from Trauma patients in Shahid rajaei Hospital of Shiraz, Iran.

	Acintobacter					
Plant oil	**From Ttauma patient 1**	**From Ttauma patient 2**	**From Ttauma patient 3**	**From Ttauma patient 4**	**From Ttauma patient 5**	**From Ttauma patient 6**
Mentha mozaffarianii	NA[a]	NA[a]	NA[a]	NA[a]	NA[a]	NA[a]
Artemisia sieberi	NA[a]	NA[a]	NA[a]	NA[a]	NA[a]	NA[a]
Salvia mirzayanii	NA[a]	NA[a]	NA[a]	NA[a]	NA[a]	NA[a]
Tanacetum dumosum	NA[a]	NA[a]	NA[a]	NA[a]	NA[a]	NA[a]
Satureja bachtiarica	21 mm	20 mm	21 mm	19 mm	21 mm	21 mm
Colistin	8 mm	8 mm	8 mm	8 mm	8 mm	8 mm

*Concentration of essential oil all medical plants is 2.5 mg/ml; [a]NA= not active

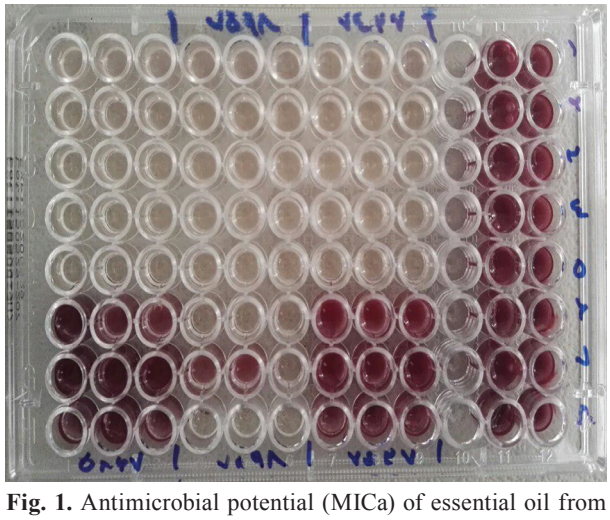


Fig. 1. Antimicrobial potential (MICa) of essential oil from *S. bachtiarica*, against *Acintobacters*. isolated from Trauma patients in Shahid rajaee Hospital of Shiraz, Iran as determined by nutrient-broth micro-dilution bioassay.

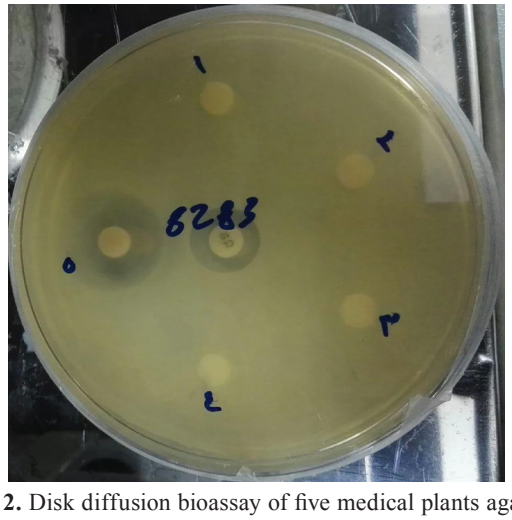


Fig. 2. Disk diffusion bioassay of five medical plants against *Acintobacter* mentioned in below.
1) *Mentha mozaffarianii*, 2) *Artemisia sieberi*, 3) *Salvia mirzayanii*, 4) *Tanacetum dumosum*, 5) *Satureja bachtiarica*, Positive control: Colestin

inhibiting completely the growth of all the clinically isolated *Acintobacters*.

The hydrodistillation of *S. bachtiarica* aerial parts led to a yellowish oil in 0.6% (w/w) yield. Then, the obtained volatile oil was analyzed by GC-FID and GC-MS. Therefore, the main constituents were identified as the oxygenated monoterpenes: carvacrol (48.57%) and linalool (5.25%); monoterpene hydrocarbons; *p*-cymene (16.55%) and *γ*-terpinene (6.86%) (Table 1) corresponding to 94.55% of the essential oil.

Discussion

Although the antibacterial activity and chemical constituents of the essential oil of *S. bachtiarica* have been previously reported [12,19,20], in agreement with the literature, the major constituents of the oil were characterized as carvacrol, *p*-cymene and thymol. Interestingly, our data revealed that the other major components after carvacrol and *p*-cymene were *γ*-terpinene and linalool. The *Satureja* species is commonly used as a spice and traditionally as a muscle pain reliever, tonic, and carminative in treating stomach and intestinal disorders such as cramps, nausea, indigestion, and diarrhea [21].

According to the phenyl backbone, the remarkable antimicrobial activity of *S. bachtiarica* may be because of the presence of mentioned compounds. It is suggested that these components could

synergistically inhibited the various bacterial strains. Therefore, the good antimicrobial activity of carvacrol is certainly justified by the hypothesis proposed by A. Ben Arfa *et al*., [22] with related to its hydrophobic behavior in the membrane along with detoxifying the unwanted free radicals. The importance of the hydroxyl group in the phenolic structure was confirmed in terms of activity when carvacrol was compared to its methyl ether. Furthermore, the relative position of the hydroxyl group exerted an influence upon the components effectiveness as seen in the difference in activity between carvacrol and thymol against Gram-negative and Gram-positive bacteria. However, the significance of the phenolic ring was demonstrated by the lack of activity of the monoterpene cyclic hydrocarbon *p*-cymene. The high activity of the phenolic components may be further explained in terms of the alkyl substitution into the phenol nucleus, which is known to enhance the antimicrobial activity of phenols .[23] These compounds were strongly active despite their relatively low capacity to dissolve in water, which is in agreement with published data 24-35]]. In another research, the antimicrobial activity of carvacrol was examined on various Gram-positive and Gram-negative bacterial strains. Results revealed that carvacrol was significantly inhibited all selected bacteria at different concentrations (400 and 800 µg/ml). The obtained data showed maximum inhibition against *Serratia* spp. (28 mm), followed by *E. coli* (26 mm) and *Enterobacter* spp. (25 mm), *Klebsiella pneumonia* (23 mm), *Proteus mirabilis* (22 mm), *S. aureus* (20 mm), *S. epidermidis* (22 mm) and *St. pneumonia* (16 mm), surprisingly, there are no effect on *Pseudomonas aeroginosa* [36]. It seems to be that the function of carvacrol as a phenolic essential oil could be due to the following manners including to disintegrate the outer membrane of bacterial cells, to be the disturbance of the cytoplasmic membrane, disrupting the proton motive force (PMF), electron flow, active transport and coagulation of cell contents [37,38]. Likewise, the potency of carvacrol in disintegrating the cell membrane of gram negative bacteria were confirmed by means of enhancing lipopolysaccharides (LPS) accompanied by the increasing permeability of the cytoplasmic membrane to ATP [39]. On the basis of a comprehensive survey on essential oils isolated from various species of *Satureja*, some biological properties such as anti-bacterial, anti-fungal, anti-viral [40-42], anti-oxidant [43], antispasmodic, anti-diarrheal, anti-nociceptive, anti-inflammatory [44,45], anti-HIV-1 [40] and immunomodulatory effects [45] have been previously mentioned. *S. bachtiarica* known as Marzeh-e-Koohi is an Iranian endemic specie and aromatic plant that commonly observed among mountain in the southwestern part of Iran [46]. In the folk medicine, the plant is used as analgesic and antiseptic by Bakhtiari and Chaharmahali tribes of Iran [47]. The major phenyl backbone components of *S. bachtiarica* were carvacrol, thymol and *p*-cymene [48].

According to the considerable antibacterial activity of essential oils noted in the literatures and some problematic features existed by some bacterial strains, paying attention to explore the natural drugs from nature in order to repair various human defeats is worthwhile.

In conclusion, the *S. bachtiarica* (known as "Marzeh" in Persian) essential oil clearly demonstrates antibacterial properties, although the mechanistic processes are poorly understood. These activities suggest potential use as chemotherapeutic agents, food preserving agents and disinfectants.

Acknowledgment

The authors are grateful to Shahid Rajaee (Emtiaz) Trauma Hospital, Medicinal and Natural Products Chemistry Research Center (MNCRC) and Shiraz University of Medical Sciences for financial and kind support.

Conflicts of Interest: None declared.

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