Internal Medicine and Medical Investigation Journal
E-ISSN: 2474-7750
Homepage: www.imminv.com

ORIGINAL ARTICLE

Evaluation of Antibacterial Activity of Essential Oil of *Ziziphora clinopodioides* and *Achillea wilhelmsii* on Antibiotic-resistant Strains of *Staphylococcus aureus*

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ARTICLE INFO

Article history
Received: Mar 29, 2017
Accepted: Apr 17, 2017
Published: May 23, 2017
Volume: 2
Issue: 2

Conflicts of interest: None
Funding: None

Key words
Antibacterial Effects,
Essential Oil,
Staphylococcus aureus

ABSTRACT

Background: The use of drugs to treat diseases led to the emergence of resistant strains of bacteria. Bacterial resistance to antibiotics is the most common problems in medical science. This study was aimed to evaluate the antimicrobial effects of essential oil of *Ziziphora clinopodioides* and *Achillea wilhelmsii* on against resistant clinical strains of *Staphylococcus aureus*. **Methods:** After collecting plants and validate its scientific name by botanists of Agricultural Organization and after drying in the shade, essential oil of *Z. clinopodioides* and *A. wilhelmsii*, extracted with steam distillation method by Clevenger and antimicrobial effects of essential oil by well diffusion on bacteria mentioned above were interpreted. The amount of essential oil were injected to gas chromatography linked to mass spectrometry (GC/Ms), and the amount and type compounds of the essential oils were identified. **Results:** The results showed that the extracted essence essential oil from the *Z. clinopodioides* and *A. wilhelmsii* has a bactericidal effect. In the obtained results by GC/Ms chromatography, in Z. clinopodioides and A. wilhelmsii essential oils, 22 and 18 compounds were identified, respectively. **Conclusion:** Our results showed *Z. clinopodioides* and *A. wilhelmsii* have considered in herbals groups with antibacterial properties and after evaluating their effects *in-vivo* condition and to identify the active ingredients, as an alternative to synthetic drugs that commonly used to treat infections are used.

INTRODUCTION

One of the most important health challenges is the treatment of infectious diseases due to their much higher prevalence and spread. Since the discovery of penicillin in the late 40's, and widely use of it, new antibiotics have been continuously presented to treat infections. The result was the increased use of clinical or synthetic antibiotics to heal clinical infectious disease. The indiscriminate use of antimicrobial drugs caused the increased drug resistance against different antibiotics in most bacteria (1-6). It has been one of the reasons of the increasing use of plants as natural, safe, accessible and affordable materials in treating bacterial infections in comparison with synthetic antibiotics. Also, the consumption of herbal medicines is more acceptable than chemical drugs (7-9). Moreover, that is why a new wave of expensive global studies has raised, and the antibacterial effects of different plants have been introduced (10). Therefore, two plant species were considered in the current study.

Ziziphora clinopodioides belongs to *Ziziphora* genus and Lamiaceae family with the Persian name of *kakuti-e kuhi* is a perennial plant. In Iran, several hundred species in 49 genera of the Lamiaceae family are scattered. The geographical distribution of *Z. clinopodioides* in the world is as following: eastern Balkan peninsular, southeast of Asia and from central Asia to Pamir-Alay and Himalaya mountains (Iran, Iraq, central and east parts of Turkey) and Africa (11). Chitsaz et al. (2005) examined the compounds and antimicrobial potential of *Z. clinopodioides* essential oil on several bacterial species. The essential oil of the plant prevents the growth of all studied gram-negative and gram- positive organisms and had the most effect on *Salmonella typhimurium* (12). Salehi et al. (2006) studied the antibacterial activity and chemical compounds of *Z. clinopodioides* essential oil.

Published by Mehrabani Publishing LLC.

http://dx.doi.org/10.24200/imminv.v2i2.58

Thirty- two components constituting 97.1 % of the essential oil were identified (13). *Achillea wilhelmsii C.Koch* is one of the most famous medicinal plants that about 85 species of it have been identified that 7 of them are exclusively found in Iran. Native Achillea species has a relatively wide distribution in different parts of the country and are commonly grown in fields, roadsides and mountainous areas (14). New flowers of Achillea are rich in chemicals and used to treat respiratory problems as anti-allergic, anti-congestion and mucus. Essential oil of them contains chamazulene, cineol and borneol which have shown anti-inflammatory, antispasmodic, anti-dandruff properties, hair growth stimulation, and topical healing of skin (14-15).

Aljancic et al. (1999) in their study showed the significant inhibitory effect of *A. wilhelmsii* on *Candida albicans* and *Bacillus subtilis*. According to them, in addition to the inhibitory effect of two above microbes, the flavonoids of *A. wilhelmsii* essential oil also prevent the growth of *Aspergillus Niger* effectively (16).

In the study by Sokmen et al. (2003), the antimicrobial effect of the essential oil, aqueous and methanol extracts of *A. sintenisii* on 12 bacteria and two yeast species were examined comparatively (17). The current study intends to investigate the chemical compounds and anti-bacterial effects of *Z. clinopodioides* and *A. wilhelmsii* on *Staphylococcus aureus* resistant to the antibiotic.

METHODS

1. *Plant sampling and investigation of its anti-microbial properties*
   Plant samples were collected from the nature areas of Eyshabad village, Marand city, and East-Azerbaijan province in two stages in late May to early June. The samples were cleaned after collection and transmission, and also, they were dried in a large and proper space under the conditions away from direct sunlight. After they had been dried completely, their aerial parts (stems and leaves) were separated from the roots and prepared to be grinded. Then, the essential oil of their aerial parts was extracted for 5 hours by a Clevenger-type apparatus. After washing with sodium sulfate and dissolving in n-hexane, their essential oil was used to investigate antibacterial properties. The bacteria employed in this study was *S. aureus* isolated from patients. Initially, a suspension equivalent to standard McFarland 0.5 turbidity was prepared from each bacterium. After spreading the bacterial suspension on Müller-Hinton agar media, wells of 5 mm in diameter were immediately made. About 50 µL and 100 µL of essential oils were added aseptically in wells. The same amount of n- hexane solvent (as control) was added to plates and then were incubated for 24 hours at 37°C (18-19).
2. *Bacterial isolates and their resistance to antibiotics*
   Thirty samples used in this study were selected from the clinical samples of Marand Razi hospital and cultured on mannitol salt agar and blood agar media. Isolated pure colonies of bacteria on culture media were detected regarding type through catalase tests and other biochemical tests. Finally, *S. aureus* isolates were recognized by using tube coagulase test, LAM test, and agglutination investigation. The antibiotics sensitivity/resistance profiles (antibiogram) of the purified isolate were determined by the methodology of Kirby-Bauer's on Mueller-Hinton Agar medium. We used antibiotics include trimethoprim/sulfamethoxazole (SXT10 + 10 µg), ampicillin (AM 10µg), ceftazidime (SAZ 30µg), ampicillin (SXT10+10 µg), ceftazidime (CF 5 µg), amikacin (AN 30 µg), penicillin (10 µg P), ceftriaxone (30 µg CRO), erythromycin (15 µg E) and tetracycline (TE 30 µg) made by Padtan Teb Co. The diameter of inhibition zone was measured by comparison to the standard isolate after 24 hours of incubation at 37˚C, and the sensitivity and resistance of isolates were determined and compared with standard table.
3. *Essential oil analysis by gas chromatography-mass spectrometry (GC/Ms)*
   The essential oil sample was injected in GC/Ms (type: Agilent GC (USA)) after finding thermal planning system. The compounds were identified by the database of the device and comparing their mass spectra. SPSS V.19 software performed variance analysis of data, and Duncan's multiple range test at the confidence level of 99% was used for the mean.

RESULTS

1. *Bacterial responses to antibiotics*
   In-vitro antibacterial activities of some antibiotics against obtained isolates were evaluated. The results revealed that in the presence of antibiotics, each bacterial isolate responded somewhat similarly, however, there were differences among the isolates in terms of antimicrobial resistance or antimicrobial sensitivity. Some differences were also observed in inhibition mechanisms of antibiotics, as the antibacterial effect of penicillin and erythromycin was much less than other antibacterial drugs (Table 1).
2. *Antimicrobial activity of essential oils*
   Because of various geographical and climatic conditions in Iran, there is a rich and varied source of plant species in it.
   Some of these herbal plants have medicinal properties

Table 1. Antibiotic susceptibility pattern of *S. aureus* isolates (%)

	CAZ	AM	TE	STX	CF	AN	CRO	E	P
R	33.3	70	23.3	76.7	80	23.3	36.7	83.3	83.3
S	50	10	26.7	20	10	76.7	16.7	10	6.7
I	16.7	20	50	3.3	10	0	46.6	6.7	10

R: Resistant; S: Sensitive; I: Intermediate such as antibacterial activity. In our study, the antibacterial effects of essential oils were as follows: essential oils of *Z. clinopodioides* and *A. wilhelmsii* could inhibit *S. aureus* growth.

The antibacterial effect of *Z. clinopodioides was more* than the one of *A. wilhelmsii.* The results of the antimicrobial activity of *Z. clinopodioides* and *A. wilhelmsii* are represented in Table 2.

The n-hexane solvent was used as control sample that showed no inhibitory effect. Given the observed clear relationship between the plant derivate (essential oils) and their antimicrobial activity, it can be said that they can be used as new biologically active substances against some virulent infectious agents.

Table 2. Antimicrobial effect of essential oils of *Z. clinopodioides* and *A. wilhelmsii* on *S. aureus* resistant to antibiotics (mean±SD)

Plant	Diameters of the inhibition zones (mm)		
	S. aureus		
	50 µl	100 µl	N-Hexane control
A. wilhelmsii	0.47±10.66	0.16±13.5	0
Z. clinopodioides	0.81±11	0.18±14.6	0

3. *Chemical compounds obtained from the essential oils of Z. clinopodioides and A. wilhelmsii*

Chemical compounds obtained from the essential oils of the aerial part of *Z. clinopodioides* and *A. wilhelmsii* were investigated *in-vitro*. The essential oils were extracted by steam distillation with Clevenger and injected to GC/Ms after purification (removing from the plant sweat), and the amount and type of the essential oil were determined. Totally, 22 compounds of *Z. clinopodioides* essential oil, including 95.4% of compounds, and 18 compounds of *A. wilhelmsii* essential oils, including 91.31% of compounds, were identified. Table3 shows the ingredients of each essential oil.

DISCUSSION

According to increased bacterial resistance to a variety of antibiotics, it has been tried to access and use the compounds of plants in the treatment of various diseases. From thousands of years ago, plants have played a critical role in maintaining health and improving the life quality of human beings. Medicinal herbs have useful properties that their antibacterial, anti-parasitic, antifungal and antioxidant properties can be noted (20).

Chalibian et al. (2003) have proved that *A. wilhelmsii* has an antimicrobial effect on *S. aureus* that the results of this study are consistent with it (21).

Table 3. Chemical compounds obtained from the essential oils of *Z. clinopodioides* and *A. wilhelmsii*

A. wilhelmsii			*Z. clinopodioides*		
#	Compounds	Percent	#	Compounds	Percent
1	alpha-Pinene	18.82	1	Alpha-thujene	0.1
2	beta-Pinene	11.2	2	alpha-Pinene	3.8
3	Caryophyllene oxide	9.21	3	Camphene	0.4
4	Alpha-thujene	8.62	4	Sabinene	4.3
5	Valencene	7.92	5	beta-Pinene	4.3
6	Chamazulene	6.31	6	Myrcene	1.8
7	ρ-Cymene	5.82	7	1,8-cineole	19.7
8	Aromadendren	5.45	8	beta oeimene	0.3
9	Camphor	4.74	9	Terpinene	0.9
10	delta-cadinene	4.12	10	Isomenthone	1.2
11	gamma-terpinene	1.94	11	Neomentho	6.9
12	Bornyl acetate	1.51	12	Neoisomenthol	0.4
13	Trans carveol	1.41	13	α-Terpineol	3.7
14	gamma- cadinene	1.32	14	Pulegone	25.9
15	Terpinolen	1.03	15	Piperitone	2.7
16	1-alpha-terpineol	0.9	16	Bornyl acetate	2.6
17	Cis carveol	0.35	17	Methyl acetate	1.1
18	Eugenol	0.32	18	Piperitenone	4.4
19	Carvacrol	0.32	19	Borneol	1.5
			20	E-Caryophyllene	3.2
			21	Spathulenol	3.6
			22	Terpinene	2.6
Total		91.31	Total		95.4

Ghaderi et al. (2012) shown that *A. wilhelmsii* had inhibitory effect on *Escherichia coli, Pseudomonas aeruginosa*, and *Bacillus cereus* and used GC/MS to separate and identify the compounds of essential oil that the results of the chemical compounds of the current study are consistent with the results of their study (22). In the study by *Aljancic* et al., it has been found that *A. wilhelmsii* prevents the growth of *Bacillus subtilis, C. albicans* and *A. niger in in-vivo* conditions. They have identified the flavonoids extracted from the essential oils as a factor of antibacterial effects (23).

Kermanshah et al. have studied the antibacterial effect of the hydroalcoholic extracts of common sage (*Salvia officinalis*) and *A. wilhelmsii* on cariogenic microorganisms and concluded that both essential oils have an inhibitory effect on the growth of S*treptococcus mutans, Lactobacillus rhamnosus* and *Actinomyces viscous* (24).

Khanaki et al. (2010) have identified the chemical compounds of *Z. clinopodioides by* GC/MS. The results of the current study are consistent with their results (25). Oussalah et al. (2006) have identified the main compounds of *Z. clinopodioides* essential oil. Pulegone is a ketone and of Monoterpene. Terpenes can damage the cell membrane and penetrate the lipid structure of the cellular wall of bacteria that this leads to protein denaturation and the collapse of the cell structure and leakage of cytoplasm and finally cell death (26). Previous studies indicated that the extracts of *Z. persica* and *Z. clinopodioides* could prevent the growth of broad range of gram-positive and gram-negative bacteria (27,28).

CONCLUSION

In general, *in-vitro* investigations showed that of the essential oils extracted from *Z. clinopodioides* and A. *wilhelmsii* have antimicrobial activity against the *S. aureus* resistant to antibiotics that would be considered as an alternative option in producing herbal medicines against the *Staphylococcus aureus* bacterium with minimal side effects. Since that they are non-toxic and natural components after performing further investigation on experimental animals.

ACKNOWLEDGMENTS

We would like to thank the Department of Microbiology, Islamic Azad University Marand and Tabriz for their laboratory equipment and technical support.

AUTHORS CONTRIBUTION

The authors had equal contributions.

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