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Antipseudomonal activity of *Artemisia quettensis* Podlech essential oil and its synergy with imipenem

[Actividad antipseudomonal del aceite esencial de *Artemisia quettensis* Podlech y su sinergia con imipenem]

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Abstract

Context: The problems associated with hospital infections caused by *Pseudomonas aeruginosa*, and the emergence of new and the re-emergence of old infectious diseases have become increasingly evident. Therefore, medicinal plants take precedence over the development of new antibacterial agents. The combination effects of antibiotics and plant compounds might be an appropriate solution for microbial resistance and useful method for assessment of synergistic interactions for inhibition of bacterial growth. This study is an experimental design for the discovery and finding of natural and harmless compounds for the treatment of infectious diseases.

Aims: To determine the antibacterial potency of *Artemisia quettensis* essential oil, and in combination with imipenem, to inhibit the growth of *Pseudomonas aeruginosa*.

Methods: The essential oil was obtained through hydrodistillation from aerial parts of the plant and analysis using GC and GC-MS. To demonstrate the *in vitro* antibacterial activity of the essential oil against *Pseudomonas aeruginosa* (ATCC 27853) disc diffusion assay was used, either alone or in combination with a standard antibiotic.

Results: The most dominant components were homoadamantane (9.38%), Camphor (7.91%) and Eugenol (10.46%). The oil and antibiotic showed high antibacterial activity against *Pseudomonas aeruginosa* with minimal inhibitory concentration (MIC) 0.5 µL/mL and 16 µg/mL and minimal bactericidal concentration (MBC) 4 µL/mL and 32 µL/mL, respectively. The synergistic effect of the oil and antibiotic showed MIC 0.2 µL/mL and 4 µg/mL and MBC 2 µL/mL and 8 µL/mL, respectively. This study showed that *Artemisia quettensis* oil has significant antibacterial activity against *Pseudomonas aeruginosa* infections.

Conclusions: The essential oil exhibited synergism with imipenem displaying the ability to enhance the activity of this compound and it may be useful in the fight against emerging microbial drug resistance.

Keywords: *Artemisia quettensis*; essential oil; imipenem; *Pseudomonas aeruginosa*; synergistic effect.

Resumen

Contexto: Los problemas asociados con las infecciones hospitalarias causadas por *Pseudomonas aeruginosa*, y la aparición de nuevas enfermedades y la reaparición de enfermedades infecciosas antiguas, se han hecho cada vez más evidentes. Por lo tanto, las plantas medicinales tienen prioridad para el desarrollo de nuevos agentes antibacterianos. Los efectos combinados de los antibióticos y los compuestos de plantas podrían ser una solución adecuada para la resistencia microbiana y un método útil para evaluar las interacciones sinérgicas para la inhibición del crecimiento bacteriano. Este estudio es un diseño experimental para la búsqueda y el descubrimiento de compuestos naturales e inofensivos para el tratamiento de enfermedades infecciosas.

Objetivos: Determinar la potencia antibacteriana del aceite esencial de *Artemisia quettensis*, y en combinación con imipenem, para inhibir el crecimiento de *Pseudomonas aeruginosa*.

Métodos: El aceite esencial se obtuvo mediante hidrodestilación de partes aéreas de la planta y análisis utilizando GC y GC-MS. Para demostrar la actividad antibacteriana *in vitro* del aceite esencial contra *Pseudomonas aeruginosa* (ATCC 27853) se utilizó el ensayo de difusión en disco, ya sea solo o en combinación con un antibiótico estándar.

Resultados: Los componentes más dominantes fueron homoadamantano (9,38%), alcanfor (7,91%) y eugenol (10,46%). El aceite y el antibiótico mostraron una alta actividad antibacteriana contra *Pseudomonas aeruginosa* con una concentración inhibitoria mínima (MIC) de 0,5 µL/mL y 16 µg/mL y una concentración bactericida mínima (MBC) de 4 µL/mL y 32 µL/mL, respectivamente. El efecto sinérgico del aceite y el antibiótico mostraron MIC 0,2 µL/mL y 4 µg/mL y MBC 2 µL/mL y 8 µL/mL, respectivamente. Este estudio demostró que el aceite de *Artemisia quettensis* tiene una actividad antibacteriana significativa contra las infecciones por *Pseudomonas aeruginosa*.

Conclusiones: El aceite esencial exhibió sinergismo con el imipenem, mostrando la capacidad de mejorar la actividad de este, y puede ser útil en la lucha contra la resistencia a los medicamentos microbianos emergentes.

Palabras Clave: aceite esencial; *Artemisia quettensis*; efecto sinérgico; imipenem; *Pseudomonas aeruginosa*.

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INTRODUCTION

Antibiotic resistance is the potential of the microbes to resist the effects of antibiotic drugs previously used to treat them. The spread of resistance to currently available antibiotics is a global concern (Lozniewski and Rabaud, 2010). With the spread of bacterial resistance to antibiotics, medicinal plants are important elements of traditional medicine in virtually all cultures.

Essential oils (EOs) are a very interesting group of secondary metabolites that are useful sources of antibacterial, antioxidants, anti-inflammatory, anticancer compounds for human diseases. Many studies have been published on the antibacterial activity of EOs proving a reduction in the bacterial resistance (Marasini et al., 2015). The *Artemisia* genus (*Asteraceae*) comprises about 500 species from South Asia, North America and European countries (100) and 34 species that are found wild all over Iran with the common Persian name of 'dermane' that provide valuable EOs notably for the pharmaceutical industry (Mozaffarian, 1998).

Artemisia quettensis Podlech belongs to the *Asteraceae* family and is narrowly distributed in the southern heights of Iran (Fig. 1).

Pseudomonas aeruginosa (PA) is recognized as one of the primary reason of infections in hospitals. The ability of this opportunistic human pathogen to acquire resistance to a broad range of antibiotics has made effective therapy more difficult (El-Shouny and Magaam, 2009).

The high level of antibiotic resistance in PA involves several mechanisms, including the overexpression of active efflux systems, the production of modifying enzymes, a decrease in outer membrane permeability and structural alterations of topoisomerases II and IV, involved in quinolone resistance (Strateva and Yordanov, 2009). Carbapenems such as meropenem and imipenem (IPM) are potent broad-spectrum antibacterial agents used to treat *Pseudomonas* infections. These antibiotics bind to critical penicillin binding proteins, and thereby disrupt the growth and structural integrity of the bacterial cell wall. However, the resistance of non-fermenting Gram-negative bacteria, including PA, to IPM and meropenem is increasing (Nicolau, 2008). Among many antibiotics against PA, IPM is considered the last option of treatment against serious infections caused by PA but IPM resistance is prevalent in many areas of the world and this problem has increased (Livermore, 2002; El-Shouny, 2006).

The use of natural products with therapeutic properties, for a long time was the main source of important therapeutic agents (Ishrat, 2011). Medicinal plants are considered a major source of new chemical compounds with curative effects (Blumenthal, 2000). They have a broad range of substances that can be used to treat infectious diseases because they are beneficial sources of antibacterial compounds such as alkaloids, flavonoids, terpenoids (Geissman, 1963). In this study was determine the antibacterial potency of *Artemisia quettensis* essential oil, in combination with imipenem, to inhibit the growth of *Pseudomonas aeruginosa*.

MATERIAL AND METHODS

Plant materials

Aerial parts of *Artemisia quettensis* Podlech (identified by the German botanist, Dietrich Podlech) were collected from Sistan & Baluchestan (N 28°36'13.9'', E 61°10'39.2'') in November 2011. The voucher specimen was prepared and deposited at the Herbarium and Botanical Lab, Research Center of Iranian Biological (national ID P1006608) Tehran, Iran.

Extraction and identification of antimicrobial activity of the *Artemisia quettensis*

The essential oil was carried out by hydrodistillation for 6 h using a Clevenger type apparatus (Clevenger, 1928). The oil (10 mg/mL) was obtained and stored in at 4°C in the dark vial and in the presence of anhydrous sodium sulphate. Different dilutions of essential oil (10, 20, 30, 40 and 50%) were prepared in a solvent containing DMSO (10%) and tween (80%).

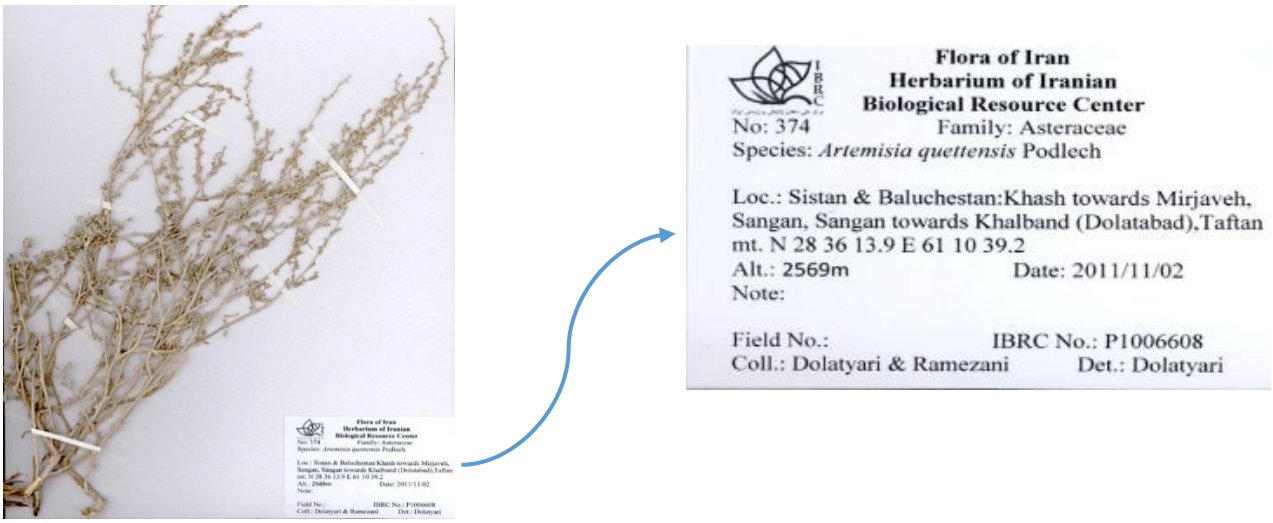

Figure 1. The photo of herbarium sample of *Artemisia quettensis.*

The analysis of the EO was performed with gas chromatography - mass spectroscopy (GC-MS). The GC apparatus was an Agilent technology HP 6980 system, with HP-5MS capillary column (60 m length; 0.25 mm I.D; 0.25 mm film thickness). Helium was used as the carrier gas at a flow rate of 1 mL/min. The oven temperature program was as follows: 1 min at 100°C, held for 1 minute, then heightened to 280°C at a rate of 5°C/minutes and held for 25 minutes. The chromatograph was equipped with a split/split less injector used in the split less mode. Relative pro- portion of each compound was expressed as percentage obtained by peak area normalization. Identification of components was assigned by comparison of their retention indices (RI) and mass spectra fragmentation with the National Institute of Standards and Technology (Adams, 2007).

Origin of *Pseudomonas aeruginosa*

The isolates of PA were obtained from pseudomonal infections and *Pseudomonas aeruginosa* ATCC 27853 were provided from the microbiology laboratory of Imam Khomeini Hospital (Fig. 2).

Antibiotics

The antibiotics–standard gentamicin (10 µg/mL), imipenem (10 µg/mL), ceftazidime (30 µg/mL), ciprofloxacin (5 µg/mL) were used. The Lister Laboratory has purchased antibiotic discs from the Bio-Analytical Company, all of which have the CE/IVD licenses and iso13485.

Antibiotic susceptibility testing of *Pseudomonas aeruginosa*

Disk diffusion method was used to determine the MIC and MBC values of oil. The Mueller– Hinton agar was poured in petri dishes and the paper discs were impregnated with 2 mL of EO and antibiotic were placed on the inoculated agar surface. The diameter of inhibition zone was measured. The larger the diameter of the area, the more sensitive the strain. To determine the minimum bactericidal concentration, all non-growing samples were cultured in a Muller Hinton Agar and incubated at 25°C for 24 hours. The lowest concentration of essential oil, which killed 99% of the bacteria, was reported as the minimum bactericidal concentration. The experiments were performed in three separate replications (Gradwohl et al., 1980).

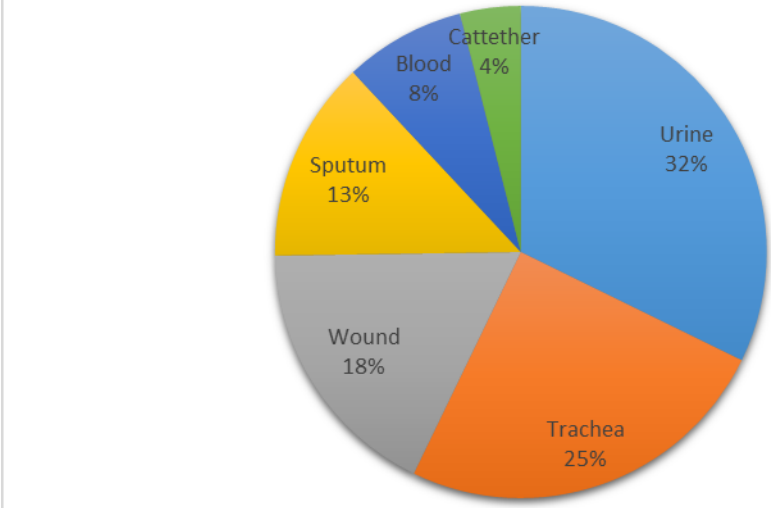

Figure 2. Frequency of *Pseudomonas aeruginosa* patterns infections.

The synergistic effect of the combination of the EO and antibiotics was assessed so that, 2 mL of EO was saturated to the antibiotic disc to determine the zones of inhibition. The obtained results were compared with those of the antibiotics tested on the same strains alone and by the same method (Moussaoui and Alaoui, 2016).

Statistical analysis

Data were analyzed using SPSS software (version 17), Kruskal-Wallis and Mann-Whitney tests. (for comparing the concentration)

Check board titer test

The checkerboard method was used to evaluate the antimicrobial interactions between *Artemisia quettensis* essential oil (AQEO) and IPM. Eight serial, twofold dilutions of AQEO and IPM was prepared and used in the MIC tests. From each dilution of oil, 100 µL were added to the wells of 96-well plates in vertical orientation and 100 µL of IPM dilutions was added in horizontal orientation. From each microbial suspension, 100 µL of (10^6 CFU/mL) were added to each well and incubated at 35°C for 24 h. Fractional inhibitory concentrations (FICs) were calculated as the MIC of the combination of *A. quettensis* oil and IPM divided by the MIC of oil or IPM alone. The results of this test were expressed as Fractional Inhibitory Concentration (FIC). The checkerboard test was used as the basis to calculate a FIC index (FICI) according to the equations [1-3]:

$$\text{FIC } A.\,quettensis \text{ oil} = \frac{\text{MIC } A.\,quettensis \text{ oil with imepenem}}{\text{MIC } A.\,quettensis \text{ oil}} \quad [1]$$

$$\text{FIC imepenem} = \frac{\text{MIC imepenem with } A.\,quettensis \text{ oil}}{\text{MIC imepenem}} \quad [2]$$

$$\text{FICI} = \text{FIC } A.\,quettensis \text{ oil} + \text{FIC imipenem} \quad [3]$$

After calculating the FICI, the interpretation of the results was carried out using the European Committee's guidelines for the antimicrobial susceptibility test (EUCAST, 2000).

Synergy (FICI ≤0.5), antagonism (FICI >4.0), and no interaction (FICI >0.5–4.0). (Wagner and Ulrich-Merzenich, 2009; Rosato et al., 2007).

RESULTS

Phytochemical composition

The chemical profile of the tested EO (Fig. 3) was performed by GC-MS. As shown in Table 1, fifteen compounds were identified. The major components were homoadamantane (9.38%), camphor (7.91%), and eugenol (7.46%) followed by geranyl acetate (6.93%), spathulenol (5.27%) and

1,8-cineole (4.14%). Although homoadamantane was the main compound found in AQEO, in addition to other constituents exhibiting relatively low proportion.

Antibiotic resistance pattern

The resistance pattern of the bacterial strains was studied by investigating their MIC using the disc diffusion test and the results achieved are reported in Table 2. The activity of IPM antibiotic fluctuated among the strains and most of them were not potent in therapeutic doses towards the tested bacteria. Among all tested MIC, only three strains numbered 5,11 and 16 were susceptible to antibiotic (Table 2). The MIC values of IPM against PA strain were varied from 2 to 64 µg/mL (Table 2).

Minimal Inhibitory Concentrations of essential oil

The results of the antibacterial activity of the EO are presented in Table 2. Twenty different strains of PA strains were used to evaluate the possible antipseudomonal activity of AQEO. The oil exhibited the antipseudomonal activity against all strains of PA with MIC values in the range of 0.5 to 64 µg/mL.

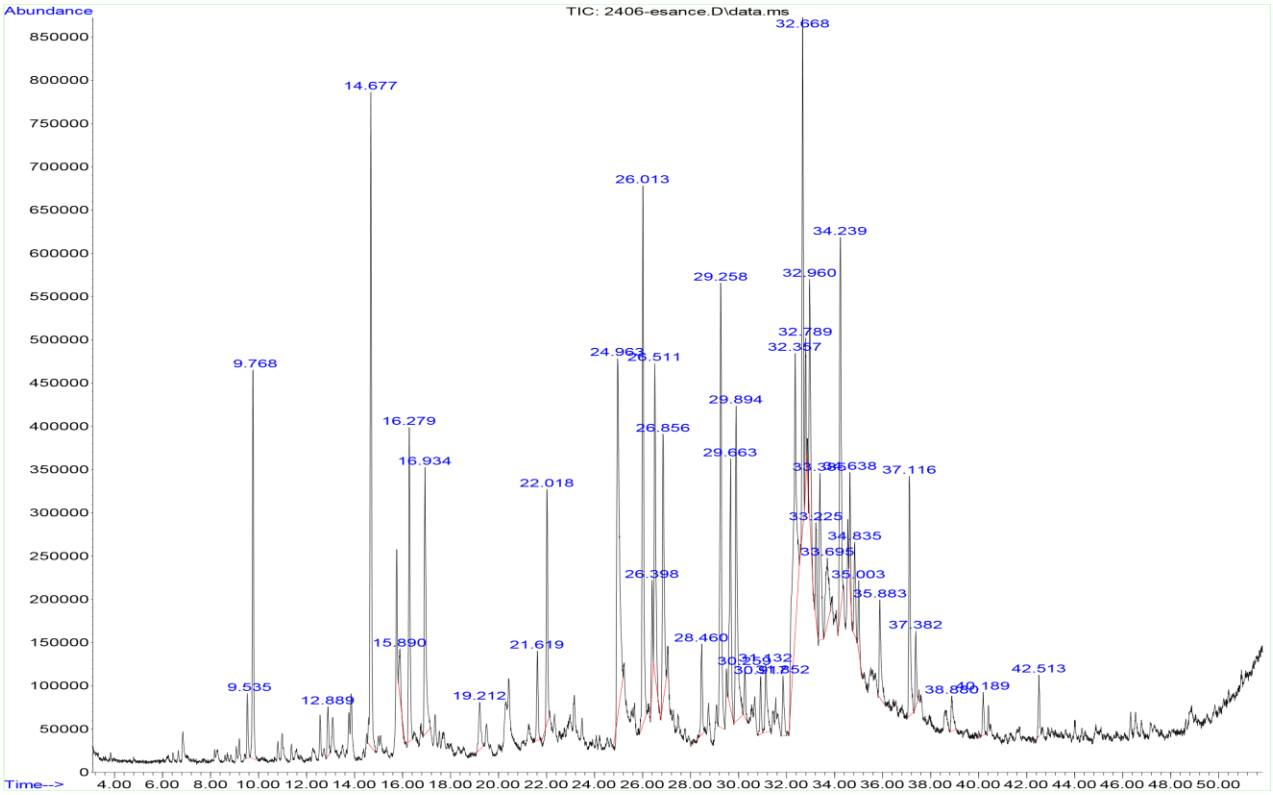

Figure 3. Chromatogram obtained by GC-MS analysis of *A. quettensis*.

Table 1. Main constituents of the essential oil isolated from aerial parts of *Artemisia quettensis* by GC-MS.

No	Retention Time (min)	Area%	Name	Quality	Retention Index
1	9.537	0.68	p-Cymene	95	1024
2	9.768	4.14	1,8-Cineole	99	1031
3	14.677	7.91	Camphor	98	1146
4	16.279	3.78	4-Terpineol	97	1177
5	16.931	3.89	alpha-Terpineol	91	1188
6	19.212	0.88	Pulegone	94	1237
7	21.62	1.09	l-Bornyl acetate	99	1288
8	22.021	2.54	Lavandulyl acetate	91	1290
9	24.963	7.46	Eugenol	98	1359
10	26.011	6.93	Geranyl acetate	91	1381
11	26.509	4.03	cis-Jasmone	98	1394
12	26.858	3.92	Methyleugenol	98	1403
13	29.256	9.38	Homoadamantane	90	Not found
14	30.915	0.67	delta-Cadinene	97	1523
15	32.666	5.27	Spathulenol	98	1576

The constituents of the essential oils were identified by comparing their retention times with available standards, RI (retention indices) values relative to those of C6–C30n-alkanes and their mass spectral fragmentation pattern with those reported in literature (Adams, 2001).

The synergy effect of essential oil and antibiotic

Considering the effect of synergism, which is the main purpose of our study, all infectious specimens had high sensitivity, so that in the concentration of one-fourth of in singular activity of EO and one-half when combined with IPM inhibited the growth of PA. In generally, the effect of EO and antibiotic combination could overcome resistant strains. The FIC of *Artemisia quettensis* and FIC of IPM and also FIC index and their antimicrobial interaction on PA strains are shown in Table 2. About 75% tested bacterial isolates were sensitive to this combination at MIC ranged from 0.25 to 64 µg/mL. Based on the results, the AQEO and IPM were studied synergistically on all pathogenic bacteria studied (Fig. 4).

Statistical analysis was performed using SPSS Version 20 Software. The diameter zones of inhibition of essential oil was measured at different concentrations (10, 20, 30, 40, and 50%). Based on the results of antibacterial activity of AQEO on tested bacteria, the diameter of the inhibition zone in positive control was higher than the diameter at 10% concentration of essential oil and negative control. By increasing the concentration of essential oil from 10 to 50%, the growth of microorganisms significantly decreased (Table 3).

DISCUSSION

In addition to slowing the treatment process, the spread of antibiotic-resistant strains jeopardizes the lives of patients who are contaminated with these resistant strains. In this study, the antibacterial effects of AQEO and its synergistic effect with IPM were evaluated and the results indicated that 0.5 µg/mL of AQEO could inhibit PA and when combined with the antibiotic it could decrease the MIC from 4 to 0.2 µg/mL and it can be considered a significant potent. Briefly, the antibacterial effect in synergism was more potent than that of IPM on the same bacteria.

Table 2. Antibacterial activity of *Artemisa quettensis*, imipenem and synergistic effect against *Pseudomonas aeruginosa* and Fractional Inhibitory Concentration (FIC) and FIC indices (FICI).

Strain number	MIC (µL/mL)		MIC (synergism) (µL/mL)		FIC		FICi
	Essential oil	Imipenem	Essential oil	Imipenem	Essential oi	Imipenem	Combination
ATCC278531	0/5	16	0/2	4	0.400	0.25	0.650 (A)
1 P	16	32	4	8	0.250	0.25	0.500 (S)
2 P	16	64	4	16	0.250	0.25	0.500 (S)
3 P	32	64	4	16	0.125	0.25	0.375 (NI)
4 P	32	64	4	16	0.125	0.25	0.375 (NI)
5 P	0/5	2	0/25	0/5	0.500	0.25	0.750 (A)
6 P	64	128	16	32	0.250	0.25	0.500 (S)
7 P	64	128	16	32	0.250	0.25	0.500 (S)
8 P	16	64	4	16	0.250	0.25	0.500 (S)
9 P	32	64	8	16	0.250	0.25	0.500 (S)
10 P	64	64	16	16	0.250	0.25	0.500 (S)
11 P	2	8	0/5	2	0.250	0.25	0.500 (S)
12 P	8	32	2	8	0.250	0.25	0.500 (S)
13 P	16	64	4	16	0.250	0.25	0.500 (S)
14 P	32	64	8	16	0.250	0.25	0.500 (S)
15 P	16	64	8	16	0.500	0.25	0.750 (A)
16 P	2	8	0/5	2	0.250	0.25	0.500 (S)
17 P	32	64	8	16	0.250	0.25	0.500 (S)
18 P	32	64	8	16	0.250	0.25	0.500 (S)
19 P	64	128	16	32	0.250	0.25	0.500 (S)
20 P	32	64	8	16	0.250	0.25	0.500 (S)

S: Synergy; A: Antagonism; NI: No interaction. FICI = FIC of *Artemisia quettensis* oil + FIC of imipenem.

Despite some information on the antibacterial activity of EO in this species (Bagheri Farahani et al., 2017; Ghanbar et al., 2017) and other plants on PA (Ahameethunisa and Hoppe, 2010; Tajehmiri et al., 2014; Al-Zubairi et al., 2017; Bajer et al., 2017), to our knowledge, this is the first report on the antipseudomonal activity of AQEO against PA using disc diffusion assay.

The results of (El-Hosseiny et al., 2014) study were similar to those of this study which *Thymus vulgaris* essential oil exerted synergistic effect with piperacillin, cefepime, meropenem on PA. In fact, the EO has been able to double the antibacterial activity of the antibiotic, but the current study demonstrated that EO in synergistic effect increased the antibacterial effect four times.

In the study by Désirée et al. (2013), antibacterial activity of the *Artemisia annua* essential oil revealed that it had antibacterial properties against most isolates tested. Inhibition zone diameters varied from 6 (*Pseudomonas aeruginosa* and *Shigella flexneri*) to 45 mm (*Vibrio cholerae*).

The AQEO used in this study has antibacterial activity against the tested strains with different diameters of inhibition zones from 1 to 22 mm.

Table 3. The antibacterial activities (zones of inhibition in mm) of essential oil of *Artemisia quettensis* and its synergistic effect with imipenem.

Microorganism	Gram strain	E1	E2	*Artemisia quettensis* essential oil concentration (%)					Control		Synergy effect
				10	20	30	40	50	Positive	Negative	
P. aeruginosa	+	12.3 ± 0.3	11.0 ± 0.0	7.8 ± 0.7	12.2 ± 2.3	14.3 ± 1.5	16.1 ± 1.1	16.6 ± 0.2	21.7 ± 0.4	0	27.0 ± 1.00

Values are represented as mean ± SEM (n=3). E1: ethanol (2:8 v/v); E2: ethyl acetate extract; positive control: gentamicin (10 µg); negative control: DMSO.

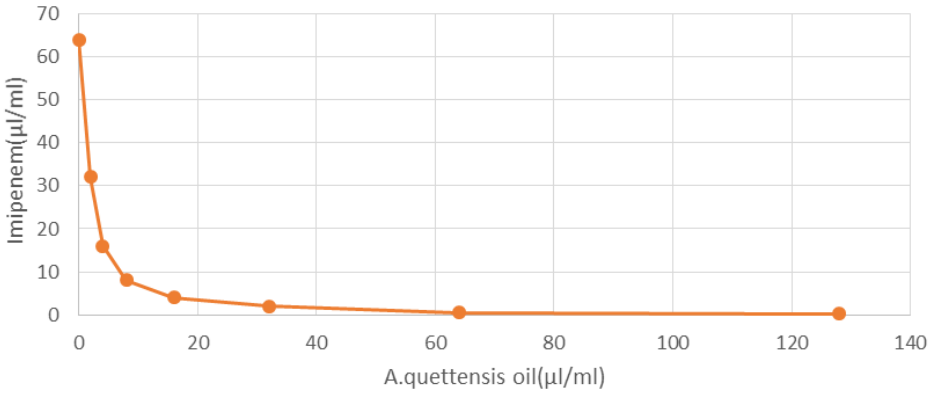

Figure 4. The isobole method describing synergy *Pseudomonas aeruginosa*. Isobole curve revealing the synergistic effect of *Artemisia quettensis* oil with imipenem against *Pseudomonas aeruginosa.*

A study on the synergistic effect of some EOs with the antibiotic showed that 544 µL/mL of *Carum copticum* essential oil displayed effect on PA growth and when combined with gentamicin, exerted no effect (Talei et al., 2017). In the present study, AQEO at 0.5 µL/mL could inhibit the growth of PA. This inconsistency in the findings can be due to the difference in the percentages of the chemical compounds among the EOs. Homoadamantane and camphor, as the main components of AQEO, seem to be responsible for the antipseudomonal effects of the oil, this oil is also equally or more effective when compared with standard antibiotics at a very low concentration.

Previously, many studies have indicated that PA resisted the action of the EO of *Thymus lanceolatus*, *C. coronarium*, *M. officinalis* (Felice et al., 2004; Carron, 2013; Khadir et al., 2013). This study revealed significant findings, which prove the efficacy of AQEO against PA.

Since the World Health Organization has rated multidrug-resistant (MDR) *Pseudomonas aeruginosa* as a critical threat to human health, many studies have been carried out in recent years on the resistance of PA to different antibiotics (Pandey and Singh, 2017).

Current results suggest that the potential use of this oil as pharmaceutical products can diminish harmful side effects and treatment costs of the synthetic drugs.

Limited studies had been carried out on the EO of this species. While researchers have identified antibacterial activities of the extract from AQEO, no synergistic study using IPM and AQEO on PA isolates has yet been published. Furthermore, it will be very important to investigate the synergistic behavior of natural products with IPM with the hope of enhancing their activity. The results of the synergistic action of oil with IPM demonstrating the potential use of AQEO to enhance IPM action.

Additional research is required to assess the practical value of the therapeutic applications.

CONCLUSIONS

From this study, we can see that *Artemsisa quettensis* Podlech showed antibacterial activity against different *Pseudomonas aeruginosa* strains, but at different levels. All the tested bacteria were more or less sensitive to standard antibiotic. According to the obtained results, the combination of essential oil of *A. quettensis* with imipenem antibiotic showed a synergistic effect against 15 tested bacteria (Table 1). Antagonistic effect was observed in three tested bacteria and two strain of bacteria exerted no interaction.

This study also showed that the utilization of this oil and antibiotic can lead to the inhibition of bacterial growth. Considering the combination effects of antibiotics and plant compounds, can prove microbial resistance. The solution to this world health issue is only feasible through further and more comprehensible investigations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Contribution	Saffari E	Khalili MNA	Mehrabadi JF
Concepts or ideas	x		
Design	x		x
Definition of intellectual content	x	x	
Literature search	x		
Experimental studies	x		x
Data acquisition	x	x	
Data analysis	x	x	
Statistical analysis		x	
Manuscript preparation	x		
Manuscript editing		x	x
Manuscript review	x	x	x

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