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Chemical composition and antimicrobial activity of *Achillea wilhelmsii* C. Koch essential oil against selected bacterial and fungal pathogens isolated fish

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ABSTRACT

Background & Aim: Increasing resistance of fish pathogens to chemical drugs, using herbal plants with antimicrobial properties is necessary. In this study, the chemical composition and antimicrobial activity against bacterial and fungal pathogens isolated from fish of the essential oil from the aerial parts of *Achillea wilhelmsii* C. Koch growing wild (Southwest of Iran) were studied.

Experimental: The aerial parts of *A. wilhelmsii* were tested against five bacterial strains including, *Streptococcus iniae*, *Yersinia ruckeri*, *Vibrio angularum*, *Aeromonas hydrophila* and *Pseudomonas aeruginosa* and four pathogenic fungi, *Saprolegnia* sp., *Fusarium solani*, *Candida albicans* and *Aspergillus flavus*. The oil constituents of *A. wilhelmsii* were analyzed by GC/MS.

Results & Discussion: The major composition of the essential oil of *A. wilhelmsii* were 1.8-cineole (25.2%), camphor (18.9%), linalool (6.9%), borneol (5.7%) and artemisia alcohol (4.3%). All microorganisms showed more sensitivity to essential oil of *A. wilhelmsii* than the control antibiotics. The highest antimicrobial activity were observed against *Y. ruckeri* (25.4 mm) and *C. albicans* (25 mm), respectively, while *S. iniae* (16.4 mm) and *A. flavus* (14 mm) exhibited the least sensitivity. In addition, MIC test indicated that minimum concentrations of the essential oil ranged from 125 to 800 µg/L were able to inhibit the growth of the selected bacterial and fungal pathogens.

Industrial and practical recommendations: Results indicated that the essential oil of *A. wilhelmsii* could be a potential source of new and effective antibacterial components in aquaculture industry. Although, more study needed to purify, fractional and characterize various antimicrobial compounds from the essential oil of *A. wilhelmsii.*

1. Introduction

Achillea is a perennial herb, belonging to the family of Asteraceae and comprises of 85 genera which seven species are endemic of Iran (Azadbakht *et al.*, 2003). *A. wilhelmsii* distributes in different parts of Iran especially Golestan, Mazandaran, Khorasan, Kerman, Fars, Isfahan, and Chaharmahal va bakhtiyari provinces (Rustayian *et al.*, 1998; Ghani *et al.*, 2008). *A. wilhelmsii* constitute of tannins, flavonoids, alkalonoids (Azadbakht *et al.*, 2003). There are several reports regarding the pharmacological properties of this genus such as anti-inflammatory, antispasmodic, antiallergic, antiulcer, antifungal, antimicrobial, and antioxidant effects (Asgary *et al.*, 2000; Ghorbani, 2005; Saeidnia *et al.*, 2011). *A. wilhelmsii* have been used for decreasing blood pressure and blood lipids, stimulating of humeral and cellular immunity and improve cardiac failure (Saeidnia *et al.*, 2011; Sharififar *et al.*, 2009). Different properties and uses of *A. wilhelmsii* is due to the different composition of essential oil and different pharmacological properties. Composition of the essential oil of *A. wilhelmsii* has been studied from different parts of Iran such as Fars (Rustayian *et al.*, 1998), Mazandran (Rustayian *et al.*, 1998), Isfahan (Ghani *et al.*, 2008), Khorasan (Jaimand & Rezaee, 2001), Sistan and Baluchestan (Shahraki & Ravandeh, 2012) and Kerman provinces (Rustayian *et al.*, 1998), but there are no reports on the composition of the essential oil of *A. wilhelmsii* growing wild in Zagros mountain (southwestern of Iran), which is an important geographical zone of medicinal plants. Since the compositions of the essential oils of these species are highly dependent on the growing location and also due to drug resistance in recent years (Saeidnia *et al.*, 2004), identifying of the major composition of the essential oil and survey antimicrobial and antifungal activity of *A. wilhelmsii* from Zagros mountain, southwestern of against common bacterial and fungal pathogens of fish is seemed necessary.

2. Materials and Methods

2.1. Plant Material

The aerial parts of *A. wilhelmsii* were collected from the Zagros mountain (in Chaharmahal VA bakhtiari province, Southwester Iran, altitude: 2000-2500 m asl; latitude: 30°-31°; longitude: 50°-51°, relative humidity: 22-45%, annual irrigation: 382-560 mm, temperature: 2.3-28.6°C), in May 2013 and the voucher specimens were deposited in the herbarium of Researches Centre of Medicinal Plants at Shahrekord University of Medical Sciences, Iran.

2.2. Essential oil preparation

The aerial parts of *A. wilhelmsii* were dried at room temperature (in a dark room), powdered and subjected to hydro-distillation using a Clevenger-type apparatus for 4 h according to the method recommended in the British Pharmacopoeia (Adams, 2001). Anhydrous sodium sulfate was used to dehydrate the essential oil. The oil was stored at 4 ºC until trials.

2.3. Gas chromatography/mass spectrometry (GC/MS) analysis

Analyses of the essential oil were performed using a Varian gas chromatography 3600 equipped with DB5 column (methyl phenyl siloxane, 30 mm × 0.25 mm i.d.) and flame ionization detector. The carrier gas was helium and split ratio was 1:15. The temperature program consisted of increasing the temperature from 60°C (for 2 min) to 240°C at a rate of 5°C/min. Temperatures of injector and detector were 250 and 260 ºC, respectively. GC-MS was performed on a cross-linked 5% methyl phenylsiloxane (HP-5, 30 m × 0.25 mm id, 0.25 µm film thickness). Carrier gas was helium, split ratio 1:15 with a quadruple mass spectrometer operating at 70ev ionization energy. The retention indices for all the components were calculated by using retention time of n-alkenes (C_8-C_{25}) that were injected after the essential oil under the same condition (Adams, 2001). The components were identified by comparing retention indices (RRI, DB-5) with those of standards and also with those reported in the literatures (Azadbakht *et al.*, 2003; Rustayian *et al.*, 1998; Ghani *et al.*, 2008; Asgary *et al.*, 2000; Ghorbani, 2005).

2.4. Preparation of bacterial and fungal strains

In vitro antibacterial activity of essential oil of *A. wilhelmsii* was examined against five bacterial strains, including *S. iniae* (LMG 14520), *Y. ruckeri* (KC291153), *V. angularum* (ATCC 22652), *A. hydrophila* (LMG 3770) and *P. aeruginosa* (ATCC 27853). These bacterial strains were obtained from the Persian Type Culture Collection, which were prepared as lyophilized stocks. *In vitro* antifungal activity was determined against four fungi comprising *Saprolegnia*

sp., *F. solani*, *C. albicans*, and *A. flavus*. Fungi strains were obtained from the Department of Aquatic Animal Health and Diseases, Research Organization of Caspian Sea.

2.5. Antimicrobial assay

The disc diffusion method as described by Goudarzi *et al*. (2011) was used to determine the growth inhibition effect of *A. wilhelmsii* essential oil on selected microbial collection. Bacterial suspensions with McFarland Standard 0.5% (equivalent to 1.5×10^7 cells/ml) were inoculated in Mueller-Hinton agar medium using sterile cotton swabs. For fungal studies PDA (potato dextrose agar) medium was dispensed in petri plates for different strains of fungi. Whatman No.1 filter paper discs with 4 mm diameter were impregnated with a known concentration of essential oil while sterile paper disc and standard antibiotic discs were applied as negative and positive controls, respectively. The impregnated discs along with the controls were kept on Agar plates which previously seeded with test bacterial and fungal cultures, separately. The bacterial plates were incubated for 24 h at 37˚C. The fungal plates (PDA) were incubated at 30 ˚C for 72-96 h to reveal any antimicrobial activity (Goudarzi *et al.*, 2011). All tests were performed in triplicate to confirm the reproducible results.

2.6. Minimum inhibitory concentration (MIC)

A. wilhelmsii antimicrobial activity was further subjected to test on minimum inhibitory concentration (MIC). The MIC is defined as the lowest concentration of essential oil of *A. wilhelmsii* at which the microorganism does not demonstrate visible growth. The essential oil of *A. wilhelmsii* was serially two-fold diluted with 100 µl with Mueller-Hinton broth (Difco Laboratories, Detroit, MI, USA) in order to determine the minimum concentration that can be used to inhibit the growth of microorganism. 50 µl of overnight inoculum was then added into each tube containing different concentrations of essential oil and incubated at 37°C for 24 hours (Hellio *et al.*, 2002). Growth inhibition was observed by visual inspection of the turbidity of the mixture. Determination of MFC for fungal collection was carried out following the method described by Hellio *et al*. (2002). Briefly, fungal suspension (2×10^6 CFU/ml) was placed in a liquid medium consisting of RPMI 1640 (with L-glutamine buffered to pH 7 with 0.165 MOPS buffer) and various known concentrations of the essential oil of *A. wilhelmsii* and/or ketoconazole (positive control). Fungal cultures were then incubated at 30 °C for 24h and MIC values were recorded.

2.7. Determination of antifungal activity

In vitro antifungal activity was determined against four fungi including *Saprolegnia* sp., *F. solani*, *C. albicans* and *A. flavus*. Fungi species were cultured on Sabouraud's dextrose agar (SDA) and incubated at 37 °C for 48 h. Several colonies of each fungi species were collected in 2 ml of sterile PBS to prepare a suspension. The suspension was adjusted to 70% transmittance by a spectrophotometer at 530 nm. This should result in a suspension containing about 1×10^7 cfu per ml. MIC was carried out according to elsewhere method (Sharif Rohani *et al.*, 2013), in summary, a serial dilution of *A. wilhelmsii* essential oil in dimethylsulfoxide (DMSO) was prepared in SDA tubes. The solvent (DMSO), at an appropriate dosage was also used as a negative control. A tube was considered as positive control without *A. wilhelmsii* essential oils and solvents. 20 ml of standardized suspension of different species of fungi were inoculated into each tube. (0, 20, 40, 80, 160, 200, 400, 800, and 1600 ppm). The tubes were incubated at 30˚C for 24 h to 5 days. The lowest *A. wilhelmsii* essential oil dosage at which tube was clear (no visible growth) defined as the minimal inhibitory dosage (MIC). For the determination of MFC, a portion of liquid (10 µl) from each clear tube was placed on SDA for further incubation at 37°C from 24 h to 5 days (Sharif Rohani *et al.*, 2013). The lowest dosage that yielded no growth after this sub-culturing was defined as the MFC, with 3 replicates for each experiment.

3. Results and discussion

The essential oil yield of *A. wilhelmsii* from the Zagros Mountain was 0.63%. GC-MS analyses of essential oil revealed 36 different compounds. The major composition of the essential oil were 1.8-cineole (25.2%), camphor (18.9%), linalool (6.9%), borneol (5.7%) and artemisia alcohol (4.3%) (Table. 1). Whereas, the main components of the species of the Mashhad province (northeastern of Iran) were camphor (19.06 %), 1,8-cineole (8.78 %), alpha-pinene (8.06%) and linalool (7.47%) (Ghani *et al.*, 2008). Isopentyl isovalerate (9.46%), alpha-pinene (8.75%), 1,8-cineole

(8.7%), 10-epi-g-eudesmol (5.65%) and spathulenol (4.94%) were the main identified compounds in *A. wilhelmsii* collected from Fars province, Iran (Ghani *et al.*, 2008), while camphor (27.99%), sabinyl acetate (6.56%), terpinene-4-ol (6.43%) camphene (6.43%) and alpha-pinene (5.47%) identified as the major components in the essential oil collected from Sistan and Balochestan province, southeastern of Iran (Shahraki & Ravandeh, 2012). In the present study, sabinene was considered as an important constituent of *A. wilhelmsii* of the Zagros Mountain, while this compound was not reported in essential oil of the studied plants from Fars and Sistan and Balochestan provinces. These variations may be attributed mainly to variation in their agroclimatic and geographical conditions, environmental and seasonal conditions, plant strain, age of plants, time of harvest, methods of drying and oil extracting and genetic differences (Esmaeili *et al.*, 2006; Javidnia *et al.*, 2004).

The *in vitro* antimicrobial activity of *A. wilhelmsii* essential oil against selected microbial collection is shown in Table 1 and 2. The maximum antibacterial activity was observed against *Y. ruckeri* with an average of 25.4 mm diameter of inhibition zone followed by *V. angularum* (24 mm), *A. hydrophila* (23 mm), *P. aeruginos*, (20.5 mm), and *S. iniae* (16.4 mm). The minimum antibacterial activity was observed against *S. iniae* (16.4 mm) (Table 2 and Fig. 1). In addition, the highest antifungal activity of essential oil of *A. wilhelmsii* was observed against *C. albicans* with 25 mm diameter of inhibition zones, followed by *F. solani* (19 mm), *Saprolegnia* sp. (17 mm) and the minimum antifungal activity was observed against *A. flavus* (14 mm) (Table 3 and Fig. 2). Amjad *et al.* (2011) tested the antibacterial activity of *A. wilhelmsii* essential oil against four pathogenic bacteria and reported high antibacterial activity against *S. aureus and Bacilus cereus*. In this study, no antibacterial activity was observed against *P. aeruginosa* which this result contrasts with the findings of other researchers. For example, in some studies low antibacterial activity of the aerial parts of *A. clavennae, A. holosericea* Sm., *A. lingulata* and *A. millefolium* was observed against bacterial strains including, *S. aureus, E. coli, K. pneumoniae, P. aeruginosa* and *Salmonella enteritidis* (Stojanovic *et al.*, 2005). In the current investigation, the results of MIC determination showed that the minimum concentration of the essential oil of *A. wilhelmsii* ranged from 125 to 800 µg/L was able to inhibit the growth of the bacterial and fungal pathogens. In the literature, essential oil of *A. wilhelmsii* inhibited the growth of *E. coli, P. aeruginosa, S. enteritidis, B. cereus* and *S. aureus* and the MIC values were in the same ranges as in the current work (Stojanovic *et al.*, 2005).

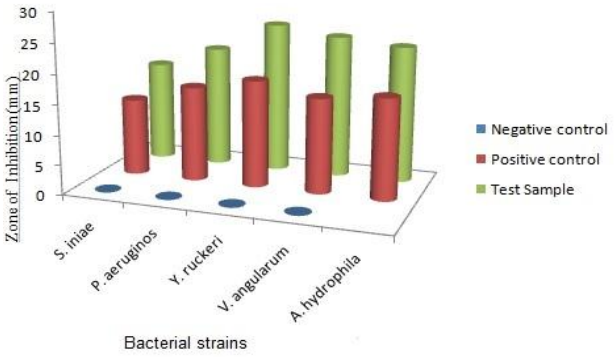

Fig 1. Antibacterial activity of *Achillea wilhelmsii*

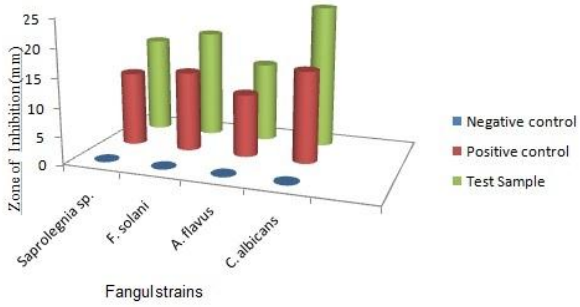

Fig 2. Antifungal activity of *Achillea wilhelmsii* essential oil

The dissimilarity in antimicrobial activity may be due to the variation in the chemical compounds that cause different anti-bacterial and anti-fungal effects. In a similar study, essential oil of *A. wilhelmsii* showed the maximum activity against *C. albicans* (12 mm) which was lower compared to our finding (Amjad *et al.*, 2012). In agreement with our findings, essential oils of *A. clavennae, A. holosericea, A. lingulata* and *A. millefolium* have shown antifungal activity against *A. niger* and *C. albicans*. The suggested antifungal and antibacterial activity of essential oil of *A. wilhelmsii* might be due to flavonoids and phenolic compounds (Mothana *et al.*, 2009; Nemeth, 2005; Yaghoubi *et al.*, 2007).

Table 1. Chemical composition (%) of the essential oils of aerial parts of *A. wilhelmsii*

Name of compounds	RI	Percentage
α-Pinene	939	2.06
Camphene	953	0.87
Sabinene	976	3.2
β- pinene	980	0.6
ρ-Cymene	1027	2.3
1,8-Cineole	1033	25.2
Cis-sabinene hydrate	1064	0.18
trans-Linalool oxide	1076	0.2
Artemisia alcohol	1083	4.3
Linalool	1098	6.7
α-Thujone	1112	0.8
Isopentyl isovalerate	1113	0.07
α-Campholenal	1123	0.23
trans-pinocarveol	1139	0.1
Camphor	1143	18.9
Pinocarveone	1161	1.1
Borneol	1165	5.7
n-Nonanol	1166	0.02
Terpinene-4-ol	1176	1.9
para-Cymen-8-ol	1180	1.2
Terpineneol	1185	2.2
Myrtenol	1194	0.8
α-Terpinolene	1201	1.84
Verbenone	1205	0.06
Fargano	1209	1.75
Cuminyl aldehyde	1235	0.8
Dihydrocarvone	1239	4.6
Thymol	1288	0.5
Bornyl acetate	1289	1.08
β- Selinene	1418	0.5
Isobornyl n-butanoate	1472	1.2
Pentyl benzoate	1475	0.1
γ-cadinene	1508	0.76
1,10-Decanediol	1547	0.06
Caryophyllene oxide	1577	2.9
Isospathulenol	1592	2.45
		97.29

Table 2. Antibacterial activity of essential oil of *Achillea wilhelmsii*

Bacterial Pathogens	Zone of Inhibition (mm)			MBC (µg/ml)	MIC (µg/ml)
	Test Sample	Positive control (Enrofloxacin)	Negative control		
S. iniae	16.4±0.5	13±0.1	-	0.5	1
P. aeruginos	20.5±0.3	16±0.9	-	0.06	0.65
Y. ruckeri	25.4±0.7	18±1.1	-	0.25	0.5
V. angularum	24±1.2	16±0.2	-	0.12	0.5
A. hydrophila	23.5±0.8	17±0.1	-	0.25	0. 5

Table 3. Antifungal activity of the essential oils of *A. wilhelmsii* against selective fungi

No	Fungal Pathogens	Zone of Inhibition (mm)			MIC (ppm)	MFC (ppm)
		Test Sample	Positive control (Ketoconazole)	Negative control		
1	*Saprolegnia* sp.	17± 0.82	13± 0.52	-	>200	400
2	*F. solani*	19± 1.2	14± 0.8	-	400	800
3	*A. flavus*	14± 0.82	11± 0.92	-	>800	1200
4	*C. albicans*	25± 1.2	10± 0.46	-	160	800

4. Conclusions

This research demonstrated the antimicrobial activities of essential oil of *A. wilhelmsii* against common fish pathogens. As a result, essential oil of this plant could be a potential source of new and effective antibacterial components. Further studies are needed to purify, fractionate and characterize various antimicrobial compounds from the essential oil of *A. Wilhelmsii* in different weather conditions in Iran.

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