Chemical composition and *in vitro* antimicrobial activity of some Iranian medical herbs against *Yersinia ruckeri*

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Abstract

Increasing bacterial resistance to commercial antibiotics has led to considering medicinal herb applications. This study aimed to identify chemical composition of essential oil of some native medical herbs and their antibacterial activity against *Yersinia ruckeri* compared with Enrofloxacin in *in vitro* experiments. The antibacterial activities of ethanolic extracts and essential oils of *Eryngium campestre, Pimpinella affinis, Mentha piperita, Achillea wilhelmsii* and *Cuminum cyminum* were analyzed by disk diffusion, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) methods in *in vitro*. Also, the oil constituents of the mentioned plants were analyzed by gas chromatography. The MIC value of the ethanolic extracts and essential oils were 31.2-500 µg/mL and 15.6-250 µg/mL, respectively, while the MBC of the mentioned extracts and essential oil were 62.4-500 µg/mL and 31.2-250 µg/mL, respectively. The results showed that the *C. cyminun*, *E. campestre* and *M. piperita* could be introduced as more effective antimicrobial candidates to aquaculture industry.

Keywords: Antibacterial activity, Chemical composition, Iranian medical herbs, *Yersinia ruckeri*

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Introduction

Yersiniosis is one of the most important bacterial infections in coldwater fish culture with significant mortalities and economical losses in the Iranian fish farms (Tobback *et al*., 2007). Yersiniosis is caused by pathogenic bacteria *Yersinia ruckeri* that has five O-serotypes (O1, O2, O5, O6 and O7), five outer membrane protein types (OMP types 1–5) and two biotypes 1 and 2 (Tobback *et al*., 2007). The bacteria is a gram negative, oxidase-negative, catalase-positive bacterium, belonging to Enterobacteriaceae. Yersiniosis is one of the most common diseases in Salmonids, especially in rainbow trout (*Oncorhynchus mykiss*) fingerlings (Tobback *et al*., 2007). Antibiotics resistance, results in increasing the medication dosage and drug side effects, which have led to considering other alternatives such as application of the medical herbs in order to minimize these problems (Alishahi *et al*., 2012). The antimicrobial activity of some Iranian medical herbs on some fish pathogens such as *Streptococcus iniae, Lactococcus garvieae, Aeromonas hydrophila and Yersinia ruckeri* have been studied by many researchers such as Ghasemi Pirbalouti *et al*., 2011; Roomiani *et al*., 2013; Akbary *et al*., 2014. Some researchers have examined antibacterial activity of *Zataria multiflora, Nigella sativa, Scutellaria multicaulis, Punica granatum, Olea europaea, Echinacea purpurea* (Alishahi *et al*., 2012) *Lavandula officinalis, Melissa officinalis, Ocimum basilicum, Origanum vulgare, Rosmarinus officinalis* and *Salvia officinalis* on *Yersinia ruckeri* in *in vitro* condition (Bulfon *et al*., 2014).

Eryngium campestre as a native plant in Mazandaran Province is an edible flowering plant belonging to the family Apiaceae (Nebija *et al*., 2009). Essential oil of *E. campestre* including phenylpropanoids, eugenol, methylisoeugenol and benzaldehyde with antibacterial and antioxidant activity have been used as a diuretic and against pertussis, urinary infections and renal calculus in traditional medicine (Nebija *et al.*, 2009).

Pimpinella affinis is another member of the family Apiaceae. This biennial herb grows up to 110 cm and is native in central and northern parts of Iran (Gulcin *et al*., 2003). In traditional medicine this herb is being used as carminative agent, appetizer, diuretic, antispasmodic drug, antimicrobial, sedative and lactation medication. It has also been distinguished as an antioxidant and antibacterial agent (Tabanca *et al*., 2007).

Mentha piperita (peppermint) is a perennial herb of the family Lamiaceae which is mainly used as antispasmodic, anti-inflammatory, antiemetic, carminative, anticancer, antibacterial and anti-fungal (Mahboubi and Haghi, 2008). The most important chemical compounds of peppermint are menthol, mentone and methyl acetate (Talpur, 2014). It has been proved that peppermint could improve the growth

and immunity of warm-blooded animals and fish (Talpur, 2014).

Achillea wilhelmsii is a flowering plant in the family Asteraceae. 85 species of the genus have been identified and 7 species are exclusively native in Iran (Javidnia *et al*., 2004). Flowers of this plant contain chamazulene cantle and burnetol, that have anti-inflammatory, antispasmodic, antimicrobial and antiparasitic effects (Javidnia *et al*., 2004). The tips of the flowered branches contain flavonoids and sesquiterpenes which have noticeable antibacterial effect on gram positive bacteria such as *Staphylococcus aureus* and *Bacillus cereus* (Amjad *et al*., 2011).

Cuminum cyminum (cumin) is a flowering plant belonging to the family Apiaceae is an aromatic species, native to many regions of Iran including Kerman, Semnan, Yazd and Mazandaran Provinces. It is used as an additive and spice in food industry. Moreover, cumin is utilized in modern and traditional medicine as a carminative and antimicrobial agent (Rafiee Pour *et al*., 2014) as well as treating indigestion problems. Antibacterial effects of *C. cyminum* on some common fish pathogens such as *L. garvieae* and *S. iniae* have been proven (Rafiee Pour *et al*., 2014; Roomiani, 2013). This study was conducted in order to identify the chemical compounds of essential oil of native herbs and their antibacterial effects on *Y. ruckeri* compared with Enrofloxacin in *in vitro* condition.

Materials and methods

Plant extractions

All five plant species were collected from their natural habitats (Table 1) and their identification were confirmed according to standard methods by Shahrekord University botany section (Table 1). 100 g of each plant were dried in darkroom, exposed to air and then were ground into fine powder by a grinder. Acquired powders were mixed in a 1 L volumetric flask by 1:5 proportion with 80% ethanol for 48 h by using a shaker. The mixture then was filtered by Büchner funnel and filter paper. Primary extract were distilled in rotary distillation in 80ºC for 4 h. The remaining dense extractions were stored at 4 ºC until to use. Essential oils were extracted by a Clevenger device and then filtered using sterilized filter (0.4 µm) and stored at 4 ºC (Sivam, 2001).

Examination of herbs essential oil composition

The essential oil composition was analyzed by using a gas chromatograph-mass spectrometry (GC-MS) in central laboratory of Sari University. The following conditions were set in order to acquire data: initial temperature 50°C; program rate 3°C; final temperature 300°C and injector temperature 290°C.

Table 1: Geographical location and environmental conditions of used medicinal herbs.

No.	Plant	Region	Altitude (m)	Latitude	Longitude
1	*Eryngium campestre*	Mazandaran Province	132	36°(N)	36°4’(E)
2	*Pimpinella affinis*	Mazandaran Province	132	36°(N)	36°4’(E)
3	*Cumminum cyminum*	Khorasan Province	1444	36°20’(N)	59°35’(E)
4	*Achillea wilhelmsii*	Chaharmahal va bakhtiary Province	2080	32°39’(N)	51°43’(E)
5	*Mentha piperita*	Yazd Province	1230	31°41’(N)	53°49’(E)

The carrier gas was helium and the split ratio was 0.8 mL/min. For GC–MS detection, an electron ionization system with ionization energy of 70 eV was used (Roomiani *et al*., 2013).

Antibacterial activity assessment of herbal extracts and essential oils

Evaluation of antimicrobial activities of the extracts and essential oils were conducted by applying disc diffusion method. Briefly, Mueller Hinton agar plates were inoculated with a *Y. ruckeri* (KC291153) at a density of 10^6 cells/mL by using sterile swabs. Then, 15 µL of crude extracts of *E. campestre, P. affinis, C. cyminum, A. wilhelmsii* and *M. piperita* were added to the sterile blank filter disks (5 mm in diameter) prior to placing disks on Mueller Hinton agar plates. Enrofloxacin disk (10 µg) and 4% DMSO disk were used as positive and negative controls, respectively. The plates were incubated at 25°C for 48 h and the antimicrobial activity was examined by measuring the diameter of the zone (mm) surrounding the paper discs (Turker *et al*., 2009). Three replicate discs were prepared for each extract and essential oil in this study.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Serial dilution assay were used for determination of MIC and MBC. First, serial dilutions of essential oils and extracts were poured into tubes containing 5 mL tryptic soy broth (TSB) and 4% DMSO, then bacterial suspension (1.5×10^6 CFU/mL) from 48 h of culture were dispensed and incubated at 25°C for 24 h. In this study, negative control was determined as a mixture without extracts and essential oils, while in the positive control, bacteria were excluded from the mixture. After incubation the MIC was determined by the lowest concentration of the essential oil at which the microorganism did not demonstrate visible growth. 10 µL of MIC and higher concentrations were reinoculating to each blood agar plates and incubated for 24 h at 25 °C. The MBC was defined as the lowest concentration of the essential oil at

which incubated microorganisms are completely killed (Roomiani, 2013).

Statistical analysis

The data were subjected to statistical analysis using the SPSS (software version no. 18). Differences between extracts and essential oil were tested by one-way analysis of variance (ANOVA). Probability value for the statistical test was 0.05%. Also, Duncan test were used in order to compare the differences of the inhibition zones between control group with plants extracts or essential oils (Turker *et al*., 2009).

Results

Chemical composition of essential oils

Chemical compositions of the essential oils are presented in Tables 2-6. Table 2 shows that the relative quantitative values of *E. campestre*. *E .campestre* had 20 different components, and the most important constituent was Bornyl acetate (17.9%). GC-MS analyses of *P. affinis* essential oil revealed 19 different compounds. Pregeijene was the main component (Table 3). The yield of the essential oil of *C. cyminum* was summarized in Table 4. Based on the GC–MS analysis 32 components were identified. The most significant compound was α-Pinene (29.1%). Other important compounds were Limonene (21.5%), 1, 8-Cineole (17.9%) and Linalool (10.4%) The essential oils from aerial parts of *A. wilhelmsii,* contained 36 different chemical compounds (Table 5). 1, 8-cineol was the most dominant component in the GC-MS analyses of *A. wilhelmsii*. Table 6 shows that the Menthol was the most abundant component (48.52%) in *M. piperta* followed by Neomenthyl acetate (15.13%) and Menthofuran (11.18%).

Antibacterial activity

According to the results, essential oil and extracts of *M. piperita, E. campestre* and *C. cyminum* had significantly higher inhibition zones than Enrofloxacin ($p<0.05$), but no significant deference was observed between inhibition zones of *P. affinis* and *A. wilhelmsii* to Enrofloxacin ($p>0.05$). Also, Negative control (DMSO 4%) could not inhibit bacterial growth. Results of this study revealed that MIC value of examined extracts and essential oils were 31.2-500 µg/ml (Table 7) 15.6-250 µg/mL respectively (Table 8), while MIC and MBC quantities for Enrofloxacin activity against *Y. ruckeri* were 100 µg/mL and 150 µg/mL, respectively.

Table 2: The profile of chemical composition of *Eryngium campestre* essential oil

Compounds	RI	Percentage
β-Ylangene	1420	0.9
Bornyl acetate	1274	17.9
Terpinen-4-ol	1164	8.7
Camphene	952	5.2
β-Pinene	978	1.3
Myrcene	991	0.2
Terpinolene	1082	0.2
γ-Terpinene	1051	0.2
α-Terpineol	1172	0.9
2,3,6-Trimethylbenzaldehyde	1293	1.4
α-Copaene	1378	0.9
β-Elemene	1389	1.2
α-Gurjunene	1412	0.1
Bicyclogermacrene	1492	1.8
Carotol	1594	0.4
γ-Muurolene	1474	1.1
Trans-Pinocarveol	1128	0.3
Linalool	1085	0.1
Limonene	1025	11.6
Neo-3-Thujanol	1148	0.05

Table 3: The profile of chemical composition of *Pimpinella affinis* essential oil

Compounds	RI	Percentage
cis-β-Ocimene	1038	1.9
-Pineneα	939	0.9
Trans-β-Ocimene	1042	3.96
Linalool	1082	0.2
3-Octanore	986	0.3
Myrcene	991	0.6
Geijerene	1145	15.7
Decanal	1192	3.6
β-Cubebene	1382	0.5
Terpiene	1018	0.4
Delta elemene	1342	0.4
Limonene	1031	11.28
Valencene	1490	1.3
Methyl cinnamate	1342	3.18
Germacrene D	1482	12.8
Trans-dictamnol	1425	1.8
Longipinanole	1565	0.3
Pregeijene	1285	27.3
Methyleeugenol	1403	1.9

Table 4: The profile of the chemical composition of *Cumminum cyminum* essential oil

Compounds	RI	Percentage
Isobutyl isoutyrate	892	0.8
α-Thujene	922	0.3
α-Pinene	931	29.1
Sabinene	971	0.6
Myrcene	981	0.2
δ-3-Carene	998	0.2
ρ-Cymene	1013	0.3
Limonene	1025	21.5
1,8-Cineole	1028	17.9
(E)-Ocimene	1038	0.1
γ-Terpinene	1051	0.6
Terpinolene	1082	0.3
Linalool	1089	10.4
α-Campholenal	1122	0.03
trans-Pinocarveole	1130	0.07
δ-Terpineole	1154	0.09
Terpinene-4-ol	1169	0.5
α-Terpineole	1180	3.17
trans-Carveole	1213	0.4
cis-Carveole	1217	0.07
Geraniol	1242	1.1
Linalyl acetate	1248	4.8
Methyl geranate	1310	0.2
α-Terpinyl acetate	1342	1.3
Neryl acetate	1351	0.09
Methyl eugenol	1369	1.6
β-Caryophyllene	1430	0.2
α-Humulene	1463	0.2
Spathulenol	1562	0.07
Caryophylleneb epoxide	1586	0.1
Humulene epoxide II	1608	0.08
Acetocyclohexanedione (2)	1704	0.4

Table 5: The profile of the chemical composition of *Achillea wilhelmsii* essential oil

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

Table 6: The profile of the chemical composition of *Mentha piperita* essential oil

Compounds	RI	Percentage
α-Pinene	939	0.31
Sabinene	975	0.26
β-pinene	979	0.58
1,8 Cineole	1031	6.69
Cis-Sabinene hydrate	1152	2.56
Menthone	998	0.23
Menthofuran	1164	11.18
Neomenthol	1165	2.79
Menthol	1171	48.52
Neomenthyl acetate	1295	15.13
Menthyl acetate	1051	0.52
Isomenthyl acetate	1305	0.61
β-Bourbonene	1089	10.34
(z)-Caryophyllene	1408	2.09
E-β-farnesene	1456	0.36
Germacrene D	1485	2.1
Bicyclogermacrene	1500	0.22
Caryophyllene oxide	1575	0.16
Linalool	1087	0.36
Pulegone	1235	4.83
Piperitone	1227	0.39
3 Octanol	978	0.08

Table 7: Antibacterial activity of the extract of some Iranian medicinal herbs on *Yersinia ruckeri*

Plant	Zone of Inhibition (mm)			MIC (µg/mL)	MBC (µg/mL)
	Test Sample	Positive control (Enrofloxacin)	Negative control		
Eryngium campestre	20.4±0.9[a]	16.8±0.1[b]	-	62.4	250
Pimpinella affinis	18.5±0.6[a]	16.9±0.9[a]	-	250	500
Cumminum cyminum	23.4±1.2[a]	17.1±1.1[b]	-	31.2	62.4
Achillea wilhelmsii	17.8±0.6[a]	16.9±0.2[a]	-	>250	500
Mentha piperita	19.8±0.8[a]	16.8±0.1[b]	-	250	250

*Values in each row with different superscripts show significant difference ($p< 0.05$).

Table 8: Antibacterial activity of the essential oils from some Iranian medicinal herbs on *Yersinia ruckeri*

Plant	Zone of Inhibition (mm)			MIC (µg/mL)	MBC (µg/mL)
	Test Sample	Positive control (Enrofloxacin)	Negative control		
Eryngium campestre	24.8±0.9[a]	17.2±0.8[b]	-	31.2	62.4
Pimpinella affinis	19.7±0.6[a]	17.3±0.7[a]	-	124	250
Cumminum cyminum	27.3±1.2[a]	17.0±0.6[b]	-	15.6	31.2
Achillea wilhelmsii	18.9±0.6[a]	16.8±0.4[a]	-	>124	250
Mentha piperita	21.6±0.9[a]	17.1±0.6[b]	-	62.4	124.8

*Values in each row with different superscripts show significant difference ($p< 0.05$).

Discussion

Yersiniosis is the second bacterial disease in the coldwater fish farms in Iran and 15 of epidemics outbreaks of this bacterial disease were reported in the country during 2012-2013 (Zorriehzahra *et al*., 2012). One of the most significant challenges of aquaculture industry was drug resistance that is mainly caused by drug over use, self-treatment and unskilled prescriptions. Drug resistance increases the rate of mortality and costs more healthcare expenses. Failure in chronic diseases treatment, antibiotics side effects and increasing bacterial resistance have led researchers to consider herbal extracts and essential oil effects on the aquaculture industry diseases, mainly because of their effectiveness and low side effects (Turker *et al*., 2009). Antibacterial activity of plants is primarily due to phenol, saponin, tannin and flavonoid

compounds that affect plasma membrane by inhibiting its enzymes (Ghasemi Pirbalouti *et al*., 2011).

Studies on the effects of medicinal herbs extracts as antibacterial agents on *Y. ruckeri* are scant in Iran. Antibacterial activity of *P. granatum*, *N. sativa* and *Z. multiflora* extracts on *Y. ruckeri* have been successfully examined by Alishahi *et al*. (2012), in which the that diameter of inhibitory zone were 22, 20 and 16 mm, respectively.

In the present study, most of the extracts and essential oils had significantly higher inhibiting activity compared to the similar studies on *Y. ruckeri*. The essential oil acquired from *C. cyminum* had the most inhibitory activity with a diameter zone of 27.3±1.2 mm. The effectiveness of *C. cyminum* on other fish pathogens such as *Lactococcus garvieae* and *Streptococcus iniae* have been approved as well (Roomiani, 2013; Rafiee Pour *et al*., 2014). Moreover, Hajlaoui *et al*. (2010) evaluated the antimicrobial activity of *C. cyminum* on the Vibrio species and they found it had the most effect against *Vibrio cholera* with an inhibitory zone of 23±1 mm in diameter. The high effectiveness of *C. cyminum* could be due to α-Pinene, Limonene and 1, 8-Cineole compounds which could increase plasma membrane permeability and cell rupture.

In current study analyses of essential oil from the aerial parts of *E. campestre* ,revealed high concentrations of Limonene and Bornyl acetate. The antibacterial activity of this species might be due to these components (Nebija *et al*., 2009). The MIC quantities of *E. campestre* essential oil and extracts on *Y. ruckeri* were 31.2 µg/mL and 62.4 µg/mL respectively, while quantities from *E. bungei* against *Streptococcus pyogenes* and *S. agalactiae* were 12.5 µg/mL and 50 µg/mL (Alipour and Khanmohammadi, 2011). In another study, MIC concentrations of *E. caucaseum* on *S. pyogenes* and *S. sanguinis* were determined as 50 µg/mL (Thiem *et al*., 2010). Thiem *et al*. (2010) reported the MIC of *E. campestre* on *Bacillus subtilis* and *Staphylococcus aureus* were 1900 µg/mL and 15000 µg/mL, respectively. The diversity in the MIC quantities from genus *Eryngium* was probably related to different chemical composition of essential oil correlated with species, geographical range, plant age, seasonal patterns, desiccation and extraction methods, genetic polymorphisms and the difference between studied bacterial strains (Ghasemi Pirbalouti *et al*., 2011).

In this study, *M. piperita* revealed less bactericide effects on *Y.ruckeri* in contrast to *C. cyminun* and *E. campestre.* Nevertheless, the diameter inhibition zone of the extract and essential oil were 19.8±0.8 and 21.6 ±0.9 showing more satisfactory results than Enrofloxacin (p<0.05). Talpur (2014) showed that the different concentrations of *M. piperita* caused to increase its resistance against *Vibrio harveyi*. Meany reports have approved

the inhibitory activity of *M. piperita* on several bacteria such as *E. coli*, *Staphylococcus*, *Pseudomonas*, *Salmonella*, *Streptobacillus*, *Listeria monocytogenes* and *Xanthomonas* (Iscan *et al*., 2002; Saeed and Tariq, 2005). The results mentioned above, confirmed that gram positive bacteria were more sensitive than gram negative bacteria to *M. piperita* (Iscan *et al*., 2002). Antibacterial activity of this species is mainly related to pulegone, isomenthone, carvone, piperitone and dehydrocarvone compounds (Tassou *et al*., 2000).

In the current study, *P. affinis* was more effective on *Y. ruckeri* compared to *A. wilhelmsii*. However no significant difference was observed between *P. affinis* essential oil and Enrofloxacin. The extract of *P. affinis* was assessed on ten different bacteria species and it had acceptable effects just on *Staphylococcus aureus* and *E. coli* (Verdian-Rizi, 2008). In another similar study, the antibacterial activity of *Pimpinella anisum* L extract on several micro-organisms such as *S. aureus*, *E. coli*, *Salmonella typhi* were evaluated, but no inhibitory effect on bacterial growth was observed (Akhtar *et al*., 2008).

Among all these herbs, *A. wilhelmsii* showed the lowest effect on *Y. ruckeri* and the MIC of its extract and essential oil were >250 µg/mL. This result was similar to the results of Bulfon *et al*. (2014), who showed that *A. millefolium* had lower effects on *Y. ruckeri*, *Photobacterium damselae* subsp. *piscicida*, and *L. garvieae* in *in vitro* condition compared with the control group (Oxytetracycline) and the MIC value for *Y. ruckeri* was 33.6 mg/ml. In another study on *S. aureus* these quantities for *Achillea santolina* were >0.573 mg/mMol and on *E. coli* were >1.146 mg/mMol (Ahmadi *et al.*, 2011). The antibacterial activity of *A. Wilhelmsii* essential oil might be due to flavonoids and phenolic compounds. In conclusion, this study approved the good antibacterial activity of *C. cyminun, E. campestre* and *M. piperita* on *Y. ruckeri* in *in vitro* condition. We suggest that more studies should be done *in vivo* condition (fish farms), in order to determine the effective dosage, safety and toxicity of these medicinal plants prior to introducing any of them as new antibacterial medication for the treatment and controlling the mortality caused by *Y. ruckeri* in fish farms.

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