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RESEARCH ARTICLE **ARAŞTIRMA MAKALESİ**

Antibacterial activity of some essential oils againts *Vagococcus salmoninarum*

Vagococcus salmoninarum'a karşı bazı uçucu yağların antibakteriyel aktivitesi

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Abstract: In this study, essential oils of thyme (*Origanum vulgare*), St. John's Wort (*Hypericum perforatum*), rosemary (*Rosmarinus officinalis*), ginger (*Zingiber officinale*), clove (*Eugenia caryophyllata*), peppermint *(Menta piperita*), lavandula (*Lavandula hybrida*) and black cumin (*Nigella sativa*) were screened for its chemical composition and *in vitro* antibacterial activity against *Vagococcus salmoninarum*. The composition of oils were analysed using GC/MS. Antibacterial effects of essential oils against *V. salmoninarum* were detected by agar diffusion and tube dilution assays. Thyme (125 µL ml^{-1} MIC) and clove (500 µL ml^{-1} MIC) essential oils were shown to possess strong antibacterial activity against *V. salmoninarum*. The main components of effective essential oils were carvacrol (63.57 %) and eugenol (85.99 %), respectively. As a result, these two plant species that tested against *V. salmoninarum* which is causative agent of vagococcosis can be used as an antibacterial agent. For this purpose, *in vivo* antibacterial activities against vagococcosis of thyme and clove essential oils should be explored in the further studies.

Keywords: Antibacterial activity, *Vagococcus salmoninarum*, essential oil, agar diffusion, minimal inhibitory concentration (MIC)

Öz: Bu çalışmada, kekik (*Origanum vulgare*), sarı kantaron (*Hypericum perforatum*), biberiye (*Rosmarinus officinalis*), zencefil (*Zingiber officinale*), karanfil (*Eugenia caryophyllata*), nane (*Menta piperita*), lavanta (*Lavandula hybrida*) ve çörek otu (*Nigella sativa*)'dan elde edilen uçucu yağların kimyasal bileşimleri ve *Vagococcus salmoninarum*'a karşı *in vitro* antibakteriyal etkileri incelenmiştir. Uçucu yağların kimyasal kompozisyonu Gaz Kromatografisi/Kütle Spektrometresi (GC/MS) ile analizlenmiştir. *Vagococcus salmoninarum*'a karşı uçucu yağların antibakteriyel etkileri agar difüzyon ve tüp dilüsyon yöntemi ile belirlenmiştir. Kekik (125 µL/ml MİK) ve karanfil (500 µL/ml MİK) uçucu yağlarının *V. salmoninarum*'a karşı güçlü antibakteriyel etkinliğe sahip olduğu görülmüştür. Etki gösteren uçucu yağlarının ana bileşenleri sırasıyla karvakrol (% 63,57) ve eugenol (% 85,99) olarak tespit edilmiştir. Sonuç olarak, vagokokkozis hastalığı etkeni *V. salmoninarum*'a karşı test edilen iki bitki türünün de antibakteriyel ajan olarak kullanılabileceği düşünülmektedir. Bu amaçla ilerki çalışmalarda, kekik ve karanfil uçucu yağlarının vagokokkozise karşı *in vivo* antibakteriyel etkinlikleri araştırılmalıdır.

Anahtar kelimeler: Antibakteriyal aktivite, *Vagococcus salmoninarum*, uçucu yağ, agar difüzyon, minimum inhibitör konsantrasyon (MİK)

INTRODUCTION

Generically known as streptococcosis, gram-positive coccal infections are now considered as one of the main problems to aquaculture production (Ghittino et al., 2003). Vagococcosis, also called cold water streptococcosis, is pathogenic to salmonid fish at water temperatures below 12 °C, and it is less widespread in aquaculture than other streptococcal infections (Eldar and Ghittino, 1999). Vagococcosis caused by *Vagococcus salmoninarum* is an important bacterial disease in the European rainbow trout industry in sub adult or adult fish, with mortality rates of 20–50%. Outbreaks in broodstocks are usually associated with post-spawning stres (Michel et al., 1997; Ghittino et al., 1999; Ruiz Zarzuela et al., 2005; Didinen et al., 2011).

V. salmoninarum has been reported in rainbow trout (*Oncorhynchus mykiss*) in many European countries and in Australia (Tasmania) (Schmidtke and Carson 1994; Michel et al., 1997; Ghittino et al., 1999, 2004; Ruiz-Zarzuela et al., 2005; Salogni et al., 2007) and in Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) in Norway (Schmidtke and Carson 1994). The disease has been also recorded in rainbow trout broodstocks in Turkey (Didinen et al., 2011; Tanrıkul et al., 2014).

Antibiotic treatment of vagococcosis in the field has not been effective, although *V. salmoninarum* isolates are susceptible to several drugs such as ampicillin, amoxycillin, erythromycin, oxytetracycline and doxycycline tested *in vitro* (Michel et al., 1997; Ruiz Zarzuela et al., 2005; Didinen et al., 2011). Treatments with antibiotics were effective only for short periods (5-7 days) and continuous application was necessary to reduce mortality.

High amounts of antibiotics are used for protection against bacterial fish diseases in the aquaculture sector in Turkey. The heavy use of antibiotics leads to the presence of its residues in fish. In addition, these antibiotic applications cause emergence of resistance in fish pathogens, including zoonotic fish bacteria. Zoonotic fish bacteria that develop resistance to antibiotics are more dangerous to humans and can cause infections that are difficult to treat (Avsever et al.,2010). Thus, it is necessary to determine and develop effective alternative protection methods against bacterial fish diseases. The use of herbal products to control of fish pathogens in aquaculture is an current practice. One of the herbal products is essential oils, which are natural components from plants that are generally recognized as safe substances. Due to their antimicrobial properties, these oils may constitute alternative prophylactic and therapeutic agents in aquaculture (Romero et al., 2012).

Phytochemicals such as phenolics, polysaccharides, proteoglycans and flavonoids may play a role in preventing or controlling infectious pathogens (Citarasu, 2010). Many essential oils and plant extracts have been shown to be effective against fish pathogens (Abutbul et al., 2005; Bansemir et al., 2006; Ekici et al., 2011; Haniffa and Kavitha, 2012; Al Laham and Al Fadel, 2014; Öntaş et al., 2016; Diler et al., 2017a,b; Metin et al., 2017). These compounds may constitute alternative prophylactic and therapeutic agents in aquaculture because of their antibacterial properties. (Turker and Birinci Yıldırım, 2015).

There are few studies on antibacterial effect of different essential oils against *Vagococcus salmoninarum* (Metin et al., 2017; Özay et al., 2018). The aim of this study, essential oils of thyme (*Origanum vulgare*), St. John's Wort (*Hypericum perforatum*), rosemary oil (*Rosmarinus officinalis*), ginger (*Zingiber officinale*), clove (*Eugenia caryophyllata*), peppermint (*Menta piperita*), lavandula (*Lavandula hybrida*) and black cumin (*Nigella sativa*) were screened for its chemical composition and *in vitro* antibacterial activity against *Vagococcus salmoninarum*.

MATERIAL AND METHODS

Essential oils

Essential oils of thyme (*Origanum vulgare*), St. John's Wort (*Hypericum perforatum*), rosemary oil (*Rosmarinus officinalis*), ginger (*Zingiber officinale*), clove (*Eugenia caryophyllata*), peppermint (*Menta piperita*), lavandula (*Lavandula hybrida*) and black cumin (*Nigella sativa*) were purchased from Botalife.

Gas chromatography-mass spectroscopy analyses of essential oils

The gas chromatographic analysis of essential oils was performed with a Hewlett-Packard 6890 series gas chromatograph (Perkin Elmer (PE) Auto System XL, Alameda, CA, USA), fitted with a flame ionization detector (FID). Relative percentage amounts were calculated from chromatograms by the Turbo Crom. Navigator computer program.

In vitro antibacterial assays

Antibacterial effects of essential oils against *V. salmoninarum* were detected by agar diffusion and tube dilution assays.

Agar difusion assay

V. salmoninarum was grown in TSB for 24 h at 25 °C, and 100 µL of bacteria culture was mixed into 100 mL of melted TSA. After solidifying and drying for 15–20 minute, wells were punched (diameter = 3 mm) and 25 µL of different concentrations prepared with 96% ethanol (the test concentrations: 1000, 500, 250, 125, 62.5, 31.25, 15.62 and 7.8 µl ml^{-1}) added to wells in triplicates. Controls were prepared using 96% ethanol. Plates were incubated at 25°C for 24h and observed for clearing zones around the wells. The concentrations of essential oils causing clearing zones were determined (NCCLS, 2001).

Tube dilution assay

The mininum inhibition concentration (MIC) of essential oils were determined by using the broth dilution method. The essential oil was dissolved in 96% ethanol and serially diluted (1000–0.01 µmL^{-1}). The ethanol was used as control. *V. salmoninarum* was cultured in TSA for 24 h at 25°C then diluted to a concentration of McFarland No.0.5. Then, *V. salmoninarum* was introduced into each tube under sterile conditions and incubated for 48h at 25 °C. After incubation, MIC values were recorded as the lowest concentration of the oils that completely inhibited bacterial growth (Park et al., 2016).

Statistical analysis

One-way analysis of variance and Duncan's multiple range tests were run to evaluate the differences using the SPSS software (Version 15, SPSS Inc., Chicago, IL, USA). The means and standard errors were calculated for each treatment. The accepted level of significance was 0.05.

RESULTS

In vitro antibacterial assays

In vitro antibacterial activities of the essential oils against *V. salmoninarum* were determineted by the agar diffusion and tube dilution methods. The antibacterial activities of different essential oils against *V. salmoninarum* were summarized in Table 1 and 2. The antibacterial activity of thyme (*Origanum vulgare*), St. John's Wort (*Hypericum perforatum*), rosemary oil

(*Rosmarinus officinalis*), ginger (*Zingiber officinale*) and black cumin (*Nigella sativa*) were studied for the first time. A broad variation in antimicrobial properties of the tested essential oils was observed in the study.

The essential oils of thyme and clove showed strong antimicrobial activity at different concentrations against *V. salmoninarum*, whereas other essential oils did not show any antibacterial activity. The maximum inhibition zones and MIC values of the essential oils of thyme and clove againts *V. salmoninarum* were in the range of 20.33-18 mm and 125-1000 µl ml^{-1}, 17.83-18.66 mm and 500-1000 µl ml^{-1}, respectively (Table 1, 2).

Table 1. Antibacterial activity of essential oils against *V. salmoninarum* by the agar diffusion assay (The diameter of the zone of inhibition, mm)

Concentration µl ml^{-1}	Thyme	Clove	Ginger	Peppermint	Rosemary	Lavandula
1000	18±2,0^{ab}	18.66±0,57^{a}	5.66 ±0,57	6.66±0,57^{ab}	-	-
500	20±1,0^{a}	17.83±0,76^{a}	5.66 ±0,57	6±1,0^{bc}	-	-
250	18±1,0^{ab}	11.66±1,52^{b}	5.66 ±0,57	7.83±0,28^{a}	-	-
125	20.33±1,52^{a}	11.83±0,28^{b}	6±00	6.83±0,76^{ab}	-	-
62.5	17±1,0^{b}	11±0^{b}	-	6.5±0,5^{bc}	-	-
31.25	12.66±1,52^{c}	8.66±0,57^{c}	-	5.33±0,57^{c}	-	-
15.62	10.66±1,52^{c}	7.16±0,28^{d}	-	-	-	-
7.8	8.16±0,28^{d}	5.83±0,76^{e}	-	-	-	-

Table 2. Minimum inhibitory concentration of essential oils against *V. salmoninarum* (µl ml^{-1})

Concentration µl ml^{-1}	Thyme	Clove	Ginger	Peppermint	Rosemary	Lavandula
1000	-	-	-	-	+	+
500	-	-	+	+	+	+
250	-	+	+	+	+	+
125	-	+	+	+	+	+
62.5	+	+	+	+	+	+
31.25	+	+	+	+	+	+
15.62	+	+	+	+	+	+
7.8	+	+	+	+	+	+
3.9	+	+	+	+	+	+
1.95	+	+	+	+	+	+
0.97	+	+	+	+	+	+
0.48	+	+	+	+	+	+
0.24	+	+	+	+	+	+
0.12	+	+	+	+	+	+
0.06	+	+	+	+	+	+
0.03	+	+	+	+	+	+
0.01	+	+	+	+	+	+

GC-MS Analysis

The results of the chemical analysis of effective essential oils are presented in Table 3. Twenty one components were identified in thyme oil and the main components were carvacrol (63.57%) and cymol (16.09%). In clove oil, only five components were detected and the main components were eugenol (85.99%) and caryophyllene (10.89%).

Table 3. Chemical composition of thyme and clove essential oils

Essential oil	Component	Composition (%)	Retention Time (RT)
Rosmarinus officinalis	Thujene<alpha->	0.11	6.298
	Alpha pinene	**12.85**	6.566
	Camphene	5.01	7.121
	Pinene<beta->	8.48	8.160
	Beta.-Myrcene	1.31	8.585
	1-Phellandrene	0.10	9.321
	.DELTA.3-Carene	0.06	9.449
	Terpinene<alpha->	0.04	9.808
	Cymol	3.04	10.154
	Limonene	2.01	10.425
	1,8-Cineole	**47.31**	10.593
	Cis-Ocimene	0.05	10.712
	Gamma.-Terpinen	0.87	11.815
	Alpha.-Terpinolen	0.07	13.253
	Linalyl acetate	0.14	14.044
	Camphor	**12.04**	16.706
	Isoborneol	0.06	17.680
	Borneol	2.62	18.229
	Trans-4-Thujanol	0.20	18.789
	.BETA. FENCHYL ALCOHOL	2.17	19.724
	Bornyl acetate	1.00	25.500
	Caryophyllene	0.47	34.107
Menta piperita	Isopulegol & p-Menthen-3-ol	1.98	16.820
	Menthone	**24.20**	17.329
	Isomenthone (CAS) p-Menthan-3-one	9.28	17.828
	Neoisomenthol	11.35	18.199
	Isopulegone	0.77	18.473
	Menthol	**44.76**	18.830
	ALPHA. TERPINEOL	0.81	19.735
	Pulegone	3.91	22.428
	Piperitone	0.80	23.409
	Neomenthol acetate	2.03	25.961
	Beta.-Bourbonene	0.05	31.855
	Trans- Caryophyllene	0.05	34.109
Origanum vulgare	Thujene<alpha->	1.67	6.290
	.ALPHA.-PINENE, (-)-	1.88	6.549
	2,4(10)-thujadien	0.14	6.869
	Camphene	1.67	7.111
	2-.BETA.-PINENE	0.75	8.147
	.beta.-Myrcene	1.30	8.576
	1-Phellandrene	0.17	9.310
	.DELTA.3-Carene	0.09	9.435
	.ALPHA. TERPINENE	1.28	9.792
	Cymol	**16.09**	10.148
	Limonene	0.56	10.378
	EUCALYPTOL (1,8-CINEOLE)	0.87	10.508
	.gamma.-Terpinene	2.65	11.808
	Trans-4-Thujanol	1.36	12.394
	.ALPHA.-TERPINOLENE	0.21	13.243
	Borneol	2.56	18.209
	Trans-Sabinene hydrate	0.84	18.780
	.BETA. FENCHYL ALCOHOL	0.26	19.722
	Thymol	0.24	26.050
	Carvacrol	**63.57**	26.715
	Caryophyllene	1.86	34.113

Zingiber officinale	.ALPHA.-PINENE, (-)-	2.07	6.538
	Camphene	7.07	7.108
	BETA.-PINENE	0.22	8.133
	6-Methyl-5-hepten-2-one	0.51	8.325
	.beta.-Myrcene	0.67	8.573
	1-Phellandrene	5.05	10.434
	EUCALYPTOL (1,8-CINEOLE)	3.05	10.496
	Linaly formate	0.10	14.031
	BORNEOL L	0.94	18.204
	.BETA. FENCHYL ALCOHOL	0.39	19.697
	Copaene <alpha->	0.47	31.390
	Geranyl butyrate	0.22	31.797
	.BETA. ELEMENE	0.74	32.344
	Sesquithujene <7-epi->	0.21	33.308
	GERMACRENE-D	1.02	37.998
	Curcumene	**11.65**	38.229
	Alloaromadendrene	1.19	38.933
	Zingiberene	**37.18**	39.215
	Farnesene <(E,E)-, alpha->	4.07	39.851
	Bisabolene	7.95	39.954
	Cedrene	**13.50**	40.898
	Patchoulene <alpha->	0.34	41.039
		0.29	42.204
	Germacrene B	0.49	42.687
	d-Nerolidol	0.27	43.160
	.alpha.-Cedrol	0.31	46.220
Eugenia caryophyllata	Chavicol$$ p-Allylphenol	0.12	23.455
	Eugenol	**85.99**	30.381
	Caryophyllene	**10.89**	34.199
	Alpha.-Humulene	2.45	36.388
	(-)-Caryophyllene oxide	0.54	44.036
Lavandula hybrida	Alpha-Pinene	0.26	6.551
	Camphene	0.26	7.116
	Beta-Pinene	0.21	8.149
	3-Octanone (CAS) EAK	0.93	8.373
	Beta-Myrceneethanoate	0.84	8.583
	Ethanoate <hexyl->	1.04	9.536
	Limonene	0.85	10.379
	1,8-Cineole	4.95	10.505
	Cis-Ocimene	0.95	10.699
	Trans-.beta.-Ocimene	2.30	11.224
	ALPHA.-TERPINOLEN	0.24	13.257
	Linalool	**42.31**	14.164
	Camphor	5.16	16.657
	Propanoic acid, 2-methyl-, hexyl ester	0.24	16.901
	BORNEOL L	1.99	18.220
	Linalool	2.40	19.698
	Linalyl acetate	**32.16**	23.515
	Lavandulyl acetate	1.22	25.671
	Neryl acetate	0.45	31.800
	Caryophyllene	0.87	34.114
	Farnesene <(E)-, beta->	0.28	36.573
	GERMACRENE-D	0.08	37.991

DISCUSSION

Essential oils isolated from different aromatic plants are known to have a wide spectrum of antimicrobial activity (Hammer et al., 1999; Baydar et al., 2004). This activity is strongly correlated with the chemical structure of the most abundant essential oil component. The chemical composition of essential oils depends on climatic, seasonal and geographic conditions, harvest period. The chemical components of thyme and clove essential oils have been studied in literatures. The phenolic monoterpens (carvacrol and thymol) are the most abundant constituent of the essential of *Origanum* species. The composition of *O. vulgare* essential oil has been characterized by several authors, with carvacrol as the major components (Goliaris et al., 2003; Viuda-Martos et al., 2007; Souza et al., 2008; De Martino et al., 2009; Derwich et al., 2010; Bejaoui et al., 2013; Stojkovic et al., 2013; Diler et al., 2017b). Other components have also been reported as important essential oil components, such as pcymene, γ-terpinene, caryophyllene, spathulenol, thymoquinene and germacrene (Milos et al., 2000; Radusiene et al., 2005; Cleff et al., 2010; Teixeira et al., 2013). In another study, the main constituents of *O. vulgare* essential oils have been reported as thymol (13.7-85.9%) and carvacrol (1.5-63.0%) (Raina and Negi, 2014). In the present study, carvacrol (63.57%) and cymol (16.09%) were detected of main components of *O. vulgare* essential oil. However, thymol content (0.24%) found to be quite low. The major component of clove oil is usually considered to be eugenol, with β-caryophyllene and lesser amounts of other components such as benzyl alcohol, but the proportions vary widely (Chaieb et al., 2007; Guan et al., 2007; Xu et al., 2016). For example, Prashar et al. (2006) found the content of eugenol to be 78%, with 13% β-caryophyllene, whereas Chaieb et al. (2007) found that eugenol was 88.58%, with β-caryophyllene at 1.39%. In this study, similarly eugenol (85.99%) and caryophyllene (10.89%) were determined as main components of clove oil.

In this study, antimicrobial effects of thyme, black cumin, ginger, rosemary oil and St. John's Wort against *Vagococcus salmoninarum* were investigated for the first time. The essential oils of thyme and clove showed strong antimicrobial activity at different concentrations against *V. salmoninarum*. However, St. John's Wort, rosemary oil, ginger, peppermint, lavandula and black cumin essential oils did not show any antibacterial activity. In another study, cyclamen L. tuber extracts had been found to have moderate antibacterial activity againts *V. salmoninarum* (Özay et al., 2018). Metin et al. (2017) noted that clove essential oil was showed a strong antimicrobial activity (MIC 1000-125 µl ml-1) againts *V. salmoninarum*.

In conclusion, thyme and clove essential oils displayed stronger inhibition zones against *V. salmoninarum*. This is a critical step in determining whether such essential oils are capable of reducing mortality caused by these pathogens. However, thyme and clove oil is needed further study to explore its therapeutic effects against vagococcosis

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