

ANTIBACTERIAL EFFECTS OF SEVEN ESSENTIAL PLANT OILS ON FISH PATHOGENS

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ABSTRACT

The use of natural products which have the least harmful effects on the environment has recently been taken as a novel approach against fish diseases. References on *in vitro* studies have demonstrated antibacterial activity of essential oils (EOs) against certain fish pathogens. The aim of this study was to evaluate the antibacterial effect of some plant essential oils against fish pathogenic bacteria *in vitro* conditions. Seven plant EOs: lavender (*Lavandula angustifolia*), clove (*Eugenia caryophyllus*), peppermint (*Mentha piperitae*), basil (*Ocimum sanctum*), rosemary (*Rosmarinus officinalis*), cinnamon (*Cinnamomum zeylanicum*) and black cumin (*Nigella sativa*) were used to identify their antibacterial properties against *Yersinia ruckeri*, *Aeromonas hydrophila*, *Vibrio anguillarum*, *Vibrio alginolyticus*, *Lactococcus garvieae* and *Vagococcus salmoninarum* at five concentrations using disc diffusion method. Especially the EOs of clove, cinnamon and rosemary showed the strongest antibacterial activities than other oils against the three most susceptible bacterial strains (*Y. ruckeri*, *A. hydrophila* and *V. salmoninarum*). Besides, the EOs of clove, rosemary, cinnamon and black cumin showed similar inhibition zones with OTC against *A. hydrophila*. The minimum inhibitory concentrations of the used EOs found between 500 and 62.5 $\mu\text{L mL}^{-1}$. As a result, three of the EOs used in this study were effective on both Gr (-) and Gr (+) bacteria.

Keywords: fish pathogens; essential oil; antibacterial activity; minimum inhibitory concentration.

EFEITOS ANTIBACTERIANOS DE SETE ÓLEOS DE PLANTAS ESSENCIAIS EM PATÓGENOS DE PEIXE

RESUMO

A utilização de produtos naturais com menos efeitos nocivos para o ambiente foi recentemente considerada uma nova abordagem contra as doenças dos peixes. Referências em estudos *in vitro* demonstraram atividade antibacteriana de óleos essenciais (OE) contra certos patógenos de peixes. O objetivo deste estudo foi avaliar o efeito antibacteriano de alguns óleos essenciais de plantas contra bactérias patogênicas de peixes em condições *in vitro*. Sete EOs de plantas: alfazema (*Lavandula angustifolia*), cravo (*Eugenia caryophyllus*), hortelã-pimenta (*Mentha piperitae*), manjeriço (*Ocimum sanctum*), alecrim (*Rosmarinus officinalis*), canela (*Cinnamomum zeylanicum*) e cominho preto (*Nigella sativa*) foram usados para identificar suas propriedades antibacterianas contra *Yersinia ruckeri*, *Aeromonas hydrophila*, *Vibrio anguillarum*, *Vibrio alginolyticus*, *Lactococcus garvieae* e *Vagococcus salmoninarum* em cinco concentrações usando o método de difusão em disco. Especialmente os OEs de cravo, canela e alecrim mostraram as atividades antibacterianas mais fortes do que outros óleos contra as três cepas bacterianas mais suscetíveis (*Y. ruckeri*, *A. hydrophila* e *V. salmoninarum*). Além disso, os OEs de cravo, alecrim, canela e cominho preto mostraram zonas de inibição semelhantes com OTC contra *A. hydrophila*. As concentrações inibitórias mínimas dos OE usados encontradas entre 500 e 62.5 $\mu\text{L mL}^{-1}$. Como resultado, três dos EOs usados neste estudo foram eficazes nas bactérias Gr (-) e Gr (+).

Palavras-chave: peixe patógeno; óleo essencial; atividade antibacteriana; Concentração Inibitória Mínima.

INTRODUCTION

The economic losses caused by infectious diseases has been a major issue in aquaculture worldwide (Flores-Kossack et al., 2020; Hwang et al., 2020; Wang et al., 2020). Antibiotics have been utilized as a successful method to control bacterial diseases but bacteria gain resistance against many antibiotics therefore utilization of numerous antibiotic agents that have been banned or using levels decreased in several countries. The use of antibiotics

in aquaculture has been implicated as a potential hazard for the development and selection of resistant bacteria and a source of these pathogens to other animals and humans (Hatha et al., 2005; Serrano, 2005; Acar et al., 2009). There is a need for new natural solutions as an alternative to antibiotics in the control and treatment of fish diseases. Essential oils (EOs) can be used as an alternative to antibiotics in aquaculture (Romero et al., 2012; Cunha et al., 2018). EOs have been exhibited to possess antimicrobial and antioxidant effects (Cunha et al., 2018). They can also serve as significant tools to reduce bacterial resistance (Stefanakis et al., 2013). Aromatic oil liquids called essential oils are obtained from plant materials (flowers, leaves, wood, roots, seeds, herbs and fruits). The highest levels of antibacterial activity were obtained with the use of EOs with substantial phenol or aldehyde content, such as cinnamaldehyde, citral, carvacrol, eugenol, or thymol (Cunha et al., 2018). This is followed by EOs that contain terpene alcohols. On the other hand, the activity levels of EOs containing ketones or esters, including β myrcene, α -thujone, or geranyl acetate, had been determined at significantly lower levels (Swamy et al., 2016). Zheng et al. (2005) established that volatile oils containing terpene hydrocarbons were usually inactive. We also know that EOs with high levels of phenolic compounds, including but not limited to carvacrol, eugenol, and thymol, hold significant antibacterial activities (Benzeria, 2006).

Rosemary, cinnamon, lavender, black cumin and clove oils have shown antibacterial and antifungal effect (Ouattara et al., 1997; Cavanagh and Wilkinson, 2002; Soltani et al., 2014). Basil oil possess anti-inflammatory property (Singh and Majumdar, 1999). Peppermint oil has shown anticancer activity (Kumar et al., 2004). The *in vitro* antibacterial effect of some EOs have been determined against a different of Gram-negative and Gram-positive strains, including prominent pathogens of aquaculture such as *Vagococcus salmoninarum* (Metin and Biçer, 2020), *Streptococcus iniae*, *Photobacterium damsela* subspecies *damsela*, *Aeromonas hydrophila* (Gholipourkanani et al., 2019); *Edwardsiella tarda*, *P. damsela*, *Vibrio harveyi*, *V. ichthyenteri*, *L. garvieae*, *Streptococcus iniae* and *S. parauberis* (Pathirana et al., 2018); *Yersinia ruckeri*, *Lactococcus garvieae*, *A. hydrophila*, *L. anguillarum* (Ontas et al., 2016; Metin et al., 2017); *Edwardsiella tarda*, *S. iniae*, *S. parauberis*, *L. garvieae*, *V. harveyi*, *V. ichthyenteri* and *P. damsela* (Park et al., 2016); *S. agalactiae* (Zhang et al., 2013).

Therefore, the aim of this study was to investigate the antibacterial effect of EOs (lavender, clove, peppermint, basil, rosemary, cinnamon and black cumin) on six fish pathogenic bacteria (*Y. ruckeri*, *A. hydrophila*, *V. anguillarum*, *V. alginolyticus*, *L. garvieae* and *V. salmoninarum*,) that are often the reason of bacterial fish infections in aquaculture.

MATERIALS AND METHODS

Plant EOs

In this study, the EOs purchased were commercial products (Arifoglu/Turkey): lavender (*Lavandula angustifolia*), clove (*Eugenia caryophyllus*), peppermint (*Mentha piperitae*), basil (*Ocimum sanctum*), rosemary (*Rosmarinus officinalis*), cinnamon (*Cinnamomum zeylanicum*) and black cumin (*Nigella sativa*).

Bacterial strains

EOs were evaluated against some bacterial strains. The bacterial strains obtained from the microorganism collection of Fisheries Faculty, Isparta University of Applied Sciences in Turkey. In this study, the bacterial strains used were already identified before (*Y. ruckeri* and *A. hydrophila* (Metin et al., 2017), *V. anguillarum* (Aysever et al., 2015), *V. alginolyticus* (Metin et al., 2017), *L. garvieae* (Altun et al., 2004) and *V. salmoninarum* (Didinen et al., 2011) by using molecular methods. Also, the antibacterial effect of EOs were tested against *Y. ruckeri*, *A. hydrophila*, *V. anguillarum*, *V. alginolyticus*, *L. garvieae* and *V. salmoninarum* (Table 1). Before using, each bacteria was identified again by using classical biochemical tests, API 20E, API 20 Strep and API 50 CH tests (Austin and Austin, 2007).

Antibacterial assay

Antibacterial activity was carried out by disc diffusion method (Andrews, 2004; CLSI, 2015). Briefly, bacteria suspensions were prepared as a fresh culture (24 h) and adjusted to 0.5 McFarland, which equals to 1.5×10^8 CFU mL⁻¹. Then inoculum (100 μ L) of each test organism was spread with a sterile spreader on Mueller Hinton Agar (Merck, Darmstadt, Germany) plates. EOs were dissolved in methanol and sterilized by filtration through a 0.45 μ m membrane filter. Under sterile conditions, blank sterile discs (Merck, Darmstadt, Germany) were impregnated with 25 μ L of different concentrations (1:1, 1:2, 1:4, 1:8, 1:16) of the EOs prepared and placed on the agar surface. In this test, as positive control oxytetracycline (20 μ g) and negative control methanol were utilized. The incubation of the inoculated plates took place at 22-24°C for 24 hours then occurred inhibition zone was measured as millimeter (mm). Inhibition zones evaluated as >15 mm were declared as strong, from 8 to 15 mm as moderate, and from 1 to 8 mm as weak antibacterial activities (Bansemir et al., 2006).

Minimum Inhibitory Concentration (MIC) assay

MIC assay was determined against the same strains by the microtitre dilution method. EOs were serially diluted in methanol, ranging from 1000, 500, 250, 125, 62.6, 31.25, 15.62, 7.8, 3.9, 1.95, 0.97, 0.48, 0.24, 0.12, 0.06, 0.03, 0.01 μ L mL⁻¹. Two-fold dilution of each compound (100 μ L) in sterile methanol was prepared in 96-well plate and 24 h bacterial culture adjusted to

Table 1. Origins of the identified bacterial strains (Turkey) which were isolated from sick fish.

Bacteria	Origin
Gram (-)	
<i>Yersinia ruckeri</i>	Rainbow trout (<i>Oncorhynchus mykiss</i>)
<i>Aeromonas hydrophila</i>	Common carp (<i>Cyprinus carpio</i>)
<i>Listonella anguillarum</i>	Rainbow trout (<i>Oncorhynchus mykiss</i>)
<i>Vibrio alginolyticus</i>	Sea bass (<i>Dicentrarchus labrax</i>)
Gram (+)	
<i>Lactococcus garvieae</i>	Rainbow trout (<i>Oncorhynchus mykiss</i>)
<i>Vagococcus salmoninarum</i>	Rainbow trout (<i>Oncorhynchus mykiss</i>)

one McFarland unit was added (100 µl) to each well. Methanol was used as a negative control. The suspensions were incubated at 22-24°C for 24 hours. After incubation was measured at OD: at 630 nm (Park et al., 2016).

Statistical analysis

The results were recorded as means ± standard error (SEM) and analyzed with SPSS version 14.0 (Tukey's comparison test using). Different letters in tables represent a significant difference at $p < 0.05$.

RESULTS

Biochemical test results

To identify the bacterial isolates, this study used conventional biochemical tests and API® 20E system (BioMerieux, France) and API 20 Strep test (Biomerieux, France). The results of the API tests was given in Table 2 and Table 3.

In vitro antibacterial activity of essential oils

Antibacterial activity of seven EOs (lavender, clove, peppermint, rosemary, cinnamon, basil and black cumin) against fish pathogen

bacterial species by the disc diffusion method was presented in Tables 4-9 respectively. Positive control of oxytetracycline (OTC) (20 µg) exhibited an inhibition zone 26.00 ± 0.57 mm against *Y. ruckeri*, 21.66 ± 1.20 mm *A. hydrophila*, 25.00 ± 0.57 mm *V. anguillarum*, 18.66 ± 0.88 mm *V. alginolyticus*, 12.00 ± 0.88 mm *L. garvieae* and 24.33 ± 1.20 mm *V. salmoninarum*.

The results revealed that the plant EOs used exhibited antibacterial activity with varying values except for basil. The basil essential oil did not show any inhibition zone against bacterial strains. Therefore, it was not included in the tables. The EOs of lavender displayed zones of inhibition against pathogens respectively; *V. salmoninarum* (19.00 ± 0.57 mm), *Y. ruckeri* (15.66 ± 0.33 mm), *A. hydrophila* (15.33 ± 0.33 mm), *V. alginolyticus* (13.66 ± 0.88 mm), *V. anguillarum* (12.00 ± 0.57 mm). The minimum were 9.00 ± 0.57 mm inhibition zone produced against *L. garvieae*. It was determined that the lavender EO formed significantly high inhibition zone (Table 4) among the concentrations against *V. salmoninarum* ($p < 0.05$). In general, it was obtained that lavender EO had moderate effects on all bacterial strains.

In general clove oil EO showed the highest inhibitory effect against *Y. ruckeri* (21.00 ± 0.57 mm), *A. hydrophila* (22.00 ± 0.57 mm) and *V. salmoninarum* (20.66 ± 0.88 mm). Statistically, the best inhibition zone (Table 5) results of 1/1 dilution of clove EO were obtained against *Y. ruckeri*, *A. hydrophila*, and *V. salmoninarum* ($p < 0.05$). Especially it was determined that clove EO formed an

Table 2. Phenotypic characteristics of the Gram (-) bacteria isolated from diseased fish.

	<i>A. hydrophila</i>	<i>Y. ruckeri</i>	<i>V. anguillarum</i>	<i>V. alginolyticus</i>
Gram	-	-	-	-
Motility	+	+	+	+
Oxidase	+	-	+	+
Catalase	+	+	+	+
O/F test	F	F	F	F
ONPG	+	+	+	-
Citrate	+	+	+	+
H ₂ S	-	-	-	+
Indole	+	-	+	+
MR	+	+	-	+
VP	+	-	+	+
O 129	R	ND	S	S
Growth at 37°C	+	+	+	+
0% NaCl, growth	+	+	-	-
Arabinose	+	-	+	-
Inositol	-	-	-	-
Lactose	-	-	-	-
Maltose	+	+	+	+
D-mannitol	+	+	+	+
Mannose	+	+	ND	+
Sucrose	+	-	+	+
Urease	-	-	-	+

+ Positive reaction; - Negative reaction; O/F: Oxidative/Fermentative; F: Fermentative; ONPG: orthonitrophenil-β-galacto pyranoside; MR: Methyl red; VP: Voges-Proskauer; O 129: *Vibrio* (which are sensitive to O/129) and *Aeromonas* (resistant to O/129). R: resistant; S: Sensitive, ND: not detected.

Table 3. Phenotypic characteristics of the Gram (+) bacteria isolated from rainbow trout.

	<i>L. garvieae</i>	<i>V. salmoninarum</i>
Gram	+	+
Motility	-	-
Haemolysis	α	α
Oxidase	-	-
Catalase	-	-
O/F test	F	F
Urease	-	-
Indole	-	-
Citrate	-	-
Gelatine	-	-
H ₂ S	-	-
Nitrate	-	-
Growth at 10°C	+	+
Growth at 45°C	+	-
Growth at 6.5% NaCl	+	-
Vogues-Proskauer	+	ND
Hippurate hydrolysis	+	-
Pyrrolidonyl arylamidase	+	+
α -Galactosidase	-	-
β -glucuronidase	-	-
β -Galactosidase	-	-
Alkaline phosphatase	-	-
Leucine arylamidase	+	-
Arginine dihydrolase	+	-
Ribose	+	+
Mannitol	+	-
Sorbitol	-	-
Lactose	-	-
Starch	+	-
Glycogen	-	-
Raffinose	-	-
Inulin	-	-

+ Positive reaction; - Negative reaction; O/F: Oxidative/Fermentative; F: Fermentative; ND: not detected.

inhibition zone similar with OTC against *A. hydrophila* strain. Besides, it has been shown that the oil has strong and moderate effects against all bacterial strains.

Peppermint EO was determined statistically different from other groups against *V. salmoninarum* (15.00 ± 0.57 mm) and was evaluated as a moderate effect ($p < 0.05$). Antibacterial activity of EO was found (Table 6) to have weak and moderate effects on the other isolates evaluated.

Table 7 presents the inhibition zone formed by the rosemary EO against the fish pathogenic bacteria. The strongest activities statistically were determined by rosemary EO with inhibition zones of 22 mm against *Y. ruckeri* and *A. hydrophila* ($p < 0.05$)

in 1/1 dilution. The minimum effects were at 11.66 mm zone inhibition observed against *L. garvieae* (Table 7). The rosemary EO showed strong activity against *Y. ruckeri*, *A. hydrophila* and *V. salmoninarum* while the rest of the strains were evaluated as moderate activity ($p < 0.05$). Also, similar inhibition zone was determined for both *A. hydrophila* strain and the positive control OTC.

The EO of cinnamon statistically exhibited maximum zone of inhibition against *A. hydrophila* (21.33 ± 0.88 mm) ($p < 0.05$). It showed strong activity (Table 8) on *Y. ruckeri* and *A. hydrophila* strains while other strains displayed moderate activity.

As presented in Table 9, it was determined that black cumin EO constitutes the highest inhibition zone against *A. hydrophila* strain ($p < 0.05$). It follows as strong activity on *V. salmoninarum*, *Y. ruckeri* and moderate effect on other strains.

Minimum Inhibition Concentration - MIC

Minimum Inhibition Concentration (MIC) of EOs (lavender, clove, peppermint, basil, rosemary, cinnamon and black cumin) which showed antibacterial activity were measured. MIC results for six selected oils ranged from 500 to 0.01 $\mu\text{L mL}^{-1}$. The results of minimum inhibitory concentration (MIC) of EOS were shown in Table 10.

Minimum Inhibition Concentration (MIC) values obtained for *Y. ruckeri* 62.5 $\mu\text{L mL}^{-1}$ (clove and rosemary EOs); *A. hydrophila* 62.5 $\mu\text{L mL}^{-1}$ (rosemary and cinnamon EOs); *V. salmoninarum* 62.5 $\mu\text{L mL}^{-1}$ (lavender, clove and rosemary EOs); *V. anguillarum* 250 $\mu\text{L mL}^{-1}$ (all EOs); *V. alginolyticus* 250 $\mu\text{L mL}^{-1}$ (lavender, peppermint, rosemary and black cumin); and *L. garvieae* 250 $\mu\text{L mL}^{-1}$ (clove, cinnamon and black cumin EOs) (Table 10).

DISCUSSION

Essential oils (EOs) have been used in pharmaceuticals, alternative medicine and natural therapies (Romero et al., 2012; Cunha et al., 2018). They have been exhibited to possess antibacterial, antiviral, antifungal and antioxidant properties (Cunha et al., 2018). The mechanisms of action of EOs depend on their chemical composition (Nazzaro et al., 2013). Numerous studies have reported that clove, cinnamon and rosemary oils have strong and consistent inhibitory activities against different pathogens (Aureli et al., 1992; Matan et al., 2006; Ekici et al., 2011; Ontas et al., 2016; Park et al., 2016; Metin et al., 2017; Majolo et al., 2018; Pathirana et al., 2018; Metin and Biçer, 2020).

In the present study, the antibacterial properties of seven EOs (lavender, clove, peppermint, basil, rosemary, cinnamon and black cumin) against six fish bacterial pathogens (*Y. ruckeri*, *A. hydrophila*, *V. anguillarum*, *V. alginolyticus*, *L. garvieae* and *V. salmoninarum*) were tested. Also, various concentrations (1/1, 1/2, 1/4, 1/8, and 1/16) of EOs were applied to these important pathogens in aquaculture. Especially the EOs of clove, cinnamon and rosemary showed stronger antibacterial activities than other oils against the three most susceptible bacterial strains (*Y. ruckeri*, *A. hydrophila* and *V. salmoninarum*). Besides, the EOs

Table 4. Antibacterial activity (mean ± standard error) of lavender EO against different bacterial fish pathogens (inhibitory zone, mm).

Bacteria	Concentrations of lavender EO				
	1/1	1/2	1/4	1/8	1/16
<i>Y. ruckeri</i>	15.66±0.33 ^b	14.00±0.57 ^{bcd}	11.00±0.57 ^{fgh}	9.66±0.88 ^{iklmn}	8.33±0.66 ^{mno}
<i>A. hydrophila</i>	15.33±0.33 ^b	11.66±0.88 ^{efgh}	10.33±0.88 ^{ghij}	9.00±1.15 ^{klmno}	7.33±0.33 ^o
<i>V. anguillarum</i>	12.00±0.57 ^{cdef}	10.66±0.33 ^{ghij}	10.33±0.88 ^{ghij}	8.66±0.33 ^{lmno}	9.00±0.57 ^{klmno}
<i>V. alginolyticus</i>	13.66±0.88 ^{bcd}	12.00±0.57 ^{cdef}	9.66±0.33 ^{ijklmn}	9.00±0.57 ^{klmno}	7.33±0.33 ^o
<i>L. garvieae</i>	9.00±0.57 ^{klmno}	8.33±0.88 ^{mno}	8.00±0.57 ^{no}	7.00±0.57 ^o	7.00±0.57 ^o
<i>V. salmoninarum</i>	19.00±0.57 ^a	14.66±0.33 ^{bc}	13.00±0.57 ^{bcd}	11.66±0.33 ^{efgh}	10.33±0.88 ^{ghijk}

Different letters represent significant differences at $p < 0.05$.

Table 5. Antibacterial activity (mean ± standard error) of clove EO against different bacterial fish pathogens (the diameter of the zone of inhibition, mm).

Bacteria	Concentrations of clove EO				
	1/1	1/2	1/4	1/8	1/16
<i>Y. ruckeri</i>	21.00±0.57 ^a	16.66±0.33 ^{bc}	15.00±0.57 ^{cd}	14.00±0.57 ^{de}	12.00±0.88 ^{fg}
<i>A. hydrophila</i>	22.00±0.57 ^a	20.00±0.57 ^a	18.00±0.55 ^b	14.66±0.87 ^d	12.33±0.33 ^{efg}
<i>V. anguillarum</i>	12.66±0.88 ^{ed}	10.66±0.33 ^{ghi}	10.00±0.57 ^{hij}	9.00±0.57 ^{jk}	8.66±0.66 ^{klm}
<i>V. alginolyticus</i>	11.66±0.33 ^{fgh}	10.66±0.66 ^{ghi}	8.66±0.33 ^{klm}	9.00±0.57 ^{jk}	7.33±0.88 ^{lm}
<i>L. garvieae</i>	10.00±0.57 ^{hij}	8.66±0.33 ^{klm}	7.00±0.57 ^m	8.33±0.33 ^{klm}	7.33±0.33 ^{lm}
<i>V. salmoninarum</i>	20.66±0.88 ^a	18.00±0.57 ^b	17.66±0.33 ^b	16.33±0.88 ^{bc}	14.00±0.57 ^{de}

Different letters represent significant differences at $p < 0.05$.

Table 6. Antibacterial activity (mean ± standard error) of peppermint EO against different bacterial fish pathogens (the diameter of the zone of inhibition, mm).

Bacteria	Concentrations of peppermint EO				
	1/1	1/2	1/4	1/8	1/16
<i>Y. ruckeri</i>	14.33±0.33 ^{ab}	12.33±0.33 ^{bcd}	9.00±0.57 ^{fgh}	8.00±0.57 ^{ghi}	8.00±0.57 ^{ghi}
<i>A. hydrophila</i>	13.00±1.15 ^{abc}	11.66±0.33 ^{cde}	8.66±0.33 ^{fgh}	8.33±0.88 ^{fghi}	6.33±0.33 ⁱ
<i>V. anguillarum</i>	9.00±1.15 ^{fgh}	8.33±0.33 ^{fghi}	7.66±0.57 ^{hi}	7.66±1.20 ^{hi}	7.33±0.33 ^{hi}
<i>V. alginolyticus</i>	10.33±0.33 ^{def}	9.00±1.15 ^{fgh}	8.33±0.33 ^{fghi}	7.33±0.88 ^{hi}	7.33±0.33 ^{hi}
<i>L. garvieae</i>	8.33±0.88 ^{fghi}	6.66±0.57 ^{hi}	7.33±0.88 ^{hi}	6.33±0.88 ⁱ	6.33±0.57 ⁱ
<i>V. salmoninarum</i>	15.00±0.57 ^a	12.00±1.00 ^{cde}	10.00±0.57 ^{efg}	8.66±0.33 ^{fghi}	7.66±0.88 ^{hi}

Different letters represent significant differences at $p < 0.05$.

Table 7. Antibacterial activity (mean ± standard error) of rosemary EO against different bacterial fish pathogens (the diameter of the zone of inhibition, mm).

Bacteria	Concentrations of rosemary EO				
	1/1	1/2	1/4	1/8	1/16
<i>Y. ruckeri</i>	22.00±0.57 ^a	18.66±0.33 ^b	18.00±0.57 ^{bc}	13.00±0.57 ^{fg}	9.66±0.33 ^{ij}
<i>A. hydrophila</i>	22.00±0.57 ^a	20.66±0.33 ^a	18.00±0.57 ^{bc}	16.66±0.33 ^{cd}	15.66±0.66 ^{de}
<i>V. anguillarum</i>	14.66±0.66 ^{ef}	12.00±0.57 ^{gh}	11.00±0.57 ^{hi}	9.66±0.33 ^{ij}	9.33±0.88 ^{ij}
<i>V. alginolyticus</i>	13.00±1.15 ^{fg}	11.66±0.33 ^{gh}	8.66±0.33 ^{jk}	8.00±0.57 ^{kl}	6.66±0.66 ^l
<i>L. garvieae</i>	11.66±0.66 ^{gh}	8.66±0.33 ^{jk}	9.00±0.57 ^{jk}	8.66±0.66 ^{jk}	7.33±0.33 ^{kl}
<i>V. salmoninarum</i>	20.33±0.88 ^a	17.33±0.33 ^{bcd}	14.66±0.66 ^{ef}	11.66±0.33 ^{gh}	9.66±0.66 ^{ij}

Different letters represent significant differences at $p < 0.05$.

Table 8. Antibacterial activity (mean \pm standard error) of cinnamon EO against different bacterial fish pathogens (the diameter of the zone of inhibition, mm).

Bacteria	Concentrations of cinnamon EO				
	1/1	1/2	1/4	1/8	1/16
<i>Y. ruckeri</i>	19.33 \pm 0.88 ^b	18.33 \pm 0.33 ^b	15.00 \pm 0.57 ^d	12.66 \pm 0.33 ^{ef}	8.66 \pm 0.33 ^{ijklmn}
<i>A. hydrophila</i>	21.33 \pm 0.88 ^a	18.00 \pm 0.57 ^{bc}	15.33 \pm 0.88 ^d	10.66 \pm 0.33 ^{ghij}	9.66 \pm 0.66 ^{hijkl}
<i>V. anguillarum</i>	12.33 \pm 0.88 ^{ef}	11.00 \pm 0.57 ^{gh}	11.33 \pm 0.33 ^{gh}	9.00 \pm 1.15 ^{ijklm}	7.66 \pm 0.33 ^{lmn}
<i>V. alginolyticus</i>	9.33 \pm 0.33 ^{hijkl}	9.00 \pm 1.15 ^{ijklm}	8.00 \pm 0.57 ^{klmn}	7.00 \pm 0.57 ^{mn}	6.66 \pm 0.66 ⁿ
<i>L. garvieae</i>	12.00 \pm 0.57 ^{fg}	10.00 \pm 0.57 ^{ghijk}	8.66 \pm 0.33 ^{ijklmn}	9.33 \pm 0.88 ^{hijkl}	7.66 \pm 0.33 ^{lmn}
<i>V. salmoninarum</i>	16.33 \pm 0.88 ^{cd}	14.33 \pm 0.33 ^{de}	12.33 \pm 0.88 ^{ef}	8.66 \pm 0.33 ^{ijklmn}	8.33 \pm 0.88 ^{klmn}

Different letters represent significant differences at $p < 0.05$.

Table 9. Antibacterial activity (mean \pm standard error) of black cumin EO against different bacterial fish pathogens (the diameter of the zone of inhibition, mm).

Bacteria	Concentrations of black cumin EO				
	1/1	1/2	1/4	1/8	1/16
<i>Y. ruckeri</i>	17.33 \pm 0.88 ^{bc}	13.66 \pm 0.33 ^{de}	11.33 \pm 0.88 ^{fgh}	9.66 \pm 0.33 ^{ghijk}	9.33 \pm 0.88 ^{ghijkl}
<i>A. hydrophila</i>	20.33 \pm 0.88 ^a	17.33 \pm 0.88 ^{bc}	15.33 \pm 1.52 ^{cd}	11.00 \pm 0.57 ^{ghi}	8.33 \pm 0.88 ^{ijk}
<i>V. anguillarum</i>	12.00 \pm 0.57 ^{ef}	10.66 \pm 0.66 ^{fghij}	8.00 \pm 0.57 ^{jk}	7.66 \pm 0.66 ^{ik}	7.00 \pm 0.57 ^k
<i>V. alginolyticus</i>	13.66 \pm 0.66 ^{de}	11.66 \pm 0.33 ^{efg}	10.00 \pm 0.57 ^{fghijk}	9.00 \pm 1.15 ^{hijkl}	7.66 \pm 0.66 ^{jk}
<i>L. garvieae</i>	11.33 \pm 0.33 ^{fgh}	10.00 \pm 1.15 ^{fghijk}	8.66 \pm 0.33 ^{hijk}	8.66 \pm 1.20 ^{hijk}	8.33 \pm 0.33 ^{ijk}
<i>V. salmoninarum</i>	18.66 \pm 0.66 ^{ab}	14.66 \pm 0.33 ^d	15.00 \pm 0.57 ^d	11.66 \pm 0.88 ^{efg}	8.66 \pm 0.33 ^{hijk}

Different letters represent significant differences at $p < 0.05$.

Table 10. The minimum inhibitory concentration of EOs against fish pathogens ($\mu\text{l mL}^{-1}$)

EO _s	<i>Y. ruckeri</i>	<i>A. hydrophila</i>	<i>V. anguillarum</i>	<i>V. alginolyticus</i>	<i>L. garvieae</i>	<i>V. salmoninarum</i>
Lavender	125	125	250	250	500	62.5
Clove	62.5	125	250	500	250	62.5
Peppermint	125	125	250	250	500	125
Rosemary	62.5	62.5	250	250	500	62.5
Cinnamon	125	62.5	250	500	250	125
Black cumin	125	125	250	250	250	125

of clove, rosemary, cinnamon and black cumin showed similar inhibition zones with OTC against *A. hydrophila*. All EOs except basil showed a wide range of antibacterial activity on used fish bacterial pathogens.

Ontas et al. (2016) determined the antibacterial effects EOs of lemon peel (*Citrus limon*) and argan (*Argania spinosa*). Both EOs possessed significant antibacterial activity against *Y. ruckeri*, *A. hydrophila*, *L. anguillarum*, *Edwardsiella tarda*, *Citrobacter freundii* and *L. garvieae*. In that study, four pathogens (*Y. ruckeri*, *A. hydrophila*, *L. anguillarum*) inhibited by EOs of lemon, and argan were evaluated as strong activity (17-19 mm) except *L. garvieae* which showed moderate activity (10.33-11.33 mm). In another study antibacterial effect of different nano-encapsulated herbal EOs against fish pathogenic bacterial strains (*A. hydrophila*, *S. iniae* and *P. damsela* subspecies *damsela*) have been studied by Gholipourkanani et al. (2019). Their results on *A. hydrophila*

showed that EOs possessed strong antibacterial effects against fish pathogenic bacteria. Hossain et al. (2018) reported that EO of *Zingiber officinale* showed antibacterial effect on Gram positive bacteria (*L. garvieae*) 13 mm inhibition zone. In this study, 12 mm of inhibition zone was determined against *L. garvieae* caused by cinnamon EO. It gave moderate antibacterial activity between all used EOs like given studies. Wimalasena et al. (2018) documented that *L. garvieae* survival rate inhibited by lavender EO (19 mm), in this study, it was found that lavender EO had moderate activity as 9 mm inhibition zone on the same pathogen. Park et al. (2016) obtained that *L. garvieae* growth was inhibited by *Eucalyptus globulus* EO at 15-24 mm. It seems that *Eucalyptus* EO has a strong effect on *L. garvieae*. These results showed that the use EOs on the bacteria causes different level of antibacterial activity.

Yıldırım and Turker (2018) examined the antibacterial effects of 24 EOs against *A. hydrophila*, *V. anguillarum*, *Y. ruckeri*,

L. garvieae determined that EOs showed high antibacterial activity against these bacteria. They reported that peppermint, rosemary, and lavender EOs possess strong antibacterial activity both Gram negative and Gram positive bacteria. A parallel result was found by Metin et al. (2017) in their study on the antibacterial activity of essential oil (*Eugenia caryophyllata*, *M. piperita* and *Lavandula hybrida*) determined that EOs incite effective strong antibacterial activity against *A. salmonicida subsp. achromogenes*, *A. hydrophila*, *V. anguillarum*, *Y. ruckeri* and *L. garvieae*.

Adel et al. (2016) reported that five EOs (*Eryngium campestre*, *Pimpinella affinis*, *Mentha piperita*, *Achillea wilhelmsii* and *Cuminum cyminum*) showed an antibacterial activity on *Y. ruckeri* pathogen. Peppermint EO exhibited 21.6 mm inhibition zone compared to the present study the EO rosemary (22 mm) caused similar results on *Y. ruckeri* pathogen. In another research, Ozogul et al. (2015) presented that rosemary EOs antimicrobial effect on *A. hydrophila* obtained as 11.5 mm inhibition zone. Whereas in this work, the same EO is measured as 22 mm on *A. hydrophila*. It showed strong antibacterial activity in this study. The reason of that is possible to use different concentrations on obtaining of antibacterial activity or using different EOs.

Antibacterial activity of rosemary, clove, peppermint, lavender and black cumin on *V. salmoninarum* have been searched by Metin and Bicer (2020) in which the diameters of inhibition zone were determined as 18.66 mm for clove, and peppermint as 7.3 mm. However, other EOs did not show any effect on the bacteria. In this paper, the antibacterial effects of EOs on *V. salmoninarum* were obtained for clove (20.66 mm), rosemary (20.33 mm), lavender (19 mm), black cumin (18.66 mm) and peppermint (15 mm). As it can be seen, five out of seven EOs showed strong activity on *V. salmoninarum* pathogen.

Minimum Inhibition Concentration (MIC) values obtained in this study were between 500 and 62.5 $\mu\text{L mL}^{-1}$ on both Gram negative and Gram positive pathogen bacteria. MIC was measured minimum as 62.5 $\mu\text{L mL}^{-1}$ on *Y. ruckeri* (lavender, clove, and rosemary EOs), *A. hydrophila* (rosemary and cinnamon EOs) and *V. salmoninarum* (clove and rosemary EOs). The *V. salmoninarum* pathogen exhibited 1000-250 $\mu\text{L mL}^{-1}$ MIC values by using EOs of clove, peppermint, lavender and rosemary. In other papers, MIC values changing between 31.25-1000 $\mu\text{L mL}^{-1}$ (Ekici et al., 2011; Ontas et al., 2016). The MIC values of EOs in present study on fish pathogens appear to be between maximum and minimum range among the published studies.

In general, results of antibacterial activity of essential oils presented in this study showed different values in the existing literature. They might be affected by factors such as the concentration of EOs, method differences, and the origins of bacteria. When these EOs were evaluated, it was detected that lavender, clove, rosemary and cinnamon EOs showed strong antibacterial activity among pathogens against Gram negative (*Y.ruckeri* and *A. hydrophila*) and Gram positive (*V. salmoninarum*) bacteria. Although they have not been completely investigated, the present study results reveal the great potential of plants for therapeutic in line with the documents on EOs from the literature.

CONCLUSIONS

In conclusion, the antibacterial activities of commercially available EOs were investigated. The results obtained from this study showed that the highest antimicrobial activity was determined in clove, cinnamon and rosemary essential oils and they inhibited the growth of both Gram negative and Gram positive bacteria. In addition, the EOs of clove, rosemary, cinnamon and black cumin showed similar inhibition zones with OTC against *A. hydrophila*. In the next study, the plan will be continued *on in vivo* studies of preventative properties of these essential oils on fish.

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