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Short Communication

Antibacterial activity of oregano (*Origanum vulgare*) essential oil, carvacrol and thymol against fish pathogenic bacteria isolated from cultured olive flounder (*Paralichthys olivaceus*)

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Introduction

In aquaculture industry, diseases of microbial origin are responsible for elevated mortality rates and decrease fish production with consequent economic losses worldwide. The crucial bacterial diseases of marine fish in Korea are edwardsiellosis caused by *Edwardsiella tarda*; streptococcosis caused by *Streptococcus iniae*, *S. parauberis*, and *Lactococcus garvieae*; and vibriosis caused by *Vibrio harveyi*, *V. ichthyoenteri*, and *Photobacterium damselae*, which have recently increased in cultured fish populations (Jee *et al*., 2014; Kim *et al.*, 2015). They have been identified as the etiological agents responsible for the most common disease outbreaks of olive flounder (*Paralichthys olivaceus*) aquaculture in Korea (Nho *et al*., 2009).

Antibiotics are widely used to prevent bacterial infections in fish. However, misuse of antibiotics leads to drug resistance and thereby to the reduced efficacy of the drugs (Wei and Wee, 2013). Therefore, it is essential to develop antibacterial treatments that are made from natural substances. Essential oils (EOs) are one kind of natural products which have been used with their aromatic, flavor, bactericidal, preservative and medicinal properties (Burt, 2004).

Oregano (*Origanum vulgare*) is an herb of the Labiatae family that has been used widely in cooking and folk healing (Soleimani Sarghashk *et al*., 2016).

Oregano essential oil (OEO) and its major phenolic components, carvacrol [2-methyl5-(1-methylethyl) phenol] and thymol (2-isopropyl-5methylphenol), are known for their wide spectrum of biological properties, such as inhibition of bacterial biofilm formation (Nostro *et al*., 2007), anti-candidal activity (Chami *et al*., 2005) and anti-fungal activities (Manohar *et al*., 2001) which has been the subject of several investigations *in vitro* and *in vivo*. Carvacrol and thymol are able to disintegrate the outer membrane of bacteria, release lipopolysaccharides, increase the permeability of the cytoplasmic membrane and allow ions to leave the cytoplasm (Ultee *et al*., 2002). In addition, other minor constituents such as the monoterpene hydrocarbons gamma-terpinene and paracymene also contribute to the antibacterial activity of OEO (Betancourt *et al*., 2012). Antimicrobial activity of paracymene has not been reported in a previous study (Aligiannis *et al*., 2001). Probably, the lack of activity of these hydrocarbon monoterpenes is due to absence of the phenolic hydroxyl group (Nostro *et al*., 2004).

However, until now no study has been conducted to investigate the antimicrobial properties of OEO and its major components against fish pathogenic bacteria. Therefore, this study was carried out to examine the potential of OEO as well as its main components, carvacrol and thymol, which could be utilized in aquaculture as potential alternatives to commercial antibiotics.

Materials and methods

Bacteria

As test strains, seven Gram-negative and nine Gram-positive bacteria strains isolated from Korean cultured olive flounder were used. The Gram-negative strains were *E. tarda* (ED47, ED45, Yoshida and FP5060), *P. damselae* (FP4101), *V. harveyi* (FP 8370) and *V. ichthyoenteri* (FP 4004), and the Gram-positive strains were *L. garvieae* (FP5245), *S. iniae* (S131, S186, S530 and FP5228) and *S. parauberis* (S124, S527, S1466 and FP5228). They were obtained from Geyongsang National University (Jinju, Korea) and National Institute of Fisheries Science (Busan, Korea).

Essential oil

The 100% pure OEO (Neumond GmbH, Raisting, Germany) was purified from oregano herb grown in Hungary and the commercial carvacrol (>99 %) and thymol (>95 %) were purchased (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan).

Disk diffusion assay

The disk diffusion assay with OEO, carvacrol and thymol was conducted to detect the antimicrobial activity. Sterile paper disks (Advantec Toyo Kaisha, Ltd., Japan) were impregnated with 20 µL of OEO, carvacrol and thymol with different dilutions; [1%, 5%, 10%, 25%, 50%, 100% (V/V)] and each disk was placed on a Mueller Hinton agar (MB Cell, LA, CA) plate smeared with the test strain. Plates were incubated for 24 h at 27ºC to determine the antimicrobial

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effect. Antibacterial activity was determined by measuring the inhibition zone diameter (IZD) (mm) against each test strain. The antibacterial activity was expressed as the percentage of relative inhibition zone diameter (RIZD %) calculated according to a previous study (Njau *et al*., 2014) using amoxicillin as standard antibiotic.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The determination of MIC was done using broth micro dilution method with some modifications using different concentrations which 5% DMSO was used to dissolve OEO, carvacrol and thymol. The MIC was measured after 24 h incubation and each test was conducted in triplicates. In order to evaluate the bactericidal/bacteriostatic effects of these oils, the cultured medium from wells which have no visible bacterial growth was smeared on separate tryptic soy agar (TSA) (MB Cell, LA, CA) plates and incubated for 24 h at 27°C. The concentration, at which no growth was observed on TSA plate, was determined as MBC. All the procedures were done as suggested by Hammer *et al*. (1999).

Determination of antibiotic sensitivity pattern of the bacterial strains

The antibiogram of the test strains was studied by disk diffusion method using fourteen antibiotics, and their multiple antibiotic resistant index (MRI %) pattern was determined in order to compare the antibacterial activity of the oils with standard antibacterial drug. Resistance profiles (resistant, intermediate or susceptible) were assigned using criteria described by Clinical and Laboratory Standards Institute (CLSI, 2014). The MRI% was determined following the method described by Das *et al.* (2012). Each test was repeated three times.

Statistical analysis

For the statistical analysis, differences were considered significant at $p<0.05$ level. The significant comparison was done with respect to MIC values and RIZD % of oregano oil, carvacrol and thymol using SPSS version 23 statistical analysis software (SPSS Inc., USA).

Results and discussion

The genus *Origanum* can be divided into three chemo types: carvacrol-type, thymol-type and sabinene-type (Betancourt *et al.*, 2012). The OEO obtained in the present study belongs to carvacrol type as it constitutes 63.3 % of carvacrol and 4.2 % of thymol (Table 1). Studies have reported about carvacrol type OEO and they showed variations of carvacrol content (Boskovic *et al*., 2015; Garvic *et al*., 2015). OEO has shown antimicrobial activity against several pathogenic bacteria where it was suggested to damage the bacterial membrane integrity, thereby altering the pH homeostasis and equilibrium of inorganic ions (Lambert *et al*., 2001).

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Table 1: Composition of oregano essential oil used in this study.

Compound name	Composition (%) [a]
Carvacrol	63.30
Pracymene	12.90
Thymol	4.20

[a]Composition of the essential oil was analysed by Neumond GmbH, Raisting, German.

According to disk diffusion test results, OEO inhibited the growth of all test bacteria at every concentration except 1 % and 5 % (V/V) (Table 2). The IZDs of major components were also found to be similar to those of OEO except inhibitions at 5 % concentration (V/V) of carvacrol and thymol against five Gram-positive strains [*S. iniae* (S186, S530, and S131) and *S. parauberis* (S124, S527)] and two Gram-positive strains [*S. iniae* (S186 and S131)], respectively. The IZDs of Gram-negative bacteria ranged from 26 to 32 mm and the IZDs of Gram-positive bacteria ranged from 24 to 35 mm at 100 % (V/V) of OEO. The IZD of Gram-negative bacteria ranged from 30 to 34 mm at 100 % (V/V) of carvacrol and 28 to 30 at 100 % (V/V) of thymol while the IZD of Gram-positive bacteria ranged from 36 to 42 mm for 100 % (V/V) of carvacrol and 26 to 40 mm for 100 % of thymol. In every tested fish pathogenic bacteria, the IZD increased in proportion to the concentration and the maximum effect was found at 100 % (V/V) concentration of OEO, carvacrol and thymol. In comparison, carvacrol showed higher IZD values than thymol and OEO. In previous studies, carvacrol was also more active than its isomer thymol against several pathogenic bacteria even at lower concentrations (Xu *et al*., 2008; Mathela *et al*., 2010). OEOs can act on bacterial proteins with different mechanisms depending on their different compositions (Betancourt *et al.*, 2012).

Table 2: Inhibition zone diameter (IZD) and the percentage of relative inhibition zone diameter (RIZD %) values of Oregano essential oil (OEO) carvacrol and thymol against fish pathogenic bacteria.

Bacteria	Agent [a]	Inhibition zone diameter (mm) and RIZD % [b]											
		1 % (V/V)		5 % (V/V)		10 % (V/V)		25 % (V/V)		50 % (V/V)		100 % (V/V)	
		IZD	RIZD	IZD	RIZD	IZD	RIZD	IZD	RIZD	IZD	RIZD	IZD	RIZD
Vibrio harveyi (FP8370)	**O**	0	0	0	0	20	83.3	22	91.7	24	100	28	117
	C	0	0	0	0	15	62.5	21	87.5	25	104	30	125
	T	0	0	0	0	12	50	14	58.3	20	83.3	28	71.4
V. ichthyoenteri (FP4004)	**O**	0	0	0	0	18	47.4	20	52.6	24	63.2	26	68.4
	C	0	0	0	0	15	39.5	20	52.6	27	71.1	32	84.2
	T	0	0	0	0	11	28.9	16	42.1	25	65.8	30	78.9
Photobacterium damselae (FP4101)	**O**	0	0	0	0	0	0	16	59.3	20	74.1	26	96.3
	C	0	0	0	0	11	40.7	20	74.1	25	92.6	30	111

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Table 2 Continued:

Bacteria	Agent [a]	Inhibition zone diameter (mm) and RIZD % [b]												
		1 % (V/V)		5 % (V/V)		10 % (V/V)		25 % (V/V)		50 % (V/V)		100 % (V/V)		
		IZD	RIZD	IZD	RIZD	IZD	RIZD	IZD	RIZD	IZD	RIZD	IZD	RIZD	
	T	0	0	0	0	11	40.7	17	63	22	81.5	30	111	
Edwardsiella tarda (FP5060)	**O**	0	0	0	0	14	51.9	23	85.2	25	92.6	28	104	
	C	0	0	0	0	11	40.7	20	74.1	25	92.6	30	111	
	T	0	0	0	0	10	37	15	55.6	24	88.9	30	111	
E. tarda (ED47)	**O**	0	0	0	0	20	83.3	24	100	28	116	32	133	
	C	0	0	0	0	18	75	23	95.8	28	116	34	141	
	T	0	0	0	0	20	83.3	22	91.7	24	100	28	116	
E. tarda (Yoshida)	**O**	0	0	0	0	18	66.7	21	77.8	23	85.2	30	111	
	C	0	0	0	0	16	59.3	20	74.1	24	88.9	30	111	
	T	0	0	0	0	14	51.9	17	63	22	81.5	28	103	
E. tarda (ED45)	**O**	0	0	0	0	16	66.6.6	24	100	26	108	30	125	
	C	0	0	0	0	18	75	22	91.7	28	116	32	133	
	T	0	0	0	0	20	83.3	24	100	25	104	28	116	
Lactococcus garvieae (FP5245)	**O**	0	0	0	0	15	60	28	112	30	120	34	136	
	C	0	0	0	0	11	44	22	88	30	120	36	144	
	T	0	0	0	0	10	40	18	72	20	80	28	112	
Streptococcus iniae (FP3287)	**O**	0	0	0	0	17	60.7	24	85.7	30	107	35	125	
	C	0	0	0	0	10	35.7	24	85.7	30	107	38	135	
	T	0	0	0	0	16	57.1	24	85.7	30	107	34	124	
S. iniae (S186)	**O**	0	0	0	0	17	48.6	20	57.1	23	65.7	32	91.4	
	C	0	0	10	28.6	30	85.7	32	91.4	34	97.1	42	120	
	T	0	0	12	34.3	20	57.1	26	74.3	34	97.1	40	114	
S. iniae (S530)	**O**	0	0	0	0	11	28.9	17	44.7	20	52.6	30	78.9	
	C	0	0	10	26.3	12	31.6	24	63.2	32	84.2	34	89.5	
	T	0	0	0	0	20	52.6	23	60.5	30	78.9	34	89.5	
S. iniae (S131)	**O**	0	0	0	0	12	34.3	16	45.7	25	71.4	34	97.1	
	C	0	0	14	40	17	48.6	24	68.6	30	85.7	38	108	
	T	0	0	10	28.6	12	34.3	20	57.1	32	91.4	35	100	
Streptococcus parauberis (FP5228)	**O**	0	0	0	0	0	0	14	50	17	60.7	30	107	
	C	0	0	0	0	16	57.1	20	71.4	27	96.4	36	128	
	T	0	0	0	0	18	64.3	20	71.4	24	85.7	34	121	
S. parauberis (S124)	**O**	0	0	0	0	10	40	14	56	22	88	26	104	
	C	0	0	10	40	20	80	30	120	34	136	40	160	
	T	0	0	0	0	17	68	21	84	22	88	30	120	
S. parauberis (S527)	**O**	0	0	0	0	0	0	18	62.1	24	82.8	30	103	
	C	0	0	10	34.5	20	69	24	82.8	28	96.6	34	117	
	T	0	0	0	0	12	41.4	13	44.8	16	55.2	26	89.7	
S. parauberis (S1466)	**O**	0	0	0	0	0	0	16	59.3	25	92.6	30	111	
	C	0	0	0	0	18	66.7	20	74.1	26	96.3	34	125	
	T	0	0	0	0	16	59.3	18	66.7	20	74.1	30	111	

[a] O- OEO, C- Carvacrol, T- Thymol. [b] IZD- Inhibition zone diameter, RIZD-Relative inhibition zone diameter percentage.

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RIZD % exhibited high values at higher concentration of OEO, carvacrol and thymol. The highest RIZD % for OEO was 136 against *L. garvieae* (FP5245) and for carvacrol and thymol it was 160 against *S. parauberis* (S124) and 124 against *S. iniae* (FP3287), respectively. It was observed that carvacrol and thymol had zero RIZD % against all Gram-negative strains at 1% and 5% (V/V) concentrations. Gram-positive strains exhibited higher RIZD % than Gram-negative bacteria and there was a significant difference between RIZD % of Gram-negative and Gram-positive strains (p<0.05).

In the present study, the MIC of OEO for bacterial strains ranged from 0.031 to 0.062 % (V/V) (Table 3). Carvacrol and thymol showed lower MIC values than OEO against every bacterium which ranged from 0.001 to 0.007 % (V/V) and 0.001 to 0.062 % (V/V), respectively. OEO showed a minor inhibitory effect because carvacrol and thymol represent only a fraction of the entire essence and they interact in an additive rather than a synergistic way (Nostro *et al.* 2007). A significant difference (p<0.05) was observed between Gram-positive and Gram-negative bacteria which is in agreement with the results of Choi *et al*. (2012). Carvacrol (MBC/MIC 1-4) and thymol (MBC/MIC 2-4) demonstrated only bactericidal activity against the pathogenic bacteria while OEO (MBC/MIC 2-8) was both bactericidal and bacteriostatic. In previous studies, the effect of OEO was both bactericidal and bacteriostatic for *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Listeria monocytogenes* (Tsigarida *et al*., 2000; Lambert *et al*., 2001). Carvacrol and thymol demonstrated bactericidal activity at lower concentrations against *Lactobacillus* strains (Du *et al*., 2015). The antimicrobial activity of OEO is mostly attributed to the action of its principal phenolic components, carvacrol and thymol, which exhibited significant bactericidal activity when tested separately against *Campylobacter jejuni*, *Escherichia coli* and *Salmonella enterica typhi* (Friedma *et al*., 2002; Nostro *et al.* 2004).

Table 3: Susceptibility pattern of oregano essential oil, carvacrol and thymol against fish pathogenic bacteria.

Bacteria	Oregano		MBC/MIC
	MIC % (V/V)	MBC % (V/V)	
Vibrio harveyi (FP8370)	0.062	0.125	2
V. ichthyoenteri (FP4004)	0.062	0.125	2
Photobacterium damselae (FP4101)	0.031	0.25	8
Edwardsiella tarda (FP5060)	0.062	0.25	4
E. tarda (ED47)	0.062	0.25	4
E. tarda (Yoshida)	0.062	0.125	2
E. tarda (ED45)	0.125	0.5	4

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Table 3 Continued:

Bacteria	Oregano		MBC/MIC
	MIC % (V/V)	MBC % (V/V)	
Lactococcus garvieae (FP5245)	0.062	0.125	2
Streptococcus iniae (FP3287)	0.062	0.25	4
S. iniae (S186)	0.062	0.125	2
S. iniae (S530)	0.062	0.125	2
S. iniae (S131)	0.031	0.125	4
S. parauberis (FP5228)	0.062	0.125	2
S. parauberis (S124)	0.062	0.125	2
S. parauberis (S527)	0.062	0.125	2
S. parauberis (S1466)	0.031	0.25	8
Bacteria	**Carvacrol**		
Vibrio harveyi (FP8370)	0.007	0.015	2
V. ichthyoenteri (FP4004)	0.007	0.015	2
Photobacterium damselae (FP4101)	0.015	0.062	4
Edwardsiella tarda (FP5060)	0.007	0.015	2
E. tarda (ED47)	0.007	0.015	2
E. tarda (Yoshida)	0.003	0.015	4
E. tarda (ED45)	0.003	0.015	4
Lactococcus garvieae (FP5245)	0.007	0.007	1
Streptococcus iniae (FP3287)	0.003	0.007	2
S. iniae (S186)	0.007	0.015	2
S. iniae (S530)	0.007	0.015	2
S. iniae (S131)	0.001	0.007	4
S. parauberis (FP5228)	0.007	0.015	2
S. parauberis (S124)	0.015	0.015	1
S. parauberis (S527)	0.001	0.003	2
S. parauberis (S1466)	0.001	0.007	4
Bacteria	**Thymol**		
Vibrio harveyi (FP8370)	0.031	0.062	2
V. ichthyoenteri (FP4004)	0.031	0.125	4
Photobacterium damselae (FP4101)	0.015	0.031	2
Edwardsiella tarda (FP5060)	0.007	0.031	4
E. tarda (ED47)	0.015	0.031	2
E. tarda (Yoshida)	0.015	0.062	4
E. tarda (ED45)	0.015	0.062	4
Lactococcus garvieae (FP5245)	0.031	0.062	2
Streptococcus iniae (FP3287)	0.031	0.125	4
S. iniae (S186)	0.031	0.062	2
S. iniae (S530)	0.001	0.007	4
S. iniae (S131)	0.001	0.007	4

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Table 3 Continued:

Bacteria	Oregano		MBC/MIC
	MIC % (V/V)	MBC % (V/V)	
Streptococcus parauberis (FP5228)	0.015	0.062	4
S. parauberis (S124)	0.007	0.015	2
S. parauberis (S527)	0.031	0.062	2
S. parauberis (S1466)	0.007	0.031	4

The antibiogram pattern indicated that all the bacterial strains excluding three strains of *S. iniae* (S186, S530, and S131) showed resistance to one or more antibiotics (Table 4). The MRI % of the isolates ranged between 0-57.1. *E. tarda* (ED45 and ED47) showed the highest MRI % (57.1), followed by both *L. garvieae* (FP5245) and *S. iniae* (FP3287) (35.7). *S. iniae* (S186, S530 and S131) showed the lowest MRI % which in turn reflects its susceptibility towards the antibiotics. It is important to mention that the degree of inhibition (in terms of zone size) by OEO, carvacrol or thymol was distinct compared to the standard antibiotics. In some strains the zones of inhibition by the antibiotics were smaller with the zones of inhibition represented by the OEO and thymol, while IZD of all Gram-positive strains at 100% (V/V) of carvacrol were larger than zones of inhibition by the antibiotics.

Table 4: Antibiogram pattern of the fish pathogenic bacteria.

Bacteria	Antibiotics [a]		MRI %
	Sensitive	Resistant	
Vibrio harveyi (FP8370)	AMX, AMP,CTX.CRO,TCI,CHL, OFX, IMI,SXT, E, DA	VA, NAL, CN	21.4
V. ichthyoenteri (FP4004)	AMX, AMP,CTX.CRO,TCI,CHL, OFX, NAL, CN, IMI,SXT, E, DA	VA,	7.1
Photobacterium damselae (FP4101)	AMX, AMP,CTX.CRO,TCI,CHL, OFX, NAL, CN,IMI,SXT, E, DA	VA,	7.1
Edwardsiella tarda (FP5060)	AMX,CTX.CRO,TCI,CHL, OFX,NAL, IMI,SXT, E, DA	AMP, CN, VAN,	21.4
E. tarda (ED47)	AMX, CTX, CRO,IMI, E, DA	AMP, TCI, CHL, VA, NAL SXT, OFX,CN	57.1
E. tarda (Yoshida)	AMX, AMP,CTX.CRO,TCI,CHL, OFX, NAL,CN, IMI, SXT, E, DA,	VA,	7.1
E. tarda (ED45)	AMX, CTX, CRO, IMI, E, DA	AMP,TC,CHL, VA, NAL, SXT, OFX, CN	57.1
Lactococcus garvieae (FP5245)	AMX, TC,DA, E, VA, NAL, CN IMI, SXT	AMP, CTX, CRO, CHL OFX	35.7

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Table 4 Continued:

Bacteria	Antibiotics [a]		MRI %
	Sensitive	**Resistant**	
Streptococcus iniae (FP3287)	AMX, TC, CHL, E, VA, NAL, CN IMI, SXT	AMP, CTX, CRO, DA, OFX	35.7
S. iniae (S186)	AMX, AMP ,CTX, CRO, TC, CHL E, DA, VA,OFX,NAL,CN,IMI,SXT	-	0
S. iniae (S530)	AMX, AMP ,CTX, CRO, TC, CHL E, DA, VA,OFX,NAL,CN,IMI,SXT	-	0
S. iniae (S131)	AMX, AMP ,CTX, CRO, TC, CHL, E DA, VA,OFX, NAL,CN, IMI, SXT	-	0
Streptococcus parauberis (FP5228)	AMX, CTX, CRO, TC E DA VA OFX,NAL,CN, IMI, SXT	AMP, CHL	14.3
S. parauberis (S124)	AMX, CTX,CRO,VA, CHL, OFX NAL, CN, IMI, SXT	AMP, TC, E, DA	28.6
S. parauberis (S527)	AMX, CTX, CRO, TC, CHL, SXT E, DA, VA, OFX, NAL, CN, IMI	AMP	7.1
S. parauberis (S1466)	AMX,CTX, CRO, TC, CHL,NAL DA, VA, OFX, CN, IMI, SXT	AMP, E	14.3

[a] Antibiotics- AMP10=ampicillin (10 µg), CTX30=cefotaxime (30 µg), CRO30=ceftriaxone (30 µg), TC15=tetracycline (15 µg), CHL30=chloramphenicol (30 µg), E15=erythromycin (15 µg), DA10=clindamycin (10 µg), VA30=vancomycin (30 µg), OFX5=ofloxacin (5 µg), NAL30=nalidixic acid (30 µg), CN10=gentamicin (10 µg), IMI10=imipenem (10 µg) and SXT25= trimethoprim-sulfamethoxazole (25 µg), AMX30=amoxicillin (30 µg).

Antimicrobial activity of the OEO, carvacrol and thymol may be due to inhibition of cell membrane synthesis, specifically due to their hydrophobic nature. Carvacrol and thymol interact with the lipid bilayer of cytoplasmic membranes causing loss of integrity and leakage of cellular material such as ions, ATP and nucleic acid (Trombetta, *et al*., 2005). A previous study reported the antibacterial activity of OEO against both *S. aureus* and *P. aeruginosa* suggesting that OEO may inhibit the cell membrane synthesis of bacteria, mostly by potassium and phosphate leakage (Lambert *et al.*, 2001).

Although, OEO, carvacrol and thymol inhibited the growth of tested fish pathogenic bacteria isolated from olive flounder, carvacrol could inhibit the growth of bacteria at lower concentrations than OEO and thymol. According to the findings of present study OEO, carvacrol and thymol are good candidates for further research to develop a new alternative antibacterial drug against fish pathogenic bacteria. Studies about pharmacokinetics of carvacrol and thymol, and acute and short-term *in vivo* effects suggested that they may not pose a risk for human and animal health (De Vincenzi *et al*., 2004;

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Chami *et al.* 2005). Therefore, in order to apply OEO, carvacrol and thymol for treatment of bacterial diseases in aquaculture, the stability in the aquatic environment, and the palatability and absorption rate in fish should be further investigated.

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