FISHERIES & AQUATIC LIFE (2018) 26: 131-139
Archives of Polish Fisheries
DOI 10.2478/aopf-2018-0014

DE GRUYTER OPEN

RESEARCH ARTICLE

Antibacterial activity of lime (*Citrus aurantifolia*) essential oil and limonene against fish pathogenic bacteria isolated from cultured olive flounder (*Paralichthys olivaceus*)

Hansani N.K.S. Pathirana, Sudu H.M.P. Wimalasena, Benthotage C.J. De Silva, Sabrina Hossain, Gang-Joon Heo

Received – 30 October 2017/Accepted – 29 May 2018. Published online: 30 June 2018; ©Inland Fisheries Institute in Olsztyn, Poland
Citation: Pathirana H.N.K.S., Wimalasena S.H.M.P., De Silva B.C.J., Hossain S., Heo G.-J. 2018 – Antibacterial activity of lime (*Citrus aurantifolia*) essential oil and limonene against fish pathogenic bacteria isolated from cultured olive flounder (*Paralichthys olivaceus*) – Fish. Aquat. Life 26: 131-139

Abstract. The antibacterial activity of lime (*Citrus aurantifolia*) essential oil (LEO) and limonene was tested against seven Gram-negative and nine Gram-positive fish pathogenic bacteria isolated from cultured olive flounder, *Paralichthys olivaceus* (Temminck & Schlegel) in Korea. Limonene was >99% concentrated and LEO consisted of eleven chemical compounds including 56.22% of limonene. Disk diffusion assay, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) tests were done. LEO and limonene inhibited the growth of both Gram-negative and Gram-positive bacteria. LEO and limonene (MBC/MIC= 2-8) were both bactericidal and bacteriostatic for the strains tested. In every fish pathogenic bacteria, the inhibition zone diameter (IZD) increased in proportion to the oil concentration and the maximum effect was found at 100% (V/V) concentrations of LEO and limonene. 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. The percentage of the relative inhibition zone diameter (RIZD %) exhibited high values at higher concentrations of all the agents. Since antibacterial activities of LEO and limonene were considerably effective against fish pathogenic bacteria, they could be used as alternatives to treat bacterial infections in aquaculture.

Keywords: antibacterial activity, lime essential oil (LEO), limonene, fish pathogenic bacteria, olive flounder

H. N.K.S. Pathirana, S. H.M.P. Wimalasena, B. C.J. De Silva, S. Hossain, G.-J. Heo [✉]
Laboratory of Aquatic Animal Medicine, Veterinary Medical Center and College of Veterinary Medicine
Chungbuk National University, Chungdae-ro 1, Seowon-gu, Cheongju, Chungbuk 28644, Korea
e-mail: gjheo@cbu.ac.kr

Introduction

Bacterial diseases pose one of the major threats to the aquaculture industry 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). Disease outbreaks are responsible for elevated mortality rates and decreased productivity, causing high economic losses in olive flounder (*Paralichthys olivaceus*) farms in Korea (Nho et al. 2009).

© Copyright by Stanisław Sakowicz Inland Fisheries Institute in Olsztyn.
© 2018 Author(s). This is an open access article licensed under the Creative Commons Attribution-NonCommercial-NoDerivs License (http://creativecommons.org/licenses/by-nc-nd/3.0/).

Antibiotics are used widely to prevent bacterial infections in fish. However, the misuse of antibiotics leads to drug resistance as well as reduced efficacy (Wei and Wee 2013) Therefore, it is essential to develop antibacterial drugs that are made from natural substances. Natural products, especially those from plants, have been investigated for their therapeutic and prophylactic effects on several fish diseases (Pongsak and Parichat 2009, Turker and Yildirim 2015, Vallado et al. 2015). Essential oils are important plant products that have been exploited for their aromatic, flavor, bactericidal, preservative, and medicinal properties (Burt 2004).

Citrus aurantifolia, which belongs to Rutaceae, was recently found to be a hybrid between citron (a cluster of *C. medica* and *C. indica*) and *C. micrantha* by phylogenetic studies. (Indo-Malayan region) (Nicolosi et al. 2000). It is considered as a native species from southeast Asia and is widespread in tropical and subtropical regions around the world such as North America (Florida, Texas, California, Mexico, etc.), India, Egypt, and Central America (Morton 1987). Lime essential oil (LEO) is used in traditional medicine, as flavoring agents in beverages and manufactured foods, and ingredients in perfumes (Morton. 1987, Apraj et al. 2011). LEO has shown antimicrobial, radical scavenging, anti-cholinesterase, anthelmintic, and anticancer activities (Gharagozloo et al. 2002, Taur et al. 2009, Jafari et al. 2011, Tundis et al. 2012) . LEO has been analyzed by GC-MS analysis in previous studies and limonene was the major component (Chisholm et al. 2003, Craske et al. 2005). However, to date no study has been conducted to investigate the antimicrobial property of LEO and its major components against fish pathogenic bacteria isolated from olive flounder, *Paralichthys olivaceus* (Temminck & Schlegel). Therefore, this study was conducted to examine the potential of LEO as well as limonene as alternatives to commercial antibiotics in aquaculture use.

Material and methods

Seven Gram-negative and nine Gram-positive bacterial strains isolated from Korean cultured olive flounder were used as the test strains. The Gram-negative strains were *E. tarda* (FP5060, ED47, Yoshida and ED45), *P. damselae* (FP4101), *V. harveyi* (FP 8370), and *V. ichthyoenteri* (FP 4004), and the Gram-positive strains were *L. garvieae* (FP5245), *S. iniae* (FP5228, S186, S530 and S131), and *S. parauberis* (FP5228, S124, S527 and S1466). The strains were obtained from Geyongsang National University (Jinju, Korea) and the National Institute of Fisheries Science (Busan, Korea). The 100% pure LEO (Aromarant Co. Ltd., Rottingen, Germany) purified from the peels of lime grown in Madagascar and the commercial trans-limonene (>99%) (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan) were purchased.

The disk diffusion assay with LEO and limonene was conducted to detect antimicrobial activity. Sterile disks (Advantec Toyo Kaisha, Ltd., Japan) were impregnated with 20 µL of LEO and limonene at 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 organism. The plates were incubated for 24 h at 27 şC to determine the antimicrobial effect. Antibacterial activity was determined by measuring the inhibition zone diameter (IZD) (mm) against each test organism. The antimicrobial activity was expressed as the percentage of the relative inhibition zone diameter (RIZD %) and was calculated according to Njau et al. (2014) using amoxicillin as the standard antibiotic. Determinations of the minimum inhibitory concentration (MIC) were done with the broth micro dilution method with some modifications using different concentrations in which 5% DMSO was used to dissolve LEO and limonene. The MIC was measured after 24 h incubation, and each test was conducted in triplicate. To determine the minimum bactericidal concentration (MBC), the cultured medium from wells that had higher concentrations of LEO and limonene than MIC was smeared on

separate tryptic soy agar (MB Cell, LA, CA) plates and incubated for 24 h at 27°C (Hammer et al. 1999). The concentration at which no growth was observed on the TSA plate was determined as the MBC.

The antibiogram of the test strains was studied with the disk diffusion method using fourteen antibiotics, and their multiple antibiotic resistant indexes (MRI %) were determined to compare the antibacterial activity of the oils with standard antibacterial drugs. Resistance profiles were assigned using criteria described by the 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.

Results

The LEO used in the present study contained 56.22% limonene (Table 1). The IZD of Gram-negative bacteria ranged from 15 to 17 mm, and the IZD of Gram-positive bacteria ranged from 14 to 25 mm at 100% (V/V) LEO, while the IZD of Gram-negative bacteria ranged from 17 to 24 mm, and the IZD of Gram-positive bacteria ranged from 20 to 30 mm in 100% (V/V) limonene. The RIZD % exhibited high values at higher concentrations of both limonene and LEO (Table 2).

Table 1
Composition of lime essential oil used in this study

Compound name	Composition (%)[a]
Limonene	56.22
gamma-Terpinene	14.31
beta-Pinene	10.96
Geranial	2.28
alpha-Pinene	2.09
Sabinene	1.79
beta-Bisabolen	1.61
Neral	1.46
Myrcene	1.4
trans-alpha-Bergamotene	1.09
Neryl acetate	0.86

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

The MIC values of the LEO for Gram-negative bacterial strains ranged from 0.0625 to 0.25% (V/V), and for Gram-positive strains it ranged from 0.031 to 0.5% (V/V) (Table 3). The MIC of limonene against Gram-negative bacteria ranged from 0.031 to 0.062% (V/V), and against Gram-positive bacteria it was 0.007 to 0.25% (V/V). The mean MBC/MIC for LEO and limonene was 2-8 (Table 3).

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, reflected its susceptibility to antibiotics.

Discussion

Many reports claim that limonene is the major compound in LEO. However, the inhibitory activity of LEO stems from the presence of several constituents, mainly limonene, beta-Pinene, gama-terpinene, and Myrcene (Craske et al. 2005, Tundis et al. 2012). As a result of lipophilicity, terpenes accumulate in the lipid structure of cell walls that causes proteins to denature and the loss of cell membrane integrity leading to membrane damage and finally bacterial death. Synergistic effects against pathogens might have resulted from the mixture of chemically different terpenes (Fisher and Phillips 2008, Galluci et al. 2009).

According to the disk diffusion test results, LEO inhibited the growth of all the bacteria tested at every concentration except 1%, 5%, and 10%. In contrast, similar IZDs were observed for two strains. Aibinu et al. (2007) observed that each bacterial strain demonstrated a significant degree of sensitivity to LEO, and extensive activity against Gram-positive bacteria, producing a clear zone of inhibition against the majority of the strains tested. In a previous study, the highest inhibitory zone was observed against *Bacillus*

Table 2
Inhibition zone diameter and the percentage of relative inhibition zone diameter (RIZD %) values of lime essential oil (LEO) and trans-limonene against fish pathogenic bacteria

		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)		
Bacteria	Agent[a]	IZD	SE	RIZD	IZD	SE	RIZD	IZD	SE	RIZD	IZD	SE	RIZD	IZD	SE	RIZD	IZD	SE	RIZD
Vibrio harveyi (FP8370)	L	0	±0.0	0	0	±0.0	0	0	±0.0	0	10	±0.1	41.7	15	±0.4	62.5	16	±0.1	66.7
	LN	0	±0.0	0	0	±0.0	0	12	±0.5	50	16	±0.0	66.7	20	±0.2	83.3	21	±0.5	87.5
V. ichthyoenteri (FP4004)	L	0	±0.0	0	0	±0.0	0	0	±0.0	0	12	±0.0	31.6	14	±0.5	36.8	17	±0.3	44.7
	LN	0	±0.0	0	0	±0.0	0	10	±0.1	26.3	15	±0.0	39.5	21	±0.4	55.3	23	±0.2	60.5
Photobacterium damselae (FP4101)	L	0	±0.0	0	0	±0.0	0	0	±0.0	0	11	±0.2	40.7	14	±0.2	51.9	18	±0.0	66.7
	LN	0	±0.0	0	0	±0.0	0	0	±0.0	0	11	±0.5	40.7	23	±0.5	85.2	24	±0.5	88.9
*Edwardsiella tarda (*FP5060)	L	0	±0.0	0	0	±0.0	0	0	±0.0	0	10	±0.1	37	15	±0.3	55.6	17	±0.1	63
	LN	0	±0.0	0	0	±0.0	0	0	±0.0	0	10	±0.1	37	13	±0.1	48.1	17	±0.2	63
E. tarda (ED47)	L	0	±0.0	0	0	±0.0	0	0	±0.0	0	12	±0.3	50	14	±0.3	58.3	15	±0.5	62.5
	LN	0	±0.0	0	0	±0.0	0	8	±0.3	33.3	10	±0.1	41.7	18	±0.1	75	19	±0.4	79.2
E. tarda (Yoshida)	L	0	±0.0	0	0	±0.0	0	0	±0.0	0	11	±0.2	40.7	13	±0.2	48.1	16	±0.3	59.3
	LN	0	±0.0	0	0	±0.0	0	0	±0.0	0	10	±0.2	37	22	±0.1	81.5	23	±0.5	88.9
E. tarda (ED45)	L	0	±0.0	0	0	±0.0	0	0	±0.0	0	8	±0.3	33.3	12	±0.5	50	15	±0.2	62.5
	LN	0	±0.0	0	0	±0.0	0	8	±0.2	33.3	14	±0.5	58.3	18	±0.3	75	20	±0.2	83.3
Lactococcus garvieae (FP5245)	L	0	±0.0	0	0	±0.0	0	10	±0.1	40	12	±0.2	48	16	±0.2	64	22	±0.3	88
	LN	0	±0.0	0	0	±0.0	0	10	±0.1	40	15	±0.1	60	16	±0.3	64	20	±0.0	80
Streptococcus iniae (FP3287)	L	0	±0.0	0	0	±0.0	0	11	±0.5	39.3	16	±0.4	57.1	18	±0.5	64.3	20	±0.4	71.4
	LN	0	±0.0	0	0	±0.0	0	12	±0.4	42.9	16	±0.5	57.1	18	±0.4	64.3	23	±0.4	82.1
S. iniae (S186)	L	0	±0.0	0	0	±0.0	0	0	±0.0	0	8	±1	22.9	12	±0.2	34.3	19	±0.1	54.3
	LN	0	±0.0	0	0	±0.0	0	16	±0.2	45.7	20	±0.1	57.1	22	±0.1	62.9	30	±0.1	85.7
S. iniae (S530)	L	0	±0.0	0	0	±0.0	0	8	±0.3	21.1	11	±0.3	28.9	15	±0.5	39.5	18	±0.2	47.4
	LN	0	±0.0	0	0	±0.0	0	16	±0.2	42	19	±0.5	50	20	±0.3	52.6	28	±0.0	73.7

		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)		
Bacteria	Agent[a]	IZD	SE	RIZD	IZD	SE	RIZD	IZD	SE	RIZD	IZD	SE	RIZD	IZD	SE	RIZD	IZD	SE	RIZD
S. iniae (S131)	L	0	±0.0	0	0	±0.0	0	0	±0.0	0	10	±0.1	28.6	13	±0.1	37.1	16	±0.3	45.7
	LN	0	±0.0	0	0	±0.0	0	10	±0.2	28.6	15	±0.1	42.9	18	±0.2	51.4	25	±0.1	71.4
Streptococcus parauberis (FP5228)	L	0	±0.0	0	0	±0.0	0	0	±0.0	0	10	±0.1	35.7	12	±0.3	42.9	14	±0.3	50
	LN	0	±0.0	0	0	±0.0	0	13	±0.7	46.4	16	±0.2	57.1	18	±0.1	64.3	24	±0.1	85.7
S. parauberis (S124)	L	0	±0.0	0	0	±0.0	0	15	±0.2	60	22	±0.5	88	23	±0.5	92	25	±0.5	96
	LN	0	±0.0	0	0	±0.0	0	16	±0.2	64	17	±0.1	68	18	±0.2	72	20	±0.2	80
S. parauberis (S527)	L	0	±0.0	0	0	±0.0	0	12	±0.1	41.4	14	±0.2	48.3	15	±0.3	51.7	22	±0.1	75.9
	LN	0	±0.0	0	0	±0.0	0	12	±0.2	41.4	15	±0.3	51.7	19	±0.1	65.5	26	±0.5	89.7
S. parauberis (S1466)	L	0	±0.0	0	0	±0.0	0	8	±0.3	29.6	13	±0.6	48.1	14	±0.6	51.9	17	±0.1	63

[a] L – lime, LN – Limonene, [b] IZD – Inhibition zone diameter, RIZD – Relative inhibition zone diameter percentage, SE – standard error

Table 3
Susceptibility pattern of lime essential oil and limonene against fish pathogenic bacteria

	lime			limonene		
Bacteria	MIC(%v/v)	MBC(%v/v)	MBC/MIC	MIC(%v/v)	MBC(%v/v)	MBC/MIC
Vibrio harveyi (FP8370)	0.125	1	8	0.031	0.25	8
V. ichthyoenteri (FP4004)	0.125	0.5	4	0.031	0.125	4
Photobacterium damselae (FP4101)	0.062	0.125	2	0.062	0.125	2
Edwardsiella tarda (FP5060)	0.125	1	8	0.062	0.5	8
E. tarda (ED47)	0.25	1	4	0.062	0.25	4
E. tarda (Yoshida)	0.25	1	4	0.062	0.5	8
E. tarda (ED45)	0.25	1	4	0.062	0.125	2
Lactococcus garvieae (FP5245)	0.125	1	8	0.031	0.25	8
Streptococcus iniae (FP3287)	0.5	1	2	0.031	0.125	4
S. iniae (S186)	0.125	0.5	4	0.031	0.25	8
S. iniae (S530)	0.5	1	2	0.125	0.25	2
S. iniae (S131)	0.5	1	2	0.25	0.5	2
Streptococcus parauberis (FP5228)	0.125	1	8	0.007	0.062	8
S. parauberis (S124)	0.031	0.125	4	0.015	0.062	4
S. parauberis (S527)	0.031	0.25	8	0.015	0.062	4
S. parauberis (S1466)	0.031	0.25	8	0.007	0.031	4

subtilis followed by *Staphylococcus aureus* (Costa et al. 2014).

Meanwhile, limonene was found to be effective against almost all Gram-positive strains at every concentration except at 1% and 5% concentrations of isolates and *Edwardsiella tarda* (FP5060). However, it was obvious with 100% of all isolates. The widest IZD was 30 mm against *S. iniae* (S186). Limonene exhibited higher activity against Gram-positive strains than Gram-negative strains, and several studies report similar results against pathogenic bacteria (Nazzaro et al. 2013, Costa et al. 2014). Limonene showed the highest effectiveness against *S. aureus* compared to Gram-negative bacterial strains (Costa et al. 2014).

In comparison, limonene exhibited the highest IZD (30 mm) against the Gram-negative *Photobacterium damselae* (FP4101) bacterial strain and LEO exhibited the highest IZD (22 mm) against the Gram-positive *S. iniae* (S186) bacterial strain. The inhibition zones induced by the LEO or limonene were relative to the concentration of limonene. LEO contains 56.22% of limonene as well as ten other components, whereas the concentration of commercial limonene was >99%. The different components of essential oils can act on bacterial proteins through several mechanisms, and they can affect cell division (Nazzaro et al. 2013). However, limonene and LEO were effective against both Gram-positive and Gram-negative microorganisms. The IZD increased in proportion to limonene and LEO concentrations with every fish pathogenic bacteria tested, and the maximum effect was found at 100% (V/V) concentrations of both limonene and LEO.

All the Gram-positive strains had a higher RIZD % at every concentration of limonene except 1% and

Table 4
Antibiogram pattern of the fish pathogenic bacteria

	Antibiotics [a]		
Bacteria	Sensitive	Resistant	MRI %
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
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]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)

5% (V/V). It was observed that limonene had a zero RIZD % against Gram-positive strains at 1% and 5% (V/V) concentrations. This indicates that Gram-positive bacteria were not susceptible to every limonene concentration tested.

Limonene had lower MIC values than LEO. The MBC/MIC ratio of limonene and LEO (MBC/MIC 2–8) demonstrated bactericidal as well as bacteriostatic activity against 16 fish pathogenic bacteria. In a previous study, MBC and MIC values were identical, indicating that the effect of LEO was mainly bactericidal against *B. subtilis* and *S. epidermidis* (Jafari et al. 2011) and limonene demonstrated bactericidal activity against *S. pneumoniae*, *S. aureus*, *Escherichia coli*, and *Proteus mirabilis* (Vimal et al. 2013).

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. It is important to mention that the degree of inhibition (in terms of zone size) by LEO and limonene was distinct compared to that of standard antibiotics. In some strains the zones of inhibition of the antibiotics were smaller than those of the limonene at 100% (V/V) concentration.

The antimicrobial activity of LEO and limonene could stem from the inhibition of cell membrane synthesis, specifically because of their hydrophobic natures. The inactivation mechanism of limonene was mediated by the tri-carboxylic acid cycle that eventually promotes hydroxyl radical formation, leading to oxidative DNA damage, as is observed in bactericidal drugs. The production of hydroxyl radicals arises from the Fenton reaction in which ferrous iron transfers electrons to hydrogen peroxide (Repine et al. 1981). Therefore, hydroxyl radical stress increases when hydrogen peroxide concentrations are high (Liu and Imlay 2013). The indirect evidence observed with 2,2'-dipyridyl and the hydroxyl radical scavengers indicated that hydroxyl radical formation and the Fenton reaction play critical roles in effective killing by limonene as observed in bactericidal antibiotics (Imlay et al. 1988). Chueca et al. (2014) suggested that limonene could be effective by targeting bacterial systems that remediate hydroxyl radical damage as proteins causing DNA damage response. Interestingly, limonene was equally active against cells in both the stationary and exponential growth phases.

LEO and limonene generally inhibited the growth of the tested fish pathogenic bacteria isolated from olive flounder. The findings of the present study highlight the promising role of LEO and limonene as good candidates for further research to develop a new alternative antibacterial drug against fish pathogenic bacteria. They can be used to prevent or treat fish diseases by fish feed supplementation or immersion treatment (Mahato et al. 2017). Therefore, to apply LEO and limonene in the treatment of bacterial diseases in aquaculture, their stability in the aquatic environment, palatability, and absorption rate in fish should be investigated further.

Acknowledgements. The authors are thankful to Professor Tae-Sung Jung of the Institute of Animal Medicine, College of Veterinary Medicine, Gyeongsang National University (Jinju, Korea) and the National Institute of Fisheries Science (Busan, Korea) for providing the bacterial strains used in this study.

Author contributions. H.N.K.S.P. and G.J.H. conceived of and designed the study and wrote the paper. H.N.K.S.P. and S.H.M.P.W. executed the experiment and finalized the data. B.C.J.D. and S.H. helped in sampling and analyzed the data. All authors read and approved the final manuscript.

References

Aibinu I., Adenipekun T., Adelowotan T., Ogunsanya T., Odugbemi T. 2007 – Evaluation of the antimicrobial properties of different parts of *Citrus aurantifolia* (lime fruit) as used locally – Afr. J. Trad. Complement Altern Med. 4(2): 185-190.

Apraj V., Thakur N.D., Bhagwat A., Mallya R., Sawant L., Pandita N.P. 2011 – Pharmacognostic and phytochemical evaluation of *Citrus aurantifolia* (Christm) Swingle PEEL – Phcog. J. 3(26): 70-76.

Burt S. 2004 – Essential oils: their antibacterial properties and potential applications in foods - a review – Int. J. Food Microbiol. 94(3): 223-253.

Chisholm M.G., Wilson M.A., Gaskey G.M. 2003 – Characterization of aroma volatiles in key lime essential oils (*Citrus aurantifolia* Swingle) – Flavor Frag. J. 18(2): 106-115.

Chueca B., Pagán R., García-Gonzalo D. 2014 – Differential mechanism of *Escherichia coli* inactivation by (+)-limonene as a function of cell physiological state and drug's concentration – PLoS ONE 9(4): e94072. doi: 10.1371/journal.pone.0094072.

CLSI 2014 – Performance standards for antimicrobial susceptibility testing of bacteria isolated from aquatic animals; second informational supplement, CLSI document. VET03/VET04-S2. Wayne, PA: Clinical and Laboratory Standards Institute.

Costa R., Bisignanoa C., Filocamo A., Grasso E., Occhiuto F., Spadaroa F. 2014 – Antimicrobial activity and chemical composition of *Citrus aurantifolia* (Christm.). Swingle essential oil from Italian organic crops – J. Essent. Oil Res. 26: 400-408.

Craske J.D., Suryadi N., Wootton M.A. 2005 – A comparison of the peel oil components of Australian native lime (*Microcitrus australe*) and Mexican lime (*Citrus aurantifolia* Swingle) – J. Sci. Food Agric. 85: 522-525.

Das M., Rath C.C., Mohapatra U.B. 2012 – Bacteriology of a most popular street food (Panipuri) and inhibitory effect of essential oils on bacterial growth – J. Food Sci. Tech. 49(5): 564-571.

Fisher K., Phillips C., 2008 – Potential application of plant essential oil as natural preservatives against *Escherischia coli* O157: H7 Pak – J. Biolog Sci. 11: 2054-2061.

Galluci M.N., Oliva M., Casero C., Dambolena J., Luna A., Zygadlo J. and Demo M. 2009 – Antimicrobial combined action of terpenes against the food-borne microorganisms *Escherichia coli, Staphylococcus aureus* and *Bacillus cereus* – Flavor Frag. J. 24: 348-354.

Gharagozloo M., Doroudchi M., Ghaderi A. 2002 – Effects of *Citrus aurantifolia* concentrated extract on the spontaneous proliferation of MDA-MB-453 andRPMI-8866 tumor cell lines – Phytomedicine 9: 475-477.

Hammer K.A., Carson C.F., Riley T.V. 1999 – Antimicrobial activity of essential oils and other plant extracts – J. Appl. Microbiol. 86(6): 985-990.

Imlay J.A., Chin S.M., Linn S. 1988 – Toxic DNA damage by hydrogen-peroxide through the Fenton reaction *in-vivo* and *in-vitro* – Science 240: 640-642.

Jafari S., Esfahani S., Fazeli M.R., Jamalifar H., Samadi M., Samadi N., Najarian-Toosi A., Shams A., Khanavi M. – 2011. Antimicrobial activity of lime essential oil against food-borne pathogens isolated from cream-filled cakes and pastries – J. Biol. Chem. 5: 258-265.

Jee B.Y., Shin K.W., Lee D.W., Kim Y.J., Lee M.K. 2014 – Monitoring of the mortalities and medications in the inland farms of olive flounder, in. *Paralichthys olivaceus,* in South Korea – J. Fish Pathol. 27: 77-83.

Kim S.M., Jun L.J., Park M.A., Jung S.H., Jeong J.B. 2015 – Monitoring of emaciation disease in cultured olive flounder in Jeju (2010-2013), Korea – Korean J. Fish Aquat. Sci. 48: 719-724.

Liu Y., Imlay J.A. 2013 – Cell death from antibiotics without the involvement of reactive oxygen species – Science 339: 1210-1213.

Mahato N., Sharma K., Koteswararao R., Sinha M., Baral E., Cho M.H. 2017 – Citrus essential oils: Extraction, authentication and application in food preservation – Crit. Rev. Food Sci. doi.org/10.1080/10408398.2017.1384716

Morton J. 1987 – Mexican lime in fruits of warm climates – In: Fruits of warm climates (Ed.) C. Morton, University of Miami, Coral Gables, Florida, USA: 168-172.

Nazzaro F., Fratianni F., Martino L.D., Coppola R., de Feo V. 2013 – Effect of essential oils on pathogenic bacteria – Pharmaceuticals 6: 1451-1474.

Nho S.W., Shin G.W., Park S.B., Jang H.B., Cha I.S., Ha M.A., Kim Y.R, Park Y.K., Dalvi R.S., Kang B.J., Joh S.J., Jung T.S. 2009 – Phenotypic characteristics of *Streptococcus iniae* and *Streptococcus parauberis* isolated from olive flounder (*Paralichthys olivaceus*) – FEMS Microbiol. Lett. 293: 20-27.

Nicolosi E., Deng Z.N., Gentile A., La Malfa S., Continella G., Tribulato E. 2000 – Citrus phylogeny and genetic origin of important species as investigated by molecular markers – Theor. Appl. Genet. 100: 1155-1166.

Njau E.A., Alcorn J., Ndakidemi P., Chirino-Trejo N., Buza J. 2014 – Antimicrobial and antioxidant activity of crude extracts of *Rauvolfia caffra* var. *caffra* (Apocynaceae) from Tanzania – Int. J. Biol. 6: 156-167.

Pongsak R., Parichat P. 2009 – Protective effect of clove oil-supplemented fish diets on experimental *Lactococcus garvieae* infection in tilapia – Biosci. Biotech. Bioch. 73: 2085-2089.

Repine J.E., Fox R.B., Berger E.M. 1981 – Hydrogen peroxide kills *Staphylococcus aureus* by reacting with staphylococcal iron to form hydroxyl radical – J. Biol. Chem. 256: 7094-7096.

Taur D.J., Kulkarni V.B., Patil R.Y., Patil R.N. 2009 – Anthelmintic activity of *Ocimum sanctum* and *Citrus aurantifolia* oils – Pharmacologyonline 3: 495-499.

Tundis R., Loizzo M.R., Bonesi M., Menichini F., Mastellone V., Colicaa C., Menichini F. 2012 – Comparative study on the antioxidant capacity and cholinesterase inhibitory activity of *Citrus aurantifolia* Swingle, *C. aurantium L.*, and *C. bergamia Risso* and Poit. peel essential oils – J. Food. Sci. 77: H40–H46.

Turker H., Yildirim A.B. 2015 – Screening for antibacterial activity of some Turkish plants against fish pathogens: a possible alternative in the treatment of bacterial infections – Biotechnol. Biotechnol. Equip. 29: 281-288.

Valladao G.M.R., Gallani S.U., Pilarski F. 2015 – Phytotherapy as an alternative for treating fish disease – J. Vet. Pharmacol. Therap. 38: 417-428.

Vimal M., Vijaya P.P., Mumtaj P., Seema M.S.F. 2013 – Antibacterial activity of selected compounds of essential oils from indigenous plants – J. Chem. Pharmaceutic. Res. 5: 248-253.

We L.S., Wee W. 2013 – Chemical composition and antimicrobial activity of *Cymbopogon nardus* citronella essential oil against systemic bacteria of aquatic animals – Iran. J. Microbiol. 5: 147-152.