J. Korean Wood Sci. Technol. 2020, 48(4): 527~547 pISSN: 1017-0715 eISSN: 2233-7180
https://doi.org/10.5658/WOOD.2020.48.4.527

Original Article

Antibacterial Activity of Essential Oils from Pinaceae Leaves Against Fish Pathogens[1]

Youngseok HAM[2] · Jiyoon YANG[2,3] · Won-Sil CHOI[4] · Byoung-Jun AHN[2] · Mi-Jin PARK[2,†]

ABSTRACT

Fish pathogens cause not only economic damages to fish farming but also infectious pathogens known as a zoonotic agent. Since the continued use of antibiotics to control fish pathogens entails side effects, materials of natural origin need to be developed. The purpose of this study is to discover coniferous essential oils with excellent antibacterial effects in order to develop antibiotic alternatives. We have extracted essential oils using hydro-distillation from the leaves of *Abies holophylla, Pinus thunbergii, Pinus parviflora, Tsuga sieboldii,* and *Pinus rigitaeda*, which are all Pinaceae family. And, we have evaluated antibacterial activity with the extracted essential oils against *Edwardsiella tarda, Photobacterium damselae, Streptococcus parauberis,* and *Lactococcus garvieae*, which are fish pathogens. As a result, the essential oils from *A. holophylla* and *P. thunbergii* showed the selectively strong antibacterial activity against *E. tarda* and *P. damselae*, which are gram-negative bacteria. From GC-MS analysis, it was identified that main component of *A. holophylla* essential oils are (-)-bornyl acetate (29.45%), D-limonene (20.47%), and camphene (11.73%), and that of *P. thunbergii* essential oils is α-pinene (59.81%). In addition, we found three compounds: neryl acetate, (-)-borneol, and (-)-carveol, which are oxygenated monoterpenes. These exist in a very small amount but exhibit the same efficacy as essential oil. Therefore, we expect that *A. holophylla* and *P. thunbergii* essential oils having excellent growth inhibitory effect against gram-negative fish pathogens can be used as biological products such as feed additives and fishery products.

Keywords: Pinaceae, essential oil, antibacterial activity, fish pathogen, neryl acetate, (-)-borneol, (-)-carveol

1. INTRODUCTION

As fishing technology has been advanced, it has become large in scale and mass production is possible. However, high density fish farming in a limited place causes problems such as environmental pollution because it is difficult to manage the surrounding water quality. This increases more frequent infection with fish pathogens and causes massive economic loss because the disease quickly spreads in small places (Oh *et al*., 2006; Yan and Kim, 2013).

Although there are differences in bacterial diseases

[1] Date Received April 7, 2020, Date Accepted June 15, 2020
[2] Division of Wood Chemistry, Department of Forest Products, National Institute of Forest Science, Seoul 02455, Republic of Korea
[3] Division of Life Sciences, School of Life Sciences, Korea University, Seoul 02841, Republic of Korea
[4] National Instrumentation Center for Environmental Management, Seoul National University, Seoul 08826, Republic of Korea
† Corresponding author: Mi-Jin PARK (e-mail: lionpmj@korea.kr, ORCID: 0000-0002-7748-3886)

according to fish species, recently, damage such as edwardsiellosis mainly caused by *Edwardsiella tarda*, streptococcosis caused by *Streptococcus parauberis* and *Lactococcus garvieae,* and vibriosis caused by *Photobacterium damselae* has been reported (Jee *et al.*, 2014; Kim *et al.*, 2015). These are representative infectious pathogenic organisms that cause bacterial diseases of olive flounder, and olive flounder infected with bacteria shows symptoms such as bleeding, abdominal distension, and enlarged kidneys, eventually leading to death (Han *et al.*, 2006; Cho *et al.*, 2007; Moon *et al.*, 2009; Nho *et al.*, 2009). In addition, some bacteria including *E. tarda*, are known as bacterial zoonoses, which can infect humans as well as fish and cause disease (Erfanmanesh *et al.*, 2012; Hundenborn *et al.*, 2013; Hirai *et al.*, 2015; Choksi and Dadani, 2017). Accordingly, antibiotics were administered to feed or water to lower the death rate of fish and increase fish production by treating or preventing diseases caused by fish pathogens (Markestad and Grave, 1997; Cabello, 2006). However, continued use of antibiotics and indiscriminate misuse and abuse are likely responsible for not only leading to emergence of resistant strains, but also disturbing environmental ecosystems (Rhodes *et al*., 2000; Cabello, 2006). Therefore, development of alternative antibiotic materials of natural origin has received attention to minimize side effects caused by the use of such antibiotics and effectively control fish pathogens (Grenni *et al*., 2017; Rossiter *et al*., 2017).

Essential oil, a natural resource, is one of the secondary metabolites isolated from scented plants, and it is a volatile mixture in the form of oil. It is known that essential oils have various biological effects such as antioxidant (Amorati *et al.*, 2013; Salgado-Garciglia *et al.*, 2018; Jeong *et al.*, 2017), antifungal (Nazzaro *et al.*, 2017; D'agostino *et al.*, 2019), insecticidal (Tripathi *et al.*, 2009; Ayvaz *et al.*, 2010), and antibacterial activity (Bakkali *et al.*, 2008; Nazzaro *et al.*, 2013; Chouhan *et al.*, 2017). Studies have also been conducted to analyze the ingredients and to evaluate the toxicity for their safety (Min *et al*., 2017; Ahn *et al*., 2018). Pinaceae is a representative plant with a high essential oil content, and it mainly consists of volatile aromatic compounds of terpene, such as monoterpenes and sesquiterpenes containing hydrocarbons and oxygenated derivatives (Koukos *et al*., 2000; Hong *et al*., 2004). Essential oils from Pinaceae family are widely used in aromatherapy (Ali *et al*., 2015), anti-inflammation (Yang *et al*., 2019), antioxidation (Xie *et al*., 2015), and antimicrobial activity (Chouhan *et al*., 2017), etc (Aziz *et al*., 2018). In particular, pine essential oil, also called phytoncide, has strong antibacterial activity against various gram-negative and gram-positive bacteria (Mohammed *et al*., 2001; Lee *et al*., 2014).

Although there have been studies on antibacterial activity against fish pathogens of natural products including essential oils (Bulfon *et al*., 2013; Park *et al*., 2016; Cunha *et al*., 2018), no demonstration has been made on the efficacy of essential oils from Pinaceae family to date. In addition, most studies have compared and evaluated the efficacy of essential oils according to the sample region or extraction method, or presented the main components through analysis. However, since various components are mixed in essential oils, there are cases where it is impossible to explain them with one component, and there have been very few studies on important active components that explain these specific phenomena.

For that reason, in this study, we evaluate antibacterial activity of essential oils from Pinaceae leaves, which are *A. holophylla, P. thunbergii, P. parviflora, T. sieboldii,* and *P. rigitaeda*, against fish pathogens and investigate their own active ingredients. Through this, we tried to investigate whether essential oils from Pinaceae leaves can be used as a prevention of fish pathogens or as a medicine for fisheries.

2. MATERIALS and METHODS

2.1. Pinaceae sample and single compounds

All five trees used in the study belong to Pinaceae family. The leaves of *A. holophylla*, *P. thunbergii*, and *P. rigitaeda* were collected from the National Institute of Forest Science in Suwon City, *T. sieboldii* was collected from the National Institute of Forest Science in Pocheon-eup, and *P. parviflora* leaves from Ulleungdo. And, all reagents used in the study are from Sigma-aldrich; (-)-bornyl acetate (99%, product number: 45855), α-pinene (97%, product number: 80604), (-)-borneol (99%, product number: 15598), neryl acetate (97%, product number: 46015), and (-)-carveol (98%, product number: 61370).

2.2. Extraction of essential oils

Essential oils were extracted using hydro-distillation from the leaves of *A. holophylla*, *P. thunbergii*, *P. parviflora*, *T. sieboldii*, and *P. rigitaeda*. 5 to 6 L of distilled water per 1 kg of sample was added to a 10 L volume round flask. Extraction was carried out until no more essential oil was extracted with a heating mantle (Model: MS-DM608, Serial number: 201602, Misung Scientific. Co. Ltd., Korea) at 100 ± 2 ˚C. The extracted essential oil was dehydrated using anhydrous Na_2SO_4 (98.5%, Samchun, Korea) and filtered using 0.45 μm pore size minisart® syringe filter (Reference number: 16555-K, Sartorius Stedim Biotech GmbH, Germany). The filtered essential oil was transferred to a light-blocked brown bottle, and then filled with nitrogen gas and stored in a 4 ˚C refrigerator until use.

2.3. Strains and culture conditions

Edwardsiella tarda FP5060, *Photobacterium damselae* FP4101, *Lactococcus garvieae* FP5245, and *Streptococcus parauberis* FP3287 were received from Marine and Fisheries Life Resources, National Institute of Fisheries Sciences, and they were stored in 25% glycerol stock at -40˚C. BHI agar was prepared by adding 15 g/L agar to BHI broth (Bacto™ brain heart infusion, Product number: 237500, BD Biosciences Korea Ltd., Korea). The frozen storage bacteria were inoculated on a BHI agar plate and cultured at 28 ˚C for 24 hours to ensure a single colony. A single colony was inoculated into 4 mL BHI broth, and then cultured with 250 rpm for overnight.

2.4. Evaluation of antibacterial activity using Paper disc diffusion

The bacteria cultured in BHI broth were measured at 600 nm using a Neo-D3117 UV-VIS spectrophotometer (NEOGEN Inc., Korea), and optical density (O.D) was adjusted to 1 ($O.D_{600}$ = 1). After inoculating 100 μL of the concentration-adjusted bacteria in the center of Mueller hinton agar (Difco, Product number: 225250, BD Biosciences Korea Ltd., Korea) plate, it was spread evenly using a glass spreader. A sterilized paper disc (ADVANTEC, Product number: 49005040, Toyo Roshi Kaisha, Ltd. Japan) was attached to the plate, and each essential oil stock solution was absorbed by 5 μL. After that, the cells were cultured at 28 ˚C for 2 days, and measure the diameter of a ring formed around the paper disc to evaluate antibacterial activity.

2.5. Evaluation of antibacterial activity at various concentration

The microdilution method of CLSI M07-A9 was modified and used in order to evaluate the antibacterial activity. *A. holophylla, P. thunbergii* essential oils and a single compound were dissolved in dimethyl sulfoxide (DMSO, extra pure grade, Product number:

3047-4460, Duksan) and were prepared at a concentration of 5% (v/v) until use. Essential oil samples were diluted to 3.5, 2, 1, and 0.5% concentration. 200 μL of BHI broth was dispensed into each well of a 96-well polystyrene microplate (Flat bottom well, Product number: 30096, SPL Life Sciences Co., Ltd., Pocheon, Korea). The prepared sample was treated with 2 μL of each well. Then, each well was inoculated so that the concentration of pre-cultured bacteria was $O.D_{600}$= 0.05, and cultured at 28 ˚C for 24 hours. After incubation for 24 hours, the absorbance was measured at 600 nm using an Epoch microplate spectrophotometer (BioTek Instruments, Inc., US) to evaluate the antibacterial activity of essential oils. The control group was treated with 1% DMSO in which the sample was dissolved, and the positive control group was tetracycline (HPLC grade, Product number: 87128, Sigma-Aldrich, Korea), an antibiotic.

2.6. Gas chromatography–mass spectrometry (GC–MS) analysis

The components of essential oils extracted from *A. holophylla* and *P. thunbergii* leaves were analyzed using GC-MS analysis. For GC-MS analysis, TRACE™ 1310 Gas Chromatograph consisting of TriPlus™ 100 LS Liquid Auto-sampler (Catalog number: IQLAAAGAAHFACMMBES), ISQ™ Series Single Quadrupole GC-MS System (Catalog number: IQLAAAGAAJFALOMAYE) was used, and all are Thermo Fisher Scientific products (Thermo Fisher Scientific Solutions LLC, Korea). For the column, VF-5MS (Silica, length 60 m x diameter 0.25 mm x thickness 0.25 μm, Agilent Technologies, Inc., US) was used. Helium was supplied as a mobile phase gas at a rate of 1 mL/min. The essential oil sample was dissolved 4 μL in 1 mL of dichloromethane and injected 1 μL. Injection temperature was maintained at 250 ˚C, and a split ratio mode of 1:20 was used. Oven temperature was initially maintained at 50 ˚C for 5 minutes, then increased to 65 ˚C at 10 ˚C/min and maintained for 30 minutes. Thereafter, the temperature was raised to 210 ˚C at 5 ˚C/min and maintained for 10 minutes. Lastly, the temperature was increased up to 325 ˚C at 20 ˚C/min and maintained for 10 minutes. The temperature of the flame ionization detector (FID) was set to 300 ˚C, the air flow was set to 350 mL/min, the hydrogen flow to 35 mL/min, and the make-up gas (helium) to 40 mL/min. MS data were collected in the range of 35 - 550 amu at 0.2 sec/scan in EI ionization mode.

Among the collected peak data, those with high match quality were first selected, and Kovats retention index (KI) was calculated using *n*-alkanes (C_8 - C_{20}, Product number: 04071, Sigma-Aldrich, Korea). The calculated KI value was compared with the National Institute of Standards and Technology (NIST, US) Chemistry WebBook (Standard Reference Database Number 69), and we finally selected compounds with a difference of less than 100.

2.7. Statistical analysis

All experimental results are presented as mean and standard deviation. One way analysis of variance was used to distinguish significant differences using IBM® SPSS software (Ver. 25.0; SPSS Inc., Chicago, Illinois, USA). Significance levels were set at 95% confidence intervals for Tukey test or paired-*t*-test.

3. RESULTS and DISCUSSION

3.1. Evaluation of antibacterial activity of essential oils from Pinaceae leaves against fish pathogens

The yields of each essential oil extracted from *A. holophylla, P. thunbergii, P. parviflora, T. sieboldii,* and *P. rigitaeda* leaves were found to be 2.99%,

0.93%, 0.08%, 0.15%, 0.72%, and the antibacterial effect was evaluated with the paper disc diffusion method. The presence of antibacterial activity of essential oils was confirmed by measuring the size of the ring formed by exposing 4 types of fish pathogens to a paper disc containing essential oils for 2 days (Table 1). A clear zone arising around the paper disc is formed because bacteria cannot grow in the area, so the size of the growth inhibiting ring indicates an index of antibacterial activity (Djabou *et al.*, 2013).

For *E. tarda*, *A. holophylla* and *P. thunbergii* essential oils showed strong antibacterial activity with 21.5 mm and 21.0 mm, respectively, followed by *T. sieboldii* (16 mm) and *P. rigitaeda* (13 mm) in order. Also, for *P. damselae*, *A. holophylla* and *P. thunbergii* essential oils formed a wide growth-inhibiting ring of size 23.5 mm and 20.5 mm, followed by *T. sieboldii* (13.25 mm), *P. rigitaeda* (11.5 mm), and *P. parviflora* (8 mm) in order. The antibacterial effect of tested essential oils against *L. garvieae* was lower than that of *E. tarda* and *P. damselae*, and among 5 essential oils, *A. holophylla* essential oil was 12.75 mm, which showed higher antibacterial activity than other essential oils. For *S. parauberis*, *P. thunbergii* essential oil formed the widest ring of 14.0 mm, while the remaining essential oil formed a growth inhibitory ring of similar size at the level of about 10 mm. On the other hand, *P. parviflora* essential oil did not form rings against *E. tarda* and *S. parauberis*. From these results, we noticed that the essential oils of *A. holophylla* and *P. thunbergii* leaves had a strong antibacterial effect against fish pathogens, and were particularly effective against *E. tarda* and *P. damselae*.

As a result of the paper disc assay, *E. tarda* and *P. damselae*, the gram-negative bacteria, showed high sensitivity to essential oils of *A. holophylla* and *P. thunbergii* leaves. In contrast, the gram-positive bacteria *L. garvieae* and *S. parauberis* were relatively less susceptible to both essential oils (Table 1). Therefore, we investigated the growth change according to the concentration of essential oils of *A. holophylla* and *P. thunbergii* leaves against 4 types of fish pathogens (Fig. 1). For *E. tarda*, *A. holophylla* leaves essential oil inhibited growth of about 5% at a concentration of 0.01%, and its effect increased rapidly at a concentration of 0.02% to inhibit 100%. *P. thunbergii* leaves essential oil showed a growth inhibition effect of about 10% and 25% at concentrations of 0.01% and 0.02%, respectively, and 100% effect at concentration of 0.035% (Fig. 1A). The growth of *P. damselae* was rapidly inhibited in the essential oil of *A. holophylla* and *P. thunbergii* leaves at 0.02% concentration, and the inhibitory effects were 74% and 83%, respectively. In addition, 0.035% concentration of *A. holophylla*

Table 1. Antibacterial activity of essential oils by the paper disc assay

	Diameter of clear zone (mm)[1)]			
	E. tarda [a)]	*P. damselae* [a)]	*L. garvieae* [b)]	*S. parauberis* [b)]
A. holophylla	21.5 ± 0.71	23.5 ± 0.71	12.75 ± 0.35	11.0 ± 0.0
P. thunbergii	21.0 ± 1.41	20.5 ± 2.12	8.5 ± 0.71	14.0 ± 0.0
P. parviflora	N.D. [2)]	8.0 ± 0.0	7.5 ± 0.0	N.D.
P. rigitaeda	13.0 ± 0.0	11.5 ± 0.71	7.5 ± 0.0	10.25 ± 1.77
T. sieboldii	16.0 ± 0.0	13.25 ± 0.35	8.5 ± 0.0	9.75 ± 0.35

[1)] Mean and standard deviation were calculated from two independent experiments.
[2)] N.D.: Not detected.
[a)] Gram-negative bacteria.
[b)] Gram-positive bacteria.

leaves essential oil showed inhibitory effect on the growth of about 93% and *P. thunbergii* leaves essential oil did about 81%. The essential oils of *A. holophylla* and *P. thunbergii* leaves had little effect on the growth of *L. garvieae* (Fig. 1C). *S. parauberis* growth was also not affected by *A. holophylla* leaves essential oil, but growth was inhibited by about 18% by 0.035% concentration of *P. thunbergii* leaves essential oil (Fig. 1D). Such antibacterial research on fish pathogens of *A. holophylla* and *P. thunbergii* leaves essential oil has not been reported so far, and it is significant in that it suggested the possibility of them as a material for preventing infectious diseases against fish pathogenic bacteria.

In general, the antimicrobial activity of essential oils is known to be more effective against gram-positive bacteria than gram-negative bacteria (Trombetta *et al.*, 2005; Nazzaro *et al.*, 2013). In gram-positive bacteria, about 90 - 95% of the cell wall is composed of peptidoglycan, and teichoic acid and lipoteichoic acid are present on the cell wall surface. Therefore, the cell wall of gram-positive bacteria allows hydrophobic substances such as essential oil to pass relatively easily, and can act on the cell wall or the cytoplasm inside the cell (Tiwari *et al*., 2009). On the other hand, the cell wall of gram-negative bacteria has a thinner layer of peptidoglycan than gram-positive bacteria, and there is one more outer membrane on the outermost side. It is known that the lipopolysaccharide present in the outer membrane gives hydrophilic properties to the surface of the cell wall, and thus exhibits resistance to hydrophobic substances such as essential oils (Vaara, 1992; Nikaido, 1994; Mann *et al*., 2000). Previous studies have also reported that essential oils extracted from herbaceous plants are more effective in inhibiting the growth of gram-positive bacteria than in gram-negative bacteria. (Aumeeruddy Elalfi *et al*., 2015; Martucci *et al*., 2015; Bouazama *et al*., 2017), Also, essential oils of woody species have been reported

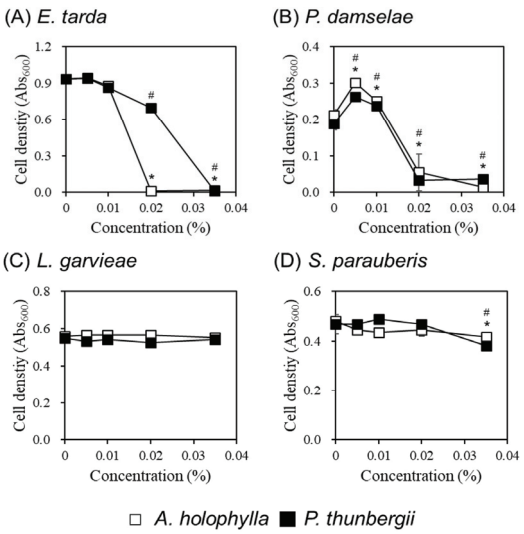

Fig. 1. Effect of various concentration two essential oils on growth of gram-negative bacteria (A and B) and gram-positive bacteria (C and D).

The essential oils were dissolved in DMSO and prepared by serial dilution. Control was treated with 1% of total volume of DMSO as the solvent in which the essential oils were dissolved. Standard deviation was calculated from four independent experiments. Statistical analysis was performed using a Tukey test. Values that differ from the control with the 95% confidence level are marked with a star (*A. holophylla*, open-square) or a sharp (*P. thunbergii*, filled-square) on the top of symbols, respectively.

to show MIC levels at lower concentrations for gram-positive bacteria (Aumeeruddy Elalfi *et al*., 2015). There was one study in which time-kill analysis was performed using essential oils extracted from *Citrus medica*. As a result, *Escherichia coli* was inhibited to a maximum growth 4 hours after exposure to essential oils, but *Staphylococcus aureus* was found to be 2 hours earlier than this (Li *et al*., 2019).

Interestingly, however, the essential oils from *A. holophylla*, *P. thunbergii*, *P. rigitaeda*, and *T. sieboldii* leaves tested in this study were more effective and showed high antibacterial activity against gram-negative bacteria (*E. tarda* and *P. damselae*) than gram-positive bacteria (*L. garvieae* and *S. parauberis*) (Table 1). Particularly, MIC concentrations of *E. tarda* and *P.*

damselae were confirmed by the essential oils of *A. holophylla* and *P. thunbergii* leaves, which showed the strongest growth inhibitory effect, but the effect was insufficient at all concentrations for gram-positive bacteria (Fig. 1). These results are in contrast to the previously reported research results, and we determined that it was necessary to analyze composition of the essential oils of *A. holophylla* and *P. thunbergii* leaves.

3.2. GC–MS analysis of essential oils from the five species of leaves belonging to Pinaceae family

We analyzed compound composition of the essential oil extracted from the leaves of the five species belonging to Pinaceae family using GC-MS analysis (Table 2). The major components of the essential oil of *P. parviflora* leaves were α-pinene (36.58%), β-pinene

Table 2. Comparative chemical composition of essential oil from five Pinaceae leaves (%)

RT[1)]	Components	Peak area (%)					KI[2)]
		A. holophylla	*P. thunbergii*	*P. parviflora*	*P. rigitaeda*	*T. sieboldii*	
24.38	Tricylene	0.82	0.11	0.21	1.96	-[3)]	917
26.00	α-Pinene	6.08	59.81	36.58	12.03	8.19	927
28.42	Camphene	11.73	0.80	2.64	8.42	0.36	941
33.36	β-Pinene	0.76	3.11	23.49	1.37	18.08	971
35.99	β-Myrcene	0.69	4.24	2.68	5.46	5.90	986
39.38	3-Carene	2.95	-	0.13	-	-	1012
41.44	o-Cymene	0.91	1.02	0.10	0.54	0.56	1033
42.01	D-Limonene	20.47	4.34	18.99	4.34	1.35	1038
42.12	Sabinene	-	0.70	-	1.22	4.75	1040
46.87	α-Terpinolene	-	-	0.54	-	-	1089
47.05	(-)-Fenchone	-	0.23	-	-	-	1091
47.22	Myroxide	-	-	-	0.12	-	1093
47.77	α-Pinene oxide	-	0.77	-	0.37	-	1098
47.80	Linalool	-	-	-	-	0.90	1099
48.45	cis-Verbenol	0.19	0.62	-	0.13	-	1107
48.66	β-Pinene oxide	-	-	-	0.11	-	1110
49.12	Fenchol	-	0.10	-	-	-	1117
49.66	α-Campholenal	0.23	0.17	-	-	-	1124
50.09	Limonene oxide, cis-	0.62	-	-	-	-	1130
50.40	(+)-(E)-Limonene oxide	0.71	-	-	-	-	1134
50.69	(-)-trans-Pinocarveol	-	0.46	0.13	0.14	0.48	1139
51.02	(S)-cis-Verbenol	-	0.63	-	-	-	1143
51.69	Camphene hydrate	-	-	0.10	0.33	-	1152
52.13	Pinocamphone	-	-	0.24	-	-	1158
52.28	Pinocarvone	-	0.15	-	0.10	-	1160
52.99	(-)-Borneol	0.40	0.14	-	0.27	-	1169
53.26	trans-2-Caren-4-ol	0.32	-	-	-	-	1173
53.65	Terpinen-4-ol	-	-	0.10	0.39	0.23	1179
54.11	Cherry propanol	0.82	0.37	-	-	-	1185
54.13	Cryptone	-	-	-	0.72	1.29	1186
54.80	L-α-Terpineol	-	0.21	1.09	1.42	2.41	1194
54.91	Myrtenal	-	0.19	-	-	-	1196
55.89	(-)-Verbenone	-	0.50	-	-	-	1209
56.82	Fenchyl acetate	-	0.12	-	-	-	1222
56.84	(-)-Carveol	0.27	-	-	-	-	1222
58.90	Cuminaldehyde	-	-	-	0.14	-	1249
59.31	Vervenone	0.30	-	-	-	-	1254
59.83	Piperitone	-	0.10	-	-	-	1261

Table 2. (Continued)

RT[1)]	Components	Peak area (%)					KI[2)]
		A. holophylla	*P. thunbergii*	*P. parviflora*	*P. rigitaeda*	*T. sieboldii*	
61.51	Phellandral	-	-	-	0.14	-	1283
61.94	(-)-Bornyl acetate	29.45	1.25	0.85	47.06	0.43	1290
62.09	Alloocimene	0.44	-	-	-	-	1291
62.78	Limonene dioxide	-	0.67	-	-	-	1300
63.84	Thymoquinone	0.40	-	-	-	-	1322
65.18	Limonene glycol	0.53	-	-	-	-	1349
65.32	α-Terpinyl acetate	-	-	0.14	0.19	-	1352
65.33	Myrtanyl acetate	-	0.13	-	-	-	1352
66.66	Neryl acetate	0.73	-	-	0.25	-	1379
66.88	α-Copaene	-	-	0.23	-	0.30	1384
67.44	β-Elemene	-	0.13	-	-	2.98	1395
67.97	Limonene oxide	0.18	-	-	-	-	1408
68.08	(-)-α-Gurjunene	0.33	-	-	-	-	1411
68.45	Longifolene	-	-	0.55	-	-	1421
68.82	β-Caryophyllene	-	2.63	1.62	0.78	4.27	1431
69.17	α-Bergamotene	-	-	-	-	0.22	1440
69.30	Butanoic acid	-	-	-	-	0.21	1444
69.55	Aromadendrene	-	-	-	-	0.85	1451
70.19	Humulene	-	2.39	0.29	1.21	0.95	1467
70.79	γ-Muurolene	-	-	0.23	0.42	1.26	1484
71.08	(-)-Germacrene D	-	-	1.48	-	0.29	1492
71.37	β-Selinene	-	-	-	-	0.68	1500
71.42	(+)-Ledene	-	-	-	-	0.91	1501
71.58	α-Muurolene	-	-	1.44	0.13	2.90	1507
71.79	β-Bisabolene	-	0.13	0.11	-	-	1513
72.11	(-)-γ-Cadinene	-	-	-	0.19	3.30	1524
72.23	(+)-δ-Cadinene	-	-	0.31	-	2.54	1528
72.35	Calamenene	-	-	-	-	0.70	1532
73.35	Nerolidol	0.22	0.22	-	-	2.31	1565
73.64	α-Calacorene	-	-	-	-	0.56	1575
73.98	Globulol	-	-	-	-	0.31	1586
74.16	(-)-Spathulenol	-	-	-	-	5.39	1592
74.38	Caryophyllene oxide	3.15	5.44	0.14	1.30	4.94	1599
74.71	Viridifloraol	-	-	-	-	0.76	1611
75.00	Rosifoliol	-	-	-	-	0.24	1622
75.16	Humulene epoxide 2	0.70	2.48	-	1.09	0.77	1627
75.54	Cubenol	-	-	0.18	0.14	0.42	1642
75.66	2-Phenylethyl hexanoate	-	-	-	-	0.92	1646
75.89	Cedrelanol	-	-	0.11	0.15	1.63	1655
75.95	τ-Muurolol	-	-	0.17	0.23	2.54	1657
76.01	δ-Cadinol	-	-	0.23	0.14	-	1659
76.31	β-Eudesmol	1.08	0.13	-	-	-	1672
76.28	α-Cadinol	-	-	0.29	0.40	5.86	1669
76.45	Epiglobulol	-	-	-	-	0.33	1676
77.03	α-Bisabolol	1.55	0.45	0.11	0.18	-	1698
87.19	Biformene	-	-	-	0.11	-	1995
87.41	(+)-Isokaurene	-	-	-	0.24	-	2002
87.84	(+)-Manoyl oxide	-	-	0.17	-	-	2024
88.57	(-)-Phyllocladene	-	-	-	0.31	-	2061
91.38	Kauran-16-ol	-	-	-	1.80	-	2259

[1)] RT: Retention time (min). [2)] KI: Kovats retention index. [3)] -: Not detected.

(23.49%), and D-limonene (18.99%). And *T. sieboldii* leaves had (-)-bornyl acetate (47.06%), α-pinene (12.03%), and camphene (8.42%) as major components. In addition, the ratio of β-pinene (18.08%) and α-pinene (8.19%) was high in *P. rigitaeda*.

Most of the components of the essential oils of *A. holophylla* and *P. thunbergii* leaves, which have a strong antibacterial activity against gram-negative bacteria, were analyzed as terpene-based compounds, including monoterpene and sesquiterpene. The main components of *A. holophylla* leaves essential oil were 22 monoterpene (79.82%) and 7 sesquiterpene (7.21%), and the main constituents of *P. thunbergii* leaves essential oil were 26 monoterpene (80.94%) and 9 sesquiterpene (14.00%). The main components of *A. holophylla* leaves essential oil were (-)-bornyl acetate (29.45%), D-limonene (20.47%), camphene (11.73%), and α-pinene (6.08%). *P. thunbergii* leaves essential oil had the highest ratio of α-pinene at 59.81%, followed by caryophyllene oxide (5.44%), D-limonene (4.34%), and β-myrcene (4.24%). A total of 17 compounds were found in both essential oils of *A. holophylla* and *P. thunbergii* leaves, and 12 were monoterpene and 5 were sesquiterpene. From these results, we identified that (-)-bornyl acetate and α-pinene account for the largest proportion of the two essential oils extracted by hydro-distillation in this study. Other studies suggest bicyclo [2.2.1] heptan- 2-ol (28.05%), δ3-carene (13.85%), and α-pinene (11.68%) as the main components of *A. holophylla* leaves essential oils (Lee and Hong, 2009), or does 3-carene (25.53%), α-pinene (17.55%), and bornyl acetate (16.22%) (Kim *et al*., 2016). *P. thunbergii* essential oil also reported 2H-benzocyclohepten-2-one (34.33%), α-humulene (19.59%), limonene (5.92%) and caryophyllene (5.32%) as the main components. (Kim *et al*., 2013). For extracts including essential oils, there are differences in components due to various extraction conditions such as the sample site, harvest time, extraction method, and temperature and time during extraction (Tongnuanchan and Benjakul, 2014; Lingan, 2018). Therefore, these factors make a difference in the compounds separated from plants, and we assume that the final composition ratio of essential oils is likely to be affected by them. Accordingly, for future research, it requires standardization of an appropriate extraction method in order for essential oils to be used as a functional material, and needs to present each of the indicators that serves as a standard for quality through an analysis of essential oils representing physiological activity.

3.3. Evaluation of antibacterial activity of single compound against fish pathogens

Various compounds exist in plant extracts including essential oils, and since they act organically and exhibit physiological activity, it is difficult to clearly present the mechanism (Sutili *et al*., 2016). In addition, there are cases where a compound known as a standard component does not match the efficacy of the extract, but rather the phenomenon may be explained by a trace amount of the compound (Chouhan *et al*., 2017; Ham and Kim, 2019).

In this study, we evaluated the activity of the terpene-based single compound constituting the essential oil, and sought to identify an active ingredient exhibiting similar physiological activity to the essential oils. We carried out antibacterial screening of various terpene-based single compounds (0.05% concentration) including the most existing (-)-bornyl acetate and α -pinene based on the results of GC-MS analysis of the essential oils of *A. holophylla* and *P. thunbergii* leaves (Data not shown). As a result, three monoterpene-based active compounds with neryl acetate, (-)-borneol, and (-)-carveol, which selectively inhibit the growth of *E. tarda* and *P. damselae*, are obtained in the same effect as essential oils of *A. holophylla* and

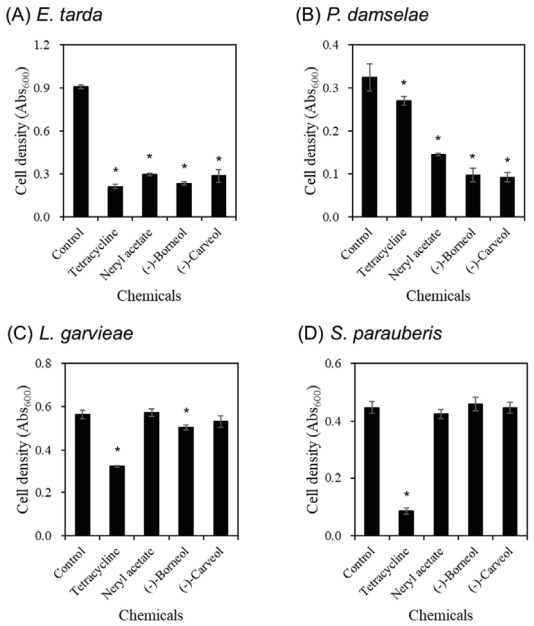

Fig. 2. Effect of three oxygenated monoterpenes on growth of gram-negative bacteria (A and B) and gram-positive bacteria (C and D).

Tetracycline was prepared at a concentration of 0.0005% as a positive control. Three oxygenated monoterpenes were prepared at concentration of 0.05%. All chemicals were dissolved in DMSO. Control was treated with 1% of total volume of DMSO as the solvent in which the chemicals were dissolved. Standard deviation was calculated from four independent experiments. Statistical analysis was performed using a paired-*t*-test. Values that differ from the control with the 95% confidence level are marked with a star on the top of bars.

P. thunbergii leaves (Fig. 2). *E. tarda* was inhibited by 68%, 75%, and 69% of growth by three compounds and 77% by tetracycline (0.0005% concentration), which was a positive control (Fig. 2A). *P. damselae* also inhibited 55%, 70%, and 71% of growth by three single compounds, but only 17% by tetracycline (Fig. 2B). On the other hand, the effects of three terpene-based compounds had insignificant impact on growth of the gram-positive bacteria *L. garvieae* and *S. parauberis*. *L. garvieae* showed a 10% of growth reduction only by (-)-borneol and 42% of inhibition by tetracycline (Fig. 2C). There was no compound that inhibited the growth of *S. parauberis*, and only tetracycline, which was a positive control, showed 81% of an inhibitory effect (Fig. 2D).

The results of the efficacy evaluation of neryl acetate, (-)-borneol, and (-)-carveol have significance in providing fundamental data that explains specific effects of essential oils of *A. holophylla* and *P. thunbergii* leaves, which shows strong and selective antibacterial activity against gram-negative fish pathogens.

Neryl acetate and (-)-carveol were found only in *A. holophylla* leaves essential oils, and (-)-borneol was detected in both *A. holophylla* and *P. thunbergii* leaves essential oils (Table 2). These results may partially explain the phenomenon that *A. holophylla* leaves essential oil has a superior growth inhibition effect against *E. tarda* than *P. thunbergii* leaves essential oil. On the other hand, neryl acetate and (-)-borneol were also detected in essential oils of *P. rigitaeda* leaves, but did not have strong antibacterial activity against fish pathogens. Some studies have combined the components present in the extract, confirming that there is a synergistic effect between single compounds. (Ham and Kim, 2019; Kim *et al*., 2016). From these studies, it also showed a synergistic effect of antibacterial activity between neryl acetate, (-)-carveol, (-)-borneol, and other components in the essential oils of *A. holophylla* and *P. thunbergii* leaves, and it is presumed to have high antibacterial activity when compared to *P. rigitaeda.*

Prior studies on (-)-borneol and (-)-carveol have been shown to have antibacterial (Knobloch *et al.*, 1989; Hammerschmidt *et al.*, 1993; Tabanca *et al.*, 2001; Cha, 2007; Jung, 2009; Lopez-Romero *et al.*, 2015; Guimaraes *et al.*, 2019) as well as antifungal effects (Tabanca *et al.*, 2001; Hussain *et al.*, 2010), and neryl acetate has also been reported to inhibit the growth of some pathogenic microorganisms found in

industry (Kotan *et al.*, 2007). However, studies on fish pathogens with these compounds have not been conducted, and such previous studies have not provided results on specific antibacterial activity by species classification. Given these results, this study suggests the possibility of selectively controlling gram-negative fish pathogens by using neryl acetate, (-)-borneol and (-)-carveol, which are classified as oxygenated monoterpene.

Generally, mechanisms of essential oils for microorganisms are known to destroy cell membranes (Li *et al*., 2014; Raeisi *et al*., 2015), loss of membrane integrity (Diao *et al*., 2014; Yang *et al*., 2015), and increase in permeability of cell membranes (Lambert *et al*., 2001; Hyldgaard *et al*., 2012) and the cytoplasm is transformed by essential oils introduced into the cell and eventually it leads to cell death (Nazzaro *et al*., 2013). In addition, essential oil is known to affect expression of pathogenic factors (biofilm, spore formation and mating) by acting on a quorum sensing system, which plays an important role in the interaction between bacteria (Bouyahya *et al*., 2017). In the case of gram-negative bacteria with high resistance to hydrophobic molecules, it has been reported that some hydrophobic compounds such as essential oils can slowly pass through the porin protein present in the cell wall (Plesiat and Nikaido, 1992; Bock and Sawers, 1996). Based on these previous studies, further studies are needed about additional mechanisms for the gram-negative fish pathogens of essential oil and three oxygenated terpenes obtained in this study. In this study, we found a material that is selectively applied only to a specific species among various fish pathogens that are problems in aquaculture. Also, we anticipate that this is a new eco-friendly material, which can replace antibiotics that may act indiscriminately and cause ecosystem disturbance.

4. CONCLUSION

The purpose of this study was to evaluate the possibility of substituting essential oil, a natural product, for antibiotics used to treat infectious fish diseases caused by bacteria to compensate for side effects such as the emergence of resistant strains, which have been pointed out as a disadvantage of using antibiotics. We evaluated antibacterial activity of the five essential oils extracted from the leaves of pine family, and noticed that *A. holophylla* and *P. thunbergii* leaves essential oils showed strong growth inhibitory effects against gram-negative fish pathogens, *E. tarda* and *P. damselae.* As a result of analyzing the components of these two essential oils by GC-MS, they mainly consisted of monoterpene-based compounds, and the main components were identified as (-)-bornyl acetate (29.45%) and α-pinene (59.81%), repectively. In addition, we found three compounds: neryl acetate, (-)-borneol, and (-)-carveol, which are oxygenated monoterpenes. These compounds exist a very small amount but exhibit the same efficacy as essential oils. We expect that these findings will effectively control gram-negative fish pathogens by utilizing essential oils extracted from Pinaceae leaves, which are natural sources. However, in order to increase the utilization of essential oils, which are fat-soluble compounds, it is considered that studies on formulations must be conducted in advance. In addition, we anticipate that it will contribute in part to solving the problem of occurrence of resistant strains in high density fish farming due to the use of antibiotics by adding essential oils to feed additives, water quality improver, etc.

REFERENCES

Ali, B., Al-Wabel, N.A., Shams, S., Ahamad, A., Khan, S.A., Anwar, F. 2015. Essential oils used in aromatherapy: A systemic review. Asian Pacific

Journal of Tropical Biomedicine 5(8): 601-611.

Ahn, C., Park, M.J., Kim, J.W., Yang, J., Lee, S.S., Jeung, E.B. 2018. Cytotoxic evaluation of plant essential oils in human skin and lung cells. Journal of the Korean Wood Science and Technology 46(2): 166-177.

Amorati, R., Foti, M.C., Valgimigli, L. 2013. Antioxidant activity of essential oils. Journal of Agricultural and Food Chemistry 61(46): 10835- 10847.

Aumeeruddy Elalfi, Z., Gurib-Fakim, A., Mahomoodally, F. 2015. Antimicrobial, antibiotic potentiating activity and phytochemical profile of essential oils from exotic and endemic medicinal plants of mauritius. Industrial Crops and Products 71: 197-204.

Ayvaz, A., Sagdic, O., Karaborklu, S., Ozturk, I. 2010. Insecticidal activity of the essential oils from different plants against three stored-product insects. Journal of Insect Science 10(1): 21-32.

Aziz, Z.A.A., Ahmad, A., Setapar, S.H.M., Karakucuk, A., Azim, M.M., Lokhat, D., Rafatullah, M., Ganash, M., Kamal, M.A., Ashraf, G.M. 2018. Essential oils: Extraction techniques, pharmaceutical and therapeutic potential - a review. Current Drug Metabolism 19(13): 1100-1110.

Bakkali, F., Averbeck, S., Averbeck, D., Idaomar, M. 2008. Biological effects of essential oils: A review. Food and Chemical Toxicology 46(2): 446-475.

Bock, A., Sawers, G. 1996. *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, 2nd edition. Fermentation, pp. 262-282.

Bouazama, S., Hicham, H., Costa, J., Desjobert, J.M., Talbaoui, A., Tabyaoui, M. 2017. Chemical composition and antibacterial activity of the essential oils of *Lavandula pedunculata* and *Lavandula dentata*. Journal of Materials and Environmental Sciences 8: 2154-2160.

Bouyahya, A., Bakri, Y., Et-Touys, A., Talbaoui, A., Khouchlaa, A., Charfi, S., Abrini, J., Dakka, N. 2017. Resistance to antibiotics and mechanisms of action of essential oils against bacteria. Phytotherapie. DOI 10.1007/s10298-017-1118-z.

Bulfon, C., Volpatti, D., Galeotti, M. 2013. Current research on the use of plant-derived products in farmed fish. Aquaculture Research 46(3): 513-551.

Cabello, F. 2006. Heavy use of prophylactic antibiotics in aquaculture: A growing problem for human and animal health and for the environment. Environmental Microbiology 8(7): 1137-1144.

Cha, J.D. 2007. Chemical composition and antibacterial activity against oral bacteria by the essential oil of *Artemisia iwayomogi*. Journal of Bacteriology and Virology 37(3): 129-136.

Cho, M.Y., Kim, M.S., Kwon, M.G., Jee, B .Y., Choi, H.S., Choi, D.L., Park, G.H., Lee, C.H., Kim, J.D., Lee, J.S., Oh, Y.K., Lee, D.C., Park, S.H., Park, M.A. 2007. Epidemiological study of bacterial diseases of cultured olive flounder, *Paralichthys olivaceus* from 2005 to 2006 in korea. Journal of Aquaculture 20(1): 61-70.

Choksi, T.T., Dadani, F. 2017. Reviewing the emergence of *Lactococcus garvieae*: A case of catheter associated urinary tract infection caused by *Lactococcus garvieae* and *Escherichia coli* coinfection. Case Reports in Infectious Diseases 2017: 1-4.

Chouhan, S., Sharma, K., Guleria, S. 2017. Antimicrobial activity of some essential oils-present status and future perspectives. Medicines 4(3): 58-79.

Cunha, J., Heinzmann, B., Baldisserotto, B. 2018. The effects of essential oils and their major compounds on fish bacterial pathogens-a review. Journal of Applied Microbiology 125(2): 328-344.

D'agostino, M., Tesse, N., Frippiat, J.P., Machouart, M., Debourgogne, A. 2019. Essential oils and their natural active compounds presenting antifungal properties. Molecules 24(20): 3713-3734.

Diao, W.R., Zhang, L.L., Feng, S.S., Xu, J.G. 2014. Chemical composition, antibacterial activity, and mechanism of action of the essential oil from *Amomum kravanh*. Journal of Food Protection 77(10): 1740-1746.

Djabou, N., Lorenzi, V., Guinoiseau, E., Andreani, S., Giuliani, M.C., Desjobert, J.M., Bolla, J.M., Costa, J., Berti, L., Luciani, A., Muselli, A. 2013. Phytochemical composition of *Corsican teucrium* essential oils and antibacterial activity against foodborne or toxi-infectious pathogens. Food Control 30(1): 354-363.

Erfanmanesh, A., Soltani, M., Pirali, E., Mohammadian, S., Taherimirghaed, A. 2012. Genetic characterization of *Streptococcus iniae* in diseased farmed rainbow trout (*Onchorhynchus mykiss*) in iran. The Scientific World Journal 7: 1-6.

Grenni, P., Ancona V., Barra Caracciolo, A. 2017. Ecological effects of antibiotics on natural ecosystems: A review. Microchemical Journal 136: 25-39.

Guimaraes, A.C., Meireles, L.M., Lemos, M.F., Guimaraes, M.C.C., Endringer, D.C., Fronza, M., Scherer, R. 2019. Antibacterial activity of terpenes and terpenoids present in essential oils. Molecules 24(13): 2471-2483.

Ham, Y., Kim, T.J. 2019. Conditions for preparing *Glycyrrhiza uralensis* extract for inhibiting biofilm formation of *Streptococcus mutans*. Journal of the Korean Wood Science and Technology 47(2): 178-188.

Hammerschmidt, F., Clark, A., Soliman, F., el-Kashoury, E.S.A., el-Kawy, M., el-Fishawy, A. 1993. Chemical composition and antimicrobial activity of essential oils of jasonia candicans and J. Montana. Planta medica 59(1): 68-70.

Han, H.J., Kim, D.H., Lee, D.C., Kim, S.M., Park, S.I. 2006. Pathogenicity of edwardsiella tarda to olive flounder, *Paralichthys olivaceus* (Temminck & Schlegel). Journal of Fish Diseases 29(10): 601-609.

Hirai, Y., Asahata-Tago, S., Ainoda, Y., Fujita, T., Kikuchi, K. 2015. *Edwardsiella tarda* bacteremia. A rare but fatal water- and foodborne infection: Review of the literature and clinical cases from a single centre. The Canadian Journal of Infectious Diseases and Medical Microbiology 26(6): 313-318.

Hong, E.J., Na, K.J., Choi, I.G., Choi, K.C., Jeung, E.B. 2004. Antibacterial and antifungal effects of essential oils from coniferous trees. Biological and Pharmaceutical Bulletin 27(6): 863-866.

Hundenborn, J., Thurig, S., Kommerell, M., Haag, H., Nolte, O. 2013. Severe wound infection with *Photobacterium damselae* ssp. *damselae* and *Vibrio harveyi*, following a laceration injury in marine environment: A case report and review of the literature. Case Reports in Medicine 2013(5): 610-632.

Hussain, A., Anwar, F., Shahid, M., Basra, S., Przybylski, R. 2010. Chemical composition, and antioxidant and antimicrobial activities of essential oil of spearmint (*Mentha spicata* L.) from Pakistan. The Journal of Essential Oil Research 22(1): 78-84.

Hyldgaard, M., Mygind, T., Meyer, R.L. 2012. Essential oils in food preservation: Mode of action, synergies, and interactions with food matrix components. Frontiers in Microbiology 3(12): 1-24.

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, *Paralichthys olivaceus*, in south korea. Journal of Fish Pathology 27(1): 77-84.

Jeong, M.J., Yang, J., Choi, W.S., Kim, J.W., Kim, S.J., Park, M.J. 2017. Chemical compositions and antioxidant activities of essential oil extracted from *Neolitsea aciculata* (Blume) Koidz leaves. Journal of the Korean Wood Science and Technology 45(1):

96-106.

Jung, E.K. 2009. Chemical composition and antimicrobial activity of the essential oil of *Chrysanthemum indicum* against oral bacteria. Journal of Bacteriology and Virology 39(2): 61-69.

Kim, H.S., Lee, B.S., Yun, K.W. 2013. Comparison of chemical composition and antimicrobial activity of essential oils from three pinus species. Industrial Crops and Products 44: 323-329.

Kim, S.H., Lee, S.Y., Cho, S.M., Hong, C.Y., Park, M.J., Choi, I.G. 2016. Evaluation on anti-fungal activity and synergy effects of essential oil and their constituents from *Abies holophylla*. Journal of the Korean Wood Science and Technology 44(1): 113-123.

Kim, S.M., Jun, L.J., Park, M.A., Jung, S.H., Jeong, H.D., Jeong, J.B. 2015. Monitoring of emaciation disease in cultured olive flounder *Paralichthys olivaceus* in Jeju (2010-2013), korea. Korean Journal of Fisheries and Aquatic Sciences 48(5): 719-724.

Knobloch, K., Pauli, A., Iberl, B., Weigand, H., Weis, N. 1989. Antibacterial and antifungal properties of essential oil components. Journal of Essential Oil Research 1(3): 119-128.

Kotan, R., Kordali, S., Cakir, A. 2007. Screening of antibacterial activities of twenty-one oxygenated monoterpenes. Zeitschrift fur Naturforschung. C, Journal of Biosciences 62(7-8): 507-513.

Koukos, P.K., Papadopoulou, K.I., Patiaka, D.T., Papagiannopoulos, A.D. 2000. Chemical composition of essential oils from needles and twigs of balkan pine (*Pinus peuce* Grisebach) grown in Northern Greece. Journal of Agricultural and Food Chemistry 48(4): 1266-1268.

Lambert, R.J., Skandamis, P.N., Coote, P.J., Nychas, G.J. 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. Journal of Applied Microbiology 91(3): 453-462.

Lee, J.H., Hong, S.K. 2009. Comparative analysis of chemical compositions and antimicrobial activities of essential oils from *Abies holophylla* and *Abies koreana*. Journal of Microbiology and Biotechnology 19(4): 372-377.

Lee, S.Y., Kim, S.H., Park, M.J., Lee, S.S., Choi, I.G. 2014. Antibacterial activity of essential oil from *Abies holophylla* against respiratory tract bacteria. Journal of the Korean Wood Science and Technology 42(5): 533-542.

Li, W.R., Shi, Q.S., Liang, Q., Xie, X.B., Huang, X.M., Chen, Y.B. 2014. Antibacterial activity and kinetics of *Litsea cubeba* oil on *Escherichia coli*. PLoS ONE 9(11): e110983.

Li, Z.H., Cai, M., Liu, Y.S., Sun, P.L., Luo, S.L. 2019. Antibacterial activity and mechanisms of essential oil from *Citrus medica* L. var. *sarcodactylis*. Molecules 24(8): 1577-1587.

Lingan, K. 2018. A review on major constituents of various essential oils and its application. Translational Medicine 8(1).
DOI: 10.4172/2161-1025.1000201.

Lopez-Romero, J.C., Gonzalez-Rios, H., Borges, A., Simoes, M. 2015. Antibacterial effects and mode of action of selected essential oils components against *Escherichia coli* and *Staphylococcus aureus*. Evidence-based Complementary and Alternative Medicine : eCAM 2015: 1-9.

Mann, C.M., Cox, S.D., Markham, J.L. 2000. The outer membrane of *Pseudomonas aeruginosa* NCTC 6749 contributes to its tolerance to the essential oil of *Melaleuca alternifolia* (tea tree oil). Letters in Applied Microbiology 30(4): 294-297.

Markestad, A., Grave, K. 1997. Reduction of antibacterial drug use in norwegian fish farming due to vaccination. Developments in Biological Standardization 90: 365-369.

Martucci, J.F., Gende, L., Neira, L., Ruseckaite, R.

2015. Oregano and lavender essential oils as antioxidant and antimicrobial additives of biogenic gelatin films. Industrial Crops and Products 71: 205-213.

Min, H.J., Kim, C.S., Hyun, H.J., Bae, Y.S. 2017. Essential oil analysis of *Illicium anistum* L. extracts. Journal of the Korean Wood Science and Technology 45(6): 682-688.

Mohammed, H., Hamamouchi, J., Zouhdi, M., Bessière, J.M. 2001. Chemical and antimicrobial properties of essential oils of five moroccan pinaceae. Journal of Essential Oil Research 13(4): 298-302.

Moon, J.S., Kim, J.Y., Joh, S.J., Kim, M.J., Son, S.W., Jang, H. 2009. Influence on efficacy of β-hemolytic *Streptococcus iniae* vaccine by mixed infections with *Edwardsiella tarda* and *Neoheterobothrium hirame* in cultured olive flounder, *Paralichthys olivaceus*. Journal of Veterinary Clinics 26(3): 226-230.

Nazzaro, F., Fratianni, F., Coppola, R., Feo, V. 2017. Essential oils and antifungal activity. Pharmaceuticals (Basel) 10(4): 86-106.

Nazzaro, F., Fratianni, F., De Martino, L., Coppola, R., De Feo, V. 2013. Effect of essential oils on pathogenic bacteria. Pharmaceuticals (Basel) 6(12): 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., 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 Microbiology Letters 293(1): 20-27.

Nikaido, H. 1994. Prevention of drug access to bacterial targets: Permeability barriers and active efflux. Science 264(5157): 382-388.

Oh, M.J., Jung, S.J., Kitamura, S.I., Kim, H.Y., Kang, S.Y. 2006. Viral diseases of olive flounder in korean hatcheries. Journal of Ocean University of China 5(1): 45-48.

Park, J.W., Wendt, M., Heo, G.J. 2016. Antimicrobial activity of essential oil of *Eucalyptus globulus* against fish pathogenic bacteria. Laboratory Animal Research 32(2): 87-90.

Plesiat, P., Nikaido, H. 1992. Outer membranes of gram-negative bacteria are permeable to steroid probes. Molecular Microbiology 6(10): 1323-1333.

Raeisi, M., Tajik, H., Yarahmadi, A., Sanginabadi, S. 2015. Antimicrobial effect of cinnamon essential oil against *Escherichia coli* and *Staphylococcus aureus*. Health Scope 4(4): e21808.

Rhodes, G., Huys, G., Swings, J., Mc Gann, P., Hiney, M., Smith, P., Pickup, R. 2000. Distribution of oxytetracycline resistance plasmids between aeromonads in hospital and aquaculture environments: Implication of tn*1721* in dissemination of the tetracycline resistance determinant tet A. Applied and Environmental Microbiology 66(9): 3883-3890.

Rossiter, S., Fletcher, M., Wuest, W. 2017. Natural products as platforms to overcome antibiotic resistance. Chemical Reviews 117(19): 12415-12474.

Salgado-Garciglia, R., López-Meza, J., Torres-Martínez, R., Ochoa-Zarzosa, A., Saavedra-Molina, A., García-Rodríguez, Y., Rios-Chavez, P. 2018. Antioxidant activity of the essential oil and its major terpenes of *Satureja macrostema* (moc. And sessé ex benth.) briq. Pharmacognosy Magazine 13(4): 875-880.

Sutili, F., Murari, A.L., Silva, L.L., Gressler, L., Heinzmann, B., Vargas, A., Schmidt, D., Baldisserotto, B. 2016. The use of *Ocimum americanum* essential oil against the pathogens *Aeromonas hydrophila* and *Gyrodactylus* sp. in silver catfish (*Rhamdia quelen*). Letters in Applied Microbiology 63(2): 82-88.

Tabanca, N., Kirimer, N., Demirci, B., Demirci, F., Baser, K.H. 2001. Composition and antimicrobial activity of the essential oils of *Micromeria cristata* subsp. phrygia and the enantiomeric distribution

of borneol. Journal of Agricultural and Food Chemistry 49(9): 4300-4303.

Tiwari, B.K., Valdramidis, V.P., O'Donnell, C.P., Muthukumarappan, K., Bourke, P., Cullen, P.J. 2009. Application of natural antimicrobials for food preservation. Journal of Agricultural and Food Chemistry 57(14): 5987-6000.

Tongnuanchan, P., Benjakul, S. 2014. Essential oils: Extraction, bioactivities, and their uses for food preservation. Journal of Food Science 79(7): 1231-1249.

Tripathi, A., Upadhyay, S., Bhuyan, M., Bhattacharya, P. 2009. A review on prospects of essential oils as biopesticide in insect-pest management. Journal of Pharmacognosy and Phytotherapy 1(5): 052-063.

Trombetta, D., Castelli, F., Sarpietro, M.G., Venuti, V., Cristani, M., Daniele, C., Saija, A., Mazzanti, G., Bisignano, G. 2005. Mechanisms of antibacterial action of three monoterpenes. Antimicrobial Agents and Chemotherapy 49(6): 2474-2478.

Vaara, M. 1992. Agents that increase the permeability of the outer membrane. Microbiol Reviews 56(3): 395-411.

Xie, Q., Liu, Z., Li, Z. 2015. Chemical composition and antioxidant activity of essential oil of six pinus taxa native to china. Molecules 20(5): 9380-9392.

Yan, L., Kim, I.H. 2013. Effects of dietary supplementation of fermented garlic powder on growth performance, apparent total tract digestibility, blood characteristics and faecal microbial concentration in weanling pigs. Journal of Animal Physiology and Animal Nutrition 97(3): 457-464.

Yang, J., Choi, W.S., Kim, J.W., Lee, S.S., Park, M.J. 2019. Anti-inflammatory effect of essential oils extracted from wood of four coniferous tree species Journal of the Korean Wood Science and Technology 47(6): 674-691.

Yang, X.N., Khan, I., Kang, S.C. 2015. Chemical composition, mechanism of antibacterial action and antioxidant activity of leaf essential oil of *Forsythia koreana* deciduous shrub. Asian Pacific Journal of Tropical Medicine 8(9): 694-700.

APPENDIX

(Korean Version)

어병 세균에 대한 소나무과 잎 정유의 항세균 효과

초록 : 어병 세균은 어류 양식업의 경제적 피해 뿐만 아니라, 인수공통감염원으로 알려진 전염성 병원균이다. 어병 세균을 제어하기 위해 지속적인 항생제의 사용은 부작용이 수반되기 때문에, 천연 유래 소재의 개발이 요구된다. 본 연구에서는 항생제대체제 개발을 위해 항세균효과가 우수한 침엽수 정유를 발굴하고자 하였다. 소나무과에 속하는 전나무 (*Abies holophylla*), 곰솔 (*Pinus thunbergii*), 섬잣나무 (*Pinus parviflora*), 솔송 (*Tsuga sieboldii*), 리기테다소나무 (*Pinus rigitaeda*)의 잎에서 hydro-distillation법을 이용하여 정유를 추출하였으며, 추출된 정유는 어병 세균인 *Edwardsiella tarda*, *Photobacterium damselae*, *Streptococcus parauberis*, *Lactococcus garivieae*에 대하여 항균력을 평가하였다. 그 결과, 전나무와 곰솔 잎 정유가 그람 음성 세균인 *E. tarda*와 *P. damselae*에 대하여 선택적으로 강한 항균력을 나타냈다. GC-MS 분석 결과, 전나무 잎 정유의 주요 성분은 (-)-bornyl acetate (29.45%), D-limonene (20.47%), camphene (11.73%)이고, 곰솔 잎 정유의 주요 성분은 α-pinene (59.81%)으로 각각 확인되었다. 또한, 미량으로 존재하지만 정유와 동일한 효능을 나타내는 유효 성분으로 oxygenated monoterpenes인 neryl acetate, (-)-borneol, (-)-carveol의 세가지 화합물을 구명하였다. 따라서 그람 음성어병세균의 생장억제효과가 우수한 전나무와 곰솔 잎 정유는 사료 첨가제, 수산용 의약품 등 생물학적 제제로 활용 될 수 있을 것으로 사료된다.

1. 서 론

어업 기술이 고도화됨에 따라 그 규모가 거대화되고 대량 생산이 가능해졌다. 그러나, 제한된 장소에서 사육하는 방식의 밀집형 양식산업은 주변의 수질 관리가 어렵기 때문에 환경 오염 등의 문제를 발생시킨다. 이로 인해 어병 세균에 대한 감염 빈도가 증가하고, 좁은 공간에서 빠르게 질병이 전염되기 때문에 막대한 경제적 손실이 야기된다(Oh *et al.*, 2006; Yan and Kim, 2013).

어종에 따른 세균성 질병에는 차이가 있지만, 주로 *Edwardsiella tarda*에 의한 edwardsiellosis, *Streptococcus parauberis*와 *Lactococcus garvieae*에 의한 streptococcosis가 있으며, 최근에는 *Photobacterium damselae*에 의한 vibriosis 등에 의한 피해가 보고되고 있다(Jee *et al.*, 2014; Kim *et al.*, 2015). 이들은 넙치의 세균성 질병을 유발하는 대표적인 감염성 병원균으로, 세균에 감염된 넙치는 출혈, 복부팽만, 신장 비대 등의 증상이 나타나고 결국에는 폐사로 이어진다(Han *et al.*, 2006; Cho *et al.*, 2007; Moon *et al.*, 2009; Nho *et al.*, 2009). 게다가, *E. tarda*를 비롯한 일부 세균은 어류뿐만 아니라 인간에게도 감염되어 질병을 유발하는 인수공통전염병(zoonosis) 세균으로 알려져 있다(Erfanmanesh *et al.*, 2012; Hundenborn *et al.*, 2013; Hirai *et al.*, 2015; Choksi and Dadani, 2017). 이에 사람들은 어병 세균에 의한 질병을 치료 또는 예방함으로써, 어류의 사망률을 낮추고 어류 생산량을 늘리기 위해서 사료나 물에 항생제를 투여했다(Markestad and Grave, 1997; Cabello, 2006). 그러나, 항생제의 지속적인 사용과 무분별한 오・남용은 내성 균주의 출현을 초래할 뿐만 아니라, 환경 생태계의 교란을 야기할 가능성이 있다(Rhodes *et al.*, 2000; Cabello, 2006). 그러므로, 이러한 항생제 사용으로 인한 부작용을 최소화하고, 어병 세균을 효과적으로 제어할 수 있는 자연 유래의 대체 항생제 소재의 개발이 주목받고 있다(Grenni *et al.*, 2017; Rossiter *et al.*, 2017).

천연 자원인 정유는 주로 향이 있는 식물에서 분리되는 2차 대사 물질 중 하나로, 기름 형태의 휘발성 혼합물이다. 정유에는 항산화(Amorati *et al.*, 2013; Salgado-Garciglia *et al.*, 2018; Jeong *et al.*, 2017), 항진균(Nazzaro *et al.*, 2017; D'agostino *et al.*, 2019), 살충(Tripathi *et al.*, 2009; Ayvaz *et al.*, 2010), 항세균(Bakkali *et al.*, 2008; Nazzaro *et al.*, 2013; Chouhan *et al.*, 2017) 효과 등 다양한 생물학적 효능이 있다고 알려져 있으며, 정유의 성분 분석과 안전성 검증을 위한 독성 평가가 이루어지고 있다(Min *et al*., 2017; Ahn *et al*., 2018). 소나무과는 정유 함량이 높은 대표적인 식물로, 그 성분은 주로 hydrocarbons과 oxygenated 유도체를 포함하는 monoterpenes과 sesquiterpenes 등 terpene류의 휘발성 방향 화합물로 구성되어 있다(Koukos *et al.*, 2000; Hong *et al.*, 2004). 이러한 화합물을 포함하는 소나무과 정유는 아로마테라피(Ali *et al.*, 2015), 항염증(Yang *et al.*, 2019), 항산화(Xie *et al.*, 2015), 항미생물(Chouhan *et al.*, 2017) 등의 다양한 분야에서 활용되고 있다(Aziz *et al.*, 2018). 특히, 피톤치드 (phytoncide)라고도 불리는 소나무과 정유는 다양한 그람 음성 세균과 양성 세균에 대하여 강한 항균 활성을 나타낸다(Mohammed *et al.*, 2001; Lee *et al.*, 2014).

정유를 포함하는 천연물의 어병 세균에 대한 항세균 활성 연구가 진행되고 있으나(Bulfon *et al.*, 2013; Park *et al.*, 2016; Cunha *et al.*, 2018), 소나무과 잎 정유에 대한 효능은 현재까지 알려진 바 없다. 또한, 대부분의 연구는 시료의 부위나 추출

방법에 따른 정유의 효능을 비교하고 평가하거나, 성분 분석을 통한 주요 성분을 제시하는 수준이다. 그러나, 정유에는 다양한 성분이 혼합되어 있기 때문에 한가지 성분으로 설명이 불가능하는 경우가 존재하며, 이런 특이적인 현상을 설명하는 중요 유효 성분에 대한 연구는 미비한 실정이다.

이에 본 연구에서는 소나무과 침엽수종인 전나무(*Abies holophylla*), 곰솔(*Pinus thunbergii*), 섬잣나무(*Pinus parviflora*), 솔송(*Tsuga sieboldii*), 리기테다소나무(*Pinus rigitaeda*) 잎 정유의 어병 세균에 대한 항세균 활성을 평가하고, 유효 성분을 구명하고자 하였다. 이를 통해, 소나무과 잎 유래 정유가 어병 세균 감염에 의한 질병의 예방이나 수산용 의약품 등으로 활용 가능한지 확인하고자 하였다.

2. 재료 및 방법

2.1. 소나무과 시료와 Terpene 계열 단일 화합물

실험에 사용한 5종의 나무는 모두 소나무과에 속한다. 전나무, 곰솔, 리기테다소나무 잎은 수원시 소재의 국립산림과학원 시험림에서, 솔송 잎은 포천읍 소재 국립산림과학원 시험림에서, 그리고 섬잣나무 잎은 울릉도에서 각각 채취하였다. 본 연구에서 사용된 (-)-bornyl acetate (99%, product number: 45855), α-pinene (97%, product number: 80604), (-)-borneol (99%, product number: 15598), neryl acetate (97%, product number: 46015), 그리고 (-)-carveol (98%, product number: 61370)은 모두 Sigma-aldrich에서 구입하였다.

2.2. 정유의 추출

전나무, 곰솔, 섬잣나무, 솔송, 리기테다소나무의 잎에서 hydro-distillation 법을 이용하여 각각 정유를 추출하였다. 10 L 부피의 둥근 플라스크에 시료 1 kg당 증류수를 5-6 L 첨가하였다. 추출은 heating mantle (Model: MS-DM608, Serial number: 201602, Misung Scientific. Co. Ltd., Korea)에서 100 ± 2˚C 온도 조건으로, 더 이상 정유가 추출되지 않을 때까지 진행되었다. 추출한 정유는 anhydrous Na_2SO_4(98.5%,Samchun,Korea)를 이용하여 수분이 제거되었고, 0.45 μm pore size minisart® syringe filter (Reference number: 16555-K, Sartorius Stedim Biotech GmbH, Germany)를 이용하여 여과되었다. 여과된 정유는 빛이 차단되는 갈색 병에 옮겨진 후, 질소를 채워 4 ˚C 냉장 보관되었다.

2.3. 세균과 배양 조건

Edwardsiella tarda FP5060, *Photobacterium damselae* FP4101, *Lactococcus garvieae* FP5245, 그리고 *Streptococcus parauberis* FP3287은 모두 국립수산과학원의 해양수산생명자원으로부터 분양 받았으며, 25% glycerol stock으로 –40 ˚C에서 보관되었다. BHI agar는 BHI agar는 BHI broth (Bacto™ brain heart infusion, Product number: 237500, BD Biosciences Korea Ltd., Korea)에 15 g/L agar를 첨가하여 제조되었다. 냉동 보관된 세균을 BHI agar plate에 접종하여 28 ˚C 배양기에서 24시간 배양하여 단일 군집을 확보되었다. 단일 군집을 4 mL BHI broth에 접종한 후, 250 rpm의 조건으로 overnight 배양되었다.

2.4. Paper disc diffusion을 이용한 항균력 평가

BHI broth에서 배양한 세균들을 Neo-D3117 UV-VIS spectrophotometer (NEOGEN Inc., Korea)를 이용하여 600 nm에서 측정되었으며, optical density (O.D)가 1이 되도록 조정되었다($O.D_{600}$ = 1). Mueller hinton agar (Difco, Product number: 225250, BD Biosciences Korea Ltd., Korea) plate의 중앙에 농도가 조정된 균 100 μL를 접종된 후, glass spreader를 이용하여 고르게 도말되었다. 멸균된 paper disc (ADVANTEC, Product number: 49005040, Toyo Roshi Kaisha, Ltd. Japan)를 균주가 도말된 plate에 부착하고, 각 정유 원액을 5 μl씩 흡수시켰다. 그 후, 28 ˚C 배양기에서 2일간 배양하여 paper disc 주위로 형성되는 환의 직경을 측정하여 항균력을 평가하였다.

2.5. 농도에 따른 전나무와 곰솔 잎 정유와 단일 화합물의 항균력 평가

항세균 활성을 평가하기 위해 CLSI M07-A9의 microdilution method를 변형하여 사용하였다. 전나무와 곰솔 잎 정유, 그리고 단일 화합물은 dimethyl sulfoxide (DMSO, extra pure grade, Product number: 3047-4460, Duksan)에 용해되어 각각 5% 농도 (v/v)로 제조되었으며, 사용 전까지 -40˚C에 보관되었다. 정유 시료는 3.5, 2, 1, 그리고 0.5% 농도로 희석되었다. 96-well polystyrene microplate (Flat bottom well, Product number: 30096, SPL Life Sciences Co., Ltd., Pocheon, Korea)의 각 well에 BHI broth를 200 μL씩 분주하였다. 준비된 시료를 각 well에 2 μL 처리하였다. 그 후, 전배양한 세균의 농도가 $O.D_{600}$ = 0.05가 되도록 각 well에 접종하였으며, 28 ˚C에서 24시간 배양하였다. 24시간 배양 후, Epoch microplate spectrophotometer (BioTek Instruments, Inc., US)기기를 이용하여 600 nm에서 흡광도를 측정하여, 정유의 항균력을 평가하였다. 대조군은 시료를 용해한 DMSO를 1% 처리하였고, 양성 대조군은 항생제인 tetracycline (HPLC grade, Product number: 87128, Sigma-Aldrich, Korea)을 사용하였다.

2.6. Gas chromatography-mass spectrometry (GC-MS) 분석

GC-MS 분석을 통하여 전나무와 곰솔 잎으로부터 추출된 정유의 성분이 분석되었다. GC-MS 분석은 TriPlus™ 100 LS Liquid Auto-sampler (Catalog number: IQLAAAGAAHFACMMBES), ISQ™ Series Single Quadrupole GC-MS System (Catalog number: IQLAAAGAAJFALOMAYE)로 구성된 TRACE™ 1310 Gas Chromatograph가 사용되었으며, 모두 Thermo Fisher Scientific 제품이다(Thermo Fisher Scientific Solutions LLC, Korea). Column은VF-5MS (Silica, 길이 60 m × 내경 0.25 mm × 두께 0.25 μm, Agilent Technologies, Inc., US)가 사용되었다. 이동상 기체로 헬륨을 1 mL/min의 속도로 공급되었다. 정유 시료는 1 mL의 dichloromethane에 4 μL를 용해되었고, 1 μL 주입되었다. Injection temperature는 250 ˚C로 유지되었고, 1:20의 비율의 분할 모드(split mode)가 사용되었다. Oven 온도는 초기 50 ˚C로 5분간 유지된 후, 10 ˚C/min으로 65 ˚C까지 증가되었으며 30분 동안 유지되었다. 그 후, 5 ˚C/min으로 210 ˚C까지 온도가 상승되었으며, 10분 동안 유지되었다. 마지막으로 20 ˚C/min으로 최고 325 ˚C까지 온도가 증가되었으며, 10분 간 유지되었다. Flame ionization detector (FID)의 온도는 300 ˚C로 설정되었고, air flow는 350 mL/min, hydrogen flow는 35 mL/min, 그리고 make-up gas (helium)은 40 mL/min의 속도로 설정되었다. MS data는 35 - 550 amu 범위의 mass를EI ionization mode에서 0.2 sec/scan 속도로 수집되었다.

수집된 peak data 중에서 match quality가 높은 것을 1차로 선별되었고, *n*-alkanes (C_8 - C_{20}, Product number: 04071, Sigma-Aldrich, Korea)를 이용하여 Kovats retention index (KI)를 계산되었다. 계산된 KI 값을National Institute of Standards and Technology (NIST, US)의NIST Chemistry WebBook (Standard Reference Database Number 69)와 비교되었으며, 그 차이가 100 미만인 화합물이 최종적으로 선정되었다.

2.7. 통계분석

모든 실험 결과는 평균 및 표준 편차로 제시되었다. 일원 분산 분석 (one way analysis of variance)는 IBM® SPSS software (Ver. 25.0; SPSS Inc., Chicago, Illinois, USA)를 사용하여 유의미한 차이를 구별하는데 사용되었으며, 유의성은 Tukey test 또는 paired-t-test에 의해 95% 신뢰 수준에서 정의되었다.

3. 결과 및 고찰

3.1. 어병 세균에 대한 소나무과 정유의 항균력 평가

전나무, 곰솔, 섬잣나무, 솔송, 그리고 리기테다 소나무 잎으로부터 추출한 각 정유의 수율은 2.99%, 0.93%, 0.08%, 0.15%, 0.72%로 확인되었으며, 항세균 효과는 paper disc diffusion법으로 평가되었다. 정유가 포함된 paper disc에 4종의 어병 세균을 2일간 노출시켜 형성되는 환의 크기를 확인하여 정유의 항균 활성 유무를 확인하였다(Table 1). Paper disc 주위로 형성되는 깨끗한 환은 세균이 자라지 못하여 형성되기 때문에, 생장억제환의 크기가 항균 활성의 지표가 된다(Djabou *et al.*, 2013).

*E. tarda*에 대하여 전나무와 곰솔 정유가 각각 21.5 mm, 21.0 mm로 강한 항균 활성을 나타냈으며, 그 다음으로 솔송(16 mm), 리기테다소나무(13 mm) 순으로 항균 효과를 보였다. *P. damselae* 또한, 전나무와 곰솔 정유가 각각 23.5 mm, 20.5 mm 크기의 넓은 생장억제환을 형성하였으며, 솔송(13.25 mm), 리기테다소나무(11.5 mm), 섬잣나무(8 mm) 순으로 환의 크기가 관찰되었다. 시험된 정유의 *L. garvieae*에 대한 항균효과는 *E. tarda*와 *P. damselae*에 대한 항균활성보다 낮으며, 5종의 정유 중 전나무 정유가 12.75 mm으로 다른 정유에 비해 항균 활성이 높게 나타났다. *S. parauberis*에 대하여 곰솔 정유가 14.0 mm의 가장 넓은 환을 형성하였으나, 나머지 정유는 약 10 mm 수준의 비슷한 크기의 생장억제환을 형성하였다. 반면, 섬잣나무 정유는 *E. tarda*와 *S. parauberis*에 대하여 환을 형성하지 않았다. 이러한 결과를 종합하여 보았을 때, 전반적으로 전나무와 곰솔 잎 정유가 어병 세균에 대하여 항균력이 강한 것으로 판단되었으며, 특히 *E. tarda*와 *P. damselae*에 대하여 효과적이었다.

앞서 paper disc assay 결과, 그람 음성 세균인 *E. tarda*와 *P. damselae*는 전나무와 곰솔 잎 정유에 대하여 높은 감수성을 나타냈다. 반면에, 그람 양성 세균인 *L. garvieae*와 *S. parauberis*는 상대적으로 두 정유에 대하여 감수성이 낮았다(Table 1). 이에 4종의 어병 세균에 대하여 전나무와 곰솔 잎 정유의 농도에 따른 성장 변화를 관찰하였다(Fig. 1). *E. tarda*에 대하여 전나무 잎 정유는 0.01% 농도에서 약 5% 성장을 억제하고, 0.02% 농도에서 그 효과가 급격하게 증가하여 100% 억제하였다. 곰솔 잎 정유는 0.01%, 0.02% 농도에서 각각 약 10%, 25%의 생장 억제 효과가 나타났으며, 0.035% 농도에서 100% 효과를 보였다(Fig. 1A). *P. damselae*는 0.02% 농도의 전나무와 곰솔 잎 정유에서 급격하게 성장이 억제되었으며, 각각 74%, 83%의 효과를 나타냈다. 또한, 0.035% 농도의 전나무 잎 정유가 약 93%, 곰솔 잎 정유가 약 81% 성장을 억제하였다(Fig. 1B). *L. garvieae*의 성장에는 전나무와 곰솔 잎 정유의 영향이 미비하였다(Fig. 1C). *S. parauberis* 성장 또한 전나무 잎 정유에 의해서는 영향을 받지 않았으나, 0.035% 농도의 곰솔 잎 정유에 의해 성장이 약 18% 억제되었다(Fig. 1D). 이와 같은 전나무와 곰솔 잎 정유의 어병 세균에 대한 항세균 연구는 현재까지 보고된 바 없으며, 전나무와 곰솔 잎 정유의 어병 세균에 대한 감염 질환 예방을 위한 소재로써

가능성을 제시하였다는 점에서 의의가 있다.

일반적으로 정유의 항미생물 활성은 그람 음성 세균보다 그람 양성 세균에 대하여 효과가 강한 것으로 알려져 있다(Trombetta *et al.*, 2005; Nazzaro *et al.*, 2013). 그람 양성 세균은 세포벽의 약 90 - 95%가 peptidoglycan으로 이루어져 있으며, 세포벽 표면에는 teichoic acid, lipotechoic acid등이 존재한다. 그러므로 그람 양성 세균의 세포벽은 정유와 같은 소수성의 물질들을 비교적 쉽게 통과시키며, 세포벽이나 세포 내부의 cytoplasm에 작용할 수 있도록 한다(Tiwari *et al.*, 2009). 반면, 그람 음성 세균의 세포벽은 그람 양성 세균보다 얇은 peptidoglycan 층을 가지고 있으며, 가장 바깥에는 outer membrane이 한 층 더 존재한다. Outer membrane에 존재하는 lipopolysaccharide는 세포벽의 표면에 hydrophilic한 특성을 부여하기 때문에, 정유와 같은 hydrophobic한 물질에 대하여 저항을 나타내는 것으로 알려져 있다(Vaara, 1992; Nikaido, 1994; Mann *et al.*, 2000). 선행 연구에서도 초본류로부터 추출한 정유가 그람 음성 세균보다 그람 양성 세균의 생장 억제에 보다 효과적이라 보고된 바 있으며(Aumeeruddy Elalfi *et al.*, 2015; Martucci *et al.*, 2015; Bouazama *et al.*, 2017), 목본류의 정유 또한, 그람 양성 세균에 대하여 보다 낮은 농도에서 MIC 수치를 나타내는 연구 결과가 있다(Aumeeruddy Elalfi *et al.*, 2015). 한 연구에서는 *Citrus medica*로부터 추출한 정유를 이용하여 time-kill analysis를 수행한 결과, *E. coli*는 정유에 노출된 지 4시간 뒤에 생장이 최대로 억제되었으나, *S. aureus*는 이보다 이른 2시간만에 균이 최대로 사멸되었다고 보고하였다(Li *et al.*, 2019).

그러나 흥미롭게도, 본 연구에서 실험된 전나무, 곰솔, 리기테다소나무, 솔송 잎 유래 정유는 그람 양성 세균(*L. garvieae*와 *S. parauberis*) 보다 그람 음성 세균(*E. tarda*와 *P. damselae*)에 대하여 높은 항균 활성을 나타냈다(Table 1). 특히, 정유 중 가장 강력한 생장 억제 효과를 나타낸 전나무와 곰솔 잎 정유 에 의해 *E. tarda*와 *P. damselae*의 MIC 농도가 확인되었으나, 그람 양성 세균에는 모든 농도에서 효과가 미비하였다(Fig. 1). 이러한 결과는 기존에 보고된 연구 결과와는 대조되는 현상으로, 전나무와 곰솔 잎 정유의 구성 화합물의 분석이 필요하다고 판단되었다.

3.2. 소나무과에 속하는 5수종 잎 유래 정유의 GC-MS 분석

소나무과에 속하는 5수종의 잎에서 추출한 정유는 GC-MS 분석을 통해 화합물 조성이 분석되었다(Table 2). 섬잣나무 잎 정유의 주요 구성 성분들은 α-pinene (36.58%), β-pinene (23.49%), D-limonene (18.99%)으로 확인되었고, 솔송 잎 정유는 (-)-bornyl acetate (47.06%), α-pinene (12.03%), camphene (8.42%)가 주요 성분으로 확인되었다. 또한, 리기테다소나무는 β-pinene (18.08%), α-pinene (8.19%)의 비율이 높았다.

그람 음성 세균에 대하여 선택적으로 강한 항균 활성을 나타내는 전나무와 곰솔 잎 정유의 구성 성분들은 대부분이 monoterpene과 sesquiterpene을 포함하는 terpene계열의 화합물로 분석되었다. 전나무 잎 정유의 주요 성분은 22개의 monoterpene (79.82%)과 7개의 sesquiterpene (7.21%) 성분으로 구성되었고, 곰솔 잎 정유의 주요 성분으로 26개의 monoterpene (80.94%)과 9개의 sesquiterpene (14.00%) 성분이 검출되었다. 전나무 잎 정유의 주요 구성 성분은 (-)-bornyl acetate (29.45%), D-limonene (20.47%), camphene (11.73%), α-pinene (6.08%) 등으로 확인되었다. 곰솔 잎 정유는 α-pinene이 59.81%로 가장 높은 비율을 차지하였으며, caryophyllene oxide (5.44%), D-limonene (4.34%), β-myrcene (4.24%)의 순으로 존재하였다. 전나무 잎 정유와 곰솔 잎 정유에 공통으로 존재하는 화합물은 총 17개로 확인되었으며, monoterpene 이 12개, sesquiterpene이 5개로 확인되었다. 이러한 결과를 통해, 본 연구에서 hydro-distillation으로 추출한 두 정유에 가장 많은 비율을 차지하고 있는 성분은 각각 (-)-bornyl acetate와 α-pinene로 확인되었다. 전나무 잎 정유의 주요 성분에 대하여 다른 연구들에서는 bicyclo [2.2.1] heptan-2-ol (28.05%), δ3-carene (13.85%), α-pinene (11.68%)을 제안하거나 (Lee and Hong, 2009), 또는 3-carene (25.53%), α-pinene (17.55%), bornyl acetate (16.22%) 를 주요 성분으로 언급하고 있다 (Kim *et al*., 2016). 곰솔 정유 또한, 2H-benzocyclohepten-2-one (34.33%), α-humulene (19.59%), limonene (5.92%), caryophyllene (5.32%)이 주요 성분으로 보고되었다 (Kim *et al.*, 2013). 정유를 비롯한 추출물은 시료의 부위, 수확 시기, 추출 방법, 추출 시 온도와 시간 등 다양한 추출 조건들에 의해 추출 성분의 차이가 존재한다 (Tongnuanchan and Benjakul, 2014; Lingan, 2018). 그렇기 때문에 식물로부터 분리되는 화합물의 차이가 발생되며, 최종적으로 정유를 구성하는 조성 비율이 달라지게 된 것으로 추정된다. 이러한 결과는 추후 정유가 기능성 소재로 활용되기 위해서는 적절한 추출 방법의 표준화가 요구되며, 생리 활성을 나타내는 정유의 성분 분석을 통해 품질의 기준이 되는 지표 성분이 각각 제시되어야 한다.

3.3. 어병 세균에 대한 단일 화합물의 항세균 활성 평가

정유를 비롯한 식물 추출물에는 다양한 화합물이 존재하고, 이들이 상호 유기적으로 작용하여 생리 활성을 나타내기 때문에, 그 기전을 명확하게 제시하기 어렵다(Sutili *et al.*, 2016). 또한, 표준 성분으로 알려진 화합물이 추출물의 효능과 일치하지 않는 경우가 존재하며, 오히려 미량의 화합물에 의해 그 현상이 설명되는 경우가 있다(Chouhan *et al.*, 2017; Ham and Kim, 2019).

이에 본 연구에서는 정유를 구성하는 terpene계열 단일 화합물에 대한 활성 평가를 진행하였으며, 정유와 유사한 생리활성을 나타내는 명확한 유효 성분을 구명하고자 하였다. 전나무와 곰솔 잎 정유의 GC-MS 분석 결과를 바탕으로, 가장 많이 존재하는

(-)-bornyl acetate와 α-pinene을 포함하여 다양한 terpene계열 단일 화합물(0.05% 농도)의 항균력 screening을 진행하였다(Data not shown). 그 결과, 전나무와 곰솔 잎 정유와 동일하게 *E. tarda*와 *P. damselae*의 생장을 선택적으로 억제하는 neryl acetate, (-)-borneol, 그리고 (-)-carveol 이상 3개의 monoterpene 계열의 유효 화합물이 확보되었다(Fig. 2). *E. tarda*는 세 화합물에 의해 각각 68%, 75%, 그리고 69% 성장이 억제되었으며, positive control인 tetracycline (0.005% 농도)에 의해 77% 억제되었다(Fig. 2A). *P. damselae* 또한, 세 단일 화합물에 의해 각각 55%, 70%, 그리고 71% 성장이 억제되었으나, tetracycline에 의해서는 17%만 감소되었다(Fig. 2B). 반면, 그람 양성 세균인 *L. garvieae*와 *S. parauberis*의 성장에는 세 terpene 계열 화합물의 영향이 미비하였다. *L. garvieae*은 (-)-borneol에 의해서만 10%의 성장 감소를 보였으며, tetracycline에 의해 42% 억제되었다(Fig. 2C). *S. parauberis*의 성장에는 모든 화합물이 억제 효과를 나타내지 않았으며, positive control인 tetracycline만 81% 억제하였다(Fig. 2D). 이러한 neryl acetate, (-)-borneol, 그리고 (-)-carveol의 효능 평가 결과는, 그람 음성 어병 세균에 대하여 선택적으로 강한 항균력을 나타내는 전나무와 곰솔 잎 정유의 특이적인 효과를 설명하기 위한 기초 자료를 제공한다는 점에서 의의가 있다.

Neryl acetate와 (-)-carveol은 전나무 잎 정유에서만 확인되었고, (-)-borneol은 전나무와 곰솔 잎 정유 모두에서 검출되었다(Table 2). 이러한 결과는 앞서 전나무 잎 정유가 곰솔 잎 정유보다 *E. tarda*에 대한 생장 억제 효과가 뛰어난 현상에 대하여 부분적으로 설명이 가능할 것으로 사료된다. 반면, 리기테다소나무 정유에서도 neryl acetate와 (-)-borneol이 검출되었으나 어병 세균에 대한 항균 활성이 뛰어나지 않았다. 몇몇 연구에서 추출물 내에 존재하는 성분들을 조합한 결과, 단일 화합물 간에 시너지 효과가 있음이 확인되었다(Ham and Kim, 2019; Kim *et al*., 2016). 이러한 연구들로 미루어 보았을 때, 전나무와 곰솔 잎 정유 또한, neryl acetate, (-)-carveol, (-)-borneol과 다른 구성 성분들간의 시너지에 의한 항균력의 상승 효과가 나타났으며, 이에 따라 리기테다소나무와 비교하여 높은 항균 활성을 나타내는 것으로 추측된다.

(-)-Borneol과 (-)-carveol에 대한 선행 연구 결과, 항세균(Knobloch *et al.*, 1989; Hammerschmidt *et al.*, 1993; Tabanca *et al.*, 2001; Cha, 2007; Jung, 2009; Lopez-Romero *et al.*, 2015; Guimaraes *et al.*, 2019)뿐만 아니라 항진균(Tabanca *et al.*, 2001; Hussain *et al.*, 2010) 효과가 있다고 알려졌으며, neryl acetate 또한 산업 분야에서 발견되는 일부 병원성 미생물들의 생장을 억제한다고 보고되었다(Kotan *et al.*, 2007). 그러나, 이러한 화합물들의 어병 세균에 대한 연구는 진행된 바 없으며, 이와 같은 선행 연구에서는 종의 구분에 따른 특이적인 항균 활성에 대하여 결과를 제시하고 있지 않다. 이러한 사실로 미루어 보았을 때, 본 연구 결과는 oxygenated monoterpene으로 구분되는 neryl acetate, (-)-borneol 그리고 (-)-carveol을 이용하여 선택적으로 그람 음성 어병 세균을 효과적으로 조절할 수 있는 가능성을 제시하는 바이다.

일반적으로 정유의 미생물에 대한 기전은 세포막 파괴(Li *et al.*, 2014; Raeisi *et al.*, 2015), 막 완전성 상실(Diao *et al.*, 2014; Yang *et al.*, 2015), 세포막의 투과성 증가(Lambert *et al.*, 2001; Hyldgaard *et al.*, 2012) 등이 알려져 있으며, 세포 내부로 유입된 정유에 의해 cytoplasm이 변형되고 결국에 세포 사멸이 유도된다(Nazzaro *et al.*, 2013). 또한, 정유는 세균 간의 상호 작용에 중요한 역할을 하는 quorum sensing system에 작용하여, 병원성 인자(바이오필름, 포자 형성 그리고 교배)의 발현에 영향을 미치는 것으로 알려져 있다(Bouyahya *et al.*, 2017). 소수성 분자에 대하여 높은 저항을 지닌 그람 음성 세균의 경우, cell wall에 존재하는 porin 단백질을 통하여 정유와 같은 소수성 화합물이 일부 천천히 통과 할 수 있다고 보고되었다(Plesiat and Nikaido, 1992; Bock and Sawers, 1996). 이러한 선행 연구들을 바탕으로, 본 연구에서 확보한 정유와 3가지 oxygenated terpene의 그람 음성 어병 세균에 대한 추가적인 기전 연구가 필요할 것으로 판단된다. 본 연구를 통하여 양식업에서 문제가 되는 다양한 어병 세균 중에서 특정 종에만 선택적으로 적용되는 소재를 발견하였으며, 이는 무분별하게 작용하여 생태계 교란을 유발할 가능성이 있는 항생제를 대체할 수 있는 새로운 친환경 소재임을 제안하는 바이다.

4. 결 론

본 연구에서는 세균에 의한 감염성 어류 질환을 치료하기 위해 사용되는 항생제의 단점으로 지적되고 있는 내성 균주의 출현 등의 부작용 문제를 보완하기 위해서 천연물 유래 소재인 정유의 대체 가능성을 평가하고자 하였다. 소나무과의 잎으로부터 추출한 5종의 정유의 항균력을 평가하였으며, 전나무와 곰솔의 잎 정유가 그람 음성 어병세균인 *E. tarda*와 *P. damselae*에 대하여 강한 생장 억제 효과를 나타냈다. 이 두 정유의 성분은 GC-MS를 통해 분석한 결과, 주로 monoterpene 계열 화합물로 이루어졌으며, 각각 주요 성분은 (-)-bornyl acetate (29.45%)과 α-pinene (59.81%)으로 확인되었다. 또한, 미량으로 존재하지만 정유와 동일한 효능을 나타내는 유효 화합물로, oxygenated monoterpenes 계열의 neryl acetate, (-)-borneol, 그리고 (-)-carveol이 구명되었다. 이러한 발견은 자연 유래 소재인 소나무과 잎으로부터 추출한 정유를 활용하여 그람 음성 어병 세균을 효과적으로 제어할 수 있을 것이라 사료된다. 그러나, 지용성 화합물인 정유의 활용도를 높이기 위해서는 제제, 제형에 대한 연구가 선행적으로 이루어져야 한다고 판단된다. 또한, 정유를 사료 첨가제, 수질 개선제 등에 첨가함으로써 항생제 사용으로 인한 밀집형 양식 산업의 내성 균주 발생 문제점을 해소하는데 일부 기여할 수 있을 것이라 기대되는 바이다.