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# Antimicrobial activity of lavender (*Lavendular angustifolia*) oil against fish pathogenic bacteria isolated from cultured olive flounder (*Paralichthys olivaceus*) in Korea

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## ABSTRACT

The antibacterial activity of lavender (*Lavendular angustifolia*) essential oil (LVO) was tested against five Gram-negative and nine Gram-positive pathogenic bacteria isolated from cultured olive flounder (*Paralichthys olivaceus*) in Korea. Disc diffusion assay, minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) tests showed that growth of both Gram-negative and Gram-positive bacteria were inhibited by LVO. The inhibition zone diameter (IZD) increased in proportion to the LVO concentration for all of the fish pathogenic bacteria and the maximum effect was found at 100% (V/V) concentration of LVO. *Streptococcus iniae* was most sensitive to LVO while *Edwardsiella tarda* was most resistant. LVO was bacteriostatic (MBC/MIC>4) for all strains of *S. iniae* and bactericidal (MBC/MIC=1-4) for rest of the isolates, except one isoate of *E. tarda.* 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. Since antibacterial activity of LVO was considerably effective against fish pathogenic bacteria, their use could be effectively used to treat bacterial infections in aquaculture.

Keywords: Antibacterial activity, Lavender oil, Olive flounder, Pathogenic bacteria

## Introduction

Olive flounder *Paralichthys olivaceus* (Termminick & Schlegel, 1846), one of the most popular and commercially important cultured marine fish species, has been intensively cultured in Korea. However, the aquaculture industry has confronted high mortalities and heavy economic losses due to bacterial pathogens. Particularly *Streptococcus iniae*, *Streptococcus parauberis* and *Lactococcus garvieae* causing streptococcosis and *Edwardsiella tarda* which causes edwardsiellosis are the major bacterial pathogens affecting olive flounder (Park *et al*., 2009, 2012). Meanwhile, several groups of antibiotics are broadly used to prevent bacterial diseases in fish. The misuse of antibiotics by fish farmers can lead to the outbreak of drug-resistant strains and poses risk to consumers, non-target animals and the environment. Since emergence of multidrug resistance bacteria poses a major threat, investigating the alternative therapies against pathogenic bacterial infection is of growing interest (Bulfon *et al*., 2014; Heo *et al*., 2015).

Accordingly, essential oils (EOs) have raised interest as sources of natural products against bacterial infections (Burt, 2004; Abutbul *et al*., 2005). Such natural substances are safe for the environment than antibiotics EOs have been tested for their potential uses as alternative medications for the treatment of many infectious diseases. In recent years, the ability of EOs to inhibit the growth of bacterial fish pathogens has been widely documented (Turker *et al*., 2009; Debbarma *et al*., 2012; Ontas *et al*., 2016; Park *et al*., 2016).

Lavender (*Lavandula angustifolia*) is a flowering plant under the family Lamiaceae. It is extensively cultivated as ornamental plants in gardens and landscapes, herbs and commercially for the extraction of lavender oil (LVO). The main components of LVO are linalool, linalyl acetate, β-ocimene, 1,8-cineole, terpinen-4-ol and camphor (Jianu *et al*., 2013). The LVO has been found to be effective against many species of bacteria, including antibiotic-resistant bacteria such as vancomycin-resistant *Enterococcus* (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) (Cavanagh and Wilkinson, 2002). The ability of LVO to disrupt the permeability of cell membrane structure, thus interrupting the chemiosmotic control is the most possible reason for its lethal action against bacteria (Miladinovic *et al*., 2012).

There have been a limited number of studies on the antibacterial activity of LVO against fish pathogenic bacteria. However, one previous study reported LVO

to be effective against *Photobacterium damselae, L. garvieae and Yersinia ruckeri* (Bulfon *et al*., 2014). The antimicrobial activity of LVO has not been evaluated against any fish bacterial pathogens in Korea. Therefore, our study sought to examine the antimicrobial activities of LVO against five species of pathogenic bacteria isolated from olive flounder by detecting the susceptibility by disc diffusion, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests.

## Materials and methods

*Fish pathogenic bacteria and EO*

A total of 14 bacterial strains comprising Gram-negative *E. tarda* (FP5060, ED47, Yoshida and ED45) and *P. damselae* (FP4101) and Gram-positive *L. garvieae* (FP5245), *S. iniae* (FP5228, S186, S530 and S131) and *S. parauberis* (FP5228, S124, S527 and S1466), isolated from farmed olive flounder in Korea, were used for this study. These bacteria were received from Institute of Animal Medicine of Gyeongsang National University (Jinju, Korea) and National Institute of Fisheries Science (Busan, Korea). The 100% pure LVO (Aromarant Co. Ltd., Rottingen, Germany) extracted and purified from the flower of lavender (*L. angustifolia*) grown in Bulgaria was procured for the study.

*Disc diffusion test*

The disc diffusion assay was performed to examine the antibacterial activity of LVO. Each bacterial suspension was adjusted to a concentration of 0.5 McFarland units ($1.5\times10^8$ cfu $ml^{-1}$) in sterile saline, then smeared on to the Mueller-Hinton agar (MHA) (MBcell, Seoul, Korea) plates using sterile cotton swabs. Subsequently, sterile discs (ADVANTEC®, Japan) were soaked with 20 µl of LVO at different dilutions of 1:1 (pure LVO), 1:2, 1:5 and 1:10 prepared by mixing 1 part of LVO in respective parts of methanolic solution. Discs were dried for 1 h to let on solvent evaporation. Then each disc was arranged aseptically onto the inoculated MHA plates and incubated for 24 h at 27°C. The test was performed in triplicates and the antibacterial activity of LVO was assessed by measuring the diameter of the inhibition zone.

The susceptibility of 16 commercially available antibiotics was examined by disc diffusion assay following the guidelines of Clinical and Laboratory Standards Institute (CLSI, 2014). Among them, 14 antibiotics excluding erythromycin and clindamycin were used for Gram-negative bacteria while 10 antibiotics excluding nalidixic acid, gentamycin, amikacin, colistin, imipenem and trimethoprim-sulfamethoxazole were used for Gram-positive bacteria. According to the disc diffusion assay results of antibiotics, all the strains were selected for calculating multiple antibiotic resistance (MAR) index. MAR index was calculated as the ratio of the number of antibiotics to which bacteria was resistant to the total number of antibiotics to which the bacteria was exposed.

Broth microdilution method was performed to determine the MIC of LVO. Prior to MIC determination, all the strains were cultured on tryptic soy agar (TSA) (MBcell, Seoul, Korea) plates and incubated at 27ºC for 24 h. The concentration of each test organism was adjusted equivalent to a concentration of 0.5 McFarland units ($1.5\times10^8$ CFU $ml^{-1}$) in sterile saline. The double strength Mueller-Hinton broth containing 5% dimethyl sulfoxide (DMSO) was prepared and 100 µl of this medium was added to each well of 96-well microtiter plates. LVO was added into the first column of wells with a final concentration of 8% (v/v) and then consecutively diluted by two-fold across the microtiter plate until a final concentration of 0.032% (v/v). After adding 100 µl of each bacterial suspension to each well, the microtiter plates were incubated at 27ºC for 24 h. The growth of each bacterial strain was examined by observing the turbidity of the wells. The assay for each of the strains was conducted in triplicates.

To determine the minimum bactericidal concentration (MBC), a bacterial culture suspension with a concentration higher than the MIC was smeared on MHA plates and incubated for 24 h at 27°C. The MBC was determined as the concentration of the maximum dilution which killed 99.9% of the initial number of inoculated strain. The differences were statistically considered significant at $p<0.05$ and correlation indices were calculated using the Pearson coefficient ($\rho$).

## Results and discussion

*Disc diffusion test, MIC and MBC*

In the disc diffusion test, LVO effectively inhibited the growth of all bacteria except two strains of *E. tarda* (ED45 and ED47) in the lowest LVO concentration (1:10). The diameter of inhibition zone increased in proportion to the LVO concentration. *S. iniae* was identified as the most susceptible species where the highest inhibition zone (30 mm) was shown to 1:1 dilution of LVO (Table 1). As the MIC results revealed, the range of MIC of LVO tested for fish pathogenic bacteria was from 0.063 to 2.0% (v/v). The MIC of LVO was $\leq$ 0.125% for most of the strains while MIC of LVO was higher than 0.5% for *E. tarda* strains.

To our knowledge, this is the first report which describes the antimicrobial activity of LVO against *E. tarda*. However, several previous reports described the antimicrobial activity of other EOs such as lemongrass,

Table 1. Susceptibility pattern of lavender oil (LVO) against fish pathogenic bacteria

| Bacterial strain | Inhibition zone[a] (mm) with different LVO dilutions[b] added on disc | | | | MIC (%) | MBC (%) | MIC:MBC |
|---|---|---|---|---|---|---|---|
| | 1:1 | 1:2 | 1:5 | 1:10 | | | |
| *Photobacterium damselae* (FP4101) | 22 | 18 | 11 | 8 | 0.125 | 0.25 | 1:2 |
| *Edwardsiella tarda* (FP5060) | 14 | 10 | 8 | 7 | 1 | 4 | 1:4 |
| *E. tarda* (ED47) | 19 | 14 | 10 | NA | 2 | 8 | 1:4 |
| *E. tarda* (Yoshida) | 18 | 12 | 9 | 7 | 0.5 | 4 | 1:8 |
| *E. tarda* (ED45) | 14 | 12 | 10 | NA | 2 | 8 | 1:4 |
| *Lactococcus garvieae* (FP5245) | 19 | 15 | 11 | 8 | 1 | 4 | 1:4 |
| *Streptococcus iniae* (FP5228) | 20 | 17 | 14 | 11 | 0.5 | 2 | 1:4 |
| *S. iniae* (S186) | 24 | 19 | 13 | 10 | 0.125 | 4 | 1:8 |
| *S. iniae* (S530) | 30 | 22 | 18 | 12 | 0.063 | 0.5 | 1:8 |
| *S. iniae* (S131) | 25 | 19 | 14 | 10 | 0.125 | 1 | 1:8 |
| *Streptococcus parauberis* (FP5228) | 18 | 14 | 11 | 9 | 0.063 | 0.5 | 1:8 |
| *S. parauberis* (S124) | 17 | 14 | 10 | 8 | 0.5 | 2 | 1:4 |
| *S. parauberis* (S527) | 19 | 15 | 10 | 8 | 0.125 | 0.5 | 1:4 |
| *S. parauberis* (S1466) | 21 | 16 | 11 | 8 | 0.25 | 1 | 1:4 |

[a]Inhibition zone; NA=No growth inhibition
[b]Concentration added on disc; 1:1=pure oil, 1:2, 1:5, 1:10 = 1 part of LVO in respective parts of diluent

eucalyptus and curcumin against *E. tarda* (Heo *et al*., 2013; Khan *et al*., 2013; Park *et al*., 2016). Furthermore, two strains of *E. tarda* (ED45 and ED47) were resistant to 6 out of 14 antibiotics and intermediate resistant to 3 antibiotics used in this study, while showing the highest MAR index value and MIC of 0.43 and 2.0% respectively. The reason could be that *E. tarda* is inherently resistant to macrolides, streptogramins, lincosamides, glycopeptides, rifampin and fusidic acid due to the presence of several antibiotic resistance genes simultaneously (Stock and Wiedemann, 2001).

Only one strain of *P. damselae* (FP4101) used in this study was sensitive to LVO which showed MIC of 0.125% and MIC:MBC ratio of 1:2. This indicates the bactericidal activity of LVO against *P. damselae.* Considering the ratio of MBC:MIC, EOs can be grouped into two types. The EOs which exhibited bacterial killing effects with MBC:MIC ≤4 are represented as bactericidal for tested bacteria, while the EOs which demonstrated inhibitory effects with MBC:MIC >4 are represented as bacteriostatic (Kone *et al*., 2004). A previous study reported that LVO showed a moderate efficacy on fish pathogenic *P. damselae* where, MIC and the ratio of MIC:MBC were 1.1 mg $ml^{-1}$ and 1:2 respectively (Bulfon *et al*., 2014).

Notably, the lower concentrations of LVO were effective against majority of Gram-positive bacteria compared to Gram-negative bacteria. The reason could be that the cell wall of Gram-positive bacteria comprises only a single layer where as the Gram negatives are having an extra outer membrane adjacent to the cell wall which restricts the diffusion of hydrophobic complexes through its lipopolysaccharide layer (Burt, 2004; Turker *et al*., 2009). In this study, one strain each of *S. iniae* (S530) and *S. parauberis* (FP5228) were the most sensitive strains which had the minimum MIC of 0.063% and MIC:MBC ratio of 1:8 which reveals that they can survive even at high LVO concentrations, although its growth is inhibited at very low LVO concentration. The *L. garvieae* FP5245 strain showed the highest MIC of 1% which was the highest among Gram-positive bacteria used in this study. A similar kind of findings was reported for LVO against fish pathogenic *L. garvieae* where, MIC was 4.2 mg $ml^{-1}$ (Bulfon *et al*., 2014).

*Antibiotic susceptibility of isolates*

Antibiotic susceptibility profile indicated that all the bacterial strains excluding one strain of *P. damselae* (FP 4101) and three strains of *S. iniae* (S186, S530 and S131) showed resistance to 2 or more antibiotics (Table 2). The MAR index values of all the species except three strains of *S. iniae* (S186, S530 and S131) were calculated as ≥0.07. More importantly, two strains of *E. tarda* (ED47 and ED45), one *L. garvieae* (FP5245), one *S. iniae* (FP5228) and one *S. parauberis* (S124) were noted as comparatively high-risk strains. MAR index, higher than 0.2 (>0.2) denotes bacteria isolated from a source with a high risk of contamination, where antibiotics have been often used (Sandhu *et al.,* 2016). Lastly, the MAR index and MIC ($\rho$=0.74) values showed positive correlation, signifying that there is a relationship between the antibacterial activity of LVO and the multidrug-resistance profile of fish pathogenic bacteria (Fig. 1).

Table 2. Antimicrobial susceptibility patterns of fish pathogenic bacteria

| Species | Isolate | Susceptibility pattern[a] of antimicrobials[b] | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | AMX10 | AMP10 | CTX30 | CRO30 | TC15 | CHL30 | E15 | DA10 | VA30 | OFX5 | NAL30 | CN10 | AK30 | CS10 | IMI10 | SXT25 |
| *Photobacterium damselae* | FP4101 | 27(S) | 18(S) | 36(S) | 40(S) | 30(S) | 40(S) | ND | ND | 0 (R) | 40(S) | 34(S) | 18(S) | 18(S) | 20(S) | 23(S) | 28(S) |
| | FP5060 | 27(S) | 14(I) | 26(S) | 28(S) | 30(S) | 34(S) | ND | ND | 0 (R) | 30(S) | 26(S) | 14(I) | 19(S) | 0 (R) | 26(S) | 30(S) |
| | ED47 | 24(S) | 16(I) | 31(S) | 36(S) | 0 (R) | 0 (R) | ND | ND | 0 (R) | 18(I) | 0 (R) | 14(I) | 17(S) | 0 (R) | 33(S) | 0 (R) |
| *Edwardsiella tarda* | Yoshida | 27(S) | 20(S) | 25(S) | 40(S) | 31(S) | 39(S) | ND | ND | 0 (R) | 40(S) | 31(S) | 18(S) | 23(S) | 7 (R) | 28(S) | 28(S) |
| | ED45 | 24(S) | 14(I) | 25(S) | 34(S) | 9 (R) | 8 (R) | ND | ND | 0 (R) | 17(I) | 8 (R) | 14(I) | 17(S) | 0 (R) | 29(S) | 0 (R) |
| *Lactococcus garvieae* | FP5245 | 25 (S) | 22 (R) | 10 (R) | 23 (R) | 24(S) | 17(R) | 28(S) | 33(S) | 21(S) | 20(I) | ND | ND | ND | ND | ND | ND |
| | FP5228 | 28 (S) | 23 (R) | 8 (R) | 23 (R) | 25(S) | 28(S) | 29(S) | 14 (R) | 21(S) | 20(I) | ND | ND | ND | ND | ND | ND |
| | S186 | 35(S) | 29(S) | 30(S) | 32(S) | 23(S) | 25(S) | 24(S) | 28(S) | 22(S) | 28(S) | ND | ND | ND | ND | ND | ND |
| *Streptococcus iniae* | S530 | 38(S) | 32(S) | 30(S) | 30(S) | 23(S) | 24(S) | 30(S) | 33(S) | 24(S) | 25(S) | ND | ND | ND | ND | ND | ND |
| | S131 | 35(S) | 30(S) | 30(S) | 36(S) | 26(S) | 30(S) | 30(S) | 29(S) | 22 (S) | 23(S) | ND | ND | ND | ND | ND | ND |
| | FP5228 | 28 (S) | 23 (R) | 28 (S) | 29 (S) | 27(S) | 18(I) | 29(S) | 18(S) | 24(S) | 23(S) | ND | ND | ND | ND | ND | ND |
| | S124 | 25 (S) | 20 (R) | 26 (S) | 28 (S) | 0 (R) | 25(S) | 0 (R) | 12 (R) | 21(S) | 29(S) | ND | ND | ND | ND | ND | ND |
| *Streptococcus parauberis* | S527 | 29(S) | 21 (R) | 27 (S) | 29 (S) | 25(S) | 24(S) | 24(S) | 25(S) | 19 (S) | 25(S) | ND | ND | ND | ND | ND | ND |
| | S1466 | 27 (S) | 20 (R) | 26 (S) | 28 (S) | 25(S) | 22(S) | 17(R) | 18(S) | 18(S) | 23 (S) | ND | ND | ND | ND | ND | ND |

[a]Susceptibility pattern; R = resistant, I = intermediate, S = susceptible (designated using breakpoints described by the Clinical Laboratory Standards Institute, CLSI, 2014)

[b]AMP10 = ampicillin (10 µg), AMX30 = amoxicillin (30 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), AK30 = amikacin (30 µg), CS10 = colistin (10 µg), IMI10 = imipenem (10 µg) and SXT25 = trimethoprim-sulfamethoxazole (25 µg).

ND: not tested

LVO exhibited antibacterial activity against strains of fish pathogenic bacteria which are resistant to erythromycin, tetracycline and nalidixic acid. These antibiotics are used in the aquaculture industry in Korea. This demonstrates the potential of LVO to be applied as an alternative antibacterial agent. Besides, the species of fish pathogenic bacteria used in this study are etiological agents of edwardsiellosis and streptococcosis in Korea (Park *et al*., 2009). LVO could be used to prevent and control these bacterial diseases in fish by mixing with fish feed or by immersion treatment. However, to gain more perceptivity into the application of LVO against fish pathogenic bacteria, the activity of LVO in the aquatic environment, their toxicity and digestibility for fish need to be further investigated.

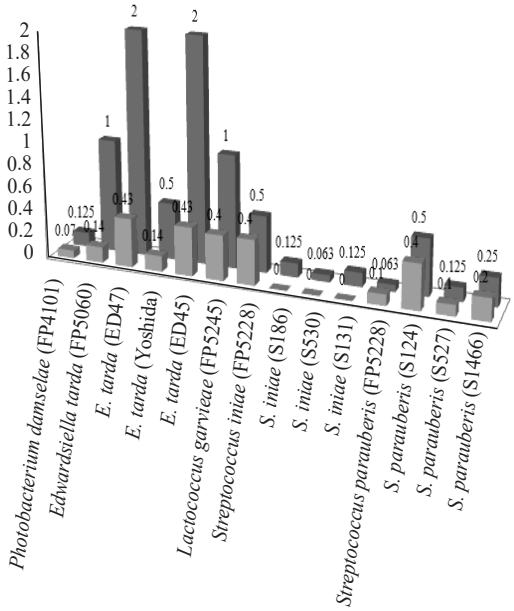


Fig. 1. Comparative expression of MAR index values and MICs of LVO detected for fourteen fish pathogenic bacteria

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