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In vitro antibacterial effect of ginger (*Zingiber officinale*) essential oil against fish pathogenic bacteria isolated from farmed olive flounder (*Paralichthys olivaceus*) in Korea

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Introduction

World population growth and food demand have been two major elements leading to the expansion of animal and fish production. Recently, many efforts advocated the extension of intensive fish farming to optimize the need for animal protein (Hussein *et al.,* 2013). In Korea, aquaculture has made rapid development in a short period of time, and many fish farms have been established in order to fulfill high consumer demand (Oh *et al.,* 2006). Meanwhile, olive flounder (*Paralichthys olivaceus*) is one of the popular marine fishes cultured in Korea, which accounts for approximately 50% of the annual fish production (Park *et al.,* 2012).

Edwardsiellosis and streptococcosis are the most prevalent bacterial diseases in olive flounder which are responsible for high stock mortality. *Streptococcus parauberis, S. iniae* and *Edwardsiella tarda* are the etiological agents of streptococcosis and edwardsiellosis, respectively (Park *et al.,* 2014). Generally, antibacterials are applied for the treatment of bacterial diseases. However, the overuse and misuse of antibacterials cause different problems such as the risk of growing bacterial resistance, accumulation of hazardous residues in fish products and severe damage to aquatic ecosystems (Chenia, 2016).

Nowadays, there is a growing interest in the screening of plant extracts and essential oils for their antibacterial properties against fish pathogens, thereby exploiting natural antimicrobials to control bacterial diseases (Bulfon *et al.,* 2014). Natural substances are eco-friendly and cheap, so they could alleviate many of the side-effects that are often related to synthetic antimicrobials. Essential oils (EOs) have widely been used to control bacterial diseases in humans (Inovye *et al.*, 2001).

Ginger (*Zingiber officinale*) is a member of the family Zingiberaceae. Ginger essential oil (GEO) extracted from ginger rhizome has been studied for biochemical and pharmacological properties, and it was reported that the major constituents are zingiberene, β-sesquiphellandrene and ar-curcumene (Nampoothiri *et al.,* 2012). The fragrance and flavoring substance of GEO composing 1-3% of the weight of fresh ginger primarily consists of zingerone, shogaols and gingerols as the major pungent compounds. The biologically active compounds of GEO are mainly gingerols and shagols which have both antibacterial and antifungal properties (Supreetha *et al.,* 2011; Shehata *et al.,* 2013). Moreover, GEO has some other biological properties such as anti-oxidative, anti-cancer, anti-parasitic, larvicidal, anti-diabetic, anti-inflammatory and nephro/hepato-protective activities (Kumar *et al.*, 2011). GEO is effective against fish-borne bacteria such as *Aeromonas hydrophila*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Lactococcus garvieae* and *S. parauberis* (Nya and Austin, 2009; Rattanachaikunsopon and Phumkhachorn, 2009; Debbarma *et al.*, 2012; Kim *et al.*, 2016).

However, the antibacterial activity of GEO against fish pathogenic bacteria has not been studied extensively. Besides, it has not been assessed against any fish bacterial pathogens in Korea. Therefore, this study aimed to investigate the *in vitro* antibacterial activity of GEO against fish pathogenic bacteria isolated from farmed olive flounder in Korea by determining the susceptibility with disk diffusion, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests.

Materials and methods

Selection of bacterial strains

Five gram-negative and nine gram-positive bacterial strains isolated from farmed Korean olive flounder (*P. olivaceus*) were used in this study. Gram-negative bacterial strains [*E. tarda* (FP5060, ED47, Yoshida and ED45) and *Photobacterium damselae* (FP4101)] and Gram-positive bacterial strains [*L. garvieae* (FP5245), *S. iniae* (FP5228, S186, S530 and S131) and *S. parauberis* (FP3287, S124, S527 and S1466)] were collected from Gyeongsang National University (Jinju, Korea) and National Institute of Fisheries Science (Busan, Korea). 100% pure ginger (*Z. officinale*) oil (Aromarant Co. Ltd., Rottingen, Germany) which was purified by steam distillation from the rhizomes of ginger grown in India was purchased for this study.

Disk diffusion test with GEO

The antibacterial activity of GEO was determined by means of disk diffusion test. Every bacterial culture was adjusted to 0.5 McFarland standard (1.5 x 10^8 CFU mL^{-1}) with sterile saline. The bacterial suspensions were spread over Mueller-Hinton agar (MHA) (MB

Cell, LA, CA) plates using a sterile cotton swab. Different concentrations of GEO (1:1= pure oil, 1:2, 1:5 and 1:10; 1 part of the GEO in respective parts of the solution) were prepared using dimethyl sulfoxide (DMSO) (OCI Co. Ltd, Seoul, Korea). Sterilized paper disks of 6 mm diameter (Advantec Toyo Kaisha, Ltd., Japan) were impregnated with 20 µL of different concentrations of GEO and placed on a MHA plate smeared with the bacterial strains. DMSO was used as the negative control and amoxicillin (10 µg) was used as the positive control. The plates were incubated for 48 h at 27°C and the diameter of the inhibition zone was measured in mm.

Multiple antibiotic resistance (MAR) index

The antimicrobial susceptibility was examined following the standards of Clinical and Laboratory Standards Institute (CLSI, 2014). A total of eleven antimicrobials including ampicillin (10 µg), cefotaxime (30 µg), ceftriaxone (30 µg), tetracycline (15 µg), chloramphenicol (30 µg), colistin (10 µg), gentamicin (10 µg), amikacin (30 µg), vancomycin (30 µg), nalidixic acid (30 µg) and ofloxacin (5 µg) (MB Cell, LA, CA) were used in this study for Gram-negative bacteria and nine antimicrobials including ampicillin (10 µg), cefotaxime (30 µg), ceftriaxone (30 µg), tetracycline (15 µg), chloramphenicol (30 µg), vancomycin (30 µg), ofloxacin (5 µg), erythromycin (15 µg) and clindamycin (5 µg) (MB Cell, LA, CA) were used for Gram-positive bacteria. Multiple antibiotic resistance (MAR) index was calculated as the ratio of the number of antimicrobials to which the bacterial strain was resistant to the total number of antimicrobials to which the bacterial strain was exposed.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests

The MICs of GEO were determined by broth microdilution method in 96-well microtiter plates using different concentrations [8%, 4%, 2%, 1%, 0.50%, 0.25%, 0.125%, 0.063% and 0.31% (V/V)] (Fournomiti *et al.*, 2015). The MICs were measured after incubation at 27°C for 48 h. For measurement of the MBC, 10 µL of medium from wells with no visible growth were transferred to tryptic soy agar (MB Cell, LA, CA) plates and incubated for 48 h at 27°C.

Results and discussion

Antibacterials have generally been used to control bacterial diseases in aquaculture for a long time. However, the frequent use of these drugs causes bacterial resistance. Recently, the demand for using plant natural products to replace antibacterial drugs has increased. The use of plant products especially essential oils for treating bacterial diseases in fish has become popular because of their strong antibacterial activities. Therefore, the present study was conducted to determine the efficacy of GEO against pathogenic bacteria isolated from olive flounder.

According to the disk diffusion test results, GEO inhibited the growth of all bacteria except *E. tarda*. The inhibition zone diameters increased with the increasing concentrations of GEO. *S. iniae* strains showed high susceptibility and the highest inhibition zone (23 mm) was observed in 1:1 dilution of GEO (Table 1). However, all *E. tarda* strains were resistant both in disk diffusion and MIC tests. In the MIC test, each of the *E. tarda* strains was observed to have MIC >8% which was the highest MIC value. In a previous study, fish pathogenic *E. tarda* was resistant to GEO (Yoon *et al.*, 2011). In contrast, GEO has been reported as an active agent against *E. tarda* infected Gourami fish (*Osphronemus goramy*) (Sarjito and Prayitno, 2015). The resistance of *E. tarda* in the present study could be due to the different composition of the chemical components in GEO. Generally, the antibacterial activity of EOs is dependent on the constitution of EOs and the bacterial strains (Shehata *et al.*, 2013). There are many variations in the chemical composition of GEO due to the environmental and genetic factors which make the antimicrobial action of GEO differ from that of bacteria (Snoussi *et al.*, 2016). Similar to previous reports, only a few *E. tarda* strains employed in the present study deter the accurate conclusion about the antibacterial activity of GEOs against *E. tarda*. In addition, two *E. tarda* isolates were resistant to multiple antimicrobials and displayed high MAR index values. The reason for such resistance could be because *E. tarda* is intrinsically resistant to macrolides, lincosamides, streptogramins, glycopeptides and fusidic acid due to the presence of different proteins in the outer membrane including porin and efflux pump systems hindering the antimicrobials from entering the cells (Stock and Wiedemann, 2001; Peng *et al.*, 2017).

Table 1: Inhibition zone with different GEO concentrations against fish pathogenic bacteria.

Bacterial strain	Inhibition zone[a] (mm) with different GEO concentrations[b]				DMSO (-ve control)	Amoxicillin (+ve control)
	1:1	1:2	1:5	1:10		
Gram-negative bacteria						
Edwardsiella tarda (FP5060)	NA	NA	NA	NA	NA	27
E. tarda (ED45)	NA	NA	NA	NA	NA	24
E. tarda (ED47)	NA	NA	NA	NA	NA	24
E. tarda (Yoshida)	NA	NA	NA	NA	NA	27
Photobacterium damselae (FP4101)	8	6.5	NA	NA	NA	27
Gram-positive bacteria						
Lactococcus garvieae (FP5245)	13	9	8	NA	NA	25
Streptococcus iniae (S186)	19	15	13	10	NA	35
S. iniae (S530)	18	13	10	9	NA	38
S. iniae (S131)	23	20	15	12	NA	35
S. iniae (FP5228)	13	8	7	NA	NA	28
Streptococcus parauberis (S124)	9	6.5	NA	NA	NA	25
S. parauberis (S527)	9.5	6.5	NA	NA	NA	29
S. parauberis (S1466)	7	NA	NA	NA	NA	27
S. parauberis (FP3287)	13	8	7	NA	NA	28

[a]Inhibition zone; NA= No growth inhibition
[b]Concentrations; 1:1= pure oil, 1:2, 1:5, 1:10= 1 part of GEO in respective parts of the dilution.

MIC results of GEO tested for fish pathogenic bacteria ranged from 0.25 to 4% except for *E. tarda* (V/V) (Fig. 1). The highest MIC value of 4% (V/V) was observed in *S. parauberis* and the lowest MIC value of 0.25% (V/V) was observed in *S. iniae* strains (S131 and S186) among the gram-positive bacteria. In a recent study, GEO was active against fish pathogenic *S. parauberis* (Kim *et al.,* 2016). A Gram-negative bacterial strain, *P. damselae* (FP4101) used in this study was sensitive to GEO in disk diffusion and MIC tests (MIC value 4%). According to the MIC results, GEO was effective against the majority of Gram-positive bacteria strains even in lower concentrations compared to gram-negative bacteria. The weak antibacterial activity could be attributed to the presence of an outer membrane in gram-negative strains which possesses hydrophilic polysaccharides, thereby hampering the diffusion of hydrophobic compounds of EOs (Inovye *et al.,* 2001).

The majority of the tested strains had MBC/MIC as ≤4. This implies the bactericidal mode of action of GEO against fish pathogenic bacteria. Considering the ratio of MBC/MIC, the EOs which showed bacterial killing effects with MBC/MIC ≤4 are designated as bactericidal for the tested bacteria, while the EOs which demonstrated inhibitory effects with MBC/MIC>4 are designated as bacteriostatic (Bulfon *et al.*, 2014) .

In antimicrobial disk diffusion tests, *S. iniae* (S186, S530 and S131) strains were susceptible to all of the tested antimicrobials whereas other strains were resistant to 1 or more antimicrobials (Table 2). The MAR index values have been calculated as ≥0.09. More importantly, two *E. tarda* (ED47 and ED45), one *L. garvieae* (FP5245), one *S. iniae* (FP5228) and two *S. parauberis* (S124, S1466) strains were denoted as high-risk strains as they had MAR index values of 0.45, 0.45, 0.44, 0.33, 0.44 and 0.22, respectively. An MAR index value of higher than 0.2 indicates that the bacteria were isolated from a source with a high risk of contamination where antimicrobials have often been used (Sandhu *et al.*, 2016).

Interestingly, GEOs exhibited antibacterial properties against the strains that resisted tetracycline, erythromycin and nalidixic acid which are common antimicrobials used in the Korean aquaculture industry (Park *et al.,* 2014). It reveals the potentiality of GEO to be used as an alternative antibacterial agent against *S. iniae*, *S. parauberis*, *L. garviae* and *P. damselae*. Since *E. tarda* strains were resistant to GEO, further studies should be focused on determining the *in vitro* antibacterial activity of GEO against more strains of pathogenic *E. tarda* in fish. Besides, the number of species and the strains should be enhanced in order to acquire a better understanding of the antibacterial property of GEOs. Also, the stability of GEO in aquatic environments, and the digestibility and the potential toxicity in fish should also be examined before the application of GEOs for control of fish bacterial infections in aquaculture systems.

Table 2: MAR index values of fish pathogenic bacteria used in this study.

Bacterial strain[a]	MAR index	No. of ineffective antimicrobials	Name of ineffective antimicrobials[b]
Gram-negative bacteria			
Edwardsiella tarda (FP5060)	0.18	2	VA, CS
E. tarda (ED45)	0.45	5	TC, CHL, VA, NAL, CS
E. tarda (ED47)	0.45	5	TC, CHL, VA, NAL, CS
E. tarda (Yoshida)	0.18	2	VA, CS
Photobacterium damselae (FP4101)	0.09	1	VA
Gram-positive bacteria			
Lactococcus garvieae (FP5245)	0.44	4	AMP, CTX, CRO, CHL
Streptococcus iniae (FP5228)	0.33	3	CTX, CRO, DA
Streptococcus parauberis (S124)	0.44	4	AMP, TC, E, DA
S. parauberis (S527)	0.11	1	AMP
S. parauberis (S1466)	0.22	2	AMP, E
S. parauberis (FP3287)	0.11	1	AMP
Bacterial strain[a]	**MAR index**	**No. of ineffective antimicrobials**	**Name of ineffective antimicrobials[b]**

[a] Bacterial strains are resistant to one or more antimicrobials
[b]Antimicrobials abbreviation; AMP=ampicillin (10 µg), CTX=cefotaxime (30 µg), CRO=ceftriaxone (30 µg), TC=tetracycline (15 µg), CHL=chloramphenicol (30 µg), E=erythromycin (15 µg), DA=clindamycin (10 µg), VA=vancomycin (30 µg), NAL=nalidixic acid (30 µg) and CS=colistin (10 µg).
Dsds

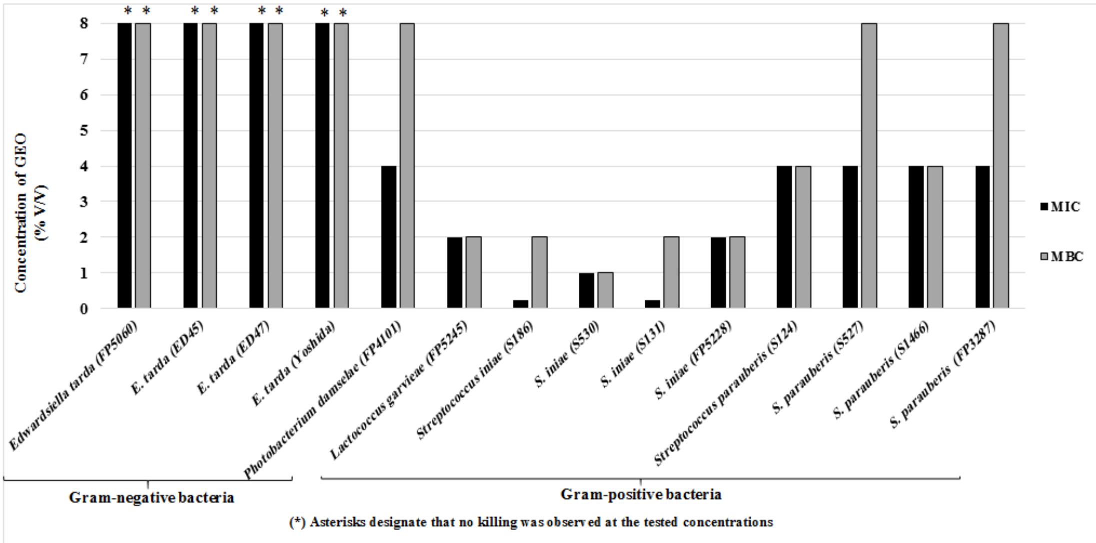

Figure 1: Comparison of MICs and MBCs of GEO against fourteen fish pathogenic bacterial strains.

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