

ANTIBACTERIAL ACTIVITY OF CLOVE ESSENTIAL OIL AND EUGENOL AGAINST FISH PATHOGENIC BACTERIA ISOLATED FROM CULTURED OLIVE FLOUNDER (*Paralichthys olivaceus*)

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Abstract: The antibacterial activity of clove (*Syzygium aromaticum*) essential oil (CEO) and eugenol was tested against seven Gram-negative and nine Gram-positive fish pathogenic bacteria isolated from cultured olive flounder (*Paralichthys olivaceus*) in Korea. Eugenol was >99% concentrated and CEO consisted of seven chemical compounds including 83.63% of eugenol. Disk diffusion assay, MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) tests showed CEO and eugenol inhibited the growth of both Gram-negative and Gram-positive bacteria. CEO (MBC/MIC= 1-2) and eugenol (MBC/MIC= 1-4) were bactericidal for all tested strains. The percentage of relative inhibition zone diameter exhibited high values at higher concentration of both CEO and eugenol. Positive correlation was observed between MAR index values and MIC values of CEO ($r = 0.61$) and eugenol ($r = 0.15$). Since antibacterial activity of CEO and eugenol were effective against fish pathogenic bacteria, the use of CEO and eugenol could be advantageous to treat bacterial infections in aquaculture.

Key words: antibacterial activity; clove essential oil (CEO); eugenol; fish pathogenic bacteria; olive flounder

Introduction

Bacterial diseases pose one of the major threats to aquaculture industry worldwide. The crucial bacterial diseases of marine fish in Korea are edwardsiellosis caused by *Edwardsiella tarda*; streptococcosis caused by *Streptococcus iniae*, *S. parauberis*, and *Lactococcus garvieae*; and vibriosis caused by *Vibrio harveyi*, *V. ichthyoenteri*, and *Photobacterium damsela*, which have recently increased in cultured fish populations (1, 2).

Disease outbreaks are responsible for elevated mortality rates and decrease of the productivity, causing a high economic loss in olive flounder (*Paralichthys olivaceus*) aquaculture in Korea (3).

Antibiotics are widely used to treat bacterial infections in fish. However, misuse of antibiotics leads to drug resistance and thereby to the reduced efficacy of the drugs (4). Antibiotics accumulate in the environment and fish, hence pose a potential risk to humans and the environment (5). Therefore, it is essential to develop antibacterial treatments that are made from natural substances. Natural products especially from plants have been investigated for their therapeutic and prophylactic

effects on several fish diseases (6). Essential oils are one kind of plant products which have been used with their aromatic, flavor, bactericidal, preservative and medicinal properties (7).

One of the most influential antimicrobial medicinal herbs is clove (*Syzygium aromaticum*) bud belonging to Myrtaceae family, indigenous to India, Indonesia, Zanzibar, Mauritius and Sri Lanka (8). Clove essential oil (CEO) was reported to possess antibacterial, antifungal, insecticidal, and antioxidant properties (9-11). These activities of CEO are mainly due to its high content of phenolic derivatives. Eugenol, a phenylpropene compound with strong biological and antimicrobial activities, is the major constituent of CEO. Eugenol was reported to play an important role in inhibiting the growth of bacteria (12). It can denature protein and react with phospholipids in the cell membrane. It also affects the transport of ions and ATP and changes the fatty acid profile of different bacteria (13, 14). Antimicrobial mechanism of eugenol has been found, affecting not only the membrane but also the envelope of fungal and bacterial cells (15).

However, until now no study has been conducted to investigate the antimicrobial property of CEO and its major component against fish pathogenic bacteria isolated from olive flounder. Therefore, this study was carried out to examine the potential of CEO as well as eugenol as alternatives to commercial antibiotics in aquaculture use.

Materials and methods

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

The disk diffusion assay with CEO and eugenol was conducted to detect the antimicrobial activity. Sterile disks (Advantec Toyo Kaisha, Ltd., Japan) were impregnated with 20 μ L of CEO and eugenol with different dilutions; [1%, 5%, 10%, 25%, 50%, 100 % (V/V)] and each disk was placed on a Mueller Hinton agar (MB Cell, LA, CA) plate smeared with the test organism. Plates were incubated for 24 h at 27 °C to determine the antimicrobial effect. Antibacterial activity was determined by measuring the inhibition zone diameter (IZD) (mm) against each test organism. The antimicrobial activity expressed as percentage of relative inhibition zone diameter (RIZD %) and it was calculated according to Njau et al. (16) using amoxicillin as a standard antibiotic. The determination of MIC was done using broth micro dilution method with some modifications using different concentrations which 5% DMSO was used to dissolve CEO and eugenol. The MIC was measured after 24 h incubation and each test was conducted in triplicates. In order to determine the MBC, the cultured medium from wells which have higher concentration of CEO or eugenol than MIC was smeared on separate Tryptic Soy Agar (TSA) (MB Cell, LA, CA) plates and incubated for 24 h at 27 °C (17). The concentration, at which no growth was observed on TSA plate, was determined as the MBC.

Antibiotic susceptibility was determined by disk diffusion method using sixteen antibiotics. Resistance profiles (resistant, intermediate or susceptible) were assigned using criteria described by Clinical and Laboratory Standards Institute (CLSI) (18). Each test was repeated three times. Following the disc diffusion test results of antibiotics, multiple antibiotic resistance (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. For the statistical analysis, differences were considered significant at $p < 0.05$. The correlation indices were calculated using the Pearson coefficient (r).

Results

The CEO obtained in the present study constituted 83.63% of eugenol and other six chemical compounds (Table 1).

Table 1: Composition of clove essential oil used in this study

Compound name	Composition %*
Eugenol	83.63
β -Caryophyllene	10.5
α -Humulene	2.78
Eugenyl-acetate	1.02
caryophyllene Oxide	0.39
Cadinene	0.37
Cadalene	0.35

*Composition of the essential oil was analysed by Neumond GmbH, Raisting, Germany

IZD of Gram-negative bacteria ranged from 19 to 27 mm and IZD of Gram-positive bacteria ranged from 15 to 25 mm in 100% (V/V) of eugenol while IZD of Gram-negative bacteria ranged from 16 to 20 mm and IZD of Gram-positive bacteria ranged from 14 to 22 mm at 100% (V/V) of CEO. RIZD % exhibits high values at higher concentration of both eugenol and CEO (Table 2).

MIC values of the CEO for Gram-negative bacterial strains ranged from 0.125 to 0.5% (V/V) and for Gram-positive strains ranged from 0.25 to 0.5% (V/V). MIC of eugenol for Gram negative bacteria ranged from 0.0312 to 0.0125% (V/V) and for Gram-positive bacteria it was 0.125% to 1 % (V/V). Mean MBC/MIC for CEO was 1 to 2 and for eugenol it was 1 to 4 (Table 3).

The MAR index of four strains [one *E. tarda* (ED47), one *L. garvieae* (FP5245), one *S. iniae* (FP3287) and one *S. parauberis* (S124)] have been calculated as ≥ 0.2 . The observed Pearson coefficient (r) between the MAR index values and MIC values of eugenol and CEO were $r = 0.15$ and $r = 0.61$, respectively (Figure 1).

Discussion

Many reports have claimed that eugenol is the major compound in the CEO. However, inhibitory activity of clove is due to the presence of several constituents, mainly eugenol, eugenyl acetate and β -caryophyllene (19, 20). Sohilaït (21) reported same composition range of eugenol in clove bud (81.13 - 84.44%).

According to disk diffusion test result, CEO inhibited the growth of all test bacteria at every

concentration except 1 % (Table 2). Chaieb, (22) observed that each bacterial strain demonstrated a significant degree of sensitivity to the CEO, and extensive activity against Gram-positive bacteria, producing a clear zone of inhibition against the majority of the tested strains.

Meanwhile, eugenol was found to be effective against almost all of Gram-negative strains except *E. tarda* (ED45) strain at every concentration, and the widest IZD was 27 mm against *V. ichthyenteri*. Eugenol exhibited higher activity against Gram-negative strains than Gram-positive strains and several studies have reported similar results against pathogenic bacteria (12, 20). In another study (23), eugenol showed the highest effect against *S. aureus* compared to the Gram-negative bacterial strains in contrast to the other previous studies.

In comparison, eugenol exhibited highest IZD (27 mm) against Gram-negative [*V. ichthyenteri* (FP 4004)] bacterial strain and CEO exhibited highest IZD (22 mm) against Gram-positive [*S. iniae* (S186)] bacterial strain. The inhibition zones induced by the CEO or eugenol were relative to the concentration of eugenol. CEO contains 86.63% of eugenol as well as six other components, whereas concentration of commercial eugenol was >99%. The different components of essential oils can act on bacterial proteins using several mechanisms and may affect cell division (14). However, eugenol and CEO were effective against both Gram-positive and Gram-negative microorganisms. In every tested fish pathogenic bacteria, the IZD increased in proportion to the eugenol or CEO concentration and the maximum effect was found at 100% (V/V) concentration of both

Table 2: Inhibition zone diameter and the percentage of relative inhibition zone diameter (RIZD %) values of clove essential oil (CEO) and eugenol against fish pathogenic bacteria

Bacteria	Inhibition zone diameter (mm) and RIZD% *																							
	1 % (V/V)		5 % (V/V)		10 % (V/V)		25 % (V/V)		50 % (V/V)		100 % (V/V)													
	CI	ER	CI	ER	CI	ER	CI	ER	CI	ER	CI	ER	CI	ER	CI	ER	CI	ER						
Gram-negative																								
<i>Vibrio harvey</i> (FP8370)	0	0	8	33.3	10	41.7	14	58.3	12	50	17	70.8	15	62.5	20	83.3	18	75	23	95.8	20	83.3	24	100
<i>V. ichthyenteri</i> (FP4004)	0	0	8	0.21	11	28.9	13	34.2	12	31.6	15	39.5	15	39.5	18	47.4	19	50	21	55.3	20	52.6	27	71.1
<i>Photobacterium damsela</i> (FP4101)	0	0	7	25.9	8	29.6	11	40.7	11	40.7	17	63	14	51.9	18	47.4	17	62.9	19	70.4	19	70.4	20	74
<i>Edwardsiella tarda</i> (FP5060)	0	0	8	29.6	8	29.6	13	48.1	10	37	14	51.9	12	44.4	20	74.1	13	48.1	21	77.8	16	59.3	22	81.5
<i>E. tarda</i> (ED47)	0	0	7	29.2	8	33.3	10	41.7	10	41.7	16	66.7	13	54.2	18	75	15	62.5	20	83.3	16	66.7	22	91.7
<i>E. tarda</i> (Yoshida)	0	0	7	25.9	10	37	10	37	11	40.7	12	44.4	14	51.9	15	55.6	16	59.3	17	63	18	66.7	19	70.4
<i>E. tarda</i> (ED45)	0	0	0	0	10	41.7	13	54.2	12	50	17	70.8	15	62.5	19	79.2	17	70.8	20	83.3	18	75	21	87.5
Gram-positive																								
<i>Lactococcus garvieae</i> (FP5245)	0	0	0	0	9	36	11	44	11	44	12	48	13	52	13	52	15	60	14	56	17	68	15	60
<i>Streptococcus iniae</i> (FP3287)	0	0	0	0	7	25	8	28.6	8	28.6	11	39.3	12	42.9	12	42.9	13	46.4	13	46.4	16	57.1	15	53.6
<i>S. iniae</i> (S186)	0	0	0	0	12	34.3	14	40	15	42.9	15	42.9	17	48.6	17	48.6	19	54.3	19	54.3	22	62.9	22	62.9
<i>S. iniae</i> (S530)	0	0	0	0	9	23.7	10	26.3	11	28.9	20	52.6	15	39.5	21	55.3	17	44.7	23	60.5	19	50	25	65.8
<i>S. iniae</i> (S131)	0	0	0	0	7	20	9	25.7	10	28.6	16	45.7	18	51.4	17	48.6	19	54.3	18	51.4	21	60	20	57.1
<i>Streptococcus parauberis</i> (FP5228)	0	0	0	0	7	25	9	32.1	9	32.1	10	35.7	10	35.7	15	53.6	12	42.9	16	57.1	14	50	17	60.7
<i>S. parauberis</i> (S124)	0	0	0	0	8	32	12	48	11	44	15	60	15	60	16	64	16	64	17	68	17	68	18	72
<i>S. parauberis</i> (S527)	0	0	0	0	7	24.1	8	27.6	13	44.8	11	37.9	15	51.7	14	48.3	18	62.1	16	55.2	19	65.5	18	62.1
<i>S. parauberis</i> (S1466)	0	0	0	0	10	37	11	40.7	12	44.4	15	55.6	15	55.6	16	59.3	16	59.3	17	63	19	70.3	19	70.4

*CI- CEO IZD, CR-CEO RIZD, EI- Eugenol IZD, ER- Eugenol RIZD

Table 3: Susceptibility pattern of clove essential oil and eugenol against fish pathogenic bacteria

Species	Clove essential oil			Eugenol		
	MIC (V/V)	MBC (V/V)	MBC/MIC	MIC (V/V)	MBC (V/V)	MBC/MIC
Gram-negative bacteria						
<i>Vibrio harveyi</i> (FP8370)	0.125	0.25	2	0.0312	0.125	4
<i>V. ichthyenteri</i> (FP4004)	0.125	0.25	2	0.0312	0.125	4
<i>Photobacterium damsela</i> (FP4101)	0.125	0.25	2	0.125	0.25	2
<i>Edwardsiella tarda</i> (FP5060)	0.25	0.5	2	0.0625	0.25	4
<i>E. tarda</i> (ED47)	0.5	0.5	1	0.125	0.5	4
<i>E. tarda</i> (Yoshida)	0.125	0.25	2	0.125	0.25	2
<i>E. tarda</i> (ED45)	0.5	0.5	1	0.125	0.5	4
Gram-positive bacteria						
<i>Lactococcus garvieae</i> (FP5245)	0.5	1	1	1	1	1
<i>Streptococcus iniae</i> (FP3287)	0.5	1	2	0.5	1	2
<i>S. iniae</i> (S186)	0.25	0.25	1	0.5	0.5	1
<i>S. iniae</i> (S530)	0.25	0.5	2	0.5	0.5	1
<i>S. iniae</i> (S131)	0.25	0.25	2	0.125	0.5	4
<i>Streptococcus parauberis</i> (FP5228)	0.5	0.5	1	1	1	1
<i>S. parauberis</i> (S124)	0.5	0.5	1	0.5	0.5	1
<i>S. parauberis</i> (S527)	0.5	0.5	1	1	1	1
<i>S. parauberis</i> (S1466)	0.25	0.5	2	0.5	0.5	1

Antimicrobial properties (MIC and MBC) were mentioned. MBC/MIC results were used to interpret bacteriostatic or bactericidal activity of oil

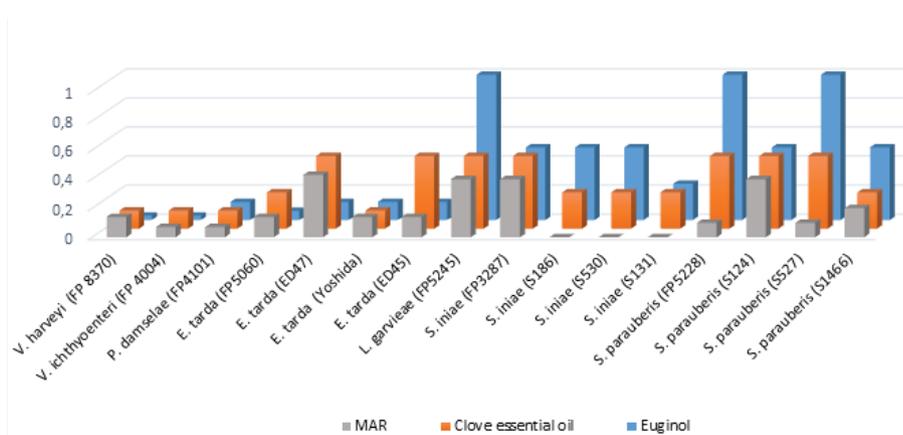


Figure 1: Comparative expression of MAR index values and MICs of clove essential oil and eugenol detected for sixteen strains of fish pathogenic bacteria

CEO and eugenol. Previous study has reported highest diameter of zone of inhibition of against *Staphylococcus aureus* followed by *Escherichia coli* and *Listeria monocytogens* which were food borne pathogens (24). Fagere (2016) has reported that CEO showed higher IZD against *Bacillus subtilis* and *S. aureus* (25).

RIZD % exhibits high values at higher concentrations. All the Gram-negative strains had higher RIZD % at every concentration of eugenol except *E. tarda* (ED45). It was observed that eugenol had zero RIZD % against Gram-positive strains at 1 % (V/V) concentration. This indicates that Gram-positive bacteria were not susceptible at every tested concentration of eugenol.

In the present study, the MIC of CEO for Gram-negative bacterial strains ranged from 0.125 to 0.5% (V/V) and for Gram-positive strains ranged from 0.25 to 0.5% (V/V) (Table 3). Eugenol showed lower MIC values than CEO against Gram-negative bacteria which range from 0.0312 to 0.0125% (V/V). According to the MBC/MIC ratio, both CEO (MBC/MIC 1-2) and eugenol (MBC/MIC 1-4) demonstrated bactericidal activity against 16 fish pathogenic bacteria. In a previous study, MBC and MIC values were identical, indicating that the effect of CEO was mainly bactericidal and not bacteriostatic (26) and eugenol demonstrated bacteriostatic activity at lower concentrations and bactericidal activity at slightly higher concentrations against *Salmonella* (27, 28).

The antibiotic resistance profile indicated that all the bacterial strains excluding three strain of *S. iniae* (S186, S530, and S131) have shown resistance to one or more antibiotics. This was facilitated by MAR index values where all the strains except the three strains mentioned above have been calculated as ≥ 0.07 . The MAR index which is higher than 0.2 (>0.2) identifies bacteria isolated from a source with a high risk of contamination where antibiotics have been often used (28). The MAR index of four strains [one *E. tarda* (ED47), one *L. garvieae* (FP5245), one *S. iniae* (FP3287) and one *S. parauberis* (S124)] have been calculated as ≥ 0.2 were noted as comparatively high-risk strains. Finally, the observed Pearson coefficient (r) between the MAR index values and MIC values of eugenol ($r = 0.15$) and CEO ($r = 0.61$) indicated that there is a positive correlation between the CEO or eugenol efficacy and the multidrug-resistance profile of fish pathogenic bacteria (Figure 1).

Although both CEO and eugenol inhibited the growth of tested fish pathogenic bacteria, eugenol could inhibit the growth of bacteria at lower concentrations than CEO. The present study established that both CEO and eugenol are effective against fish pathogenic bacteria isolated from olive flounder and are good candidate for further research to develop a new antibacterial drug against fish pathogenic bacteria. CEO and eugenol can be used to prevent fish diseases by adding to fish feed or immersion treatment. Moreover, in order to apply CEO and eugenol for treatment of bacterial diseases in aquaculture, the stability of them in the aquatic environment, their digestibility and the toxicity in fish should be further investigated.

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PROTIBAKTERIJSKA AKTIVNOST ETERIČNEGA OLJA NAGELJNOVIH ŽBIC IN EVGENOLA PROTI PATOGENIM BAKTERIJAM RIB, IZOLIRANIH IZ GOJENEGA MORSKEGA LISTA (*Paralichthys olivaceus*)

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Povzetek: Protibakterijsko delovanje eteričnega olja nageljnovih žbic (*Syzygium aromaticum*) (CEO) in evgenola je bilo testirano pri sedmih vrstah po Gramu negativnih in devetih vrstah po Gramu pozitivnih patogenih bakterij, ki so bile izolirane iz gojenega morskega lista (*Paralichthys olivaceus*) v Južni Koreji. Evgenol je bil 99-odstotno koncentriran, CEO pa je sestavljalo sedem kemičnih spojin, med njimi je bilo največ evgenola, ki je predstavljal 83, odstotka eteričnega olja.

Metoda difuzije v trdem gojišču, minimalna zaviralna koncentracija (MIC) in minimalna baktericidna koncentracija (MBC) so pokazale, da sta CEO in evgenol zavirala rast tako po Gramu negativnih kot po Gramu pozitivnih bakterij. CEO (MBC/MIC = 1-2) in evgenol (MBC/MIC = 1-4) sta bila baktericidna za vse testirane seve. Odstotek velikosti relativne zaviralne cone je bil visok pri višji koncentraciji CEO in evgenola. Pozitivna soodvisnost je bila opažena med vrednostmi indeksa MAR in vrednostmi MIC pri CEO ($r=0,61$) in evgenolu ($r=0,15$). Ker sta CEO in evgenol pokazala učinkovito delovanje proti patogenim bakterijam rib, bi bila uporaba CEO in evgenola lahko uporabna za zdravljenje bakterijskih okužb v ribogojništvu.

Ključne besede: antibakterijsko delovanje; eterično olje klinčkov (CEO); evgenol; patogene bakterije rib; morski list