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Antimicrobial Activities of Diallyl Disulfide Against Fish Pathogenic Bacteria

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Abstract: As a major pathogen for fish, the antimicrobial activity of Diallyl Disulfide (DADS) was examined for the following bacteria, *Aeromonas hydrophila*, *A. salmonicida* ssp. *masoucida*, *A. salmonicida* ssp. *salmonicida*, *Edwardsiella tarda*, *Vibrio vulnificus*, *V. paraheamolyticus* and *L. anguillarum*. About 10 ug mL^{-1} and more of DADS formed a clear inhibitory zone to all pathogenic bacteria in a disk diffusion test. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values were in the ranges of 160-640 and 640-1280 ug mL^{-1} of DDS, respectively. The most sensitive pathogen to DDA was *V. vulnificus* (160 ug mL^{-1} for MIC and 640 ug mL^{-1} for MBC) followed by *E. tarda* (320 ug mL^{-1} for MIC and 640 ug mL^{-1} for MBC). These results suggest bioavailability of DADS for controlling bacterial pathogens in aquaculture.

Key words: Diallyl disulfide, fish pathogenic bacteria, antimicrobial activity, aquaculture, disk, diffusion test

INTRODUCTION

The aquaculture has rapidly advanced for producing fish food which is a major source of protein as an essential nutrient for humans. However, the growing industry has its own potential risk factors such as severe infectious diseases. The aquaculture industry has continually been threatened by several bacterial pathogens, *Aeromonas hydrophila*, *Edwardsiella tarda* and *Vibrio* sp. (Toranzo *et al*., 2005). Recently, there were several outbreaks involving a typical *Aeromonas* sp. from cultured fish (Han *et al*., 2011; Kim *et al*., 2011). Since, these bacterial pathogens are autochthonous bacteria for aquatic environment and opportunistic bacteria for fish, it is difficult to control them in aquaculture.

Although, vaccination is an effective method for preventing bacterial pathogens, it has its own inherent problems such as a limited specificity of them under a pathogens' pool environment, the difficult of vaccine administration and the short duration of vaccine immunity (Plant and Lapatra, 2011). At the same time, antibiotics has been widely used to treat and prevent bacterial pathogens in fish managements but it has resulted in negative impacts such as the emergence of antibiotic resistant bacteria and environmental contamination (Kummerer, 2009a, b). The dially sulfides are major components for oils extracted from shallot (*Allium ascalonicum* L.), Chinese chive (*Allium tuberosum*) and garlic (*Allium sativum*), all of which belong to the Family Alliaceae (Rattanachaikunsopon and Phumkhachorn, 2009a, b; Harris *et al*., 2001). These dially sulfides contain Diallyl Sulfide (DMS), Dially Disulfide (DADS), Diallyl Trisulfide (DTS) and Diallyl Tetrasulfide (DTTS) (Harris *et al*., 2001). Of these sulfides, DADS is an abundant organosulfur compound found in garlic oil and it possesses antioxidant and anticancer activities (Song *et al*., 2009; Koh *et al*., 2005). In addition, DADS has been examined as a natural antibiotic for food preservatives against the contamination of food-borne pathogens. Lu *et al*. (2011) have shown that its antibiotic mechanism was associated with damage on cell membrane of *Listeria monocytogenes* and *Escherichia coli* O157:H7. Meanwhile, several studies have shown differences in sensitivities to DADS between bacterial species (Lu *et al*., 2011; Rattanachaikunsopon and Phumkhachorn, 2008, 2009b). However, the compound has rarely been studied with its antibiotic effects to bacterial pathogens for fish. In this study, we investigated the capacity of DADS as a natural product for controlling bacterial pathogens in the aquaculture industry.

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MATERIALS AND METHODS

Bacteria: Antimicrobial activity of DADS was examined in 7 species of 4 genera from gram-negative bacteria for fish: *A. hydrophila* (ATCC7966), *V. vulnificus* (ATCC 33148), *V. paraheamolyticus* (ATCC 33844), *A. salmonicida* ssp. *salmonicida* (ATCC 33658) and *masoucida* (ATCC 27013), *E. tarda* and *L. anguillarum*. Of the bacteria, *E. tarda* and *L. anguillarum* was isolated from the diseased fish. All bacteria were separately stocked in Tryptic Soy Broth (TSB) with 10% glycerol and stored -70°C until required.

Disk diffusion test: The stocked bacteria were cultured on Tryptic Soy Agar (TSA) at 20°C for disk diffusion and broth dilution tests. For the disk diffusion test, the generated colonies were adjusted to the turbidity of a McFarland 0.5 standard with saline. The bacterial suspension (5×10^5 CFU mL^{-1}) was separately spread on Muller-Hinton Agar (MHA). The paper disk (10 mm in diameter, Advantec Toyo, Japan) was independently soaked with 4 different concentrations of DADA, 5 ,10, 15 and 20 mg mL^{-1}. Thereafter, the disks were attached on MHA plates inoculated with the bacteria and the plate was then incubated for 48 h at 20°C. After incubation, antimicrobial activity of the thymol was examined by measuring diameters of the clear zones formed on the MHA plate. All tests were performed in triplicate and the mean values were calculated. Amoxicillin (30 ug), tetracycline (30 ug), erythromycin (15 ug), trimethoprim-sulfamethoxazole (1.25-23.75 ug), lincomycin (15 ug), nalidixic acid (30 ug) and chloramphenicol (30 ug) were used as reference antibiotics to control the sensitivity of bacteria. Of the antibiotic disks, a disk for trimethoprim-sulfamethoxazole was purchased from Biolab (Hungary) and the other antibiotic disks were from Liofilcam (Italy).

MIC and MBC: The MIC and MBC values of DADS were determined by the Broth Dilution Method using Muller-Hinton Broth (MHB). In brief, DADS was adjusted to 2.5 mg mL^{-1} as stock solution and the solution was then diluted 2-fold to 0.005 mg mL^{-1} in MHB. Each dilution was mixed with an equal volume of the bacterial suspension with approximately 10^6 CFU mL^{-1} in MHB. All tests were performed on 96 well microplate. After incubation for 48 h at 20°C, the MIC was determined at the lowest concentration that completely inhibited viable bacteria growth. Therefore, the MBC was determined in the mixtures below the MIC value of DADS to each bacterium from the broth dilution test. The mixture was separately re-inoculated on TSA and the plates were then incubated for 48 h at 20°C. The number of colonies was calculated by viability counts. The definition of the MBC was the lowest concentration for killing 99.9% or more of the initial inoculums.

RESULTS AND DISCUSSION

The results for the disk diffusion test are shown in Table 1. Amoxicillin resistance was exhibited in all bacteria except *V. paraheamolyticus*. Moreover, *A. hydrophila*, *V. vulnificus* and *L. anguillarum* did not form any inhibitory clear zone to amoxicillin on MHA. Tetracycline and chloramphenicol showed susceptibility in all bacteria except *L. anguillarum*. Erythromycin had an intermediate resistance to *A. hydrophila*, *L. anguillarum* and *E. tarda* whereas it was susceptible to *A. salmonicida*, *V. vulnificus* and *V. paraheamolyticus*.

On the other hand, all bacteria were susceptible to trimethoprim-sulfamethozaxole and nalidixic acid but were resistant to lincomycin. In the case of DADA, the lowest concentration (5 mg) was not shown with an inhibitory clear zone to *A. salmonicida* ssp. *masoucida* and *E. tarda*. In addition, the remaining bacteria exhibited a narrow range of clear zone (~11 mm). However, the increased concentrations of DADS were seen to cause broader inhibitory zones to all bacteria used. Table 2 shows the MIC and MBC values of DADS to each bacterial pathogen. In the results, MIC values for *V. vulnificus* and *E. tarda* were 160 and 640 ppm of DADS, respectively. The remaining bacteria had MIC values of 640 ppm. All bacteria except *V. vulnificus*

Table 1: Antimicrobial activity of DADS and antibiotics by the disk diffusion test

							DADS (mg)				
Bacteria	AML	TE	E	SXT	MY	NA	C	5	10	15	30
A. hydrophila	R* (0#)	S (35)	I (16)	S (39)	R (0)	S (38)	S (45)	10	16	25	31
A. salmonicida ssp. *masoucida*	R (10)	S (40)	S (31)	S (38)	R (13)	S (41)	S (50)	0	13	22	29
A. salmonicida ssp. *salmonicida*	R (8)	S (38)	S (25)	S (41)	R (0)	S (40)	S (46)	9	15	24	30
V. vulnificus	R (0)	S (35)	S (29)	S (42)	R (0)	S (40)	S (52)	8	19	31	41
V. paraheamolyticus	R (30)	S (35)	S (25)	S (39)	R (16)	S (38)	S (52)	11	18	30	40
L. anguillarum	R (0)	I (11)	I (14)	S (33)	R (0)	S (40)	I (13)	10	21	31	46
E. tarda	R (7)	S (35)	I (15)	S (37)	R (10)	S (39)	S (45)	0	11	16	25

Disc zone diameters were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) for Tetracycline (TE), Erythromycin (E), Sulfamethoxazole/Trimethoprim (SXT; 19:1), Nalidixic Acid (NA), Chloramphenicol (C) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) for Amoxicillin (AML) and lincomycin (MY). R: Resistance; I: Intermediate resistance; S: susceptibility; # 0 = No growth inhibition

Table 2: MIC and MBC values (ug mL^{-1}) of DADS against 7 different bacteria using broth dilution tests

Strains	MIC	MBC
A. hydrophila	640	1280
A. salmonicida ssp. *masoucida*	640	1280
A. salmonicida ssp. *salmonicida*	640	1280
V. vulnificus	160	640
V. paraheamolyticus	640	1280
L. anguillarum	640	640
E. tarda	320	640

showed MBC values at 2 volumes MIC of DADS. MBC value of *V. vulnificus* was 640 ppm. The garlic oil contains 4 different dially sulfides, DMS, DADS, DTS and DTTS with antimicrobial activities to food-borne pathogenic bacteria (Harris *et al*., 2001). Of these sulfides, DADS was abundantly present in oils extracted from plants belonging to the allium family oils and had a lower toxicity for hemolytic anemia compared with the other dially sulfides. In addition, a previous study has suggested the bioavailability of DADS for the chemoprotective action of garlic (Munday *et al*., 2003).

This reason justifies our studying the antimicrobial effects of DADS to pathogenic bacteria for fish. The present findings indicated that DADS exhibited antimicrobial effects in 7 different pathogenic bacteria for fish. In addition, DADS inhibited the growth of bacteria with resistance to certain antibiotics such as amoxicillin, tetracycline, liconmycin and chloramphenicol. The finding was in agreement with previous studies, showing its effects to food-borne pathogens and antibiotic resistant pathogens (Rattanachaikunsopon and Phumkhachorn, 2008; Tsao and Yin, 2001; Yin *et al*., 2002). Contrary to previous studies, however, the present MIC values of DADS ranged from 160-640 ppm.

These MIC values were higher than those of the food-borne pathogens were. This could be due to different culture conditions such as growth temperature and inoculum volume of bacteria tested for MIC (Rattanachaikunsopon and Phumkhachorn, 2008, 2009b; Tsao and Yin, 2001; Tsao *et al*., 2007). Although, researchers did not perform an inhibitory effect of DADS to bacterial pathogens *in vivo* test using fish, there were several papers about its therapeutic usefulness on animal models for example, the compound was reported to the therapeutic agent for the infection of Methicillin-Resistant *Staphylococcus aureus* (MRSA) in diabetic and BALB/cA mice (Tsao *et al*., 2003, 2007). In addition, Rattanachaikunsopon and Phumkhachorn (2009a) have reported that DADS was able to decrease the cumulative mortality of Nile tilapia (*Oreochromis niliticus*) markedly due to an infection of *Flavobacterium columnare*. Based on all studies, DADS may be considered a useful natural compound instead of the chemical antibiotics in fish farms.

CONCLUSION

DADS has antimicrobial activity in 7 different bacteria: *A. hydrophila*, *V. vulnificus*, *V. paraheamolyticus*, *A. salmonicida* ssp. *salmonicida* and *masoucida* and *E. tarda* and *L. anguillarum*. The researchers suggest bioavailability of DADS for preventing bacterial infectious diseases in the aquaculture industry.

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