Efficacy of Condensed Tannin on Antibacterial Activities against Pathogenic Bacteria of Aquatic Animals

ประสิทธิภาพของคอนเดนส์แทนนินในการต้านเชื้อแบคทีเรียก่อโรคของสัตว์น้ำ

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บทคัดย่อ

คอนเดนส์แทนนินมีฤทธิ์ต้านเชื้อแบคทีเรีย และถูกนำมาใช้มากขึ้นในการเพาะเลี้ยงปลานิล อย่างไรก็ตามข้อมูลเกี่ยวกับฤทธิ์ของคอนเดนส์แทนนินในการต้านเชื้อแบคทีเรียก่อโรคของสัตว์น้ำยังมีค่อนข้างจำกัด การศึกษาครั้งนี้ต้องการประเมินประสิทธิภาพของคอนเดนส์แทนนินในการต้านเชื้อแบคทีเรียก่อโรคของสัตว์น้ำ โดยการทดสอบ microplate broth dilution test และ agar diffusion test เพื่อวัด minimal inhibitory concentration (MIC) และ minimal bactericidal concentration (MBC)

ตามลำดับ เชื้อแบคทีเรียก่อโรคของสัตว์น้ำที่ใช้ศึกษาในครั้งนี้ได้แก่ *Aeromonas hydrophila*, *Streptococcus agalactiae*, *Edwardsiella tarda*, *Flavobacterium columnare*, *Vibrio harveyi*, *V. parahaemolyticus* และ *V. cholerae* ผลการศึกษาพบว่าค่า MIC และ MBC ของคอนเดนส์แทนนินในการต้านเชื้อแบคทีเรียก่อโรคของสัตว์น้ำในการศึกษาครั้งนี้อยู่ในช่วง 200 – 2000 พีพีเอ็ม และ 400 – 2000 พีพีเอ็ม ตามลำดับ โดยคอนเดนส์แทนนินมีค่า MIC (200-400 พีพีเอ็ม) และ MBC (400-600 พีพีเอ็ม) ต่อเชื้อ *Strep. agalactiae* ต่ำที่สุด และมีค่า MIC (1800-2000 พีพีเอ็ม) และ MBC (1800-2000 พีพีเอ็ม) ต่อเชื้อ *V. harveyi* สูงที่สุด

คำสำคัญ: ฤทธิ์ต้านเชื้อแบคทีเรีย คอนเดนส์แทนนิน minimal bactericidal concentration, minimal inhibitory concentration

ABSTRACT

Condensed tannin has antimicrobial activities, and has been increasingly used by Nile tilapia producers. However, data concerning its effects on antimicrobial activities against pathogenic bacteria of aquatic animals is limited. Therefore, the efficacy of condensed tannin on *in vitro* antibacterial activities against pathogenic bacteria of aquatic animals was determined by microplate broth dilution test, agar diffusion test for minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC), respectively. Pathogenic bacteria, including *Aeromonas hydrophila*, *Streptococcus agalactiae*, *Edwardsiella tarda*, *Flavobacterium columnare*, *Vibrio harveyi*, *V. parahaemolyticus* and *V. cholerae*, were used. Results indicated that the ranges of MIC and MBC of condensed tannin against all studied pathogenic bacteria were 200-2,000 and 400-2000 ppm, respectively. Condensed tannin had the lowest MIC (200-400 ppm) and MBC (400-600 ppm) against *Strep. agalactiae* and had the highest MIC (1800-2000 ppm) and MBC (1800-2000 ppm) against *V. harveyi*

Key words: antibacterial activity, condensed tannin, minimal bactericidal concentration, minimal inhibitory concentration

INTRODUCTION

Aquaculture plays a significant role in Thai economy, and Thailand has been a leading exporting country of aquacultural products. Recently, aquacultural production system is well-developed into an intensive agriculture or cultivation, which could manage the limited farm resources to optimize the yields. Proficiency of an intensive cultivation depends on good farm management, particularly feed and feeding management, broodstock management, health management, water and environmental management, and prudent use of drugs and chemicals. Failures or mismanagement in an intensive cultivation

may critically induce stress, which will affect the health of cultured aquatic animals (Barton and Iwama, 1991). These stressed aquatic animals will have a decreased immune response, and will be vulnerable to infection by pathogenic and opportunistic bacteria (Naylor and Burke, 2005)

Antibiotics are natural or synthetic substances that could inhibit or kill bacteria. In order to reduce the production losses due to bacterial infection, the antibiotics are used for disease prevention and therapeutic purposes. As the aquacultural production increases following the great demand from the increasing world human population, antibiotics used for prevention, control and treatment of bacterial infections are also dramatically increasing in aquatic animal farming. As a consequence, residues of antibiotics in aquatic animal products, antibiotics contamination in the environment, and antibiotics-resistant bacteria are the key problematic concerns. Several studies reported antibiotics-resistant bacteria contaminated in the environment of aquaculture industry (Aarestrup, 2000; Alderman and Hastings 1998; Furushita *et al*., 2005; Miranda *et al*., 2003; Petersen *et al*., 2002; Samuelsen *et al*., 1992; Schmidt *et al*., 2000; Tendencia and Pena, 2001; Wegener *et al*., 1999). In addition, antibiotic-resistant genes have been transferred to the commonly found environmental bacteria (Miranda and Zemelman, 2002; Petersen and Dalsgaard, 2003). Kerry *et al*. (1996) cultured bacteria from the aquaculture area and reported that two species of gram-negative bacteria, *Plesiomonas shigelloides* and *Aeromonas hydrophila*, were resistant to tetracycline, oxytetracycline, chloramphenicol, ampicillin and nitrofuran. Furthermore, Le and Munekage (2004) reported that the residues of antibiotics, including trimethoprim, sulfamethoxazole, norfloxacin and oxolinic acid, are contaminated in the water nearby the aquaculture farms. Jones *et al*. (2004) also reported some antibiotic residues in water flea and atremia.

Due to the antibiotic residues problems, researches have been focused on alternative agents used for inhibiting or killing the bacteria. Natural crude extracts from plants, phenolic compounds, have long been studied for their antibacterial properties. The phenolic compounds, such as robientic, myricetin and epigallocatechin, could inhibit DNA synthesis of *Proteus vulgaris* and RNA synthesis of *Staphylococcus aureus* (Mori *et al*., 1987). Catechin is abundantly found in green tea, which could inhibit growth of gram-positive bacteria more than gram-negative bacteria (Ikigai *et al*., 1993). Apart from antibacterial activity, phenolic compounds also have antiviral and antifungal properties. For example, propolis, which is found in honey, could inhibit growth of dermatophytes and *Candida* spp. (Cafarchia *et al*., 1999).

Quercetin, morin, rutin, dihydroquercetin, dihydrofisetin, leucocyanidin, pelargonidin chloride as well as catechin possess antiviral properties against herpes simplex virus (HSV), respiratory syncytial virus, poliovirus, and Sindbis virus (Middleton *et al*., 1993). Phenolic compounds also have antioxidant activities, which could enhance cell membrane integrity and immune responses (Facino *et al*,. 1999; Hertog *et al*., 1993; Alonso *et al*., 2007). As previously mentioned, failures or mismanagement in an intensive cultivation may induce aquatic animals vulnerable to infection by pathogenic and opportunistic bacteria, including *Aeromonas hydrophila, Flavobacterium* spp*., Edwardsiella tarda, Sterptococcus agalactiae, Vibrio harveyi* and *V. parahaemolyticus*. It could be hypothesized that application of phenolic compounds, such as condensed tannin supplemented in the diet, could inhibit the growth of bacteria in aquatic animals.

In this study, the objectives were, firstly to determine condensed tannin properties, including solubility and heat stability on *in vitro* antibacterial activities against *E. coli* (ATCC25922) and *S. aureus* (ATCC25923), and secondly to determine *in vitro* antibacterial activities of condensed tannin against aquatic pathogenic bacteria, including *A. hydrophila*, *Strep. agalactiae*, *E. tarda*, *F. columnare*, *V. harveyi*, *V. parahaemolyticus* and *V. cholerae*.

MATERIALS AND METHODS

Condensed tannin properties on *in vitro* antibacterial activities

Solubility of condensed tannin

Solubility of condensed tannin with 3 different solvents, including sterile water, acetone (30%) (Sripad *et al*., 1982) and dimethyl sulfoxide (DMSO) was evaluated. All solvents were used to dissolve the stock solution of 6000 ppm of condensed tannin, which was then prepared and diluted with phosphate buffer solution (PBS) to the serial working solutions of 4000, 3600, 3200, 2800, 2400, 2000, 1600, 1200, 800, 400 and 200 ppm. All dilutions were tested for antibacterial activities against the reference strains of *E. coli* (ATCC25922) and *S. aureus* (ATCC25923). These two strains were multiplied in Mueller-Hinton broth (MHB) at 28°C for 12 h; thereafter the broths were adjusted to 0.5 Mcfarland standard, and were diluted to the final concentration of 10^6 cfu/ml. These bacterial solutions were used to determine minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of each dilution of condensed tannin. For each dilution of condensed tannin, 9 repeated wells were conducted.

Heat stability of condensed tannin

Condensed tannin was heated in the heat block for 5 min with 3 different

temperatures; 50°C, 100°C and 150°C. Thereafter, the heated condensed tannin dissolved with 30% acetone, was prepared to serial dilutions from 200 – 6000 ppm, and was used to determine MIC and MBC using the same procedure as described previously.

In vitro antibacterial activities of condensed tannin against aquatic bacteria

Preparation of condensed tannin solution

Condensed tannin was dissolved with 30% acetone to prepare for the stock solution of 6000 ppm. The stock solution was diluted with phosphate buffer solution (PBS) to the serial working solutions of 4000, 3600, 3200, 2800, 2400, 2000, 1600, 1200, 800, 400 and 200 ppm.

Antibacterial activities determination

Minimum inhibitory concentration was measured by microplate broth dilution test (CLSI, 2011). Bacteria were prepared to the concentration of 10^6 cfu/ml, and condensed tannin was prepared to serial dilutions of 0, 400, 800, 1200, 1600, 2000, 2400, 2800, 3200, 3600, 4000 and 6000 ppm. A 96-well microplate was used in this test. In general, 100 µl of bacteria solution was added to the wells and 100 µl of serial dilutions of condensed tannin was added. Therefore, the final dilutions of each well were 0, 200, 400, 600, 800, 1000, 1200, 1400, 1600, 1800, 2000 and 3000 ppm, respectively. The tested microplate was incubated at 28°C for 12 h; thereafter, 50 µl of resazurin was added. Resazurin was used as an indicator of bacterial growth by observing the color change; blue indicated no bacterial growth whereas red-pink indicated bacterial growth. The lowest concentration of condensed tannin at which the well appeared blue was recorded as MIC. Agar diffusion test was used to determine minimum bactericidal concentration (MBC), by taking 100 µl of mixed solution in each well and spreading on the blood agar for gram-positive bacteria; MacConky agar for gram-negative bacteria; on cytophaga agar for *F. columnar*e; and on thiosulfate-citrate bile salts sucrose agar (TCBS) for *Vibrio* spp. All agar plates were incubated 28°C for 12 h. The lowest concentration of condensed tannin at which the bacteria did not grow on the agar plate was recorded as MBC. For each dilution of condensed tannin, 16 repeated wells were conducted.

Bacteria sources

Pathogenic and opportunistic bacteria of aquatic animals were collected from Aquatic Diagnostic Unit, Faculty of Veterinary Medicine, Kasetsart University. In total, seven species, including *A. hydrophila*, *E. tarda*, *F. columnare*, *V. cholerae*, *V. harveyi*, *V. parahaemolyticus* and *Strep. agalactiae* were used.

Bacterial culture, isolation, identification

The collected samples were cultured and isolated on blood agar, MacConkey agar, cytophaga agar and TCBS agar according to different bacteria. Identification of these studied bacteria was performed using biochemical characteristics test. Bacteria were kept for further use on Mueller-Hinton agar at 28°C, except *Vibrio* spp. were kept on Mueller-Hinton agar supplemented with 2% NaCl.

Working bacterial solution

Seven bacterial strains were multiplied in Mueller-Hinton broth (MHB) at 28°C for 12 h; thereafter the broths were adjusted to 0.5 Mcfarland standard, and were diluted to the final concentration of 10^6 cfu/ml. For *Vibrio* spp., 2% NaCl was added into the broth during preparation.

Statistical analysis

Antibacterial activities of condensed tannin expressed as MIC and MBC against 7 pathogenic bacteria of aquatic animals were reported using descriptive statistics.

RESULTS

The MIC and MBC of condensed tannin dissolved in sterile water, 30% acetone, and DMSO *E. coli* and *S. aureus* are presented in Table 1. For the standard strain of *E. coli*, the MIC and MBC of condensed tannin were greater than 3000 ppm for all 3 solvents. For the standard strain of *S. aureus*, the MIC ranged from 800 to 1200 ppm, and the MBC were 3000 ppm. It was obvious that condensed tannin dissolved with DMSO had slightly higher MIC for *S. aureus* than that dissolved with sterile water and acetone.

The MIC and MBC of condensed tannin that was unheated; and heated at 50°C, 100°C and 150°C against *E. coli* and *S. aureus* are presented in Table 2. For the standard strain of *E. coli*, the MIC and MBC of condensed tannin were greater than 3000 ppm for unheated and

Table 1 Comparison of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of condensed tannin that were dissolved with sterile water, 30% acetone and dimethyl sulfoxide (DMSO) against *E. coli* and *S. aureus*.

Bacteria (10^6 cfu/ml)	Solvents					
	Sterile water		30 % Acetone		DMSO	
	MIC	MBC	MIC	MBC	MIC	MBC
E. coli (n = 9)	> 3000	> 3000	> 3000	> 3000	> 3000	> 3000
S. aureus (n = 9)	800-1000	3000	800-1000	3000	800-1200	3000

heated conditions. For the standard strain of *S. aureus*, the MIC ranged from 600 to 1200 ppm and the MBC were 3000 ppm. Condensed tannin heated at 150°C had higher MIC for *S. aureus* than that unheated, and heated at 50°C and 100°C.

Efficacy of *In vitro* antibacterial activities of condensed tannin against 7 aquatic bacteria is demonstrated in Table 3. The MIC of condensed tannin against 7 aquatic bacteria ranged from 200 to 1200 ppm, and the MBC ranged from 400 to 2000 ppm. The lowest MIC and MBC of condensed tannin were for *Strep. agalactiae*, and the highest MIC and MBC were for *V. haveyi*.

DISCUSSION

According to the solubility, acetone was practically an appropriate solvent for dissolving condensed tannin. Acetone also did not impair the antibacterial activities of

Table 2 Comparison of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of condensed tannin that was unheated, and heated at 50°C, 100°C and 150°C against E. coli and S. aureus.

Bacteria (106 cfu/ml)	Heating temperature							
	Un-heated		50°C		100°C		150°C	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. coli (n = 9)	> 3000	> 3000	> 3000	> 3000	> 3000	> 3000	> 3000	> 3000
S. aureus (n = 9)	600-1000	3000	800-1000	3000	600-1000	3000	800-1200	3000

Table 3 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of condensed tannin against 7 aquatic bacteria.

Bacteria (10^6 cfu/ml)	Condensed tannin	
	MIC (ppm)	MBC (ppm)
Strep. agalactiae (n = 16)	200-400	400-600
A. hydrophila (n = 16)	1000-1200	1000-1200
E. tarda (n = 16)	800-1000	800-1200
F. columnare (n = 16)	800-1000	800-1200
V. haveyi (n = 16)	1800-2000	1800-2000
V. parahaemolyticus (n = 16)	800-1200	1000-1200
V. cholerae (n = 16)	800-1000	800-1200

dissolved condensed tannin. Due to the MIC and MBC of condensed tannin for the standard *E. coli*, which were greater than those for the standard *S. aureus*, it was suggested that gram-negative bacteria required a higher concentration of condensed tannin for their inhibition. In this study, the MIC and MBC of condensed tannin for *E. coli* could not be determined, because the final dilution in the tested well was 3000 ppm. In literature, it is reported that gram-negative bacteria required greater MIC and MBC of fluoroquinolone for their inhibition (Duggirala *et al*., 2007). As compared to antibiotics such as amoxicillin, the MIC and MBC were 4 ppm and 0.5 ppm for *E. coli* and *S. aureus*, respectively (Andrews, 2001). Therefore, application of condensed tannin for its antibacterial activities required a larger dose as compared to antibiotics. However, condensed tannin caused less residual effects and less environmental contamination.

Exposure of condensed tannin to high temperature could reduce its efficacy on antibacterial activities. In this study, heating condense tannin upto 150°C for 5 min significantly decreased antibacterial properties. It has been reported that phenolic compounds extracted at 80 - 145°C had reduced their functional properties (Vergara-Salinas *et al*., 2012; Monica *et al*., 2009).

Results of MIC and MBC of condensed tannin against 7 bacteria species indicated that condensed tannin had a good efficacy to inhibit growth of pathogenic bacteria in aquaculture. However, the efficacy would vary on the solvents that could effectively dissolve condensed tannin. Moreover, environmental factors such as temperature and acid-base balance could also impair the antibacterial activities of condensed tannin. Therefore, application of condensed tannin in aquaculture to control or prevention of bacteria should take into account the appropriate dosage for antibacterial activities, dissolving solvents for feed or water supplementation, and temperature storage and usage. The use of condensed tannin was expected to have an advantage not only to control bacterial infection, but also it could reduce the use of antibiotics that would increase adverse effects on consumer's health as well as environmental health (Taguri *et al*., 2004; Smullen *et al*., 2007)

In conclusion, condensed tannin was confirmed its antibacterial activities against aquatic bacteria. It could be easily dissolved in acetone, and practically stable at temperature less than 100°C. Though the MIC and MBC of condensed tannin were relatively higher than those of antibiotics, condensed tannin extracted from natural plants was considered its high safety for aquaculture system. Condense tannin would therefore be an alternative substance to control and prevention of bacterial infection.

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