The Thai Journal of Veterinary Medicine

Volume 43
Issue 1 *March, 2013*

Article 1

3-1-2013

Antibacterial Activity of Tannin from Sweet Chestnut Wood Against Aeromonas and Streptococcal Pathogens of Tilapia (Oreochromis niloticus)

Hathairat Maisak

Sasibha Jantrakajorn

Mintra Lukkana

Janenuj Wongtavatchai

Original Article

Antibacterial Activity of Tannin from Sweet Chestnut Wood Against Aeromonas and Streptococcal Pathogens of Tilapia (*Oreochromis niloticus*)

Hathairat Maisak[1] Sasibha Jantrakajorn[2] Mintra Lukkana[3] Janenuj Wongtavatchai[1*]

Abstract

The present study determined the inhibitory effect of tannin from sweet chestnut wood against tilapia *Oreochromis niloticus* bacterial pathogens, *Aeromonas hydrophila* (20 isolates) and *Streptococcus agalactiae* (20 isolates). Minimum Inhibitory Concentrations (MICs) corresponding to tannin and oxytetracycline (OTC) for *A. hydrophila* were 18.75-300 µg/ml and 0.125-128 µg/ml; and for *S. agalactiae* were 600 µg/ml and 0.5-8 µg/ml, respectively. The antibacterial potency of tannin from sweet chestnut wood was found in the test system of pH: 6.5-7.0, suggesting that the inhibitory effect of tannins is unrelated to their acidity property. The OTC-resistant *A. hydrophila* and *S. agalactiae* (MIC of OTC > 4 µg/ml) responded to lower MICs of OTC in the test system with sub-inhibitory concentration of tannins (150 µg/ml). Concerning the trend towards the evolution of antimicrobial resistant strains, the antimicrobial activity of tannins may be a possible additive compound against fish pathogens treated with antibiotics.

Keywords: *Aeromonas,* antibacterial activity, *Streptococcus*, tannin, tilapia

[1]*Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand*
[2]*Faculty of Veterinary Science, Prince of Songkla University, Songkla, Thailand*
[3]*National Bureau of Agricultural Commodity and Food Standards, Ministry of Agriculture and Cooperatives, Bangkok, Thailand*
***Corresponding author E-mail:** Janenuj.w@chula.ac.th*

บทคัดย่อ

การต้านเชื้อแบคทีเรียแอโรโมแนสและสเตรปโตคอคคัสที่แยกจากปลานิล (*Oreochromis niloticus*) โดยสารแทนนินจากไม้เกาลัด

หทัยรัตน์ ไม้สัก[1] ศศิภา จันทร์ขจร[2] มินตรา ลักขณา[3] เจนนุช ว่องธวัชชัย[1*]

การศึกษานี้เป็นการทดสอบคุณสมบัติของสารแทนนินที่สกัดจากต้นเกาลัดในการต้านเชื้อแอโรโมแนสและเชื้อสเตรปโตคอคคัสซึ่งเป็นแบคทีเรียก่อโรคสำคัญในฟาร์มปลานิล *Aeromonas hydrophila* และ *Streptococcus agalactiae* ที่แยกจากปลานิลป่วยจำนวนอย่างละ 20 เชื้อ และตรวจยืนยันชนิดเชื้อด้วยวิธีชีวเคมีและปฏิกิริยาห่วงโซ่พอลิเมอเรส ถูกนำมาทดสอบกับสารแทนนินและยาออกซิเตตร้าไซคลิน ความเข้มข้นต่ำสุดของสารแทนนินและยาออกซิเตตร้าไซคลินในการยับยั้งการเจริญของ *A. hydrophila* เท่ากับ 18.75-300 ไมโครกรัม/มล. และ 0.125-128 ไมโครกรัม/มล. *และ S. agalactiae* เท่ากับ 600 ไมโครกรัม/มล. และ 0.5-8 ไมโครกรัม/มล. ตามลำดับ นอกจากนี้พบว่าความเข้มข้นต่ำสุดของยาออกซิเตตร้าไซคลินในการต้านเชื้อ *A. hydrophila* และ *S. agalactiae* ลดลงเมื่อผสมแทนนิน (150 ไมโครกรัม/มล.) ในอาหารเลี้ยงเชื้อ การทดสอบทางห้องปฏิบัติการแสดงว่าสารประกอบแทนนินมีคุณสมบัติในการต้านเชื้อแอโรโมแนสไฮโดรฟิลาและเชื้อสเตรปโตคอคคัสคุณสมบัติดังกล่าวไม่สัมพันธ์กับความเป็นกรดของสารประกอบแทนนิน โดยพบว่าอาหารเลี้ยงเชื้อที่ผสมแทนนิน (600 µg/ml) มีค่า pH 6.5-7.0 คุณสมบัติของแทนนินในการต้านเชื้อแบคทีเรียแอโรโมแนสและเชื้อสเตรปโตคอคคัสที่ปรากฏในการศึกษานี้สามารถนำไปสู่การทดสอบการใช้แทนนินเป็นสารที่เติมในอาหารปลาเพื่อต้านเชื้อแบคทีเรีย และอาจร่วมกับการใช้ยาต้านจุลชีพในการควบคุมการติดเชื้อแบคทีเรีย

คำสำคัญ: เชื้อแอโรโมแนส ต้านเชื้อแบคทีเรีย เชื้อสเตรปโตคอคคัส สารแทนนิน ปลานิล

[1]*ภาควิชาอายุรศาสตร์ คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย กรุงเทพฯ*

[2]*คณะสัตวแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ สงขลา*

[3]*สำนักงานมาตรฐานสินค้าเกษตรและอาหารแห่งชาติ กรุงเทพฯ*

****ผู้รับผิดชอบบทความ E-mail:** Janenuj.w@chula.ac.th*

Introduction

Among the diseases in intensive fish farming, bacteria are the most significant pathogens of cultured fish. *Aeromonas hydrophila*, *Streptococcus agalactiae* and *S. iniae* are bacterial pathogens causing infectious diseases in Thai farmed tilapia (Lukkana et al., 2011). *A. hydrophila* is common bacteria in freshwater habitats throughout the world and is present in normal microflora and inhabiting fish reservoirs (Cipriano, 2001; Floyd, 2009). The gram positive cocci, *Streptococcus*, are pathogenic bacteria which have been reported to be responsible for severe economic losses in tilapia production caused by high mortality and poor meat quality yield (Yanong and Floyd, 2006; Floyd, 2009).

Although bacterial infection generally responds to antibiotic therapy, fish bacterial disease is not effectively controlled by antibiotics all the way to market. Oral medication with medicated diet is the most common route of antibiotic therapy in fish, but such application causes leaching of the compound to water, consequently the bio-availability of the compound may be inconsistent. In addition, some bacterial strains in fish, aquatic environments or sediments that break out in different areas may develop resistance to some antibiotics (Bowser, 1999; Najiah et al., 2011). As many natural products that possess antibacterial activity can resolve the problems of chemical and antibiotic resistances, they have potential to be an alternative choice (Najiah et al., 2011; Haniffa and Kavitha, 2012).

Tannins are water-soluble polyphenols commonly found in higher herbaceous and woody plants (Scalbert, 1991). They are categorized as being generally recognized as safe (GRAS) food additives (Chung et al., 1998). Tannins can be classified into hydrolysable and non-hydrolysable (condensed) tannin, tannic acid is a substantial hydrolysable class (Akiyama, 2001). Tannins extracted from different plant species have specific physical and chemical properties. On the antimicrobial activity, tannin has a positive effect on different bacterial strains that are

associated with food borne diseases and animal disease outbreaks. Antibacterial actions of tannins have been reported as bacteriostatic and bactericidal against different harmful bacteria, including *A. hydrophilla, E. coli, Listeria, Pseudomonas, Salmonella, Staphylococcus* and *Streptococcus* (Chung et al., 1998; Banso and Adeyemo, 2007; Doss et al., 2009). The ability of plant polyphenols to complex with polymers and minerals is suggested to be the principle reason for their inhibitory effect on bacteria. The complexation induced by tannins may account for tannin toxicity and the inhibition of microbial enzymes (Bossi et al., 2007).

Due to global consultation over the use of antibiotics in farmed animals, natural substances are being used as feed additives for several purposes including the antimicrobial action and promoting growth in animal farming. At the same time, the use of natural feed additives has no negative impact on the farming environment and accords with consumers' concerns for safe and healthy products. Dietary tannins from sweet chestnut *Castanea sativa* Mill have shown to have benefits such as increasing feed efficiency in poultry production and antimicrobial growth promoter in swine industry (Jamroz et al., 2009; Van Parys et al., 2010). In view of the growing aquaculture industry, the production of fish feed additives is driven to make use of environmentally-friendly feed additives. Several natural feed additives are currently used by the aquaculture industry including probiotics, micro-algae and herbal extracts (Lovatelli and Chen, 2009). Some phytochemicals such as tannins, alkaloids and flavonoids have demonstrated their antibacterial activity in fish bacterial pathogens (Haniffa and Kavitha, 2012). The purpose of this study was to examine the *in vitro* antibacterial potency of hydrolysable tannin from sweet chestnut wood *C. sativa* Mill against *A. hydrophila* and *S. agalactiae* obtained from disease outbreaks in farmed tilapia.

Materials and Methods

Tannin substance: A commercial feed additive, Globatan® (supplied by Global Nutrition, France), was the testing tannin substance used in the study. The product is a hydrolysable tannin extract from sweet chestnut wood *C. sativa* Mill containing approximately 75% (w/w) active tannin substance as a mixture of esteric and glycosidic tannins. A serial two-fold dilution of Globatan® dissolved in 3 ml absolute ethanol and then diluted with 17 ml distilled water was processed with distilled water, giving a series of tested concentrations from 25 to 1,600 µg/ml corresponding to 18.75 to 1,200 µg/ml hydrolysable tannin.

Antimicrobial agent: Oxytetracycline (OTC; Wako Pure Chemical Industries, Japan) was included as a standard antibiotic and positive control. OTC dissolved in 1% HCL and then was processed with distilled water as suggested by Clinical and Laboratory Standard Institute (CLSI, 2010), giving a series of tested concentrations from 0.125 to 128 µg/ml.

Bacterial strains:

Test strains: *A. hydrophila* and *S. agalactiae*, each of 20 clinical isolates were from disease cases occurring in most regions of tilapia farming; in north-eastern, eastern and the central part of Thailand, between 2007-2010. All bacterial strains were stored in maintenance broth containing 40% glycerol and supplemented with 10% fetal bovine serum, at -70^{0}C. Before each experiment for the MIC was carried out, the stored bacterial strains were transferred to tryptic soy agar (TSA; Oxoid®, UK) supplemented with 10% sheep blood. After incubation at 30±2^{0}C for 18-24 hours, colonies from the pure culture material were resolved in 4 ml volumes of sterile normal saline and turbidity of the inoculum was adjusted to 0.5 McFarland standard or approximately 10^8 Colony Forming Unit (CFU)/ml. The inoculum was then diluted ten-fold in sterile normal saline, giving a final cell density of approximately 10^7 CFU/ml. Quality control strains: Additional bacterial organisms were obtained from the American Type Culture Collection (ATCC) for use as quality controls; *A. hydrophila* ATCC 35654 and *S. agalactiae* ATCC 13813.

Bacterial identification: Identification of bacterial isolates was performed by using conventional biochemical methods described in the API® system (BioMérieux®, France). The commercial kit was applied following the manufacturer's instructions with a modification of the incubating temperature to 30±2^{0}C for the suitable bacterial growth. Bacterial isolates were further confirmed by polymerase chain reaction (PCR). The chromosomal DNA from bacterial cells was separated with a NucleoSpin® Extract I kit (MACHEREY-NAGEL, Germany) following the manufacturer's instructions. PCR identification of the streptococcal DNA was amplified by genus specific oligonucleotide primers and species specific primers as shown in Table 1. PCR was carried out in PCR Thermal Cycler (Whatman Biometra®, UK), and the PCR reaction mixture (20 µl) contained 2 µl of 10x PCR buffer (100 mM Tris HCl (pH 8.3), 500 mM KCl, 20 mM $MgCl_2$), 2 µl of dNTP 2.5 mM, 0.2 µl of Tag polymerase 5 U (iNtRON Biotechnology, USA), 1 µl of forward primer 10 µM, 1 µl of reverse primer 10 µM, and 5 µl of DNA 50 ng/µl (Meiri-Bendek et al., 2002). PCR was conducted with the following program: 94^{0}C denaturation step), 30 cycles at 94^{0}C for 20 sec (denaturation step), at 56^{0}C for 10 sec (annealing step), and at 72^{0}C for 30 sec (extension step), followed by a final extension at 72^{0}C for 2 min. The PCR products were determined by the electrophoresis in 2% agarose gel at 100 volt for 40 min and 100 bp DNA ladder as a molecular marker (SibEnzyme, Russia). Gels were soaked in 0.5 µg/ml ethidium bromide (Sigma-Aldrich, USA) for 30 min and visualized under UV illumination (Vilber Lourmat, Germany) and photographed. S. agalactiae ATCC13813 and A. hydrophila ATCC 35654 were used as positive controls and distilled water was used as negative control of the reaction.

Table 1 Oligonucleotide primers for identification of *A. hydrophila* and *S. agalactiae*

Primer	Sequence (5′-3′)	Gene	Size	Reference
Aeromonas				
AERF	CTA CTT TTG CCG GCG AGC GG	16S rRNA	953 bp	Lee et al. (2002)
AERR	TGA TTC CCG AAG GCA CTC CC			
A. hydrophila				
Aero1a	CCA AGG GGT CTG TGG CGA CA	Aerolysin	209 bp	Pollard et al. (1990)
Aero1b	TTT CAC CGG TAA CAG GAT TG			
Streptococcus				
C1	GCG TGC CTA ATA CAT GCA A	16S rRNA	202 bp	Meiri-Bendek et al. (2002)
C2	TAC AAC GCA GGT CCA TCT			
S. agalactiae				
F1	GAG TTT GAT CAT GGC TCA G	16S rRNA	220 bp	Martinez et al. (2001)
IMOD	ACC AAC ATG TGT TAA TTA CTC			

Minimum Inhibitory Concentration (MIC): The MIC procedures are in accordance with the international recommendations provided by the Clinical and Laboratory Standards Institute (CLSI, 2010). Mueller Hinton Agar (MHA; Oxoid®, UK) added with serial two-fold dilutions of the antimicrobial agent, tannin substance or OTC, was inoculated with a standardized inoculum of the test strains (10^7 CFU/ml). Using a standard multipoint inoculator, bacteria from mature cell cultures were allocated at approximately 10^4 CFU/spot on the surface of the MHA. After 18-20 hours incubation, the MIC was recorded as the lowest concentration of antimicrobial with no visible growth bacteria. Quality control and purity control of the methods were regularly performed for each test.

Results

The biochemical identification of *A. hydrophila* and *S. agalactiae* is shown in Table 2 and 3. Confirmative identification with PCR assays was employed to all tested isolates. *Aeromonas spp*. amplified with primers AERF/AERR presented 953 bp amplicon and the amplification of *A. hydrophila* with species specific primers Aero1a/Aero1b yielded 207 bp amplicon. PCR assay for *Streptococcus spp.* using primers C1/C2 presented 202 bp amplicon and species specific primers F1/IMOD yielded 220 bp amplicon for *S. agalactiae* (Fig 1).

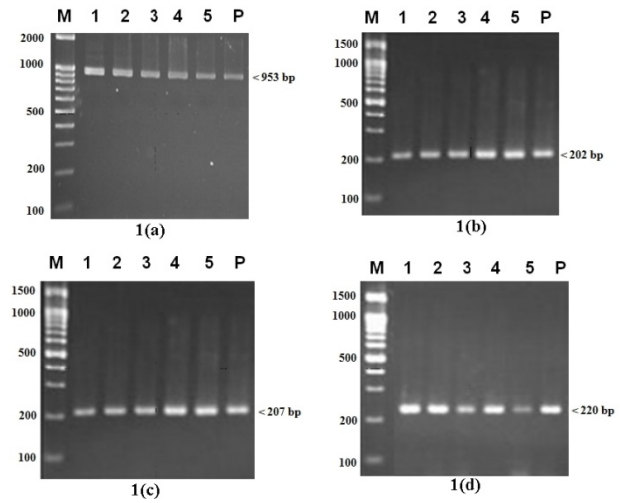

Figure 1 Direct PCR assay using specific primers to identify *A. hydrophila* and *S. agalactiae*. The amplification of *Aeromonas spp.* with primers AERF/AERR showed 953 bp amplicon, 1(a), and the amplification of with species specific primers Aero1a/Aero1b yielded 207 bp amplicon, 1(c). PCR assay for *Streptococcus spp.* using primers C1/C2 presented 202 bp amplicon, 1(b), and species specific primers F1/IMOD yielded 220 bp amplicon for *S. agalactiae*, 1(d). Lane M: 100-bp DNA ladder, Lane P: positive control (*A. hydrophila* ATCC 35654 or *S.agalactiae* ATCC 13813).

Table 2 Biochemical characteristics of *A. hydrophila* ATCC35654 and *A. hydrophila* isolated from diseased tilapia tested with API®20E (BioMérieux®)

Aeromonas hydrophila	Test																				
	ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX
ATCC35654	+	+	+	-	-	-	-	-	-	+	+	+	+	-	-	-	+	-	-	-	+
Clinical isolate1 (n=10)	+	+	+	-	-	-	-	-	+	+	+	+	+	-	-	-	+	-	-	-	+
Clinical isolate2 (n=6)	+	+	+	-	+	-	-	-	+	+	+	+	+	-	-	-	+	-	-	-	+
Clinical isolate3 (n=4)	+	+	+	-	-	-	-	-	-	+	+	+	+	-	-	-	+	-	-	-	+

ONPG: beta-galactosidase, ADH: arginine dihydrolase, LDC: lysine decarboxylase, ODC: ornithine decarboxylase, CIT: citrate utilization, H_2S: H_2S production, URE: urease, TDA: tryptophane deaminase, IND: indole production, VP: acetoin production (Voges Proskauer), GEL: gelatinase, GLU: fermentation/oxidation (glucose), MAN: fermentation/oxidation (mannitol), INO: fermentation/oxidation (inositol), SOR: fermentation/oxidation (sorbitol), RHA: fermentation/oxidation (rhamnose), SAC: fermentation/oxidation (saccharose), MEL: fermentation/oxidation (melibiose), AMY: fermentation/oxidation (amygdalin), ARA: fermentation/oxidation (L-arabinose), OX: cytochrome-oxidase, + : positive reaction, - : negative reaction

Table 3 Biochemical characteristics of *S. agalactiae* ATCC13813 and *S. agalactiae* isolated from diseased tilapia tested with API® 20 STREP (BioMérieux®)

Streptococcus agalactiae	Test																			
	VP	HIP	ESC	PYRA	αGAL	βGUR	βGAL	PAL	LAP	ADH	RIB	ARA	MAN	SOR	LAC	TRE	INU	RAF	AMD	GLYG	βHEM
ATCC13813	+	-	-	-	-	+	-	-	-	+	+	-	-	-	+	+	-	-	-	-	+
Clinical isolate (n=20)	+	-	-	-	+	-	-	+	+	+	+	-	-	-	-	+	-	-	-	-	+

VP: acetoin production (Voges Proskauer), HIP: hydrolysis (hippuric acid), ESC: β-glucosidase hydrolysis (esculin), PYRA: pyrrolidonyl arylamidase, αGAL: α-galactosidase, βGUR: β-glucuronidase, βGAL: β-galactosidase, PAL: alkaline phosphatase, LAP: leucine arylamidase, ADH: arginine dihydrolase, RIB: acidification (ribose), ARA: acidification (L-arabinose), MAN: acidification (mannitol), SOR: acidification (sorbitol), LAC: acidification (lactose), TRE: acidification (trehalose), INU: acidification (inulin), RAF: acidification (raffinose), AMD: acidification (starch), GLYG: acidification (glycogen), βHEM: β-hemolysis, + : positive reaction, - : negative reaction

Agar dilution method was used to determine the MIC values of tannin substance and OTC for 20 A. hydrophila and 20 *S. agalactiae* strains in MHA plates. The lowest concentration of OTC that inhibited growth of quality control strains used in the test system, *A. hydrophila* ATCC 35654 and *S. agalactiae* ATCC 13813, was 1 µg/ml. The assay showed that both quality control strains were susceptible to OTC (CLSI, 2010). The MICs for *A. hydrophila* varied from 18.75-300 µg/ml of tannin substance and 0.125-128 µg/ml of OTC (Table 4). Five *Aeromonas* strains were resistant to OTC (MIC > 4 µg/ml). Table 5 shows the MIC of OTC and tannin substance for 20 strains of *S. agalactiae.* More confining MIC ranges were observed for *S. agalactiae*, the MIC of tannin substance for 20 *S. agalactiae* strains was 600 µg/ml while the MICs of OTC varied between 0.5-8.0 µg/ml and were > 4 µg/ml in 4 strains. For both *A. hydrophila* and *S. agalactiae*, the high MIC values of OTC observed in some strains did not carry through the increased MICs of tannin substance.

The distribution of MIC values of OTC and tannin substance presented in Fig 2, narrow MIC ranges for *S. agalactiae* compared to MIC ranges for *A. hydrophila* indicating a less variable susceptibility for *S. agalactiae*. Frequencies of MIC observed for each compound also showed that *A. hydrophila* and *S. agalactiae* displayed more consistent susceptibility to tannin substance than OTC. The antibacterial activity

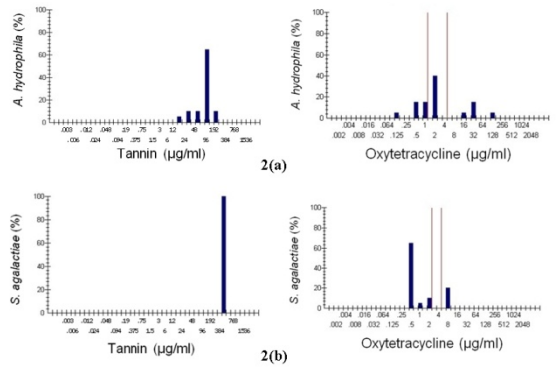

Figure 2 Frequencies of Minimum Inhibitory Concentrations (MICs) observed for tannin substance (TNN) and oxytetracycline (OTC) that were tested against 20 *A. hydrophila* isolates, 2(a), and 20 *S. agalactiae* isolates clinical isolates, 2(b).

the combination of OTC and 150 µg/ml tannin of tannin substance was subsequently demonstrated in strains which were resistant to OTC (MIC of OTC > 4 µg/ml). The incubation of OTC-resistant strains in substance lowered the amount of OTC required for the antibacterial action (Table 4 and 5).

Discussion

The antimicrobial potency of tannin extracts on a number of microorganisms has been reported in several plants which are rich in tannins. There are probably several mechanisms involved in tannin toxicity to microorganisms. A review of the literature indicates that the ability of plant polyphenols to complex with polymers and minerals is the basis of inhibitory effect on bacteria. Inhibitory mechanisms of tannins are explained as a direct inhibition caused by interacting with membranes, cell walls and/or extracellular proteins (Scalbert, 1991; Doss et al., 2009). A complexation of metal ions by tannins also

Table 4 Minimum Inhibitory Concentrations (MICs, µg/ml) of tannin substance (TNN) and oxytetracycline (OTC) against *A. hydrophila* clinical isolates associated with tilapia disease

Clinical isolates	**MIC (µg/ml)**		
A. hydrophila	**TNN**	**OTC**	**OTC#**
AhTh07-2007	150	2	-
AhTh12-2008	300	0.5	-
AhTh13-2008	150	0.5	-
AhTh14-2008	150	0.5	-
AhTh15-2008	300	128	32
AhTh16-2008	150	1	-
AhTh17-2008	18.75	1	-
AhTh18-2008	150	16	4
AhTh19-2008	150	1	-
AhTh20-2008	75	2	-
AhTh28-2009	37.5	32	16
AhTh29-2009	150	2	-
AhTh30-2009	150	2	-
AhTh31-2009	150	2	-
AhTh32-2009	150	2	-
AhTh33-2010	150	2	-
AhTh34-2010	150	2	-
AhTh35-2010	75	32	16
AhTh36-2010	150	32	16
AhTh37-2010	37.5	0.125	-
ATCC 35654	150	1	-

For the OTC-resistant isolates, the test system was added with 150 µg/ml tannin substance.

Table 5 Minimum Inhibitory Concentrations (MICs, µg/ml) of tannin substance (TNN) and oxytetracycline (OTC) against *S. agalactiae* clinical isolates associated with tilapia disease

Clinical isolates *S. agalactiae*	MIC (µg/ml)		
	TNN	OTC	OTC#
SaTh01-2007	600	0.5	-
SaTh03-2007	600	0.5	-
SaTh04-2007	600	0.5	-
SaTh06-2007	600	0.5	-
SaTh07-2008	600	0.5	-
SaTh08-2008	600	0.5	-
SaTh09-2008	600	0.5	-
SaTh10-2008	600	0.5	-
SaTh11-2008	600	0.5	-
SaTh12-2008	600	0.5	-
SaTh13-2008	600	0.5	-
SaTh14-2008	600	0.5	-
SaTh17-2009	600	0.5	-
SaTh18-2009	600	8	4
SaTh22-2009	600	2	-
SaTh24-2009	600	8	4
SaTh25-2009	600	8	4
SaTh26-2010	600	8	4
SaTh27-2010	600	1	-
SaTh28-2010	600	2	-
ATCC 13813	600	1	-

For the OTC-resistant isolates, the test system was added with 150 µg/ml tannin substance.

results in an indirect inhibition by making nutrients unavailable for bacteria and may account for in vitro antibacterial activity (Akiyama et al., 2001). Of the two types, hydrolysable and non-hydrolysable (condensed) tannins, the antimicrobial effect of hydrolysable tannins is stronger compared to that of condensed tannin (Smith et al., 2005). Tannin substances obtained from different plant species may produce different degree of the inhibitory effect, for instance, the MIC values of tannin for *E. coli* were 5,000 µg/ml from *Dichrostachys cinerea* (Banso and Adeyemo, 2007) and 4,000 µg/ml from *Solanum trilobatum* Linn. (Doss et al., 2009). The present study determines *in vitro* inhibitory activity of tannin substance from sweet chestnut wood against fish bacterial pathogens. Sweet chestnuts contain tannins over 10% of their weight and sweet chestnut tannins in animal feed have shown beneficial effects on animal performance, including the inhibitory functions on pathogenic bacteria (Jamroz et al., 2009). The wide range MICs of tannins for bacteria was evident in many studies and different bacterial mechanisms to overcome inhibitory effects of tannins were proposed. Smith et al. (2005) suggested that the gram negative bacteria possessed outer lipopolysaccharide membrane which might be capable of repelling the phenolic compound and, hence, they were not well susceptible to tannin. A further mechanism involving tannin detoxification may be attributed to the synthesis of tannin-complexing polymers, oxidation and membrane modification of the bacteria. Antibacterial activities of tannins evaluated *in vitro* showed large differences in MIC values between fungi, bacteria or yeasts. MICs for bacteria were found between 12-1,000 µg/ml whereas fungi and yeasts were reported to be more resistant to tannins (Scalbert, 1991).

Our study showed a bacteriostatic effect of sweet chestnut tannin on *A. hydrophila* at level of 18.75-300 µg/ml and a higher level of 600 µg/ml was observed for *S. agalactiae* clinical isolates. The moderate MIC values may be partially attributed to the potent anti-microbial effect of hydrolysable tannins which is the testing tannin substance in this study. In spite of the acidity quality claimed for polyphenolic compounds such as tannin, the present study demonstrated antibacterial activity of tannin substance in a test system posing pH: 6.5-7.0 (MHA with ≤ 600 µg/ml tannin substance), thus, *in vitro* antibacterial activity of sweet chestnut tannins did not contribute to their acidity property.

This study also represents that antibiotic oxytetracycline inhibited the growth of *A. hydrophila* at MIC range of 0.125-128 µg/ml and *S. agalactiae* at MIC range of 0.5-8.0 µg/ml. The apparent broad MIC values of OTC also imply that the tested isolates were not entirely susceptible to OTC. Considering the inhibitory break point of OTC (4 µg/ml; CLSI, 2010), susceptibility of the tested isolates to OTC differed significantly and OTC-resistance was evident in some strains. It is notable that antimicrobial resistance in fish pathogens and environmental bacteria occurs consequently to therapeutic applications in aquaculture (Alderman and Hastings, 1998). Few antimicrobial agents are allowed for therapeutic use in Thai aquaculture and OTC is one of the most widely used antimicrobial due to its relatively low cost and availability of generic commercial products. With the trend towards the evolution of antimicrobial resistance strains, natural products having antimicrobial activities have been further investigated. To investigate the suitability of tannins as an alternative to control pathogenic bacterial infection in tilapia, the inhibitory potency of tannins was tested on OTC-resistant *A. hydrophila* and *S. agalactiae* strains. The incubation of OTC-resistant isolates in the combination of OTC and tannin substance below the MIC (150 µg/ml) markedly decreased the MIC of OTC for *A. hydrophila* and *S. agalactiae*. The increased inhibitory effects of OTC could be compatible with changes of organism morphology and growth conditions occurring in the presence of tannin. Impairment of growth and morphology of sensitive organisms following an incubation of tannin extracts was formerly reported in many food-borne bacterial pathogens (O'Donovan and Brooker, 2001). The present findings indicate that hydrolysable tannin extract of sweet chestnut wood is a potential antibacterial agent, even against OTC-resistant bacteria. Although it has a marked *in vitro* inhibitory effect on fish bacterial pathogens, *A. hydrophila* and *S. agalactiae*, the inhibition is necessary to be confirmed in an *in vivo* trial. This result will further our understanding as to how tannins may be useful additives for the antibacterial treatment in aquaculture.

Acknowledgements

We wish to thank the Royal Golden Jubilee Ph.D. Program (RGJ), the Thailand Research Fund and the Graduate School, Chulalongkorn University for research funding.

References

Akiyama H, Fujii K, Yamasaki O, Oono T and Iwatsuki K 2001. Antibacterial action of several tannins against *Staphylococcus aureus*. J Antimicrob Chemother. 48: 487-491.

Alderman DJ and Hastings TS 1998. Antibiotic use in aquaculture: Development of antibiotic resistance - potential for consumer health risks. Int J Food Sci Technol. 33: 139-155.

Banso A and Adeyemo SO 2007. Evaluation of antibacterial properties of tannins isolated from *Dichrostachys cinerea*. Afr J Biotechnol. 6(15): 1785-1787.

Bossi A, Rinalducci S, Zolla L, Antonioli P, Righetti PG and Zapparoli G 2007. Effect of tannic acid on *Lactobacillus hilgardii* analysed by a proteomic approach. J Appl Microbiol. 102: 787-795.

Bowser PR 1999. Diseases of fish. In: Aquatic Animal Health Program at Department of Microbiology and Immunology, Cornell university, USA: 1-10.

Chung KT, Lu Z and Chou MW 1998. Mechanism of inhibition of tannic acid and related compounds on the growth of intestinal bacteria. Food Chem Toxicol. 36: 1053-1060.

Cipriano RC 2001. *Aeromonas hydrophila* and motile aeromonad septicemias of fish. Fish Dis Leafl. 68. 24 pp.

Clinical and Laboratory Standards Institute. 2010. Performance standards for antimicrobial disk susceptibility tests. In: Twentieth Information Supplement. 30(1): 92-95.

Doss A, Mubarack HM and Dhanabalan R 2009. Antibacterial activity of tannins from the leaves of *Solanum trilobatum* Linn. Indian J Sci Technol. 2(2): 41-43.

Floyd RF 2009. Aeromonas infection. The institute of Food and Agricultural Sciences (IFAS) document, University of Florida. USA.

Haniffa MA and Kavitha K 2012. Antibacterial activity of medical herbs against the fish pathogen *Aeromonas hydrophila*. J Agric Technol. 8(1): 205-211.

Jamroz D, Wiliczkiewicz A, Skorupińska J, Orda J, Kuryszko J and Tschirch H 2009. Effect of sweet chestnut tannin (SCT) on the performance, microbial status of intestine and histological characteristics of intestine wall in chickens. Br Poult Sci. 50(6): 687-699.

Lee C, Cho JC, Lee SH, Lee DG and Kim SJ 2002. Distribution of *Aeromonas spp*. as identified by 16S rDNA restriction fragment length polymorphism analysis in a trout farm. J Appl Microbiol. 93: 976-985.

Lovatelli A and Chen J 2009. Use of environmental friendly feed additives and probiotics in Chinese aquaculture. FAO Aqua. Newsletter. 42: 32-35.

Lukkana M, Wongtavatchai J and Chuanchuen R 2011. Expression of AheABC efflux system and plasmid profile of *Aeromonas hydrophila* isolates from farmed nile tilapia (*Oreochromis niloticus*). Thai J Vet Med. 41(4): 529-533.

Martinez C, Harel J and Gottschalk M 2001. Specific detection by PCR of *Streptococcus agalactiae* in milk. Can J Vet Res. 65: 68-72.

Meiri-Bendek I, Lipkin E, Friedmann A, Leitner G, Saran A, Friedmans S and Kashi Y 2002. A PCR-based method for the detection in milk. J Dairy Sci. 85: 1717-1723.

Najiah M, Nadirah M, Arief Z, Zahrol S, Tee LW, Ranzi AD, Amar AS, Laith AA, Mariam M, Suzana S and Aida RJ 2011. Antibacterial activity of Malaysian edible herbs extracts on fish pathogenic bacteria. Res J Med Plant. 5(6): 772-778.

O'Donovan L and Brooker JD 2001. Effect of hydrolysable and condensed tannins on growth, morphology and metabolism of *Streptococcus gallolyticus* (*S. caprinus*) and *Streptococcus bovis*. Microbio. 147: 1025-1033.

Pollard DR, Johnson WM, Lior H, Tyler SD and Rozee KR 1990. Detection of the aerolysin gene in *Aeromonas hydrophila* by the polymerase chain reaction. J Clin Microbiol. 28(11): 2477-2481.

Scalbert A 1991. Antimicrobial properties of tannins. Phytochem. 30: 3875-3883.

Smith AH, Zoetendal E and Mackie RI 2005. Bacterial mechanism to overcome inhibitory effects of dietary tannins. Microbiol Ecol. 50: 197-205.

Van Parys A, Boyen F, Dewulf J, Haesebrouck F and Pasmans F 2010. The use of tannins to control *Salmonella Typhimurium* infections in pigs. Zoonos Pub Health. 57: 423-428.

Yanong RPE and Floyd RF 2006. Streptococcal infections of fish. The institute of Food and Agricultural Sciences (IFAS) document, University of Florida, USA.