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Short Communication

In Vitro Efficacy of Red Kwao Krua (*Butea superba* Roxb.) Extract Against Streptococcal bacteria Isolated from Diseased Tilapia (*Oreochromis niloticus*)

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Abstract

Streptococcal infection is a major problem in intensive tilapia farming. This study was conducted to investigate the antimicrobial activity of mucilage, distilled water- and 70% methanol-crude extract from *Butea superba* Roxb. against Streptococcal bacteria. The antimicrobial activity using disc diffusion assay showed that *B. superba* Roxb. had a broad antimicrobial range against twelve isolates of streptococcal bacteria: *Streptococcus iniae* (n=2) and *Streptococcus agalactiae* (n=10). The minimum inhibitory concentration (MIC) of the 70% methanol-crude extract varied between 128 and 512 µg/ml depending on the tested streptococcal strains. Mucilage has a higher potency in the inhibition of streptococcal bacteria when compared with the 70% methanol-crude extract. However, distilled water-crude extract had no efficiency of inhibiting the growth of microorganism.

Keywords: *Butea superba* Roxb., disc diffusion assay, MIC, Streptococcal bacteria, tilapias

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บทคัดย่อ

ประสิทธิภาพของสารสกัดกวาวเครือแดง (*Butea superba* Roxb.) ต่อเชื้อแบคทีเรียสเตรปโตคอคคัสที่แยกได้จากปลานิลป่วย

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โรคติดเชื้อแบคทีเรียสเตรปโตคอคคัส ถือเป็นปัญหาหลักสำหรับการเลี้ยงปลานิลในเชิงอุตสาหกรรม การศึกษาครั้งนี้ทดสอบความสามารถในการต้านจุลชีพก่อโรคในปลานิลของน้ำยางกวาวเครือแดง (*Butea superba* Roxb.) สารสกัดด้วยน้ำกลั่น และ 70% เมทานอล สารสกัดกวาวเครือแดงมีความสามารถในการต้านจุลชีพสเตรปโตคอคคัส 12 เชื้อที่นำมาทดสอบได้แก่ สเตรปโตคอคคัส อินิเอ้ (n=2) และ สเตรปโตคอคคัส อะกาแลคติเอ้ (n=10) ด้วยวิธี disc diffusion assay สารสกัดจากกวางเครือแดงที่สกัดด้วย 70% เมทานอลมีค่าความเข้มข้นต่ำสุดที่สามารถยับยั้งการเจริญเติบโตของเชื้ออยู่ในช่วง 128 ถึง 512 ไมโครกรัมต่อมิลลิลิตร ขึ้นกับสายพันธุ์หรือสเตรนของเชื้อแบคทีเรียที่นำมาทดสอบ น้ำยางมีประสิทธิภาพในการยับยั้งการเจริญเติบโตเชื้อแบคทีเรียในห้องปฏิบัติการสูงกว่าสารสกัดกวาวเครือแดงที่สกัดด้วย 70% เมทานอล อย่างไรก็ตาม สารที่สกัดด้วยน้ำกลั่นไม่มีประสิทธิภาพในการยับยั้งการเจริญเติบโตของเชื้อที่นำมาทดสอบ

คำสำคัญ: กวาวเครือแดง disc diffusion assay ความเข้มข้นต่ำสุดที่สามารถยับยั้งการเจริญเติบโตของเชื้อจุลชีพ แบคทีเรียสเตรปโตคอคคัส ปลานิล

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Introduction

Bacterial diseases are still the major cause of infectious problem in an intensive tilapia culture worldwide including Thailand. It is generally accepted that streptococcosis is the most important bacterial diseases in tilapia farming causing a significant economic loss due to the high morbidity and mortality rates (Bromage et al., 2002; Pasnik et al., 2006). *Streptococcus agalactiae* and *S. iniae* are the species that were mostly reported (Evan et al., 2002). In Thailand, streptococcosis is mainly caused by *S. agalactiae* while *S. iniae* can be found in a few numbers (Suanyuk et al., 2008). Conventional method to prevent and to control the streptococcosis is the use of antibiotics. However, an extensive deposition of antibiotic residues in fish body and various environments is now a highly public concern (Miranda and Zemelman, 2001). The alternative methods such as probiotics, medicinal herbs or toxin binders, are ongoing developed.

Red Kwao Krua (*Butea superba* Roxb.), Thai medicinal plants in the family Luguminosae (Cherdshewasart et al., 2008), has been recently focused on its potential for the treatment of erectile dysfunction and maintaining sexual performance in mature male (Roengsumran et al., 2000). In aquaculture, many attempts to use Red Kwao Krua extracts as a natural synthetic hormone for musculinization or mono-sex production have been documented (Cagauan et al., 2004; Mengumphan et al., 2006). Red Kwao Krua's tuber contains several ingredients including flavonoid and flavonoid glycoside, which have antimicrobial potential against pathogenic fungi and bacteria (Yadava and Reddy, 1998). However, the information of an antimicrobial property of red kwao krua against pathogenic bacteria in fish is still limited. The inhibitory effect against streptococcus has never been elucidated as well. The objective of the present study was to determine the *in vitro* efficacy of Red Kwao Krua extracts against streptococcal bacteria from diseased tilapia.

Materials and Methods

Preparation of red Kwao Krua extraction: Red Kwao Krua tubers were prepared into three forms; mucilage and distilled water- and 70% methanol-extract. Fresh Red Kwao Krua tubers were transversally cut. Red mucilage was collected from its cut surface using sterile trip and kept at -20^{0}C before tested. To prepare the distilled water- and 70% methanol-crude extract form, the tuber roots of Red Kwao Krua were washed, sliced and minced into small pieces, dried at 60^{0}C and powdered. The dried powder was extracted by distilled water and 70 % methanol (8/10 w/v) for 24 hours at 4^{0}C. Supernatant was collected and filtrated

using 0.22 µm-disc filters. The filtrate was evaporated under reduced pressure at 30^{0}C. Sticky content was collected and kept at -20^{0}C until examined.

***Bacterial strain and culture medium*:** *Streptococcus iniae* (n=2), *Streptococcus agalactiae* (n=10) from laboratory collection of Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn were cultured on Tryptic soy agar (TSA) at 30^{0}C. All isolated bacteria were identified by the conventional biochemical method and confirmed by polymerase chain reaction (PCR) (Martinez et al., 2001).

Antimicrobial assay

Disc diffusion assay: The red Kwao Krua (B. superba Roxb.) solutions (mucilage, distilled water- and 70% methanol-crude extract) were prepared by adding distilled water to make a concentration of 1/2 (w/v). Sterile blank paper discs were placed on the Muller Hilton agar, which was inoculated with strains of streptococcal bacteria. Then 20 µl of each solution of Red Kwao Krua were applied on the paper discs. Plates were incubated at 30^{0}C for 24 hours and observed for their inhibition zone (Wei et al., 2008).

Minimal Inhibitory Concentration (MIC): Antimicrobial activity of different concentrations of Red Kwao Krua distilled water- and 70% methanol-crude extract was performed using agar dilution method as described in the guidelines of Clinical Laboratory Standard Institute (CLSI) (2009). Stock solution (10.24 mg/ml) of distilled water- and 70% methanol-crude extract was subjected to two-fold serial dilution in dilution tubes containing distilled water (DW) by CLSI. The concentrations of the compound obtained from the serial dilution ranging from 512 to 0.125 ug/ml were prepared in Mueller Hinton agar. All strains of tested microbe suspension with concentration of 10^8 CFU were determined by Mcfarlane. Plates were inoculated with 10-15 µl spots containing approximately 10^7 CFU of each organism, using a multipoint replicator. *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *P. aeroginosa* ATCC 27853 were use as the quality control organisms. Enrofloxacin was used for positive MIC control. After incubation at 37^{0}C for 24 hours, bacterial growth was determined by measuring colony appearance. The MIC was determined as the lowest concentration of distilled water- and 70% methanol-crude extract at which no colony on the agar plate was observed after incubation. The presence of one colony was disregarded. (CLSI, 2009)

Results and Discussion

In this study, the disc diffusion assay of *B. superba* Roxb. extract (70% methanol and distilled water) and mucilage form was evaluated against streptococcal bacteria. The results from disc diffusion assay are shown in Table 1. Both mucilage and 70% methanol-crude extract of Red Kwao Krua (*B. superba* Roxb.) had broad antimicrobial activity against streptococcal bacteria. Mucilage has a higher in potency than 70% methanol-crude extract. This might be suggested that the active inhibitory compounds were accumulated in mucilage more than other parts of *B. superba* Roxb. The main active ingredients of *B. superba* Roxb. are flavonoids and flavonoid glycosides with cAMP phosphodiesterase inhibitory activity (Roengsumran et al., 2000). *B. superba* Roxb. stem extract was able to inhibit several bacterial and fungal species due to the presence of a novel active compound, chemically called 3,5,7,3',4'-pentahydroxy-8-methoxy-flavonol-3-O-beta-D-xylopyranosyl(1-2)-alpha-L-rhamnopyranoside according to Yadava and Reddy (1998). However, Chukeatirote and Saisavoey (2009) reported that *B. superba* Roxb. tuber root extract using 100% methanol could not show the effective antimicrobial activity. Our study showed a different result. It might be due to different methods of extraction which our study used 70% (50 mg/ml) methanol extraction but Chukeatirote and Saisavoey (2009) used pure (100 mg/ml) methanol extraction. In addition, the geographical conditions including soil, sun light precipitation and air also influence the different chemical compositions of *Butea superba* Roxb.

Table 1 Antimicrobial activity of *B. superba* extract against streptococcal bacteria determined by the disc diffusion method.

Microorganisms	Inhibition zone			
	Control(DW)	Mucilage (mm.)	70% methanol-crude extract (mm.)	Distilled water-crude extract
S. agalactiae I	-	14	9.5	-
S. agalactiae II	-	15	9.75	-
S. agalactiae III	-	16	12	-
S .agalactiae IV	-	15	11.5	-
S. agalactiae V	-	13	10.25	-
S. agalactiae VI	-	13	9.75	-
S. agalactiae VII	-	13	10	-
S. agalactiae VIII	-	18	11.75	-
S. agalactiae IX	-	14.4	9.25	-
S. agalactiae X	-	14.8	10.25	-
S. inae I	-	16	12	-
S. inae II	-	16	11	-

- : No inhibition zone

Table 2. MIC of *B. superba* Roxb. crude extract (70 % methanol-crude extract) against various tested streptococcal bacteria.

Microorganisms	MIC (μg/ml)	
	70% methanol extraction	Enrofloxacin
S. agalactiae I	256	0.5
S. agalactiae II	256	0.5
S. agalactiae III	256	0.5
S. agalactiae IV	256	0.5
S. agalactiae V	256	0.5
S. agalactiae VI	256	0.5
S. agalactiae VII	256	0.5
S. agalactiae VIII	256	0.5
S. agalactiae IX	128	0.5
S. agalactiae X	> 512	0.25
S. inae I	128	1.0
S. inae II	256	0.5
S. aureus ATCC 25923	128	0.125
E. coli ATCC 25922	> 512	0.125
P. aeruginosa ATCC 27853	> 512	2.0

The minimum inhibitory concentrations of *B. superba* Roxb. crude extract (70%methanol extraction) was also evaluated. The result of the MIC test is shown in Table 2. The MIC of methanol extract varied between 128 and 512 μg/ml depending on the tested bacterial strains. The MIC of 70% methanol-crude extract was 128 μg/ml for *S. iniae* I, *S. agalactia* IX and *S. aureus*. Tested organisms that were inhibited at 256 μg/ml were *S. iniae* II and *S. agalactiae* I-VIII. Tested organisms that were inhibited at the concentration of more than 512 μg/ml were *S. agalactiae* X, *E. coli* and *P. aeruginosa* .

The present study revealed the antimicrobial activity of *B. superba* Roxb. over all streptococcal bacteria used in the study. The mucilage form and 70% methanol- crude extract exhibited the inhibitory properties against streptococcal bacteria from diseased tilapias. The mucilage form showed the superb inhibitory activities. MIC of the 70% methanol extraction showed bactericidal activity between 128-512 μg/ml for Gram-positive and >512 μg/ml for Gram-negative bacteria. The reason for different antimicrobial activities between Gram-positive and Gram-negative bacteria could be described to the cell wall morphological differences between these microorganisms. The Gram-positive bacteria are much susceptible. This could be explained that they have only an outer peptidoglycan layer which is not an effective permeability barrier. In contrast with Gram-negative bacteria, an outer phospholipidic membrane carries the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic solutes (Arias et al., 2004).

In conclusion, the crude extract of *Butea superba* Roxb. showed strong antimicrobial ability against aquatic pathogenic bacteria. Red Kwao Krua extracts might be an alternative herb applied for the prevention and control streptococcal diseases in sustainable tilapia culture.

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