The Effects of Heartwood Extracts from *Biancaea sappan* L. against Coagulase-Negative Staphylococci (CoNS) and *Staphylococcus aureus* Isolated from Subclinical Mastitis in Dairy Goats

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Abstract

Subclinical mastitis is an important disease affecting milk quality in dairy goats, and slow to detect because of non-clinical signs. Subclinical mastitis will progress to clinical mastitis which requires the treatments. The treatment mainly uses the antimicrobial intra-mammary infusion, and it may cause antimicrobial resistance problems. Thus, this study aimed to determine the change of milk composition and the antibacterial activity of ethanolic extracts from *Biancaea sappan* L. heartwood against bacteria isolated from milk of subclinical mastitis goats. Eighty-eight milk samples were classified into two groups; control (n=34) and subclinical mastitis group or SCM (n=54) according to California mastitis test (CMT) score and somatic cell count (SCC). The study showed that the percentage of lactose significantly decreased (*p*=0.002) in the subclinical mastitis goats, whereas the fat contents significantly increased (*p*=0.014). The changing of milk protein, solids not fat (SNF) and total solid (TS) contents were not observed. The bacterial identifications from milk samples revealed that the most commonly isolated bacteria were coagulase-negative staphylococci (CoNS) (n=41, 78.9%) and *Staphylococcus aureus* (n=7, 13.5%). The ethanolic extracts from *B. sappan* showed their antibacterial activity against isolated CoNS and *S. aureus* with minimum inhibitory concentration (MIC) range were 0.125-4 mg/ml and 0.125-1 mg/ml, respectively. These findings indicated that *B. sappan* ethanolic extracts potentially effected against major pathogens of subclinical mastitis in dairy goats.

Keywords: antibacterial activity, *Biancaea sappan* L., goats, sappan, subclinical mastitis

ประสิทธิภาพของสารสกัดหยาบจากแก่นฝางในการต้านเชื้อแบคทีเรีย coagulase-negative staphylococci (CoNS) และ *Staphylococcus aureus* ที่เพาะแยกได้จากโรคเต้านมอักเสบชนิดไม่แสดงอาการในแพะนม

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บทคัดย่อ

โรคเต้านมอักเสบชนิดไม่แสดงอาการเป็นโรคสำคัญที่ส่งผลต่อคุณภาพน้ำนมของแพะ และตรวจสอบได้ช้า เนื่องจากแพะไม่แสดงอาการของโรค โรคมักจะพัฒนาไปเป็นเต้านมอักเสบชนิดแสดงอาการซึ่งต้องการการรักษา การรักษานั้นจะใช้ยาต้านจุลชีพในรูปแบบสอดเต้านมเป็นหลัก และอาจก่อให้เกิดปัญหาการดื้อยาต้านจุลชีพ ดังนั้น การศึกษานี้มีจุดประสงค์เพื่อตรวจวัดการเปลี่ยนแปลงขององค์ประกอบสำคัญในน้ำนม และเพื่อทดสอบฤทธิ์ของสารสกัดด้วยเอทานอลจากแก่นฝาง ที่มีต่อเชื้อแบคทีเรียที่เพาะแยกได้จากน้ำนมแพะที่เป็นโรคเต้านมอักเสบชนิดไม่แสดงอาการ น้ำนมทั้งหมด 88 ตัวอย่างถูกแบ่งออกเป็น 2 กลุ่ม ได้แก่ กลุ่มควบคุม (n=34) และกลุ่มเต้านมอักเสบชนิดไม่แสดงอาการ (n=54) จากคะแนนการทดสอบด้วยวิธีแคลิฟอร์เนีย (CMT) และผล somatic cell count (SCC) ผลการศึกษาพบว่า ปริมาณของแลคโตสลดลงอย่างมีนัยสำคัญ (p=0.002) ในแพะที่เป็นโรคเต้านมอักเสบชนิดไม่แสดงอาการ ขณะที่ปริมาณไขมันเพิ่มขึ้นอย่างมีนัยสำคัญ (p=0.014) ส่วนปริมาณโปรตีน ของแข็งไม่รวมไขมันนม และเนื้อนมทั้งหมดไม่พบการเปลี่ยนแปลง การจำแนกเชื้อแบคทีเรียจากตัวอย่างน้ำนมพบว่า เชื้อส่วนใหญ่ที่แยกได้ คือ coagulase-negative staphylococci (CoNS) (n=41, ร้อยละ 78.9) และ *Staphylococcus aureus* (n=7, ร้อยละ 13.5) ซึ่งสารสกัดด้วยเอทานอลจากแก่นฝางมีฤทธิ์ต้านเชื้อแบคทีเรียชนิด CoNS และ *S. aureus* ที่เพาะแยกได้ โดยมีค่าความเข้มข้นต่ำสุดในการยับยั้งการเจริญเติบโตของเชื้อแบคทีเรียเท่ากับ 0.125-4 และ 0.125-1 มิลลิกรัม/มิลลิลิตร ตามลำดับ ผลการศึกษาดังกล่าวแสดงให้เห็นว่า สารสกัดด้วยเอทานอลจากแก่นฝาง มีฤทธิ์ต้านเชื้อแบคทีเรียสำคัญที่ก่อโรคของโรคเต้านมอักเสบชนิดไม่แสดงอาการในแพะนม ต้นฝางจึงเป็นพืชที่ควรนำมาศึกษาเพิ่มเติม เพื่อเป็นยารักษาทางเลือกของโรคเต้านมอักเสบในสัตว์เคี้ยวเอื้องขนาดเล็กในประเทศไทย

คำสำคัญ : ฤทธิ์ต้านแบคทีเรีย แก่นฝาง แพะนม องค์ประกอบน้ำนม เต้านมอักเสบชนิดไม่แสดงอาการ

Introduction

The mastitis is one of the most frequently diagnosed conditions in dairy goat farms which silently impact to the economic values of goat industry and human food safety (Turkmen 2017). Subclinical mastitis is usually underestimate because of non-typical clinical signs, which is slowly diagnosed and treated. The prevalence of subclinical mastitis approximately 15-53% in dairy goats that have the pathogenic bacteria colonized in the mammary gland (Gabli et al., 2019; Hussein et al., 2020). The predominant pathogen contaminated in dairy milk from small ruminants with subclinical mastitis was *Staphylococcus aureus* (Hussein et al., 2020; Merz et al., 2016). The other causative pathogens include coagulase-negative staphylococci (CONS), *Streptococci agalactiae, Streptococci* Group C, *Mycoplasma* spp. and *Corynebacterium pseudotuberculosis* (Nabih et al., 2018). In Thailand, CoNS and *S. aureus* are the most common pathogens associated with subclinical bovine mastitis (Suriyasathaporn 2011; Jarassaeng et al., 2012; Pumipuntu et al., 2019). But the incidence data of pathogenic bacteria in subclinical mastitis in dairy goat is still limited.

After subclinical infection, the subclinical mastitis usually progresses to clinical mastitis which the treatments are urgently need. The changes of milk quality and quantity arise consequently from inflammation of the mammary glands and tissues (Bossis 2018). During the intra-mammary infections, the somatic cell counts increased and the more oxidative stress on milk components happens, including the reduction of milk antioxidant capacity and enzymatic activity (Gelasakis et al., 2018; Novac and Andrei 2020). The common uses of antimicrobial agents in dairy goat farms may cause the increasing number of multiple antimicrobial resistant microorganisms. These problems will aggravate many losses in both live-stock farms and human public health (Yuan et al., 2017; Rana et al., 2020). Therefore, the development of alternative, cost-effective, and efficient natural agents is urgently required. The ethnoveterinary practice base on Ayurveda medicine is one of the alternative treatment of livestock diseases in animal husbandry systems for a long time (Boonmasawai 2012; Syakalima and Simuunza 2018) included the mastitis in ruminants (Mushtaq et al., 2018). Several plant extracts revealed the antibacterial activities on both clinical and subclinical mastitis such as *Atractylodis macrocephalae* (Xu et al., 2015), *Prosopis juliflora* (Shah et al., 2018) and *Brassica oleracea* (Sobrinho et al., 2019). Leaf extracts of *Ocimum sanctum* was also used as supportive therapy with ceftriaxone against chronic staphylococcal mastitis in goat (Dash et al., 2016).

Biancaea sappan L. (Sappan wood) belongs to Fabaceae family. Its heartwood extracts compose of many potential compositions include xanthone, coumarin, chalcones, flavones, and homoisoflavonoids. Brazilin is the major active compound found in the heartwood (Nirmal et al., 2015). The extracts from sappan tree express the high potential as anti-microbial agents, especially on *Staphylococcus* spp. (Zuo et al., 2014; Jun et al., 2015; Settharaksa et al., 2019). Thus, the study was aimed to evaluate the characteristics of major components and isolate the major pathogenic bacteria from subclinical mastitis dairy goat milks. Then the antimicrobial susceptibility and the antimicrobial activity of ethanolic extracts from *B. sappan* heartwood on isolated bacteria was determined. The potential of *B. sappan* as the antimicrobial agents of the mastitis treatment might be useful for reducing the economic loss of livestock farming system and antimicrobial resistance problem in the future.

Materials and Methods

Plant material and extraction

Biancaea sappan (L.) Tod. was obtained from Kanchanaburi Province, Thailand. The plant was identified and housed at Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University (PBM No. 005497-5499). The sappan heartwood was chopped into small pieces and dried at room temperature for 5 days, then macerated in 95% ethanol (1 kg per 1 liter) for 30 days at room temperature in the dark cabinet. The extract solution was filtered by using sterile gauzes and vaporized solvent by using a rotary evaporator (Büchi® Rotavapor R-205/Vacuum pump V-800) at 40°C and 175 mbar pressure. The crude extracts were dried out with the freeze-dry system (Labconco FreeZone® Freeze Dry) and crushed into the fine powder. The crude extracts were measured as the total weight and calculated the extracted yield by using the following formula before stored at -20°C until use.Extract yield (%) = W1/W2 x 100 where W1; weight of extracted plant residues and W2; weight of plant raw sample (Anokwuru et al., 2011).

Milk sample collection

The animal care and use protocol was approved by the Faculty of Veterinary Science-Animal Care and Use Committee (FVS-ACUC no. MUVS-2018-05-15). The milk samples (n=88) were randomly collected during lactation period from 2-5 years old Saanen or Saanen crossbred goats living in four dairy goat farms in Ratchaburi province, Thailand. All goats were not given any antimicrobial agents for 7 days before milk collection. Firstly, the udder cleaned by sterile water and discharged some foremilk. The milk samples of subclinical mastitis were determined by using the California mastitis test (CMT) (Giss marketing, Thailand). The CMT scores were justified in 0, trace, 1+, 2+ and 3+ according to the degree of milk precipitation or gelling formation (Souza et al., 2012; Abdalhamed et al., 2018). Then 10 ml of milk from each sample was collected in sterile tubes and immediately stored at 0-4°C before evaluating the somatic cell count (SCC), milk composition and bacterial isolation. The control goat milk might be CMT zero score. The 1+ and 2+ score samples were categorized as the SCM group. The samples from goat that showed clinical mastitis symptoms within 7 days after milk collections would be excluded.

The evaluation of milk composition and somatic cell count (SCC)

The milk composition was determined the levels of the fat, protein, lactose, solids not fat (SNF) and total solids (TS) by using the automatic milk analyzer (MilkoScan™) at Kanchanaburi Campus, Faculty of Veterinary Science, Mahidol University. The somatic cell was counted by flow cytometry technology (Fossomatic™). The SCC values > $1x10^6$ cells/ml and CMT ≥ 1+ were considered as SCM group. In addition, the quality grading of raw goat milk was categorized based on SCC according to Thai Agricultural Standard (TAS 6006-2008) as followed; premium = $7x10^5$ cells/ml, good = 0.7-$1x10^6$ cells/ml, standard = 1-$1.5x10^6$ cells/ml and poor > $1.5x10^6$ cells/ml (TAS 2008).

Bacterial isolation and identification

The collected milk samples from SCM group (n=54) were isolated and identified the pathogenic bacteria. Each sample was inoculated on Columbia sheep blood agar (Oxoid, UK), MacConkey agar and selective mannitol salt agar (Oxoid, US) and incubated at 37°C for 16-20h. The colony identifications based on Gram staining, routine phenotypic classification by light microscope and biochemistry methods were performed by the Center for Veterinary Diagnosis, Faculty of Veterinary Science, Mahidol University. The conventional method which include culture characteristic on selective media, *S. aureus* is capable of fermenting mannitol and Beta-hemolysis on sheep blood agar, gram-staining and biochemical reactions. Biochemical tests used to confirm *S. aureus* were coagulase test, catalase test, indole production, methyl red test, Voges-proskauer reaction, urease production, citrate utilization and sugar fermentation. The coagulase-negative staphylococci (CoNS) were confirmed by catalase, coagulase, oxidase, indole, methyl red, urease, VogesñProskauer, lecithinase production, mannitol, and glucose fermentation by the coagulase test (Bhattacharyya et al., 2016; Windria et al., 2016).

Antimicrobial susceptibility testing of isolated bacteria

Antimicrobial susceptibility tests and their interpretations of the isolated bacteria (n=51) from SCM groups were performed by disc agar diffusion technique according to the Clinical and Laboratory Standards Institute (CLSI) guideline (CLSI 2020). The 1.5x10^8 CFU/ml of bacterial suspension (0.5 McFarland) were streaked on Muller-Hinton agar (MHA) by sterile cotton swabs and incubated with the selected antimicrobial disks from several antimicrobial classes, including penicillins: ampicillin (10 μg) and oxacillin (1 μg); cephalosporins: cefoxitin (30 μg), ceftriaxone (30 μg), cefepime (30 μg) and ceftaroline (10 μg); fluoroquinolones: ciprofloxacin (5 μg) and enrofloxacin (30 μg); aminoglycosides: gentamicin (10 μg); tetracycline: tetracycline (30 μg); doxycycline (5 μg) and oxytetracycline (30 μg); macrolides: azithromycin (15 μg) and erythromycin (15 μg); glycopeptides:vancomycin (30 μg); lincosamides: clindamycin (2 μg); and sulfonamide/dihydrofolate reductase inhibitor: sulfamethoxazole/trimethoprim (1.25/23.75 μg). Then agar plates with antimicrobial disks were incubated at 35 ± 2°C for 16-20h. The inhibition zone diameter values were recorded (mm). The interpretation of antimicrobial susceptibility results was categorized as susceptible, intermediate and resistant following the breakpoint criteria recommended by CLSI.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of ethanolic extracts from sappan heartwood

The ethanolic extracts from sappan heartwoods were determined anti-microbial activity by using the broth microdilution method according to the CLSI guidelines (CLSI 2018). *S. aureus* ATCC®29213 was used as quality control strain in the MIC determinations. The bacteria colonies were suspended in 0.85% saline to obtain a 0.5 McFarland standard and then diluted by Muller-Hinton. The recommended inoculum size for broth microdilution susceptibility testing was 3x10^5 CFU/ml (Andrews 2001; Egervärn et al., 2007; Ross et al., 2015). The ethanolic extracts from sappan heartwood were diluted by two-fold serial dilutions to a final concentration of 16, 8, 4, 2, 1, 0.5, 0.25, 0.125 mg/ml. Each dilution of extracts was added into triplicate wells of sterile 96-well plate and incubated with bacteria at 37°C for 16-20h. Then resazurin solution (0.015%) was added to all wells (30 μl per well), and further incubated for 2h. The MIC was defined as the lowest concentration of plant extracts without any color change of blue resazurin (Elshikh et al., 2016).

The ceftriaxone was used as positive control to verify the sterility of procedure. After incubation time, the MBC was determined by transferring the contents of the MIC in each dilution to subculture on MHA and incubated at 37 °C for 16-20h (Ekhuemelo et al., 2019). Then the average of MIC and MBC and the geometric means of MICs were used to compare the susceptibilities of different organisms or the activities of different antimicrobial agents (Davies 1990; Hamilton-Miller 1991) by using SAS software, version 9.2 (Kaniga et al., 2016).

Statistical analysis

The data were statistically analyzed the normal distribution characteristics by the Kolmogorov-Smirnov test and Shapiro-Wilk test. The independent t-test was used to determine the significant difference of various milk component between groups. Furthermore, the differentiation of MIC and MBC were described as descriptive statistics and analyzed by Mann-Whitney U test. The IBM SPSS statistic version 21 was used for all data analysis. The significance statistical different was considered at $p < 0.05$

Results

The composition of milk and bacterial isolated from subclinical mastitis goats

The CMT scores of milk samples (n=88) from subclinical mastitis goats were 0 (17.1%: n=15), trace (21.6%: n =19), 1+ (38.6%: n =34) and 2+ (22.7%: n =20). The average SCC of control groups (n=34) was 1.6×10^6 cells/ml (Range: $0.4\text{-}4.3 \times 10^6$ cells/ml) and SCM groups (n=54) was 3.2×10^6 cells/ml (Range: $1.0\text{-}5.4 \times 10^6$ cells/ml). The levels of fat content in SCM groups were significantly higher than control group (p=0.014). While the lactose in the SCM groups was significantly lower (p=0.002). Whereas protein, SNF and TS were not significantly different between both groups (Table 1). The milk from SCM groups (n=54) were classified into poor (92.6% n=50) and standard level (7.4%: n=4) (Table 2). The average SCC of poor and standard level samples was 3.8×10^6 cells/ml (Range: $1.5\text{-}5.4 \times 10^6$ cells/ml) and 1.05×10^6 cells/ml (Range: $1.0\text{-}1.4 \times 10^6$ cells/ml), respectively. The bacteria could be isolated from 52 SCM milk samples. The number of bacteria isolations from the poor and standard quality milk was shown in Table 2. Only one standard milk sample was found isolated *S. aureus*. The highest number of isolated bacteria was CoNS, followed by *S. aureus*, Gram negative bacilli and Beta-hemolytic streptococci (Table 2).

The antimicrobial susceptibility of CoNS and *S. aureus* isolated from subclinical mastitis goats

The antimicrobial susceptibility of CoNS isolated from 52 SCM samples found that total 41 CoNS isolations were completely susceptible to oxacillin, cefepime, ceftaroline, ciprofloxacin, vancomycin and gentamicin (Table 3). Ampicillin was the most resistance antimicrobial of isolated CoNS (36.6%) and *S. aureus* (42.8%). Some CoNS (n=4) were resistant to more than 2 antimicrobial classes and one of them was resistant to cefoxitin. Only one CoNS isolation resisted to 4 antimicrobials from 3 classes (Beta-lactam: ampicillin, Tetracycline: tetracycline and oxytetracycline and Lincosamides: clindamycin). Whereas *S. aureus* isolation had high susceptibility to almost antimicrobials. The 4 of 7 isolations (57.2%) were resistant to ampicillin and one isolation (14.3%) was resistant to sulfamethoxazole-trimethoprim.

Antimicrobial effects of the ethanolic extracts from *B. sappan* heartwoods on CoNS and *S. aureus* isolated from subclinical mastitis goats

The MIC and MBC of the sappan heartwood extracts (% yield = 4.69) on *S. aureus* ATCC 25923 were 0.125 mg/ml and 0.25 mg/ml, respectively. The ethanolic extracts also showed the antimicrobial activities on CoNS (MIC range: 0.125-4 mg/ml) and *S. aureus* (MIC range: 0.125-1 mg/ml) isolated from subclinical mastitis goats. The MIC and MBC values of *B. sappan* extracts were summarized in Table 4 and 5. The range MBC/MIC ratio of CoNS was 1-8 and *S. aureus* was 1-4. These were not significantly different between MIC and MBC of isolated CoNS and *S. aureus*.

Discussion

The results of study revealed the changes of goat milk composition in subclinical mastitis goats. The lactose in milk collected from SCM group was lower than standard milk (Table 1). The reduction of lactose might result from the decreased secretory activities of the damaged mammary cells and the increased lactose utilization by bacteria after udder infection (Hassan 2013; Chhalgari 2014; Novac and Andrei 2020). Conversely from Gelasakis (2018), the fat content was significantly increased (Gelasakis et al., 2018). The increased fat content might be the consequence of the reduction of total milk volume after udder infection in subclinical levels (Bruckmaier et al., 2004; Leitner et al., 2004) include the increased levels of peroxides on fat (Silanikove et al., 2014). The protein, SNF and TS levels of control and SCM did not change as same as previous reports (Teleb et al., 2009; Hassan 2013; Silanikove et al., 2014). From this study, the major pathogens found in milk from SCM goats were CoNS (78.9%) and *S. aureus* (13.5%). Consistent with previous study in Italy, China, Malaysia and Kenya, the major pathogens were CoNS (20.7-95.9%) and *S. aureus* (4.1-18%) (Virdis et al., 2010; Zhao et al., 2015; Omar 2018; Mahlangu et al., 2018). By quality grading, the bacterial isolations could be isolated from the poor quality milk (SCC > 1.5×10^{6} cells/ml) more than standard quality milk (SCC = $1\text{-}1.5\times10^{6}$ cells/ml). The presence of bacteria in goat milk with subclinical mastitis might have the relationship with the increase of total SCC (Bagnicka et al., 2011) that refer to a large number of leukocytes and epithelial cells in goat milk. Elevated leukocytes were due to the non-specific immune responses to intra-mammary infection, mammary tissue injury and oxidative stress. Thus, the increased SCC was still used as the screening indicator of intra-mammary infection (Sharma et al., 2011; Alekish 2015). For CONS and *S. aureus* isolation and identification, the automated systems such as the Vitek 2 and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Becker et al., 2014) as well as various typing methods, such as pulse-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), SCCmec typing, and spa typing (Lakhundi and Zhang 2018) may recently use as the routine diagnostics for differentiation at the species level of CoNS and distinguishing between the species of the *S. aureus* complex (Kosecka-Strojek et al., 2019).

Manny previous study found that CoNS and *S. aureus* isolated from mastitis goats were mostly resistant to beta-lactam classes (Silva et al., 2004; Virdis et al., 2010; Alian et al., 2012). The report in Taiwan showed that most of *S. aureus* was resistant to ampicillin (more than 60%) (Chu et al., 2012). Likewise, the study in South India, the ampicillin resistance in CoNS and *S. aureus* were 69.6% and 71.4%, respectively (Preethirani et al., 2015). Similarly, the antimicrobial susceptibility of the CoNS and *S. aureus* isolates in this study also showed the markedly resistant to ampicillin. The erythromycin, penicillin, oxytetracycline, cephapirin and ceftiofur were antimicrobials that commonly used for mastitis treatment in ruminants (Garrett et al., 2015). While the commercial intra-mammary infusion products using for mastitis treatments in the goat farms in Thailand are the combination of penicillin-streptomycin, penicillin-neomycin and cloxacillin-ampicillin etc. The most antimicrobial combination consists of drugs in beta-lactam class. The injectable antimicrobial, especially the penicillin-streptomycin combination is also usually uses in goat farms. The overuse of these antimicrobials probably caused the problem of beta-lactam resistance (Kong et al., 2010). Interestingly, some CoNS (4 isolates) were resistant to more than two antimicrobial classes. These isolated bacteria might be considered as the multidrug resistance (MDR) characteristic. The one isolation among these CoNS was resistant to cefoxitin, which was the antimicrobial in reliable phenotypic method for detecting the methicillin resistance (MR) CoNS (Surase et al., 2015). Cefoxitin is a stronger inducer of the *mecA* gene of MR *S. aureus* (MRSA) and MR CoNS (Rostami et al., 2013; Johnson et al., 2014). Therefore, this isolation might be MR CoNS due to a surrogate marker characteristic of cefoxitin (Fernandes et al., 2005) and required the further evaluation. The sensitivity and specificity of cefoxitin by disk diffusion methods on MR CoNS were 91.8% and 94%, respectively. However, detection of the mecA gene by PCR is the gold standard method of MR CoNS determination (Bhatt et al., 2016).

The previous study showed the antibacterial activities of *B. sappan* ethanolic extracts on various bacteria including *S. aureus, S. epidermidis, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus* spp. (Nirmal and Panichayupakaranant 2015). From this study, ethanolic extracts of *B. sappan* heartwood harvested from Kanchanaburi Province, Thailand could inhibit the major pathogen: CoNS and *S. aureus* isolated from subclinical mastitis in dairy goats (MIC of CoNS: 0.125-4 mg/ml, *S. aureus*: 0.125-1 mg/ml). Whereas, the MIC of MDR CoNS (1 isolate) was 0.125 mg/ml. The concurrent study reported that the MIC of *B. sappan* ethanolic extracts against *S. aureus* isolated from human wound was higher (0.156-10 mg/ml) (Temrangsee and Itharat 2011). Thus, this result firstly demonstrated that *B. sappan* ethanolic extracts had antimicrobial activity for all isolated CoNS and *S. aureus* from SCM goats. The MBC/MIC ratio of *B. sappan* ethanolic extracts showed the bacteriostatic activity on isolated CoNS but the mechanism was not clearly known. The bactericidal activity on *S. aureus* was likely more effective to inhibit bacterial growth. The major active ingredient, brazilin from sappan heartwood extracts had bactericidal effect by inhibiting DNA and protein synthesis (Xu and Lee 2004).

In conclusion, this study found that SCM in dairy goats was an important status that effect on milk quality because of the changes of milk composition and must be consider for the appropriate treatments. The major pathogens in subclinical mastitis in dairy goats were CoNS and *S. aureus* with the high resistant to ampicillin. Furthermore, *B. sappan* ethanolic extracts expressed the antibacterial activities against both CoNS and *S. aureus* isolated from subclinical mastitis in dairy goats. Thus, the future development of *B. sappan* ethanolic extracts as intra-mammary preparation is suggested for solving the silent antimicrobial resistance problem in goat farm.

Table 1. The milk composition from control (n=34) and SCM group (n=54).

Milk composition	Average values (%)		*P*-value
	Control	SCM	
Fat	3.34	3.8*	($p = 0.014$)
Protein	3.02	2.94	
Lactose	4.09	3.75*	($p = 0.002$)
SNF	8.18	7.82	
TS	11.47	11.58	

Note*, significantly different compared with control group ($p < 0.05$); SNF, solids not fat; TS, total solids.

Table 2. The number of bacteria isolations from SCM group categorized according to the quality grading of raw goat milk.

Isolated bacteria	Number of bacterial isolations (%)		
	Standard (n=1)	Poor (n=51)	Total (n=52)
CoNS	-	80.4	78.9
S. aureus	100	11.8	13.5
Gram negative bacilli	-	3.9	3.8
Beta-hemolytic streptococci	-	3.9	3.8

Table 3. The antimicrobial susceptibility of CoNS (n=41) and *S. aureus* (n=7) isolated from SCM group (n=48).

Antimicrobials	The antimicrobial susceptibility (%)	
	CoNS	*S. aureus*
OX	100	100
FEP	100	100
CPT	100	100
CIP	100	100
VA	100	100
CN	100	100
FOX	97.6	100
CRO	97.6	100
ENR	97.6	100
DO	97.6	100
AZM	92.7	100
E	92.7	100
SXT	90.2	85.7
TE	87.8	100
OT	87.8	100
DA	82.9	100
AMP	36.6	42.8

Abbreviations are: OX, oxacillin; FEP, cefepime; CPT, ceftaroline; CIP, ciprofloxacin; VA, vancomycin; CN, gentamicin; FOX, cefoxitin; CRO, ceftriaxone; ENR. enrofloxacin; DO, doxycycline; AZM, azithromycin; E, erythromycin; SXT, sulfamethoxazole/trimethoprim; TE, tetracycline; OT, oxytetracycline; DA, clindamycin; AMP, ampicillin.

Table 4. The MIC of the ethanolic crude extracts from sappan heartwood on CoNS and *S. aureus* isolated from SCM group.

Isolated bacteria	MIC of sappan heartwood extracts (mg/ml)			
	MIC_{50}	**MIC_{90}**	**GM of MIC**	**MIC range**
CoNS	0.25	1	0.29	0.125-4
S. aureus	0.5	1	0.41	0.125-1

MIC_{50}, MIC requires to inhibit the growth of 50% of pathogens; MIC_{90}, MIC requires to inhibit the growth of 90% of pathogens; GM, Geometric mean.

Table 5. The MBC of the ethanolic crude extracts from sappan heartwood on CoNS and *S. aureus* isolated from SCM group.

Isolated bacteria	MIC of sappan heartwood extracts (mg/ml)			
	MBC_{50}	**MBC_{90}**	**GM of MBC**	**MBC range**
CoNS	0.5	1	0.61	0.125-4
S. aureus	0.5	2.2	0.61	0.125-4

MBC_{50}, MBC requires to inhibit the growth of 50% of pathogens; MBC_{90}, MBC requires to inhibit the growth of 90% of pathogens; GM, Geometric mean.

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