Antibacterial and anti-biofilm effects of a polyherbal formula and its constituents against coagulase-negative and -positive staphylococci isolated from bovine mastitis

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ABSTRACT
Concerns about antibiotic residues in milk and emergence of antimicrobial resistant pathogens necessitate exploration of alternative therapeutic strategies for the treatment of mastitis. This study aims to investigate anti-infective properties of a Thai traditional polyherbal formula, namely Ya-Sa-Marn-Phlae (YSMP), its herbal components (Curcuma longa, Areca catechu, Oryza sativa, and Garcinia mangostana), and representative chemical constituents (catechin, α-mangostin, and curcumin). Ethanol extracts of YSMP and G. mangostana, and α-mangostin exhibited potent antibacterial effects against Staphylococcus spp. isolated from mastitis cows with MIC values of 1–32 µg/mL. These tested agents inhibited biofilm formation of the isolates on both polypropylene (hydrophobic) and glass (hydrophilic) surfaces. The current study indicated that YSMP had strong antibacterial activity and anti-biofilm abilities against the tested isolates similar to that of α-mangostin and G. mangostana. The antibiofilm effects were confirmed with both scanning and transmission electron microscopes. The main abnormalities in the microstructure of the treated cells were the severe alterations of the cell wall with the formation of holes and morphological disorganization. Therefore, it might be proposed that G. mangostana is the major active component of YSMP and α-mangostin may be used as an active marker compound for YSMP to indicate its activity against bovine mastitis-isolated staphylococci.

1. Introduction
Mastitis is an inflammation of the mammary gland most often caused by bacterial intramammary infections. Based on bacteriological etiologic agents, mastitis can be categorized as contagious mastitis, mainly caused by Staphylococcus aureus and Streptococcus agalactiae and environmental mastitis, generally infected by Escherichia coli, Streptococcus dysgalactiae, and Streptococcus uberis (Gruet et al. 2001). This disease is considered one of the most significant causes of economic loss in the dairy industry due to reduced milk production, discarded milk, and additional inputs to reduce the level of mastitis (Hogeveen et al. 2011). Global economic losses due to mastitis are estimated more than US$ 2 billion per year (Huijps et al. 2008). The national economic loss in India due to mastitis was approximately US$ 2.6 billion annually (Joshi & Gokhale 2006), whereas in the United Kingdom and Northern Ireland the annual losses were £300 million and £14 million, respectively (Hillerton & Berry 2005). Although the uses of both disinfectant preparations and antibiotics to treat and prevent udder infections play a key role in bovine mastitis control, this method was cited as a major reason for milk contamination (Erskine et al. 2003). The presence of drug residues in milk could lead to the selection of antibiotic-resistant strains of bacteria (Sandegren 2014) as well as provoke allergic reactions in some hypersensitive individuals (Tan et al. 2009). According to the aforementioned concerns, effective alternative strategies are needed for controlling mastitis in dairy cows.

An increasing interest has emerged for therapeutic use of non-toxic traditional medicinal plants as alternative and inexpensive drugs for treating bovine mastitis. A few studies indicate that plant-derived compounds such as trans-cinnamaldehyde, eugenol, carvacrol, thymol, citral, and geraniol exhibited good results for inhibiting mastitis-causing pathogens (Ananda Baskaran et al. 2009; Aiemsaard et al. 2011). Ya-Sa-Marn-Phlae (YSMP) consists of Curcuma longa (rhizome), Areca catechu (seed), Oryza sativa (seed), and Garcinia mangostana (pericarp). This polyherbal preparation has been traditionally used for treatment of wounds and inflammatory skin conditions by topical application. Because of its in vitro-notable antibacterial activity against clinical isolates of S. aureus with minimum inhibitory concentration (MIC) value of 4 µg/mL, YSMP might be a potential natural antibacterial agent when used as a disinfectant (Chusri, Jittanon et al. 2013). An ethanol extract of YSMP had no cytotoxic effect and exhibited good anti-inflammatory and anti-oxidant activities. Its ethanol extract additionally possessed anti-biofilm activity against Staphylococcus epidermidis (Chusri, Settharaksa et al. 2013) and Pseudomonas aeruginosa (Chusri, Sompetch et al. 2013).
Table 1. Antibiotic susceptibility patterns of coagulase-positive staphylococci and coagulase-negative staphylococci isolated from bovine mastitis.

| Tested isolates          | AN  (30) | AM  (10) | E   (15) | GM  (10) | OX  (1) | P   (10) | RD  (5) | TE  (30) | VA  (30) |
|--------------------------|----------|----------|----------|----------|--------|----------|--------|----------|----------|
| Coagulase-positive staphylococci (CPS) |          |          |          |          |        |          |        |          |          |
| NPRC BCP531              | S        | S        | S        | S        | S      | S        | S      | S        | I        |
| NPRC BCP552              | S        | R        | R        | S        | I      | R        | I      | S        | I        |
| Coagulase-negative staphylococci (CNS) |          |          |          |          |        |          |        |          |          |
| NPRC BCNS31              | S        | S        | S        | S        | R      | S        | R      | S        | I        |
| NPRC BCNS19              | S        | R        | S        | S        | I      | R        | S      | S        | I        |
| NPRC BCNS20              | S        | S        | I        | S        | R      | S        | R      | S        | I        |
| NPRC BCNS21              | S        | S        | I        | S        | R      | S        | R      | S        | I        |
| NPRC BCNS22              | S        | S        | I        | S        | R      | S        | R      | S        | I        |
| NPRC BCNS27              | S        | R        | S        | S        | R      | S        | R      | S        | I        |
| NPRC BCNS28              | S        | R        | I        | S        | R      | S        | R      | S        | I        |
| NPRC BCNS29              | S        | R        | I        | S        | R      | S        | R      | S        | I        |
| NPRC BCNS40              | S        | S        | I        | S        | R      | S        | R      | S        | I        |
| NPRC BCNS43              | S        | R        | I        | S        | R      | S        | R      | S        | I        |
| NPRC BCNS44              | S        | R        | I        | S        | R      | S        | R      | S        | I        |
| NPRC BCNS45              | S        | S        | I        | S        | S      | S        | S      | R        | I        |

*AN = amikacin; AM = ampicillin; E = erythromycin; GM = gentamicin; OX = oxacillin; P = penicillin; RD = rifampicin; TE = tetracycline; VA = vancomycin.

| Tested agents          | MICs (µg/mL) | BCP531 (n = 2) | BCNS19 (n = 12) | S. aureus ATCC 25923 | S. epidermidis ATCC 35984 |
|------------------------|--------------|----------------|-----------------|----------------------|--------------------------|
| YSMP                   |              | 32             | 15–32           | 4                    | 7                        |
| A. catechu             |              | 250            | 250             | 250                  | 250                      |
| C. longa               |              | 250            | 250             | 32                   | 125                      |
| G. mangostana          |              | 7              | 7–15            | 3                    | 3                        |
| O. sativa              |              | >1000          | >1000           | >1000                | >1000                    |
| α-Mangostin            |              | 10             | 10              | 1                    | 10                       |
| Curcumin               |              | >250           | >250            | 32                   | 125                      |
| Catechin               |              | >250           | >250            | >250                 | >250                     |

In this study, we aimed to compare antibacterial effects of an ethanol extract of YSMP, ethanol extracts of its individual plant components, and their major representative components which were catechin (Wang et al. 1997), α-mangostin (Mahabusarakam et al. 1987), and curcumin (Arjo & Leon 2001) against coagulase-positive and -negative staphylococci isolated from subclinical and clinical mastitis cows in dairy farms in Phatthalung province, Thailand. Biofilm-forming ability of Staphylococcus spp. is considered to be a major virulence factor affecting their pathogenesis in mastitis. Therefore in vitro testing of the activity of effective agents on the staphylococcal isolates was additionally carried out.

2. Materials and methods

2.1. Plant materials and preparation of extracts

A selected herbal recipe, YSMP was first prescribed by Mr Somporn Chanwanisakul, a traditional Thai medical doctor at a traditional Thai medicine hospital, Prince of Songkla University, Hat Yai, Thailand. This recipe is traditionally used for wound healing and consists of C. longa (rhizome), A. catechu (seed), O. sativa (seed), and G. mangostana (pericarp). The medicinal plants were washed with distilled water, dried at 60°C overnight, and separately ground into fine powder. The formulation of YSMP consisting of 75 g of each herbal component was prepared as previously described (Chusri, Settharaksa et al. 2013). Powders (300 g) of each medicinal plants and the recipe were then separately soaked in 95% ethanol (1500 mL) for 7 days at 25°C. After filtrations through a Whatman No. 1 paper, the filtrates were concentrated using a rotary evaporator, and kept at 55°C until they were completely dry. Extraction yields (%; w/w) of YSMP, A. catechu, G. mangostana, and C. longa were 6.45, 9.88, 7.89, 0.46, and 3.54, respectively. Catechin (Sigma-Aldrich, USA), alpha-mangostin (Sigma-Aldrich, USA), and curcumin (Sigma-Aldrich, USA), were additionally tested as active chemical constituents of A. catechu, G. mangostana, and C. longa, respectively. Stock solutions at concentration of 200 mg/mL for dried ethanol extracts and 1 mg/mL for the active compounds were prepared by dissolving in 1 mL of dimethyl sulfoxide (DMSO) (Merck, Germany) and stored at −20°C.

2.2. Tested pathogens

In this study 14 isolates of Staphylococcus spp., S. aureus ATCC 25923, and a biofilm-positive strain, S. epidermidis ATCC 35984 were provided by the Department of Microbiology, Faculty of Science, Prince of Songkla University. The tested pathogens were isolated from milk samples of subclinical mastitis cows in dairy farms in Phatthalung province, Thailand in June-July 2010. The subclinical mastitis cases were considered when cows had bacteriologically positive milk samples with at least one the California Mastitis Test (CMT)-positive quarter (Ajariyakajorn et al. 2003). Colonies were cultured on blood agar plates and tentatively identified according to morphological features, pigment production, type of haemolysis produced, Gram staining, catalase test, and characteristic growth on mannitol salt agar (Oxoid, UK) which was used as a selective and differential medium for isolation and identification (Asfour & Darwish 2011). The coagulase test was performed with rabbit plasma and the results were recorded after 4 and 24 h of incubation at 37°C. S. aureus ATCC 25923 and a biofilm-positive strain, S. epidermidis ATCC 35984 were used for quality control. Antibiotic susceptibility test was performed by the disk diffusion method using a panel of antimicrobial agents, including amikacin (30 µg/disc), ampicillin (10 µg/disc),
erythromycin (15 µg/disc), gentamicin (10 µg/disc), oxacillin (1 µg/disc), penicillin (10 unit/disc), rifampicin (5 µg/disc), tetracycline (30 µg/disc), and vancomycin (30 µg/disc) (CLSI 2008). The antibiotics were purchased from Becton Dickinson Microbiology Systems (Sparks, MD, USA).

2.3. Determination of MIC

Cultures for experiments were prepared from 24 h Mueller-Hinton broth (MHB, Difco, France) and the suspensions were subsequently diluted with fresh MHB to achieve a bacterial culture.
concentration corresponding to 10^6 CFU/mL. MICs of the formula extracts were tested by the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method (2011). A 96-well sterile microtiter plate (Nunc, Denmark) was prepared by dispensing 100 μL of the inoculated broth plus an aliquot of 100 μL of twofold serial dilutions of the tested extracts, catechin, curcumin, or alpha-mangostin. The tested final concentrations of the extracts, catechin, curcumin, and alpha-mangostin in the microtitre plate were in the range of 15.6-1000, 3.9-250, 3.9-250, and 0.2–10 μg/mL, respectively. An aliquot of 100 μL of 1% DMSO was employed as a negative control. The plate was incubated for 24 h at 37°C and the bacterial growth was measured by recording the absorbance at 620 nm, using a microplate reader (Tecan Sunrise, Tecan Austria).

2.4. Time kill assay

Assays for the rate of killing bacteria by both the ethanol extracts and the active constituents were performed using a modified plating technique. Bacterial inoculum at concentration of 10^6 CFU/mL (1 mL) was mixed with 1 mL of MHB containing each tested agent at final concentrations of 0.5MIC, MIC, 2MIC, and 4MIC. The MICs of YSMP, A. catechu, C. longa, G. mangostana, α-mangostin, and curcumin against representative isolates, S. epidermidis ATCC 35984 were investigated (Chusri, Settharaksa et al. 2013). The bacterial cultures were diluted 200-fold in TSBGl, divided into 100-μL aliquots, and added into a flat-bottomed 96-well polystyrene microtiter plate (Nunc, Roskilde, Denmark). An aliquot of 100 μL of fresh TSBGl was used to exclude a possible contamination and serve as a blank control. All experiments were performed at least in triplicate. The biofilm-forming ability of the isolates could be classified into three groups (Taponen al. 2003), isolates that formed (i) fully established biofilm with >75% of the biomass of the positive control, (ii) moderately adherent biofilm with 25–75% biomass, or (iii) weak biofilm with <25% of the biomass of the positive control.

2.6. Anti-biofilm property

Effects of the recipe extract and its effective constituents on biofilm formation of coagulase-positive staphylococci NPRC BCPS031 and the biofilm-positive strain, S. epidermidis ATCC 35984 were investigated (Chusri, Settharaksa et al. 2013). The bacterial cultures were diluted 200-fold in TSBGl, divided into 100-μL aliquots, and added into a flat-bottomed 96-well polystyrene microtiter plate (Nunc, Roskilde, Denmark). An aliquot of 100 μL of YSMP extract (3.9–250 μg/mL), G. mangostana extract (3.9–250 μg/mL), or alpha-mangostin (0.2–10 μg/mL) prepared in TSBGl was subsequently added into the 96-well microtiter plate. After incubation at 37°C for 48 h, the effect of the tested agents on the biofilm mass of the pathogens was evaluated using a colorimetric assay, as described above. The wells containing the media and the tested agents were included as control. In parallel experiments, the bacterial growth after the treatment of these agents was additionally quantified using the microplate reader.

2.7. Their effects on bacterial cell morphology

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) images were taken to elucidate the morphology of bacterial cells. Coagulase-negative staphylococci cells (BCNS 18) were pretreated with the ethanol extracts of YSMP (15 μg/mL), G. mangostana (7 μg/mL), A. catechu (250 μg/mL), C. longa (250 μg/mL), and alpha-mangostin (10 μg/mL) for 18 h. The treated cells were harvested (5000 rpm for 5 min), washed twice with PBS pH7.4, and then prepared for the transmission electron microscope (TEM; JEM 100 CX II; JEOL, Japan) and SEM (5800LV, JEOL, Japan), as previously described (Chusri and Voravuthikunchai 2009; Chusri, Jitthanon et al. 2013).

### Table 3. Biofilm-forming ability of coagulase-positive staphylococci and coagulase-negative staphylococci isolates.

| Tested isolates | Biofilm production (OD<sub>492 nm</sub> ± SE) |
|-----------------|---------------------------------------------|
| NPRC BCNS018    | 0.793 ± 0.013                               |
| NPRC BCNS019    | 0.349 ± 0.002                               |
| NPRC BCNS020    | 0.143 ± 0.002                               |
| NPRC BCNS021    | 0.197 ± 0.007                               |
| NPRC BCNS022    | 0.178 ± 0.013                               |
| NPRC BCNS027    | 0.580 ± 0.009                               |
| NPRC BCP028     | 0.374 ± 0.016                               |
| NPRC BCP039     | 0.490 ± 0.005                               |
| NPRC BCP040     | 0.188 ± 0.005                               |
| NPRC BCP043     | 0.193 ± 0.014                               |
| NPRC BCP044     | 0.660 ± 0.016                               |
| NPRC BCP045     | 0.140 ± 0.008                               |
| S. epidermidis ATCC 35984 | 1.974 ± 0.003 |
3. Results and discussion

3.1. Anti-staphylococcal activity of YSMP and its components

Our ongoing research aimed to investigate the antibacterial properties of traditional herbal formulations and to analyse the activity of the individual compound or the herbal components in the formulations, with the aim of informing eventual quality control analysis of the formulation for use as medicines. We found that the ethanol extract of ‘YSMP’ possessed the ability to inhibit and eradicate human clinical isolates of *S. aureus*. This study further showed that the formula extract as well as the ethanol extract of *G. mangostana*, its medicinal plant component, showed excellent results for inhibiting coagulase-negative and -positive staphylococci isolated from bovine mastitis cases.

The majority of the tested isolates were coagulase-negative staphylococci. It may be because of coagulase-negative staphylococci were the predominant bacteria involved in bovine mastitis (Mordmuang & Voravuthikunchai 2015; Suriyasathaporn 2011).

The isolates of *Staphylococcus* used in this study were susceptible to amikacin, gentamicin, and tetracycline (Table 1). A multidrug resistant pattern, which meant that an isolate was resistant to three or more classes of antimicrobials, was not seen in all *Staphylococcus* spp. isolates, but 78% of the isolates were resistant to at least one drug. Most of the tested isolates were resistant to penicillin, oxacillin, and ampicillin which occurred in 50–71% of the tested isolates.

As shown in Table 2, the ethanol extracts of YSMP and *G. mangostana*, and α-mangostin exhibited a notable antibacterial effect towards all tested staphylococcal isolates with MIC values ranging between 1 and 32 µg/mL. The ethanol extracts of *C. longa* and *A. catechu*, and curcumins were less effective against the bovine pathogens (MIC values = 32 to >250 µg/mL) than the ethanol extracts of YSMP, whereas *O. sativa* ethanol extract and catechin did not show any antimicrobial activity (MIC values >1000 µg/mL). Exposures of BCPS, BCNS, and *S. epidermidis* to these agents at 0.5–4 times of their MIC caused bacteriostatic effects, whereas the extracts of YSMP and *G. mangostana* at 4x MIC were able to kill the tested pathogens at approximately 2–4 log reduction within 2 h (Figure 1).

As proposed by Cos et al. (2006) and Kuete (2010), MIC values of a plant extract should be below 100 µg/mL and below 25 µg/mL for plant-derived pure compounds. Based on those criteria, only YSMP and *G. mangostana* extracts can be categorized as a useful antibacterial agent, as they have developed MIC varying between 3–15 and 4–32 µg/mL, respectively. Although bovine mastitis-isolated staphylococci appeared to be more resistant to the tested agents than the reference strains, the MIC values of the disinfectants against coagulase-positive staphylococci was the same as that of coagulase-negative staphylococci. Similar results have been found by our group when testing the activity of the ethanol extract of YSMP against methicillin-resistant *S. aureus* (MRSA) and -susceptible *S. aureus* involved in nosocomial infections (Chusri, Jittanon et al. 2013). The results from MIC values showed that *C. longa*, *A. catechu*, curcumin, and catechin had the anti-staphylococcal activity approximately 10 times less than that of YSMP extracts. Thus, combining more than one plant may increase the effectiveness of this formula and this might be due to the synergistic interaction of the herbal components. This finding was also

![Figure 2](image-url)
similar to the previous ethnobotanical surveys indicated that traditional medical practitioners use a combination of more than one plant for treating diseases (Simbo, 2010; Neamsuvan et al. 2012). Although considerable information is available on the anti-staphylococcal properties of *G. mangostana* (Chomnawanget al. 2009), *C. longa* (Kim et al. 2005), and *A. catechu* (Zhanget al. 2009), those studies were carried out to evaluate antibacterial activity of the plants against human-isolated clinical strains of *Staphylococcus* spp. Similar to our study, ethyl acetate extract of *C. longa* (Kim et al. 2005), curcumin (Guneset al. 2014), and turmeric oil (Negi et al. 1999) were found to possess moderate antibacterial activity against MRSA (MIC = 100–1000 µg/mL). Our results are similar to that reported by Karphrom et al. (2009) as the ethanol extract of *A. catechu* also exhibited moderate anti-staphylococcal activity (MIC = 780 µg/mL), whereas *G. mangostana* extracts exhibited remarkable antibacterial effect on MRSA (MIC = 39–80 µg/mL) (Asai et al. 1995; Voravuthikunchai & Kitpipit 2005). α-Mangostin, the major constituent and most studied bioactive xanthone from this plant, had an activity against MRSA with MIC of 1.57–12.5 µg/mL (Asai et al. 1995). It is interesting to note that YSMP had strong antibacterial activities against the tested

Figure 3. Scanning electron micrograph of BCNS 18 after treated with four MIC of YSMP (b), *G. mangostana* (c), *A. catechu* (d), *C. longa* (e), and alpha-mangostin (f). BCNS 18 (a) was growth in TS8 used as a control. MICs of YSMP, *G. mangostana*, *C. longa*, *A. catechu*, and alpha-mangostin against BCNS 18 were 15, 7, 250, 250, and 10 µg/mL, respectively.
isolates similar to that of α-mangostin and G. mangostana. It might be proposed that pericarp of G. mangostana is the major active component in the formulation of YSMP. Alpha-mangostin may be used as an active marker compound for YSMP to indicate its activity against bovine mastitis-isolated staphylococci.

3.2. Biofilm inhibition potency of YSMP and its components

According to several reports, the biofilm-forming ability of Staphylococcus spp. is considered as an important virulence factor that facilitates the persistence of the pathogen in the udder, contributes to the evasion of host immunological defense, and impairs antibiotic therapy (Oliveira et al. 2006; Babra et al. 2013). Biofilm-forming ability of 14 isolates of Staphylococcus spp. isolated from mastitis cows is presented in Table 3. Most isolates were characterized as weak or non-biofilm producers, whereas coagulase-negative staphylococci NPRC BCNS018, 027, and 044 and coagulase-positive staphylococci NPRC BCPS031 were categorized as moderate biofilm producers.

Coagulase-positive staphylococci NPRC BCPS031 was the most efficient strain in terms of biofilm production, it was thus selected for assessment of anti-biofilm activity of YSMP, G. mangostana, and α-mangostin. Their effects on biofilm production are shown in Figure 2. The inhibitory effects of the agents on biofilm formation of coagulase-positive staphylococci NPRC BCPS031 and the biofilm producer, S. epidermidis ATCC 35984 (data not shown) had a similar pattern. At concentrations ranging from 3.9 to 250 µg/mL, both YSMP and G. mangostana extracts inhibited >50% of the biofilm formation of the isolates in a dose-dependent manner. At the tested
3.3. Morphological changes in coagulate-negative staphylococci on exposure to YSMP and its components

Regarding YSMP and G. mangostana extracts, SEM and TEM images demonstrated that the treated cells seemed to burst upon exposure, open holes, and deep craters in the bacterial cells and/or their envelope were found (Figures 3(b) and (c) and 4(b) and (c)). Treatment of staphylococci with A. catechu and C. longa extracts were slightly changed their cell morphology compared to untreated control (Figures 3(a) (d) and (e) and 4(a), (d) and (e)). The mechanism of action of YSMP and the interactions of its components are still unclear, but Koh et al. (2013) showed that α-mangostin may serve as a biological marker of YSMP directly affects cytoplasmic membrane of S. aureus. We showed for the first time that YSMP which is commonly applied for wound treatment possesses a potential as an alternative compound for the action against staphylococci involved in bovine mastitis.

4. Conclusion

The results presented in this article also indicate that G. mangostana is the major active component of YSMP and further investigation will be needed to correlate the activity of either YSMP, G. mangostana, or their constituents. More studies investigating the effects of this antibacterial substance in milk and in vivo mastitis model are therefore warranted and currently being carried out in our laboratory.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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