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**Antimicrobial Activities of Plant Extracts Against Bacteria
Associated with Bovine Mastitis**

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ABSTRACT

Bovine mastitis is mainly caused by bacterial infection of cow's udders, resulting in milk of lower quality. Antibiotic treatment of mastitis can leave antibiotic residues in milk and can increase antibiotic resistant bacteria in the food chain. The aims of this study were to investigate antimicrobial activities of selected natural extracts from traditional Thai plants against bovine mastitis-associated bacteria, in order to evaluate the possibility of using plant extracts as alternatives to antibiotics for bovine mastitis treatment and control. Rhizome and leaf plants (two types for each category) were chosen in this study, including *Curcuma mangga* (Mango ginger), *Zingiber montanum* (J. Koenig) Link ex A. Dietr. (Cassumunar ginger), *Azadirachta indica* A. Juss (Neem leaf) and *Piper betle* Linn. (Betel Pepper leaf). Antimicrobial activities of the aqueous and 70% ethanolic extracts of these plants were determined against 12 species of bovine mastitis-associated genera: *Staphylococcus* and *Streptococcus*, using disc diffusion, MIC and MBC assays. Extracts from *P. betle* and *C. mangga* showed inhibitory effects against the mastitis-associated bacterial isolates/strains tested. The 70% ethanolic extract of *P. betle* exhibited the highest antimicrobial activities, having the inhibition zone of 8.00 ± 0.00 to 24.33 ± 0.58 mm. The MIC and MBC values of the *P. betle* extract ranged from 0.07 to 1.17 and 0.29 to 9.38 mg/ml, respectively. *P. betle* extracts also had wide scope of inhibition against all bacterial species tested, and particularly displayed promising inhibitory effects against *Staphylococcus aureus*, one of

the most significant infectious and drug-resistant bacteria in dairy farming. These results will be used for further development of natural therapeutic products for bovine mastitis treatment and control, which is hoped to be alternative products to commercial antibiotics.

Keywords: Plant extracts, Bovine mastitis, Antibiotic resistance

INTRODUCTION

Mastitis is one of the most common diseases of dairy cows, which causes great concerns and great economic loss among dairy industries (Petrovski et al., 2006; Suriyasathaporn et al., 2012 and Hogeveen et al., 2011). The main cause of the disease is the infection in the cow udders and the formation and release of toxic substances from bacterial pathogens. The major mastitis pathogens, such as *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, and coliform bacteria, are usually considered more virulent and can cause greater damage to the udder than minor mastitis pathogens such as *Corynebacterium bovis* and coagulase-negative staphylococci (CNS) (Amin et al., 2011 and Cressier et al., 2011). Mastitis reduces milk quality, thus causes great economic loss to farmers. In addition, treatment of mastitis requires the use of antibiotics, which can leave antibiotic residues in milk and can increase dairy cattle reservoir for antibiotic resistant bacteria in the food chain. It adversely affects the environment, animal and human health, economy, and international food trade (Galal Abdel Hameed et al., 2006). In West Bengal, India, Gram-negative bacteria have been recently found to be resistant to antibiotics like β -lactams and tetracyclines (Das et al., 2017). A study conducted in China showed that fluoroquinolones and sulfonamides were respectively detected in 47.2% and 20.1% of milk from 10 provinces (Zheng et al., 2013).

Natural plant extracts are natural sources of antimicrobial agents and they are regarded as nutritionally safe and easily degradable (Berahou et al., 2007; Chika et al., 2007). The use of plant extracts and herbal therapy are considered an alternative way to reduce the problems of bovine mastitis and contamination of antibiotic residues in milk. For example, in British Columbia, Canada, mastitis is treated with *Achillea millefolium*, *Arctium lappa*, *Salix alba*, *Teucrium scorodonia* and *Galium aparine* (Lans et al., 2017). Mordmuang et al. (2015) demonstrated the antibacterial activity of *Rhodomyrtus tomentosa* leaf ethanolic extract against staphylococcal bovine mastitis isolates with MIC and MBC values ranged from 16–64 $\mu\text{g/ml}$ and 64–

128 µg/ml, respectively. Das et al. (2009) found that the methanol extract of *Spathodea campanulata* inhibited the growth of *S. agalactiae*, *S. uberis*, *E. coli* and *S. aureus* (Mubarack et al., 2011). Furthermore, many studies have also reported that traditional Thai plants had antimicrobial activities against different types of microbes, including bacteria pathogens (Hara-Kudo et al., 2004 and Mordmuang et al., 2015). However, the studies on the antimicrobial activities of traditional Thai plants against mastitis-causing bacteria were rare. Our study, therefore, was aimed to evaluate antimicrobial activities of selected Thai plants: *P. betle*, *A. indica*, *C. mangga*, and *Z. montanum*, against mastitis-associated bacteria. The results are expected to serve as a basis for development of a natural therapeutic product for mastitis control in dairy cows, which can be a safe alternative to commercial antibiotics.

MATERIAL AND METHODS

Bacterial strains

Field isolates and a reference strain of Staphylococcus and Streptococcus (12 in total) were used in this study. Staphylococcus isolates/strain included *S. epidermidis*, *S. aureus*, *S. capitis* subsp. *capitis*, *S. chromogenes*, *S. cohnii* subsp. *cohnii*, *S. cohnii* subsp. *urealyticus*, *S. haemolyticus*, *S. hominis* subsp. *novobioseptiae*, *S. sciuri*, and *S. simulans*. Streptococcus isolates included *Str. dysgalactiae* subsp. *dysgalactiae* and *Str. uberis*. The isolates were obtained from mastitis milk from a Dairy Farm in Chai Prakan, Chiang Mai, Thailand, apart from *S. aureus* which was a reference strain. The bacterial isolates/strain were deposited in the culture collection of the Food and Environmental Protection Laboratory of the Microbiology Division, Department of Biology, Chiang Mai University, Chiang Mai, Thailand. The bacterial isolates/strain were maintained on Trypticase soy agar (TSA) or glycerol.

Plants preparation

Four plant species used in this study (Table 1) were purchased from an organic local market of Chiang Mai, Thailand. The plant rhizomes and leaves were washed and rinsed with distilled water. After washing, they were cut into small pieces and dried overnight in a hot air oven at 55 °C. The dried plant materials of each plant species was grounded with a blender into the powdered form.

Extraction procedures

Water and 70 % (v/v) ethanol were used to prepare the extracts from the dried plant parts. A forty-gram portion of the fine powder from each sample was extracted with 200 ml distilled water and 70 % ethanol at room temperature (~23 °C) for 24 h, using shaking condition. The extract was filtered through Whatman filter paper No. 1 to attain a clear filtrate under vacuum at room temperature. The filtrates were concentrated using vacuum rotary evaporator and freeze-dried. The extracts were weighted, stored in small bottles at 4 °C and their yield percentages were calculated using the following formula: $\text{Extract yield\%} = R/S \times 100$; where R is the weight of extracted plants residues and S is the weight of the original sample.

The 70 % ethanolic extract of each plant species was dissolved in dimethyl sulfoxide (DMSO) to make a 300 mg/ml stock solution, while the aqueous extracts were dissolved in distilled water to the same concentration. The extracts were stored at 4 °C until use

Antimicrobial activity evaluation

Microbial culture preparation. Fresh 3-5 colonies of Staphylococcus and Streptococcus on TSA were inoculated in Mueller-Hinton broth (MHB) (Difco, USA) and incubated at 37 °C for 18 h. The bacterial suspensions were adjusted to approximately 10^8 cfu/ml, using McFarland turbidity standard No. 5.

Disk diffusion assay. The disk diffusion method was employed for screening of the antimicrobial activity of each plant extract. Briefly, a suspension of each bacterial test isolate/strain (10^8 cfu/ml) was swabbed on the surface of Mueller Hinton agar (MHA). The sterile filter paper discs (6 mm in diameter) was placed on the top of inoculated Mueller-Hilton agar plates and 10 µl of the 70% ethanolic and aqueous extracts from the stock solution (300 mg/ml) were loaded onto those sterile filter paper discs. Sterile distilled water was used as the negative control and Mastilex[®], an antibacterial drug (10 µl of 20 mg/ml), containing 35 mg/ml cephalexin monohydrate and 3.5 mg/ml gentamycin sulfate, was used as the positive control. The inoculated plates were incubated at 37 °C for 24 h. Antibacterial activities were evaluated by the diameter of inhibition zone (in millimeters) of the tested bacteria. All tests were performed in triplicate.

Minimal inhibitory concentration (MIC) and Minimal bactericidal concentration (MBC) determination. For initial screening, crude extracts that showed 100% inhibition (no visual growth of bacterial colonies compared to control) were further evaluated for minimum inhibitory

concentration (MIC) and minimum bactericidal concentration (MBC) values with the each of the 70% ethanolic and aqueous extracts according to Reller et al. (2009). In 96-well microtiter plate, 50 µl of MHB was added in each well. After adding 50 µl of the 70% ethanolic and aqueous extracts to the first well containing 50 µl MHB, two-fold serial dilutions were made to obtain final concentration ranging from 300 mg to 0.07 mg. The 50 µl portion of each bacterial suspension (10^8 cfu/ml) was added into each well. The 96-well microtiter plates were incubated at 37 °C for 18 h. Mastilex[®] was used as a positive reference. After incubation, the first well that had visually no growth of bacteria was determined for the MIC values. Then, the clear wells were selected and a 10 µl portion was dropped on TSA and incubated at 37 °C for 24 h. The MBC was defined from the concentration which yielded no colony.

RESULTS

Plant Extraction Yields

The percentage yields of the plant extracts were illustrated in Table 1. The 70% Ethanol extraction gave higher yields of the plant extracts than those resulted from the aqueous extraction.

Table 1. The yields of the aqueous and 70% ethanolic extracts of the plants used in this study

Scientific name	Common name	Plant part used	Extract yield (%)	
			Aqueous extract	70% Ethanolic extract
<i>Piper betle</i> Linn.	Betel Pepper	leaf	16.84	29.44
<i>Azadirachta indica</i> A. Juss.	Neem	leaf	20.68	25.12
<i>Curcuma mangga</i> Valetton & Zijp.	Mango ginger	rhizome	18.56	27.92
<i>Zingiber montanum</i> (J. Koenig) Link ex A. Dietr.	Cassumunar ginger	rhizome	20.2	33.96

Antimicrobial Activity Evaluation

The antimicrobial activities of the plant extracts were tested against mastitis-associated bacteria, using disk diffusion assay (Reller et al., 2009). From the 8 plant extract preparations (300 mg/ml), four preparations displayed inhibitory activities against all bacterial isolates tested. These four preparations included the aqueous extract of *P. betle* and the 70% ethanolic extracts of *P. betle*, *A. indica*, and *C. mangga*. The degree of inhibition varied depending on the bacterial isolates (Figure 1 and 2). The highest antibacterial activity was detected in 70% ethanolic extract of *P. betle*, having the average inhibition zone of 13.42 mm (calculated using data from 12 isolates tested), followed by the aqueous extract of *P. betle* (11.83 mm), *C. mangga* (10.28 mm), and *A. indica* (9.86 mm). The extracts from *Z. montanum* did not show an inhibitory activity against the mastitis-associated bacterial isolates tested.

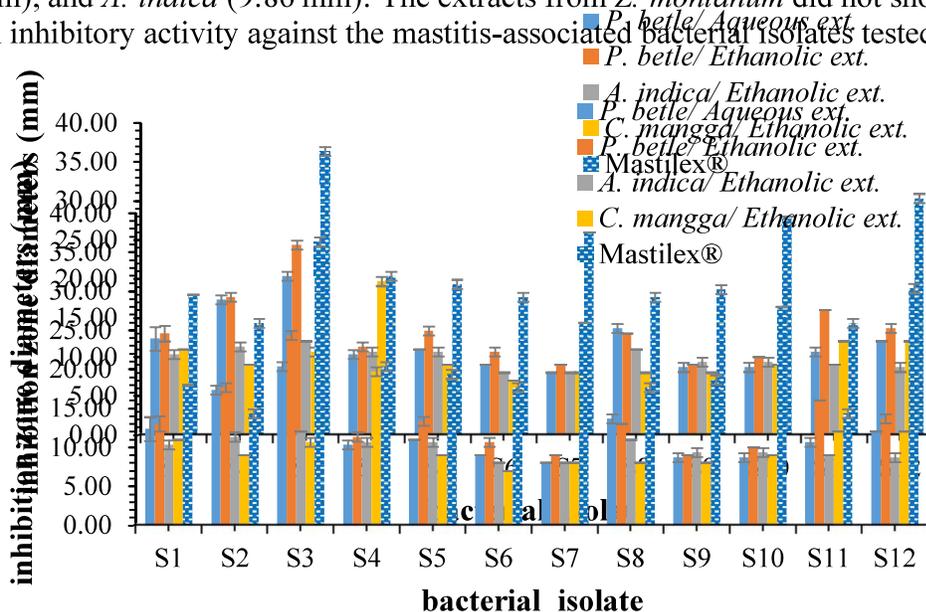


Figure 1. Inhibition zone diameters (mm) of the plant extracts against mastitis-associated bacterial isolates. S1: *S. epidermidis*, S2: *S. aureus*, S3: *S. capitis* subsp. *capitis*, S4: *S. chromogenes*, S5: *S. cohnii* subsp. *cohnii*, S6: *Staphylococcus cohnii* subsp. *urealyticus*, S7: *S. haemolyticus*, S8: *S. hominis* subsp. *novobiosepticus*, S9: *S. sciuri*, S10: *S. simulans*, S11: *Str. dysgalactiae* subsp. *dysgalactiae*, S12: *Str. uberis*

The plant extracts that were positive in the disk diffusion assay were determined for their MIC and MBC for each isolate. The results are shown in Table 2. These results indicated that all of the test isolates were most sensitive

to the 70% ethanolic extract of *P. betle*, having the MIC and MBC values ranged from 0.07-1.17 and 0.29-9.38 mg/ml, respectively. Interestingly, the antibacterial activities of *P. betle* and *C. mangga* ethanolic extracts against *S. aureus* were better than Mastilex[®] drug, a commercial antibiotic formula, used as the positive control.

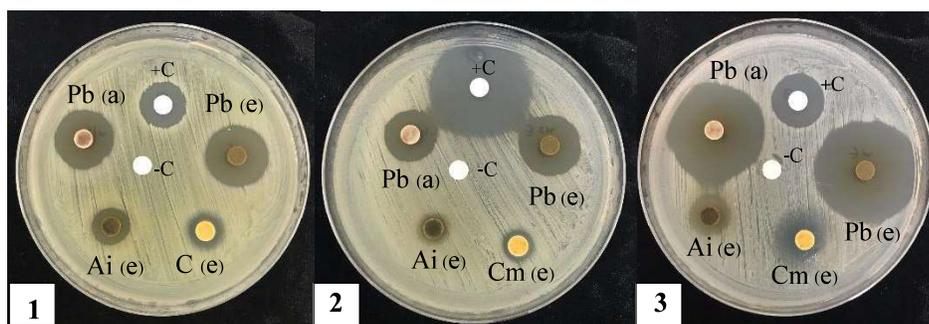


Figure 2. Inhibitory effects of plant extracts on some mastitis-associated bacterial strains. 1: *S. aureus*, 2: *S. haemolyticus*, 3: *S. epidermidis*; Pb (a): *P. betle* (aqueous extract), Pb (e): *P. betle* (ethanolic extract), Ai (e): *A. indica* (ethanolic extract), Cm (e): *C. mangga* (ethanolic extract), -C: water (Negative control) and +C: Mastilex[®] (positive control)

Table 2. MIC and MBC of plant extracts against mastitis-associated bacteria

Test isolate	MIC and MBC of samples (mg/ml)					
	<i>P. betle</i>				<i>C. mangga</i>	
	aqueous extract		70% ethanolic extract		70% ethanolic extract	
	MIC	MBC	MIC	MBC	MIC	MBC
S1	2.34	9.38	1.17	4.69	1.17	4.69
S2	1.17	9.38	0.29	1.17	2.34	18.75
S3	0.59	2.34	0.07	0.29	2.34	4.69
S4	4.69	18.75	1.17	9.38	9.38	18.75
S5	1.17	9.38	1.17	4.69	4.69	9.38
S6	4.69	18.75	2.34	9.38	9.38	9.38
S7	2.34	9.38	1.17	4.69	9.38	37.5
S8	2.34	9.38	1.17	4.69	9.38	37.5
S9	4.69	18.75	1.17	4.69	9.38	18.75
S10	4.69	18.75	1.17	9.38	4.69	4.69
S11	0.59	2.34	0.07	0.29	0.59	2.34
S12	0.59	4.69	0.07	0.29	1.17	2.34
S1	1.17	4.69	37.5	150	0.15	1.17
S2	2.34	18.75	37.5	150	2.34	18.75
S3	2.34	4.69	18.75	75	<0.07	<0.07
S4	9.38	18.75	18.75	75	0.29	1.17
S5	4.69	9.38	4.69	8.75	0.07	0.59
S6	9.38	9.38	37.5	150	0.07	0.29
S7	9.38	37.5	37.5	150	0.59	4.69
S8	9.38	37.5	18.75	75	<0.07	0.15
S9	9.38	18.75	37.5	50	0.29	2.34
S10	4.69	4.69	37.5	150	0.15	0.59
S11	0.59	2.34	0.59	1.17	0.59	2.34
S12	1.17	2.34	0.15	0.59	0.15	1.17

DISCUSSION

This study investigated the antibacterial potential of the different plant extracts against bacteria associated with bovine mastitis. Interestingly, we found that the aqueous and 70% ethanolic extracts of *P. betle* had antibacterial activities against all of the bacterial isolates/strains tested. The MIC and MBC values of *P. betle* ethanolic extracts ranged from 0.07-2.34 and 0.29-9.38 mg/ml, respectively. Other studies demonstrated many bioactive components were present in the leaf of *P. betle*. Examples of the bioactive compounds were tannins, anthraquinones, flavonoids, alkaloids, terpenoids, saponins, cardiac glycosides, glycosides, phlobatanins, hydroxychavicol acetate, isoeugenol, allylpyrocatechol piperbetol, anethole, stearic acid, methyl eugenol, carvacrol, chavicol, and allylpyrocatechol (Dwivedi et al., 2014; Rekha et al., 2014). Some of these compounds have antimicrobial effect on some bacteria. Taukoorah et al. (2016) showed that the ethylacetate (EAE) and acetone (ACE) extracts of *P. betle* leaves gave the highest antimicrobial potential by inhibiting the growth of many bacterial species, such as *Staphylococcus aureus*, *S. epidermidis*, *S. pyogenes*, *Escherichia coli*, and *Pseudomonas aeruginosa*. As indicated by the correlation analysis, the high antibacterial activities of *P. betle* leaf can be attributed to the total phenolic contents of both EAE and ACE extracts as well as the high flavonoid contents.

The aqueous and the ethanolic extracts of *P. betle* leaves had slight differences in their antibacterial activities. The differences between the activities of the aqueous and ethanolic extracts of *C. mangga* and *A. indica* were more obvious. While 70% ethanolic extract of *C. mangga* and *A. indica* had mild antibacterial activities, their aqueous extracts had no antibacterial activity at all. Solvents of varied polarities have been reported to influence the amount of phytochemicals retrieved from plants. Polar solvents (such as water and ethanol) and medium-polar solvents (such as ethyl acetate, acetone, and dichloromethane) will yield different amount of bioactive compounds. For example, the phenolic contents of *Bridelia retusa* Spreng. were highest when extracted with acetone (Tatiya et al., 2011). In our study, we limited the types of solvents used, because the plant extracts or their active components are intended for use as a safe, natural alternative antibacterial agent in dairy cattle.

As for *Z. montanum*, the aqueous and ethanolic extracts had no antibacterial activity against *S. aureus*, coagulase-negative Staphylococci, and Streptococci associated with bovine mastitis. These results were in agreement with the work of Kamazeri et al. (2012), in which *Z. cassumunar* oil is reported to display weak or no antimicrobial activity. It failed to inhibit the growth of

the gram-positive *S. aureus*, gram-negative *E. coli*. It has been reported that the dichloromethane (DCM) and methanol extracts of *Z. cassumunar* rhizome and root were also inactive against *E. coli* and *C. albicans* (Habsah et al., 2000). *Z. montanum* oil, however, is known to have anti-inflammatory effect (Jeenapongsa et al., 2003) and the use of this medicinal plant for mastitis therapy may employ its anti-inflammatory characteristics rather than the antimicrobial properties.

It is interesting to observe, from the MIC values, that the antibacterial activities of the aqueous and 70% ethanolic extracts of *P. betle* could be better in suppressing some bacterial species than the antibiotic drug (Mastilex[®]) at the concentration used. Their effect on *S. aureus* and *Str. dysgalactiae* were especially worthy to note. Because, *S. aureus* and *Str. dysgalactiae* are one of the most common mastitis pathogens causing contagious and environmental mastitis (Saidi et al., 2013). They are associated with clinical and sub-clinical bovine mastitis. Recently, *S. aureus* has been recognized as facultative intracellular pathogen by its abilities to produce adherence factors and toxins such as endotoxin, exotoxins, and α -toxin (Atalla et al., 2010). Intramammary infection of *S. aureus* often causes tissue damage and inflammatory process in host (Otto, 2014 and Wall et al., 2005). Moreover, it is also known as one of the most important agents in food borne diseases (Braga et al., 2005). Thus, the extracts from *P. betle* leaves which showed the antibacterial activities against *S. aureus* and *Str. dysgalactiae* can potentially be used as a natural alternative to antibiotics to prevent or control mastitis in dairy cattle. The therapeutic or antibacterial products are being developed.

CONCLUSION

This study demonstrates that the extracts of some traditional Thai plants, especially of *P. betle* leaves, possess the antimicrobial activities against major bacterial species associated with bovine mastitis. The aqueous and 70% ethanolic extracts of *P. betle* leaves can be used for further development of natural products for mastitis treatment and control in dairy cows. Since the extensive and excessive uses of antibiotics have caused many negative effects on dairy cattle's health, the environment, and food safety, use of potential antibiotic substitutes such as *P. betle* extracts can offer an alternative approach to sustainable, organic, or environmental-friendly farming.

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