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Antimicrobal Activity of Ethnotraditional Herb Extracts against Coagulase-Negative Staphylococci (CNS) Isolated From Dairy Cows with Mastitis in Lopburi Province, Thailand

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ABSTRACT

This study aimed to investigate the efficacy of traditional herb extracts on inhibition of coagulase-negative staphylococci (CNS) isolated from dairy cows with mastitis. Milk samples were collected and diagnosed subclinical mastitis by California mastitis test (CMT), then followed by CNSisolation and identification. Leaves herbal plants, Syzygium cumini, Millingtonia hortensis, and Zizyphus mauritiana were extracted with water or 95% methanol. Crude extracts were analyzed phytochemical compounds and determined the effectiveness of CNS inhibition using the disc diffusion technique, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Seventeen isolates of CNS were recovered. Aqueous extracts of S. cumini and methanol extracts of Z. mauritiana showed the most pronounced inhibition zone on the tested CNS isolates, with a range of 14.3 to 21.0 mm and 14.0 to 20.0 mm, respectively. The MIC and MBC of all extracts ranged from 1.56 to 12.5 mg/ml. However, the aqueous extracts of S. cumini leaves and the methanol extracts of Z. mauritiana leaves showed strong antimicrobial effects (1.56 mg/ml) against CNS isolated from bovine mastitis milk samples. The ethnotraditional herb extracts of S. cumini and Z. mauritiana leaf could be effectively used against bovine mastitis CNS bacteria and their antibacterial activity were confirmed by phytochemical compounds presence.

Keywords
Dairy cows, Coagulase-negative staphylococci, Herb; Antibacterial activity, Mastitis

INTRODUCTION

Dairy cows are an important economic animal in Thailand. Lopburi, a province in the central part of Thailand, is one of the top three regions for raw milk production in the country. Mastitis in dairy cows continues to be the most costly disease in the dairy industry. Thai dairy farmers suffer from increasing production costs and loss of income [1]. Mastitis is caused mainly by a bacterial infection. Coagulase-negative staphylococci*(*CNS) are one of the most frequently isolated groups of bovine mastitis-causing bacteria [2].These bacteria are of great interest because they are currently the most commonly isolated microorganisms in cows and heifers in herds, and are currently considered emerging pathogens of bovine mastitis [3]. Although CNS infections are usually mild or subclinical, they can cause more severe and persistent issues, causing an increase in somatic cell counts and a decrease in milk quality and production. Therefore, controlling subclinical mastitis can reduce the losses in milk production substantially**.**

CNS produce biofilms, a group of cells that adhere to a surface, and are frequently embedded within a self-produced matrix of an extracellular polymeric substance that causes a dramatic decrease in susceptibility to the antimicrobial agents**.** This formation is considered an important virulence factor that is frequently associated with clinical infections [4]. Subclinical and clinical cases of mastitis are routinely treated with antibiotics; however, treatments are not always effective. The use of antibiotics over long periods has triggered the development of multidrug-resistant strains, which has resulted in the use of increasing doses of antibiotics, risking increasing amounts of drug residues in milk [5].

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Herbal extracts have increased widespread interest in the search for alternative antimicrobial agents because natural products of higher plants may be a new source of antimicrobial agents with possibly novel mechanisms of action [6]. Some secondary metabolites such as tannins, alkaloids, and flavonoids in herbal plants have been found *in vitro* to have antimicrobial properties [7-9]. Several reports also reported the antimicrobial activity of different herbal extracts [10-13]. Phyotherapy manuals contend that various natural plants able to treat infectious diseases have fewer side effects and low toxicity [14].
Syzygium cumini (family Myrtaceae) is known as Java plum. It has been used in folk medicine to treat dysentery, bloody diarrhea, and skin diseases [15]. *Millingtonia hortensis* belongs to family Bignoniaceae. The leaves have carotene, dinatin, and hispidulin which are antimicrobial [15]. *Zizyphus mauritiana* (family Rhamnaceae), known as jujube, has been reported to have antimicrobial activity against the staphylococci group [15]. Therefore, this study investigated the antimicrobial activity of aqueous and methanol extracts of leaves from these three traditional herbs against CNS isolated from subclinical bovine mastitis. Effective microbial action of these extracts may contribute to reduction in the use of antibiotics.

MATERIALS AND METHODS

2.1 Sample collection and subclinical mastitis diagnosis

Sixty milk samples were collected from a dairy farm in Phatthana Nikhom District, Lopburi province, Thailand. The California mastitis test (CMT) was carried out on the samples as a screening test to diagnose the presence of subclinical mastitis in the collected samples**.** A few drops of milk from each quarter of the udder were placed in each of four cups in the CMT paddle and an equal amount of the reagent was added. Positive samples showed gel formation within a few seconds after a gently circular motion. The result was scored based on the gel formation as positive or negative; the cow was considered to have mastitis if one or more of the quarters were CMT positive. Thirty-two positive samples from subclinical bovine mastitis cases was further used for isolation of CNS isolation

2.2 Isolation and identification of CNS

A loop of milk sample was streaked onto plates of nutrient agar media and incubated at 37°C for 24–48 h. Suspected colonies were picked and examined microscopically in Gram stained films before being transferred to semisolid agar to be subjected to further identification, including the Gram stain procedure and catalase testing to distinguish between *Streptococci* and staphylococci. Standard protocols for mannitol fermentation, the coagulase test and DNase reaction were used to identify CNS.

2.3 Plant collection

Leaves of *Syzygium cumini*, *Millingtonia hortensis* and *Zizyphus mauritiana* (Fig. 1) were collected from Phatthana Nikhom District, Lopburi, in June 2018. The leaves were washed with distilled water, dried in air at room temperature, chopped into small pieces and subjected to extraction.

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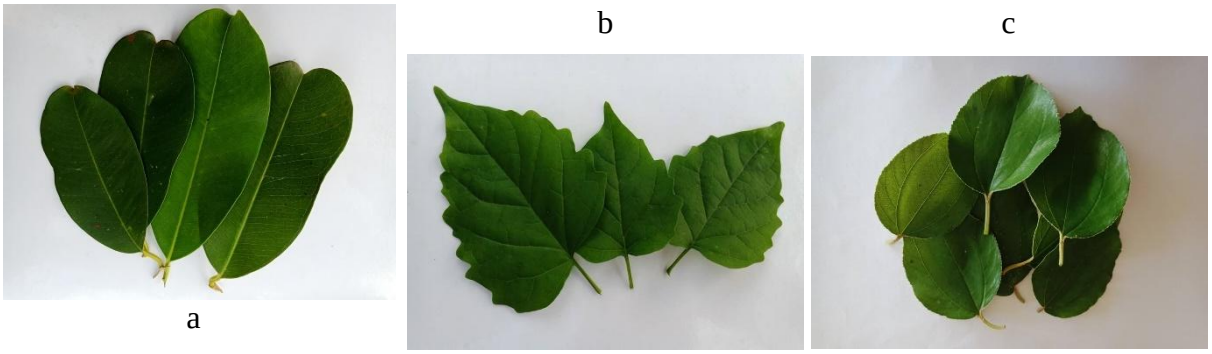

Figure 1. Fresh leaves of *Syzygium cumini* (a), *Millingtonia hortensis* (b) and *Zizyphus mauritiana* (c)

2.4 Crude extraction

Extraction from the leaf pieces was carried out using one of twosolvents: water or 95% methanol. The leaf pieces (200g) were placed in water or methanol (400 ml) and incubated at room temperature for 3 days. The extract was filtered through eight layers of muslin cloth**.** The extract was dried by distilling off the solvents under pressure using a rotary evaporator [16].

2.5 Phytochemical screening

The aqueous and methanol extracts of the employed herbs were qualitatively screened for some bioactive phytochemical constituents such as alkaloids, anthraquinones, coumarins, cardiac glycoside, flavonoids, phenols, saponins, steroids, tannins, and terpenoid by using standard procedures [17]. These phytochemicals were identified by characteristics colour change using standard procedures.

2.6 Antimicrobial plant extract assays

A modified disc diffusion method was used to determine the antimicrobial activity of plant extracts, as described in the guidelines of the National Committee for Clinical Laboratory Standards [18]. Mueller-Hinton agar was prepared as per the manufacturer's instructions and checked for sterility by incubating the plates overnight at 37°C. Modified discs (6 mm diameter) were prepared using a Whatman filter paper. 100 discs were punched, put in vials and sterilized in an oven at 170°C for 30 min. The discs were then impregnated with 20 µl of a single 200 mg/ml plant extract. The discs were dried at 37°C for 24 h. The prepared discs containing the various extracts were placed on the $1\boldsymbol{0}^{8}$CFU/ml CNS-inoculated plates using sterilized forceps according to Wiegand et al [19]. The disc with solvent alone was the negative control and an antibiotic disc (ampicillin) was the positive control. The plates were incubated at 37°C for 24 h. The zone of growth and inhibition of the isolates by the test extracts was recorded. Antimicrobial activity was evaluated by measuring the diameter of the inhibition zone around the disc as mean ± standard deviation (SD) of the triplicates of each condition.

2.7 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC of *S. cumini*, *M. hortensis* and *Z. mauritiana*extracts against all of the CNS isolates were determined by the broth dilution assay described by the National Committee for Clinical Laboratory Standards[18]**.** The MIC was defined as the lowest concentration of the

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compound to inhibit the growth of microorganisms**.** The 96-well plates were prepared by dispensing 50 µl of Mueller-Hinton broth into each well**.** 50 µl of extract ***(***200 mg*/*ml***)*** was added into the first row of the plate and two-fold serial dilutions were performed, to a final concentration range of 100–0**.**195 mg*/*ml. 10 µl of CNS inocula (adjusted to approximately 1 × 10^8 CFU/ml) was added**.** Plant extract with media was used as a positive control and inocula with media was used as a negative control**.** Microtiter plates were incubated at 37°C for 24 h**.** Lack of turbidity (indicating growth inhibition) was further confirmed by pouring a 100 µl suspension aliquot onto pre-sterilized nutrient agar plates**.** The lowest concentration of the treatment that prevented the growth of the organism after subculture on tryptic soy agar (TSA) following serial dilution and plating was taken as the MBC. Triplicate samples were included for each treatment.

2.8 Statistical analysis

All data were subjected to statistical analysis including the calculation of the mean and standard deviation (SD) of the triplicate samples of each condition. Significant differences between the means of different extracts with the same CNS isolate were evaluated using one-way analysis of variance ***(***ANOVA***)***, followed by post-hoc analysis ***(***Duncan**'**s test***)*** using the statistical package SPSS 22.0**.** The significance level was $p < 0.05$**.**

RESULTS AND DISCUSSIONS

The ethnotraditional data of the employed herbs and their extract percentage yield are illustrated in Table 1**.** The extract of 200g of dried plant leaves with water and methanol yielded plant extract residues ranged from 2**.**3 to 6**.**1% and 4**.**4 to 13**.**5%, respectively**.** The highest yield of plant extract was obtained from methanol *M. hortensis*extract ***(***13**.**5%***)*** followed by methanol *Z. mauritiana* extract ***(***9**.**5%***)*** while aqueous *S. cumini* extract gave the lowest extract yield**.** Phytochemicals (secondary metabolites) screening of aqueous and methanol leaf extracts revealed the presence of coumarins, cardiac glycoside, flavonoids, phenols, saponins, steroids, and tannins (Table 2). Alkaloids and terpenoids were detected in *S. cumini* extracts and anthraquinones were detected in aqueous *S. cumini* extracts

Thirty-two bacterial isolates from seven subclinical mastitis milk samples were Gram-positive and catalase test-positive (staphylococci). Seventeen of these isolates were CNS, classified as positive coagulase reaction, positive mannitol fermentation and negative DNase test (Table 3). All aqueous and methanol extracts of *S. cumini, M. hortensis,* and *Z. mauritiana* showed antimicrobial activity against isolated CNS (Table 4). Of the three herb extracts in this study, aqueous extracted *S. cumini* and methanol extracted *Z. mauritiana* showed the most pronounced activity with inhibition zones on all isolates of more than 14.0 mm (14.0–21.0). The others showed moderate activity with inhibition zones ranging from 7.3 to 17.3 mm. MIC and MBC of all extracts ranged from 1.56 to 12.5 mg/ml. The aqueous *S. cumini* extracts and methanol *Z. mauritiana* extract had the best MIC and MBC at 1.56 mg/ml (Table 5).

This study identified 17 CNS isolates from a total of 32 staphylococci isolates from bovine mastitis samples. CNS have been identified as the most frequently isolated bacteria causing bovine mastitis [2].CNS are normally found on the healthy skin of the nipple. They are often called opportunistic microorganisms because they live in areas where it is easy to colonize the teat canal and penetrate the secretory tissue. CNS infection adversely affects milk production due to subclinical mastitis [3]. Dry cow therapy, using a long-acting antibiotic, is considered to be one of the most effective methods for preventing mastitis during the dry period [20]. However, mastitis treatment with antibiotics leads to the development of antibiotic-resistant strains and

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consumer health problems. The use of antibiotics and other chemical products are banned for animal healthcare in a number of countries because of the effect on human healthcare. Development of alternative compounds to antibiotics is needed. Traditional ethno-veterinary medicinal practices are followed by rural populations to manage a number of veterinary diseases in the developing countries. The World Health Organization (WHO) states that 74% of the medicines derived from plant resources have a modern indication that correlates with their traditional and cultural uses [2].

This study showed the efficacy of the antibacterial action of traditional herb extracts on mastitis-associated CNS *in vitro*. Different extraction solvents (water and methanol) altered the antibacterial activity of the herbal extract against CNS isolated from bovine mastitis. Aqueous extracted *S. cumini* and methanol extracted *Z. mauritiana* demonstrated strong antibacterial activity against CNS. Similarly, a previous study [21] showed an inhibitory effect of crude methanolic extract of *Z. mauritiana* leaves on pathogenic bacteria and fungus. Our findings on MIC or MBC of the various extracts against the bovine mastitis-associated CNS confirmed that aqueous *S. cumini* and methanol *Z. mauritiana* extracts have the potential to be developed as treatments against bovine mastitis caused by CNS. This result is in agreement with Zeedan et al. [22] report on the antibacterial activity of selected some Sinai medicinal plant extracts against Gram-positive bacteria, *S. aureus, Streptococcus* spp*.*, *S. Agalactiae* and CNS, isolated from bovine mastitis. The observed antibacterial activity is attributed to the presence of bioactive compounds in the extracts of plants tested. The presence of these bioactive compounds in plant extracts confirm antibacterial activity against disease causing microorganisms offer protection to plants themselves against pathogenic microbial infections [8,9,23-26]. The presence of these phytochemical capacity supported the use of this plant as an antibacterial agent against the bovine mastitis CNS.

Table 1. The ethnotraditional data of employed herbs and their extract yield percentage

Herb species	Family	Local name	Common name	Extract pH		Extract yield (%)	
				Aqueous	Methanol	Aqueous	Methanol
S. cumini	Myrtaceae	Hwa	Java plum	5.24	4.32	2.3	4.4
M. hortensis	Bignoniaceae	Pib	tree jasmine	5.20	4.88	6.1	13.5
Z. mauritiana	Rhamnaceae	Phuthra	jujube	5.39	5.05	2.5	9.5

Table2. Phytochemicalconstituents of aqueous and methanol extract of employed herbs leaves

Phyto-constituents	*S. cumini*		*M. hortensis*		*Z. mauritiana*	
	Aqueous	Methanol	Aqueous	Methanol	Aqueous	Methanol
Alkaloids	+	+	-	-	-	-
Anthraquinones	+	-	+	-	-	-
Cardiac glycosides	+	+	+	+	+	+
Coumarins	+	+	+	+	+	+
Saponins	+	+	+	+	+	+
Steroids	+	+	+	+	+	+

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Flavonoids	+	+	+	+	+	+
Phenols	+	+	+	+	+	+
Saponins	+	+	+	+	+	+
Steroids	+	+	+	+	+	+
Tannins	+	+	+	+	+	+
Terpenoids	+	+	-	-	-	-

+, Present; -, Absent

Table 3. Morphological and biochemical characteristics of isolated CNS

Isolate no.	Gram Stain	Morphology	Catalase	Mannitol	Coagulase	DNase
A1	Positive	Coccus	+	+	-	-
A2	Positive	Coccus	+	+	-	-
A3	Positive	Coccus	+	+	-	-
A4	Positive	Coccus	+	+	-	-
A5	Positive	Coccus	+	-	ND	ND
B1	Positive	Coccus	+	+	-	-
B2	Positive	Coccus	+	+	-	-
B3	Positive	Coccus	-	ND	ND	ND
B4	Positive	Coccus	+	+	-	-
B5	Positive	Coccus	-	ND	ND	ND
C1	Positive	Coccus	+	-	ND	ND
C2	Positive	Coccus	+	-	ND	ND
C3	Positive	Coccus	+	+	-	-
C4	Positive	Coccus	+	-	ND	ND
D1	Positive	Coccus	+	+	-	-
D2	Positive	Coccus	+	+	-	-
D3	Positive	Coccus	+	-	ND	ND
D4	Positive	Coccus	+	+	-	-
D5	Positive	Coccus	+	+	-	-
E1	Positive	Coccus	+	-	ND	ND
E2	Positive	Coccus	+	+	-	-
E3	Positive	Coccus	-	ND	ND	ND
E4	Positive	Coccus	-	ND	ND	ND
E5	Positive	Coccus	+	+	-	-
F1	Positive	Coccus	+	-	ND	ND
F2	Positive	Coccus	+	-	ND	ND
F3	Positive	Coccus	-	ND	ND	ND
G1	Positive	Coccus	+	+	-	-
G2	Positive	Coccus	+	-	ND	ND
G3	Positive	Coccus	+	+	-	-
G4	Positive	Coccus	+	+	-	-
G5	Positive	Coccus	+	-	ND	ND

ND, Not determined.

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Table 4. Antimicrobial activity of crude extracts of *S. cumini*, *M. hortensis* and *Z. mauritiana* against mastitis associated CNS

Isolate no.	Zone of Inhibition *(*mm*)*					
	Aqueous			95% methanol		
	SC	MH	ZM	SC	MH	ZM
S. aureus	12.0 ± 0.2[a]	9.0 ± 0.3[a]	8.0 ± 0.3[b]	11.3 ± 0.2[a]	12.7 ± 0.2 [a]	16.0 ± 0.2[c]
A1	19.0 ± 0.2[a]	16.5 ± 0.7[b]	10.4 ± 0.3[c]	13.7 ± 0.3[d]	15.3 ± 0.6[bd]	18.0 ± 0.2[a]
A2	20.0 ± 0.2[a]	17.3 ± 0.2[b]	11.6 ± 0.2[c]	14.7 ± 0.2[d]	16.3 ± 0.2[b]	19.0 ± 0.2[a]
A3	21.0 ± 0.2[a]	18.3 ± 0.3[b]	12.6 ± 0.6[c]	15.7 ± 0.2[d]	17.3 ± 0.2[b]	20.0 ± 0.3[a]
A4	20.0 ± 0.2[a]	17.3 ± 0.2[b]	11.6 ± 0.2[c]	14.7 ± 0.2[d]	16.3 ± 0.2[b]	19.0 ± 0.2[a]
B1	18.0 ± 0.2[a]	18.7 ± 0.2[a]	10.0 ± 0.2[b]	12.0 ± 0.2[c]	16.0 ± 0.2[a]	18.3 ± 0.7[a]
B2	19.0 ± 0.2[a]	19.7 ± 0.2[a]	11.0 ± 0.2[b]	13.0 ± 0.2[c]	17.0 ± 0.2[a]	19.3 ± 0.2[a]
B3	18.0 ± 0.2[a]	18.7 ± 0.2[a]	10.0 ± 0.3[b]	12.0 ± 0.2[b]	16.0 ± 0.2[a]	18.3 ± 0.2[a]
C3	14.7 ± 0.2[a]	11.0 ± 0.2[b]	10.0 ± 0.2[b]	12.6 ± 0.3[a]	14.7 ± 0.2[ac]	15.7 ± 0.2[c]
D1	17.0 ± 0.3 [a]	16.3 ± 0.2[a]	14.7 ± 0.2[b]	12.7 ± 0.2[b]	14.3 ± 0.2[b]	15.0 ± 0.7[ab]
D2	16.0 ± 0.2 [a]	15.3 ± 0.2[a]	13.7 ± 0.4[b]	11.7 ± 0.2[b]	13.3 ± 0.2[b]	14.0 ± 0.2[a]
D3	15.0 ± 0.7 [a]	14.3 ± 0.2[a]	12.7 ± 0.2[a]	10.7 ± 0.6[b]	12.3 ± 0.3[b]	14.5 ± 0.5[a]
D4	15.0 ± 0.2 [a]	15.3 ± 0.2[a]	13.7 ± 0.2[ab]	11.7 ± 0.2[b]	13.3 ± 0.2[b]	14.0 ± 0.2[a]
E2	15.6 ± 0.6[a]	11.5 ± 0.2[b]	8.0 ± 0.3[c]	7.3 ± 0.2[c]	10.6 ± 0.2[b]	14.0 ± 0.2[a]
E5	16.6 ± 0.2[a]	12.7 ± 0.2[b]	9.0 ± 0.2[c]	8.3 ± 0.7[c]	11.6 ± 0.2[b]	15.0 ± 0.3[a]
G1	15.3 ± 0.2[ac]	14.3 ± 0.2[a]	11.0 ± 0.5[b]	11.0 ± 0.3[b]	16.0 ± 0.3[ac]	17.0 ± 0.2[c]
G3	14.3 ± 0.3[ac]	13.3 ± 0.2[a]	10.0 ± 0.2[b]	10.0 ± 0.2[b]	15.0 ± 0.2[ac]	16.0 ± 0.3[c]
G4	17.6 ± 0.2[a]	16.0 ± 0.2[a]	12.3 ± 0.2[b]	12.0 ± 0.2[b]	12.3 ± 0.2[b]	14.3 ± 0.2[c]

Mean± SD of triplicates. SC, *S. cumini*; MH, *M. hortensis*; ZM, *Z. mauritiana.*
[a, b, c, d] Significant difference between means with different letter superscripts in the same row ($p < 0.05$) using ANOVA and post-hoc analysis *(*Duncan*'*s test*)*.

Table 5. MIC and MBC of crude extracts of *S. cumini*, *M. hortensis* and *Z. mauritiana* against CNS

	Aqueous			95% methanol		
	SC	MH	ZM	SC	MH	ZM
MIC mg/ml	1.56	6.25	3.13	3.13	12.5	1.56
MBC mg/ml	1.56	12.5	6.25	6.25	12.5	1.56

SC, *S. cumini*; MH, *M. hortensis*; ZM, *Z. mauritiana*.

CONCLUSION

It can be concluded that *S. cumini* leaves and *Z. mauritiana* leaves extracts have found to be efficient and have a strong antimicrobial effect against CNS bacteria**.** From this perspective, natural products (these herb extracts) can be used to effectively prevent the occurrence of mastitis in dairy cows with cost and antibiotic residue reduction.Further, molecular epidemiological studies for exploring the different types of CNS-causing mastitis in dairy cows should be done**.**

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