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Antibacterial Activity of Aqueous and Ethanol Extracts of *Caesalpinia Sappan* L. Against Coagulase-Negative Staphylococci Isolated from Subclinical Bovine and Caprine Mastitis

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Abstract: This study aimed to investigate the effect of *Caesalpinia sappan* L. hearth wood extract against Coagulase-Negative Staphylococci (CNS) causing subclinical mastitis in dairy cows and goats. Seventeen and thirty CNS isolates were isolated from bovine and caprine mastitis samples, respectively. The phytochemicals tests performed on aqueous and ethanol extracts of *C. sappan* heartwood presented total phenolic (150 and 187 mg GAE/g dw), flavonoids (28 and 21 mg RE/g dw), tannins, alkaloid, saponin, and steroid. The extracts were used to determine the antibacterial effect on CNS using disc diffusion, Minimum Inhibitory Concentration (MIC), and Minimum Bactericidal Concentration (MBC) methods. The aqueous extract and ethanol extract of *C. sappan* contained effective compounds, including phenol, flavonoid, tannin, alkaloid, saponin, and steroid. Ethanol extract of *C. sappan* had a greater content of phenol, while aqueous extract of *C. sappan* had a greater content of flavonoid. Ethanol extracts of *C. sappan* showed the strongest inhibition on the CNS isolated from bovine (14.3-21.0 mm) and caprine (24.3-30.0 mm). The antimicrobial activity of ethanol *C. sappan* extract showed significantly higher than aqueous extract against bovine CNS (8.0 to 14.7 mm) and caprine CNS (22.3 to 29.0 mm). The *C. sappan* ethanol extract had the best MIC and MBC of bovine CNS (6.24 mg/mL) and caprine CNS of (1.56 mg/mL). The ethanol extracts of *C. sappan* showed strong antimicrobial effects against CNS isolated from both bovine and caprine mastitis.

Keywords: *Caesalpinia Sappan*, Coagulase-Negative Staphylococci, Bovine, Caprine, Mastitis

Introduction

Dairy products derived from bovine and caprine milk are an important part of the economy of Thailand. Mastitis is an important health problem in dairy husbandry, with great economic losses worldwide (Contreras and Rodríguez, 2011). The direct effects of mastitis are temporary or permanent loss in milk production, poor milk quality, reduction in price, treatment costs, labor costs, and premature culling. Mastitis is categorized as clinical or subclinical .Subclinical mastitis predominates in the herds of small ruminants, presenting a prevalence between 5 and 30%. Clinical mastitis is prevalent at levels below 5% (Contreras *et al.*, 2007). In subclinical mastitis, there are no evidencing indications of inflammation in the mammary halves, but there are alterations in milk composition .Subclinical mastitis presents a positive result in California Mastitis Test CMT (or other indicative tests, confirmed by microbial isolation (Fragkou *et al.*, 2014; Machado *et al.*, 2018).

The most commonly identified etiologic agents are found similarly in bovine and caprine mastitides, such as Coagulase-Negative Staphylococci) CNS), *Streptococcus* spp., *Escherichia coli*, *Corynebacterium* spp., *Pseudomonas* sp., and some species of fungi (Contreras *et al.*, 2007). The coagulase-negative staphylococci CNS are the most prevalent microorganisms in subclinical mastitis in cows and goats

(Contreras *et al*., 2007; Zigo *et al*., 2021). The most frequently isolated bacterial species was CNS species (72%) in dairy goats (Persson and Olofsson, 2011). CNS infection causes subclinical mastitis that negatively affects milk production (Pyorala and Taponen, 2009). The CNS intramammary infections have been also associated with an increase in somatic cell counts SCC **(**of affected cows (Zigo *et al*., 2021). Recent studies propose that infections with CNS cause more serious harm than thought before (Taponen *et al.*, 2006). CNS infections were investigated in prepartum heifers and results revealed an association between bacteriological treatment and SCC decrease (Borm *et al.*, 2006). Even though individual CNS infections may have a moderate effect on SCC. It could be exhibited that any infection with CNS caused an SCC increase in goat and cow milk (Aulrich and Barth, 2008).

The increased use of antibacterial drugs caused antibiotic residues in milk (Erskine *et al.*, 2003) and bacterial resistance to antibiotics, however, cure rates obtained with such antibacterial drugs are not always effective and may determine the emergence of resistant bacteria (Oliver and Murinda, 2012). Therefore, it is essential to explore the antibacterial action of local plants, as an alternative drug to control CNS isolated in the local area, Lopburi**.** The antimicrobial action of plant extracts may promote a reduction in antibiotic use.

Currently, ethnoveterinary medicinal procedures are followed by agricultural peoples to control a lot of animal illnesses in developing countries. Seventy-four percent of medicines developed from plants in the remark of the World Health Organization (WHO) indicated a correlation with their traditional and cultural applications (Myllys, 1995). Several plants are globally used in traditional medicine as cures for bacterial infections. Some of them were subjected to *in vitro* screening but their efficacy was rarely tested faithfully in controlled clinical trials (Severino and Ambrosio, 2012). The largest number of used medicinal plants in Thailand are used for curing many diseases. *Caesalpinia sappan* L. in the Leguminosae family is one of the Thai medicine plants in the Phra Narai folk medicine textbook that is empirically used to cure some diseases such as inflammation, dysentery diarrhea, and cancer (Wang *et al.*, 2011; Limsuwan and Voravuthikunchai, 2013). Moreover, this plant is commonly consumed in traditional herbal beverages and as a component of a food coloring agent (Toegel *et al.*, 2012). However, there has been rarely exploration for using *C. sappan* as alternative remedies against animal mastitis.

For these reasons, many researchers have attempted to find natural materials to replace antibiotics to treat bacterial infections. Therefore, the present research determined the antibacterial activity of *C. sappan* hearth wood extracted with water and ethanol against CNS isolated from subclinical bovine and caprine mastitis.

Materials and Methods

Collection of Milk Sample and Assessment of Subclinical Mastitis

Bovine and caprine milk samples were obtained from animals raised in Phatthana Nikhom, Lopburi, Thailand. The California Mastitis Test (CMT) was used as a screening test for subclinical mastitis. Milk from each udder and an equivalent load of CMT testing agent was put in each shallow cup and gently swirled in the mixture for 30 sec. A positive reaction showed gel formation during swirling. The CMT result was graded based on the gel formation as positive or negative results; One or more of the CMT positive quarters indicated mastitis. Positive milk samples from subclinical bovine and caprine mastitis. Were further subjected to CNS isolation.

Isolation of CNS

The bacteria of milk samples were cultured on nutrient agar media (Oxoid Ltd., Basingstoke, Hampshire, England (at 37°C for 24-48 h. Considered colonies were collected and carried out further identification based on Gram staining and catalase testing to pick out between streptococci and staphylococci. For CNS characterization, the mannitol fermentation test, the coagulase test, and DNase reaction test were used (Christopher and Bruno, 2003).

Preparation of Crude Extracts

The shade-dried hearth wood powder of *C. sappan* was purchased from a traditional Thai pharmacy in Muang, Lopburi province. The powder extraction was carried out using one of two solvents: Water or 95% ethanol (powder: Solvent 1:5). The powder (200 g) was placed in water or 95% ethanol (1,000 mL), mixed well, incubated at room temperature, and shaken once a day for 7 days. The extract was sieved through cheesecloth, what man paper, no.4, and 0.45 mm millipore filter, respectively. The extract solvents were evaporated under pressure using a rotary evaporator at a temperature of 40 50°C. Viscous and sticky extracts were dissolved with water or ethanol for a concentration of 256 mg/mL, filtered through a 0.45 mm millipore filter, and kept at 4°C (Boonkusol, 2017).

Analysis of Plant Phytochemicals

A qualitative phytochemical examination of the aqueous and ethanol *C. sappan* extracts was conducted using standard procedures (Trease and Evans, 2002) . Bioactive compounds including alkaloids, flavonoids, phenols, saponin, steroids, and tannins were detected. Total phenolic content of *C. sappan* extracts was determined by the Folin-Ciocalteu procedure and data were represented as mg gallic acid equivalents (Noreen *et al*., 2017), while total flavonoid content of *C. sappan* extracts was measured by colorimetric technique and data were represented as mg Rutin (RE) (Nabavi *et al.*, 2008).

Antibacterial Analysis of Plant Extracts

The antimicrobial activity of *C. sappan* extracts was determined using a disc diffusion technique, as explained in the guidelines of the NCCLS (National Committee for Clinical Laboratory Standards, 1997). Discs (6 mm diameter) modified from Whatman filter paper were sterilized, soaked with 20 μL of each 200 mg/mL *C. sappan* extract, dried at 37°C for 24 h, and put on the 10^8 CFU/mL CNS-inoculated plates described in Wiegand *et al.* (2008). The disc with pure solvent (water or ethanol) and antibiotic disc (ampicillin) were applied as negative control and positive control, respectively. The plates were then incubated at 37°C for 24 h. The diameter of the zone of inhibition produced by each antibacterial disc was measured in terms of millimeters. The antibacterial potential was recorded for each extract in triplicated treatment.

The Minimum Inhibitory Concentration (MIC), the lowest concentration of the extract to inhibit the growth of CNS isolates, of *C. sappan* extracts was tested by the broth dilution method according to the NCCLS (1997). A final concentration of different extracts ranged from 100 to 0.195 mg/mL. Ten microliters of CNS inocula (adjusted to approximately 1×10^8 CFU/mL) were added .The *C. sappan* extract with media was applied as a positive control and inocula with media was applied as a negative control .The test tubes were incubated at 37°C for 24 h. The lowest concentration of the tested extract that exhibited no visible turbidity when compared with the control tubes was considered MIC. The Minimum Bactericidal Concentration (MBC) was determined by subculturing the test dilutions that showed no turbidity. The plates were incubated further at 37°C for 18-42 h. The least concentration that yielded no single bacteria colony was addressed as MBC. Each treatment was performed in triplicates.

Statistical Analysis

The inhibition diameter of bacterial growth was expressed as the mean and Standard Deviation (SD) of each triplicated treatment. Significant differences within the means of various extracts in each CNS isolate were analyzed by one-way analysis of variance ANOVA, followed by Duncan's test (SPSS 22.0). Values with $p<0.05$ were considered statistically significant.

Results

Plant Extraction Yield and CNS Isolation

Two solvents in this study, water, and ethanol exhibited a positive effect qualitatively on secondary metabolite compounds consisting of tannins, flavonoids, alkaloids, saponins, and steroids Table 1. The total phenol and flavonoid contents in *C. sappan* aqueous and ethanol extracts were shown in Table 2. The highest total phenolic content was detected in ethanol extract of (*C. sappan)* 187 mg GAE/g dw (while the highest total flavonoid content was determined in the aqueous extract of (*C. sappan*) 28 mg RE/g dw. The extract yield percentage of aqueous and ethanol *C. sappan* heartwood were 2.68 and 4.90%, respectively.

The CMT diagnosis for subclinical mastitis of bovine and caprine milk samples from bovine and caprine showed that the percentage of positive CMT in bovine milk samples was 75% (6/8) and caprine milk samples were 66.7% (6/9) As presented in Table 3, milk samples which were positive by CMT from bovine (n = 6) and caprine (n = 6) gave a positive bacterial culture 45 and 37 isolates of bacteria. The results of the isolated number of coagulase-negative staphylococci identified by morphological and biochemical characteristics isolated from bovine and caprine with subclinical mastitis are given in Table 3. Twenty-nine bovine bacteria isolates and thirty caprine bacteria isolates from subclinical mastitis milk samples were positive Gram staining, spherical in shape (cocci), and positive catalase test (staphylococci). For these isolates, 17/29 bovine isolates and 13/30 caprine isolates were CNS were identified as negative coagulase reaction, positive mannitol fermentation, and negative DNase test (Table 3).

Antibacterial Activity

All aqueous and ethanol extracts of *C. sappan* showed antibacterial activity against tested CNS (Table 4). Ethanol-extracted *C. sappan* showed the greatest activity with inhibitory diameters in all bovine CNS isolates of 14.3-21.0 mm and caprine CNS isolates of 14.3-30.0. The aqueous extracts expressed mild action with inhibitory diameters ranging from 8.0 to 14.7 mm in bovine but showed elevated action with inhibitory diameters ranging from 12.3 to 19.0 mm in caprine.

The growth of all isolated CNS strains was inhibited by the crude extract of *C. sappan* heartwood with the water and ethanol Table 4. However, the ethanol extract was greater antibacterial activity against all CNS strains than the aqueous extract .

Among the test bacteria, the standard strain of *S. aureus* was susceptible to aqueous extract with a mean zone of inhibition of 24.5 mm, followed by the caprine CNS strains with a mean zone of inhibition ranging from 12.3 to 19.0 mm, In addition, among the bovine CNS strains, the most susceptible isolated CNS of the aqueous extract with the maximum mean zone of inhibition of 14.7 mm (Table 4).

Similar to the aqueous extract, the most sensitive CNS against ethanol extract was caprine CNS strains with a mean inhibitory zone ranging from 24.3 to 30.0 mm, followed by the standard strain of *S .aureus* with a mean zone of inhibition of 26.0 mm. In addition, among the bovine CNS strains, the most susceptible isolated CNS of the aqueous and ethanol extract with the maximum inhibition diameters of 14.7 and 21.0 mm, respectively (Table 4).

The MIC and MBC of *C. sappan* extracts were in the range of 1.56 to 12.5 mg/mL against the growth of the susceptible bacterial isolates. The *C. sappan* aqueous extracts and ethanol *C. sappan* extract exhibited the best MIC and MBC at 1.56 mg/mL (Table 5). The ethanol extract showed more potent than the aqueous extract.

Table 1: Phytochemical analysis of aqueous and ethanol *C. sappan* heartwood extract

	C. sappan hearth wood	
Secondary metabolite	Aqueous	Ethanol
Tannin	+	+
Flavonoid	+	+
Alkaloid	+	+
Saponin	+	+
Steroid	+	+

Table 2: Total phenolic and flavonoid contents in aqueous and ethanol *C. sappan* heartwood extract in mg/g dry weight (dw)

Plant extract	Total phenolic (mg GAE/g dw)	Flavonoids content (mg RE/g dw)
Aqueous extract of *C. sappan*	150	28
Ethanol extract of *C. sappan*	187	21

Table 3: Isolate number of CNS identified by morphological and biochemical characteristics isolated from bovine and caprine with subclinical mastitis (n = 6)

	Isolate number	
	Bovine	Caprine
Sample isolated no.	45	37
Positive gram	29	30
Coccus morphology	29	30
Positive catalase	29	30
Positive mannitol	23	30
Negative coagulase	17	13
Negative DNase	17	13

Table 4: Antibacterial activity of aqueous and ethanol *C. sappan* extracts against mastitis-associated bovine and caprine CNS

Isolate no. of bovine CNS	Inhibition zone mm		Isolate no. of caprine CNS	Inhibition zone mm	
	Aqueous extract	Ethanol extract		Aqueous extract	Ethanol extract
BA1	11.4±0.3[a]	19.0±0.5[b]	CA1	13.3±0.4[a]	26.3±0.3[b]
BA2	11.6±0.2[a]	20.0±0.9[b]	CC1	16.3±0.3[a]	28.3±0.5[b]
BA3	12.6±0.6[a]	21.0±0.6[b]	CC3	14.3±0.7[a]	27.7±0.4[b]
BA4	11.6±0.2[a]	20.0±0.2[b]	CC4	14.0±0.3[a]	27.7±0.3[b]
BB1	10.0±0.3[a]	18.0±0.2[b]	CD1	16.7±0.9 [a]	30.0±0.3[b]
BB2	11.0±0.3[a]	19.0±0.4[b]	CE1	16.3±0.8[a]	29.0±0.7[b]
BB3	10.0±0.3[a]	18.0±0.2[b]	CF1	14.3±0.3[a]	28.0±0.5[b]
BC3	10.0±0.2[a]	14.7±0.5[b]	CF2	13.3±0.3[a]	27.3±0.9[b]
BD1	14.7±0.2[a]	17.0±0.3[b]	CF3	12.3±0.2[a]	26.0±0.3[b]
BD2	13.7±0.4[a]	16.0±0.2[b]	CG1	13.3±0.4[a]	24.3±0.2[b]
BD3	12.7±0.2[a]	15.0±0.7[b]	CG2	12.3±0.3[a]	25.3±0.4[b]
BD4	13.7±0.5[a]	15.0±0.2[b]	CI1	17.0±0.2[a]	29.3±0.3[b]
BE2	8.0±0.3[a]	15.6±0.6[b]	CI2	19.0±0.5[a]	27.3±0.5[b]
BE5	9.0±0.2[a]	16.6±0.2[b]			
BG1	11.0±0.5[a]	15.3±0.2[b]			
BG3	10.0±0.4[a]	14.3±0.3[b]			
BG4	12.3±0.2[a]	17.6±0.5[b]			
S. aureus	24.5±0.3[a]	26.0±0.5[b]			

Table 5: MIC and MBC of aqueous and ethanol *C. sappan* extracts against bovine and caprine CNS

	MIC mg/ml			MBC mg/ml		
	Aqueous extract	Ethanol extract	Ampicillin	Aqueous extract	Ethanol extract	Ampicillin
S. aureus	6.24	6.24	0.25	12.50	12.50	0.25
Bovine CNS	12.48	6.24	0.25	12.48	6.24	0.25
Caprine CNS	3.12	1.56	0.25	3.12	1.56	0.25

Discussion

The phytochemical determination of the aqueous and ethanol extracts of *C. sappan* heartwood was found positive for bioactive compounds including tannins, flavonoids, alkaloids, saponins, and steroids Phytochemical study of plants was used to explore potentially bioactive substances in plants. Reports were showing that the aqueous and ethanol extracts were qualitatively equal to the investigation of bioactive substances in plants and *in vivo* examinations could be used for reference (Raipuria *et al.*, 2018; Amadioha and Chidi, 2019). Phytochemicals are generally synthesized by plants to assist them to control diseases or pathogens (Amadioha and Chidi, 2019) .

Phenol substances of plants are common secondary metabolites that can be actively used for the protection of plant microbial infections (Tamokou *et al.*, 2017). Flavonoids are commonly observed in plants and they have different effects on plant health (Montoro *et al.*, 2005) .The reports on flavonoid derivatives confirmed that flavonoids had antibacterial, anthelmintic, antiviral, anti-inflammatory, anti-allergic, and anticancer activities. Plant-active substances were confirmed to control pathogenic bacteria in livestock (Doss *et al.*, 2012; Padhi and Panda, 2015; Carvalho *et al.*, 2019; Famuyide *et al.*, 2019).

The antibacterial activities of the aqueous extract in terms of its zone of inhibition were lower than that of the ethanol extract against each bacterial isolate for which it was active, even though the difference at the corresponding concentration were not statistically significant. Our presented study exhibited the effectiveness of the antibacterial action of medicinal plant extracts on *in vitro* CNS-associated mastitis. Different extraction solvents, water, and ethanol modified the antibacterial activity of the plant extracts against CNS isolated from bovine and caprine mastitis. Ethanol-extracted *C. sappan* demonstrated strong antibacterial activity against CNS in both cattle and goats. Similarly, a previous study (Zeedan *et al.*, 2014) showed an inhibitory effect of some Egyptian medicinal plant extracts on Gram-positive bacteria *S .aureus*, *Streptococcus* spp., *S. Agalactiae*, and CNS with an inhibitory zone of 25.6±0.60, 20.66±0.57, 18.67±0.57 and 19.66±0.58 mm respectively. The data on MIC or MBC of *C. sappan* extracts against CNS causing bovine and caprine mastitis supported that both aqueous and ethanol *C. sappan* extracts have the efficacy to be produced as antibacterial medication against bovine and caprine CNS associated mastitis. This is consistent with the bacterial inhibitory effect of most Thai herbal extracts from the report of Parekh *et al.* (2006). There are also some reports on alternative treatments of bovine mastitis with bacteriocins (Klostermann *et al.*, 2010) and plant-derived compounds (Wiegand *et al.*, 2008; Othman *et al.*, 2019). Medicinal plant extracts have expanded global importance in the exploration of alternative antibacterial compounds because plant compounds are a valuable resource of antibacterial agents with feasibly new mechanisms of action (Ahmad and Aqil, 2007). There has been *in vitro* detection of antimicrobial properties of medicinal plants containing some secondary metabolites such as tannins, alkaloids, and flavonoids (Othman *et al.*, 2019). Many reports also confirmed the antimicrobial activity of various plant extracts (Toegel *et al.*, 2012; Atef *et al.*, 2017; Rekha *et al.*, 2018; Kachhawa *et al.*, 2019; Fatmawati *et al.*, 2020).

Conclusion

This study demonstrates that *C. sappan* heartwood extracts can be used to effectively kill CNS bacteria causing subclinical bovine and caprine mastitis. Our results showed that natural products can have a strong antimicrobial effect against CNS bacterial isolates. The phytochemicals tests performed on the extracts of *C. sappan* heartwood confirm the presence of flavonoids, phenolic compounds, tannins, alkaloids, saponin, and steroids also exhibited antimicrobial activity. Thus, *C. sappan* extract could be used as an alternative source of production of the antibiotic drug against mastitis in cattle and goats.

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Author's Contribution

Duangjai Boonkusol: Planned the experiments, interpreted the results, statistically analyzed the data, and finally approved the manuscript.

Janejira Detraksa: Interpreted the results, and final approval of the manuscript.

Kansuda Duangsrikaew: Statistically analyzed the data, final approval of the manuscript.

Ethics

This article is original and contains unpublished material .The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues are involved.

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