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by Vincentia Rizke Ciptaningtyas

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Phytochemical properties and antibacterial activity of *Ageratum conyzoides*, *Piper betle*, *Muntinga calabura* and *Curcuma domestica* against mastitis bacteria isolates

Dian Wahyu Harjanti^{1*}, Rizke Ciptaningtyas², Fajar Wahyono¹

¹Faculty of Animal and Agricultural Sciences, Diponegoro University, Indonesia

²Faculty of Medicine, Diponegoro University, Indonesia

*Email: dianharjanti@undip.ac.id

Abstract. Mastitis also known as inflammation of the mammary gland. It is a costly disease, which often caused by bacterial infection. Antibiotic have been use mastitis treatment for decades, however it potentially caused an antibiotic residue in milk. This study was aimed evaluate the potency of local resources as natural remedies for mastitis. The ethanol extract of *Ageratum conyzoides* leaves, *Piper betle* leaves, *Muntinga calabura* leaves and *Curcuma domestica* were screened for their phytochemical properties and antibacterial activity against mastitis causing bacteria. The bacteria were isolated from sub-clinical mastitis milk in smaller dairy farms in Central Java, then identified by VITEK. Antibacterial activity of seven extracts were determined by agar well diffusion method. Experimental findings revealed that high proportion of test organisms were all susceptible to *Piper betle*, *Ageratum conyzoides* and *Curcuma domestica*. However, for *Muntinga calabura*, only *S. simulans*, *S. chromogens*, *S. dysgalactiae* and *S. sanguinis* were able to be inhibited, whereas *S. mitis*, *S. agalactiae* and *S. uberis* were resistant. The minimum inhibitory concentrations of all extracts were 12.5 mg/ml. Phytochemical screening revealed the presence of alkaloid, flavonoid, tannin and saponin with various concentration in all extracts. In conclusions, *Ageratum conyzoides* leaves, *Piper betle* leaves, *Muntinga calabura* leaves and *Curcuma domestica* rhizomes are potential sources of antibacterial agents for mastitis treatment.

1. Introduction

Antibiotic therapy for dairy cows has caused public concern worldwide due to the potential of antibiotic residues in milk. In another hand, dairy industry facing an economically devastating disease named mastitis. Economic losses due to mastitis are reduction of milk, veterinary treatment, animal culling and an increase in mortality rate. A study has been done to isolate pathogens from infected udders and found *Staphylococcus simulans*, *Staphylococcus chromogens*, *Streptococcus uberis*, *Streptococcus sanguinis*, *Streptococcus dysgalactiae ssp dysgalactiae*, *Streptococcus mitis* and *Streptococcus agalactiae* [1]. Previously, Salasia et al. [2] isolated *Staphylococcus aureus* from mastitis cows and Sudarwanto et al. [3] isolated *Klebsiella pneumonia* from bulk tank milk in West Java region of Indonesia. The use of antibiotic for mastitis treatment is not only causing residue in milk, but also associated with the problem of antibiotic resistance [4,5].



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There is a need to explore alternative approaches for the treatment of mastitis. Recently, an interest in discovering new natural antimicrobials from plants is rising. Phytochemical analysis of ethnomedical plants for secondary metabolites is an important area of fundamental research because of its relevance for the discovery of therapeutic agents. Evidence found through research in human showed that *Piper betle*, *Ageratum conyzoides*, *Muntinga calabura* and *Curcuma domestica* are useful for various disease treatments, such as fever, respiratory disease, gastritis and inflammation by injuries due to their bioactive compounds, such as saponin, flavonoid, tannin, alkaloid and essential oils [6,7]. In line with above evidence, current study was aimed to determine the phytochemical compounds and to investigate the antibacterial effectiveness of *Piper betle*, *Ageratum conyzoides*, *Muntinga calabura* and *Curcuma domestica* against Mastitis causing pathogens as an alternative treatment to mastitis infection.

2. Material and method

2.1. Herbal collection and extraction.

Piper betle, *Ageratum conyzoides* and *Muntinga calabura* leaves, also *Cucuma domestica* rhizome were collected from Central Java region Indonesia. The medicinal plants were dried in oven 50°C for 24-48 h, then finely ground into powder. Ethanol 96% was then added to each herbal separately and homogenize for 1 h. Maceration for each herbal was done for 24 h following method described by Harjanti et al. [8]. Ethanol extract of each herbal was dissolve in 10% dimethyl sulphoxide (DMSO) (Merck) just before use to make concentration of 100, 50, 25 and 12.5 mg/ml.

2.2. Bacterial isolates.

Bacterial isolates used in this study were the pathogens isolated from sub-clinical cases of bovine mastitis which have been identified by VITEK 2 compact (bioMérieux, UK) as *Staphylococcus simulans*, *Staphylococcus chromogens*, *Streptococcus uberis*, *Streptococcus sanguinis*, *Streptococcus dysgalactiae ssp dysgalactiae*, *Streptococcus mitis* and *Streptococcus agalactiae*. All isolates were obtained from Diponegoro University National Hospital. The isolates were maintained on nutrient agar slants at 4°C and subcultured onto nutrient broth for 24 h prior to testing. These bacteria served as test pathogens for antibacterial activity assay.

2.3. Antibacterial activity assay

Antibacterial activity of seven extracts were determined by agar well diffusion method [9]. Inoculum from each bacterial tested which containing 10⁶ cfu/ml each isolate was spread on Mueller Hinton agar plates for *Staphylococcus simulans* and *Staphylococcus chromogens*, and on the Mueller Hinton Blood agar plates for *Streptococcus uberis*, *Streptococcus sanguinis*, *Streptococcus dysgalactiae ssp dysgalactiae*, *Streptococcus mitis* and *Streptococcus agalactiae*. Subsequently, wells with diameters of 8 mm were punched into the agar medium and filled with each extract, then allowed to diffuse at room temperature for 2 h. Each extract with different concentration (100, 50, 25 and 12.5 mg/ml) were analyzed with five replications. All plates were then incubated in the upright position at 37°C for 24 h. Wells containing the same volume of 10% DMSO and 96% ethanol were served as negative control, while Mastilak (Procaine penicillin and dihydrostreptomycin, SANBE, Indonesia) was selected to be used as positive control because it is commonly used by veterinarian to cure mastitis in dairy farms. Minimum inhibitory concentrations (MICs) were determined after 24 h incubation and measured in mm. Five replicates were carried out for each extract with each concentration against each of the tested bacteria. Data were expressed as mean ± standard deviation.

2.4. Phytochemical properties analysis

Ethanol extract of *Piper betle*, *Ageratum conyzoides* and *Muntinga calabura* leaves, also *Cucuma domestica* rhizome were subjected to phytochemical analysis to ascertain the presence metabolites such as saponin, alkaloid, steroid, flavonoid, tannin, phenol.

3. Results and discussion

Table 1 shows the results of the zones inhibition diameter produced by each extract on the various test organisms. The result of antibacterial activity assay indicated that high proportion of test organisms were all susceptible to *Piper betle*, *Ageratum conyzoides* and *Curcuma domestica*. However, for *Muntinga calabura*, only *S. simulans*, *S. chromogens*, *S. dysagalactiae* and *S. sanguinis* were able to be inhibited, whereas *S. mitis*, *S. agalactiae* and *S. uberis* were resistant (table 1). Ethanol extract of *Piper betle* had inhibition zones ranging from 14.2 to 28.7 mm. *Ageratum conyzoides* extract had inhibition zones ranging from 10.4 to 21.5 mm. *Curcuma domestica* extract had inhibition zones ranging from 7.0 to 21.0 mm, whereas *Muntinga calabura* extract had inhibition zone ranging from 8.0 to 17.4 mm. The minimum inhibitory concentrations of all extracts were 12.5 mg/ml.

Various publications have documented the antimicrobial activity of *Ageratum conyzoides*, *Piper betle* and *Muntinga calabura* leaves extracts, also *Curcuma domestica* rhizome extract. Previous publications showed that ethanol, ethyl acetate, methanol and supercritical CO₂ extract of four varieties of *Piper betle* had significant activities against *Vibrio cholera*, *Staphylococcus aureus* and *Streptococcus pneumoniae* and methicillin-resistant *Staphylococcus aureus* (MRSA) [10,11]. *Ageratum conyzoides* leaves was reported to possess antimicrobial activity, anti-inflammatory activity, analgesic activity, anti-cancer and radical scavenging activity [6]. Regarding to *Muntinga calabura* leaves, we found that *S. simulans*, *S. chromogens*, *S. dysagalactiae* and *S. sanguinis* could be inhibited by 12.5 mg/ml of *Muntinga calabura* ethanol extract. Another study using aqueous, petroleum ether and ethyl acetate extracts of *Muntinga calabura* leaves reported antibacterial activity against *Staphylococcus aureus* [12] and MRSA [13] by micro-broth dilution method. The antibacterial activity of curcumin as bioactive compound of *Curcuma domestica* also have been investigated previously by Mun et al. [14] against MRSA and by de Oliveira [15] against *Escherichia coli* and *Listeria innocua*. The antibacterial activity in both studies were assessed by broth microdilution method. Curcumin is a natural polyphenolic flavonoid from the *Curcuma* rhizome [14].

Furthermore, phytochemical investigations in this study revealed that *Ageratum conyzoides*, *Piper betle* and *Muntinga calabura* leaves extracts, also *Curcuma domestica* rhizome extract possessed active compounds such as saponin, alkaloid, steroid, flavonoid, tannin and phenol in various concentration. Phytochemical profiles of each extract presented in Table 2. *Ageratum conyzoides* extract had the highest flavonoid (6.15 %w/v) and phenol (3.86%w/v) contents. Alkaloid, steroid and tannin contents in *Muntinga calabura* extract (0.56, 2.48 and 53.98%w/v) were the highest whereas saponin content in *Piper betle* extract (4.80%w/v) was the highest among plant extracts. Saponins have a soapy characteristic and characterized as glycosides, which could facilitate the absorption of food and medicine. Tannins have been reported to prevent the development of bacteria by precipitating microbial protein and making the bacteria could not able to use the nutritional protein for their growth [16]. Tannins also could disturb the extracellular microbial enzymes and oxidative phosphorylation which in turn initiate iron deprivation, the most important material for bacterial growth [17]. Flavonoid from plant extract may inhibit the cytoplasmic membrane function and energy metabolism of bacteria [18].

Comparison of the data of antibacterial activity and phytochemical property obtained from current study with previously published are problematic. The composition of plant extract is known to vary according to local climatic, environmental conditions and different regions [19]. Some plants with the same common name may be derived from different plant species. Furthermore, the method used to evaluate antibacterial activity varies between publications. Agar disc diffusion, agar dilution and broth dilution methods are commonly used. The results obtained by each of these methods may differ as many factors vary between assay. These include the microbial growth differences, the exposure of bacteria to plants extracts, solubility of plant extract, and the use and quantity of an emulsifier.

Table 1. Antibacterial activity of five herbals against Mastitis causing bacteria

	Minimum inhibitory concentration (cm)				Positive control
	100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	
Piper betle					
<i>Staph. simulans</i>	26.5 ± 1.6	25.8 ± 1.5	24.5 ± 1.5	22.0 ± 1.4	40.0 ± 0
<i>Staph. chromogenes</i>	28.7 ± 1.6	28.0 ± 1.5	25.3 ± 0.9	24.3 ± 0.9	48.0 ± 0
<i>Staph. mitis</i>	24.5 ± 0.5	22.7 ± 0.7	21.0 ± 0.3	19.5 ± 0.4	40.0 ± 0
<i>Strep. dysagalactiae</i>	23.8 ± 1.2	21.6 ± 1.3	19.4 ± 1.4	18.2 ± 1.0	38.0 ± 0
<i>Strep. agalactiae</i>	17.7 ± 1.8	17.3 ± 1.5	16.0 ± 1.4	14.2 ± 1.7	32.0 ± 0
<i>Strep. uberis</i>	20.0 ± 2.0	19.0 ± 1.6	17.4 ± 1.6	16.0 ± 1.3	29.8 ± 0.3
<i>Strep. sanguinis</i>	19.6 ± 1.8	18.2 ± 1.7	16.4 ± 1.7	14.8 ± 1.8	29.5 ± 0.6
Ageratum conyzoides					
<i>Staph. simulans</i>	18.0 ± 0	16.2 ± 0.2	16.0 ± 0	14.2 ± 14.2	40.0 ± 0.0
<i>Staph. chromogenes</i>	20.3 ± 1.0	18.7 ± 0.9	16.5 ± 0.7	15.0 ± 0.8	48.0 ± 0.0
<i>Staph. mitis</i>	21.5 ± 0.8	20.3 ± 0.6	19.2 ± 0.4	17.8 ± 0.4	37.0 ± 0.0
<i>Strep. dysagalactiae</i>	17.4 ± 2.3	16.0 ± 2.3	14.8 ± 2.1	14.0 ± 2.1	36.0 ± 0.0
<i>Strep. agalactiae</i>	17.7 ± 1.8	17.3 ± 1.5	13.7 ± 1.8	13.5 ± 1.6	32.0 ± 0.0
<i>Strep. uberis</i>	13.2 ± 3.3	10.4 ± 3.8	12.6 ± 2.6	12.2 ± 2.2	29.0 ± 0.0
<i>Strep. sanguinis</i>	19.4 ± 1.6	18.4 ± 1.6	17.0 ± 1.4	15.2 ± 1.3	28.7 ± 0.2
Muntinga calabura					
<i>Staph. simulans</i>	17.0 ± 0.9	16.0 ± 0.9	14.8 ± 0.9	13.6 ± 0.9	42 ± 0.4
<i>Staph. chromogenes</i>	17.4 ± 1.0	16.2 ± 0.9	15.2 ± 0.8	14.2 ± 0.9	45.4 ± 1.3
<i>Staph. mitis</i>	0	0	0	0	44.3 ± 0.9
<i>Strep. dysagalactiae</i>	12.2 ± 0.9	11.6 ± 0.8	10.6 ± 0.7	10.6 ± 0.7	38.2 ± 0.2
<i>Strep. agalactiae</i>	0	0	0	0	37.3 ± 0.5
<i>Strep. uberis</i>	0	0	0	0	35.2 ± 0.2
<i>Strep. sanguinis</i>	13.0 ± 1.0	12.0 ± 0	10 ± 0	8.0 ± 0	34.7 ± 1.3
Curcuma domestica					
<i>Staph. simulans</i>	17.4 ± 1.6	15.2 ± 1.6	12.8 ± 1.5	12.4 ± 1.9	40.0 ± 0
<i>Staph. chromogenes</i>	20.6 ± 0.7	20.0 ± 0	18.0 ± 0.7	16.4 ± 0.4	47.6 ± 0.4
<i>Staph. mitis</i>	15.0 ± 0	13.2 ± 0.2	11.8 ± 0.4	10.6 ± 0.2	36.0 ± 1.1
<i>Strep. dysagalactiae</i>	21.0 ± 0.4	20.0 ± 0.4	19.2 ± 0.4	18.0 ± 0.5	36.4 ± 0.4
<i>Strep. agalactiae</i>	12.7 ± 0.7	12.7 ± 0.7	10.7 ± 0.7	9.0 ± 1.1	33.6 ± 1.8
<i>Strep. uberis</i>	12.8 ± 1.0	14.0 ± 0	11.0 ± 1	10.0 ± 0	30.7 ± 0.7
<i>Strep. sanguinis</i>	11.0 ± 0.9	13.0 ± 0	9.0 ± 0	7.0 ± 0	30.8 ± 0.8

Although in this study *Muntinga calabura* extracts were resistant to some organism (*S. mitis*, *S. agalactiae* and *S. uberis*), our previous *in vivo* study in sub-clinical mastitis cows showed an antibacterial potency. *In vivo* experiment using the aqueous extract of *Muntinga calabura* leaves as the main ingredient of teat dip antiseptic for sub-clinical mastitis cows showed that the concentration of 3% (w/v) could inhibit the penetration and the growth of bacteria into teat canals of mammary gland and reduce the somatic cell counts in milk [20].

4. Conclusion ³

In conclusions, *Ageratum conyzoides* leaves, *Piper betle* leaves, *Muntinga calabura* leaves and *Curcuma domestica* rhizomes are potential sources of antibacterial agents for mastitis treatment.

Table 2. Phytochemical profile of plants extracts

	<i>Ageratum conyzoides</i>	<i>Piper betle</i>	<i>Curcuma domestica</i>	<i>Muntinga calabura</i>
Saponin (% w/v)	3.47	4.80	3.73	3.42
Alkaloid (% w/v)	0.14	0.42	0.24	0.56
Steroid (% w/v)	1.72	1.21	1.55	2.48
Flavonoid (% w/v)	6.15	4.97	1.92	4.69
Tannin (% w/v)	42.0	36.85	41.33	53.98
Fenol (% w/v)	3.86	3.70	1.71	3.62

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