

Antibacterial Activity of Crude Extracts of *Centella asiatica* against *Staphylococcus aureus* in Bovine Mastitis

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ABSTRACT

Crude extracts of Asiatic Pennywort (*Centella asiatica* (Linn.) Urban) extracted with either ethanol or water were tested for antibacterial activity against 30 isolates of *S. aureus* from milk samples of dairy cows. The antibacterial sensitivity of crude extracts were tested by the disc diffusion test, and results showed that ethanol extracts and water extracts had average inhibition zones ranged from 6.44-6.49 and 6.54-17.72 mm in diameter, respectively. The modified resazurin microtiter-plate was used to determine the minimum inhibitory concentration (MIC), and the minimum bactericidal concentration (MBC) was determined by touching the loop from each well of MIC plate and streaking it on a mannitol salt agar. Results showed that the ethanol extracts had an MIC₅₀ value of 8 mg/mL, the water extracts of leaf powder had an MIC₅₀ value of 32 mg/mL, and the water extracts of fresh leaves had an MIC value of 32-256 mg/mL. The ethanol extracts had an MBC value of 16 mg/mL. The water extracts could not kill *S. aureus*. In conclusion, the ethanol extracts had more potential antibacterial activity than the water extracts.

Key words: antibacterial activity, bovine mastitis, *S. aureus*, *Centella asiatica*,

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INTRODUCTION

Mastitis is a common and costly disease in the dairy farm. Infection of the udders is predominantly caused by bacteria, and induces inflammation of the mammary gland leading to decreased milk production and impaired milk composition (Harmon, 1996). Bacterial infected quarters release several mediators, many of which are chemotactants for leucocytes, especially neutrophils. The neutrophils move rapidly from the blood circulation into the infected quarters, causing an increase of somatic cells in milk. Clinical mastitis is characterized by abnormal milk, udder swelling and systemic signs including fever, lethargy and anorexia (Harmon, 1996). Subclinical mastitis shows no visible changes in the milk or the udder but decreased milk production, and bacteria are presented in the secretion (Erskine, 2001). *Staphylococcus aureus* is one of the most important and prevalent contagious pathogens. It causes both clinical and subclinical mastitis and spread directly from one infected quarter to another or between cows during the milking process (Harmon, 1996). Antibiotics for treatment of *S. aureus* infection in bovine mastitis include amoxicillin - clavulanic acid, penicillin and cephalosporins. The most common antibiotic treatment is intramammary infusion into infected quarters of the udder and intramuscular injection (Barkema et al., 2006). However, the success rate of antibiotic treatment is low because *S. aureus* usually penetrates the mammary gland tissue, forms abscesses and finally forms scar

tissues (Belschner et al., 1996, Erskine et al., 2003). This may impair penetration of antibiotics to the infected tissue of the udder. The conventional antibiotic treatment has been incriminated as a catalyst for resistance in bacteria isolated from treated animals, other animals within the herd, and food derived from cattle for human consumption (NMC, 2004) and as a cause of illegal antibiotic residue in marketed milk. Because of the increasing occurrence of antibiotic resistance, the use of medical herbs could be an alternative treatment as to replace antibiotics, which is preferable for health of animals and humans.

Centella asiatica (Linn.) Urban is the herb that has a wide range of medical properties for curing illness or disease. Its common name is Asiatic Pennywort and is known as Buabok in Thai. *Centella* is in the family apiaciae or umbelliferae, genus centella and species asiatica. It has been used for centuries as traditional medicine in India and oriental countries for treatment of mental fatigue, anxiety, epidermal wound, eczema and leprosy (Guo et al., 2004). It also inhibits growth of *Staphylococcus spp.* and reduces inflammation (Department of Pharmaceutical Botany, 1996). The most prominent group of biologically active compounds is the triterpenes (Wijeweera et al., 2006) which consist of asiatic acid, madecassic acid and asiaticoside. The asiatic acid is an aglycone of asiaticoside isolated from the plant *Centella asiatica*, commonly used for wound healing, anti-bacterial and potentially anti-fungal, anti-oxidizing and anti-free radical protection, dermis

reconstruction by stimulates the collagens synthesis, anti-aging by reinforcing the bio-mechanic properties of mature skins. (www.mdidea.com, 2006)

The main objective of this study was to evaluate antibacterial activity of *Centella asiatica* crude extracts against *Staphylococcus aureus*.

MATERIALS AND METHODS

Preparation of Crude Extracts

Centella asiatica was collected from Nakhon-Pathom Province, Thailand. Only the leaf portion was used. The fresh leaves were dried at room temperature, and were further dried at 50°C in the hot air oven for 24 hours. The dried leaves were finely ground into powder. The leaf powder (500 g) was extracted with 95% ethanol and macerated. To obtain the crude extracts (242 g), ethanol was removed under vacuum by using the rotary evaporators (BUCHI Rotavapor R-205, Switzerland). For the water crude extracts, the leaf powder (500 g) and the fresh leaves (500 g) were boiled in distilled water under reflux for one hour. The solution was then filtered and freeze-dried (Telstar Lyoalfa 6, Spain) to obtain the powder of the extracts of 19 g and 13 g for leaf powder and fresh leaves, respectively. All crude extracts were kept at -20°C until use.

Culture of *S. aureus*

Four isolates of *S. aureus* were collected from milk samples of dairy cows and twenty six

isolates were obtained from the National Institute of Animal Health, Thailand. *S. aureus* ATCC 25923 (Medimark, France) was cultured and used as a reference strain. The strains were confirmed using coagulase plasma rabbit with EDTA (BBL, U.S.A.) and mannitol salt agar (MSA; Merck, Germany).

Antibacterial activity test

The antibacterial sensitivity was tested by the disc diffusion test. The modified resazurin assay in microtiter-plate was used to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) according to the guidelines recommended by the NCCLS and CLSI (NCCLS, 1993, CLSI, 2007).

Screening of crude extract for antibacterial activity by disc diffusion test

Disc diffusion test was performed using sterile 6 mm-diameter filter paper discs (Whatman, Maidstone, UK). The discs were prepared using 10 µL of crude extract diluted in the solvent to concentrations of 1,000 mg/mL, 800 mg/mL, 400 mg/mL, 200 mg/mL, 100 mg/mL and 50 mg/mL respectively; thus, each disc contained 10 mg, 8 mg, 4 mg, 2 mg, 1 mg and 0.5 mg of crude extract, respectively. All discs were dried at room temperature overnight. At least three isolated colonies of the same morphological type were selected from blood agar culture. The top of each colony was touched with a wire loop and the growth transferred to a tube containing 5 mL of Mueller-Hinton broth (MHB; Merck, Germany). The broth

culture was incubated at 37 °C (usually 3 to 5 hours) until the turbidity of the 0.5 McFarland standard using the McFarland densitometer (Grant-bio, U.K.), this resulted in a suspension containing approximately 1 to 2×10^8 CFU/mL. The 0.5 McFarland suspension should be 150 μ L in 5 mL of Mueller-Hinton agar (MHA; Merck, Germany) and mixed to pour plate, the final inoculum on the agar would be approximately 10^6 CFU/mL. The discs were placed on the surface of the inoculum Mueller-Hinton agar (MHA) and incubated at 37 °C 18 to 24 hours. Pure DMSO and sterile water were used as a negative control while amoxicillin-clavulanic acid (30 μ g; Oxoid) and cephalotin (30 μ g; Oxoid) were used as a positive control. The disc diffusion test was determined by measuring the diameter of the inhibition zone. Tests were performed in triplicate and the mean of the diameters of the inhibition zones was calculated.

Determination of the MIC of crude extracts by the modified resazurin assay

The modified resazurin assay was performed using sterile 96 well plates (Greiner bio-one, Germany) for determining the minimum inhibitory concentration (MIC). Fifty microliters of Mueller-Hinton broth (MHB) was added into all wells. The stock concentration of Asiatic acid was 10.24 mg/mL in dimethylsulfoxide (DMSO; AMRESCO, U.S.A.). Fifty microliters of the initial concentration of Asiatic acid and crude extracts prepared at 10.24 mg/mL and 1024 mg/mL, respectively, were added into the first well. Then, 50 μ L from their serial

dilutions were transferred into nine consecutive wells using a multichannel pipette. The positive control was added with DMSO, 95% ethanol and sterile water, and the last blank well is the negative control. Finally, 50 μ L of the 0.5 McFarland suspensions were inoculated into all wells. The plates were incubated at 37°C for 18 to 24 hours. After incubation, bacterial growth was evaluated by adding 50 mL of 5 mg of one resazurin tablet (BDH, England) in 50 μ L of sterile water and the plates were left for one hour for extending incubation. The colour change was then assessed visually from purple to pink and compared to the negative control plate, which appeared visually in dark green colour, while pink and orange colours were a positive control plate. The lowest concentration at which colour change was recorded as the MIC values that inhibited the bacteria growth.

Determination of the MBC of crude extracts by the mannitol salt agar

The minimum bactericidal concentration (MBC) was determined by touching the loop from each well of MIC plate and streaking it on a Mannitol Salt Agar (MSA) and incubated at 37°C for 18 to 24 hours. Unchanged media colour was defined as the lowest concentration that killed the bacteria. The yellow colour of media showed *S. aureus* growth.

Statistical analysis

All statistical analyses were performed using NCSS 2007 software. Comparison of the inhibition

zone between control and the extracts was performed using ANOVA with Duncan's multiple comparison test. Inhibition zone are expressed as mean \pm SD.

RESULTS

The yields (%) of crude extracts from *Centella asiatica* are presented in Table 1. The ethanol extraction of leaf powder yielded crude extracts twice as high as the water extraction of fresh leaves and about six times of the water extraction of leaf powder.

Disc diffusion test

The antibacterial activity of crude extracts against *S. aureus* ATCC 25923 was investigated by disc diffusion test. The results of inhibition zone are presented in Table 2. The DMSO and sterile water used as negative controls showed no inhibitory effect. The positive controls of amoxicillin-clavulanic acid and cephalotin showed inhibition zones ranging from 24 to 29 mm. The inhibition zone of ethanol extracts differed from that of control at 8 mg/disc. The inhibition zone of water extracts of leaf powder differed from that of control at 4 mg/disc. The inhibition zone of water extracts of

Table 1 The yields of crude extracts from *Centella asiatica*.

Crude extracts	Extract yield (%) of fresh leaves
The ethanol extracts of leaf powder	6.05
The water extracts of leaf powder	0.47
The water extracts of fresh leaves	2.60

Table 2 Antibacterial activity, measured as mean (\pm SD) of inhibition zone (mm), of crude extracts against *S. aureus* ATCC 25923.

Crude Extracts	Concentration of crude extracts (mg/disc)					
	0.5	1	2	4	8	10
Control	6.00 \pm 0.00 ^a	6.00 \pm 0.00 ^a	6.00 \pm 0.00 ^a	6.00 \pm 0.00 ^a	6.00 \pm 0.00 ^a	6.00 \pm 0.00 ^a
The ethanol extracts	6.00 \pm 0.00 ^a	6.00 \pm 0.00 ^a	6.00 \pm 0.00 ^a	6.00 \pm 0.00 ^a	6.44 \pm 0.73 ^{*a}	6.49 \pm 0.73 ^{*a}
The water extracts of leaf powder	6.00 \pm 0.00 ^a	6.00 \pm 0.00 ^a	6.00 \pm 0.00 ^a	6.54 \pm 1.10 ^{*a}	10.22 \pm 3.95 ^{*b}	10.24 \pm 1.11 ^{*b}
The water extracts of fresh leaves	6.98 \pm 2.05 ^{*a}	9.99 \pm 1.39 ^{*b}	12.59 \pm 2.93 ^{*b}	15.06 \pm 1.00 ^{*b}	17.72 \pm 1.22 ^{*c}	17.16 \pm 1.47 ^{*c}

* shows significant differences between each extract and control.

^{a, b, c} different superscripts within the same column show significant differences between concentrations in each plant extract.

fresh leaves differed from that of control at 0.5 mg/disc and the most effective inhibition was observed at 8 and 10 mg/disc.

Minimum inhibitory concentration

The MIC values of asiatic acid and crude extracts were determined by the modified resazurin microtiter-plate (Figure 1). The DMSO and 95% ethanol used as controls showed no inhibitory

effect. The asiatic acid showed the strongest inhibitory effect against all strains with the MIC₅₀ value of 0.02 mg/mL (Figure 1A). The ethanol extracts show MIC₅₀ value of 8 mg/mL (Figure 1B), the water extracts of leaf powder showed MIC₅₀ value of 32 mg/mL (Figure 1C) and the water extracts of fresh leaves showed MIC value of 32-256 mg/mL (Figure 1D).

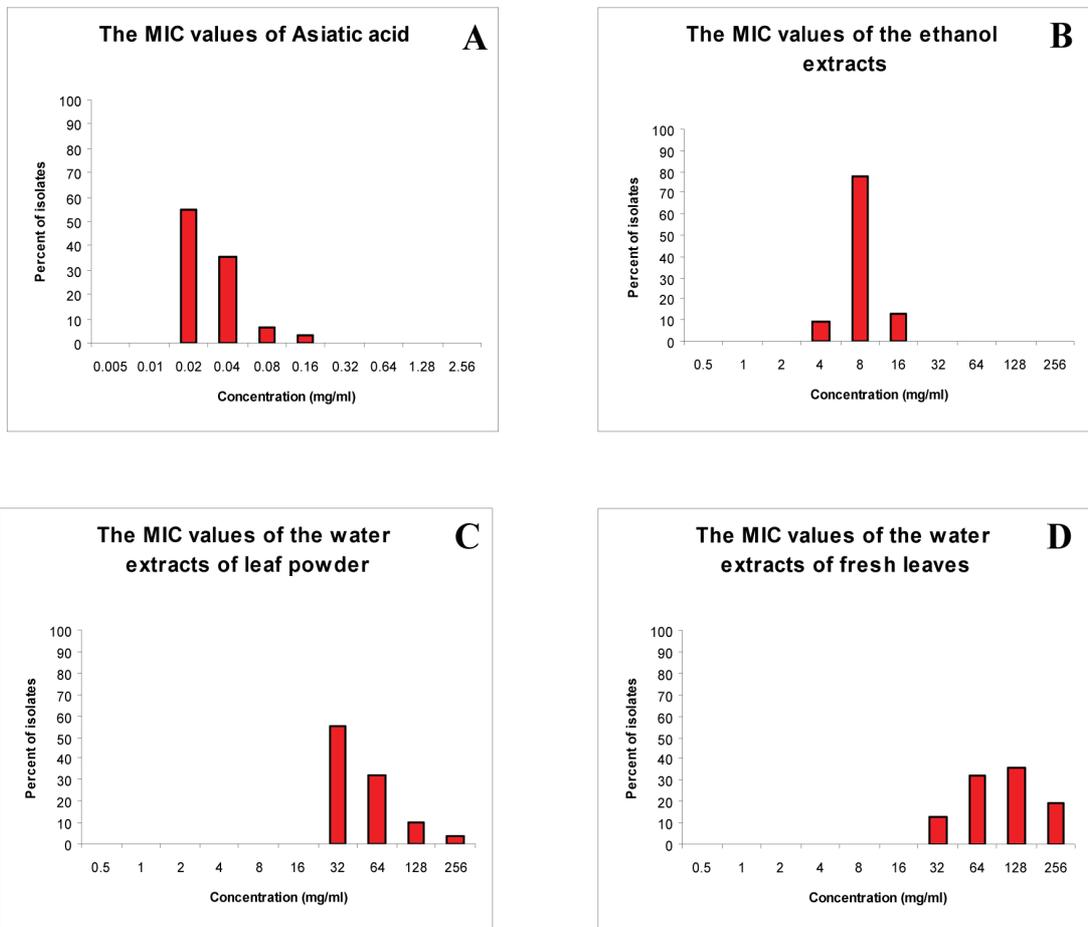


Figure 1 The MIC values of Asiatic acid (A), and crude extracts (B, C, D) against 31 isolates of *S. aureus*

Minimum bactericidal concentration

The MBC values of asiatic acid and crude extracts were determined by touching the loop from each well of MIC plate and streaking it on a Mannitol Salt Agar (MSA) and were incubated at 37°C for 18 to 24 hours. The DMSO and 95% ethanol as controls could not kill *S. aureus*. The MBC value of Asiatic acid ranged from 0.02-0.04 mg/mL (Figure 2A) and the MBC value of ethanol extracts was 16 mg/mL (Figure 2B). The water extracts of leaf powder and the water extracts of fresh leaves could not kill *S. aureus*.

DISCUSSION

In this study, *Centella asiatica* could be extracted with either ethanol or water. However, extraction using ethanol had a greater yield as compared to water extraction. The ethanol could be, therefore, an effective solvent for the extraction of *C. asiatica*.

For the antibacterial activity test, the water extracts of fresh leaves had inhibition zones of 6.98 mm at 0.5 mg, 9.99 mm at 1 mg, 12.59 mm at 2 mg, 15.06 mm at 4 mg, 17.72 mm at 8 mg and 17.16 mm at 10 mg. The strongest inhibitory effect was observed at 8 and 10 mg/disc. The ethanol extracts had inhibition zones of 6.44 mm at 8 mg and 6.49 mm at 10 mg which was ineffective.

Conversely, the ethanol extracts had an inhibitory effect against all studied strains of *S. aureus* with the MIC₅₀ value of 8 mg/mL and the MBC value of 16 mg/mL. The water extracts had MIC value of 32-256 mg/mL and these concentrations could not kill *S. aureus*.

The disc diffusion is a conventional method for screening antibacterial activities test of natural product. The microdilution broth in microtiter-plate is the method for determine the minimum inhibitory concentration (MIC). Results of the disc diffusion test differed from results of modified resazurin

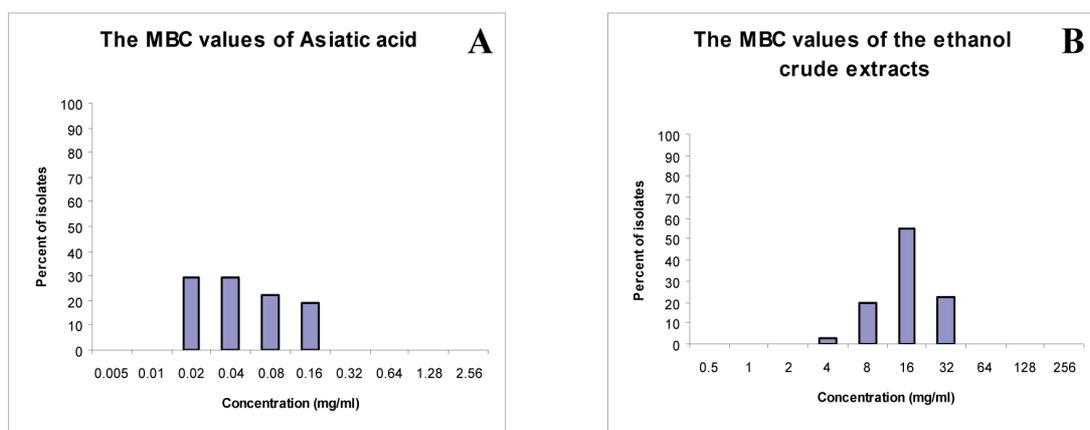


Figure 2 The MBC values of Asiatic acid (A), and crude extracts (B) against 31 isolates of *S. aureus*

microtiter-plate, and a possible explanation was due to the diffusion factor of substance through the agar. The substance in the disc diffusion test did not directly touch *S. aureus*, while in the well of microtiter-plate, the substance directly touched *S. aureus*. Moreover, the disc diffusion method used a larger amount of the crude extracts as compared to the microtiter-plate method. Resazurin is an indicator for detect the growth of *S. aureus* in microtiter-plate because it can cope with the conceal from the colour of crude extracts (Sarker et al., 2007). Another report used INT (*p*-iodonitrotetrazolium violet) as an indicator to study antibacterial activity of medicinal plants (Pithai et al., 2003).

Phadet et al. (2002) reported that the inhibition zones of *C. asiatica* crude extracts against *S. aureus* were 6.95 mm of the ethanol extracts and 10.80 mm of the water extracts. For *S. aureus* ATCC 25923, they reported that the inhibition zones of the ethanol extracts were 7.15 mm and that of the water extracts were 10.20 mm, which were similar to our results. The results of MIC and MBC values were less than 5 mg/mL for the ethanol extracts and 2.5 mg/mL for the water extracts (Phadet et al., 2002), which were lower than MIC and MBC values in our study. The inhibitory effect will have lower MIC and MBC values. The differences in MIC and MBC values may be resulted from the method of extraction, purity of crude extracts, method for studied MIC and MBC values (the agar dilution method) and strain of pathogens.

Furthermore, Thai medical plants have been

also studied for antibacterial activity against *S. aureus* and other bacterial strains from mastitic pathogens such as *Garcinia mangostana*, *Punica granatum* and *Psidium guajava*. Mullika et al (2008) reported *Garcinia mangostana* extracts showed the inhibition zones of 11.3 mm against *S. aureus*, 10.50 mm against *S. epidermidis* and 10.00 mm against methicillin-resistant *Staphylococcus aureus* (MRSA). The MIC value of 39 µg/mL against all strains of *S. aureus* (Mullika et al., 2008) which is in agreement with the report of Voravuthikunchai and Kitpipit (2005) that the inhibition zones of *Garcinia mangostana* ethanol extracts were 10.43 mm against MRSA and 11.00 mm against *S. aureus* ATCC 25923. The inhibition zones of *Punica granatum* ethanol extracts were 16.70 mm against MRSA and 17.00 mm against *S. aureus* ATCC 25923. The MIC values of *Garcinia mangostana* ethanol extracts were 0.05-0.4 mg/mL against MRSA and 0.1 mg/mL against *S. aureus* ATCC 25923. The MIC values of *Punica granatum* ethanol extracts were 0.2-0.4 mg/mL against MRSA and 0.2 mg/mL against *S. aureus* ATCC 25923.

In conclusion, results from extract yield percentage indicated that the 95% ethanol could be the most effective solvent for the extraction. For antibacterial test, the modified resazurin microtiter-plate was appropriate to test antibacterial activity in crude extracts. Then, the ethanol extracts could inhibit and kill *S. aureus* rather than the water extracts. However, the present report of antibacterial activity of crude extracts from *C. asiatica* was based on an *in vitro* study. It is

therefore necessary to test this activity in an *in vivo* study. Furthermore, to have better application of the crude extracts in bovine mastitis, other bacterial strains from mastitis pathogens should be tested in the future.

ACKNOWLEDGEMENTS

This research is funded by the Center of Excellence on Agricultural Biotechnology Science and Technology, Postgraduate Education and Research Development Office, Commission on Higher Education, Ministry of Education and the Kasetsart University Research and Development Institute. We would like to thank the staffs of Faculty of Veterinary Medicine, Kasetsart University for the technical assistance.

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