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Rhodomyrtus tomentosa (Aiton) Hassk. leaf extract: An alternative approach for the treatment of staphylococcal bovine mastitis

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ABSTRACT

Antibiotic residues in dairy products as well as emergence of antimicrobial resistance in foodborne pathogens have been recognized as global public health concerns. The present work was aimed to study a potent antibacterial extract from natural product as an alternative treatment for staphylococcal bovine mastitis. Staphylococcal isolates (n = 44) were isolated from milk samples freshly squeezed from individual cows. All staphylococcal isolates were resistant to ampicillin, ciprofloxacin, erythromycin, gentamicin, penicillin, except vancomycin. *Rhodomyrtus tomentosa* leaf ethanolic extract was accessed for its antibacterial activity and anti-inflammatory potential. The extract exhibited profound antibacterial activity against all of staphylococcal isolates with MIC and MBC values ranged from 16–64 μg/ml and 64–>128 μg/ml, respectively. Moreover, the extract also exerted anti-protein denaturation and human red blood cell membrane stabilizing activity. The results support the use of *R. tomentosa* extract that could be applied to cure bovine mastitis and to reduce inflammatory injury caused by the bacterial infections.

1. Introduction

Bovine mastitis is a frequent cause of economic loss in dairy cows, which adversely affects animal health, impinges on quality of milk, and reduces milk production. The disease is defined as inflammation of mammary gland tissue. It is also characterized by abnormalities of udders and changes in milk composition including increase in somatic cell counts (Galal Abdel Hameed et al., 2006). Mastitis begins after organisms pass through the teat canal. Invasion of the teat usually occurs during milking. Major mastitis causing microorganisms are coagulase-negative staphylococci, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Escherichia coli*, *Corynebacterium* sp., and *Klebsiella* sp. (Sharma et al., 2012).

S. aureus is one of the most common mastitis pathogens causing contagious and environmental mastitis (Saidi et al., 2013). It is associated with sub-clinical and clinical bovine mastitis. Recently, *S. aureus* has been recognized as facultative intracellular pathogen by its abilities to adhere and internalization within bovine mammary epithelial cell and phagocytic cells (Atalla et al., 2010). It can produce adherence factors and virulence toxins involved in pathogenesis of bovine cells such as endotoxin, exotoxin, α-toxin, and haemolysin. Intramammary infection of *S. aureus* is often causing tissue damage and inflammatory process in host (Craven et al., 2009). Moreover, it is also known as one of the most important agents in dairy products by accidentally mixed into bulk milk and can be transmitted to human (Sasidharan et al., 2011).

Emergence of antibacterial resistance in foodborne pathogens has increased mainly as a result from irrational antibiotics used in livestock. The problem of *S. aureus* bovine mastitis is becoming worse due to widespread of multidrug-resistant strains (Sampimon et al., 2011). There have been reports on staphylococcal isolates obtained from mastitis resistant to existing antibiotics (Unakal and Kaliwal, 2010; Wang et al., 2013). Antibiotic residue and disinfectant agents accumulate in milk due extensive use in the treatment and control of the disease. It adversely affects environment, animal, human health, economy, and international food trade (Galal Abdel Hameed et al., 2006). Several non-traditional antimicrobial agents or medicinal plants have been tested in the recent years, in order to reduce the settlement of antibiotic residue in milk. *Rhodomyrtus tomentosa* (Aiton) Hassk. is a plant species belonging to the family Myrtaceae. Many recent studies have reported that ethanolic extract of *R. tomentosa* leaves has pronounced effect against Gram-positive bacteria such as *S. aureus*, *Staphylococcus epidermidis*, methicillin-resistant *S. aureus* (MRSA) (Saising et al., 2008), *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Streptococcus mutans*, *Enterococcus faecalis*, *Bacillus subtili*, and *Bacillus cereus* (Limsuwan et al., 2009; Voravuthikunchai et al., 2010). However, anti-inflammatory potential of the ethanolic extract has not been studied.

Therefore, the present work was aimed to further investigate the ability of *R. tomentosa* extract to eradicate staphylococci in bovine mastitis as well as its anti-inflammatory potential.

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2. Materials and methods

2.1. Bacterial isolation and culture condition

Staphylococcal isolates (n = 44) were isolated from milk samples freshly squeezed from individual cows in dairy farms located in Phatthalung province, Thailand. Identification of isolates was based on colony characteristics on mannitol salt agar (MSA, Merck, Germany), Gram staining, and catalase reaction. Coagulase test was used to differentiate between coagulase-positive and coagulase-negative staphylococcal isolates. Out of 44 isolates, 18 and 26 isolates were identified as coagulase-positive and coagulase-negative staphylococci, respectively. In addition, *S. aureus* ATCC 29213 was included as a reference strain. All bacterial isolates were cultured on tryptic soy agar (TSA, Becton Dickinson, Difco, France) at 37 °C for 18–24 h.

2.2. Antibiotic susceptibility testing

All staphylococcal isolates were tested for susceptibility to antibiotic drugs by agar disk diffusion method according to Clinical and Laboratory Standards Institute guideline (CLSI, 2011). Six antibiotics were included in this study, which are most prescribed and used for treatment of bovine mastitis in dairy farms. Aminoglycosides were represented by gentamicin (10 μg), fluoroquinolones by ciprofloxacin (5 μg), while beta-lactamic drugs were represented by penicillin (10 units) and ampicillin (10 μg). Macrolides were represented by erythromycin (15 μg) and glycopeptide by vancomycin (30 μg). All of the drugs were purchased from Oxoid, UK. After measuring the growth inhibition zone, the isolates were categorized as sensitive or resistant by following the recommendations of CLSI (2011). Statistically significant difference between the two groups of staphylococci was analyzed using one-way analysis of variance (ANOVA). A probability *p*-value less than 0.05 was considered different significantly.

2.3. Preparation of R. tomentosa ethanolic extract

R. tomentosa leaves were dried in oven at 60 °C for 48 h and ground in an electric blender. Dried leaf powder was extracted with 95% ethanol at room temperature for 7 days. The extract was evaporated using a rotary evaporator (BUCHI Rotavapor R-114, Büchai Labortechnik AG, Switzerland) until it was completely dried and kept at 4 °C until use. The ethanol extract was dissolved in 10% dimethyl sulphoxide (DMSO, Sigma, Germany) and used for further study.

2.4. Antibacterial activity of R. tomentosa extract

Antibacterial activity of *R. tomentosa* ethanolic extract was determined by agar disk diffusion method (CLSI, 2011). Fresh 3–5 colonies of staphylococci on TSA were inoculated in Mueller–Hinton broth (MHB, Difco) and incubated at 37 °C for 3–5 h. The bacterial suspensions were adjusted to McFarland turbidity standard No. 0.5 and spread on Mueller–Hinton agar (MHA, Difco) plates. The ethanol extract was dissolved in DMSO. Ten microliter of the extract from the stock solution (250 mg/ml) was loaded onto sterile filter paper disk (diameter 6 mm). The disks containing the ethanol extract were placed on seeded plates and incubated at 37 °C for 18 h. The diameters of inhibition zones (mm) were measured and the mean was calculated. The disk containing DMSO was used as a negative control. The assay was performed in triplicate.

2.5. Determination of minimum inhibitory concentration (MIC)

Antimicrobial activity was measured by determining the minimum inhibitory concentration of the extract needed to inhibit the growth of bacteria, a value called MIC, was evaluated by broth microdilution method according to CLSI (2011). In 96-well microtiter plate, 20 μl of the ethanolic extract was added and diluted by two-fold serial dilution to obtain final concentration ranging from 4–256 μg/ml. Total volume was adjusted to 100 μl by adding 80 μl of MHB in each wells, followed by 100 μl of the bacterial suspensions (10^6 CFU/ml). The plates were incubated at 37 °C for 18 h. Vancomycin was used as a standard drug. The experiment was performed in triplicate.

2.6. Determination of minimum bactericidal concentration (MBC)

Minimum bactericidal concentration (MBC) was determined subsequently to the MIC assays. MBC is the lowest concentration of the extract necessary for the complete suppression of bacterial growth. An aliquot of 0.1 ml from the wells with MIC was seeded on TSA and incubation at 37 °C for 18 h. The appearance of bacterial growth on the agar plate was evaluated for the MBC determination. The experiment was carried out in triplicate.

2.7. Human red blood cell (HRBC) membrane stabilizing activity

Membrane stabilizing activity assay was carried out as reported previously (Debnath et al., 2013). Alsever's solution was prepared by dissolving 2% dextrose, 0.8% sodium citrate, 0.5% of 1 M citric acid, and 0.42% sodium chloride in distilled water followed by sterilization. Blood was collected from healthy volunteers and mixed with equal volumes of Alsever's solution. The blood was centrifuged (Centrifugen 5804, Eppendorf, Hamberg, Germany) at 3000 rpm for 20 min. Debris of packed cells was washed with 0.9% sodium chloride and suspended in normal saline solution (0.85% sodium chloride) to attain a suspension density of 6×10^{12} red blood cells/ml. *R. tomentosa* ethanolic extract was tested with concentration ranging from 8–256 μg/ml. The assay mixture contained 1 ml of the extract, 1 ml phosphate buffer, 2 ml hyposaline (0.25% sodium chloride), and 0.5 ml HRBC suspension. Diclofenac sodium was used as a standard drug and 2 ml of distilled water was used as a control which produces 100% of haemolysis. All the assay mixtures were incubated at 37 °C for 30 min and centrifuged at 3000 rpm for 20 min. The hemoglobin content in the supernatant solution was estimated using UV–Visible spectrophotometer (*GENESYS* 10S UV–vis) at 560 nm. Percentage inhibition of haemolysis was calculated using the following equation: (OD of control − OD of treated sample / OD of control) × 100. Statistical analysis was performed using *t*-test for dependent samples. *p*-Values less than 0.05 were considered significant difference between the standard drug and the ethanolic extract effect.

2.8. Anti-protein denaturation activity

The method of Williams et al. (2008) was employed for anti-protein denaturation assay. A solution of 0.2% (w/v) bovine serum albumin (BSA) was prepared in Tris buffer saline and pH was adjusted to 6.8 using 1 M glacial acetic acid. Solutions of 8–256 μg/ml of *R. tomentosa* ethanolic extracts were prepared using DMSO as a solvent. Five hundred microliters of each concentration of the extract was added to tubes containing 4.5 ml of BSA solution. The control consists of 4.5 ml of BSA solution and 0.5 ml of 1% DMSO. The standard consists of 0.5 ml diclofenac sodium (8–256 μg/ml) in 4.5 ml of BSA solution. Test mixtures were heated at 72 °C for 5 min and then cooled for 10 min at room temperature. The absorbance of the mixtures was determined by UV–Visible spectrophotometer (*GENESYS* 10S UV–vis) at 660 nm. The percentage inhibition of protein denaturation was determined on a % basis relative to the control using the following formula: % inhibition of protein denaturation = 100 − (OD of control − (OD of tested − OD of extract) / OD of control) × 100. The experiment was carried out in triplicate.