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NAME: Mrs. Duangkamol Taemchuay

THIS THESIS HAS BEEN ACCEPTED BY

(Associate Professor Theera Rukkwamsuk, Ph.D.)
THESIS ADVISOR

(Assistant Professor Thavajchai Sakpuaram, Ph.D.)
THESIS CO-ADVISOR

(Assistant Professor Nongluck Ruangwises, Ph.D.)
THESIS CO-ADVISOR

(Associate Professor Pongthep Akratanakul, Ph.D.)
GRADUATE COMMITTEE CHAIRMAN

APPROVED BY THE GRADUATE SCHOOL ON _____

(Associate Professor Gunjana Theeragool, D.Agr.)
DEAN

THESIS

A STUDY ON *CENTELLA ASIATICA* LINN. (URBAN) LEAF EXTRACTS AGAINST *STAPHYLOCOCCUS AUREUS* IN BOVINE MASTITIS

DUANGKAMOL TAEMCHUAY

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The study aimed to identify asiatic acid in crude extracts of *C. asiatica* and to test the antibacterial activity of crude extracts against *S. aureus*.

Thin Layer Chromatography (TLC) method was developed to determine asiatic acid in *C. asiatica* using chloroform, methanol, ethyl acetate and water (30:5:5:1) as the mobile phase. The developed plate was sprayed with anisaldehyde-sulphuric acid reagent and the plate was determined asiatic acid with densitometer. The percentage of recovery was 99.70%, the detection limit was 0.76 ng and the quantitation limit was 2.60 ng.

Crude extracts of *C. asiatica* were tested for antibacterial activity against 30 isolates of *S. aureus* from milk samples of dairy cows. The antibacterial activity of crude extracts was tested by the disc diffusion test. The ethanol extracts and water extracts of *C. asiatica* had average inhibition zones ranged from 6.44-6.49 and 6.54-17.72 mm in diameter, respectively. The minimum inhibitory concentration (MIC) was determined by the modified resazurin microtiter-plate. 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. The ethanol extracts had an MIC_{50} value of 8 mg/ml, the water extracts of leaf powder had an MIC_{50} 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*. Three DNA patterns of *S. aureus* were identified from 30 isolated from bovine mastitis by polymerase chain reaction (PCR) technique based on *coa* gene. The sequences of 30 isolates, representing 10 groups were determined. The 30 isolated were also tested for susceptibility to antimicrobial drugs using the agar disc diffusion test. The highest resistance was observed in β-lactam antibiotics, penicillin and ampicillin.

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Duangkamol Taemchuay

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LIST OF ABBREVIATIONS

µl	=	microliter
ml	=	milliliter
L	=	liter
ng	=	nanogram
µg	=	microgram
mg	=	milligram
g	=	gram
kg	=	kilogram
ppm	=	part per million
nm	=	nanometer
mm	=	millimeter
µM	=	micromolar
mM	=	millimolar
M	=	molar
bp	=	base pair
°C	=	Degree Celsius
sec	=	second
min	=	minute
hr	=	hour
rpm	=	round per minute
SCC	=	Somatic Cell Count
BTSCC	=	Bulk tank (milk) somatic cell count
DNA	=	Deoxyribonucleic acid
dNTP	=	deoxynucleotide triphosphate
DNase	=	deoxynuclease
PCR	=	Polymerase Chain Reaction
UV	=	ultraviolet
CFU	=	colonies forming unit
EDTA	=	ethylenediamine tetraacetic acid
TLC	=	Thin Layer Chromatography

LIST OF ABBREVIATIONS (continued)

HPTLC	=	High Performance Thin Layer Chromatography
CV	=	coefficient of variation
RSD	=	relative of standard deviation
USP	=	United States Pharmacopoeia
r^2	=	correlation coefficient
R_f	=	retention factor
ANOVA	=	Analysis of Variance
US	=	United State
EU	=	European Union

A STUDY ON *CENTELLA ASIATICA* LINN. (URBAN) LEAF EXTRACTS AGAINST *STAPHYLOCOCCUS AUREUS* IN BOVINE MASTITIS

INTRODUCTION

Mastitis is a common and costly disease in the dairy farm. The costs associated with the disease include discarded abnormal milk and milk withheld from cows treated with antibiotic, early replacement of affected cows, reduced sale value of culled cows, drug costs and veterinary services, increased labor costs and most important, reduced milk production and decreased quality of milk (DeGraves and Fetrow, 1993). Infection of the udders is predominantly caused by bacteria, and induce inflammation of the mammary gland leading to decreased milk production and impaired milk composition (Harmon, 1996). In the dairy cattle, mastitis may be in clinical and subclinical forms. 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 in milk production, and bacteria are presented in the secretion (Erskine, 2001). Both clinical and subclinical mastitis caused an increase in milk somatic cell count and changes in milk composition (Forsback *et al*., 2009).

The most common bacteria causing udder infection are *Staphylococcus aureus*, *Streptococcus agalactiae*, *St. dysgalactiae*, *St. uberis* and *Escherichia coli*, though other pathogens such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Corynebacterium pyogenes* can cause occasional herd outbreaks. The pathogenic bacteria can be devided in to two groups based on their sources, which are contagious pathogens and environmental pathogens. Contagious pathogens are well adapted to survive and grow in the mammary gland and frequently cause infections lasting weeks, months or years. The infected quarter is the main source of these organisms in a dairy herd, and transmission to uninfected quarters and cows occurs mainly during milking time.

S. aureus is one of the most important and prevalent contagious pathogens. It causes both clinical and subclinical mastitis and can infect udders, and spread directly from one infected quarter to another or between cows during the milking process (Harmon, 1996). These pathogens can colonise and multiply in teat ducts and this greatly increases the bacteria in the udder. They usually cause chronic infections which persist in the subclinical form and occasionally become clinical mastitis (Sutra and Poutrel, 1994). The most common antibiotic treatment is intramammary infusion into infected quarters of the udder and intramuscular injection (Barkema *et al*., 2006). Antibiotics for treatment of *S. aureus* infection include amoxicillin-clavulanic acid, penicillin and cephalosporins. 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.

Currently, medical herbs are interested in searching for a new alternative treatment from natural resources. Many medicinal plants have been employed since prehistoric times. Those plants are continued to be used within the framework of traditional medicine as an effective disease remedy. The use of medical herbs could be replaced antibiotics for treatment the disease, and also resulted in the reduction of antibiotic resistance and antibiotic residue in marketed milk which is preferable for health of animals and humans. Moreover, it is a good way to save the money and promote the usage of available natural resources within the country.

Centella asiatica (Linn.) Urban is the herb that has a wide range of medical properties for treatment of illness or disease. Its common name is Asiatic Pennywort and is known as Buabok in Thailand. *Centella* is in the family apiaciae or umbelliferae, genus centella and species asiatica. The most prominent group of biologically active compounds are the triterpenes (Wijeweera *et al*., 2006), which

consist of asiatic acid, madecassic acid and asiaticoside. 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).

Moreover, medicinal plant extracts have been increasingly used in the food supplements, cosmetics, pharmaceutical and medicines. However, it is important to prove the efficacy of medical herbs or isolated natural compounds which are considered to be the active compounds and to study their antibacterial activity of medicinal plant. To develop the herbal drug scientifically, the active compounds containing in the herb should be identified and quantified. For identification and quantitation of the interest active compounds, an appropriate analytical method which can simultaneously detect the analyte, must be developed. The chromatographic method is usually applied to separation and quantitation of the analyte in natural product extract. In addition, thin layer chromatographic (TLC) method is also widely used due to the simplest and rapid analysis.

Therefore, this experiment developed appropriate method of extraction, quantification methods for analyte the asiatic acid in *C. asiatica* and studied antibacterial activity of *C. asiatica* against *S. aureus*. The results of this study can be applied for treatment of the bovine mastitis in the future.

OBJECTIVES

1. To develop the method for crude extraction of *Centella asiatica*

2. To identify and quantify an active compound in the crude extracts of *Centella asiatica*

3. To study antibacterial activity of crude extracts against *Staphylococcus aureus*

4. To study antimicrobial susceptibility test of *Staphylococcus aureus*

5. To study genotype of *Staphylococcus aureus* isolated from bovine mastitis

LITERATURE REVIEW

1. Bovine Mastitis Disease

1.1 Biology of the disease

Mastitis is an inflammation of the mammary gland that most frequently develops in cows in response to intramammary bacterial infection. Mastitis remains one of the most prevalent and costly production diseases in dairy herds worldwide (Douglas and Robert, 2005). Mastitis results in decreased milk production and impaired milk quality. The magnitude of these changes in individual cows varies with the severity and duration of the infection and the causative microorganisms. Mastitis is most frequently caused by bacteria. These microorganisms produce toxins that can directly damage milk producing tissue of the mammary gland, and the presence of bacteria initiates inflammation within the mammary tissue in an attempt to eliminate the invading microorganisms. 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 (Erskine, 2001). In general, compositional changes involve an increase in blood components present in milk and a decrease in normal milk constituents.

Clinical syndromes of mastitis are based upon the severity of the inflammatory response. Clinical mastitis is characterized by the presence of gross inflammation signs include redness udder, udder swelling, heat, pain and loss of function including decreased milk production, change in composition, and change in appearance showing the abnormal milk (Harmon, 1996). The clinical syndromes are peracute, acute, subacute and chronic mastitis. Peracute mastitis results in severe inflammation of the udder. The inflammation may result from the organism itself, enzymes from the tissue or the bacteria, endotoxin or exotoxin, and leukocyte products. The systemic illness is due to septicemia resulting in fever, anorexia, depression, decreased rumen motility, dehydration, and sometimes death of the cow.

Acute mastitis could be moderate to severe inflammation of the udder, decreased milk production, and serous milk or fibrin clots. Subacute mastitis is mild inflammation, there may be no visible changes in udder. There are generally small flakes or clots in the milk. Chronic mastitis may persist in subclinical form for months or years with occasional clinical flareups. Subclinical mastitis is the most common form of mastitis. It shows no visible changes in the milk or the udder, but decreased in milk production and bacteria are presented in the secretion (Erskine, 2001).

1.2 Mammary gland

A basic knowledge of mammary gland anatomy and physiology is necessary to understand how mastitis develops. The mammary gland of the dairy cow consists of four separate glands, each with a teat. Structure of the mammary gland showing the teat and the gland cisterns, milk ducts, and glandular tissue in Figure 1. Glandular tissue is made up of many small microscopic sacs called alveoli that are lined by milk-producing epithelial cells. There are millions of alveoli within each mammary gland. Milk synthesized in one gland cannot pass over to any of the other glands. So, milk is synthesized in the secretory cells, which are arranged as a single layer on a basal membrane in a spherical structure called alveoli. Several alveoli together form a lobule. Milk is continuously synthesized in the alveolar area, is stored in the alveoli, milk ducts, udder and teat cistern between milking. The teat consists of a teat cistern and a teat canal. Where the teat cistern and teat canal meet, 6-10 longitudinal folds form called Furstenberg's rosette, which is involved in the local defense against mastitis. The teat canal is surrounded by bundles of smooth muscle fibers, longitudinal as well as circular. Between milkings the smooth muscles function to keep the teat canal closed. The teat canal is also provided with keratin as a barrier for the pathogenic bacteria. The mammary gland is very well supported with blood vessels, arteries and vein. Blood vessels bring nutrients to each alveolus where epithelial cells convert them to milk (Schroeder, 1997).

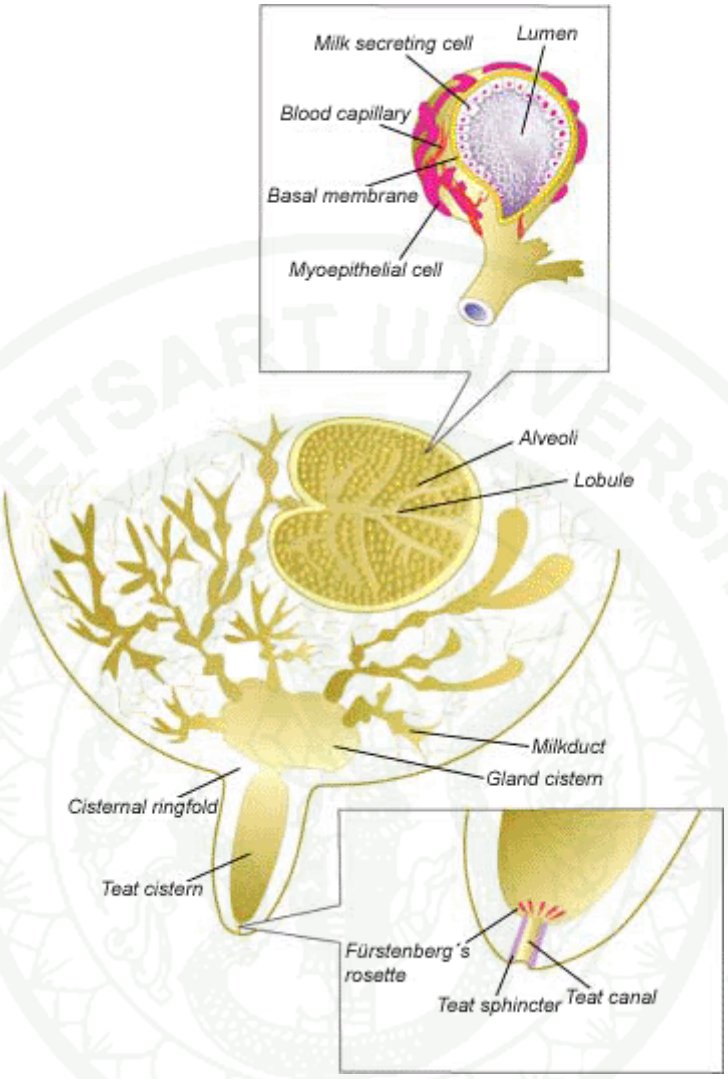


Figure 1 Structure of the mammary gland.

Source: Anonymous (2003)

1.3 Development of mastitis

Bacteria penetrate to the teat canal in several ways between milkings, bacteria pass through the teat canal by multiplying inside the canal. During machine milking, bacteria may be propelled into the teat canal, teat cistern and udder. The potential invasion is greatly increased by bacteria that reside in or colonize the teat duct. Such colonizations occur in both lactating and dry cows, and the colonizing

bacteria may survive for months, serving as sources of bacteria for infecting the gland (Auldist and Hubble, 1998).

The inherent virulence of the bacterial species is often associated with an ability to adhere to mammary epithelium. *St. agalactiae* and *S. aureus* adhere well but *E. coli* does not; however, multiplies rapidly (Auldist and Hubble, 1998). As part of the cow's defence mechanism, the new intramammary infection is quickly followed by an influx of leukocytes into udder and an increase of the somatic cell counts (Bruckmaier and Blum, 2004). The increase in permeability may be an important part of the inflammatory process as it allows immune components to reach the infection site (Nguyen *et al*., 1998). Benites *et a*l. (2002) state that the enhanced paracellular diapedesis of leukocytes through the epithelial cells causes reduced tight junction integrity and hence exchange of constituents between the blood and the milk through the paracellular pathway (Pyorala, 2003). The predominant leukocytes present in milk under such circumstances are polymorphonuclear neutrophils (PMN). They are responsible for the high somatic cell counts (SCC) that are characteristic of mastitic milk and are associated with many of the changes to milk composition.

Furthermore, the bacteria produce virulence factors, toxins and irritants causing swelling and death of alveoli (Bruckmaier and Blum, 2004). The cellular damage can produce holes within the mammary epithelium that can lead to changes in milk composition and short circuit the blood-milk electrical potential in the same manner as opening of tight junctions. For example, lactose which is synthesized by mammary epithelial cells, partially leaks into blood circulation through the damaged blood-milk barrier (Bruckmaier and Blum, 2004). Simultaneously, there is an increase of the concentrations of blood borne components in the milk of affected quarters, such as serum albumin, sodium and chloride ions. The concentration of caseins is reduced in infected quarters due to reduced secretion and increased destruction by blood borne proteases on milk proteins and fat such as plasmin.

The causative organisms adhere to the surface of the epithelial cells and form colonies. Localised areas are proably exposed to higher concentrations of toxic products than other parts and while endotoxins failure to affect the mammary tissue directly. The cytokines and arachidonate metabolites are produced by leukocytes upon interaction with the endotoxin which induces inflammatory response (Shuster *et al*., 1991).

The repair process in the damaged mammary gland tissue is accomplished by the proliferation of fibrous tissue. The repair process replaces permanently the glandular tissue by connective tissue and consequently leads to reduction in milk production (Benites *et a*l., 2002). In addition, tissue debris, causative organisms and fibrinogen leaking from the interstitial spaces are converted to fibrin and leukocytes from clots that occlude ducts draining the areas. Then, the secretion accumulates in the lumen and causes local involution of the affected area, and alveoli are permanently destroyed and replaced by scar tissue (Roux *et al*., 2003).

1.4 Somatic cell counts

Somatic cells are leukocytes or white blood cells. Somatic cell counts (SCC) are a very useful tool to increase the awareness of subclinical mastitis and its effect on production and milk quality. Furthermore, SCC are useful both in individual cows and on a herd level. Bulk tank milk somatic cell counts (BTSCC) are a measure of the prevalence of mastitis in a dairy herd (Harmon, 1994) and are used by regulatory agencies as an indicator of the wholesomeness, safety and suitability of raw milk for human consumption (Smith and Hogan, 1999). The number of somatic cells increases in response to pathogenic bacteria like *S. aureus*, a cause of mastitis. The SCC are quantified as cells per milliliter. The EU, New Zealand, Australia, Switzerland and Norway all accept 400,000 cells/ml as the upper limit (Hillerton, 2001). Many countries are able to determine a national average SCC, based on all producers in the country and indicate considerable progress in control of subclinical mastitis or control the SCC of the herd bulk milk. The national average SCC is currently less than 300,000 cells/ml in most of the EU countries as well as New

Zealand (Smith *et al*., 2000). The national average in the US is estimated to be 350,000 cells/ml. The national average SCC of less than 200,000 cells/ml in countries such as Switzerland, Norway, Finland, United Kingdom, West Germany and New Zealand.

1.5 Effects on milk composition

The intramammary inflammatory response associated with mastitis not only results in a decrease in milk production and quality of milk but also an increase of whey proteins, serum albumin, immunoglobulins, chloride, sodium, pH, free fatty acids and somatic cell count (SCC) in the milk. Mastitis also results in a reduction in synthesis of the main components of milk, such as lactose, milk fat, solids not fat and casein (NMC, 1996).

Changes in milk composition accompany the increase in SCC following infection of the mammary gland. The compositions of normal milk, low SCC milk and high SCC milk are presented in Table 1. These comparisons are frequently made between high and low SCC milk from opposite quarters of the same cow to reduce cow to cow variation. Elevated SCC is associated with a decrease in the content of lactose and fat in milk because of a reduced ability of the mammary gland to produce these components. Some studies have shown no change in fat percentage, yet total fat production declines with the decrease in milk production.

During mastitis, it is generally accepted that milk proteins are increased. It has been attributed to the influx of circulating proteins into the milk coupled with a decrease in caseins (Auldist *et al*., 1995), the major milk protein. This protein has high nutritional qualities and is very important in cheese manufacturing. However, in high SCC milk, there is a decrease in α-lactalbumin and β-lactoglobulin, because of the leakage of these proteins out of milk into the extra-cellular fluid via the paracellular pathways. The lactoferrin concentrations are increased during mastitis, possibly related to the immune function of this protein.

Table 1 Changes in milk compositions associated with mastitis.

Milk compositions	Normal milk	Mastitis milk	% of normal
Solid-not-fat	8.98	8.80	99
Fat	3.50	3.20	91
Lactose	4.90	4.40	90
Total protein	3.61	3.56	99
Total casein	2.80	2.30	82
Whey protein	0.80	1.30	162
Serum albumin	0.02	0.07	350
Lactoferrin	0.02	0.10	500
Immunoglobulins	0.10	0.60	600
Sodium	0.06	0.105	184
Chloride	0.09	0.147	161
Potassium	0.17	0.157	91
Calcium	0.12	0.04	33

Source: Viroj (2003)

The increase in fat concentration indicates that there is a reduction of lactose synthesis and therefore reducing milk volume, while the fat synthesis is slightly depressed (Bruckmaier and Blum, 2004). In addition, the leakage of lactose from the milk takes its water, resulting in decreased volume of secretion in the gland. The fat droplets are large relative to the gaps between the cells and are contained within the alveoli and consequently increase their concentrations. Milk fat globule membranes are susceptible to the action of lipase enzymes, produced by leukocytes that invade the mammary gland in response to infection, resulting in breakdown of triglycerides oxidation to fatty acids and off-flavours (Auldist and Hubble, 1998).

The lactose concentration is reduced as the osmotic regulator of milk volume. There is an increase influx of electrolytes during mastitis because water would be drawn into the cells only in sufficient quantities to maintain osmotic

equilibrium. The plasma level of lactose is elevated because of the leakage of lactose from the lumen of the mammary gland into the blood stream, which is finally excreted in urine of mastitis cows (Bruckmaier and Blum, 2004).

Sodium and chloride increase in high SCC milk due to increased passage of these minerals from blood into milk. Potassium, normally the predominant mineral in milk, declines due to its passage out of milk to lymphoid system between damaged secretory cells. Most of the calcium in milk is associated with casein, and disruption of casein synthesis results in reduced calcium levels in milk from mastitis cows. These alterations in mineral content affect the pH and conductivity of milk.

1.6 Causative organism

The causative organisms can be categorized as contagious and environmental pathogens. The reservoir of contagious pathogens is the udder of infected cows which can spread the bacteria from one infected quarter to another or between cows, most often during the milking process. The reservoir of environmental pathogens is the housing environment of the cow and infection may occur at any time. The most often infection occurs during milking or after milking process.

1.6.1 Contagious pathogen

The main contagious pathogens are *Staphylococcus aureus*, *Streptococcus agalactiae*, *Coronybacterium bovis* and *Mycoplasma* species (spp.). *S. aureus* is generally considered to be the most prevalent cause of mastitis. The major reservoir of this pathogen is the infected udder, and infections are spread among cows or between quarters during the milking process by contaminated milking equipment, milker's hands, cloths or spongy used to wash or dry more than one cow. Thus, contagious mastitis results in decreases in milk production and increases in bulk tank SCC (Harmon, 1996).

1.6.2 Environmental pathogen

The main environmental pathogens are gram-negative bacteria, which include the coliforms and environmental streptococci. The gram-negative bacteria include *Escherichia coli, Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp., *Pseudomonas* spp., *Proteus* and *Actinomyces pyogenes*. The environmental streptococci include *S. uberis*, S. *dysgalactiae* and *S. equinus*. Environmental mastitis is a major problem in many dairy herds resulting in increased SCC and increased incidence of mastitis. The primary sources of environmental pathogens are from bedding materials in the close-up, pre-calving and calving areas as well as cow stalls. This results in teat colonization and occasionally teat canal infection. Another likely source of this pathogen is the cow's own skin and intestinal tract (Hillerton and Berry, 2003).

1.7 Treatment and control

The prevention and control of mastitis include proper milking hygiene based on reducing the number of bacteria to which the teat end is exposed. The basic management which has the greatest effectiveness is teat dipping and dry cow therapy (Harmon, 1996). An effective teat dip should be applied to all teats at the end of milking to kill contagious pathogens that are deposited on the skin during milking. The intramammary treatment of all quarters is recommended to use after the last milking of the lactation with a commercial intramammary antibiotic. Dry cow therapy is more effective than lactation therapy in eliminating existing infections. Dry cow therapy will also reduce the number of new infections in dry period and a higher concentration of antibiotic can be used without risk of antibiotic residues in milk.

2. Importance of *Staphylococcus aureus*

Staphylococcus aureus is the most important and prevalent contagious pathogen. It causes clinical and subclinical intramammary infection with serious

economic loss and herd management problems in dairy cows (Dego *et al*., 2002). This pathogen is very effective at causing extensive damage to milk secretory tissue, and then it is able to live within the udders of many cows for the remainder of the cows live (Douglas and Robert, 2005). The infection is spread at milking time, when *S. aureus* contaminated milk from infected cows comes into contact with teats of uninfected cows and bacteria penetrate the teat canal (Jones *et al*., 1998). *S. aureus* usually does not respond to antibiotic treatment, it forms abscesses in the tissue which may result in fibrosis. The end result is decreased milk production and increased somatic cell count (Roberson *et al*., 1994).

2.1 Biology of *S. aureus*

S. aureus are gram-positive, cocci about 0.5-1.0 μm in diameter. They grow in clusters resembling grapes, pairs and occasionally in short chains. *S. aureus* forms a fairly large yellow colony on rich medium and they are often hemolytic on blood agar. They are facultative anaerobes that grow by aerobic respiration or by fermentation that the yields of lactic acid. They can grow at temperature range from 15 to 45 °C and at high NaCl concentrations. Nearly all strains of *S. aureus* produce the enzyme (Kenneth, 2005).

The outer layers of pathogens are important in the infection process (Anthony and Hill, 1988). The gram positive cell wall is differed from the gram negative bacteria in two major characteristics, the gram positive cell wall has a thicker and highly peptidoglycan layer and it lacks the outer membrane (Baddiley, 1989 and Beveridge, 1999). The peptidoglycan of cell wall allows the organism to attach to host's cell membranes and is resistant to unfavorable environmental conditions. The peptidoglycan consists of glycan strands of N-acetylglucosamine-N-acetylmuramic acid (GlcNAc-MurNAc) disaccarides, crosslinked by the tetrapeptides consisting of L-alanine, D-glutamine, L-lysine, and D-alanine (Giesbrecht *et al*., 1998). In *S. aureus,* a pentaglysine inter-bridge links the tetrapeptide units of adjacent glycan strands. *S. aureus* produces four penicillin-binding proteins, PBP1-4, involving in the cell wall peptidoglycan assembly (Labischinski, 1992). The biological activity of

these native PBPs is similar to that of serine proteases, which act as transpeptidase in the crosslinking of the glycan chains (Murakami *et al*., 1994). PBP2 is a bifunctional protein which in addition to transpeptidase activity, which act as transglycosylase (Goffin and Ghuysen, 1998). PBPs bind effectively to beta-lactam antibiotics.

2.2 Isolation and identification

S. aureus is isolated by streaking from the clinical specimen or from a blood culture onto solid media such as blood agar, tryptic soy agar and baird-parker selective media. Specimens likely to be contaminated with other microorganisms can be plated on mannitol salt agar containing sodium chloride, which allows the halo-tolerant staphylococci to grow. Ideally, a gram stain of the colony should be performed and tested for catalase and coagulase production, allowing the coagulase-positive *S. aureus* to be identified quickly (Quinn *et al*., 1994).

Coagulase is an extracellular protein which binds to prothrombin in the host to form a complex called staphylothrombin. The protease activity characteristic of thrombin is activated in the complex, resulting in the conversion of fibrinogen to fibrin. This is the basis of the coagulase test. A clot is formed in plasma after incubation with the *S. aureus* broth culture. Therefore, coagulase is a traditional marker for identifying *S. aureus* in the clinical microbiology laboratory (Quinn *et al*., 1994).

2.3 Pathogenesis of *S. aureus*

S. aureus cells express on their surface proteins that promote attachment to host proteins that form the extracellular matrix of epithelial and endothelial surfaces. In addition, most strains express a fibrin or fibrinogen binding protein which promotes attachment to blood clots and traumatized tissue. *S. aureus* can express protein toxins which are probably responsible for clinical symptoms during infections. The membranes of erythrocyte is damage causing hemolysis. The leukocidin causes membrane damage to leukocytes.

2.3.1 Membrane damaging toxins

α-toxin is a most potent membrane damaging toxin of *S. aureus*. It is expressed as a monomer that binds to the membrane of susceptible cells (Bhakdi and Tranum-Jensen, 1991).

β-toxin is a sphingomyelinase which damages membranes rich in this lipid. The classical test for β-toxin is lysis of sheep erythrocytes. The majority of isolates from bovine mastitis express β-toxin, that the toxin is important in the pathogenesis of mastitis (Prevost *et al*., 1995).

δ-toxin is a very small peptide toxin produced by most strain of *S. aureus* (Prevost *et al*., 1995).

γ-toxin and the leukocidins are two component protein toxins that damage membranes of susceptible cells. Leukocidin is a toxin that specifically action on polymorphonuclear leukocytes (Prevost *et al*., 1995).

2.3.2 Staphylokinase

This factor lyses fibrin. A complex formed between staphylokinase and plasminogen activates plasmin-like proteolytic activity which causes dissolution of fibrin clots. Furthermore, *S. aureus* can express proteases, a lipase, a deoxyribonuclease (DNase) and a fatty acid modifying enzyme (FAME). The first three probably provide nutrients for the bacteria. However, the FAME enzyme may be important in abscess formation, where it could modify antibacterial lipids and prolong bacterial survival (Kenneth, 2005).

2.3.3 Capsular polysaccharide and protein A

S. aureus strains isolated from infections express high levels of polysaccharide. Although, it is impede phagocytosis in the absence of complement. Protein A is a surface protein of *S. aureus* which binds IgG molecules by their Fc region. The bacteria will bind IgG molecules in the wrong orientation on their surface which disrupts opsonization and phagocytosis (Kenneth, 2005).

2.4 Damage to the host

S. aureus produces toxins that destroy cell membranes and can directly damage milk producing tissue. Leukocytes are attracted to the area of inflammation where they attempt to fight off the infection. Initially, the bacteria damage the tissue lining the teat and the gland cisterns within the quarter. Then, they move up in to the duct system and establish deep of infection in the alveoli. This is followed by walling-off of bacteria by scar tissue and the formation of abscesses, which is responsible for the poor response to antibiotic treatment. Alveolar and duct cells may be destroyed and milk yield is reduced. These degenerated cells may combine with leukocytes and clog the milk ducts that drain the alveolar areas and contributing to further scar tissue formation. The ducts may reopen and release *S. aureus* to other areas of the mammary gland (Jones et al., 1998).

2.5 Transmission of *S. aureus* infection

The major reservoirs are infected udders, teat canals and teat lesions. The bacteria are spread to uninfected quarters by teat cup liners, milker's hands and washing cloths. *S. aureus* multiplies in infected lesions or colonized teat canals and can enter to the udder (Jones et al., 1998).

2.6 Detection of *S. aureus* in mastitis

Culture of bulk tank milk is easy and is an important aid in determining the microbiological cause of mastitis in the herd. This should be conducted in herds where SCC is above 250,000 cells/ml of milk withheld from the bulk tank on any day. California Mastitis Test (CMT) is used in cows with elevated SCC to determine which quarters may be infected. In herds with acute mastitis problems, milk samples from clinical mastitis quarters should be collected aseptically and cultured. Early identification of the infection is important before the bacteria have an opportunity to invade deeply into the udder and form abscesses (Jones *et al*., 1998).

2.7 Antibiotic treatment of *S. aureus* cows

S. aureus infections were found in 36% of clinical mastitis cases in Finish herds (Pyorala and Pyorala, 1997). Of these, only 39% responded to treatment. Successful treatment during lactation is greater if detected and treated early and response is lower when treating chronic infections. *S. aureus* infection is difficult to be treated, the bacteria penetrate the mammary gland tissues and the cow attempts to wall-off the area, forming an abscesses and eventual scar tissue (Belschner *et al*., 1996). These areas of scar tissue are difficult for antibiotics to penetrate in effective concentrations. The bacteria also escape the killing effects of some antibiotics in the neutrophils. As these white blood cells attempt to remove bacteria through phagocytosis, many organisms become inactive and are not killed by the neutrophils or by antibiotics which penetrate the cell. The bacteria may remain inactive inside the neutrophils. When the cells die, the bacteria are released to resume cell division and the infection process. The development of antibiotic resistance and formation of L-forms during treatment with some beta-lactam antibiotics are additional reasons for therapy failures.

2.8 Prevalence of *S. aureus* in bovine mastitis

For Prince Edward Island, Keefe *et al*. (1997) studied herd prevalence of *S. aureus* and *St. agalactiae* and they found herd prevalences of 70% and 18%, respectively, and similar to Richard et al. (2006). They found the cumulative prevalence for *S. aureus*, *St. agalactiae* and *Mycoplasma spp*. was 74, 1.6 and 1.9%, respectively. *S. aureus* -positive herds had a geometric mean BMSCC of 169,000 cells/ml compared to 129,000 cells/ml in herds with no contagious pathogen. The results in this study agree with earlier studies where herd prevalence of *S. aureus* raged from 31 to almost 100% in North America (Kelton *et al*.,1999a; Khaitsa *et al*., 2000; Jayarao *et al*., 2003).

Kelton *et al*. (1999) found *S. aureus* in 58 out of 59 bulk milk samples from Ontario, while 92% of the herds had at least one *S. aureus* culture-positive cow.

Pitkala *et al.* (2004) conducted a survey in Finland to estimate prevalence of bovine mastitis, distribution of mastitis pathogens, and antimicrobial susceptibility to different mastitis pathogens. The penicillin resistant among the staphylococci was still at a relative high level in Finland (52.1 and 32.0%) for *S. aureus* and coagulase-negative staphylococci.

Workineh *et al*. (2004) studied prevalence of mastitis in hand-milked cows in 186 samples. The California Mastitis Test and culturing for bacteria revealed that 21.5% of the cows were clinically infected and 38.2% had subclinical mastitis. Most mastitis pathogens isolated from milk samples testing positive by the California Mastitis Test were Gram-positive cocci. Staphylococci constituted 57% of the isolates, of which the predominant cause of bovine mastitis was *S. aureus* (40.5%). Other mastitis pathogens isolated include streptococci (16.5%), coliforms (9%) and corynebacteria (5%).

Ekgachai *et al*. (2001) survey for subclinical mastitis in 336 samples from Nong-Pho dairy cooperative, Thailand, and results showed 91 samples (35.27%) infected with major pathogens including *S. aureus*, *St. uberis*, *St. agalactiae*, *St. disgalactiae* and coliform bacteria.

Anonymous (2001) Laboratory of microbiology in Nong –Pho animal's hospital, Thailand survey in 1036 samples in mastitis cases from the western region, and results showed 34.27% of *Staphylococcus spp.*, 21.43% of *Streptococcus spp*., 21.04% of *Klebsiella* spp., 8.01% of *Candida*, 6.67% of *E. coli*, 2.42% of *Pseudomonas* spp., 1.35% of *Enterobacter*, 1.35% of *Bacillus spp*. and 1.06% of *Coronybacterium spp*.

Fianally, *S. aureus* causes disease that varies from peracute, through acute and chronic to subclinical, indicating the complexity of the organism and its pathogenesis. Therefore, *S. aureus* mastitis is considerable concern for welfare in dairy cow (FAWC, 1997). *S. aureus* often causes infection of long duration, compared to infection caused by *E. coli* as environmental pathogen (Fitzpatrick, 2000). The persistence of *S. aureus* often remains undetected in its subclinical manifestation and it can result in high SCC and financial penalties (Fitzpatrick, 2000). Treatment of *S. aureus* will not control this mastitis but it may shorten the duration of the infection (Jones, 2009). Intramammary antibiotic treatment curing rates were 70% when infections were less than two weeks of duration but only 35% when duration exceeded four weeks (Owens *et al*., 1995). If new *S. aureus* infections are untreated, it is likely that abscesses will form followed by scar tissue, making it difficult for drugs to penetrate and causing low curing rates (Belschner *et al*.,1996).

3. *Centella asiatica*

The common name of *Centella asiatica* is Asiatic pennywort and it is known as Buabok in Thai. *Centella* is in the family Apiaciae or Umbelliferae, genus Centella and species Asiatica as shown in Table 2. This plant has many common names such as Indian pennywort, Gotu-kola and Indian water navelwort (English) (McGuffin and

Kartesz, 2000), hydrocotyle asiatique (French), Asiatischer Wassernable and Indischer Wassernable (German), Idrocotyle (Italian), Brahma-manduki and Brahmi-Buti (Hindi), Tsubo-Kusa (Japanese), and Tungchian and Luei Gong Gen (Chinese) (WHO, 1999: De Padua and Bunyapraphatsara, 1999; Brinkhaus *et al*., 2000).

C. asiatica is a tropical medicinal plant that has been used for several centuries as a traditional medicine in oriental countries. It has a wide range of applications for mental fatique, anxiety, epidermal wound, eczema and leprosy (Guo *et al*., 2004). *C. asiatica* and its derivatives are also used to lower blood pressure, reduce oxidative stress, improve microcirculation and decrease capillary permeability in chronic venous insufficiency (Incandela *et al*., 2001). It can help to shrink swollen membranes and aid in the elimination of excess fluids. It also reduces scarring when applied during the inflammatory period of wound (Shukla *et al*., 1999).

Table 2 Systemic classification (Taxonomy) of *Centella asiatica.*

Classification	**Name**
Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Rosidae
Order	Apiales
Family	Apiaceae or Umbelliferae
Genus	Centella
Species	*Centella asiatica* (L.) Urban

Source: WHO (1999)

3.1 Botanical description

Centella asiatica is a perennial herb. It has long, creeping stems with rooting at the nodes. The leaf has long petioles arising rosette-like from the node, and the individual leaf rosettes are connected by slender aerial stolons (Figure 2). The leaf (diameter of 2-5 cm) is thin and soft, with palmate nerves. The leaf margin is crenate or slightly lobed. The petioles are slender and have a length between 5 and 25 cm (Brinkhaus *et al*., 2000).

Figure 2 *Centella asiatica* (Linn.) Urban.

3.2 Chemical constituents

The most prominent group of biologically active compounds is the triterpenoid glycosides (Wijeweera *et al*., 2006). The major triterpenoid glycosides are asiaticoside, medecassoside, centelloside, brahminoside, brahmoside and thankuniside. Aglycones are asiatic acid, madecassic acid, brahmic acid, isobrahmic acid, butulic acid, centoic acid, centellic acid, thankunic acid and isothankunic acid

(Tables 3 and 4). Moreover, it also contains phytosterols, volatine oils, alkaloids, amino acids, flavonoids, and miscellaneous compounds (Srivastava *et al*., 1997).

Although, *C. asiatica* contains a wide range of active substances, many researchs indicated that the active ingredients of *C. asiatica* are asiaticoside, asiatic acid, medecassic acid and medecassoside (Figures 3 and 4) (Brinkhaus *et al*., 2000; Cheng and Koo, 2000; WHO, 1999)

Table 3 Triterpenoid glycosides isolated from *Centella asiatica*

.

Triterpenoid glycoside	Constituent of glycoside	Melting point
Asiaticoside	Asiatic acid, glucose, rhamnose	230-233 °C
Medecassoside	Medecassic acid, glucose, rhamnose	ND*
Centelloside	Centellic acid, glucose, fructose	ND*
Brahmoside	Brahmic acid, glucose, rhamnose, arabinose	242 °C
Brahminoside	Brahmic acid, glucose, rhamnose, arabinose	223 °C
Thankuniside	Thankunic acid, glucose, rhamnose	239 °C
Isothankuniside	Isothankunic acid, glucose, rhamnose	250 °C
Asiaticoside A	6β-Hydroxyasiatic acid, glucose, rhamnose	ND*
Asiaticoside B	Terminolic acid, glucose, rhamnose	ND*

* ND = Not determined

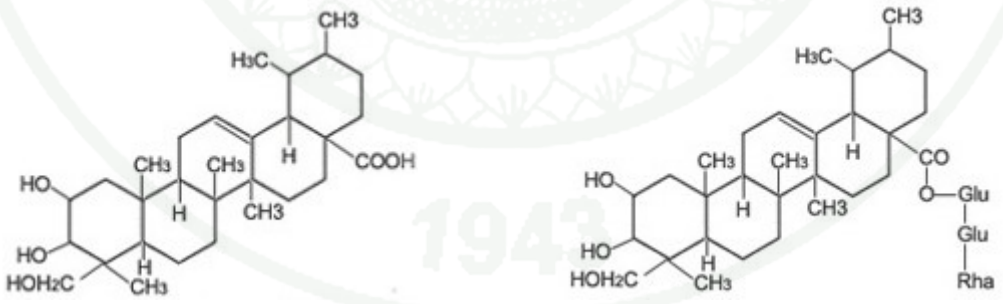
Source: Srivastava *et al*. (1997)

Table 4 Triterpenic acids isolated from *Centella asiatica* (Srivastava *et al*., 1997).

Acid	Melting point
Asiatic acid	241 °C
Madasiatic acid	ND*
Brahmic acid	293 °C
Isobrahmic acid	263 °C
Thankunic acid	314 °C
Isothankunic acid	288 °C
Betulic acid	308 °C
Centoic acid	256-261 °C
Centellic acid	ND*
6β-Hydroxyasiatic acid	285-288 °C
Terminolic acid	>300 °C

* ND = Not determined

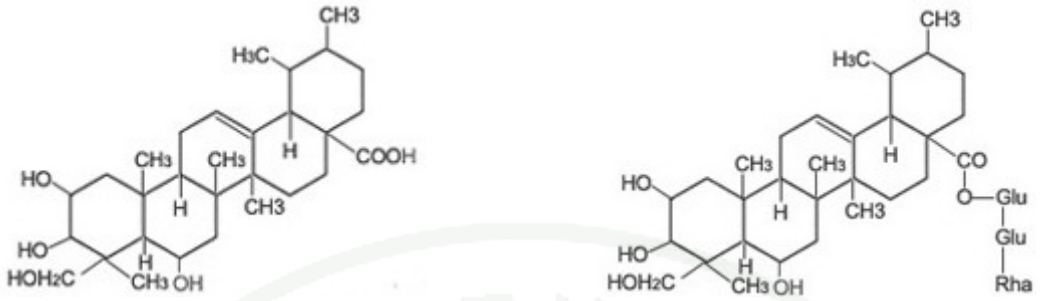
Source: Srivastava *et al*. (1997)



Asiatic acid **Asiaticoside**

Figure 3 The chemical structures of asiatic acid and asiaticoside.

Source: Anonymous (2006)



Medecassic acid **Medecassoside**

Figure 4 The chemical structures of medecassic acid and medecassoside.

Source: Anonymous (2006)

3.3 Nutrient composition

The analysis of *C. asiatica* showed that 100 g of the leaves contain 34 kilocalories of energy, 89.3 g of water, 6.9 g of carbohydrate, 2 g of fiber, 1.6 g of protein, 0.6 g of fat, 1.6 g of ash, 170 mg of Ca, 30 mg of P, 3.1 mg of Fe, 414 mg of K, 6.58 mg of beta carotene, 0.15 mg of thiamine, 0.14 mg of riboflavin, 1.2 mg of niacin and 4 mg of ascorbic acid (Brinkhaus *et al*., 2000). The monograph of *C. asiatica* in WHO 1999 has limits of foreign organic matter for not more than 2%, total ash for not more than 19%, acid-insoluble ash for not less than 6%, water-soluble extractive for not less than 6%, alcohol-soluble extractive for not less than 9.5%, and triterpene ester glycosides for not less than 2%. Patarapanich *et al*., 2004 reported that the leaf part of *C. asiatica* has larger amount of triterpene glycosides than the stem part. Jacinda et al. (2008) reported that *C. asiatica* leaves contained the highest triterpenoid levels ranging from 1.8 to 5% dry weight of *C. asiatica* for the triterpenoid acids and their glycosides, respectively. The largest amount of triterpene glycoside in *C. asiatica* was found in May and June while the smallest amount was found in February (Luangchonlathan *et al*., 2004).

3.4 Effect of *Centella asiatica* on antibacterial activity

Mamtha *et al*. (2004) studied the effect of *C. asiatica* on enteric pathogens *in vitro*. The pathogens used were *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *Vibrio parahaemolyticus* ATCC 17802 and *Pseudomonas aeruginosa* ATCC 27853, 45 isolates *Vibrio cholerae*, *Shigella spp.*, *Salmonella typhimurium*, Enteroaggregative *E. coli*, *Aeromonas hydrophila* and *Candida albicans*. Punch well and agar dilution methods with viable cell count were carried out. The working concentrations of the ethanol extract were 100, 200, 300 and 400 mg/ml, respectively. The inhibitory effect of the extract was best demonstrated at a concentration 400 mg/ml of the agar. Of the two methods used, the punch well method yielded better results than the agar dilution mehod. Viable cell count method was used to study whether the observed inhibition was bactericidal or bacteriostatic in action. The ethanol extract inhibited *Vibrio cholerae*, *Shigella spp.*, *S. aureus* ATCC 25923 and was bactericidal within two hours. They reported that the plant consisted of triterpenoid glycosides with the phytochemical activity against the microorganisms. The triterpenes weakened the membranous tissues which resulted in dissolving cell walls of the microorganisms.

Oyedeji and Afolayan (2005) studied chemical compositions and antibacterial activity of *C. asiatica* growing in South Africa. Analyses of the essential oil of this medicinal plant revealed 11 monoterpenoid hydrocarbons (20.20%), nine oxygenated monoterpenoids (5.46%), 14 sesquiterpenoid hydrocarbons (68.80%), five oxygenated sesquiterpenoids (3.90%), and one sulfide sesquiterpenoid (0.76%). α.-Humulene (21.06%), β.-caryophyllene (19.08%), bicyclogermacrene (11.22%), germacrene B (6.29%), and myrcene (6.55%) were the predominant constitutes. They also found that the essential oil extract exhibited a broad spectrum of antibacterial activities against gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*) and gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa* and *Shigella sonnei*).

Zaidan *et al*. (2005) studied five local medicinal plants for antibacterial activity *in vitro*. The objective of the study was to determine the presence of antibacterial activity in the crude extracts of plants in Malaysia. They reported the presence of antibacterial activity in the crude extract of *C. asiatica*, using standard protocol of disc diffusion method. The antibacterial activities were assessed by the presence of inhibition zones and MIC values. The methanol extract of *C. asiatica* have potential antibacterial activities to both gram positive *S. aureus* and Methicillin Resistant *S. aureus* (MRSA)

Phadet *et al*. (2002) studied crude extracts of *C. asiatica* from fifteen difference types of Thai medicinal plants using various solvents i.e., n-hexane, chloroform, 95% ethanol and water. The study was performed to evaluate their antibacterial activities. Pathogens of mastitis such as *S. aureus* and *E. coli* were determined by microbiological assay techniques using disc diffusion method. The inhibition zones of *C. asiatica* crude extracts against *S. aureus* were 6.95 mm for the ethanol extract and 10.80 mm for the water extract. For *S. aureus* ATCC 25923, they reported that the inhibition zones of the ethanol extract were 7.15 mm and that of the water extract were 10.20 mm. The MIC values of *C. asiatica* from water and hexane extracts showed the highest antibacterial efficiency of 1.25 mg/ml and the MBC value of 5 mg/ml

Chaidate *et al*. (2005) studied the medicinal plants against *S. aureus* causing bovine mastitis *in vitro*. They reported that *C. asiatica* crude extracts inhibited both sensitive and resistant *S. aureus* in dairy cows with mastitis.

Table 5 Antibactreial activity tests for crude extracts of *Centella asiatica*.

Microorganism	Method	Extract of *C. asiatica*	Result	Reference
Bacillus cereus	Disc diffusion	Chloroform	Average inhibition zone:	Obayed M.
Bacillus megaterium		n-haxane	Chloroform (8-16 mm)	Ullah, 2009
Bacillus subtilis		Methanol	n-haxane (7-15 mm)	
Staphylococcus aureus		Carbon tetrachloride	Methanol (8-13 mm)	
Sarcina lutea			Carbon tetrachloride (8-12 mm)	
Escherichia coli				
Pseudomonas aeruginosa				
Salmonella paratyphi				
Salmonella typhi				
Shigella boydii				
Shigella dysenteriae				
Vibrio mimicus				
Vibrio parahemolyticus				

Table 5 (Continued)

Microorganism	Method	Extract of *C. asiatica*	Result	Reference
Pseudomonas vulgaris *Staphylococcus aureus* *Escherichia coli* *Bacillus subtilis*	Agar disc diffusion	Petroleum ether Ethanol Water	Average inhibition zone: Petroleum ether (9-19 mm) Ethanol (6-22 mm) Water (8-17mm)	Jagtap NS. *et al*., 2009
Klebsiella pneumoniae *Escherichia coli* *Staphylococcus aureus* *Aspergillus fumigatus*	Agar disc diffusion, Agar well diffusion	Ethanol Acetone Ethyl acetate	*Klebsiella pneumoniae* (20 mm) *Escherichia coli* (17 mm) *Staphylococcus aureus* (21-22mm) *Aspergillus fumigatus* (30-31mm)	Thenmozhi M. *et al*., 2009

Table 5 (Continued)

Microorganism	Method	Extract of *C. asiatica*	Result	Reference
Vibrio alginolyticus *Vibrio parahaemolyticus* *Vibrio harveyi* *Vibrio vulnificus* *Vibrio cholerae* *Escherichia coli* *Citrobacter freundii* *Edwardsiella tarda* *Aeromonas hydrophilla* *Salmonella spp.* *Schewanella putrifaciens* *Streptococcus spp.*	Agar disc diffusion	Water Methanol	Average inhibition zone of water extract: 11 mm for *Citrobacter freundii* 8 mm for *Vibrio alginolyticus* 9 mm for *Vibrio cholerae* 8 mm for *Vibrio harveyi* 8 mm for *Vibrio parahaemolyticus* Average inhibition zone of methanol extract: 7 mm for *Streptococcus spp., Vibrio alginolyticus, Vibrio vulnificus*	Lee Seong Wei *et al*., 2008

Table 5 (Continued)

Microorganism	Method	Extract of *C. asiatica*	Result	Reference
Staphylococcus aureus Escherichia coli Pseudomonas aeruginosa Klebsiella pneumoniae	Agar disc diffusion, Agar well diffusion	Aqueous (50 mg/ml) Ethanol (50 mg/ml) Acetone (50 mg/ml) Ethyl acetate (50 mg/ml) Saponin fraction(10 mg/ml)	*Staphylococcus aureus* (2-12mm) *Escherichia coli* (2-19 mm) *Pseudomonas aeruginosa* (2-18 mm) *Klebsiella pneumoniae* (5-21mm)	Krishnan *et al*., 2009
E. coli ATCC 25922 *S. aureus* ATCC 25923 *Vibrio parahaemolyticus* ATCC 17802 *Pseudomonas aeruginosa* ATCC 27853 *Vibrio cholerae* *Shigella spp.* *Salmonella typhimurium* Enteroaggregative *E. coli Aeromonas hydrophila Candida albicans*.	Punch well method, Agar dilution	Ethanol 100 mg/ml 200 mg/ml 300 mg/ml 400 mg/ml	Average inhibition zone: *S. aureus* ATCC 25923 (less than 15 mm)	Mamtha *et al*., 2004

3.5 The analytical method

Thin-layer chromatography (TLC) is the method for qualitative and quantitative determination of active constituents of *C. asiatica*. The method can be regarded as an almost universal separation technique based on the affinities of the components. The standard method of ASEAN Herbal Medicine (1993) uses TLC plate (silica gel GF_{254}) and a mixture of hexane: ethylacetate: diethylamine (8: 2: 0.2) as mobile phase for qualitative determination of constituents in *C. asiatica*. Arunya (1997) used a mixture of chloroform: methanol: water (15: 7: 1) to resolve asiatic acid, medecassic acid, asiaticoside and medecassoside and detected with 0.2% anthrone reagent for qualitative determination in crude extracts. For quantitative determination of active constituents of *C. asiatica*, Tanwarat *et al*. (2003) reported a mixture of chloroform: methanol: water (40: 30: 4) as developing solvent used for isolation of asiaticoside and medecassoside. The developed plate was sprayed with 10% sulfuric acid in ethanol and heated at 110 °C for 10 min and finally scanned with HPTLC scanner.

MATERIALS AND METHODS

Materials

1. Plant materials

Centella asiatica (Linn.) Urban were obtained from Nakhon Pathom Province in May, 2009 in the single batch collection.

2. Microorganisms

Staphylococcus aureus isolates from three hundred of milk samples from dairy cows with subclinical mastitis and *S. aureus* isolates obtained from the National Institute of Animal Health, Thailand were used.

S. aureus ATCC 25923 (Medimark, France) was used as the reference strain.

3. Enzyme

Lysozyme was purchased from Sigma L7651, Sigma-Aldrich, Germany

4. Primers

Specific primers used in this study were synthesized by First base, Malaysia

5. Chemicals and Substances

Chemical and substances used in this study were presented in Appendix A.

Methods

1. Phytochemistry

1.1 Preparation of plant samples

Fresh *Centella asiatica* (10 kg) were cleaned with water and cut for leaves. The fresh leaves (4 kg) were dried at room temperature for one day. Thereafter, it was dried at 50°C in the hot air oven for 24 hr. and finely ground into a powder (650 g) by cutting mill machine.

1.2 Preparation of ethanol extracts of leaf powder

The leaf powder (500 g) was extracted with 5 L of 95% ethanol and macerated at room temperature for 72 hr. The extract was hand-squeezed through a thin cloth and a filter paper (Whatman No.1), respectively. To obtain the crude extract (242 g), ethanol was removed using a rotary evaporator. The extract was kept at -20°C until use.

1.3 Preparation of water extracts of leaf powder

The leaf powder (500 g) was boiled in distilled water 4 L, (1:8) for one hour. The solution was then filtered and freeze-dried to obtain the powder. The extract (19 g) was kept at -20°C until use.

1.4 Preparation of water extracts of fresh leaves

The fresh leaves (500 g) were boiled in distilled water (2 L, 1:4) for one hour. The solution was then filtered and freeze-dried to obtain the powder. The extract (13 g) was kept at -20°C until use.

1.5 Identification of asiatic acid in crude extracts

The asiatic acid was identified by Thin Layer Chromatography (TLC) on silica gel plates. A mixture of chloroform, methanol, ethyl acetate and water (30:5:5:1) was used as the mobile phase to separate this compound from the extract. The developed plate was sprayed with anisaldehyde-sulphuric acid reagent followed by heating at 110°C for 5 min. The amount of asiatic acid on the plate was determined using a densitometer at the wavelength of 525 nm.

1.6 Quantitative analysis by TLC densitometry

1.6.1 Preparation of the standard solution

The asiatic acid solution (1,000 ppm) was prepared by accurately weighing about 0.01 mg of asiatic acid and dissolving in 10 ml of methanol. The solution was sonicated for 10 min and filtered through a 0.45-μm nylon syringe filter.

1.6.2 Preparation of sample solutions

The ethanol extracts and the water extracts of leaf powder (10,000 ppm) were prepared by accurately weighing about 0.1 mg of the extract and dissolving in 10 ml of methanol. The solution was sonicated for 30 min and filtered through a filter paper (Whatman No.1). The solution volume was adjusted to 10 ml with methanol in a volumetric flask and the solution was filtered through a 0.45-μm nylon syringe filter.

The water extracts of fresh leaves (10,000 ppm) were prepared by accurately weighing about 0.1 mg of the extract and dissolving in 10 ml of methanol. The solution was sonicated for 30 min and filtered through a filter paper (Whatman No.1). The solution volume was adjusted to 10 ml with methanol in a volumetric flask and the solution was filtered through a 0.45-μm of nylon syringe filter.

1.6.3 Condition of TLC

The TLC condition to separate asiatic acid from crude extract of *C. asiatica* was as follow:

Stationary phase:	Silica gel GF_{254} precoated on aluminium sheet (10 × 20 cm).
Solvent system:	chloroform: methanol: ethyl acetate: water (30:5:5:1)
Applied:	10 μl
Detection:	anisaldehyde-sulphuric acid reagent
Examination:	White light
Detection of:	Triterpene

1.6.4 Spraying reagent

Anisaldehyde-sulphuric acid reagent was used as a spraying reagent. It was prepared by dissolving 0.5 ml of anisaldehyde in a mixture of 10 ml of glacial acetic acid, 5 ml of concentrated sulfuric acid and adjusting to 100 ml with cool methanol.

1.6.5 Method validation

The developed TLC method was validated according to the International Conference on Harmonization (ICH) guideline (ICH, 2006). The method validation parameters for quantitative analysis of asiatic acid in *C. asiatica* leaf extracts were specificity, repeatability, intermediate precision, accuracy, limit of detection, limit of quantitation, linearity and range.

1.6.5.1 Specificity

The specificity of a method can be evaluated by the peak developed from the densitometry at a unique wavelength for the absorption of the

targeted compound. One hundred percent specificity of the method is demonstrated by a single peak from the densitometry.

1.6.5.2 Precision

The precision of a method is the extent to which the individual test results of multiple injections of a series of standards agree. The precision was determined in term of percent of coefficient of variation (%CV) or percent relative standard deviation (%RSD) by using below equation.

$$\%RSD = (SD/\bar{x}) \times 100$$

Where,

SD = standard deviation of peak area

$\bar{x}$ = mean of peak area

1) Repeatability

The repeatability or intra-day precision was evaluated by repeating the same spots of asiatic acid standard solution 1,000 ppm and applied 1 μg/spot in five times (n=4) on the same day. The result was expressed as %RSD.

2) Intermediate precision

The intermediate precision or inter-day precision was evaluated by repeating the same spots of asiatic acid standard solution 1,000 ppm and applied 1 μg/spot in five times (n=4) on the different days. The result was expressed as %RSD.

3) Accuracy

The accuracy of a measurement is defined as the closeness of the measured value to the true value. In a method with high accuracy, a sample is analyzed and the measured value should ideally be identical to the true value. Typically, accuracy is presented and determined from recoveries study performed by standard addition method.

The accuracy was determined by comparing the mean calculated with the spiked asiatic acid standard solution with a known concentration. Accuracy should be reported as percent recovery, calculated and averaged from asiatic acid standard solution were added. Standard asiatic acid in concentrations 50, 100, 150 ng were spiked into the samples. Each sample was spotted in three replicates (n=3).

Percent recoveries were calculated by the following equation:

$$\% \text{ recovery} = \frac{Xfound - Xinitial}{Xadded} \times 100$$

Where,

Xfound = The concentration of standard found in the spiked sample

Xinitial = The concentration of standard found in the sample

Xadded = The concentration of standard added

4) Limit of detection

The LOD is the smallest quantity of analyte that can be shown to be significantly greater than the measurement (random) error of the blank at a prescribed level of confidence.

The LOD was determined from the following equation:

$$LOD = 3.3\ \sigma/S$$

Where,

σ = standard deviation of the response

S = slope of the calibration curve

5) Limit of quantitation

The LOQ is the smallest amount of analyte in a test sample that can be quantitatively determined with suitable precision and accuracy under previously established method conditions. The LOQ is a crucial parameter in assays of low levels of compounds and in the determination of impurities, contaminants, or degradation products.

The LOQ was determined from the following equation:

$$LOQ = 10\ \sigma/S$$

Where,

σ = standard deviation of the response

S = slope of the calibration curve

6) Linearity and range

Linearity is the ability of an analytical method to elicit test results that are directly, or by a well defined mathematical transformation, proportional to the concentration of analyte in samples within a given range.

The asiatic standard solutions was prepared for five concentration levels (0.1, 0.4, 0.8, 1.2 and 1.6 μg/spot) and applied on the TLC plate in triplicate. The linearity was calculated from the slope by plotting peak areas against concentrations within the range of 0.1-1.6 μg/spot using the winCATS software. The result was expressed as the correlation coefficient (r^2).

A calibration curve was described by the equation:

$$y = ax + b$$

where,

y =peak area ratio between peak area of an analyte and peak area of internal standard

a =the slope

x =the concentration of an analyte

b =the intercept of a line fit to the data

The correlation coefficient (r^2) should be 0.995 or greater.

1.6.6 Quantitative analysis of asiatic acid in *C. asiatica* leaves extract

The asiatic acid standard solutions was prepared for five concentration levels (0.1, 0.4, 0.8, 1.2 and 1.6 μg/spot) and each crude extracts of *C. asiatica* leaf extract were applied 10 μg/spot on the TLC plate in triplicate. The plates were spotted using the Linomat V Automatic sample spotter. The plate was then developed with a mixture of chloroform: methanol: ethyl acetate: water (30:5:5:1) as solvent system. The developed plate was sprayed with anisaldehyde-sulphuric acid reagent, followed by heating at 110°C for 5 min. Thereafter, the plate was scanned at the wavelength of 525 nm with the TLC Scanner. The amount of asiatic acid present in the crude extract solution was determined by comparing with the peak areas of standard. asiatic acid.

2. Isolation and identification of *Staphylococcus aureus*

Prior to milk sample collection, data of bulk tank somatic cell count from dairy farms in Ratchaburi Province were selected using SCC greater than 500,000 cell/ml. At the farm, the CMT (California Mastitis Test) was used to screening the quarters of the udder. Three hundred milk samples (CMT positive) were collected from quarters by aseptic technique.

2.1 Isolation of *S. aureus*

Pipette 1 ml of milk sample into 10 ml of Tryptic Soy Broth (TSB) with 1% pyruvate and 10% sodium chloride, and incubated at 37 °C for 24 to 48 h. Then, touching 2-3 loops from Tryptic Soy Broth (TSB) and streaking it onto Baird-Parker agar (BPA) and incubated at 37 °C for 24 to 48 h. Colonies of *S. aureus* are circular, smooth, convex, and gray to jet-black and surrounded by opaque zone.

2.2 Identification of *S. aureus*

S. aureus can be confirmed by testing colonies for coagulase-positive. Transfer suspected colonies of *S. aureus* into small tubes containing 2 ml of Brain Heart Infusion Broth (BHI), and incubate at 37 °C for 18 to 24 h. Pipette 20 µl of solution from Brain Heart Infusion Broth (BHI) into 200 µl of coagulase plasma solution, and incubate at 37 °C for 24 h. *S. aureus* was confirmed using coagulase plasma rabbit with EDTA and mannitol salt agar (MSA) (Quinn *et al*., 1994).

3. Antibacterial activity test of crude extracts of *Centella asiatica*

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).

3.1 Screening of crude extract for antibacterial activity by disc diffusion test

Disc diffusion test was performed using sterile 6 mm-diameter filter paper discs. The discs were prepared using 10 µl of crude extract diluted in the solvent (dimethylsulfoside; DMSO for ethanol extracts and sterile water for water extracts) 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). The broth culture was incubated at 37 °C (usually 3 to 5 h) until the turbidity of the 0.5 McFarland standard using the McFarland densitometer, this resulted in a suspension containing approximately 1 to 2 × 10^8 CFU/ml. A 150 µl of the 0.5 McFarland suspensions were added to 5 ml of Mueller-Hinton agar (MHA) 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 h. Pure dimethylsulfoside (DMSO) and sterile water were used as negative controls while amoxicillin-clavulanic acid (30 µg, Oxoid) and cephalotin (30 µg, Oxoid) were used as positive controls. The disc diffusion test was determined by measuring the diameter of the inhibition zone. Experiments were performed in triplicate and the mean of the diameters of the inhibition zones was calculated.

3.2 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 µl of Mueller-Hinton Broth (MHB) was added into all wells. The stock concentration of asiatic acid was 10.24 mg/ml in dimethylsulfoside (DMSO). Fifty µl

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 µl 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 µl of the 0.5 McFarland suspensions were inoculated into all wells. The plates were incubated at 37 °C for 18 to 24 h. After incubation, bacterial growth was evaluated by adding 50 µl of 5 mg of one resazurin tablet in 50 ml 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.

3.3 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 h. Unchanged media colour was defined as the lowest concentration that killed the bacteria. The yellow colour of media showed *S. aureus* growth.

4. Antimicrobial susceptibility test for isolates of *S. aureus*

Antimicrobial susceptibility test was performed by disc diffusion test using the Mueller-Hinton Agar (MHA) following antimicrobial drugs used in the dairy farm (Guler *et al*., 2005), Penicillin G (10 unit), Ampicillin (10 µg), Methicillin (5 µg), Vancomycin (30 µg), Cephalotin (30 µg), Amoxycillin-Clavulanic acid (30 µg), Oxytetracycline (30 µg), Cloxacillin (5 µg), Enrofloxacin (5 µg), Kanamycin (30 µg), Gentamicin (10 µg), Lincomycin (2 µg), Sulfamethoxazole-Trimethoprim (25 µg), Streptomycin (10 µg), Neomycin (30 µg) and Novobiocin (30 µg). Reference strain

S. aureus ATCC 25923 was used as the quality control organism and included of isolates tested.

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). The broth culture was incubated at 37 °C (usually 3 to 5 h) until the turbidity of the 0.5 McFarland standards using the McFarland densitometer, this resulted in a suspension containing approximately 1 to 2×10^8 CFU/ml. The suspensions were swabbed to the Mueller-Hinton agar (MHA). The discs of antimicrobial drugs were placed on the surface of the Mueller-Hinton agar (MHA) and incubated at 37 °C 18 to 24 h. The susceptibility test was determined by measuring the diameter of the inhibition zone.

5. Polymerase chain reaction and sequencing of isolates of *S. aureus*

5.1 DNA manipulation

5.1.1 Bacterial strains

A thirty isolates of *S. aureus* field strains and the reference strain were cultivated in Blood agar, and incubated at 37 °C for 24 to 48 h. Transferred colonies of *S. aureus* into tubes containing 2 ml of TSB, and incubated at 37 °C for 18 to 24 h using shaking incubator.

5.1.2 DNA isolation and purification

The *Staphylococcus aureus* DNA was extracted from overnight cultures in 2 ml of Tryptic Soy Broth. DNA was extracted by using QIAamp DNA Mini Kit (modified by Guler *et al*., 2005). Briefly, bacterial cells were collected by centrifugation at 20,000 × *g* for 30 sec. The pellets were washed in distilled water (PCR grade) and resuspended in 100 µl of distilled water. The pellets were suspended in 180 µl of lysozyme (stock concentration 20 mg/ml) and incubated at 37 °C for 30

min. Subsequently, the 20 μl of proteinase K and 200 μl of buffer AL were added to the solution and incubated at 56 °C for 30 min, and then for a further 15 min at 95 °C. It was centrifuged at 20,000 × *g* for 30 sec. The 200 μl of ethanol (96-100%) were added to the solution, and mix by pulse-vortexing for 15 sec. After mixing, carefully applied the mixture to the QIAamp Mini spin column in a 2 ml collection tube and centrifuged at 6,000 × *g* for 1 min. The QIAamp Mini spin column was placed in a clean 2 ml collection tube and discarded the tube containing the filtrate. The 500 μl of buffer AW1 were added to the QIAamp Mini spin column and centrifuged at 6,000 × *g* for 1 min. The QIAamp Mini spin column was placed in a clean 2 ml collection tube and discarded the tube containing the filtrate. The 500 μl of buffer AW2 were added to the QIAamp Mini spin column and centrifuged at 20,000 × *g* for 3 min. The QIAamp Mini spin column was placed in a microcentrifuge tube and discarded the tube containing the filtrate. The 150 μl of buffer AE were added to the QIAamp Mini spin column and centrifuged at 6,000 × *g* for 1 min. The solutions were then store at -20 °C until used for DNA amplification.

5.2 Polymerase chain reaction

The polymerase chain reaction analysis of the *coa* gene was performed by using the primers COAG2 (5′CGAGACCAAGATTCAACAAG3′) and COAG3 (5′AAAGAAAACCACTCACATCA3′) described by Goh *et al*. (1992). The PCR reactions were performed in a final volume of 50 μl of the mixture as follow: 1x PCR buffer, 0.2 mM dNTPs, 3 μM $MgCl_2$, 1 μM of each primer, and 1.25 unit of *Taq* polymerase (Intron). The amplification of *coa* gene was performed using the following condition: initial denaturation at 94 °C for 2 min, and then 34 cycles of heat denaturation at 94 °C for 30 sec, primer annealing at 63 °C for 30 sec, and DNA extension at 72 °C for 1 min, and finally extension step at 72 °C for 5 min. The PCR products were analyzed by electrophoresis through a 2% agarose gel and after which the gel was stained with ethidium bromide and photographed. With this protocol, the presence of the 800 bp fingerprint pattern bands indicated the successful amplification of *coa* gene.

5.3 Sequencing and nucleotide analysis

The amplified product of *coa* gene was extracted using QIAquick Gel Extraction Kit. Briefly, the DNA fragment was excised from the agarose gel with a clean,sharp scalpel. The 100 µl of buffer QG was added to the microcentrifuge tube and incubated at 50 °C for 10 min. After the gel slice was completely dissolved, the color of the mixture is yellow. Then, the 100 µl of isopropanol was added to the tube and mixed. After mixing, the mixture was carefully applied to the QIAquick spin column in a 2 ml collection tube. The tube was centrifuged at 17,900 × *g* for 1 min. The tube was discarded and the QIAquick spin column was placed in the same collection tube. The 500 µl of buffer QG was added to the QIAquick spin column. The tube was centrifuged at 17,900 × *g* for 1 min and discarded. The 750 µl of buffer PE was added to the QIAquick spin column and centrifuged at 17,900 × *g* for 1 min. The QIAquick spin column was placed in a microcentrifuge tube and discarded the tube containing the filtrate. The 20 µl of buffer EB was added to the QIAquick spin column and the tube was centrifuged at 17,900 × *g* for 1 min. The purified DNA was analyzed by electrophoresis using a 1.5% agarose gel the gel was stained with ethidium bromide and photographed.

The DNA sequences would be then analysed using BioEdit program version 7.0.5.2 (Tom Hall, http://www.mbio.ncsu.edu/BioEdit/ bioedit.html) and deposited in GenBank.

6. Statistical analysis

The statistical analysis was 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 zones were expressed as mean ± SD.

RESULTS AND DISCUSSION

1. Extract yields

The leaf powder of *Centella asiatica* (500 g) was obtained from fresh leaves (4 kg). The leaf powder (500 g) was extracted with 95% ethanol and giving crude extracts of *C. asiatica* 242 g. The ethanol extracts was very sticky and had a dark brown color. The leaf powder (500 g) was extracted with water and giving crude extracts of *C. asiatica* 19 g. The fresh leaves (500 g) were extracted with water and giving crude extracts of *C. asiatica* 13 g.

The yields (%) of crude extracts from *C. asiatica* calculated on weight of fresh leaves are presented in Table 6. The ethanol extraction of leaf powder yielded crude extracts twice as much as that from the water extraction of fresh leaves and about six times of the water extraction of leaf powder. However, extraction using ethanol had a greater yield as compared to water extraction. Because of, the ethanol had non-polarity that can dissolve non-polar substance.

Table 6 The yields of crude extracts from *Centella asiatica*.

Crude extract	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

Phadet *et al*. (2002) reported that the yield (%) of crude extract from the whole parts of *C. asiatica*, the ethanol extraction percent yield are 6.79, the water extract extraction percent yield are 9.34, the chloroform extraction percent yield are 1.22 and the n-hexane extraction percent yield are 0.69. The ethanol extraction percent yield result was similar to this study but the water extract percent yield was different from this study. The different results of percent yield differed from method of extraction.

In this study, the method used a single extraction with one solvent. Phadet *et al*. (2002) used continuous extraction with different solvents such as chloroform, n-hexane, ethanol and water.

2. TLC Densitometry method

The developed TLC-Densitometry method for quantitative analysis of asiatic acid in crude extracts of *C. asiatica* were validated. The validation parameters consisted of specificity, repeatability, intermediate precision, accuracy, limit of detection, limit of quantitation, linearity and range (Table 7).

Table 7 Method validation parameters for quantification of asiatic acid.

Parameter	Asiatic acid
Specificity	specific
Range	0.1-1.2 μg/spot
Linearity (Correlation coefficient; r^2)	0.998
Precision (%RSD)	
Intra-day precision (n=4)	0.90
Inter-day precision (n=4)	0.31
Accuracy (%recovery)	99.70±0.76
Level 1 (50 ng)	99.22
Level 2 (100 ng)	100.58
Level 3 (150 ng)	99.30
Limit of detection	0.76 ng/spot
Limit of quantification	2.60 ng/spot

2.1 Linearity

The linearity plotting of peak areas against concentrations was obtained within the range of 0.1-1.2 μg/spot for asiatic acid. The correlation coefficient (r^2) was 0.9983 and the linear regression equation was Y = 3551.2X + 376.88 (Table 8 and Figure 5) that was within the acceptance criteria ($r^2 \geq 0.995$) (AOAC, 1993). Therefore, the calibration curve could be used to analyze asiatic acid in *C. asiatica* crude extracts.

Table 8 Peak area of standard asiatic acid at various amount per spot.

Amount (μg/spot)	Peak Area 1st Track	2nd Track	3rd Track	Mean	%RSD
0.1	663.94	670.09	678.32	670.78	1.08
0.4	1866.39	1863.16	1806.42	1845.32	1.83
0.8	3273.95	3276.91	3319.83	3290.23	0.78
1.2	4555.86	4604.04	4577.49	4579.13	0.53

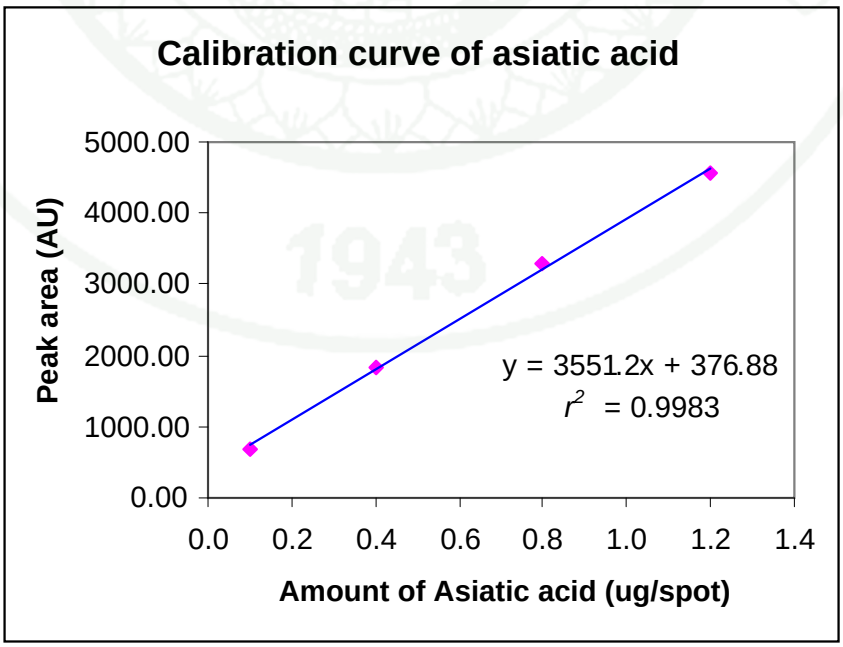


Figure 5 The calibration curve of standard asiatic acid.

2.2 Precision

The precision of the method was determined by repeatability (intra-day precision) and intermediate precision (inter-day precision). Precision was expressed as %RSD and the method with a good precision should have an acceptable value of %RSD (AOAC, 1993).

2.2.1 Intra-day precision

Intra-day precision was evaluated by repeated analysis the same asiatic acid standard solutions in four times (n=4) on the same day. The %RSD of peak area and R_f value of standard asiatic acid were showed in Table 9. The results showed that the %RSD of standard asiatic acid was 0.90. It met the acceptance criteria of USP 2001 that allowed %RSD of not more than 2.

Table 9 Intra-day precision of standard asiatic acid.

No. of Spot	Amount of asiatic acid /spot	R_f value[a]	Area of asiatic acid peak
1	1.0 μg	0.45	8411.48
2	1.0 μg	0.45	8595.53
3	1.0 μg	0.45	8542.45
4	1.0 μg	0.46	8511.72
		Mean	**8515.30**
		SD	**77.38**
		%RSD	**0.90**

[a] R_f value = Retention factor value

2.2.2 Inter-day precision

Inter-day precision was evaluated by repeating analysis the same asiatic acid standard solutions in four times (n=4) on the different day. The %RSD of peak area and R_f value of standard asiatic acid are showed in Table 10. %RSD of standard asiatic acid was 0.31 of peak area that met the acceptance criteria of USP 2001 that allowed %RSD of not more than 2.

Table 10 Inter-day precision of standard asiatic acid.

Day	Amount of asiatic acid /spot	R_f value[a]	Area of asiatic acid peak
1	1.0 μg	0.45	8471.43
2	1.0 μg	0.46	8511.72
3	1.0 μg	0.47	8530.45
4	1.0 μg	0.47	8484.70
		Mean	**8499.58**
		SD	**26.54**
		%RSD	**0.31**

[a] R_f value = Retention factor value

2.3 Accuracy

The accuracy of the method was determined by using standard addition on the basis of the recovery. The spiked asiatic acid standards at known amounts of three levels as 50, 100 and 150 ng were analysed each in triplicate. The percent recovery was calculated. The average percent recovery value was 99.70±0.76 (Table 11). The results obtained were within the range of 80-120% that met the acceptance criteria by USP requirement. Therefore, this method is accurate for the analysis of asiatic acid in *C. asiatica* crude extracts.

Table 11 Percent recovery of standard asiatic acid.

Level	Amount of asiatic acid in sample (ng)	Amount added (ng)	Amount found (ng)*	Recovery (%)	Average Recovery (%)
1	222.21	50	271.82± 0.63	99.22	
2	222.21	100	322.79±0.15	100.58	**99.70**±0.76
3	222.21	150	371.16±0.35	99.30	

*Average ± SD (n=3)

2.4 Limit of detection (LOD) and Limit of quantification (LOQ)

The LOD was defined as the lowest concentration of the analyst in a sample that can be detected, but not necessarily quantified. Determination based on the standard deviation of the response and the slope by using below equation.

$$LOD = 3.3\,\sigma/S$$

Where,

σ = standard deviation of the response;

S = slope of the calibration curve.

The calculated LOD value for asiatic acid was 0.76 ng/spot.

The LOQ was defined as the lowest concentrations of the analyst in a sample that can be determined with acceptable precision and accuracy. Determination was based on the standard deviation of the response and the slope by using below equation.

$$LOQ = 10\,\sigma/S$$

Where,

σ = standard deviation of the response;

S = slope of the calibration curve.

The calculated LOQ value for asiatic acid was 2.60 ng/spot.

3. Quantitative analysis of asiatic in *C. asiatica* leaf extract

The quantitative analysis of asiatic acid in crude extracts of leaf of *C. asiatica*. was determined using the validated TLC-Densitometry, each applied in triplicate. The asiatic acid contents in various crude extracts were shown in Table 12.

Table 12 The quantity of asiatic acid in *C. asiatica* from each crude extract.

Crude extract	Asiatic acid in crude extract (ng)	%Yield of asiatic acid
Ethanol extract of leaf powder	930	0.93
Water extract of leaf powder	208.70	0.21
Water extract of fresh leaves	131.70	0.13

The highest quantity (0.93% w/w) of asiatic acid in crude extracts of leaves of *C. asiatica* was obtained from the ethanol extracts of leaf powder. This method is the most appropriate extraction method for high yielding of asiatic acid that was the marker compound in this study. Then, each crude extract was further tested antibacterial activity against *S. aureus*.

Bungon (2004) reported the method for analysis madecassoside, asiaticoside, madecassic acid and asiatic acid in dried *C. asiatica* plant. The HPLC method was used to determine the active compounds in leaves and stems of *C. asiatica*. In leaf, madecassoside, asiaticoside, madecassic acid and asiatic acid were found in the range of 0.919-1.552%, 0.712-1.006%, 0.027-0.309% and 0.120-0.455%, respectively. In

stem, madecassoside, asiaticoside, madecassic acid and asiatic acid were found in the range of 0.029-1.062%, 0.069-0.098%, 0.007-0.010% and 0.008-0.058%, respectively.

TLC method was used to determination of madecassoside and asiaticoside in *C. asiatica* using silica gel GF_{254} and chloroform: methanol: water (30: 15: 2) mixture as developing solvent. The developed TLC plate was sprayed with 0.2% anthrone reagent. The average percent of madecassoside and asiaticoside in dried *C. asiatica* plant materials were 0.759% and 0.733%, respectively (Bungon, 2004).

The developed TLC-Densitometry method for determination of the quantity of asiatic acid in crude extracts of leaves of *C. asiatica* was a simple, precise and accurate technique. The TLC solvent system containing chloroform: methanol: ethyl acetate: water (30:5:5:1) gave the best resolution of asiatic acid (R_f = 0.45) which also resolved from other components of the extracts and enabled their simultaneous quantification. The identified band of asiatic acid was confirmed by spraying with anisaldehyde-sulphuric acid reagent, that band of asiatic acid appeared as blue-violet spot (Figure 6). Detection and quantification were performed by densitometry at 525 nm (Figure 7, 8).

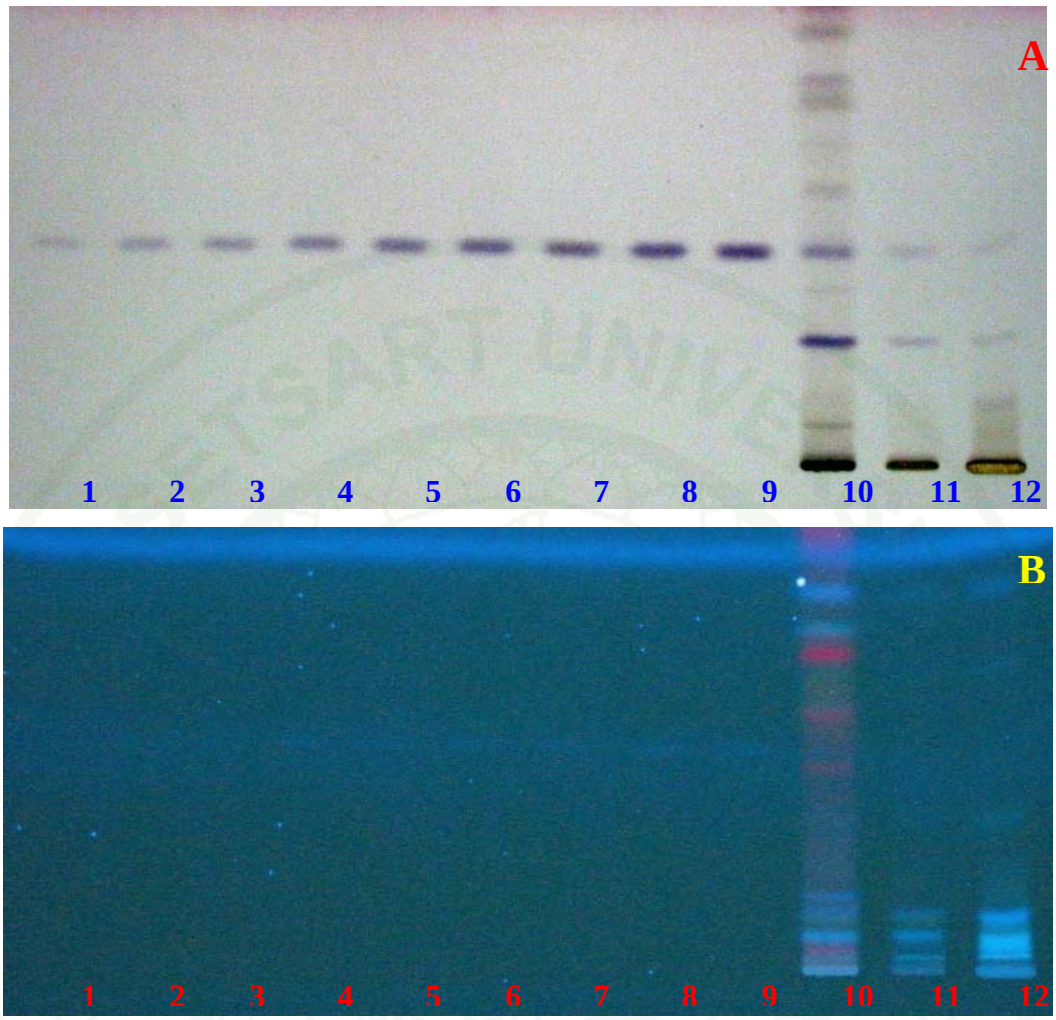


Figure 6 TLC chromatograms of asiatic acid and crude extracts of *C. asiatica.*
(A) Anisaldehyde-sulphuric acid under visible light
(B) Anisaldehyde-sulphuric acid under UV 366 nm

Chromatographic condition:

Stationary phase: Silica gel GF_{254} precoated on aluminium sheet (10 × 20 cm).

Solvent system: chloroform: methanol: ethyl acetate: water (30:5:5:1)

Sample application:

1 -9 = Standard asiatic acid 11 = the water extracts of leaf powder

10 = the ethanol extracts 12 = the water extracts of fresh leaves

Detection: Anisaldehyde-sulphuric acid reagent, heat at 110°C for 5 mins.

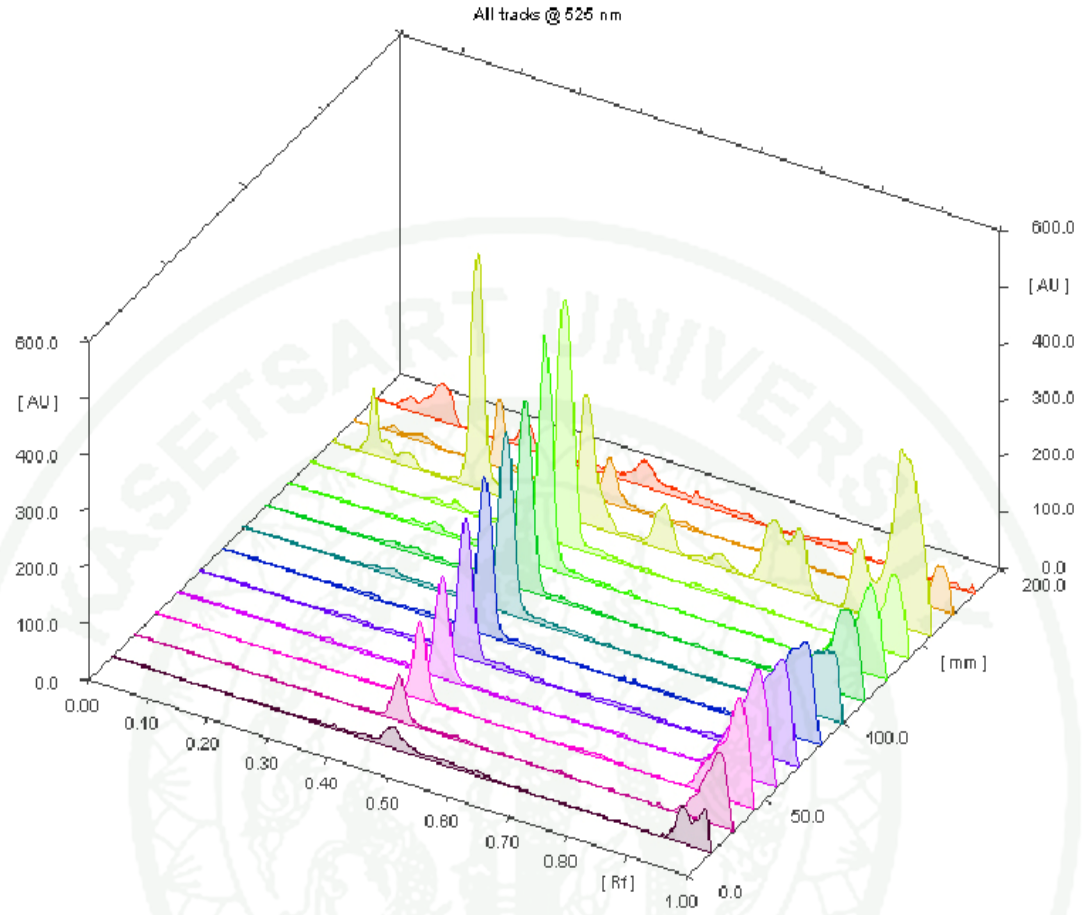


Figure 7 TLC Densitogram of asiatic acid and crude extracts of *C. asiatica.*

Chromatographic condition:

Stationary phase: Silica gel GF_{254} precoated on aluminium sheet

Solvent system: chloroform: methanol: ethyl acetate: water (30:5:5:1)

Sample application:

1 -9 = Standard asiatic acid 11 = the water extracts of leaf powder

10 = the ethanol extracts 12 = the water extracts of fresh leaves

Detection: After sprayed with anisaldehyde-sulphuric acid reagent, the plate was scanned at the wavelength of 525 nm with the TLC Scanner.

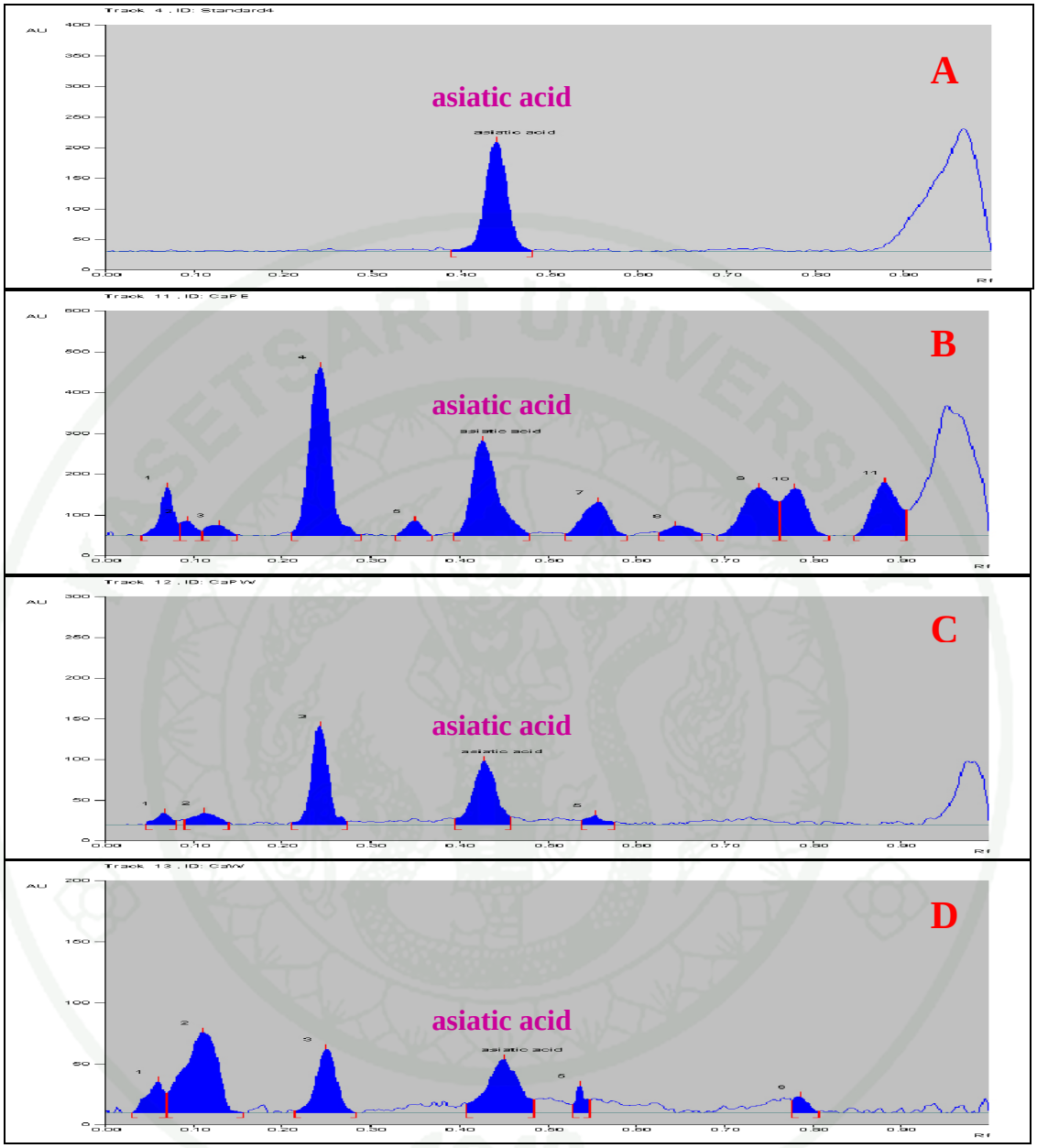


Figure 8 Typical TLC chromatogram obtained from asiatic acid (A), the ethanol extract (B), the water extract of leaf powder (C) and the water extract of fresh leaves (D).

Chromatographic condition:

Detection: The amounts of Asiatic acid present in the crude extracts solutions were determined from the peak areas.

4. Disc diffusion test

Only 3 isolates of *S. aureus* were obtained from milk sample. Therefore, other 26 isolates of *S. aureus* were requested from the National Institute of Animal Health, Thailand. In total, 29 isolates of *S. aureus* and *S. aureus* ATCC 25923 as the reference strain were used to test antibacterial activity.

The antibacterial activity of crude extracts against *S. aureus* ATCC 25923 and 29 isolates of *S. aureus* were investigated by disc diffusion test. The results of inhibition zone are presented in Table 13 and 14. 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.

Table 13 Antibacterial activity, measured as mean (± SD) of inhibition zone (mm), of crude extracts against *S. aureus* ATCC 25923.

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

* shows significant differences between each extract and control.

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

In *S. aureus* ATCC 25923, 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 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.

Table 14 Antibacterial activity, measured as mean (± SD) of inhibition zone (mm), of crude extracts against 29 isolates of *S. aureus* field strain.

Concentration of crude extracts (mg/disc)	Control	The ethanol extracts	The water extracts of leaf powder	The water extracts of fresh leaves
0.5	**6.00** ± 0.00[a]	**6.00** ± 0.00[a]	**6.00** ± 0.00[a]	**6.00** ± 0.00[a]
1	**6.00** ± 0.00[a]	**6.00** ± 0.00[a]	**6.00** ± 0.00[a]	**6.00** ± 0.00[a]
2	**6.00** ± 0.00[a]	**6.00** ± 0.00[a]	**6.00** ± 0.00[a]	**6.28** ± 1.03*[a]
4	**6.00** ± 0.00[a]	**6.00** ± 0.00[a]	**6.00** ± 0.00[a]	**8.61** ± 1.37*[b]
8	**6.00** ± 0.00[a]	**8.01** ± 0.86*[a]	**6.00** ± 0.00[a]	**9.44** ± 1.82*[b]
10	**6.00** ± 0.00[a]	**9.75** ± 1.09*[a]	**6.00** ± 0.00[a]	**10.48** ± 2.13*[b]

* shows significant differences between each extract and control.

[a, b] different superscripts within the same row show significant differences between concentrations in each plant extract.

In *S. aureus* 29 isolated from milk samples, the average inhibition zone of ethanol extracts differed from that of control at 8 mg/disc. The inhibition zone of water extracts of fresh leaves differed from that of control at 2 mg/disc and the most effective inhibition was observed at 8 and 10 mg/disc. The water extracts of leaf powder no show inhibition zone.

5. Minimum inhibitory concentration

The MIC values of asiatic acid and crude extracts were determined by the modified resazurin microtiter-plate (Figure 9). 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 MIC_{50} value of 0.02 mg/ml (Figure 9A). The ethanol extracts show MIC_{50} value of 8 mg/ml (Figure 9B), the water extracts of leaf powder showed MIC_{50} value of 32 mg/ml (Figure 9C) and the water extracts of fresh leaves showed MIC value of 32-256 mg/ml (Figure 9D).

6. 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 hrs. 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 10A) and the MBC value of ethanol extracts was 16 mg/ml (Figure 10B). The water extracts of leaf powder and the water extracts of fresh leaves could not kill *S. aureus*.

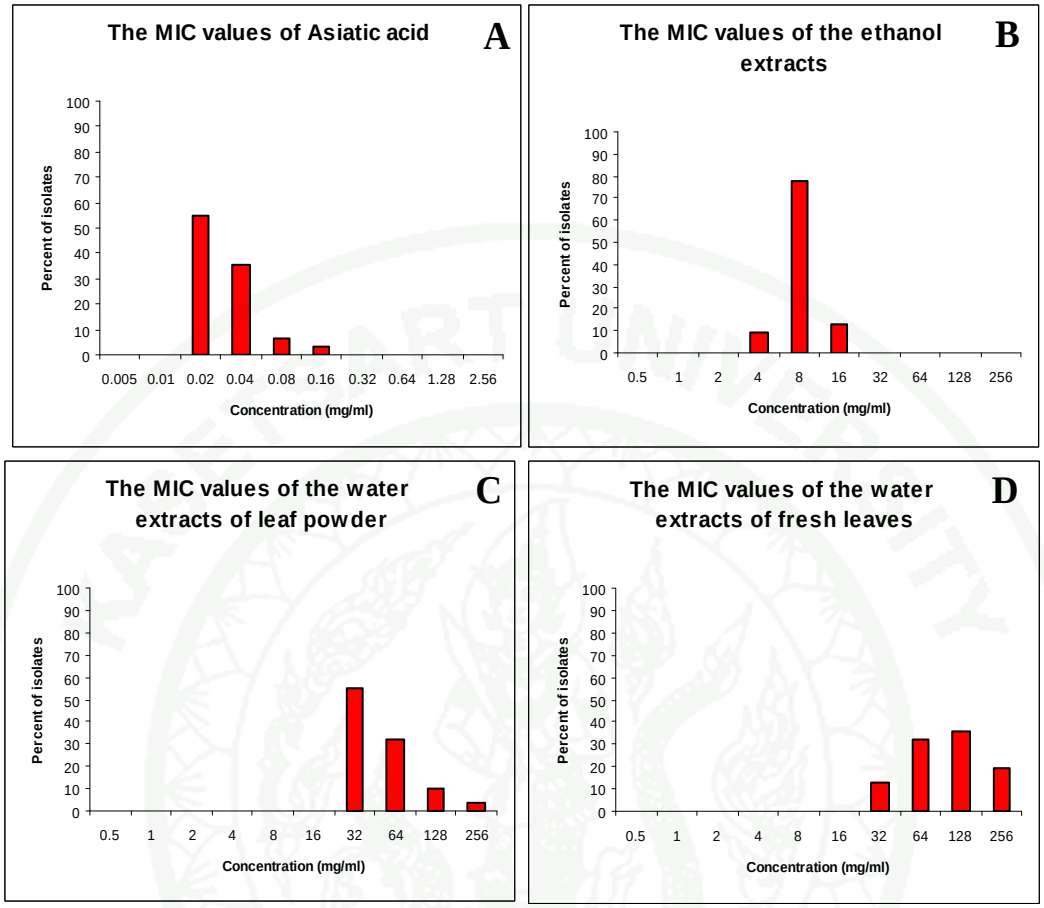


Figure 9 The MIC values of asiatic acid (A), and crude extracts (B, C, D) against 30 isolates of *S. aureus*.

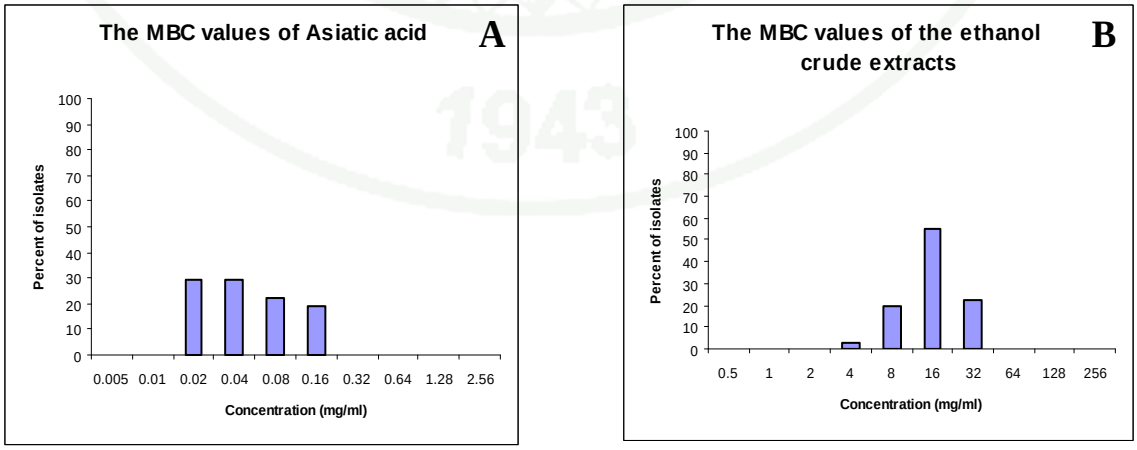


Figure 10 The MBC values of asiatic acid (A), and crude extracts (B) against 30 isolates of *S. aureus*.

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 MIC_{50} 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 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.

7. Antimicrobial susceptibility test for isolates of *S. aureus*

Antimicrobial susceptibility test results of thirty isolates of *S. aureus* are shown in Table 15. A total of thirty (100%) *S. aureus* isolates were susceptible to methicillin, vancomycin, cephalotin, amoxycillin-clavulanic acid, enrofloxacin and gentamicin. A total of thirteen (43.33%) isolates were resistant and two (6.66%) isolates were intermediate to ampicillin. A total of eight (26.66%) isolates were resistant and two (6.66%) isolates were intermediate to penicillin. A total of six (20%) isolates were resistant and only one (3.33%) isolates were intermediate to oxytetracycline. Only one (3.33%) was intermediate to cloxacillin, lincomycin and novobiocin that it was resistant to kanamycin, sulfamethoxazole-trimethoprim and neomycin. A total of twenty-nine (96.66%) isolates were intermediate to streptomycin.

Table 15 Antimicrobial susceptibility of thirty isolates *S. aureus*.

Antimicrobial agents	Resistant isolates	
	Number	%
Ampicillin	13	43.33
Penicillin G	8	26.66
Oxytetracycline	6	20.00
Kanamycin	1	3.33
Sulfamethoxazole-Trimethoprim	1	3.33
Neomycin	1	3.33

Table 16 Antimicrobial susceptibility and antimicrobial activity of the extracts in each isolates of *S. aureus*.

Isolates of *S. aureus*	β-lactam Resistant	MIC value of the extract (mg/ml)				MBC value of the extract (mg/ml)			
		asiatic acid	Ethanol	Water of leaf powder	Water of fresh leaves	asiatic acid	Ethanol	Water of leaf powder	Water of fresh leaves
ATCC 25923 (gr.1)	No	0.04	4	32	64	0.04	8	0	0
TD 1 (gr.2)	No	0.16	8	64	64	0.16	32	0	0
TD 3 (gr.2)	No	0.02	8	32	128	0.08	8	0	0
TD 4 (gr.2)	No	0.02	4	32	128	0.16	8	0	0
No. 8 (gr.4)	**Yes**	0.02	8	32	128	0.02	16	0	0
No. 149 (gr.5)	**Yes**	0.08	8	32	64	0.04	16	0	0
A1 (gr.3)	**Yes**	0.04	8	64	32	0.04	16	0	0
Pud C (gr.10)	**Yes**	0.02	8	64	64	0.02	8	0	0
1 (gr.9)	No	0.02	8	32	32	0.04	16	0	0
6 (gr.6)	**Yes**	0.02	8	32	32	0.02	16	0	0
8 (gr.5)	**Yes**	0.04	16	32	64	0.04	32	0	0
9	No	0.04	8	64	256	0.08	16	0	0
11 (gr.5)	No	0.02	8	64	64	0.16	8	0	0

Table 16 (Continued)

Isolates of *S. aureus*	β-lactam Resistant	MIC value of the extract (mg/ml)				MBC value of the extract (mg/ml)			
		asiatic acid	Ethanol	Water of leaf powder	Water of fresh leaves	asiatic acid	Ethanol	Water of leaf powder	Water of fresh leaves
13 (gr.5)	**Yes**	0.02	8	64	64	0.02	8	0	0
14 (gr.10)	**Yes**	0.02	8	32	64	0.02	4	0	0
15 (gr.5)	**Yes**	0.04	8	64	128	0.16	16	0	0
16 (gr.6)	No	0.04	8	128	256	0.04	16	0	0
17 (gr.7)	No	0.04	8	32	128	0.08	16	0	0
18 (gr.7)	No	0.02	8	32	128	0.04	16	0	0
19 (gr.7)	No	0.02	8	32	32	0.02	16	0	0
20 (gr.7)	No	0.04	8	32	128	0.04	32	0	0
21 (gr.7)	No	0.02	8	32	64	0.08	32	0	0
22 (gr.7)	No	0.02	16	32	64	0.02	16	0	0
23 (gr.7)	No	0.02	16	64	128	0.02	16	0	0
25 (gr.7)	**Yes**	0.08	8	32	256	0.04	16	0	0
26 (gr.7)	No	0.02	8	32	128	0.02	16	0	0
27 (gr.7)	**Yes**	0.04	4	64	128	0.08	32	0	0

Table 16 (Continued)

Isolates of *S. aureus*	β-lactam Resistant	MIC value of the extract (mg/ml)				MBC value of the extract (mg/ml)			
		asiatic acid	Ethanol	Water of leaf powder	Water of fresh leaves	asiatic acid	Ethanol	Water of leaf powder	Water of fresh leaves
28 (gr.7)	**Yes**	0.02	8	128	256	0.08	32	0	0
29 (gr.8)	No	0.04	8	128	256	0.16	32	0	0
30 (gr.7)	**Yes**	0.02	8	256	256	0.08	16	0	0

The penicillin and ampicillin are β-lactam antibiotics which are the most widely used treatment of bovine mastitis. The highest resistance was observed against penicillin, a β-lactam antibiotic in *S. aureus* strains isolated from bovine mastitis cases (Erskine *et al*., 2002; Vintov *et al*., 2003 and Sabour *et al*., 2004). Carla *et al* (1999) reported that 29 (43.9%) of *S. aureus* isolated from 66 milk samples of subclinical mastitis in Brazil were resistant to penicillinG/ampicillin. Guler *et al* (2005) reported that *S. aureus* from clinical mastitis cases in Turkey had the highest resistance against the β-lactam antibiotics, penicillin and ampicillin. Vintov *et al* (2003) reported the penicillin resistance with an average of 32.4% among *S. aureus* isolates from 9 European countries and the United States. Penicillin resistance was high among *S. aureus* isolates from Ireland (71.4%), the United Kingdom (67.3%), and the United States (50%) whereas it was low from Norway (2%). However, the frequency of penicillin resistance varies among countries which it may be linked to many factors, such as the difference in animal production systems and national policies for the use of antimicrobial drugs in each country.

8. Polymerase chain reaction

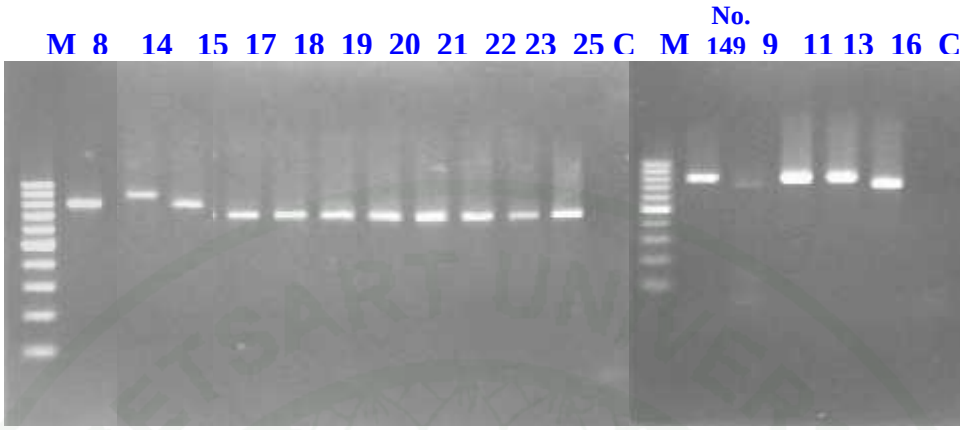
The *coa* gene coding for coagulase protein has been used for the development of DNA based diagnostic assays for *S. aureus*. This gene has highly polymorphic because of variable sequences at its 3′ coding region that can be used for differentiation of *S. aureus* isolates (Goh *et al*., 1992)

The PCR amplification of *coa* gene and subsequent agarose gel analysis of the amplification products showed a single band for each isolates. *S. aureus* isolate 9 could not give amplification product but the remaining 29 isolates produced four different types of PCR products which ranged from 600 bp to 900 bp (Table 17 and Figure 11). The 600 bp and 900 bp PCR products were observed in 2 isolates (6.66%) whereas the 800 bp PCR products were found in 9 isolates (30%) and the 700 bp PCR products were found in 16 isolates (53.33%).

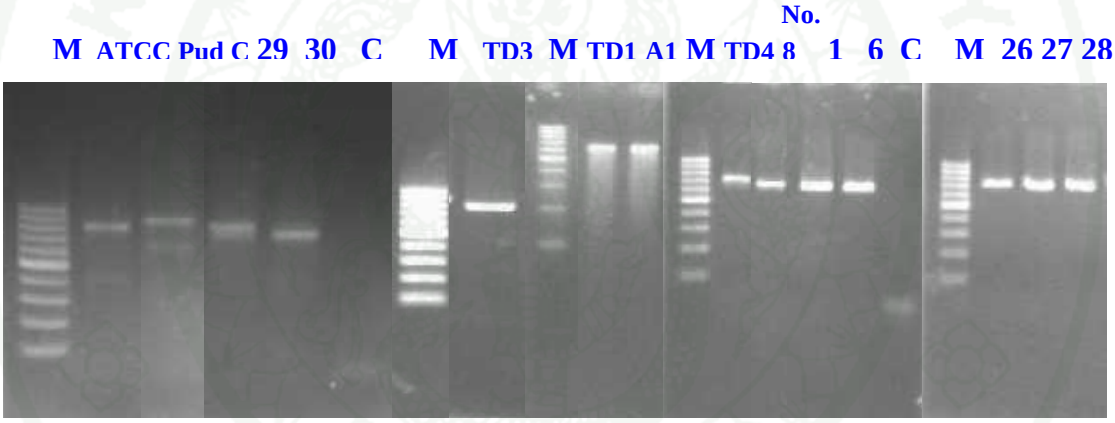
Table 17 *Coa* gene amplicons of *S. aureus* isolates from bovine mastitis.

Serial No.	Isolate No. (Total isolate)	*Coa* gene amplicon (bp)	Pattern
1	TD1, A1	600	I
2	TD4, No.8, 1, 6, 16, 17, 18, 19, 20, 21, 22, 23, 25, 26, 27, 28	700	II
3	ATCC 25923, TD3, No.149, 8, 11, 13, 15, 29, 30	800	III
4	Pud C, 14	900	IV

In this study, a 700 bp PCR product was the predominant type, accounting for 53.33% of the isolates. Similarly, Schlegelova *et al*. (2003) identified three coagulase genotypes among the 24 milk isolates in a farm, and 730 bp was predominant in 83.3% of the isolates. Su *et al*. (1999) reported that predominant types of *S. aureus* might be varied in different geographical areas or herds, and that predominant types were more resistant to neutrophil bactericidal activities than rare genotypes. Kumar *et al* (2008) studied 21 *S. aureus* isolates of mastitic milk that reported 3 different types of *coa* gene products (600, 680 and 850 bp), and 3 distinct RFLP patterns were obtained with *Alu* I digests of PCR products. Guler *et al* (2005) studied 265 isolates of *S. aureus* from clinical mastitis cases using the same primer pairs of COAG2 and COAG3 obtained 4 different types of *coa* gene PCR products (700, 800, 900 and 1,000 bp) for *S. aureus* isolated from bovine clinical mastitis and 2 different patterns of fragments were detected after *Alu* I digestion. Carla *et al* (1999) studied 66 isolates of *S. aureus* from bovine mastitis cases and results showed that 7 different types of *coa* gene PCR products ranged from 580 to 1060 bp and had 14 different patterns by *Alu* I digestion.



A



B

Figure 11 Coagulase gene PCR products with the COAG2 and COAG3 on 2% agarose gel. A: M-100 bp marker, C-Negative control (sterile distilled water). 8, 14, 15, 17, 18, 19, 20, 21, 22, 23, 25, No.149, 9, 11, 13 and 16 are PCR products obtained with the *S. aureus* cultures. B: M-100 bp marker, C-Negative control (sterile distilled water). ATCC 25923, Pud C, 29, 30, TD3, TD1, A1, TD4, No.8, 1, 6, 26, 27 and 28 are PCR products obtained with the *S. aureus* cultures.

The nucleotide sequence of *coa* from 29 isolates were determined using a multiple sequence alignment editor BioEdit version 7.0.5.2. The alignment of *coa* gene sequences were demonstrated in Figure 12 see in appendix E.

Multiple alignments of DNA sequences of 29 sequences were represented in 10 groups. The first group was consisted of *S. aureus* ATCC 25923. The second group was consisted of *S. aureus* TD1, TD3, and TD4. The third group was consisted of *S. aureus* A1. The fourth group was consisted of *S. aureus* No.8. The fifth group was consisted of *S. aureus* No.149, 8, 11, 13, and 15. The sixth group was consisted of *S. aureus* 6 and 16. The seventh group was consisted of *S. aureus* 17, 18, 19, 20, 21, 22, 23, 25, 26, 27, 28, 29, and 30. The eighth group was consisted of *S. aureus* 29. The ninth group was consisted of *S. aureus* 1. The last group was consisted of *S. aureus* Pud C and 14. The groups of DNA sequences are shown in Figure 13 (see in Appendix E).

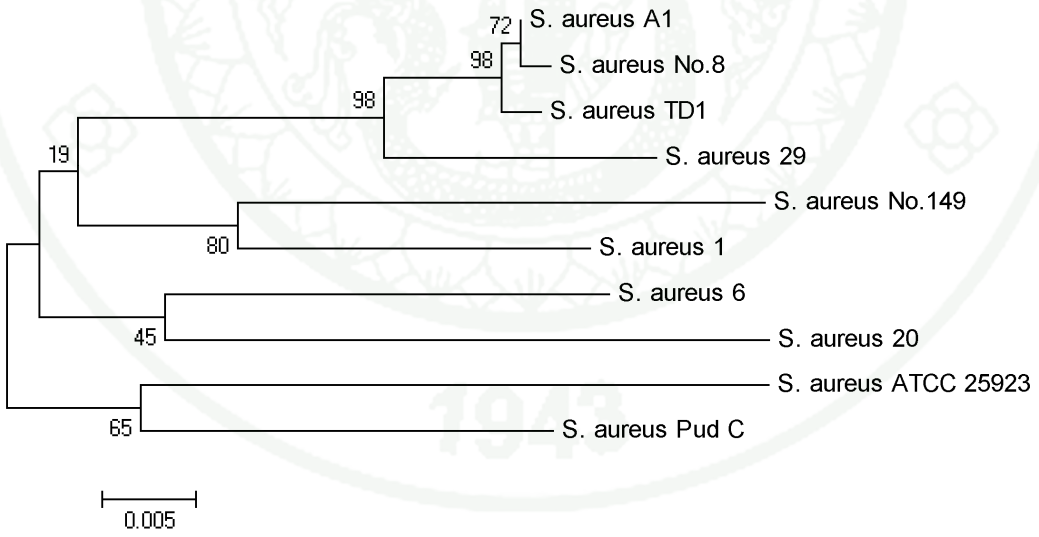


Figure 12 Dendrogram representing phylogenetic tree relationship of different types of coagulase gene. This dendrogram was constructed using MEGA 4 version 4028 software (neighbor joining method). The support each branch, as determined from 1000 bootstrap samples, is indicated by the value at each node (%).

The *coa* sequences were used to construct the phylogenetic tree using MEGA 4 program version 4028. A dendrogram of the genetic relationship of all coagulase types (Figure 14) shows that the *coa* sequences of *S. aureus* isolates were different in 10 groups. The *coa* sequences of *S. aureus* from 29 isolates were aligned with the sequence from *S. aureus* ATCC 25923. The *S. aureus* A1 showed a relationship with the *S. aureus* No.8, with a homology of 72%, and this group showed a relationship with the *S. aureus* TD1, with a homology of 98%. The group of *S. aureus* A1, No.8, TD1 showed a relationship with the *S. aureus* 29, with a homology of 98%. The *S. aureus* No.149 showed a relationship with the *S. aureus* 1, with a homology of 80%, the *S. aureus* ATCC 25923 showed a relationship with the *S. aureus* Pud C, with a homology of 65% and the *S. aureus* 6 showed a relationship with the *S. aureus* 20, with a homology of 45%.

The coagulase is an extracellular product of most strains of *S. aureus*. It is capable of clotting plasma from a number of different species and this ability is often used for the rapid identification of potentially pathogenic staphylococci. Coagulase can also bind fibrinogen and this activity is responsible for the clumping of *S. aureus* in plasma (Carter *et al*., 2003). However, the analysis of coagulase encoding *S. aureus* DNA (*coa* genes) has demonstrated variable sequences in the 3′-end coding region (Goh *et al*., 1992). This region contains a polymorphic repeat region that can be used to type *S. aureus* isolates of bovine origin (Guler *et al*., 2005).

Previously, several different phenotyping and genotyping techniques have been applied for subtyping of *S. aureus* isolates of both bovine and human origin, such as phage typing, plasmid analysis, ribotyping, pulse-filed gel electrophoresis, multilocus enzyme electrophoresis, PCR-based fingerprinting, binary typing, and amplification of specific gene regions. The development of gene amplification and sequencing has simplified the taxonomy and identification of *S. aureus* (Guler *et al*., 2005).

The results of this study suggested that PCR technique was appropriately used for detection of *S. aureus* because the technique was rapid, sensitive and accurate. The technique could indicate that the *S. aureus* specific *coa* gene between coagulase-positive *S. aureus*. Sequencing techniques were simplified and could be an epidemiological marker with high discriminatory value. Because of the heterogeneity, the coagulase gene would be a potential target for typing classification on the basis of DNA sequence analysis.

CONCLUSION

From the experimental results and discussion of this study, the conclusion can be drawn as follow:

1. The ethanol extraction of leaf powder yielded crude extracts twice as much as the water extraction of fresh leaves and about six times of the water extraction of leaf powder. However, extraction using ethanol had a greater yield as compared to water extraction. The ethanol could be an effective solvent for the extraction of asiatic acid from *C. asiatica*.

2. The identification and the quantification of asiatic acid in crude extracts of *Centella asiatica* were developed and analytical method was successfully validated by TLC-Densitometry. The TLC solvent system containing chloroform: methanol: ethyl acetate: water (30:5:5:1) gave the best resolution of asiatic acid (R_f = 0.45). The identified band of asiatic acid was sprayed with anisaldehyde-sulphuric acid reagent, that band of asiatic acid appeared as blue-violet spot. The detection and quantification were performed by TLC scanner at the wavelength of 525 nm. Validation of TLC method for quantifying asiatic acid in the crude extracts showed a good correlation coefficient (0.998), the intra-day precision (0.90), the inter-day precision (0.31), the average recovery percentage (99.7±0.76), the limit of detection (0.76 ng/spot) and the limit of quantitation (2.60 ng/spot).

3. The highest quantity of asiatic acid in crude extracts of leaves of *C. asiatica* was 0.93 w/w, which was obtained from the ethanol extracts of leaf powder. This method was the appropriate extraction method for high yielding of asiatic acid that was the marker compound in this study. The asiatic acid content in water extracts of leaf powder and the water extracts of fresh leaves were 0.21 and 0.13 w/w, respectively.

4. Crude extracts of *C. asiatica* were tested for antibacterial activity against 29 isolates of *S. aureus* from milk samples of dairy cows. The antibacterial activity of

crude extracts were tested by the disc diffusion test, and the results showed that ethanol extract and water extract had average inhibition zones ranged from 6.44-6.49 and 6.54-17.72 mm in diameter, respectively.

5. 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 extract had an MIC_{50} value of 8 mg/ml, the water extract of leaf powder had an MIC_{50} value of 32 mg/ml, and the water extract of fresh leaves had an MIC value of 32-256 mg/ml. The ethanol extract had an MBC value of 16 mg/ml. The water extract of *C. asiatica* could not kill *S. aureus*.

6. For antibacterial activity test, the modified resazurin microtiter-plate was appropriate to test antibacterial activity in crude extracts.

7. Three DNA patterns (900, 800, 700 bp) of *S. aureus* were identified from 30 isolated from bovine mastitis by polymerase chain reaction (PCR) technique based on *coa* gene. The *coa* gene coding for coagulase protein has also been used for the development of DNA based diagnostic assay for *S. aureus*. The *coa* gene is polymorphism, it can be used for differentitation of S. aureus isolates.

8. The *coa* sequences of 30 isolates, representing 10 isolates were determined. The 30 isolated were also tested for susceptibility to antimicrobial drugs using the agar disc diffusion test. The highest resistance was observed in β-lactam antibiotics, penicillin and ampicillin.

9. *S. aureus* susceptibility to antimicrobial drugs except penicillin and ampicillin, they remain no problem of methicillin resistance in animals.

The present research on antibacterial activity of crude extracts from *C. asiatica* was solely based on *in vitro* studies. It is therefore necessary to test this

activity in an *in vivo* study to have better application of the crude extracts of *C. asiatica* in bovine mastitis. Product development of *C. asiatica* crude extracts was studied for teat dipping solution and intramamary drug to use in dairy farm. Therefore, further research should be studied as follows :

1. Mechanism of asiatic acid and crude extracts against *S. aureus*

Previous studies demonstrated that *C. asiatica* extracts can enhance collagen synthesis in *vitro* and extracellular matrix accumulation in *vivo*, and can enhance tensile strength in wound tissue and facilitate the wound healing process. The bioactive compounds in *C. asiatica* are asiatic acid, asiaticoside, medecassoside and medecassic acid. Thus the synthesis and pharmacological mechanism of asiatic acid and derivatives have drawn considerable interest for effect on antibacterial activity and cytotoxic activity on fibroblast cells.

2. Identification and isolation of other fraction in crude extracts

Typically, bioactive compounds in herbal plants are presented in low concentrations. Therefore, it is very important to develop more effective, selective extraction and isolation and identification of the fraction of bioactive compound from the herb materials. Then, the fraction should be studied for antibacterial activity and then considering purification of the fraction before application to animals.

3. Toxicity of crude extracts for udder of the cow

The residual solvents may be a problem of using crude extracts because residual toxic of organic solvents in the extracts can deteriorate the quality of the extracts and may cause serious health problems when the extracts are applied to the udder of the cow. Moreover, the toxicity should be studied for dosage limits or dose respond and adverse side effects of *C. asiatica*.

LITERATURE CITED

Anonymous. 2001. **Laboratory report in Kaset save the nation project, as the project of technology instructed for increase efficiency and improve raw milk quality in the western region phase 3 at the animal's hospital of Kasetsart University, Kamphaengsaen campus**. October 2000-September 2001.

_____. 2003. Available Source: www.milkproduction.com/Library/Articles/ Efficient_Milking_The_Mammary_Gland.htm., 17 January 2003.

_____. 2006. Available Source: www.mdidea.com/products/herbextract/ gotukola/data.html., 17 December 2006.

Anthony, B.F. and H.R. Hill. 1988. Gram positive bacteria: an overview and summary of session. **Rev Infec Dis** 10: S345-350.

AOAC. 1993. AOAC Peer Verified methods Program, Manual on policies and procedures, **Arlington.** VA, Nov 1993.

ASEAN Countries. 1993. **Standard of ASEAN Herbal Medicine Vol. I.** Aksara Buana Printing, Jakarta, Indonesia. P. 141-153.

Auldist, M.J., S. Coats, G.L. Rogers, and G.H. McDowell. 1995. Changes in the composition of milk from healthy and mastitic dairy cows during the lactation cycle. **Australian Journal of Experimental Agriculture.** 35: p.427-436

_____. and I.B. Hubble. 1998. Effects of mastitis on raw milk and dairy products. **The Australian Journal of Dairy Technology.** 53: 28-36.

Barkema, H.W., Y.H. Schukken and R.N. Zadoks. 2006. Invited Review: The Role of Cow, Pathogen and Treatment Regimen in the Therapeutic Success of Bovine *Staphylococcus aureus* Mastitis. **J Dairy Sci**. 89: 1877-1895.

Baddiley, J. 1989. Bacterial cell walls and membranes. Discovery of the teichoic acids. **Bioessays** 10: 207-210.

Belschner, A.P., J.W. Hallberg, S.C. Nickerson and W.E. Owens. 1996. ***Staphylococcus aureus* mastitis therapy revisited in Proceedings.** 35th Annual. Meeting., National Mastitis Council., Madison, WI, pp. 116-122.

Benites, N.R. 2002. Aetiology and histopathology of bovine mastitis of spontaneous occurrence. **Journal of Veterinary Medicine.** 49(8): 366.

Beveridge, T.J. 1999. Structures of gram-negative cell walls and their derived membrane vesicles. **J. Bacteriol.** 181: 4725-4733.

Bhakdi, S. and J. Tranum-Jensen. 1991. **Alpha-toxin of *Staphylococcus aureus*.** Microbiol Rev 55: 733-751.

Brinkhaus, B., M. Lindner, D. Schuppan and G. Hahn. 2000. Chemical pharmacological and clinical profile of the East Asian medical plant *Centella asiatica*. **Phytomedicine.** 7(5): 427-448.

Bruckmaier, R.M., C.E. O. and J.W. Blum. 2004. Fractionized milk composition in dairy cows with subclinical mastitis. **Veterinary medicine-Czech.** 8(283-290).

Carter P.E., K. Begbie and F.M. Thomson-Carter. 2003. Coagulase gene variants associated with distinct populations of *Staphylococcus aureus*. **Epidemiol. Infect** 130: 207-219.

Chaidate I., A. Kittisak and S. Sukuma. 2005. **Antibacterial activity of some medicinal plants against *Staphylococcus aureus* as a causes of bovine mastitis.** In Abstracts: The 4th Veterinary Science Conference. 60th building, Veterinary Science faculty, Chulalongkorn University, Thailand, 15 February 2005. p.103.

Cheng, C.L., and M.W.L. Koo. 2000. Effect of *Centella asiatica* on ethanol induced gastric mucosal lesions in rats. **Life Science.** 67: 2647-2653

CLSI. 2007. **Performance Standards for Antimicrobial Susceptibility Testing; Seventeenth Informational Supplement. CLSI document M100-S17.** Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania, USA.

Dego, O. K., J.E. Van Duk and H. Nederbragt. 2002. A review: Factors involved in the early pathogenesis of bovine *Staphylococcus aureus* mastitis with emphasis on bacterial adhesion and invasion. **Veterinary quartery.** vol 24, pp. 182-198.

DeGraves, F.J. and J. Fetrow. 1993. Economics of mastitis and mastitis control. Veterinary Clinics of North America. **Food animal Practice.** 9: 421-434.

De Padua, LS., N. Bunyapraphatsara and Lemmens RHMJ (eds). 1999. **Plant Resources of South-East Asia No. 12(1).** Medicinal and poisonous plants1. Prosea Foundation, Indonesia. P. 190-194.

Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University. 1996. **Medicinal Plants in Thailand: Volume I.** Amarin Printing and Publishing Public Co. Ltd., Bangkok.

Douglas, D. B. and J. W. Robert. 2005. **A novel strategy for the prevention of *Staphylococcus aureus* induced mastitis in dairy cows.** n.p.

Ekgachai, S., W. Srisamai, B. Somphong, C. Suksan, K. Sunan, T. Suwanna, S. Piyawan and D. Mangorn. 2001. **Survey in subclinical bovine mastitis of member of Nong-Pho dairy co-operative.** In proceeding of Kasetsart university 39th Veterinary program. 5-7 February 2001. p. 543-549.

Erskine, R. J. 2001. **Mastitis control in dairy herds**, p. 397-433. *In* O. M. Radostits (ed.), Herd health: food animal production medicine, 3rd ed. W. B. Saunders Co., Philadelphia, PA..

Erskine, R.J., R.D. Walker, C.A. Bolin, P.C. Barlett and D.G. White. 2002. Trends in antibacterial susceptibility of mastitis pathogens during as even-year period. **J. Dairy Sci.** 85: 1111–1118.

Erskine, R.J., S.A. Wagner and F.J. DeGraves. 2003. Mastitis therapy and pharmacology. Vet. Clin. **North Am. Food Anim. Pract.** 19: 109-138.

Farm Animal Welfare Council. 1997. Report on the Welfare of Dairy Cattle, Tolworth, Surrey.

Fitzpatrick, J.L. 2000. *Staphylococcus aureus* Mastitis: Past Problems, Future Risks. **Cattle Practice in The Journal of the British Cattle Veterinary Association.** Vol.8 pp.273-277.

Forsback, L., H. Lindmark-Mansson, A. Andren, M. Akerstedt and K. Svennersten-Sjaunja. 2009. Udder quarter milk composition at different levels of somatic cell count in cow composite milk. **Journal Animal.** 3:5. pp. 710-717.

Giesbrecht, P., T. Kersten, H. Maidhof and J. Wecke. 1998. Staphylococcal cell wall: morphogenesis and fatal variations in the presence of penicillin. **Microbiol Mol Biol Rev.** 62: 1371-1414.

Goffin, C. and J.M. Ghuysen. 1998. Multimodular penicillin-binding proteins: an enigmatic family of orthologs and paralogs. **Microbiol Mol Biol Rev.** 62: 1079-1093.

Goh, S.H., S.K. Byrne, J.L. Zhang and A.W. Chow. 1992. Molecular typing of *Staphylococcus aureus* on the basis of coagulase gene polymorphisms. **J. Clin. Microbiol.** 30: 1642-1645.

Guler, L., U. Ok, K. Gunduz, Y. Gulcu and H.H. Hadimli. 2005. Antimicrobial Susceptibility and Coagulase Gene Typing of *Staphylococcus aureus* Isolated from Bovine Clinical Mastitis Cases in Turkey**. J. Dairy Sci.** 88:3149-3154.

Guo, J.S., C.L. Cheng and M.W.L. Koo. 2004. Inhibitory Effects of *Centella asiatica* Water Extract and Asiaticoside on Inducible Nitric Oxide Synthase During Gastric Ulcer Healing in Rats. **Planta Med.** 70: 1150-1154.

Harmon, R.J. 1994. Physiology of mastitis and factors affecting somatic cell counts. **J. Dairy Sci.** 77: 2103-2112.

_____. 1996. **Controlling Contagious Mastitis.** Presented at the 1996 National Mastitis Council Regional Meeting, Queretero, Mexico. Published in the 1996 National Mastitis Council Regional Meeting Proceedings, pp 11.

Hillerton, J.E. 2001. **Meeting somatic cell count regulations in the European Union.** Proc. 40th Annual Meeting of the National Mastitis Council. pp 47-53.

Hillerton, J.E. and E.A. Berry. 2003. The management and treatment of environmental streptococcal mastitis. **Vet Clin North Am Food Pract.** 19(1): 157-169.

International Conference on Harmonization. 2006. **Note for Guideance on Validation of Analytical Methods.** Text and Methodology. Available Source: http://www.emea.eu.int/pdfs/human/ich/038195en.pdf, July 28, 2006.

Incandela L. 2001. Treatment of diabetic microangiopathy and edema with total triterpenic fraction of *Centella asiatica*: a prospective, placebo-controlled, randomized study. **Angiology** 52(2): 27-31.

Jacinda, T. J., R. Meyer and A.D. Ian. 2008. Characterisation of two phenotypes of *Centella asiatica* in Southern Africa through the composition of four triterpenoids in callus, cell suspensions and leaves. **Plant Cell Tiss Organ Cult.** 94: 91-99.

Jagtap, NS, S.S. Khadabadi, D.S.Ghorpade, N.B. Banarase, S.S. Naphade. 2009. Antimicrobial and Antifungal Activity of *Centella asiatica* (L.) Urban, Umbeliferae. **Research J. Phar. And Tech.** 2 (2): 328-330.

Jayarao, B.M. and D.R. Wolfgang. 2003. Bulk-tank milk analysis. A useful tool for improving milk quality and herd udder health. **Vet Clin North Am Food Anim Pract**. 19: 75–92.

Jones, G.M., T.L. Bailey and J.R. Roberson. 1998. ***Staphylococcus aureus*** **Mastitis: Cause, Detection, and Control.** Virginia Cooperative Extension.

Kannabiran, K., T. Mohankumar and V. Gunaseker. 2009. Evaluation of Antimicrobial Activity of Saponin Isolated From Solanum Xanthocarpum and *Centella asiatica*. **International Journal of Natural and Engineering Sciences** 3 (1): 22-25.

Keefe, G.P., I.R. Dohoo, E. Spangler. 1997. Herd prevalence and incidence of *Streptococcus agalactiae* in the dairy industry of Prince Edward Island. **J Dairy Sci.** 80: 464–470.

Kenneth, T. 2005. ***Staphylococcus*.** Todar's Online Textbook of bacteriology.

Khaitsa, M.L., T.E. Wittum, K.L. Smith, J.L. Henderson and K.H. Hoblet. 2000. Herd characteristics and management practices associated with bulk-tank somatic cell counts in herds in official dairy herd improvement association programs in Ohio. **Am J Vet Res.** 61: 1092–1098.

Kongthong, B. 2004. **Development of the quantitative determination of Asiaticoside, Madecassoside, Asiatic acid and Madecassic acid in *Centella asiatica* (Linn.).** Urban by High Performance Liquid Chromatography, a master thesis of faculty of pharmaceutical sciences, Chulalongkorn University.

Labischinski, H. 1992. Consequences of the interaction of beta-lactam antibiotics with penicillin binding proteins from sensitive and resistant *Staphylococcus aureus* strains. **Med Microbiol Immunol (Berl).** 181: 241-265.

Lange, C., M. Cardoso, D. Senczek and S. Schwarz. 1999. Molecular subtyping of *Staphylococcus aureus* isolates from cases of bovine mastitis in Brazil. **Veterinary Microbiology.** 67 (Issue 2): 127-141.

Lee, S.W., N. Musa, T. S. Chuah, W. Wee and Noor Azhar Mohd Shazili. 2008. Antimicrobial properties of tropical plants against 12 pathogenic bacteria isolated from aquatic organisms. **African Journal of Biotechnology** 7 (13): 2275-2278,

Luangchonlathan, S., B. Kongthong, and C. Patarapanich. 2004. A TLC Method Determination of Active Constituents, Madecassoside and Asiaticoside, from *Centella asiatica* Linn. In various seasons in Thailand. **Thai J. Pharm. Sci.** Vol.28 (Suppl.)

Mamtha, B., K. Kavitha, K.K. Srinivasan and P.G. Shivananda. 2004. An in vitro study of the effect of *Centella asiatica* (Indian pennywort) on enteric pathogens. **Indian J. Pharmacol.** 36: 41.

McGuffin M., J.T. Kartesz, *et al*. 2000. **Herbs of Commerc.** 2nd edition. The American Herbal Product Association, USA. P. 36.

Mullika T., S. Surassmo, K. Wongsariya and N. Bunyapraphatsara. 2008. Antibacterial activity of Thai medicinal plants against Methicillin-resistant *Staphylococcus aureus*. **Fitoterapia** 80: 102-104

Murakami, K., T. Fujimura and M. Doi. 1994. Nucleotide sequence of the structural gene for the penicillin-binding protein 2 of *Staphylococcus aureus* and the presence of a homologous gene in other staphylococci. **FEMS Microbiol Lett.** 117: 131-136.

National Committee for Clinical Laboratory Standards NCCLS, 1993. **Approved Standard, M7-A3: Methods for dilution Antimicrobial Susceptibility test for bacteria that grow aerobically.** NCCLS, Villanova, PA.

National Mastitis Council Research Committee Report. 1996. **Milk Quality and Mastitis Control in Canada: Progress and Outlook.** NMC Annual Meeting proceedings.

_____. 2004. **Bovine Mastitis Pathogens and Trends in Resistance to Antibacterial Drugs.** NMC Annual Meeting proceedings. pp 400-414.

Nguyen, D. A.D. and M.C. Neville. 1998. Tight Junction Regulation in the Mammary Gland. **Journal of Mammary Gland Biology and Neoplasia**. 3 (3): 233-246.

Obayed, U., S. Shapna, H. Afroza and T. Saira. 2009. Antimicrobial, Cytotoxic and Antioxidant Activity of *Centella asiatica*. **European Journal of Scientific Research** Vol.30 No.2 (2009), pp.260-264.

Owen, S., P. Segall, M. Lisowski, M. Murray, M. Bevis and J. Foster. 1995. The January 30, 1997 eruptive event on Kilauea Volcano, Hawaii, as monitored by continuous GPS. **Geophys. Res. Lett.** 27, 2757-2760.

Oyedeji, O.A. and A.J. Aflolayan. 2005. Chemical Composition and Antibacterial Activity of the Essential Oil of *Centella asiatica* Growing in South Africa. **Pharmaceutical Biology.** 43 (3): 249-252.

Patarapanich, C., B. Kongthong and S. Luangchonlathan. 2004. A HPLC Method for Determination of Asiaticoside, Madecassoside, Asiatic acid and Medecassic acid in the extract of *Centella asiatica* Linn.: Development, Validation and Application. **Thai J. Pharm. Sci.** Vol.28 (Suppl.)

Pithai, K. P., S. Porntrakulpipat, P. Borisuthpetch, K. Sarachoo, J. Jivakanon, C. Aromdee and S. Wongkham. 2003. **Anti-bacterial activity of Thai Medicinal Plants on Escherichia coli (F18+).** P479-481. In Proceedings: The 2nd International Conference on Medicinal Mushroom and the International Conference on Biodiversity and Bioactive Compounds, Peach, Pattaya, Thailand, 17-19 July 2003. With Poster presentation

Pitkala, A., M. Haveri, S. Pyorala, V. Myllys and T.Honkanen-Buzalski. 2004. Bovine mastitis in Finland 2001 prevalence, distribution of bacteria, and antimicrobial resistance. **J Dairy Sci.** 87:2433–2441.

Prevost, G., P. Couppie and P. Prevost. 1995. Epidemiological data on *Staphylococcus aureus* strains producing synergohymenotropic toxins. **J Med Microbiol** 42: 237.

Pyorala, S. and Pyorala, E. 1997. Accuracy of methods using somatic cell count and N-acetyl-B-D-glucosaminidase activity in milk to assess the bacteriological cure of bovine clinical mastitis. **J. Dairy Sci.** 72: 1057-1061.

Pyorala, S. 2003. Indicators of inflammation in the diagnosis of mastitis. **Veterinary Research.** 34(5): 565-568.

Quinn, P.J., M.E. Carter, B.K. Markey and G.R. Carter. 1994. Clinical Veterinary Microbiology. Wolfe Publishing, an imprint of Mosby-Year Book Europe Limited.

Richard, G.M., R. Olde, W. B. Herman, V. Stefan, E. P. Doris, T. D. Randy and Gregory P. Keefe. 2006. Prevalence of contagious mastitis pathogens in bulk tank milk in Prince Edward Island. **Can Vet J.** 47(6): 567–572.

Roberson, J.R., L.K. Fox, , Hancock, D.D, G.M. Gay and T.E. Besser. 1994. Ecology of *Staphylococcus aureus* isolated from various sites on dairy farms. **J. Dairy. Sci.**

Roux, Y.I., F. Laurent and F. Moussaoui. 2003. Polymorphonuclear proteolytic activity and milk composition change. **Veterinary research.** 34(5): 629-645.

Sabour, P.M., J.J. Gill, D. Lepp, J.C. Pacan, R. Ahmed, Dingwell and K. Leslie. 2004. Molecular typing and distribution of *Staphylococcus aureus* isolates in Eastern Canadian dairy herds. **J.Clin.Microbiol.** 42:3449–3455.

Sangkhapaitoon, P., P. Sangkhapaitoon and S. Kummee. 2002. **Study on Fifteen Medicinal Plant Extracts for Antibacterial Activity of Mastitis in Dairy Cow.** In Proceedings of Thai Medical herb Opportunity and Alternatives of Animal Production Industry. Maruay Garden Hotel, Chatuchak, Bangkok. pp. 298-308.

Sanjiv, K., K.K. Anil, R. Sharma and G. Singh. 2008. Epidemiological typing of *Staphylococcus aureus* by DNA restriction fragment length polymorphism of *coa* gene. **Veterinarski Arhiv** 78 (1): 31-38.

Sarker S.D., L. Nahar and Y. Kumarasamy. 2007. Microtitre plate - based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the *in vitro* antibacterial screening of phytochemicals. **Method.** 42(4): 321-324.

Schlegelova, J., M. Dendis, J. Benedik, V. Babak and D. Rysanek. 2003. *Staphylococcus aureus* isolates from dairy cows and humans on a farm differ in coagulase genotype. **Vet.Microbiol.** 92:327–334.

Schroeder J.W. 1997. **Mastitis Control Programs: Bovine Mastitis and Milking Management.** Available Source: www.ag.ndsu.edu, January 16, 2007.

Shukla, et al. 1999. In vitro and in vivo wound healing activity of asiaticoside isolated from *Centella asiatica*. **Journal of Ethnopharmacology** 65 (1): 1-11.

Shuster, D.E. *et al.* 1991. Suppression of milk production during endotoxin-induced mastitis. **J. Dairy Sci.** 74(11): 3763-3774.

Smith, K.L. and J.S. Hogan. 1999. **A world standard for milk somatic cell count: is it justified?**. Bulletin of the International Dairy Federation. No345. pp. 7-10.

_____, K. Takemura and J.S. Hogan. 2000. **International progress in mastitis control.** Proc. Pacific Congress on milk quality and mastitis control. Nagano, Japan. November 13-16. pp.245-252.

Sribusarakum, A. 1997. **Chromatographic determination of active constituents of *Centella asiatica* (Linn.).** Urban in Thailand, a master thesis of faculty of pharmacy, Mahidol University.

Srivastava, R., Y.N. Shukla and S. Kumar. 1997. Chemistry and pharmacology of *Centella asiatica* : A review. **J. Med. Aro. Sci.** 19: 1049-1056.

Su, C., C. Herbelin, N. Frieze, O. Skardova and L.M. Sordillo. 1999. Coagulase gene polymorphism of *Staphylococcus aureus* isolates from dairy cattle in different geographical areas. **Epidemiol. Infect.** 122:329–336.

Supplement to Thai Herbal Pharmacopoeia. 2004. **Department of Medical Sciences.** Ministry of Public Health, Nonthaburi, Thailand. P. 9-17, 41-47.

Sutra, L. and B. Poutrel. 1994. Virulence factors involved in the pathogenesis of bovine intramammary infections due to *Staphylococcus aureus*. **J. Med. Microbiol.** 40, p.79-89.

Tanwarat K., L. Pongsatorn, N. Manti, S. Taweesak. 2003. **Quantitative Determination of Asiaticoside and Madecassoside in *Centella asiatica* Linn.** by TLC-Densitometry Technique, Poster Presentation in The 6th JSPS-NRCT Joint Seminar on Recent Advances in Natural Medicine Research, Bangkok, Thailand, December 2-4, 2003.

Thenmozhi, M., G. Vinitha and K. Kannabiran. 2009. **Evaluation of antimicrobial activity of saponin extracts from *Solanum xanthocarpum* and *Centella asiatica*.** International Conference on Biotechnology (INCOB - 2008), VIT University.

United States Pharmacopeial Convention, Inc. 2001. **The United States pharmacopeia 25/The national formulary 20 – 2002.** United States Pharmacopeial Convention, Inc., Rockville, Md.

Vintov J., F.M. Aarestrup, C.E. Zinn and J.E. Olsen. 2003. Association between phage types and antimicrobial resistance among bovine *Staphylococcus aureus* from 10 countries. **Vet. Microbiol.** 95:133–147.

Viroj patarajinda. 2003. **Dairy Cattle.** Khon-Khan University Printing and Publishing Public Co. Ltd., Khon-khan. Page 450.

Voravuthikunchai, S. and L. Kitpipit. 2005. Antibacterial activity of crude extracts of Thai medicinal plants against clinical isolates of methicillin-resistant *Staphylococcus aureus*. Songklanakarin **J. Sci. Technol.** 27 (suppl. 2): 525-534

Wijeweera, P., J.T. Arnason, D. Koszycki and Z. Merali. 2006. Evaluation of anxiolytic properties of Gotukola (*Centella asiatica*) extracts and asiaticoside in rat behavioral models. **Phytomedicine.** 13: 668-676.

Workineh, S., M. Bayleyegn, H. Mekonnen and L.N.D. Potgieter. 2004. Prevalence and Aetiology of Mastitis in Cows from Two Major Ethiopian Dairies. **Tropical Animal Health and Production.** Vol. 34 No.1 pp. 19-25.

World Health Organization. 1999. **Herba Centellae.** WHO monographs on selected medicinal plants. Volume 1. Geneva. pp.77-85.

Zaidan, MR, R.A. Noor, A.R. Badrul, A. Adlin, A. Norazah and I. Zakiah. 2005. In vitro screening of five local medicinal plants for antibacterial activity using disc diffusion method. **Trop Biomed.** 22 (2): 165-170.

APPENDICES

Appendix A

Chemical and Substances

Chemical	**Company**
Asiaticoside standard purity	Fluka, Switzerland
Asiatic acid standard purity	Sigma-Aldrich, Germany
Anisaldehyde	Fluka, Switzerland
Chloroform, HPLC grade	Labscan, Thailand
Deionized water	J.T. Baker, USA
95% Ethanol, Commercial grade	Excise Department, Thailand
Ethyl acetate, AR grade	J.T. Baker, USA
Glacial acetic acid, AR grade	J.T. Baker, USA
Methanol, AR grade	J.T. Baker, USA
Methanol, HPLC grade	Labscan, Thailand
Sulfuric acid, AR grade	J.T. Baker, USA
Filter paper Whatman Number 1	Maidstone, England
Nylon filter 0.45 μm	Vertical Chromatography, Thailand
TLC aluminium sheets silica gel 60 F_{254}	E.Merck, Germany
Baird-Parker Agar	Difco, USA
Brain Heart Infusion Broth	Difco, USA
Blood Agar	Oxoid, England
California Mastitis Test solution	Thailand
Coagulase plasma rabbit with EDTA	BBL, USA
Dimethylsulfoside	AMRESCO, USA
Mannitol Salt Agar	Pronadisa, Spain
Mueller-Hinton Agar	Merck, Germany
Mueller-Hinton Broth	Merck, Germany
Sodium pyruvate	BDH, England
Resazurin tablet	BDH, England
Sodium chloride	BDH, England
Tryptic Soy Broth	Oxoid, England
96 well plates (Microtiter-plate)	Greiner bio-one, Germany

Chemical	**Company**
Amoxycillin-Clavulanic acid (30 μg)	Oxoid, England
Ampicillin (10 μg)	Oxoid, England
Cephalotin (30 μg)	Oxoid, England
Cloxacillin (5 μg)	Oxoid, England
Enrofloxacin (5 μg)	Oxoid, England
Gentamicin (10 μg)	Oxoid, England
Kanamycin (30 μg)	Oxoid, England
Lincomycin (2 μg)	Oxoid, England
Methicillin (5 μg)	Oxoid, England
Neomycin (30 μg)	Oxoid, England
Novobiocin (30 μg)	Oxoid, England
Oxytetracycline (30 μg)	Oxoid, England
Penicillin G (10 unit)	Oxoid, England
Streptomycin (10 μg)	Oxoid, England
Sulfamethoxazole-Trimethoprim (25 μg)	Oxoid, England
Vancomycin (30 μg)	Oxoid, England
Absolute ethanol	Merck, Germany
Agarose	First base, Malaysia
Distilled water, PCR grade	Invitrogen
Ethidium bromide	Biobasic Inc.
Isopropanol	Merck, Germany
QIAamp®	QIAGEN
QIAquick®	QIAGEN
PCR buffer	Intron, Pacific Science
dNTPs	Intron, Pacific Science
$MgCl_2$	Intron, Pacific Science
Taq polymerase	Intron, Pacific Science
Chemical	**Company**
Primers	First base, Malaysia

Instrument	Company
Analytical balance	Mettler Toledo, Thailand
Autopipette	LABMATE
Freeze dried	Telstar Lyoalfa 6, Spain
Hot air oven Model UL 50	Memmert, Germany
Membrane Filter apparatus	Duran, Germany
Rotary vacuum evaporator	Buchi, Switzerland
Sample Application: Linomat V	Camag, Muttenz, Switzerland
Chromatogram Evaluation: TLC Scanner	Camag, Muttenz, Switzerland
Computer Integrator: winCATS 1.2.6 software	Camag, Muttenz, Switzerland
TLC Twin trough chamber 10×20 cm.	Camag, Muttenz, Switzerland
TLC heater	Camag, Switzerland
TLC Syringe	Camag, Switzerland
Ultrasonic Bath	Cole Parmer, USA
Water Bath with thermostat	Memmert, Germany
Incubator	Memmert, Germany
Laminar flow	Boss Tech
Gel document	Bio-Rad Gel doc 1000
Gel electrophoresis	COSMO BIO
McFarland densitometer	Grant-bio, UK
Microcentrifuge	Denville Scientific
Shaker incubator	Bio-active
Thermo cycle	Biometra
Vortex mixer	LMS, Japan

Appendix B

Bacterial media and general buffer

1. Bacterial media

1.1 Tryptic Soy Broth (TSB)

Approximate Formula Per 1 L	
Pancreatic digest of casein	17.0 g
Papaic digest of soybean mea	l3.0 g
Sodium chloride	5.0 g
Di-potassium hydrogen phosphate	2.5 g
Glucose	2.5 g
Final pH 7.3 ± 0.2 at 25°C	

Directions for Preparation from

1. Suspend 30 g of the powder in 1 L of purified water. Mix thoroughly.

2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.

3. Autoclave at 121°C for 15 minutes.

1.2 Baird-Parker Agar (BPA)

Approximate Formula Per 950 ml	
Pancreatic Digest of Casein	10.0 g
Beef Extract	5.0 g
Yeast Extract	1.0 g
Glycine	12.0 g
Sodium Pyruvate	10.0 g
Lithium Chloride	5.0 g
Agar	20.0 g

Tellurite Enrichment

Egg yolk emulsion containing potassium tellurite consists of 30% egg yolk suspension with 0.15% potassium tellurite.

Directions for Preparation from

1. Suspend 63 g of the powder in 950 ml of purified water. Mix thoroughly.
2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
3. Autoclave at 121°C for 15 minutes.
4. Cool to 45-50°C and aseptically add 50 ml of Egg Yolk Tellurite Enrichment.

Mix thoroughly but gently.

1.3 Mannitol Salt Agar (MSA)

Approximate Formula Per 1L	
Proteose Peptone No. 3	10.0 g
Beef Extract	1.0 g
D-Mannitol	10.0 g
Sodium Chloride	75.0 g
Agar	15.0 g
Phenol Red	25.0 mg

Directions for Preparation from

1. Suspend 111 g of the powder in 1 L of purified water. Mix throughly.
2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
3. Autoclave at 121°C for 15 minutes.

1.4 Brain Heart Infusion Broth (BHI)

Approximate Formula Per 1L	
Calf Brains, Infusion from 200 g	7.7 g
Beef Heart, Infusion from 250 g	9.8 g
Proteose Peptone	10.0 g
Dextrose	2.0 g
Sodium Chloride	5.0 g
Disodium Phosphate	2.5 g

Directions for Preparation from

1. Suspend 37 g the powder in 1 L of purified water. Mix thoroughly.

2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.

3. Autoclave at 121°C for 15 minutes.

1.5 Mueller-Hinton Agar (MHA)

Approximate Formula Per 1L	
Beef Extract Powder	2.0 g
Acid Digest of Casein	17.5 g
Starch	1.5 g
Agar	17.0 g

Directions for Preparation from

1. Suspend 38 g of the powder in 1 L of purified water. Mix throughly.

2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.

3. Autoclave at 121°C for 15 minutes.

4. Check prepared medium to ensure the final pH is 7.3 ± 0.1 at 25°C.

1.6 Mueller-Hinton Broth (MHB)

Approximate Formula Per 1L

Beef Extract	3.0 g
Acid Hydrolysate of Casein	17.5 g
Starch	1.5 g

Adjusted supplemented as required with appropriate salts to provide 20-25 mg/L of calcium and 10-12.5 mg/L of magnesium and as additionally required to meet performance

Directions for Preparation from

1. Suspend 22 g of the powder in 1 L of purified water. Mix throughly.
2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
3. Autoclave at 116-121°C for 10 minutes.

2. General buffers and reagent

2.1 Resazurin solution

5 mg of one resazurin tablet in 50 mL of sterile water

2.2 Buffer for agarose gel electrophoresis

50 X Tris-acetate buffer (TAE)

Tris	242 g
Glacial acetic	57.1 g
0.5 M EDTA	100 ml

Adjust the volume to 1,000 ml with DW, sterile by autoclaving

2.3 10 X loading buffer/dye

20% glycerol, 0.01% bromphenol blue, added TE to final volume

2.4 Ethidium Bromide (EtBr) 5 mg/ml

EtBr 100 mg

Add distilled water to 100 ml

2.5 2% agarose gel

Dissolve 2 g of agarose in 1X TAE buffer then make up volume to 100 ml and melt by microwave.

Appendix C

Method validation

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results. The parameters for method validation defined as specificity, repeatability, intermediate precision, accuracy, detection limit, quantitation limit, linearity and range.

1. Specificity

The specificity of a method can be evaluated by the peak developed from the densitometry at a unique wavelength for the absorption of the targeted compound. One hundred percent specificity of the method is demonstrated by a single peak from the densitometry.

2. Precision

The precision of a method is the extent to which the individual test results of multiple injections of a series of standards agree. The precision was determined in term of percent of coefficient of variation (%CV) or percent relative standard deviation (%RSD) by using below equation.

$$\%RSD = (SD/\bar{x}) \times 100$$

Where,

SD = standard deviation of peak area

$\bar{x}$ = mean of peak area

2.1 Repeatability

The repeatability or intra-day precision expresses the precision under the same conditions in a short time interval or same day.

2.2 Intermediate Precision

The intermediate precision or inter-day precision expresses the precision within different days and different analysts.

3. Accuracy

The accuracy is the closeness of the test results to the true value. It was determined by comparing the mean calculated with the spiked standard solution with a known concentration. The accuracy should be reported as percent recovery, calculated and averaged from standard solution were added.

$$\% \text{ recovery} = \frac{Xfound - Xinitial}{Xadded} \times 100$$

Where,

Xfound = The concentration of standard found in the spiked sample

Xinitial = The concentration of standard found in the sample

Xadded = The concentration of standard added

4. Limit of detection

The limit of detection (LOD) was defined as the lowest concentrations of the analyst in a sample that can be detected, but not necessarily quantified. The detection limit is reported as percentage and parts per million.

Determination based on signal-to-noise ratio between 3:1 is generally considered acceptable for estimating the detection limit.

Determination based on the standard deviation of the response and the slope by using below equation.

$$LOD = 3.3\,\sigma/S$$

Where,

σ = standard deviation of the response

S = slope of the calibration curve

5. Limit of quantitation

The limit of quantitation (LOQ) was defined as the lowest concentrations of the analyst in a sample that can be determined with acceptable precision and accuracy. The quantitation limit is reported as percentage and parts per million.

Determination based on signal-to-noise ratio between 10:1 is generally considered acceptable for estimating the quantitation limit.

Determination based on the standard deviation of the response and the slope by using below equation.

$$LOQ = 10\,\sigma/S$$

Where,

σ = standard deviation of the response

S = slope of the calibration curve

6. Linearity

The linearity of the method is ability to test results that are directly proportional to the concentration of analytes in samples within a given range or proportional by mean of well-defined mathematical transformations. The linearity should be evaluated by graphically. The evaluation is made by visually inspecting a plot of peak area of analyte concentration. The first is to plot the deviations from the

regression line versus the concentration, if the concentration range covers several decades. The correlation coefficient (r^2) less than 0.9995 should be submitted.

7. Range

The range of the method is the interval between the upper and lower levels that have been demonstrated to be determined with precision, accuracy and linearity. The range is normally expressed in the same units as the test results that percentage, parts per million.

Appendix D

Antibacterial activity of *C. asiatica* against
bovine mastitis causative agents

Antibacterial activity of *C. asiatica* against bovine mastitis causative agents

The disc diffusion test for *Streptococcus agalactiae*, *Escherichia coli* and *Klebsiella pneumoniae spp*. was similar to the method for *S. aurerus*. 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. Result of the test showed no inhibitory effect.

The Minimum Inhibitory Concentration tested for *Streptococcus agalactiae*, *Escherichia coli* and *Klebsiella pneumoniae spp*. The MIC values of asiatic acid and crude extracts were determined by the modified resazurin microtiter-plate. The asiatic acid showed the inhibitory effect against *St. agalactiae* with the MIC and MBC value of 0.5 and 0.5 mg/ml. The ethanol extracts showed MIC and MBC value of 4 and 8 mg/ml, the water extracts of leaf powder showed MIC value of 32 mg/ml and the water extracts of fresh leaves showed MIC value of 64 mg/ml. The water extracts could not kill the *St. agalactiae*.

The asiatic acid showed the inhibitory effect against *E.coli* with the MIC and MBC value of 128 and 128 mg/ml. The ethanol extracts showed MIC and MBC value of 64 and 128 mg/ml, the water extracts of leaf powder showed MIC value of 64 mg/ml and the water extracts of fresh leaves showed MIC value of 128 mg/ml. The water extracts could not kill the *E.coli*.

The asiatic acid showed the inhibitory effect against *Klebsiella pneumoniae spp* with the MIC and MBC value of 128 and 128 mg/ml. The ethanol extracts showed MIC and MBC value of 64 and 128 mg/ml, the water extracts of leaf powder showed MIC value of 64 mg/ml and the water extracts of fresh leaves showed MIC value of 256 mg/ml. The water extracts could not kill the *Klebsiella pneumoniae spp*.

Appendix E

The alignment of *coa* nucleotide sequences

The nucleotide sequence of *coa* from 29 isolates were determined using a multiple sequence alignment editor BioEdit version 7.0.5.2. The alignment of *coa* gene sequences are demonstrated in Figure 12

```
                          10        20        30        40        50
                    ....|....|....|....|....|....|....|....|....|....|
S. aureus ATCC 25923  GTAACAACACATGCAAACGGCCAAGTATCATATGGCGCCCGCCCAACATA
S. aureus TD1         ............G.....................T.........C.
S. aureus TD3         ............G.....................T.........C.
S. aureus TD4         ............G.....................T.........C.
S. aureus No.8        ............G.....................TT........C.
S. aureus No.149      ..........C.G.....................T.....G...C.
S. aureus A1          ............G.....................T.........C.
S. aureus Pud C       ..................T...........C.....T..T..G.....
S. aureus 1           ................T..T..............T.....G...C.
S. aureus 6           .....G...A..CA.G.T...AC....A.....T.........C.
S. aureus 8           ..........C.G.....................T.....G...C.
S. aureus 11          ..........C.G.....................T.....G...C.
S. aureus 13          ..........C.G.....................T.....G...C.
S. aureus 14          ..................T...........C.....T..T..G.....
S. aureus 15          ..........C.G.....................T.....G...C.
S. aureus 16          .....G...A..CA.G.T...AC....A.....T.........C.
S. aureus 17          .....G...A..CA.G.T...AC..............T.....G...C.
S. aureus 18          .....G...A..CA.G.T...AC..............T.....G...C.
S. aureus 19          .....G...A..CA.G.T...AC..............T.....G...C.
S. aureus 20          .....G...A..CA.G.T...AC..............T.....G...C.
S. aureus 21          .....G...A..CA.G.T...AC..............T.....G...C.
S. aureus 22          .....G...A..CA.G.T...AC..............T.....G...C.
S. aureus 23          .....G...A..CA.G.T...AC..............T.....G...C.
S. aureus 25          .....G...A..CA.G.T...AC..............T.....G...C.
S. aureus 26          .....G...A..CA.G.T...AC..............T.....G...C.
S. aureus 27          .....G...A..CA.G.T...AC..............T.....G...C.
S. aureus 28          .....G...A..CA.G.T...AC..............T.....G...C.
S. aureus 29          ................T..T..........C..A..T.........C.
S. aureus 30          .....G...A..CA.G.T...AC..............T.....G...C.


                         60        70        80        90       100
                    ....|....|....|....|....|....|....|....|....|....|
S. aureus ATCC 25923  CAAGAAGCCAAGCGAAACAAACGCATACAACGTAACGACAAATCAAGATG
S. aureus TD1         A..C.........A.......T..............A...C..GC.A...
S. aureus TD3         A..C.........A.......T..............A...C..GC.A...
S. aureus TD4         A..C.........A.......T..............A...C..GC.A...
S. aureus No.8        A..C.........A.......T..............A...C..GC.A...
S. aureus No.149      A..C........TA.......T.....T........A...C..GC.A...
S. aureus A1          A..C.........A.......T..............A...C..GC.A...
S. aureus Pud C       ...C.........A.......T.....T........A...C..GC.A.C.
S. aureus 1           A..C.........A.......T.....T........A...C..GC.A...
S. aureus 6           A..C..A......A.......T..............A...C..GC.A...
S. aureus 8           A..C........TA.......T.....T........A...C..GC.A...
S. aureus 11          A..C........TA.......T.....T........A...C..GC.A...
S. aureus 13          A..C........TA.......T.....T........A...C..GC.A...
S. aureus 14          ...C.........A.......T.....T........A...C..GC.A.C.
S. aureus 15          A..C........TA.......T.....T........A...C..GC.A...
S. aureus 16          A..C..A......A.......T..............A...C..GC.A...
S. aureus 17          A..C.........A..............T........A...C..GC.A.C.
```

Appendix Figure E1 Nucleotide sequence alignment of *coa* gene.

```
S. aureus 18          A..C.........A.............T........A...C..GC.A.C.
S. aureus 19          A..C.........A.............T........A...C..GC.A.C.
S. aureus 20          A..C.........A.............T........A...C..GC.A.C.
S. aureus 21          A..C.........A.............T........A...C..GC.A.C.
S. aureus 22          A..C.........A.............T........A...C..GC.A.C.
S. aureus 23          A..C.........A.............T........A...C..GC.A.C.
S. aureus 25          A..C.........A.............T........A...C..GC.A.C.
S. aureus 26          A..C.........A.............T........A...C..GC.A.C.
S. aureus 27          A..C.........A.............T........A...C..GC.A.C.
S. aureus 28          A..C.........A.............T........A...C..GC.A.C.
S. aureus 29          A..C.........A.......T.............A...C..GC.A...
S. aureus 30          A..C.........A.............T........A...C..GC.A.C.

                                 110       120       130       140       150
                      ....|....|....|....|....|....|....|....|....|....|
S. aureus ATCC 25923  GCACAGTATCATATGGCGCTCGCCCGACACAAAACAAGCCAAGCGAAACA
S. aureus TD1         .TCA.....................................A.....
S. aureus TD3         .TCA.....................................A.....
S. aureus TD4         .TCA.....................................A.....
S. aureus No.8        .TCA.....................................A.....
S. aureus No.149      .TCA...........A.....T.....T.C..G...........G
S. aureus A1          .TCA.....................................A.....
S. aureus Pud C       .TCA............C.........T.C...........A.....
S. aureus 1           .TCA.........C..A...........T.C..G..............
S. aureus 6           .TCA.....................................A.....
S. aureus 8           .TCA...........A.....T.....T.C..G...........G
S. aureus 11          .TCA...........A.....T.....T.C..G...........G
S. aureus 13          .TCA...........A.....T.....T.C..G...........G
S. aureus 14          .TCA............C.........T.C...........A.....
S. aureus 15          .TCA...........A.....T.....T.C..G...........G
S. aureus 16          .TCA.....................................A.....
S. aureus 17          .TCA.........C..T.........................A.....
S. aureus 18          .TCA.........C..T.........................A.....
S. aureus 19          .TCA.........C..T.........................A.....
S. aureus 20          .TCA.........C..T.........................A.....
S. aureus 21          .TCA.........C..T.........................A.....
S. aureus 22          .TCA.........C..T.........................A.....
S. aureus 23          .TCA.........C..T.........................A.....
S. aureus 25          .TCA.........C..T.........................A.....
S. aureus 26          .TCA.........C..T.........................A.....
S. aureus 27          .TCA.........C..T.........................A.....
S. aureus 28          .TCA.........C..T.........................A.....
S. aureus 29          .TCA.....................................A.....
S. aureus 30          .TCA.........C..T.........................A.....

                                 160       170       180       190       200
                      ....|....|....|....|....|....|....|....|....|....|
S. aureus ATCC 25923  AACGCATATAACGTAACAACACATGCAAACGGCCAAGTATCATACGGAGC
S. aureus TD1         ..T.........................T..T.................
S. aureus TD3         ..T.........................T..T.................
S. aureus TD4         ..T.........................T..T.................
S. aureus No.8        ..T.........................T..T.................
S. aureus No.149      ..T.....C..T.................................C..
S. aureus A1          ..T.........................T..T.................
S. aureus Pud C       ..T.....C.....................................T..C..
S. aureus 1           ..T.....C...................T..T..........T..C..
S. aureus 6           ..T.........................T..T.................
S. aureus 8           ..T.....C..T.................................C..
S. aureus 11          ..T.....C..T.................................C..
```

Appendix Figure E1 (Continued)

```
S. aureus 13          ..T.....C..T.................................C..
S. aureus 14          ..T.....C.....................................T..C..
S. aureus 15          ..T.....C..T.................................C..
S. aureus 16          ..T.........................T..T................
S. aureus 17          ....AG......................T.............T..T..
S. aureus 18          ....AG......................T.............T..T..
S. aureus 19          ....AG......................T.............T..T..
S. aureus 20          ....AG......................T.............T..T..
S. aureus 21          ....AG......................T.............T..T..
S. aureus 22          ....AG......................T.............T..T..
S. aureus 23          ....AG......................T.............T..T..
S. aureus 25          ....AG......................T.............T..T..
S. aureus 26          ....AG......................T.............T..T..
S. aureus 27          ....AG......................T.............T..T..
S. aureus 28          ....AG......................T.............T..T..
S. aureus 29          ..T.........................T..T................
S. aureus 30          ....AG......................T.............T..T..

                                 210       220       230       240       250
                      ....|....|....|....|....|....|....|....|....|....|
S. aureus ATCC 25923  TCGTCCGACACAAAACAAGCCAAGCGAAACGAACGCATATAACGTAACAA
S. aureus TD1         ...C......T.C..G.............A..T.....C..........
S. aureus TD3         ...C......T.C..G.............A..T.....C..........
S. aureus TD4         ...C......T.C..G.............A..T.....C..........
S. aureus No.8        ...C......T.C..G.............A..T.....C..........
S. aureus No.149      ..................G..........A...................
S. aureus A1          ...C......T.C..G.............A..T.....C..........
S. aureus Pud C       ...C.........................A...................
S. aureus 1           ...C.........................A...................
S. aureus 6           ...C.......................A....A..T.............
S. aureus 8           ..................G..........A...................
S. aureus 11          ..................G..........A...................
S. aureus 13          ..................G..........A...................
S. aureus 14          ...C.........................A...................
S. aureus 15          ..................G..........A...................
S. aureus 16          ...C.......................A....A..T.............
S. aureus 17          ..........T.C..G........T.............................
S. aureus 18          ..........T.C..G........T.............................
S. aureus 19          ..........T.C..G........T.............................
S. aureus 20          ..........T.C..G........T.............................
S. aureus 21          ..........T.C..G........T.............................
S. aureus 22          ..........T.C..G........T.............................
S. aureus 23          ..........T.C..G........T.............................
S. aureus 25          ..........T.C..G........T.............................
S. aureus 26          ..........T.C..G........T.............................
S. aureus 27          ..........T.C..G........T.............................
S. aureus 28          ..........T.C..G........T.............................
S. aureus 29          ...C......T.C..G.............A..T.....C..........
S. aureus 30          ..........T.C..G........T.............................
```

Appendix Figure E1 (Continued)

```
                                 260       270       280       290       300
                          ....|....|....|....|....|....|....|....|....|....|
S. aureus ATCC 25923      CACATGCAAACGGTCAAGTGTCATACGGAGCTCGCCCAACACAAAACAAG
S. aureus TD1             .........T........A.....T..C........G............
S. aureus TD3             .........T........A.....T..C........G............
S. aureus TD4             .........T........A.....T..C........G............
S. aureus No.8            .........T........A.....T..C........G........A...
S. aureus No.149          ..........................................G...T.C..G...
S. aureus A1              .........T........A.....T..C........G........A...
S. aureus Pud C           .........T.................G........G............
S. aureus 1               ...................A........C..C.........T.C..G...
S. aureus 6               ....C.....................................G...T.C..G...
S. aureus 8               ..........................................G...T.C..G...
S. aureus 11              ..........................................G...T.C..G...
S. aureus 13              ..........................................G...T.C..G...
S. aureus 14              .........T.................G........G............
S. aureus 15              ..........................................G...T.C..G...
S. aureus 16              ....C.....................................G...T.C..G...
S. aureus 17              ...................A........C........T............
S. aureus 18              ...................A........C........T............
S. aureus 19              ...................A........C........T............
S. aureus 20              ...................A........C........T............
S. aureus 21              ...................A........C........T............
S. aureus 22              ...................A........C........T............
S. aureus 23              ...................A........C........T............
S. aureus 25              ...................A........C........T............
S. aureus 26              ...................A........C........T............
S. aureus 27              ...................A........C........T............
S. aureus 28              ...................A........C........T............
S. aureus 29              .........T........A.....T..C........G........A...
S. aureus 30              ...................A........C........T............


                                 310       320       330       340       350
                          ....|....|....|....|....|....|....|....|....|....|
S. aureus ATCC 25923      CCAAGTAAAACAAATGCATACAATGTAACAACACATGCAGATGGTACTGC
S. aureus TD1             .....CG.......C.....T..C...........................
S. aureus TD3             .....CG.......C.....T..C...........................
S. aureus TD4             .....CG.......C.....T..C...........................
S. aureus No.8            .....CG.......C.....T..C...........................
S. aureus No.149          .....CG............................................
S. aureus A1              .....CG.......C.....T..C...........................
S. aureus Pud C           ..............C.....T..C...........................
S. aureus 1               .....CG....G........T..C...........................
S. aureus 6               ....................................................
S. aureus 8               .....CG............................................
S. aureus 11              .....CG............................................
S. aureus 13              .....CG............................................
S. aureus 14              ..............C.....T..C...........................
S. aureus 15              .....CG............................................
S. aureus 16              ....................................................
S. aureus 17              ....................................................
S. aureus 18              ....................................................
S. aureus 19              ....................................................
S. aureus 20              ....................................................
S. aureus 21              ....................................................
S. aureus 22              ....................................................
S. aureus 23              ....................................................
S. aureus 25              ....................................................
```

Appendix Figure E1 (Continued)

```
S. aureus 26          ..................................................
S. aureus 27          ..................................................
S. aureus 28          ..................................................
S. aureus 29          .....CG.......C.....T..C..........................
S. aureus 30          ..................................................


                                 360       370       380       390       400
                      ....|....|....|....|....|....|....|....|....|....|
S. aureus ATCC 25923  GACATATGGTCCTAGAGTAACAAAATAAGTTTATAACTCTATCCATAGAC
S. aureus TD1         .........G..................G...........A....
S. aureus TD3         .........G..................G...........A....
S. aureus TD4         .........G..................G...........A....
S. aureus No.8        .........G..................G...........A....
S. aureus No.149      .........G.................A.............A....
S. aureus A1          .........G..................G...........A....
S. aureus Pud C       ..................................................
S. aureus 1           .........G..................G...........A....
S. aureus 6           .........G..................G...........A....
S. aureus 8           .........G.................A.............A....
S. aureus 11          .........G.................A.............A....
S. aureus 13          .........G.................A.............A....
S. aureus 14          ..................................................
S. aureus 15          .........G.................A.............A....
S. aureus 16          .........G..................G...........A....
S. aureus 17          .........C........................................
S. aureus 18          .........C........................................
S. aureus 19          .........C........................................
S. aureus 20          .........C........................................
S. aureus 21          .........C........................................
S. aureus 22          .........C........................................
S. aureus 23          .........C........................................
S. aureus 25          .........C........................................
S. aureus 26          .........C........................................
S. aureus 27          .........C........................................
S. aureus 28          .........C........................................
S. aureus 29          .........G.................A.............A....
S. aureus 30          .........C........................................


                                 410       420       430       440       450
                      ....|....|....|....|....|....|....|....|....|....|
S. aureus ATCC 25923  ATACAGTCAATACAAAACATTATGTATCTTTACAACAGTAATCATGCATT
S. aureus TD1         ......................C...........................
S. aureus TD3         ......................C...........................
S. aureus TD4         ......................C...........................
S. aureus No.8        ......................C...........................
S. aureus No.149      ................GA.........A.....................
S. aureus A1          ......................C...........................
S. aureus Pud C       ..................................................
S. aureus 1           ......................C...........................
S. aureus 6           ......................C...........................
S. aureus 8           ................GA.........A.....................
S. aureus 11          ................GA.........A.....................
S. aureus 13          ................GA.........A.....................
S. aureus 14          ..................................................
S. aureus 15          ................GA.........A.....................
S. aureus 16          ......................C...........................
S. aureus 17          ......................C...........................
S. aureus 18          ......................C...........................
S. aureus 19          ......................C...........................
S. aureus 20          ......................C...........................
S. aureus 21          ......................C...........................
S. aureus 22          ......................C...........................
```

```
S. aureus 23          ......................C.........................
S. aureus 25          ......................C.........................
S. aureus 26          ......................C.........................
S. aureus 27          ......................C.........................
S. aureus 28          ......................C.........................
S. aureus 29          ................GA..........A....................
S. aureus 30          ......................C.........................

                                460       470       480       490       500
                              ....|....|....|....|....|....|....|....|....|....|
S. aureus ATCC 25923  CTATGATGCTTCTAACTGAATTAAAGCATCGAACAATCGGAAGCATATTT
S. aureus TD1         ..................................................
S. aureus TD3         ..................................................
S. aureus TD4         ..................................................
S. aureus No.8        ..................................................
S. aureus No.149      ..................................................
S. aureus A1          ..................................................
S. aureus Pud C       ..................................................
S. aureus 1           ..................................................
S. aureus 6           ..................................................
S. aureus 8           ..................................................
S. aureus 11          ..................................................
S. aureus 13          ..................................................
S. aureus 14          ..................................................
S. aureus 15          ..................................................
S. aureus 16          ..................................................
S. aureus 17          ..................................................
S. aureus 18          ..................................................
S. aureus 19          ..................................................
S. aureus 20          ..................................................
S. aureus 21          ..................................................
S. aureus 22          ..................................................
S. aureus 23          ..................................................
S. aureus 25          ..................................................
S. aureus 26          ..................................................
S. aureus 27          ..................................................
S. aureus 28          ..................................................
S. aureus 29          ..................................................
S. aureus 30          ..................................................

                                510       520       530       540       550
                              ....|....|....|....|....|....|....|....|....|....|
S. aureus ATCC 25923  CTAAATTATTTATTCATTATAGTCTTAAACATAACATGACCTAATATATT
S. aureus TD1         ..................................................
S. aureus TD3         ..................................................
S. aureus TD4         ..................................................
S. aureus No.8        ..................................................
S. aureus No.149      ..................................................
S. aureus A1          ..................................................
S. aureus Pud C       ..................................................
S. aureus 1           ..................................................
S. aureus 6           ..................................................
S. aureus 8           ..................................................
S. aureus 11          ..................................................
S. aureus 13          ..................................................
S. aureus 14          ..................................................
S. aureus 15          ..................................................
S. aureus 16          ..................................................
S. aureus 17          ..................................................
S. aureus 18          ..................................................
S. aureus 19          ..................................................
S. aureus 20          ..................................................
```

```
S. aureus 21          ..................................................
S. aureus 22          ..................................................
S. aureus 23          ..................................................
S. aureus 25          ..................................................
S. aureus 26          ..................................................
S. aureus 27          ..................................................
S. aureus 28          ..................................................
S. aureus 29          ..................................................
S. aureus 30          ..................................................

                               560       570       580       590       600
                      ....|....|....|....|....|....|....|....|....|....|
S. aureus ATCC 25923  ACTAACCTATTAAAATAAACCACGCACATCTATGTGACATACGACAATCA
S. aureus TD1         ..............................A....T............
S. aureus TD3         ..............................A....T............
S. aureus TD4         ..............................A....T............
S. aureus No.8        ..............................A....T............
S. aureus No.149      ..............................A....T............
S. aureus A1          ..............................A....T............
S. aureus Pud C       ..................................................
S. aureus 1           ..............................A....T............
S. aureus 6           ..............................A....T............
S. aureus 8           ..............................A....T............
S. aureus 11          ..............................A....T............
S. aureus 13          ..............................A....T............
S. aureus 14          ..................................................
S. aureus 15          ..............................A....T............
S. aureus 16          ..............................A....T............
S. aureus 17          ..................................T............
S. aureus 18          ..................................T............
S. aureus 19          ..................................T............
S. aureus 20          ..................................T............
S. aureus 21          ..................................T............
S. aureus 22          ..................................T............
S. aureus 23          ..................................T............
S. aureus 25          ..................................T............
S. aureus 26          ..................................T............
S. aureus 27          ..................................T............
S. aureus 28          ..................................T............
S. aureus 29          ..............................A....T............
S. aureus 30          ..................................T............

                               610       620       630       640
                      ....|....|....|....|....|....|....|....|.
S. aureus ATCC 25923  CAGCAATAATAATTGCTTTAGAAAGTCGTACCGAACTGGAA
S. aureus TD1         ............................G.T.........
S. aureus TD3         ............................G.T.........
S. aureus TD4         ............................G.T.........
S. aureus No.8        ............................G...........
S. aureus No.149      ....................A.....................
S. aureus A1          ............................G...........
S. aureus Pud C       ..................................................
S. aureus 1           ...............T..........................
S. aureus 6           ............................G...........
S. aureus 8           ....................A.....................
S. aureus 11          ....................A.....................
S. aureus 13          ....................A.....................
S. aureus 14          ..................................................
S. aureus 15          ....................A.....................
S. aureus 16          ............................G...........
S. aureus 17          ..................................................
S. aureus 18          ..................................................
S. aureus 19          ..................................................
S. aureus 20          ..................................................
```

```
S. aureus 21        ....................................
S. aureus 22        ....................................
S. aureus 23        ....................................
S. aureus 25        ....................................
S. aureus 26        ....................................
S. aureus 27        ....................................
S. aureus 28        ....................................
S. aureus 29        ....................................
S. aureus 30        ....................................
```

Appendix Figure E1 (Continued)

Multiple alignments of DNA sequences of 29 sequences were represented 10 groups. The groups of DNA sequences are demonstrated in Figure 13.

```
                              10        20        30        40        50
                     ....|....|....|....|....|....|....|....|....|....|
S. aureus ATCC 25923 GTAACAACACATGCAAACGGCCAAGTATCATATGGCGCCCGCCCAACATA
S. aureus TD1        ............G.......................T.........C.
S. aureus A1         ............G.......................T.........C.
S. aureus No.8       ............G......................TT.........C.
S. aureus No.149     ..........C.G.......................T.....G...C.
S. aureus 6          .....G...A..CA.G.T...AC....A.........T.........C.
S. aureus 20         .....G...A..CA.G.T...AC..............T.....G...C.
S. aureus 29         ................T..T..........C..A..T.........C.
S. aureus 1          ................T..T..............T.....G...C.
S. aureus Pud C      ..................T..........C.....T..T..G.....

                              60        70        80        90       100
                     ....|....|....|....|....|....|....|....|....|....|
S. aureus ATCC 25923 CAAGAAGCCAAGCGAAACAAACGCATACAACGTAACGACAAATCAAGATG
S. aureus TD1        A..C.........A.......T.............A...C..GC.A...
S. aureus A1         A..C.........A.......T.............A...C..GC.A...
S. aureus No.8       A..C.........A.......T.............A...C..GC.A...
S. aureus No.149     A..C........TA.......T.....T........A...C..GC.A...
S. aureus 6          A..C..A......A.......T.............A...C..GC.A...
S. aureus 20         A..C.........A............T........A...C..GC.A.C.
S. aureus 29         A..C.........A.......T.............A...C..GC.A...
S. aureus 1          A..C.........A.......T.....T........A...C..GC.A...
S. aureus Pud C      ...C.........A.......T.....T........A...C..GC.A.C.

                             110       120       130       140       150
                     ....|....|....|....|....|....|....|....|....|....|
S. aureus ATCC 25923 GCACAGTATCATATGGCGCTCGCCCGACACAAAACAAGCCAAGCGAAACA
S. aureus TD1        .TCA.....................................A.....
S. aureus A1         .TCA.....................................A.....
S. aureus No.8       .TCA.....................................A.....
S. aureus No.149     .TCA............A.....T.....T.C..G............G
S. aureus 6          .TCA.....................................A.....
S. aureus 20         .TCA.........C..T........................A.....
S. aureus 29         .TCA..............................A.........A.....
S. aureus 1          .TCA.........C..A............T.C..G..............
S. aureus Pud C      .TCA..............C.........T.C............A.....

                             160       170       180       190       200
                     ....|....|....|....|....|....|....|....|....|....|
S. aureus ATCC 25923 AACGCATATAACGTAACAACACATGCAAACGGCCAAGTATCATACGGAGC
S. aureus TD1        ..T..........................T..T................
S. aureus A1         ..T..........................T..T................
S. aureus No.8       ..T..........................T..T................
S. aureus No.149     ..T.....C..T.....................................C..
S. aureus 6          ..T..........................T..T................
S. aureus 20         ....AG.......................T.............T..T..
S. aureus 29         ..T..........................T..T................
S. aureus 1          ..T.....C....................T..T...........T..C..
S. aureus Pud C      ..T.....C.....................................T..C..
```

Appendix Figure E2 Base differences in coa genes of 10 groups from 29 isolated sequences.

Center dots indicate the same bases as *S. aureus* ATCC 25923.

```
                                 210       220       230       240       250
                          ....|....|....|....|....|....|....|....|....|....|
S. aureus ATCC 25923      TCGTCCGACACAAAACAAGCCAAGCGAAACGAACGCATATAACGTAACAA
S. aureus TD1             ...C......T.C..G.............A..T.....C..........
S. aureus A1              ...C......T.C..G.............A..T.....C..........
S. aureus No.8            ...C......T.C..G.............A..T.....C..........
S. aureus No.149          ...................G..........A...................
S. aureus 6               ...C.....................A....A..T................
S. aureus 20              ..........T.C..G........T.........................
S. aureus 29              ...C......T.C..G.............A..T.....C..........
S. aureus 1               ...C.........................A....................
S. aureus Pud C           ...C.........................A....................


                                 260       270       280       290       300
                          ....|....|....|....|....|....|....|....|....|....|
S. aureus ATCC 25923      CACATGCAAACGGTCAAGTGTCATACGGAGCTCGCCCAACACAAAACAAG
S. aureus TD1             ..........T........A.....T..C........G............
S. aureus A1              ..........T........A.....T..C........G........A...
S. aureus No.8            ..........T........A.....T..C........G........A...
S. aureus No.149          ...................................G...T.C..G...
S. aureus 6               ....C..............................G...T.C..G...
S. aureus 20              ...................A........C........T............
S. aureus 29              ..........T........A.....T..C........G........A...
S. aureus 1               ...................A........C..C.........T.C..G...
S. aureus Pud C           ..........T..................G........G............


                                 310       320       330       340       350
                          ....|....|....|....|....|....|....|....|....|....|
S. aureus ATCC 25923      CCAAGTAAAACAAATGCATACAATGTAACAACACATGCAGATGGTACTGC
S. aureus TD1             .....CG.......C.....T..C.....................
S. aureus A1              .....CG.......C.....T..C.....................
S. aureus No.8            .....CG.......C.....T..C.....................
S. aureus No.149          .....CG.....................................
S. aureus 6               .....................................................
S. aureus 20              .....................................................
S. aureus 29              .....CG.......C.....T..C.....................
S. aureus 1               .....CG....G........T..C.....................
S. aureus Pud C           ..............C.....T..C.....................


                                 360       370       380       390       400
                          ....|....|....|....|....|....|....|....|....|....|
S. aureus ATCC 25923      GACATATGGTCCTAGAGTAACAAAATAAGTTTATAACTCTATCCATAGAC
S. aureus TD1             .........G.....................G............A....
S. aureus A1              .........G.....................G............A....
S. aureus No.8            .........G.....................G............A....
S. aureus No.149          .........G.................A.................A....
S. aureus 6               .........G.....................G............A....
S. aureus 20              .........C.....................................
S. aureus 29              .........G.................A.................A....
S. aureus 1               .........G.....................G............A....
S. aureus Pud C           .....................................................
```

Appendix Figure E2 (Continued)

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                                410       420       430       440       450
                       ....|....|....|....|....|....|....|....|....|....|
S. aureus ATCC 25923   ATACAGTCAATACAAAACATTATGTATCTTTACAACAGTAATCATGCATT
S. aureus TD1          ......................C.........................
S. aureus A1           ......................C.........................
S. aureus No.8         ......................C.........................
S. aureus No.149       ................GA..........A.....................
S. aureus 6            ......................C.........................
S. aureus 20           ......................C.........................
S. aureus 29           ................GA..........A.....................
S. aureus 1            ......................C.........................
S. aureus Pud C        ..................................................

                                460       470       480       490       500
                       ....|....|....|....|....|....|....|....|....|....|
S. aureus ATCC 25923   CTATGATGCTTCTAACTGAATTAAAGCATCGAACAATCGGAAGCATATTT
S. aureus TD1          ..................................................
S. aureus A1           ..................................................
S. aureus No.8         ..................................................
S. aureus No.149       ..................................................
S. aureus 6            ..................................................
S. aureus 20           ..................................................
S. aureus 29           ..................................................
S. aureus 1            ..................................................
S. aureus Pud C        ..................................................

                                510       520       530       540       550
                       ....|....|....|....|....|....|....|....|....|....|
S. aureus ATCC 25923   CTAAATTATTTATTCATTATAGTCTTAAACATAACATGACCTAATATATT
S. aureus TD1          ..................................................
S. aureus A1           ..................................................
S. aureus No.8         ..................................................
S. aureus No.149       ..................................................
S. aureus 6            ..................................................
S. aureus 20           ..................................................
S. aureus 29           ..................................................
S. aureus 1            ..................................................
S. aureus Pud C        ..................................................

                                560       570       580       590       600
                       ....|....|....|....|....|....|....|....|....|....|
S. aureus ATCC 25923   ACTAACCTATTAAAATAAACCACGCACATCTATGTGACATACGACAATCA
S. aureus TD1          ..............................A....T.....T.....T.....
S. aureus A1           ..............................A....T.....T.....T.....
S. aureus No.8         ..............................A....T.....T.....T.....
S. aureus No.149       ..............................A....T.....T.....T.....
S. aureus 6            ..............................A....T.....T.....T.....
S. aureus 20           ..................................T.....T.....T.....
S. aureus 29           ..............................A....T.....T.....T.....
S. aureus 1            ..............................A....T.....T.....T.....
S. aureus Pud C        ..................................................
```

Appendix Figure E2 (Continued)

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                              610       620       630       640
                         ....|....|....|....|....|....|....|....|.
S. aureus ATCC 25923     CAGCAATAATAATTGCTTTAGAAAGTCGTACCGAACTGGAA
S. aureus TD1            ............................G.T.........
S. aureus A1             ............................G...........
S. aureus No.8           ............................G...........
S. aureus No.149         ....................A.....................
S. aureus 6              ............................G...........
S. aureus 20             ........................................
S. aureus 29             ........................................
S. aureus 1              ..............T.........................
S. aureus Pud C          ........................................
```

Appendix Figure E2 (Continued)

CIRRICULUM VITAE

NAME : Mrs. Duangkamol Taemchuay

BIRTH DATE : July 2, 1979

BIRTH PLACE : Ratchaburi, Thailand

EDUCATION :

YEAR	INSTITUTE	DEGREE/DIPLOMA
2001	Rajmangala Institute of Technology	B.Sc.
2003	Chulalongkorn University	M.S.

POSITION/TITLE : Lecturer

WORK PLACE : King Mongkut's Institute of Technology
Ladkrabang, Chumphon Campus