Effect of *Quercus infectoria* G. Olivier Extracts On Antibiotic-resistant *Staphylococcus aureus*

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ABSTRACT

The continuing emergence and development of resistance to existing antibiotics by *Staphylococcus aureus* has created the need for new compounds that exhibit antibacterial activity against these resistant strains. To overcome the problem of antibiotic resistance, medicinal plants have been extensively studied as an alternative treatment for this infectious agent. In this present communication, we investigate the anti-MRSA activity and its mechanisms of actions of Thai traditional medicine, *Quercus infectoria* G. Olivier nutgalls, and determine the effects of the nutgall extract and its components on staphylococcal colonization. In addition, we examine resistant modifying ability of the plant extract and its components on MRSA. An important Gram-negative nosocomial pathogen, multidrug-resistant *Acinetobacter baumannii* has been included in the finishing part of this study with the aim to extend an application of natural products to troublesome Gram-negative organism.

Acetone, ethyl acetate, ethanol, and water extracts of *Quercus infectoria* nutgall demonstrated significant antibacterial activities against all isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *Staphylococcus aureus* (MSSA). Inhibition zones ranged from 11.75-16.82 mm. Both MRSA and MSSA isolates exhibited the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values at 0.13 and 0.13-1.00 mg/mL, respectively. At 2MIC, the growth of two representative MRSA isolates was continually inhibited for at least 20 h. The survival cells of MRSA were not detected within 12-14 h after treated with the extract at 4MIC concentration. *S. aureus* ATCC 25923 demonstrated similar results.

We further establish modes of action of nutgall ethanol extract as well as its main constituents to induce anti-MRSA activity. The ethanol extract was fractionated by quick column chromatography. The MIC/MBC values of ethyl acetate I, ethyl acetate II, 95% ethanol, and 30% ethanol fractions against MRSA were 0.06/0.25, 0.13/0.25, 0.25/0.5, and 0.5/1.00 mg/mL, respectively. Ellagic acid, gallic acid, syringic acid, and tannic acid were included in the study as major components of *Quercus infectoria* nutgall extract. The results demonstrated the MIC/MBC values of gallic acid and tannic acid at 0.06/0.06 and 0.13/0.25 mg/mL. A lysis experiment showed that the plant extract, the ethyl acetate fraction I, and the main components failed to lyse MRSA cells. Additionally, the ethanol extract, the partially-purified fraction, and the components did not appear to alter membrane permeability that assessed by measuring the leakage of small UV-absorbing compounds. Both MRSA and *S. aureus* ATCC 25923 treated with the extract, the ethyl acetate fraction I, gallic acid, and tannic acid displayed significant loss of tolerance to low osmotic pressure and high salt concentration. Clumps of partly-divided cocci with thickened cell wall were observed by scanning and transmission electron microscopy in the cultures of MRSA incubated in the presence of the ethanol extract, the ethyl acetate fraction I, and tannic acid.

The biofilm formation, cell surface hydrophobicity (CSH), and virulence factors of infective origin, MRSA and colonization origin, MSSA were investigated. More than 70% of MRSA and MSSA isolates were identified as moderate adherence. Most of MRSA isolates (79%) were classified as low hydrophobic isolates, while 88% of MSSA isolates were described as high hydrophobic isolates. Although, there was no significant difference in biofilm formation of MRSA and MSSA isolates, the nature of CSH of infective and colonization origin *S. aureus* were dramatically different. Mean values of degree of hydrolysis by enzyme production including haemolysin, lipase, and proteases of MSSA isolates were significantly higher than in those MRSA isolates ($P<0.05$). Although the cell surface property of MRSA and MSSA isolates were significantly different, similar MIC_{90} from the ethanol extract and tannic acid were remarked against all test isolates.

Nasal colonization with *S. aureus* is a significant risk factor for *S. aureus* infections, eradicating *S. aureus* colonization has remained an attractive strategy for preventing infections and transmission. As a consequence of the well recognized activity of *Quercus infectoria* nutgalls is anti-ulceration. Therefore, the effect of the extract from the nutgall as well as tannic acid, its major component with anti-MRSA activity, on the biofilm formation and CSH of MRSA and MSSA were further discussed as an alternative decolonization agent**.** The ethanol extract at MIC and sub-MIC (1/2MIC-1/16MIC) were significantly able to reduce the biofilm formation of MRSA. Even though, tannic acid is the main component in the extract, the compound is found to be a less effective anti-biofilm than the extract. Surprisingly, when exposed to sub-MIC of both the ethanol extract and tannic acid, there was no reduction in the biofilm formation of the MSSA isolates. However, the biofilm formation of some test MSSA isolates was inhibited by the ethanol extract and tannic acid at MIC. It should be stressed that at the sub-MIC of the plant extract prevent bacterial adhesion and biofilm formation by MRSA only. The effects of supra-MIC, MIC, and sub-MIC on CSH were further examined by bacterial adherence to hydrocarbon method (BATH). The hydrophobicity index of all bacterial isolates increased after treated with supra-MIC, MIC, and sub-MIC values of both the extract and tannic acid. It is noteworthy that pure tannic acid, as tested by BATH assay, showed similar results to the plant extract, suggesting that tannic acid may be the component of the herb extract with highest effect in relation to its consequence on CSH of MRSA and MSSA. The significant effect of the extract and its main component on *S. aureus* cell surface hydrophobicity that may cause to reduced staphylococcal biofilm formation, leads the extract to be a promising plant material for further development as an alternative staphylococcal decolonization agent.

One approach to the restoration of antibiotics is to administer them in conjunction with non-antibiotic compounds that depress resistance mechanisms. Since staphylococcal cell wall seemed to be one possible site for anti-MRSA activity, their effect with representative β-lactam antibiotics were determined. Synergistic effects were observed with 76% and 53% of the test isolates for the

combination of ethanol extract and amoxicillin and penicillin G, respectively. Although restoration of oxacillin activity by the plant extract was not observed, most of the test isolates demonstrated a synergistic effect between the nutgall ethanol extract and the β-lactamase-susceptible penicillins.

We additionally describe the activity of ellagic acid, the component of *Quercus infectoria* extract with low antibacterial activity, as a resistant modifying agent (RMA) that enhances the activity of aminocoumarin antibiotics against an important nosocomial pathogen, *Acinetobacter baumannii*. The RMA activity of various plant phenolics was tested in growth inhibition assays in combination with subinhibitory concentrations of novobiocin. The antibacterial susceptibilities of multidrug resistant *A. baumannii* and *A. baumannii* ATCC19606 to a variety of antibiotics were determined in the absence and presence of antibiotic adjuvants, ellagic acid and tannic acid. In addition, the effect of ellagic acid on bacterial outer membrane function was examined by using the hydrophobic fluorescence probe, 1-*N*-phenylnaphthylamine (NPN). The intracellular accumulation of ethidium bromide (EtBr) and pyronin Y was measured to confirm the efflux pump inhibition mechanism. Growth inhibition studies using in a combination of subinhibitory concentration of antibiotics and the plant phenolics, ellagic and tannic acids were carried out. At 40 μM, the plant phenolics enhanced the activity of novobiocin, related aminocoumarins (coumermycin and chlorobiocin), rifampicin, and fusidic acid against *A. baumannii*. There were no increases in the uptake of NPN or in the accumulation of EtBr after the isolates were treated with these adjuvants. But, the intracellular accumulation of pyronin Y by the treated cells was significantly increased.

Taken together, crude extract as well as some components from *Quercus infectoria* nutgall are promising alternative agent for eradication of *S. aureus* and *A. baumannii*, both as a disinfectant and as a resistant modifying agent.

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LIST OF ABBREVIATIONS

%	=	Percent
μg	=	Microgram
μl	=	Microlitre
ATCC	=	American type culture collection
^{o}C	=	Degree Celsius
CDC	=	Centers for Disease Control and Prevention, USA
CNS	=	Central nerve system
cfu	=	Colony forming unit
DNA	=	Deoxyribonucleic acid
DMSO	=	Dimethylsulfoxide
g	=	Gram
HPBI	=	Hydrophobicity index
h	=	Hour
kDa	=	Kilo Daltal
LPS	=	Lipopolysaccharide
L	=	Liter
MATH	=	Microbial adhesion test to hydrocarbon assay
mRNA	=	Messenger Ribonucleic acid
MRSA	=	Methicillin-resistant *Staphylococcus aureus*
MIC	=	Minimum inhibitory concentration
MBC	=	Minimum bactericidal concentration
min	=	Minute (s)
ml	=	Millitre
mm	=	Millimetre
M	=	Molar
nm	=	Nanometre
OD	=	Optical density
OMP	=	Outer membrane protein

LIST OF ABBREVIATIONS (CONTINUED)

OsO_4	=	Osmium tetroxide crystal
PBS	=	Phosphate buffer saline
PCR	=	Polymerase chain reaction
RNA	=	Ribonucleic acid
rpm	=	Rounds per minute
SEM	=	Scanning electron microscope
TEM	=	Transmission electron microscope
TLC	=	Thin layer chromatography

CHAPTER 1
INTRODUCTION

1. Background and rationale

Staphylococcus aureus has long been recognized as an important pathogen in human diseases. It is the leading cause of bacterial infections in both affluent (Kramer *et al.*, 2010; Valsesia *et al.*, 2010) and developing countries (Bell and Turnidge, 2002; Nickerson *et al.*, 2009; Ghaznavi-Rad *et al.*, 2010), including Thailand (Nickerson *et al.*, 2009; Pawun *et al.*, 2009). The pathogen produces a wide spectrum of diseases, ranging from minor skin infections (Kurlenda *et al.*, 2009) to fatal bloodstream infections and necrotizing pneumonia (Rosenthal *et al.*, 2006; Grisaru-Soen *et al.*, 2007). Penicillin was initially highly effective against staphylococcal infections, but penicillinase-producing *S. aureus* emerged in the mid 1940s, and its prevalence rose dramatically within a few years (Barber and Rozwadowska-Dowzenko, 1948). This increase was completely attributable to widespread use of penicillin, which selected for bacteria containing resistance genes. Penicillin-resistant *S. aureus* was pandemic in the 1950s and early 1960s (Rountree and Beard, 1958; Robinson *et al.*, 2005). These infections, both in hospitals and in the community, were caused primarily by one *S. aureus* clone known as phage-type 80/81 (Rountree and Beard, 1958). After the introduction of methicillin in 1959, pandemic phage-type 80/81 infections declined (Jevons and Parker, 1964). Within two years, the emergence of methicillin-resistant *S. aureus* (MRSA) was reported (Jevons *et al.*, 1963) and the first cluster of cases was established shortly thereafter (Stewart and Holt, 1963). However, penicillinase stable β-lactams such as cloxacillin and flucloxacillin have been mainstay of treatment of *S. aureus* infections for more than 35 years (Valencia *et al.*, 2004). MRSA has since spread and is pandemic in most hospitals. In Asia, *S. aureus* is the number one cause of hospital-acquired infections, and a high percentage of these are caused by MRSA (Tantracheewathorn *et al.*, 2007; Rosenthal *et al.*, 2008; Nickerson *et al.*, 2009).

The emergence of community-acquired MRSA (CA-MRSA) infections occurrence in healthy people is one of the most surprising events in infectious diseases in recent years (Elston and Barlow, 2009; Leclercq, 2009; Stanforth *et al.*, 2010). Community-acquired *S. aureus* infections were traditionally caused by methicillin-susceptible *S. aureus* (MSSA), rather than the antibiotic-resistant strains (Skiest *et al.*, 2007). In the 1990s, the first report of CA-MRSA was established in Western Australia (Udo *et al.*, 1993; Nimmo and Coombs, 2008) and has emerged worldwide (Elston and Barlow, 2009; Leclercq, 2009; Stanforth *et al.*, 2010) and become epidemic in the USA (Bratu *et al.*, 2005; Wiersma *et al.*, 2009). The emergence of CA-MRSA has led to an increase in the overall trouble of staphylococcal diseases (CDC, 2003; Wiersma *et al.*, 2009; Ramsetty *et al.*, 2010). Most common types of CA-MRSA infection are skin and soft tissue infections constitute approximately 90% of cases (DeLeo and Chambers, 2009). Moreover, CA-MRSA strains can cause virtually any infection, including lethal necrotizing pneumonia (Bradley, 2005; Maltezou and Giamarellou, 2006; DeLeo and Chambers, 2009).

During the past decades, vancomycin remains a standard treatment for MRSA infection (Lowy, 1998), however vancomycin-resistant isolates of MRSA have emerged in the USA (CDC, 2002) and vancomycin-intermediate isolates are being increasingly reported worldwide (CDC, 2000; Song *et al.*, 2004; Lulitanond *et al.*, 2009). Moreover, adverse effects, the need for intravenous access, and growing resistance to vancomycin limit its use. New antimicrobial agents such as linezolid, daptomycin, and tigecycline have been approved for clinical use for treatment of specific infections caused by MRSA in the USA (Eliopoulos, 2005). However, the resistant isolates are being reported (Hill *et al.*, 2010; Putnam *et al.*, 2010).

To overcome the problem of antibiotic resistance, medicinal plants have been extensively studied as alternative treatments for infectious diseases. Natural products and their derivatives have become the most significant sources of new leads into the development of new pharmaceutical agents. Approximately 25% of modern medications have been derived from previously used plant remedies (Liu and Wang, 2008). More than 50% of both anti-infectious and anticancer agents were developed from natural plant compounds (Newman *et al.*, 2003; Cragg and Newman,

2005). Although a number of herbs has a long history of use as cures for many human ailments, often there has been very little scientific evidences to substantiate their claims. According to WHO, herbal medicines serve the health needs of about 80% of the world's population, especially for millions of people in the vast rural areas of developing countries (WHO, 2001). Thai medicinal plants have been applied to treat many infectious diseases. They are available locally, inexpensive, and become increasingly popular. In traditional Asian medicine, bark of *Quercus infectoria* G. Olivier (Fagaceae) and its nutgalls, a result of attack by gall wasp *Cypnis gallae-tintoriae*, are locally used in the treatment of skin infections, gastrointestinal disorders, and vaginal infections (Kaur *et al.*, 2004; Basri and Fan, 2005; Voravuthikunchai *et al.*, 2006; Kaur *et al.*, 2008). Especially with the nutgall, it has been been pharmacologically documented on their antiamoebic (Sawangjaroen *et al.*, 2004), anticariogenic (Hwang *et al.*, 2004), and anti-inflammatory (Kaur *et al.*, 2004) activities. A series of experiments in our laboratory have demonstrated the spectrum of inhibitory activity of *Quercus infectoria* against a wide range of important pathogenic bacteria (Voravuthikunchai *et al.*, 2004; Voravuthikunchai and Kitpipit, 2005; Voravuthikunchai and Mitchell, 2008). It is obvious that the medicinal plant can be used as alternative medication against antibiotic-resistant bacteria. However, there were very few reports that investigate the principle of its anti-MRSA activity.

The aims of this research were to (i) study the anti-MRSA activity and its mechanisms of actions of *Quercus infectoria* nutgalls, (ii) determine the effects of the nutgall extract and its components on staphylococcal colonization, (iii) investigate resistant modifying ability of the plant extract and its components on MRSA, (iv) extend application of resistant modifying agents to treat an important Gram-negative nosocomial pathogen, multidrug-resistant *Acinetobacter baumannii*, and (v) expose other biological properties of *Quercus infectoria* extract.

2. Literature review

2.1 Methicillin-resistant *Staphylococcus aureus*: Potent pathogenic Gram-positive bacteria

2.1.1 Introduction to *Staphylococcus aureus*

Taxonomists currently separate Gram-positive pathogens into two major groups: (i) low G+C Gram-positive bacteria and (ii) high G+C Gram-positive bacteria. Low G+C Gram-positive bacteria include three genera of pathogenic spherical cells; Staphylococcus, Streptococcus, and Enterococcus. The genus *Staphylococcus* is in the bacterial family Staphylococcaceae, which includes three lesser known genera, Gamella, Macrococcus, and Salinicoccus.

Staphylococci are facultative anaerobes capable of generating energy by aerobic respiration and by fermentation which yields mainly lactic acid. *Staphylococcus* sp. is catalase-positive, a feature differentiating them from *Streptococcus* sp., and they are oxidase-negative and require complex nutrients for growth. Staphylococcal cells are 0.5-1.0 μm in diameter and typically clustered in grape like arrangements. This arrangement results from two characteristics of cell division which cell divisions occur in successively different planes and daughter cells remain attached to one another. Staphylococci are very tolerant of high concentrations of NaCl, up to 1.7 molar, which explains how the bacteria resist the salt deposited on human skin by sweat glands. Additionally, they are tolerant to desiccation, radiation, and heat up to 60 °C for 30 min, allowing the microorganism to survive on environmental surfaces in addition to skin (Schleifer, 2009). Another characteristic of the *Staphylococcus* genus is the cell wall peptidoglycan structure that contains multiple glycine residues in the crossbridge, which causes susceptibility to lysostaphin (Kumar, 2008).

There are two pigmented colony types of staphylococci and proposed the appropriate nomenclature: *Staphylococcus aureus* (yellow) and *S. albus* (white) (Cowan *et al.*, 1954). The golden pigmentation of *S. aureus* colonies is caused by the presence of carotenoids and has been reported to be a virulence factor protecting the pathogen against oxidants produced by the immune system (Liu *et al.*, 2005).

S. aureus produces coagulase which interacts with prothrombin in the blood causing plasma to coagulate by converting fibrinogen into fibrin. Blood coagulation is used to distinguish *S. aureus* from others members of the genus, which are collectively designated as coagulase-negative staphylococci (Ryan and Ray, 2004). Since this microorganism is a common cause of human diseases and exhibits resistance to increasing number of therapeutic agents, *S. aureus* is also one of the most intensively studied bacterial species.

The first genome sequences of *S. aureus* strains Mu50 and N315 were published in 2001 (Kuroda *et al.*, 2001). At present, complete genomic sequences from 10 strains of *S. aureus* are available, and the genomes of several others have been partially determined (Reviewed by Plata *et al.*, 2009). The genome of *S. aureus* is a circular chromosome that is 2.8-2.9 Mbp in size, with a G+C content of about 33% (Crossley and Archer, 1997). The chromosome encodes approximately 2700 protein coding sequences as well as structural and regulatory RNAs. It has been proposed that the *S. aureus* genome is composed of the core genome, accessory component, and foreign genes. The core genes are present in more than 95% of isolates, represent 75% of any *S. aureus* genome and determine the backbone of the genome. The organization of the core component is highly conserved and the identity of individual genes between isolates is 98-100%. The majority of core genes are associated with fundamental functional categories of housekeeping functions and central metabolism. The accessory component includes genetic regions present in 1-95% of isolates and accounts for about 25% of any *S. aureus* genome. It typically consists of mobile genetic elements that have or previously had the ability of horizontal transfer between strains. These genetic elements include pathogenicity islands, genomic islands, prophages, chromosomal cassettes, and transposons (Lindsay and Holden, 2004).

2.1.2 *Staphylococcus aureus* infections

Colonization provides a reservoir from which bacteria can be introduced when host defences are breached, whether by shaving, aspiration, insertion of an indwelling catheter, or surgery. Colonization also allows *S. aureus* to be transmitted among individuals in both health care and community settings. Approximately 80% of nosocomial *S. aureus* infections are caused by patients own flora which are already present on their skin or mucosal membranes before admission (Critchley, 2006). The anterior nares are the main ecological niche for this pathogen. However, numerous other sites may be colonized, including the axillae, groin, and gastrointestinal tract **(Figure 1.1)** (Wertheim *et al.*, 2004). Three types of nasal carriers have been described in the general population (Kluytmans *et al.*, 1997). Persistent carriers include those who permanently harbor one strain of *S. aureus* and represent approximately 20% of the population. Intermittent carriers consist of 30% of the population and might carry varying strains of *S. aureus* intermittently over time. The remaining 50% of the population are never colonized with the organism and are referred to as noncarriers (Critchley, 2006).

In starting an infection, *S. aureus* expresses a large number of cell surface proteins named microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), that mediate adherence to host tissues and initiate colonization leading to an infection (Gordon and Lowy, 2008; Foster, 2009). MSCRAMMs bind molecules such as collagen, fibronectin, and fibrinogen, and different MSCRAMMs may adhere to the same host-tissue component. MSCRAMMs appear to play a key role in initiation of endovascular infections, bone and joint infections, and prosthetic-device infections. Fibronectin binding proteins A and B contribute in attachment of bacterial cells to fibronectin and to plasma clot. Collagen binding protein is essential for adherence of *S. aureus* to collagenous tissues and cartilage (Foster and McDevitt, 1994) and it has been shown that antibodies against collagen binding protein block the bacteria attachment to those tissues (Mamo *et al.*, 1994). Clumping factor A and B mediate the adherence of bacterial cells to fibrinogen in the presence of fibronectin those play a significant role in wound infections (Foster and McDevitt, 1994). It has been shown that the strain that have mutation on clumping factor encoding gene is less virulent than the

wild type strain (Higgins *et al.*, 2006). Plasma sensitive surface protein participates in binding to both fibrinogen and fibronectin (Huesca *et al.*, 2002).

A well-characterized staphylococcal surface protein is protein A which is a cell wall-associated and is encoded by the *spa* gene protein. This protein binds to the Fc domain of immunoglobin G in wrong orientation which is thought to disrupt opsonization and phagocytosis (Gomez *et al.*, 2006). Protein A also exhibits an ability to bind to a protein that present at sites of damage of endothelium, and as a result, it can play a role in adherence and induction of endovascular diseases (Hartleib *et al.*, 2000; Chavakis *et al.*, 2005).

Once *S. aureus* adheres to host tissues or prosthetic materials, it is able to grow and persist in various ways. *S. aureus* can form biofilms on host and prosthetic surfaces, enabling it to persist by evading host defenses and antimicrobials (Donlan, 2002). Biofilms are complex bacterial populations which are surface-attached and enclosed in a polysaccharide matrix, composed of poly-*N*-acetylglucosamine (PNAG). The production of PNAG depends on proteins encoded by the *ica* operon (O'Gara, 2007). Biofilm-associated bacteria are resistant to the host immune responses and to antimicrobials, which often complicates treatment. It was reported that almost 50% of MRSA and 70% of MSSA isolates were able to produce biofilm *in vitro* (Grinholc *et al.*, 2007). However, all of the MRSA isolates possess the *icaADBC* genes.

In addition, *S. aureus* has other characteristics that help it evade the host immune system during an infection (reviewed by Foster, 2009). Its main defense is production of a zwitterionic capsule that is detectable by serologic methods but is not visible by negative staining. Serotype 5 and 8 capsular polysaccharides predominate among clinical isolates of *S. aureus* (O'Riordan and Lee, 2004; Roghmann *et al.*, 2005; Watts *et al.*, 2005) while most of MRSA strains (>60%) showed type 5 capsules (Roghmann *et al.*, 2005).

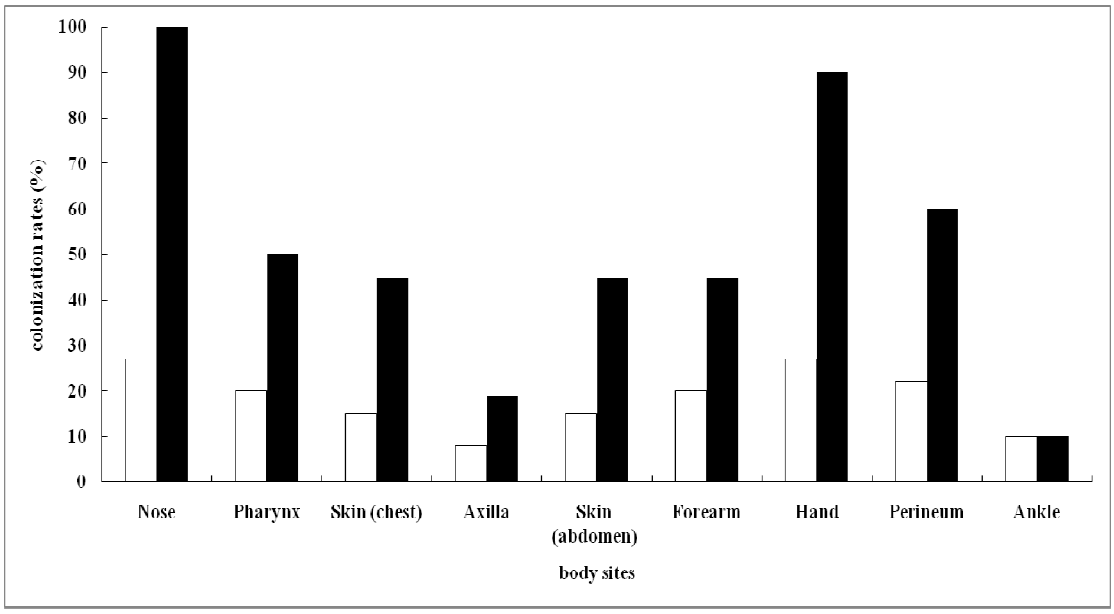

Figure 1.1 *Staphylococcus aureus* colonization rates on different body sites in the general population and nasal carriers (Wertheim *et al.*, 2004)

Staphylococci can produce both through their ability to multiply and spread widely in tissues and through their production of many extracellular substances that selected examples of these factors are described in **Table 1.1** (Gordon and Lowy, 2008). The armamentarium of virulence factors of *S. aureus* is extensive, with both structural and secreted products participating in pathogenesis of infections. Staphylococcal virulence factor may have several functions in pathogenesis while multiple virulence factors may perform the same function. Expression of MSCRAMMs generally occurs during logarithmic growth, whereas secreted proteins, such as toxins, are produced during the stationary phase. During infection, the early expression of the MSCRAMM proteins facilitates initial colonization of tissue sites, whereas the later elaboration of toxins facilitates spread.

During infection, *S. aureus* produces numerous enzymes, such as proteases, lipases, and elastases that enable it to invade and destroy host tissues and metastasize to other sites. This pathogen also has capability to secrete toxins that disrupt membranes of host cells. Cytolytic toxins form β-barrel pores in the cytoplasmic membranes and cause leakage of the cell's content and lysis (Foster, 2005). *S. aureus* secrets several cytolytic toxins including α-hemolysin, β-

hemolysin, γ-hemolysin, leukocidin, and Panton-Valentine leukocidin (PVL) (Kaneko and Kamio, 2004). α-hemolysin, encoded by the *hla* gene, inserts into eukaryotic membranes and oligomerizes into a β-barrel that forms a pore which causes osmotic cytolysis. α-hemolysin is particularly cytolytic toward human platelets and monocytes (Ferreras *et al.*, 1998; Craven *et al.*, 2009). PVL is classified as a bicomponent cytolysin because it is dependent on two secreted proteins (LukF-PV and LukS-PV) that insert into the host's cytoplasmic membrane and hetero-oligomerize to form a pore (Kaneko and Kamio, 2004). PVL exhibits a high affinity toward leukocytes and is mostly associated with CA-MRSA which causes severe necrotizing pneumonia and contagious skin infections (Labandeira-Rey *et al.*, 2007; Daskalaki *et al.*, 2010). *S. aureus* produces leukocidins that cause leukocyte destruction by the formation of pores in the cell membrane (Inden *et al.*, 2009). Some strains also produce epidermolysins or exfoliative toxins capable of causing scalded skin syndrome or bullous impetigo (Iwatsuki *et al.*, 2006; Yamasaki *et al.*, 2006).

S. aureus generates a group of powerful immuno-stimulatory proteins associated in gastroenteritis (Imanifooladi *et al.*, 2007; Schmid *et al.*, 2007) and toxic shock syndrome (Bader *et al.*, 2007; Walden *et al.*, 2008). These proteins are resistant to heat denaturation and proteases. They have the ability to cross-link MHC class II molecules located on antigen-presenting cells with T-cell receptors, forming a trimolecular complex. Formation of the complex induces intense T-cell proliferation in an antigen-independent manner resulting in massive cytokine production and release which causes capillary leak, epithelial damage and hypotension (Baker and Acharya, 2004). The primary function of superantigens is thought to weaken the host's immune system sufficiently to allow the pathogen to propagate and the disease to progress (Shimonkevitz *et al.*, 1996). The staphylococcal enterotoxins A, B, C, D, E, G, and Q are responsible for staphylococcal food borne diseases and toxic shock syndrome, while TSST-1 is the cause of toxic shock syndrome (Baker and Acharya, 2004).

Table 1.1 Selected *Staphylococcus aureus* virulence factors (Gordon and Lowy, 2008)

Type of virulence factors	Selected factors
Involved in attachment	MSCRAMMs (clumping factors/fibronectin-binding protein/collagen binding protein/bone sialoprotein-binding protein)
Involved in persistence	biofilm accumulation/small colony variants/intracellular persistent
Involved in evading/destroying host defences	leukocidins/capsular polysaccharide/protein A
Involved in tissue invasion/penetration	proteases/lipases/nucleases/hyaluronatelyase/ phospholipase C/metalloproteases
Involved in toxin-mediated disease and/or sepsis	enterotoxin/toxic shock sysdrome toxin/ exfoliative toxin

2.1.3 Antibiotic resistant mechanisms in *Staphylococcus aureus*

Antibiotic consumption encourages the overgrowth of microorganisms on skin and mucosal surfaces (Dancer, 2004). These organisms survive because they are resistant to the drugs prescribed and they proliferate quickly, assisted by the sudden access to nutrients and space (Dancer, 2008). The mechanisms of acquisition of resistance in *S. aureus* are classified into two main categories: (i) mutation of a bacterial gene on the chromosome and (ii) acquisition of a resistance gene from other organisms by some form of genetic exchange (conjugation, transduction, or transformation) (Ito *et al.*, 2003).

β-lactam antibiotics are analogues of D-alanyl-D-alanine the terminal amino acid residues on the precursor N-acetylglucosamine/N-acetlymuramic acid-peptide subunits of the nascent peptidoglycan layer. These antibiotics are bactericidal, cell wall-active agents that target the transpeptidation step of the peptidoglycan synthesis. They bind to the active site of traspeptidases known as penicillin binding proteins (PBPs), responsible for inserting the peptidoglycan precursors into the nascent cell wall (Ghuysen, 1994; Goffin and Ghuysen, 1998). β-lactams compete with the wall precursor for binding to the active site of the enzyme, however, unlike natural D-alanyl-D-alanine, the β -lactam-PBP acyl adduct is stable, resulting in irreversible blockage of PBP function. The features of β-lactams which make them attractive antimicrobial agents stem from the fact that their targets are easily accessible and specific to bacteria (Wilke *et al.*, 2005). β-lactamases are enzyme that contribute to β-lactam resistance by inactivation of many of these antibiotics in a reaction similar to the one β-lactams use to inhibit PBPs. β-lactamases bind β-lactams, which results in formation of an acylated intermediate and the cleavage of the amide bond of the β-lactam ring. The gene coding for β-lactamase (*blaZ*) is usually carried on a plasmid or located on a transposon. Expression of β-lactamase is induced by the presence of β-lactam antibiotics through a regulatory system composed of a repressor, BlaI, and a signal transducer, BlaRI. Based on sequence similarity four different types of β-lactamases have been described so far in *S. aureus* which differ in their substrate specificity (Zygmunt *et al.*, 1992). Because of this, penicillin is no longer effective treatment of

most *S. aureus* infections. β-lactamase resistant penicillins, such as oxacillin and methicillin, are the drugs of choice against β-lactamase producing *S. aureus*.

Methicillin, oxacillin and nafcillin are semisynthetic β-lactamase resistant β-lactams. *S. aureus* has developed resistance to this class of β-lactams by acquiring the *mecA* gene carried on the staphylococcal cassette chromosome *mec* (SCC*mec*) element. Strains containing *mecA* are known as MRSA, although they are, in fact, resistant to all β-lactam antibiotics (Chambers, 2001; Katayama *et al.*, 2004). *mecA* encodes an alternative penicillin binding protein PBP2a, that has low affinity for β-lactams. PBP2a belongs to the group of high molecular mass (78 kDa) family of PBPs and consists of a transpeptidase domain and a non-penicillin binding domain of unknown function (Goffin and Ghuysen, 1998). This enzyme is known to posse low affinity for β-lactams that allows MRSA strains to grow in antibiotic concentrations that inactivate all native PBPs (Gaisford and Reynolds, 1989). PBP2a is an absolute requirement for high-level β-lactam resistance in MRSA (Chambers, 2001). Blocking its activity in isolation, as in PBP2a negative mutants, restores susceptibility to β-lactams.

The other class of antibiotics playing an important role in the therapy of serious staphylococcal infections is aminoglycosides although reports of increased resistance to these drugs in many countries (Janknegt, 1997; Schmitz and Jones, 1997; Yoshikawa *et al.*, 2004). The main mechanism of aminoglycoside resistance is drug inactivation by aminoglycoside modifying enzymes encoded within mobile genetic elements (Wildemauwe *et al.*, 1996; Ida *et al.*, 2001). These aminoglycoside modifying enzymes including aminoglycoside-6'-N-acetyltransferase/2''-O-phosphoryltransferase [AAC(6') and APH(2'')] encoded by *aac(6')-Ie+aph(2'')* gene, aminoglycoside-4'-O-nucleotidyltransferase I ANT(4')-I encoded by *ant(4')-Ia* gene, and aminoglycoside-3'-O- phosphoryltransferase III encoded by *aph(3')-IIIa* gene are of particular significance among staphylococci. They modify and inactivate the traditional aminoglycosides of therapeutic importance. Resistance to gentamicin and concomitant resistance to tobramycin and kanamycin in staphylococci are mediated by bifunctional enzyme displaying AAC(6') and APH(2'') activity. Resistance to neomycin, kanamycin, tobramycin, and amikacin is mediated by ANT(4')-I enzyme.

Kanamycin and neomycin is also inactivated by APH(3')-III enzyme (Shaw *et al.*, 1993; Hauschild *et al.*, 2008)

The macrolide, lincosamide, and streptogramin antibiotics are chemically distinct but they similarly display their effects by inhibiting bacterial protein synthesis. For this reason, genes conferring resistance to one of the macrolide-lincosamide-streptogramin (MLS) antibiotics may confer cross-resistance to others. Increased rates of resistance to MLS antibiotics among clinical staphylococcal isolates are considered a consequence of the expanded use of these antibiotics in the treatment of Gram-positive infections (Patel *et al.*, 2006; Otsuka *et al.*, 2007; Gul *et al.*, 2008). Several authors have screened clinical isolates of erythromycin-resistant *S. aureus* for genes encoding resistance to macrolides, lincosamide, and streptogramin B (Kim *et al.*, 2004; Patel *et al.*, 2006; Otsuka *et al.*, 2007; Gul *et al.*, 2008). Three main resistant mechanisms including (i) an alteration of the target on ribosome due to dimethylation of a specific adenine residue in the 23S ribosomal RNA by the product of the *erm* gene, and consequently a decrease in binding of MLS antibiotics, (ii) an inactivation of streptogramin B and lincosamide by streptogramin B hydrolase and 3-lincomycin-4-clindamycin-*O*-nucleotidyltransferase respectively, and (iii) an active efflux of macrolides and streptogramin-B determined by the *msrA* and *msrB* genes in *Staphylococcus epidermidis* and *Staphylococcus xylosus*, respectively, both of which appear to act as an ATP-dependent efflux pump are responsible for MLS resistance.

Vancomycin is a useful antibiotic against Gram-positive pathogens. In 1996, the first *S. aureus* strain with reduced susceptibility to vancomycin was isolated from a Japanese patient who contracted vancomycin-refractory surgical incision site infection, designated VRSA for vancomycin-resistant *S. aureus* or GISA for glycopeptide-intermediate *S. aureus* (Hiramatsu *et al.*, 1997). (Chang *et al.*, 2003) described a patient infected with fully VRSA that contained the *vanA*, vancomycin-resistance gene. Although the acquired vancomycin-resistance genes including *vanA*, *vanB*, *vanD*, *vanE*, *vanF*, and *vanG* have been reported in vancomycin-resistant enterococci, (Woodford, 1998) these genes have not previously been identified in any clinical isolates of *S. aureus*. However, conjugative transfer of the *vanA* gene from enterococci to *S. aureus* has been demonstrated *in vitro* (Noble

et al., 1992). They suspected the vanA detected in the current patient's VRSA isolate probably originated in vancomycin-resistant *Enterococcus faecalis*, which was also isolated from the patient.

The pathogen *S. aureus* may use various strategies to resist antibiotic therapy. One of these strategies is the formation of small colony variants (SCVs), a naturally occurring, slow-growing subpopulation with distinctive phenotypic characteristics and pathogenic traits. SCVs are defined by mostly non-pigmented and non-haemolytic colonies approximately 10 times smaller than the parent strain. SCVs have been reported to cause recurrent, persistent infections many years after the initial infection had been cured (Proctor *et al.*, 1995). Very often they reside inside human cells avoiding host defenses and antimicrobial chemotherapeutics. SCVs are defective in their electron transport pathways and usually form non-pigmented, non-hemolytic tiny colonies on agar (von Eiff *et al.*, 2006). The small colony variants display marked auxotrophisms for thymidine, menadione, and/or hemin (von Eiff *et al.*, 2006; Besier *et al.*, 2007; Lannergard *et al.*, 2008). They exhibit reduced rate of metabolism and are less virulent, but due to their slow growth and reduced cell wall synthesis, they are more tolerant of β-lactam antibiotics than their wild type parents. Their low membrane potential makes them also resistant to aminoglycoside antibiotics (Proctor *et al.*, 2006). Clinically, small colony variants are better able to persist in mammalian cells and are less susceptible to antibiotics than their wild type counterparts, and can cause latent or recurrent infections on emergence from the protective environment of the host cell.

2.1.4 Hospital-acquired methicillin-resistant *Staphylococcus aureus*

Before the introduction of antibiotics, the mortality rate of staphylococcal bacteraemia was approximately 70%. Because of the widespread availability of penicillin, this rate dropped to 25% in 1944. However, mortality rates rose to reach 45% as *S. aureus* became resistant to penicillin, within the next 10 years. The isoxazolyl group of penicillins, including methicillin and flucloxacillin, was then introduced into clinical use and once again, the mortality of staphylococcal bacteraemia dropped to 25% (Reviewed by (Dancer, 2008)). The capacity for methicillin resistance was documented almost as soon as these drugs became available. A meta-analysis which performed to compare the impact of

methicillin-resistance on mortality in *S. aureus* bacteremia revealed that MRSA bacteremia is associated with significantly higher mortality rate than is MSSA bacteremia (Blot *et al.*, 2002; Cosgrove *et al.*, 2003). In critically ill patients, an MRSA bacteraemia mortality rate was over 70% (Ho and Robinson, 2009)

In the 1950s, years before MRSA was reported for the first time, three independent developments led to appreciation of the potential public-health implications that *S aureus* might have in store. The first was slow but persistent rise in penicillin resistance, which was noted soon after its first clinical trials. By the end of the 1940s, hospitals in the UK and the USA reported that 50% of *S. aureus* was resistant to penicillin. In 1957, the hospital staphylococcus, 80/81 strain (according to its bacteriophage susceptibility pattern) was responsible for nearly all the epidemics in maternity units in the USA and half of all hospital outbreaks in the UK. A third of hospital patients, who were nasal carriers of this strain, went on to develop septicaemia compared with only 2.5% who were colonised with other strains. There was concern not only because of the high death rate in these patients but also because outbreaks were not confined to patients and often caused invasive skin infections in health-care workers. The 80/81 strain began to decline in the 1960s when methicillin, the first semisynthetic penicillinase-stable penicillin was introduced (Reviewed by (Grundmann *et al.*, 2006)). In October 1960, methicillin was marketed in and three meticillin-resistant isolates were reported after the screening of 5000 clinical isolates, six months later (Jevons *et al.*, 1963). These isolates were from the same hospital in southern UK, had the same phenotype, and were only resistant to β-lactam antibiotics. However, the situation started to change in 1967, and multidrug-resistant MRSA was reported from Switzerland, France, Denmark, England, Australia, and India. Most isolates belonged to the same phage type 83A complex. There were renewed concerns in the early 1980s, when a rise in the frequency of gentamicin-resistant MRSA was reported from several countries, including USA, Ireland, and UK. In Australia, an epidemic multidrug-resistant MRSA strain was noted in the state of Victoria, which, after 1982, started to cause outbreaks in patients in UK hospitals and was believed to have been imported by an Australian health-care worker. When a second MRSA strain became prevalent in

hospitals, a numerical prefix for epidemic MRSA was introduced by the staphylococcal reference laboratory of the UK Public Health Laboratory Service. On the basis of this definition, 16 epidemic types of MRSA were identified in England and Wales until 1995. However, only UK EMRSA-3, UK EMRSA-15, and UK EMRSA-16 were still reported in the 1990s. The epidemic types that showed the most dynamic behaviour are UK EMRSA-15 and UK EMRSA-16 (Review by (Crisostomo *et al.*, 2001; Hiramatsu *et al.*, 2001; Johnson *et al.*, 2001; Grundmann *et al.*, 2006)).

Methicillin resistance is conferred by the *mecA* gene, which encodes a penicillin-binding protein (PBP2a) with decreased affinity for *β*-lactam antibiotics. *mecA* is part of a mobile genetic element, SCC*mec* which is flanked by cassette chromosome recombinase genes (*ccrA*/*ccrB* or *ccrC*) that permit intra- and interspecies horizontal transmission of this genetic element. The initial reservoir of SCC*mec* is unclear but may have been a coagulase-negative staphylococcal species (IWG-SCC, 2009). HA-MRSA infections historically have been caused by internationally disseminated 5 major clones, including the Iberian, Brazilian, Hungarian, New York/Japan, and Pediatric clones. These multidrug-resistant clones were disseminated globally and accounted for the majority of HA-MRSA infections in several regions (Oliveira *et al.*, 2002; Rodriguez-Noriega *et al.*, *In press*).

2.1.5 Community-acquired methicillin-resistant *Staphylococcus aureus*

The worldwide emergence of CA-MRSA during the last decade represents a significant change in the biology of MRSA strains and is changing the epidemiology of MRSA infections. Since, the first description of MRSA in 1961 and until the late 1990s, MRSA was almost exclusively HA infections. Multi-locus sequence typing has shown that >90% of these MRSA infections were caused by MRSA strains that belonged to only five genetic lineages/clonal complexes (CC) including CC5, CC8, CC22, CC 30, and CC45 carrying SCC*mec* cassettes I–VI (Feil *et al.*, 2003; Lindsay, 2010). The epidemiology of MRSA infections has changed significantly, while CA-MRSA infections are caused by strains belonging to lineages distinct from HA-MRSA. New SCC*mec* types and subtypes are emerging rapidly and currently at least nine SCC*mec* types plus several subtypes have been described (Conceicao *et al.*; Blanc *et al.*, 2007; Deurenberg and Stobberingh, 2009).

CA-MRSA was firstly described in 1982 when MRSA spread among intravenous drug abusers (Saravolatz *et al.*, 1982a; Saravolatz *et al.*, 1982b). This outbreak was followed by secondary cases of HA infections (Saravolatz *et al.*, 1982b). The endemic presence of CA-MRSA in aboriginal communities in Western Australia was reported in 1993 (Udo *et al.*, 1993) and followed by a document from New Zealand. Common to all these reports was that three causative strains, ST74-IVa (in USA), ST8-IV (in Australia), and ST30-IV (in New Zealand), were all different from the types causing HA infections (Nimmo and Coombs, 2008). A marked increase in the number of CA-MRSA infections in children admitted to the University of Chicago Children's Hospital with no established HA risk factors (Gonzalez *et al.*, 2005). The MRSA isolates differed from MRSA isolates causing nosocomial infections in the same institution. However, as stated in the accompanying editorial, this was, at that time, not perceived as indicative of a general change in the epidemiology of MRSA. CA-MRSA from the same time period was shortly reported as retrospective cases or case series from a number of European countries including The Netherlands, Denmark, Finland, Greece, and France (Reviewed by Diederen and Kluytmans, 2006).

However, the general change in the perception that CA-MRSA actually constitutes a separate entity began with a report of the death of four children caused by CA-MRSA (CDC, 1999). Since then, a steadily increasing number of papers have confirmed this significant change in the epidemiology of MRSA transmission and infection. Cases of CA-MRSA differ from the traditional HA-MRSA in several aspects, both microbiologically and epidemiologically (Millar *et al.*, 2007) (**Table 1.2**). CA-MRSA strains belong to distinct lineages and are not just feral descendants of HA-MRSA strains but seem to have evolved separately. Presently, five CA-MRSA lineages are found worldwide that contain (i) ST1-IV, (ii) ST8-IV, (iii) ST30-IV, (iv) ST59-IV and ST59-V, and (v) ST80-IV (Enright *et al.*, 2002). CA-MRSA is characterised by possession of the smaller SCC *mec* type IV and, to a lesser degree, type V or new variants. Depending on the lineage they are often less resistant to other classes of antibiotics than HA-MRSA strains. Furthermore, CA-MRSA very often produces potent toxins/virulence factors such as

PVL, arginine catabolic mobile element, and phenol-soluble modulins (Diep and Otto, 2008).

Epidemiologically, the type of infections, the age distribution of patients with CA-MRSA infections and the risk factors differ significantly from HA-MRSA. Risk factors for CA-MRSA are (i) history of colonisation/infection with CA-MRSA, (ii) close contact with a person colonised/infected with CA-MRSA, and (iii) membership of special communities such as participation in contact sports, injection drug use, living in correctional facilities or shelters, military personnel, and men who have sex with men (Skov and Jensen, 2009).

Although characteristic microbiological and epidemiological features, none of these are specific and there is no clear definition of CA-MRSA. This has led to the use of several different definitions including strain characteristics and/or epidemiological criteria including (i) susceptibility to clindamycin/ciprofloxacin, (ii) being non-multidrug resistant, (iii) carrying SCC*mec* IV or V, (iv) being PVL positive, and (v) community onset combined with a lack of HA risk factors and without previous history of being MRSA positive (David *et al.*, 2008). As mentioned previously, none of the above criteria are exclusive for CA-MRSA and among the microbiological features it is worth noting that EMRSA-15 (ST 22), which is one of the five large traditional HA-MRSA lineages, carries SCC*mec* IV. Similarly, the characterisation of MRSA infections in persons previously found MRSA positive as healthcare-related was very valuable in the beginning of the CA-MRSA epidemic, however, it has become increasingly problematic and, in several cases, directly misleading with the increasing number of CA-MRSA cases (Skov and Jensen, 2009).

Table 1.2 Comparison of clinical, epidemiological and microbiological characteristics of community acquired (CA-) and hospital acquired (HA)-methicillin-resistant *Staphylococcus aureus* (MRSA) (Millar *et al.*, 2007)

Characteristics	HA-MRSA	CA-MRSA
Population affected	Hospital/healthcare/nursing home	Usually young healthy individuals in the community
Site of infection	Bacteraemia/wound infections Symptomatic infections of respiratory and urinary tracts	Skin and severe skin and soft tissue infections In severe cases, septic shock, bacteraemia, necrotising pneumonia
Transmission	(i) Person-to-person spread (healthcare staff, visitors, patients) (ii) Environment-to-patient spread (hospital equipments)	(i) Person-to-person spread (shared facilities) (ii) Environment-to-person spread (shared sports equipments)
Risk factors	Indwelling devices, catheters, lines, haemodialysis, prolonged hospitalisation, long-term antibiotic use	Close physical contact, abrasion injuries, activities associated with poor hygiene
Susceptibility to methicillin/other antibiotics	No/No	No/Yes (in majority of cases)
Presence of *pvl* gene	Low (<5%)	High (>95%)
SCCmecA type	Predominantly subclasses I, II or III	Mainly IV (and subtypes a–h), V
agr genotype	Predominantly II	Predominantly I and III

2.1.6 Vancomycin-resistant methicillin-resistant *Staphylococcus aureus*

Glycopeptides have a pivotal role in the present therapy of MRSA infections, thus glycopeptide resistance in MRSA strains is of potentially great importance and may further compromise treatment. Vancomycin tolerance in *S. aureus* has been reported in several studies. Even in the original paper describing β-lactam tolerance in *S. aureus* (Sabath *et al.*, 1977), most of the strains also showed cross-tolerance to vancomycin. The mechanism of tolerance in *S. aureus* is unclear, although it is sometimes associated with autolysis deficiency. *S. aureus* isolates are usually inhibited *in vitro* by vancomycin concentrations of 0.5-2 µg/mL. Isolates with vancomycin MICs of 8-16 µg/mL are referred to as VISA, and those with vancomycin MICs of ≥32 µg/mL are designated VRSA (Appelbaum, 2006). Vancomycin-resistant or -intermediate isolates usually show similarly reduced susceptibilities to teicoplanin and may be referred to as glycopeptide-resistant or glycopeptide-intermediate *S. aureus* (GRSA or GISA).

Intermediate vancomycin resistance in MRSA was first reported from Japan in 1997 (Hiramatsu *et al.*, 1997a; Hiramatsu *et al.*, 1997b). Mu50, showed true homogeneous VISA, with a vancomycin MIC of 8 µg/mL. A second strain, Mu3, showed heterogeneous resistance, when grown in a drug-free medium, Mu3 produced subpopulations with varying degrees of vancomycin resistance; when grown in the presence of vancomycin ≥8 µg/mL, Mu3 produced subclones with vancomycin MICs of 8 µg/mL at a frequency ≥ 10^6 CFU/mL. A survey of more than 1000 MRSA isolates from 203 Japanese hospitals in 1996 found no further homogeneous vancomycin resistance. However, the prevalence of MRSA heterogeneously resistant isolates was 10-20% in Japanese University hospitals and around 1% in non-University hospitals (Hiramatsu *et al.*, 1997a). Intermediate resistance to vancomycin in *S. aureus* is associated with a variety of alterations in cell wall structure and metabolism. These result in thickened cell walls with reduced peptidoglycan cross-linking that trap or sequester vancomycin molecules, impeding their antimicrobial function. Since 1997, VISA has been found throughout the world, but they remain uncommon, and only about 100 such isolates have been reported. Approximately 90% of these strains show heterogeneous resistance and 10% are

homogeneous. In contrast to Hiramatsu's report of 1997, a study carried out by Ike *et al.* in 2001 found no VISA or hVISA among more than 6000 MRSA isolates from nearly 300 Japanese hospitals (Ike *et al.*, 2001). Similarly, an analysis of more than 600 MRSA isolates from 33 US hospitals in 1997 failed to identify any homogeneous VISA and only two hVISA (Hubert *et al.*, 1999). Although so far they have occurred infrequently, vancomycin-intermediate strains can arise from MRSA during treatment with vancomycin, especially when low concentrations of the drug are used. hVISA have appeared in all five of the major pandemic clones of MRSA and homogeneous VISA has arisen in two of these (Howe *et al.*, 2004). It is likely that heterogeneous VISA will continue to emerge independently from existing circulating MRSA strains and that homogeneous VISA strains will develop from heterogeneous ones.

Fully vancomycin- and teicoplanin-resistant MRSA isolates are rare: only four such isolates have been reported from the USA between 2002 and 2005 (Miller and Rudoy, 2000; Tenover *et al.*, 2004). High-level resistance is encoded in these strains by the *vanA* transposon, probably acquired from vancomycin-resistant enterococci (Appelbaum, 2006).

2.1.7 Global epidemiology of methicillin-resistant *Staphylococcus aureus*

The widespread emergence of MRSA, especially in various types of nosocomial infections, is a serious clinical problem worldwide. Numerous surveillance studies confirm that methicillin resistance is common among *S. aureus* isolates in Africa, Asia, Australia, Europe, and the USA, in both hospital- and community-acquired infections.

In Asia, there are several research teams collected the prevalence data on both HA-MRSA and CA-MRSA (**Table 1.3**). The incidence of methicillin resistance among nosocomial isolates of *S. aureus* is higher than 50% in some Asian countries such as Taiwan, Hong Kong, and Singapore (Christiansen *et al.*, 2004). Among Asian countries, Japan appears to be one of those mostly impacted with the occurrence of MRSA. The proportion of methicillin-resistant strains among S. *aureus* isolates from 43 university hospitals was as high as 60% in 1991. An even higher prevalence of MRSA was reported in the surgery department of another university hospital. During a two-year study period the average rate of clinical

MRSA isolates was 83%. Similarly, the proportion of MRSA among all *S. aureus* strains causing skin infections at the Medical University of Osaka was 41.5% in 1991, indicating a high proportion even among strains only causing infections. In Hong Kong, MRSA has been endemic in public hospitals since the mid-1980s. A recent investigation documented that MRSA accounts for 58.2% of *S. aureus* isolated from blood cultures (Voss and Doebbeling, 1995). In Thailand, a study at a provincial 1000-bed hospital demonstrated that *S. aureus* was the third most common cause of bacteraemia, and was associated with a mortality rate of 48% (Nickerson *et al.*, 2009).

In Europe, MRSA prevalence rates vary between countries, from <1% in northern Europe to >40% in southern and western Europe (Tiemersma *et al.*, 2004). There is a north-south gradient, MRSA strains being rare in Scandinavia and far more prevalent in southern Europe (Stefani and Varaldo, 2003). A survey of 3,051 *S. aureus* isolates from 25 university hospitals distributed among 15 countries of central and southern Europe (Fluit *et al.*, 2001), MRSA isolates constituted 25% of all isolates; the highest prevalence was seen in hospitals in Portugal (>50%) and Italy (approximately from 40-60%), whereas the lowest prevalence was observed in hospitals in Switzerland and The Netherlands (2%). In the European Prevalence of Infection in Intensive Care study, the highest prevalence of MRSA strains was found in Italy and France (>70%) (Vincent, 2000). Bacteraemia data from England and Wales show that proportion of MRSA among *S. aureus* bacteraemia increased from under 2% in 1990 to around 40% in 2004 (Das *et al.*, 2007). Increasing mortality from MRSA in England and Wales has been reported in parallel with the increase in bacteraemia (Crowcroft and Catchpole, 2002).

In the USA, the prevalence of MRSA in even higher than in much of Europe, approximately 60% of staphylococcal infections in the intensive care unit are now cause by MRSA (Rice, 2006). Almost all of USA hospitals reported having patients with MRSA between 1987 and 1989 (Boyce, 1990). The proportion of hospitals with large numbers of MRSA cases (> 50 per year) increased significantly from 18% to 32% during the same period. The proportion of MRSA among all USA hospitals rose from 2% in 1975 to almost 30% in 1991, although the rate of increase varied significantly among different sized hospitals (Voss and Doebbeling, 1995). In

2004 data from the LEADER program, which collected Gram positive clinical isolates from 50 USA laboratories in 33 states, found a prevalence of 54.2% (Draghi *et al.*, 2005). Data from the LEADER program recorded a similar rate, 58.1% (Jones *et al.*, 2008). It is obvious that the increasing prevalence of MRSA is a worldwide problem, affecting both affluent and poor countries (Woodford and Livermore, 2009).

Table 1.3 The prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in hospital- and community- acquired infection in Asian countries

Nation	Percentage of MRSA (Reported year)	References
China	27.8% (1998-1999); 37% (2001)	(Bell and Turnidge, 2002; Zhang *et al.*, 2006)
Hong Kong	69.8% (1998-1999); 1.9-4.2% (2005)	(Bell and Turnidge, 2002; Ho *et al.*, 2007)
India	40-50% (2003-2004); 1.4% (2004)	(Arakere *et al.*, 2005; Patil *et al.*, 2006)
Japan	69.5% (1998-1999); 4.3% (2001)	(Bell and Turnidge, 2002; Hisata *et al.*, 2005)
Jordan	19%; 57% (2002)	(Al-Zu'bi *et al.*, 2004)
Korea	58.4% (2005)	(Kim *et al.*, 2007)
Kuwait	9% (2001)	(Zhang *et al.*, 2006)
Malaysia	35.4% (1990-1991)	(Cheong *et al.*, 1994)
Philippines	5% (1998-1999); 18.2% (1999-2003)	(Bell and Turnidge, 2002; Ontengco *et al.*, 2004)

Table 1.3 The prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in hospital- and community- acquired infection in Asian countries *(Contd.)*

Nation	Percentage of MRSA (Year)	References
Saudi Arabia	33% (2001)	(Madani *et al.*, 2001)
Shri Lankan	27.5% (1994)	(Thevanesam *et al.*, 1994)
Singapore	62.3% (1998-1999); 43.14% (2004)	(Bell and Turnidge, 2002; Hsu *et al.*, 2005)
Taiwan	59.6% (1998-1999); 13.2% (2007)	(Bell and Turnidge, 2002; Lo *et al.*, 2007)
Thailand	30.3% (2002-2003); 68.8% (2000-2002)	(Thongpiyapoom *et al.*, 2004; Danchaivijitr *et al.*, 2005)
Turkey	32.8% (2000-2002); 0.3% (2007)	(Ciftci *et al.*, 2007; Savas *et al.*, 2007)

2.1.7 Morbidity rate, mortality rate, and economic impact of methicillin-resistant *Staphylococcus aureus* infections

In common with other serious infections, appropriate antibiotic therapy improves outcome in MSSA bacteraemia, and methicillin resistance certainly increases mortality, probably by the use of inappropriate treatments. (Lodise and McKinnon, 2005) studied the morbidity, mortality, and hospital costs caused by MRSA in patients with *S. aureus* bacteremia. Patients with MRSA had a 1.5 fold longer length of stay and a 2 fold increased cost of hospitalization compared with MSSA. Confirming this clinical significance, the present 15 year study involving 1148 patients documented that MRSA accounted for 74.1% of all nosocomial *S. aureus* bacteremia in a large tertiary care hospital (Wang *et al.*, 2008). The difference between the mortality rates of MRSA (49.8%) and MSSA bacteremia (27.6%) was almost 2 fold. MRSA bacteremia express a significantly higher mortality compared with MSSA bacteremia, with increased mortality rates of 20%-64%. The study shows that the risk of mortality for MRSA bacteremia was 1.68-2.9 times higher than MSSA bacteremia. A current study registered 160 subjects with USA300 MRSA, the clone that commonly found in the community and is being increasingly reported in the healthcare setting infections. Failure in the HA-MRSA group was higher (38.1%) compared with the CA-MRSA group (23.7%) (Moore *et al.*, 2009).

The main reasons for this increased mortality are delayed appropriate treatment and the use of glycopeptides which, at least as monotherapy, may be given in inadequate doses, are poorly bactericidal and have poor deep-tissue penetration. In addition, duration of hospital stay after bacteremia, use of mechanical ventilator, and central venous catheter are independent factors of mortality. The length of stay before MRSA bacteremia for risk and the length of stay after MRSA bacteremia for mortality were the important attributes.

2.2 New sources of anti-methicillin-resistant *Staphylococcus aureus* agents

Plants are not only important to the millions of people to whom traditional medicine serves as the only opportunity for health care and to those who use plants for various purposes in their daily lives, but also as a source of new pharmaceuticals. According to WHO, herbal medicines serve the health needs of about 80% of the world's population especially for millions of people in the vast rural areas of developing countries (WHO, 2001). To overcome the problem of antibiotic resistance, medicinal plants have been extensively studied as alternative treatments for infectious diseases. Since, *S. aureus* and MRSA are major causes of hospital- and community-acquired infections, and can result in serious consequence as well as high healthcare costs. Therefore in recent year, increasing attention has been focused on phytochemicals as alternative anti-MRSA agents. Although, many hundreds of plants worldwide are used in traditional medicine as treatments for bacterial infections, very few of them have been validated by scientific data. Very limited repots have been focused on the antibacterial properties and the mechanisms of actions of certain plant species and plant derived compounds.

Essential oils are volatile, natural, complex compounds characterized by a strong odor and are formed by aromatic plants as secondary metabolites. They are liquid, volatile, limpid and rarely colored, soluble in lipid and organic solvents with a generally lower than density than that of water and usually obtained by steam or hydro-distillation. The oils can be sensitized by all plant organs including buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood, and bark (Hartford and Zug, 2005). Up to the present day, these characteristics have not changed much except that more is now known about some of their mechanisms of action, particularly at antimicrobial level. Tea tree oil (TTO) is a complex mixture of terpene hydrocarbons and tertiary alcohols distilled from the leaves of the Australian native plant, *Melaleuca alternifolia* (Carson *et al.*, 2006). The pharmacology of TTO has been identified in part through bacteriologic and animal studies. Several published reported have addressed minimum inhibitory and bactericidal concentrations of this oil against clinical isolates of *S. aureus*. The plant compounds are considered an effective decolonization agent for MRSA both *in vitro* and *in vivo* (Carson *et al.*, 1998; Caelli *et al.*, 2000; Thompson *et al.*, 2008).

Tea, a beverage has been safety consumed worldwide for centuries. Chinese green tea (*Camellia sinensis* L.) and its extract in particular have shown many health benefits to human and animal, including chemopreventive, anti-carcinogenic, anti-atherogenic, and anti-microbial activities (Amin and Buratovich, 2007; Ferrazzano *et al.*, 2009). In term of anti-microbial activity, most pathogen such as *S. aureus*, *E. coli*, *Salmonella* Typhimurium, *Listeria monocytogenes*, and *Campylobacter jejuni* have been reported to be inhibited by tea component which may be from different types of tea or tea extract (Hatano *et al.*, 2005; Friedman, 2007). Although, the effectiveness of tea catechins as anti-staphylococcal activity is compromised by relative chemical instability and poor bioavailability, but tea catechins will be apply as a viable option as alternative medicine or as a synergistic combination therapy. The commercial product of green tea extract was particularly rich in epicatechin gallate and epigallocatechin gallate, at 26 and 15%, respectively (Yam *et al.*, 1997; Si *et al.*, 2006). ECG and EGCG were the most active, particularly EGCG against *S. aureus*. EGCG had the lowest MIC_{90} values against MSSA (58 µg/mL) and its MRSA (37 µg/mL) (Si *et al.*, 2006). Several studies have further indicated that epicatechin gallate and epigallocatechin gallate have the capacity to reduce oxacillin resistance in *S. aureus* (Shiota *et al.*, 1999; Hamilton-Miller and Shah, 2000; Stapleton and Taylor, 2002; Stapleton *et al.*, 2004a).

Berries are traditionally an important part of many countries diet. The most well known and also commercially most important wild berries are lingonberry, bilberry, raspberry, cloudberry, buckthorn berry, and crowberry. Berry fruits are rich sources of bioactive compounds, such as phenolics and organic acids, which have antimicrobial activities against human pathogens. Puupponen-Pimiä and teams found that phenolic compounds such as ellagitannins present in berries selectively inhibit the growth of human pathogens (Puupponen-Pimia *et al.*, 2005a; Puupponen-Pimia *et al.*, 2005b). Especially cranberry, cloudberry, raspberry, strawberry and bilberry possess clear the effects. Flavonoids and phenolic acids, and eight extracts from common Finnish berries, was measured against selected Gram-positive and Gram-negative bacteria species, including probiotic bacteria and the intestinal pathogen bacteria (Puupponen-Pimia *et al.*, 2005a; Puupponen-Pimia *et al.*, 2005b). Myricetin inhibited the growth of all lactic acid bacteria derived from the

human gastrointestinal tract but it did not affect the pathogenic strain. These berry extracts inhibited the growth of Gram-negative but not Gram-positive bacteria. Contrastly, antimicrobial activity and mechanisms of phenolic extracts of 12 Nordic berries were studied against selected human pathogenic microbes. The most sensitive bacteria on berry phenolics were *Bacillus cereus* and *Helicobacter pylori* and *S. aureus* cells adhered to berry extracts were dead on the basis of their fluorescence and plate count (Nohynek *et al.*, 2006).

2.3 *Quercus infectoria*: a promising Thai medicinal plant for control multidrug-resistant pathogen infections

Herbs have been found to have antimicrobial properties for over 4,500 years. However, herb resistant bacteria strains have never been reported, and are in stark contrast to resistance often seen in pathogens treated with modern antibiotic (Lai and Roy, 2004). In Asian countries, the nutgalls of *Quercus infectoria* G. Olivier have been used for centuries in oriental traditional medicines for treating inflammatory diseases. *Quercus infectoria* is a small tree or a shrub. This species is most abundant in Asia Minor, and extends to middle Asia. The tree capitulate nutgalls that emerge on its shoots as a consequence of assault of gall wasp, *Cypnis gallae tincotoriae* (Samuelson *et al.*, 1996) (**Figure 1.2**). The nutgalls of *Quercus infectoria* have a great medicinal value and have pharmacologically been deciphered to be used because of their anti-amoebic (Sawangjaroen *et al.*, 2004; Sawangjaroen and Sawangjaroen, 2005), anti-inflammatory (Kaur *et al.*, 2004), and anti-cariogenic (Hwang *et al.*, 2004) activity. The main constituents found in the nutgalls of *Quercus infectoria* are tannin (50-70%) and small amount of free gallic acid and ellagic acid (Ikram and Nowshad, 1977; Dar and Ikram, 1979; Hwang *et al.*, 2000; Hamid *et al.*, 2005). However, there were very few reports that investigate the application and mechanisms with the antibiotic-resistant *S. aureus* of this medicinal plant.

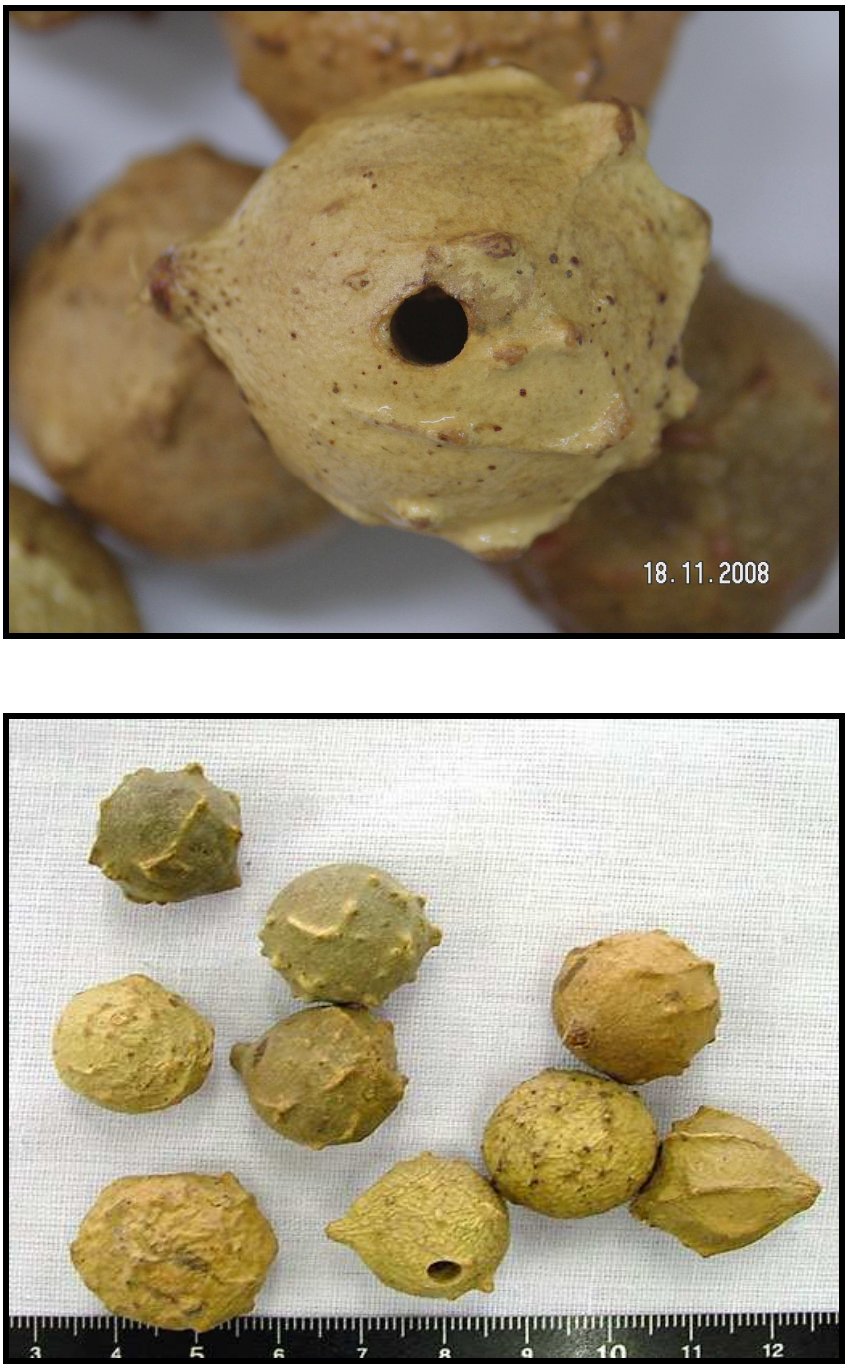

Figure 1.2 *Quercus infectoria* G. Olivier (nutgall)

2.3.1 Description

Quercus infectoria is a member in the family Fagaceae. This specie is most abundant in Asia Minor, and extends to middle Asia. A small tree from four to six feet high, crooked and shrubby-looking, with smooth and bright-green leaves. This tree is valued for excrescences which are formed upon the young branches, and which are known in market under the names of galls or nut galls. They are the result of a puncture made in the bark by an insect (*Cypnis gallae tincotoriae*) for the purpose of depositing its egg (Samuelson *et al.*, 1996). A small tumor soon follows the puncture, and forms a very dense mass about the egg. The egg hatches into the fly while in these tumors, eating its way by a small opening. The excrescences vary from the size of a large pea to that of a small hickory-nut, are nearly round, hard, and quite smooth with the exception of small tubercles scattered over the surface. Those in which the egg has not yet turned into larva are most compact and heavy, of a dark blue or bluish-green color externally, grayish-brown internally, and of an almost flinty fracture. When the larva has been developed, the external color lightens; and those of large size and grayish appearance are more or less fed upon internally by the grub, and depreciate in value. Nutgalls are purely and powerfully astringent, scarcely stimulant. They may be used as a wash and gargle in aphthous sores and putrid sore throat, and as an injection in bad leucorrhea; in which cases they arrest putrefactive tendencies, and may be combined with suitable stimulants. By coagulating the blood, they frequently will arrest hemorrhage from small vessels; and sometimes are used for bleeding piles, both as ointment and suppository, but are inadmissible when the tumors are sensitive. A tincture is made by macerating four ounces of nutgalls in diluted alcohol for two days, and then treating them by percolation till a quart has passed. It is rarely used. Age impairs its astringency. One ounce of powdered nutgalls rubbed into seven ounces of lard, forms the usual ointment (Dar *et al.*, 1976; Alkofahi *et al.*, 1996; Voravuthikunchai *et al.*, 2006).

2.3.2 Components of *Quercus infectoria*

The components in *Quercus infectoria* have been studies since 1977. The main constituents found in the nutgalls of *Quercus infectoria* are tannin (50-70%) and small amount of free gallic acid and ellagic acid (Ikram and Nowshad, 1977; Dar and Ikram, 1979; Hwang *et al.*, 2000; Hamid *et al.*, 2005) (**Figure 1.3**). Ikram and Nowshad (1977) isolated syringic, gallic, and ellagic acid from ethanol extract from the nutgalls. Syringic acid showed central nervous system activity. In 1983, two structural isomer of pentagalloylglucose, four isomers of hexagalloylglucose, and tetragalloylglucose were isolated from *Quercus infectoria* (Nishizawa *et al.*, 1983). Hexagalloylglucose, which was isolated from methanol extract of this medicinal plant, significantly inhibited α-glycosidase such as sucrase, maltase, and isomaltase (Hwang *et al.*, 2000). Because of tannin is the main component in nutgalls, therefore Regerat and team hydrolyzed the tannin extract in 1989. They found that the hydrolysis process was complete in 22 to 30 h, and high yields of gallic acid were obtained (Regerat *et al.*, 1989). Ahmad and team (1991) have been reported three types of flavonoids from *Quercus infectoria* that included β-sitosterol, amentoflavone hexamethyl ether, and isocryptomerin (Ahmad *et al.*, 1991).

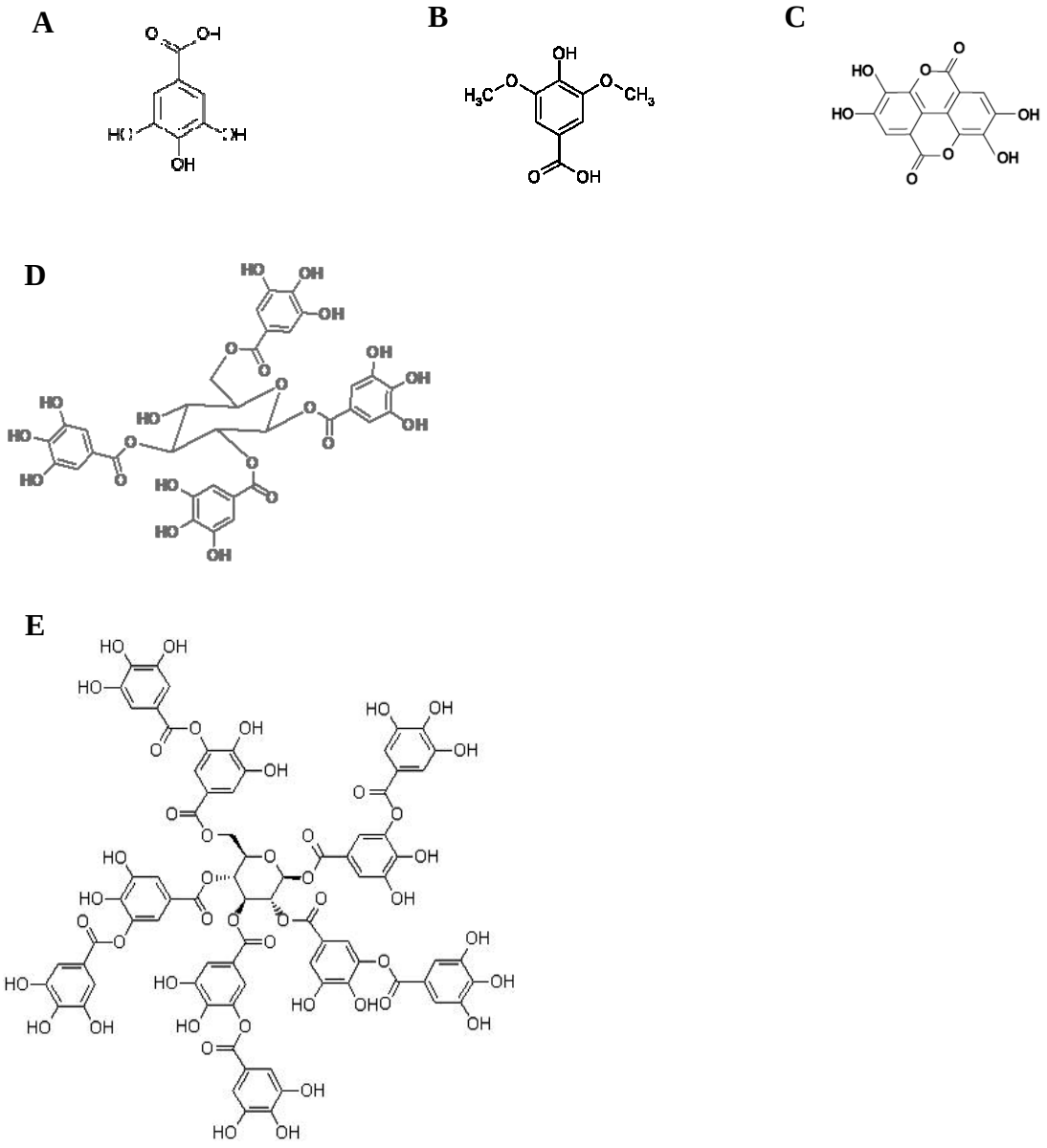

Figure 1.3 Some reported compounds from *Quercus infectoria* nutgall that include gallic acid (A), syringic acid (B), ellagic acid (C), ellagitannin (D), and tannic acid (E)

2.3.3 Therapeutic used

Because of an increasing of problem with infections that caused by antibiotic resistance bacteria, medicinal plants have been extensively studied as alternative treatments for the diseases. The nutgalls of *Quercus infectoria* have been studied the pharmacology since 1976. Two fractions were prepared, including a dried acetone-treated methanol extract dissolved in water (Fraction A) and a chloroform-methanol extraction (Fraction B). Fraction A was active as an analgesic in rats and significantly reduced blood sugar levels in rabbits. Fraction B had central nervous system depressant activity (Dar *et al.*, 1976). In 1977, the syringic acid was isolated from the nutgalls and showed the central nervous system depressant activity (Ikram and Nowshad, 1977).

A report in term of antibacterial activity of *Quercus infectoria* nutgalls was demonstrated in Jordan. Alkofahi *et al*. (1996) found that ethanol extract from this medicinal plant showed the strongest activity against *Pseudomonas mirabilis*, *Pseudomonas aeruginosa*, and *S. aureus* (Alkofahi *et al.*, 1996). Natural dye prepared from *Quercus infectoria* showed the maximum zone of inhibition thereby indicating best antimicrobial activity against *Escherichia coli*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Proteus vulgaris*, and *P. aeruginosa* (Singh *et al.*, 2005). *Quercus infectoria* was used in different tooth power (Farooqi *et al.*, 2001). Hwang *et al.* (2004) reported this plant showed the anticariogenic activity against *Streptococcus mutans*. In Thailand, recent reports were investigated the antibacterial activity of the medicinal plant against MRSA and enterohaemorrhagic *E. coli* O157: H7. An ethanol extract of the galls presented the antibacterial activity against all 35 clinical isolates of MRSA with MICs and MBCs were 0.2-0.4 and 0.4-1.6 mg/mL, respectively (Voravuthikunchai and Kitpipit, 2005). With Gram-negative pathogenic bacteria, both water and ethanol extracts of *Quercus infectoria* were highly effective against *E. coli* O157:H7 with the best MIC and MBC values of 0.09, 0.78, and 0.19, 0.39 mg/mL, respectively (Voravuthikunchai *et al.*, 2004; Voravuthikunchai and Limsuwan, 2006). This plant may provide alternative but bioactive medicine for the treatment of both Gram-positive and Gram-negative bacterial infections.

3. Objectives of the research

3.1 To determine the antibacterial activity of crude extract, partially purified fractions, and pure compounds from *Quercus infectoria* against MRSA and MSSA.

3.2 To examine possible mechanisms of actions of the crude extract, the partially purified fractions, and pure compounds on MRSA

3.3 To study effects of *Quercus infectoria* on staphylococcal colonization

3.4 To investigate the resistant modifying activity of *Quercus infectoria* extract and components against MRSA and multidrug-resistant *Acinetobacter baumannii*

3.5 To establish other biological activities of *Quercus infectoria* extract

CHAPTER 2

RESEARCH METHODOLOGY

1. Materials and equipments

1.1 Microorganisms

1.1.1 Methicillin-resistant *Staphylococcus aureus* (MRSA) NPRC R001-R047

1.1.2 Methicillin-susceptible *Staphylococcus aureus* (MSSA) NPRC S001-S050

1. 1.3 *Staphylococcus aureus* ATCC 25923

1.1.4 Multidrug-resistant *Acinetobacter baumannii* JVC 1053

1.1.5 *Acinetobacter baumannii* ATCC 19606

1.2 Antibiotic discs and antibiotics

1.2.1 Antibiotic discs (Oxoid, UK)

Ciprofloxacin 30 µg

Clindamycin 2 µg

Erythromycin 15 µg

Gentamicin 10 µg

Penicillin 1 µg

Oxacillin 10 µg

Teicoplanin 30 µg

Tetracycline 30 µg

Trimethoprim-sulfamethoxazole 1.25/23.75 µg

Vancomycin 30 µg

1.2.2 Antibiotics

Amoxicillin (Sigma-Aldrich, Switzerland)

Oxacillin (Sigma-Aldrich, Switzerland)

Penicillin G (General Drugs House Co., Ltd, Thailand)

Vancomycin (Eli Lilly, USA)

1.3 Media

1.3.1 MacConkey agar (Difco, France)

1.3.2 Mannitol salt agar (Difco, France)

1.3.3 Mueller-Hinton agar (Difco, France)

1.3.4 Mueller-Hinton broth (Difco, France)

1.3.5 Nutrient agar (Difco, France)

1.3.6 Tryptic soy agar (Difco, France)

1.3.7 Tryptic soy broth (Difco, France)

1.4 Chemicals

1.4.1 Acetone (Fisher Scientific, USA)

1.4.2 Casein (Sigma, USA)

1.4.3 Catechol (Sigma, USA)

1.4.4 Carbonyl cyanide *m*-chlorophenlyhydrazone (Sigma, USA)

1.4.5 Cinnamic acid (Sigma, USA)

1.4.6 *p*-Coumaric acid (Sigma, USA)

1.4.7 Dimethylsulfoxide (Merck, USA)

1.4.8 2,2-Diphenyl-1-picrylhydrazyl (Sigma, USA)

1.4.9 Ethidium bromide (Sigma, USA)

1.4.10 Ellagic acid (Sigma, USA)

1.4.11 Ethylenediaminetetraacetic acid (Fisher Scientific, USA)

1.4.12 95% Ethanol (Merck, Germany)

1.4.13 Ferric chloride (Merck, Germany)

1.4.14 Ferulic acid (Sigma, USA)

1.4.15 Folin-Ciocalteu’s phenol reagent

1.4.16 Gallic acid (Fluka, Spain)

1.4.17 Glycerol (Vidhyasom, Thailand)

1.4.18 Glucose (Merck, Germany)

1.4.19 Glutaraldehyde (Merck, Germany)

1.4.20 1-*N*-Phenylnaphthylamine (Sigma, USA)

1.4.21 Propylene oxide (Merck, Germany)

1.4.22 Pyronin Y (Sigma, USA)

1.4.23 Quercetin (Sigma, USA)

1.4.24 Rabbit plasma (Difco, France)

1.4.25 Reserpine (Sigma, USA)

1.4.26 Silica gel (Merck, Germany)

1.4.27 Sodium chloride (Merck, Germany)

1.4.28 Syringic acid (Sigma, China)

1.4.29 Tannic acid (Fluka, Switzerland)

1.4.30 Thin layer chromatography (Merck, Germany)

1.4.31 Tributyrine (Rosco Diagnostica, Denmark)

1.4.32 Toluene (Merck, Germany)

1.5 Equipments

1.5.1 Autoclave ES 315 (Tomy, USA)

1.5.2 Balance BP 210S (Sartorius, USA)

1.5.3 Black microtiter plates (BD Biosciences-Labware, USA)

1.5.4 Filter paper disc 6 mm (Whatman, Germany)

1.5.5 Fluorescence plate reader (Tecan, Germany)

1.5.6 Hot air oven T410340 (Binder,Germany)

1.5.7 Hot plate stirrer HP 3000 (Lab Companion, USA)

1.5.8 Incubator B 5100E (Heraeus,Germany)

1.5.9 Light microscope CX31RBSFA (Olympus, USA)

1.5.10 Micropipette 1-10 µL, 2-20 µL, 20-200 µL, 100-1000 µL (Eppendorf, Germany)

1.5.11 Microtiter plate reader Wallac 1420 Victor multilabel counter (Perkin-Elmer, Finland)

1.5.12 Microtiter plate 96 wells (Corning Life Sciences, USA)

1.5.13 Multichannel micropipette 20-200 µl (Thermo Scientific, USA)

1.5.15 pH Meter (Beckman, USA)

1.5.16 Scanning electron microscope 5800LV (JEOL, Japan)

1.5.17 Transmission electron microscope JEM 100 CX II (JEOL, Japan)

1.5.18 Ultramicrotome MTXL (RMC, USA)

1.5.20 UV/VIS spectrophotometer UV-1601 (Shimadzu, Japan)

1.5.21 Vernier caliper (Whale, China)

2. Methods

2.1 Preparation of *Quercus infectoria* extracts and their components

2.1.1 Preparation of crude extracts and fractionations

A voucher specimen of *Quercus infectoria* (nutgall) was deposited at the Herbarium of the Faculty of Pharmaceutical Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand. The nutgalls were washed with distilled water, cut into small pieces and crushed in a mechanical mortar. The plant powder was extracted (20%, w/v) with the solvents of increasing polarity beginning with acetone, ethyl acetate, 95% ethanol, and water at room temperature for 7 days. After filtration, the excess of solvent was removed with a rotatory evaporator kept at 60 °C until they were completely dry and stored in a sterile screw-capped bottle at -20 °C.

The ethanol extract with strong anti-MRSA activity was further partially purified as follows. The dried nutgall extract (10 g) was dissolved in 95% ethanol at concentration of 10% (w/v), and submitted to quick column chromatography on silica gel (column i.d., 5 cm). The column was eluted using an increasing polarity gradient (chloroform, ethyl acetate, 95% ethanol, and 30% ethanol). Each fraction (75 mL) was monitored by separating it by thin layer chromatography (TLC) on silica gel (60GF254 70-230 mesh) TLC aluminium sheets (layer thickness 0.2 mm) with chloroform: methanol: H_2O (6: 3.7: 0.3, v/v/v) as the mobile phase. After air drying, spots on the aluminium sheet were visualized with a UV light (200-400 nm). The fractions with similar TLC R_f values were combined and then concentrated to complete dryness. The nutgall extract yielded 97 fractions including a chloroform fraction (fractions 1-8), ethyl acetate fraction I (fractions 9-21, 2.26 g), ethyl acetate fraction II (fractions 22-33, 0.21 g), 95% ethanol (fractions 34-72, 0.52 g) and 30% ethanol (fractions 73-97, 0.66 g). The chloroform fraction was not further investigated because it inability to visualize under UV light and low yield for antibacterial activity assay.

Tannic acid, gallic acid, ellagic acid, and syringic acid were included as its main components. For bioassay, the extracts, the fractions**,** and the components were dissolved in 10% dimethylsulfoxide (DMSO) before use.

2.1.2 Phytochemical screening methods

For conducting the different chemical tests, both the ethanol extract and the ethyl acetate fraction I were dissolved in 45% ethanol (1: 1, w/v) and divided into a number of separate fractions. Phytochemical screening tests for alkaloids, cardiac glycosides, flavonoids, tannins, and triterpene were qualitatively analysed by standard colour tests according to previously described methods (Houghton and Raman, 1998; Ahmad and Beg, 2001; Woo, 2001). Dragendorff' s reagent and Mayer's reagent were used for alkaloids, Kedde's reagent and 2% potassium hydroxide in ethanol for cardiac glycosides, the Shinoda test for flavonoids, 5% ferric chloride and bromine water for hydrolysable and condensed tannins and the Liebermann-Burchard test for triterpenes.

2.2 The general characteristics of test strains and culture conditions

2.2.1 Test strains

Methicillin-resistant *S. aureus* (MRSA) NPRC R001-R047 including 29, 8, 6, and 4 isolates were isolated from sputum, blood, pus, and urine, respectively. All strains were isolated from patients at Hat Yai hospital and Songklanagarind hospital, Hat Yai, Thailand. Methicillin-susceptible *S. aureus* (MSSA) NPRC S001-S050 were isolated from healthy people in Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai, Thailand. The bacteria were grown on tryptic soy agar (TSA) at 37 °C for 18-24 h. Antibacterial susceptibility patterns of both MRSA and MSSA isolates were conducted by disc diffusion method (CLSI, 2009).

2.2.2 Virulent factors

Both MRSA and MSSA isolates were screened for the production of several virulent-factors including haemolysin, lipase, and proteases. The bacteria were prepared by subculturing on TSA and incubating overnight at 37 °C. Well-isolated colonies were selected and patched on the surface of each modified TSA plates. The plate was prepared by adding 2% (w/v) casein, 5% (v/v) blood, and 1% (v/v) tributyrine to TSA for the detection of proteases, haemolysin, and lipase productions, respectively. After incubation for 48 h, the digested substrates formed

clear area adjacent to the bacterial growth. The diameter ratio of clear zones and colonies was calculated as the degree of hydrolysis. For coagulase production, the bacteria were grown separately in tryptic soy broth (TSB) for 24 h and then adjusted to $5x10^9$ CFU/mL (McFarland standard No. 5). Bacterial suspensions were added into 500 µL of rabbit plasma (1: 1, v/v). A positive result is denoted by fibrin clot formation in the test tube after incubation at 37°C for 4 and 24 h.

2.2.3 Biofilm formation

Microtiter plate method was carried out according to the protocol of Karaolis and team with a modification (Karaolis *et al.*, 2005). Briefly, an overnight culture grown in TSB supplemented with 0.25% glucose (GTSB) was adjusted to McFarland standard No. 2 ($6x10^8$ CFU/mL). Two hundred µL of bacterial suspension in GTSB, approximately $5x10^5$ CFU/mL, was transferred to 96-well microtiter plate and incubated at 37 °C for 24 h. GTSB (200 µL) was used as negative control. After incubation, the broth was discarded from each well and the wells were then washed twice with phosphate buffer saline (PBS) to remove non-adherent cells and air-dried for 30 min. The wells were stained with 200 µL 0.1% crystal violet for 30 min. After that, the dye was removed and the test plate was rinsed with PBS. The air-dried plates were added with 200 µL of DMSO and the OD at 570 nm was measured by microtiter plate reader.

All strains were classified the cut-off OD (ODc) for the microtiter plate test as three standard deviations above the mean OD of the negative control. The MRSA and MSSA isolates were categorized as follows (Christensen *et al.*, 1985):

$OD \leq ODc$	non-adherent
$ODc < OD \leq 2ODc$	weakly adherent
$2ODc < OD \leq 4ODc$	moderately adherent
$4ODc < OD$	strongly adherent

2.2.4 Cell surface hydrophobicity (CSH)

Cell hydrophobicity was measured by bacterial adherence to hydrocarbon (BATH) according to the previous method (Rosenberg *et al.*, 1986;

Nostro *et al.*, 2004). Briefly, the bacterial suspension (100 µL), approximately $5x10^6$ CFU/ mL, were grown in 900 µL TSB at 37 °C for 18 h and the cells were collected by centrifugation at 6,000 rpm for 5 min. The pellets were washed twice and then resuspended in 0.85% (w/v) sodium chloride to determine an initial optical density (OD) of the cell suspension at 600 nm. The cell suspension (3 mL) after the addition of toluene (0.25 mL) was agitated uniformly by using a vortex mixer for 2 min and allowed to equilibrate at room temperature for 30 min. After toluene phase was separated from the aqueous phase, the OD of the aqueous phase was then determined at 600 nm. The hydrophobicity index (HPBI) was calculated as: (OD initial-OD final/OD initial) x 100%. Low hydrophobic isolates was defined as a HPBI index >0<25 and high hydrophobic isolates was classified as a HPBI index ≥25 (Akiyama *et al.*, 1998).

2.3 Antibacterial activities of *Quercus infectoria*

2.3.1 Disc diffusion method

The paper disc agar diffusion method (CLSI, 2009) was used for primary screening of antibacterial activity of the extracts. Ten microlitres of crude extracts (250 mg/mL) were applied to sterile filter paper discs (Whatman no. 1; 6 mm in diameter). Dry discs (dried at 37 °C overnight) were applied to the surface of MHA plates seeded with 5 h broth culture of the tested bacteria. The plates were then incubated for 18 h at 37 °C. Antibiotic susceptibility discs including amikacin, erythromycin, oxacillin, and vancomycin were used as controls. The antibacterial activity was evaluated by measuring the diameter of inhibition zones. The experiment was performed in duplicate and the means of inhibition zone diameters were calculated.

2.3.2 Broth microdilution method

A modified broth microdilution method (CLSI, 2009) was used to obtain the MICs and MBCs of *Quercus infectoria* extracts. Vancomycin (0.8-25.0 µg/mL) was used as reference standards. Twenty microlitres of a 3-5 h culture of each bacterial strain, containing approximately $5x10^4$ CFU/mL, were applied into MHB supplemented with the medicinal plant extracts at concentrations ranging from

0.03-1.00 mg/mL. The microtiter plates were incubated at 37 °C for 18 h. Minimum inhibitory concentrations were observed at least in duplicate as the lowest concentration of plant extracts that produce a complete suppression of bacterial growth. Minimum bactericidal concentrations were performed with the extracts that gave significant MIC values by sub-culturing on fresh MHA.

2.3.3 Time kill assay

Bactericidal activity of the plant extracts was studied using the time-kill assay (CLSI, 1999). One hundred microlitres of bacterial culture ($5x10^5$ CFU/mL) were added to 0.9 mL of MHB containing 100 µL of plant extract at MIC, two times MIC, and four times MIC. The tubes were incubated at 37 °C with shaking. The samples were collected every two hour intervals until 20 h, control tubes without extract were incubated under the same conditions. Surviving bacteria were cultured on TSA and incubated under the same condition for 24 h. All assays were carried out in duplicate.

2.4 The anti-MRSA mechanisms of *Quercus infectoria* extract and its components

2.4.1 Bacteriolytic assay

Since the ethyl acetate fraction I gave anti-MRSA activity and the highest percentage yield, therefore it was performed in further set of experiments. The whole-cell lysis activity of the crude extract and its fraction were compared with the commercial compounds (Carson *et al.*, 2002). The bacterial suspensions ($3x10^8$ CFU/mL) were exposed to ethanol extract, ethyl acetate fraction I, gallic acid, and tannic acid at concentrations equivalent to 4MIC, 2MIC, and MIC (10: 1, v/v). The OD at 620 nm was measured immediately at 0, 2, 6, 12, and 24 h after incubation. Each concentration of the extract and the components in 0.85% sodium chloride were used as negative controls. The experiment was performed in duplicate. The results were express as a ratio of the OD at 620 nm at each time interval versus the OD at 620 nm at 0 h (in percent).

2.4.2 Effects on the tolerance of MRSA to low osmotic pressure

To assess the tolerance of the bacterial cells to low osmotic pressure, twenty microlitres of the suspensions ($1.5x10^8$ CFU/mL) were incubated in sterile

distillation water containing the ethanol extract, ethyl acetate fraction I, tannic acid, and gallic acid at 1/2MIC, MIC, and 2MIC at 37 °C for 0, 2, 6, 12, and 24 h. The viability was confirmed by culturing the cells on TSA plates for additional 48 h.

2.4.3 Effects on the salt tolerance of MRSA

The ability of *S. aureus* cells after treated with *Quercus infectoria* extract and its components to grow on TSA and TSA supplemented with NaCl was investigated. Suspensions of bacteria ($1.5x10^8$ CFU/mL) were prepared as described above and treated with ethanol extract, ethyl acetate fraction I, gallic acid, and tannic acid at 1/2MIC, MIC, and 2MIC. Samples were removed and incubated onto TSA, TSA supplemented with 5% NaCl, and TSA supplemented with 7.5% NaCl. The numbers of CFU per milliliter on each TSA-NaCl and TSA plates were evaluated after incubated at 37 °C for 48 h.

2.4.4. Effect of the *Quercus infectoria* extract on cell membranes of MRSA

A bacterial suspension of $1.5x10^9$ CFU/mL in 0.85% NaCl was added into 100 μL of the ethanol extract, the ethyl acetate fraction I, gallic acid, and tannic acid (10: 1, v/v). The bacterial suspension added to 10% DMSO was used as a positive control. The cell pellets were separated by centrifugation at 10,000 rpm for 5 min, after incubation at 37 °C for 0, 4, 8, 12, 16, and 20 h (Oonmetta-aree *et al.*, 2006). Nucleotides and their component structures, amino acids, and inorganic ions present in the supernatant were reported as the low molecular weight metabolites, leaking from the bacterial cells. The physical properties of nucleic acid result in the absorption of light in the UV range (260 nm) of the light spectrum. The levels of purines, pyrimidines, and their derivatives in the supernatant were determined at each time interval by measuring the OD at 260 nm, using a UV/VIS spectrophotometer according to the method described earlier (O'Neill *et al.*, 2004). Each concentration of the extract and its components without the bacterial suspension were used as blanks. For accurate results, absorbance should be in the range of 0.05-0.10, which for a 1.0 mL assay requires 2.5-5.0 μg of DNA. The OD at 260 nm at each time point was expressed as a proportion of the initial OD at 260 nm. Mean ratios for each treatment agent and time were calculated and compared with the means for the corresponding untreated samples (Carson *et al.*, 2002).

The formula for converting absorbance to nucleic acid concentration is 1 OD at 260 nm = 50 μg/mL for DNA; 40 mg/mL for RNA and 33.3 μg/mL for oligonucleotides (Sambrook and Russell, 2001).

2.4.5 The effect of the extract and its component on cell morphology

The bacteria was cultured in TSB at 37 °C for 5 h and then 800 μL of the bacterial suspension was transferred into TSB supplemented with the ethanol extract and tannic acid. The suspensions were incubated at 37 °C for 8 h. Bacterial cells were collected by centrifugation at 6,000 rpm for 10 min. They were fixed in 2.5% (w/v) of glutaraldehyde at 4 °C for 2 h. For scanning electron microscopy (SEM), the bacterial cells fixed in glutaraldehyde were isolated on millipore filters and post-fixed with osmium tetraoxide. Cells were dehydrated by passage through graded acetone/water mixtures and treated with tetramethylsilane. The air-dried cells were coated with gold and examined using a SEM. For transmission electron microscopy (TEM), the fixed cells were washed three times with 0.1 mol/L phosphate buffer solution, pH 7.2 and post-fixed with 1% (w/v) osmium tetraoxide at room temperature overnight. The pellets were then dehydrated through serial concentrations of ethanol (70, 80, 90, and 100 mL in 100 mL distilled water, respectively). They were infiltrated in a propylene oxide for 15 min twice, propylene oxide: Epon 812 resin, 1: 1 for 30 min, followed by Epon 812 resin for 60 min and polymerized in a hot air oven at 80 °C for 10-12 h. The polymerized samples were sliced with an ultramicrotome and observed using a TEM.

2.5 Effect *Quercus infectoria* extract and its components on staphylococcal colonization

2.5.1 Effect of *Quercus infectoria* and tannic acid on the biofilm formation of MRSA and MSSA

The biofilm formation of the representative MRSA and MSSA isolates after treated with the ethanol extract and tannic acid were investigated by the microtiter plate method. Overnight cultures were prepared by inoculating 3-5 colonies into GTSB and incubated at 37 °C for 18 h. Cultures were suspended in 0.85% NaCl to the opacity of a 0.5 McFarland turbidity standard and then diluted 1:

30 (v/v) with GTSB to obtain an initial concentration of approximately $5x10^6$ CFU/mL. Two-fold serial dilutions of the extract and tannic acid were prepared in a 96-well microtiter plate in GTSB to get final concentrations of the test compounds ranged from MIC-1/8MIC. One hundred and eighty µL of the bacterial suspension was transferred to 96-well microtiter plate and the test plate was then incubated at 37 °C for 24 h. After incubation, the changes in biofilm forming ability of treated cells were examined by the method as described above.

2.5.2 Modification of staphylococcal-CSH by the extract and tannic acid

The properties of cell surface of the representative low hydrophobic and high hydrophobic isolates after treated with the ethanol extract and tannic acid were investigated by BATH test (Nostro *et al.*, 2004). The cell suspension ($1.5x10^9$ CFU/mL) was incubated into 0.85% (w/v) NaCl supplemented with the extract and tannic acid at 4MIC, 2MIC, MIC, 1/2MIC, 1/4MIC, and 1/8MIC at 37 °C for 24 h (10:1, v/v). The changes of cell-surface hydrophobicity of treated cells were observed by the method as described above.

2.6 Other biological activities of *Quercus infectoria*

Some additional biological activities of the ethanol extract from *Quercus infectoria* such as anti-viral, anti-*Mycobacterium tuberculosis*, anti-malarial, anti-cancer, anti-oxidant, and anti-inflammation activities were evaluated using various methods as describe in **Table 2.1.**

Table 2.1 Methods for determining the biological activities of ethanol extract of *Quercus infectoria*

Test biological activities	Methods	Positive control	Negative control
Anti-microbial			
Anti-HSV-1 Herpes simplex virus type 1	Sulforhodamine B assay	Acyclovir	0.5% DMSO
Anti-*Mycobacterium tuberculosis*	Resazurin microplate assay	Rifampicin/Kanamycin/ Isoniazid	0.5% DMSO
Anti-*Plasmodium falciparum* K1	Microculture Radioisotope Technique	Dihydroartemisinine	0.1% DMSO
Anti-cancer			
Anti-KB-Oral human epidermal carcinoma	Sulforhodamine B assay	Ellipticine/Doxorubicin	0.5% DMSO
Anti-MCF7-Breast cancer	Sulforhodamine B assay	Ellipticine/Doxorubicin	0.5% DMSO
Anti-NCI-H187-Human lung cancer	MTT assay	Ellipticine/Doxorubicin	0.5% DMSO
Free radical scavenging	1, 1-diphenyl-2-picrylhydrazyl radicals	BHT	1% DMSO
	Hydroxyl radical	Tannin	1% DMSO
	Superoxide dismutase	Trolox	1% DMSO
Anti-inflammation			
Anti-COX-1/Anti-COX-2	Radioimmunoassay	Aspirin	0.1% DMSO

2.7 *Quercus infectoria* extract: a source of resistant modifying agents for multidrug-resistant Gram-positive and Gram-negative bacteria

2.7.1 Effect of *Quercus infectoria* extract and its components in combination with antibiotics against MRSA

Representative β-lactam antibiotics including amoxicillin, oxacillin, and penicillin G were dissolved in 10% DMSO and used as control antibiotics. The β-lactam antibiotics were serially diluted twofold with 10% DMSO to obtain solutions of 512-0.06, 640-0.06, and 256-0.02 μg/mL for amoxicillin, oxacillin, and penicillin G, respectively. Their MIC values were measured as described above. The combined effects of the ethanol extract, the ethyl acetate fraction I, gallic acid, and tannic acid with these β-lactam antibiotics were evaluated by the checkerboard method to obtain the fractional inhibitory concentration (FIC) index (Sato *et al.*, 2004). The checkerboard consists of columns in which each well contains the same amount of antimicrobial agents diluted twofold along the *x*-axis, and rows in which each well contains the same amount of the plant extract and its components diluted twofold along the *y*-axis on a 96-well plate. The FIC index was calculated according to the equation: FIC index = $FIC_A + FIC_B$ = (MIC of drug A in combination/MIC of drug A alone) + (MIC of drug B in combination/MIC of drug B alone). Synergism was defined as an FIC index □ 0.5, the additive effect as an FIC index of 0.5-2.0, and antagonism as an FIC index □2.0 (Eliopoulos and Moellering, 1996).

2.7.2 Ellagic acid: a resistant modifying agent for multidrug-resistant *Acinetobacter baumannii*

Plant-derived phenolic compounds

Plant-derived compounds including catechol, cinnamic acid, *p*-coumaric acid, ellagic acid, ferulic acid, gallic acid, quercetin, tannic acid, and syringic acid were purchased from Sigma-Aldrich, ellagitannin was isolated from *Quercus infectoria* extract and kindly provided by Dr. B.E. Yingyongnarongkul (Ramkhamhaeng University, Bangkok, Thailand). All test compounds were dissolved in DMSO to a concentration of 10 mM.

Carbonyl cyanide *m*-chlorophenlyhydrazone (CCCP), reserpine, ethidium bromide (EtBr), 1-*N*-phenylnaphthylamine (NPN), and pyronin Y were obtained from Sigma-Aldrich and EDTA from Fisher Scientific.

Bacterial strains and antibacterial assay

Clinical isolate MDR *A. baumannii* JVC1053 was kindly provided by Dr. D. Roscoe (Vancouver General Hospital, BC, Canada). This strain is resistant to levofloxacin, aztreonam, ciprofloxacin, and trimethoprim/sulfamethoxazole but susceptible to amikacin and ceftazidime. *A. baumannii* ATCC19606 was included in this study as an antibiotic susceptible, non-MDR reference strain. MIC was determined by serial dilution of test compounds in MHB with bacteria at a density of $5x10^5$ CFU/mL. Plates were incubated at 37 °C for 18 h and growth was assayed with a microtiter plate reader, monitoring the OD at 595 nm.

Primary Screening for antibiotic adjuvants

The bacterial culture was inoculated at 10^6 CFU/mL into 96-well microtiter plate containing novobiocin at a concentration of one-fourth the MIC (8 μg/mL), either alone or with 40 μM of the test compound. The extent of growth inhibition was determined by comparison of the OD at 595 nm of the test cultures to those of controls after 18 h of incubation at 37 °C.

Effect of antibiotic adjuvants on antibiotic susceptibility patterns of A. baumannii

The susceptibility of bacterial cultures to a panel of selected antibiotics including aminoglycosides (neomycin, amikacin, tobramycin, and gentamicin), β-lactams (ampicillin and imipenem), fusidic acid, macrolides (erythromycin and azithromycin), rifampicin, tetracycline, and aminocoumarins was conducted on MHA with or without 40 μM of ellagic and tannic acids supplementation. Antibiotic susceptibility test discs were purchased from Becton Dickinson Microbiology Systems, Difco, or from the laboratory collection of antibiotics. Susceptibility was determined on MHA supplemented with efflux pump inhibitors, CCCP and reserpine and a permeabilizer, EDTA. The diameters of inhibition zones were measured after incubation of the plates at 37 °C for 18 h and compared to the inhibition zones on MHA alone.

Adjuvant activity of ellagic and tannic acids with antibiotics

MIC values and growth inhibition assays of aminocoumarins (novobiocin, chlorobiocin, and coumermycin), fusidic acid, rifampicin, and tetracycline in the presence of ellagic or tannic acids were established in MHB in the presence of 10-40 µM of ellagic and tannic acids by broth microdilution; six concentrations of each antibiotic ranging from 1MIC-1/32MIC were tested. Adjuvant (50 µL) was added to 50 µL of various concentrations of the test antibiotics. Bacterial suspensions at an OD at 600 nm of 0.4 corresponding to 10^8 CFU/mL were diluted and a final inoculum of 10^6 CFU/mL in MHB (100 µL) was then added into microtiter plate. The plates were incubated for 18 h at 37 °C and growth observed using the microtiter plate reader. Growth studies were performed as described above. Samples were taken every hour for 8 h.

Permeabilization of the outer membrane

NPN fluorescence is associated with the presence of this hydrophobic probe in a glycerophospholipid environment such as the lipid bilayers of biological membranes; increased fluorescence values indicate weakening of the outer membrane. NPN uptake by bacterial suspensions was measured using black microtiter plates in a Wallac 1420 Victor Multilabel counter (excitation 355 nm, emission 460 nm) (Nohynek *et al.*, 2006). The bacterial cultures (OD at 600 nm ≈ 0.5) were collected by centrifugation at 3,500 rpm for 10 min and resuspended in an equal volume of 5 mM HEPES buffer (pH 7.2). Aliquots (100 µL) of this cell suspension were added to the microtiter plate, containing 50 µL of ellagic acid (40 µM), tannic acid (40 µM), EDTA (1 mM and 0.25 mM), or HEPES buffer (control). NPN (40 µM) was then added immediately to a total volume of 200 µL. Fluorescence was monitored within 3 min and continuously recorded every 10 min for 3 h. Each assay was performed at least three times.

Ethidium bromide and Pyronin Y efflux

The inhibition of bacterial efflux pumps was established by measurement of the level of EtBr and pyronin Y accumulation using the method described previously (Kern *et al.*, 2006; Schumacher *et al.*, 2006). Overnight cultures of *A. baumannii* JVC 1053 were diluted in PBS + 0.4% glucose (pH 7.4) and incubated to OD at 600 nm of 0.5. The suspension was transferred to a 96-well plate and pretreated with ellagic acid, CCCP, or 1% DMSO for 5 h. EtBr was added to a final concentration of 4 mg/L, and the relative fluorescence intensity was measured in a Safire fluorescence plate reader (excitation 518 nm, emission 605 nm). A similar assay was performed with pyronin Y (excitation 545 nm, emission 570 nm).

2.8 Statistical analysis

Data were subjected to analysis of variance (ANOVA). Values were expressed as mean±SD and mean±SE. A Duncan-ANOVA test was used to compare the parameters between the groups and a Dunnett-ANOVA test to compare between the tests and control.

CHAPTER 3
RESULTS

1. *Quercus infectoria* extracts

The percentage yields of the extract from the various solvents used are presented in **Table 3.1**. The highest extract yield was obtained from 95% ethanol extract, followed by water, acetone, and ethyl acetate extracts. The 95% ethanol extract was further separated by column chromatography using silica gel 60GF254 eluted with chloroform, ethyl acetate, 95% ethanol, and 30% ethanol. The percentage yields of the partially-purified fractions from the ethanol extract are summarized in **Table 3.1**. The highest fraction yield was achieved from the ethyl acetate fraction I, followed by 30% ethanol, 95% ethanol, and ethyl acetate fraction II fractions.

Preliminary phytochemical analysis of the ethanol extract revealed the presence of flavonoids, tannins, and steroidal compounds as has been previously reported (Ahmad and Beg, 2001; Pithayanukul *et al.*, 2005; Kaur *et al.*, 2008). Tannins were detected in the ethyl acetate fraction I.

Table 3.1 Percentage yield of various crude extracts of *Quercus infectoria* and partially-purified fractions from the ethanol extract

Quercus infectoria extracts or fractions	Percentage yield
Crude extracts	
Acetone	18.9
Ethyl acetate	8.0
95% Ethanol	57.2
Water	24.1
Partially-purified fractions from 95% ethanol extract	
Ethyl acetate fraction I	22.6
Ethyl acetate fraction II	2.1
95% Ethanol	5.2
30% Ethanol	6.6

2. The general characteristics of *Staphylococcus aureus*

2.1 Antibacterial susceptibility patterns of the test isolates

The antibacterial susceptibility patterns of both MRSA and MSSA are shown in **Table 3.2**. Most MRSA isolates (>70%) are resistant to the aminoglycoside (gentamicin), the β-lactam antibiotics (oxacillin and penicillin), the macrolide (erythromycin), quinolone (ciprofloxacin), tetracycline, clindamycin, and co-trimoxazole. However, the test isolates are still susceptible to the glycopeptides (teicoplanin and vancomycin). With the exception of penicillin, most of MSSA isolates are susceptible to all test antibiotics.

2.2 Virulence factors

For coagulase production, all MRSA isolates exhibited a complete fibrin formation within 4 h but only 32 isolates (64%) of the test MSSA demonstrated this activity. Positive haemolysin in MSSA isolates were more frequently observed than those from infective origin ($P<0.05$). Furthermore, MSSA isolates significantly evinced higher degree of hydrolysis of all extracellular enzymes, compared with the infective origin isolates ($P<0.05$) (**Table 3.3**).

2.3 Biofilm formation

Table 3.4 presents the results of the biofilm formation of MRSA isolates from infective origins and MSSA isolates from carriers at 24 hours. There was a negative correlation (R^2=0.093 and 0.001 for MRSA and MSSA, respectively) between the adherence ability and the cell surface properties of the test isolates. Most of the test isolates (>70%) were classified as moderate adherence strains. About 10% of MRSA isolates were categorized as strong adherent, while only 4% of MSSA isolates were found with this ability.

2.4 Cell surface hydrophobicity

The cell surface properties of both MRSA and MSSA isolates are demonstrated in **Table 3.5**. In the BATH test, the HPBI of MRSA isolates were significantly lower than MSSA isolates ($P<0.05$). Most of MRSA isolates were classified as low hydrophobic strains (HPBI index <25), while 88% of MSSA isolates were described as high hydrophobic strains (HPBI index ≥25). A reference strain, *S. aureus* ATCC 25923 was identified as a high hydrophobic strain.

Table 3.2 Antibacterial susceptibility patterns of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *Staphylococcus aureus* (MSSA) isolates

Antibiotics (μg/disc)	Percentage of resistant isolates		
	MRSA (n=47)	MSSA (n=50)	*S. aureus* [a] ATCC 25923
Aminoglycosides			
Gentamicin (10)	74	0	23.00±2.83
β-lactam penicillins			
Oxacillin (1)	100	0	27.50±0.71
Penicillin (10)	100	86	44.00±2.83
Glycopeptides			
Teicoplanin (30)	0	0	19.50±0.71
Vancomycin (30)	0	0	20.50±0.71
Macrolides			
Erythromycin (15)	100	6	28.00±2.83
Quinolones			
Ciprofloxacin (5)	98	2	30.50±0.71
Tetracyclines			
Tetracycline (30)	98	20	29.50±4.95
Others			
Clindamycin (2)	74	10	26.00±5.66
Co-trimoxazole (25)	100	2	30.00±1.41

[a] Mean values±SE of duplicate results of inhibition zones

Table 3.3 Comparison of extracellular enzyme production by methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *Staphylococcus aureus* (MSSA) isolates

Extracellular enzymes	Percentage of positive isolates (Mean values of degree of hydrolysis±SE)		
	MRSA (n=47)	MSSA (n=50)	*S. aureus* ATCC 25923
Coagulase [a]	100	64*	
Haemolysin	61 (1.15±0.23)	90* (2.63±0.69) **	2.77±0.06
Lipase	97 (1.89±0.36)	96 (2.60±0.50) **	2.53±0.10
Protease	93 (2.73±0.94)	88 (4.11±0.78) **	4.65±0.05

[a] Formation of fibrin clot in positive isolates was observed within 4 h

* Percentage of positive isolates are significantly different from MRSA isolates (P<0.05)

** Mean values are significantly different from MRSA isolates (P<0.05)

Table 3.4 Biofilm formation of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *Staphylococcus aureus* (MSSA) isolates (modified microtiter-plate method)

Test isolates [a]	Percentage of isolates showing;			
	No adherence	Weak adherence	Moderate adherence	Strong adherence
MRSA (n=47)	-	15	74	11
MSSA (n=50)	-	18	78	4

[a] *S. aureus* ATCC 25923 was identified as strongly adherent

Table 3.5 Comparison of cell surface hydrophobicity of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *Staphylococcus aureus* (MSSA) isolates

Hydrophobicity index (HPBI)	Percentage of tested isolates		
	MRSA (n=47)	MSSA (n=50)	*S. aureus* ATCC 25923
Low hydrophobicity ($0 \leq HPBI < 25$)	79	12	
High hydrophobicity ($25 \leq HPBI \leq 100$)	21	88	
Mean values±SE	15.78±14.04	55.26±19.41*	81.44±2.89

* Mean values are significantly different from MRSA isolates ($P<0.05$)

3. Antibacterial activity of *Quercus infectoria*

3.1 Anti-staphylococcal activity of *Quercus infectoria* extracts by disc diffusion

In this study, acetone, ethyl acetate, ethanol, and water extracts from *Quercus infectoria* demonstrated significant antibacterial activities against all MRSA and MSSA strains (**Table 3.6**). The results showed similar activities among all strains of MRSA, MSSA, as well as *S. aureus* ATCC 25923, with inhibition zones ranged from 11.75-16.82 mm. Since the water extract did not dissolve well in 10% DMSO, only the acetone, ethyl acetate, and ethanol extracts were performed in further set of experiments.

3.2 Anti-staphylococcal activity of the extracts, partially-purified fractions, and pure compounds by broth microdilution method

Table 3.7 shows the anti-staphylococcal activity of the acetone, ethyl acetate, and ethanol extracts. Significant antibacterial effects, expressed as MIC and MBC of the crude extracts against MRSA, MSSA isolates, and *S. aureus* ATCC 25923, were in the range of 0.13-1.00 mg/mL. Comparisons of the MIC and MBC values of the acetone, ethyl acetate, and ethanol extracts showed no difference.

As indicated in **Table 3.8**, with the exception of ellagic acid and syringic acid, the ethanol extract, the partially purified fractions, and the commercial pure compounds possessed anti-MRSA activity. Comparison of the MIC values of these compounds demonstrated that their MICs were very similar, ranging from 0.06-0.13 mg/mL, with the exception of the 95% ethanol fraction and 30% ethanol fraction that exhibited slightly lower anti-MRSA activity, with the MIC ranges from 0.25-0.50 mg/mL.

3.3 Time kill assay

Time kill curves are examples of bactericidal activity expressed as the rate of killing by a fixed concentration of an antimicrobial agent and are one of the most reliable methods for determining tolerance. The studies were performed over a

period of 20 h with bacteria being exposed to the MIC, two times the MIC, and four times the MIC of the ethanol extract. The results of the time kill curves for two representative MRSA isolates (NPRC R004 and NPRC R009), and a reference strain are presented in **Figure 3.1**. At the MIC concentration, a number of MRSA and reference strains declined significantly (3 Log(CFU/mL)) after 6 h. The clinical isolates showed bacterial re-growth after 8 h while *S. aureus* ATCC 25923 was still inhibited at 20 h. At 2MIC, all strains decreased approximately 3-4 Log(CFU/mL) and remain suppressed until 20 h. At 4MIC, the ethanol extract exhibited complete eradication of NPRC R004 and R009 within 12-14 h, and 16 h for *S. aureus* ATCC 25923. The time kill curves of the extract showed a clear relationship between the extent of inhibition and the concentration of *Quercus infectoria*. Both the MIC values depicted in **Table 3.7** and **3.8** and the time kill curves described in **Figure 3.1** confirmed the potency of the ethanol extract from nut galls to inhibit *S. aureus* and MRSA isolates.

Table 3.6 Antibacterial activity of the crude extracts of *Quercus infectoria* against methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *Staphylococcus aureus* (MSSA)

Quercus infectoria extracts	Mean values±SE (mm)		
(2.5 mg/disc)	MRSA	MSSA	*S. aureus*
	(n=17)	(n=33)	ATCC 25923
Acetone	16.59±0.23	14.61±1.09	15.50±0.50 [a]
Ethyl acetate	16.47±0.19	14.54±0.88	15.25±0.25
95% Ethanol	16.47±0.27	14.68±0.72	15.25±0.75
Water	13.64±0.19	13.25±1.80	11.75±0.75

[a] Mean values±SE of duplicate results

Table 3.7 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the crude extracts from *Quercus infectoria* against methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *Staphylococcus aureus* (MSSA)

Quercus infectoria extracts	MIC/MBC (mg/mL)		
	MRSA (n=17)	MSSA (n=33)	*S. aureus* ATCC 25923
Acetone	0.13/0.13-1.00	0.13-0.25/0.13-1.00	0.13/0.50
Ethyl acetate	0.13/0.13-1.00	0.13-0.25/0.13-1.00	0.13/0.50
95% Ethanol	0.13/0.13-0.50	0.13-0.25/0.13-0.50	0.13/0.50
Vancomycin (μg/mL)	1.25/1.25	0.60/1.25	0.60/1.25

Table 3.8 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of partially-purified fractions from the 95% ethanol extract of *Quercus infectoria* and its components against methicillin-resistant *Staphylococcus aureus* (MRSA)

Quercus infectoria components	MIC/MBC (mg/mL)	
	MRSA (n=17)	*S. aureus* ATCC 25923
Ethyl acetate fraction I	0.13/0.50	0.13/0.25
Ethyl acetate fraction II	0.06/0.25	0.13/0.50
95% Ethanol fraction	0.25/0.50	0.50/0.50
30% Ethanol fraction	0.50/1.00	0.50/>1.00
Ellagic acid	>1.00/NA[a]	>1.00/NA
Gallic acid	0.06/0.06	0.06/0.06
Syringic acid	>1.00/NA	>1.00/NA
Tannic acid	0.13/0.25	0.13/0.50
Vancomycin (µg/mL)	1.25/1.25	0.60/1.25

[a] NA=Not applicable

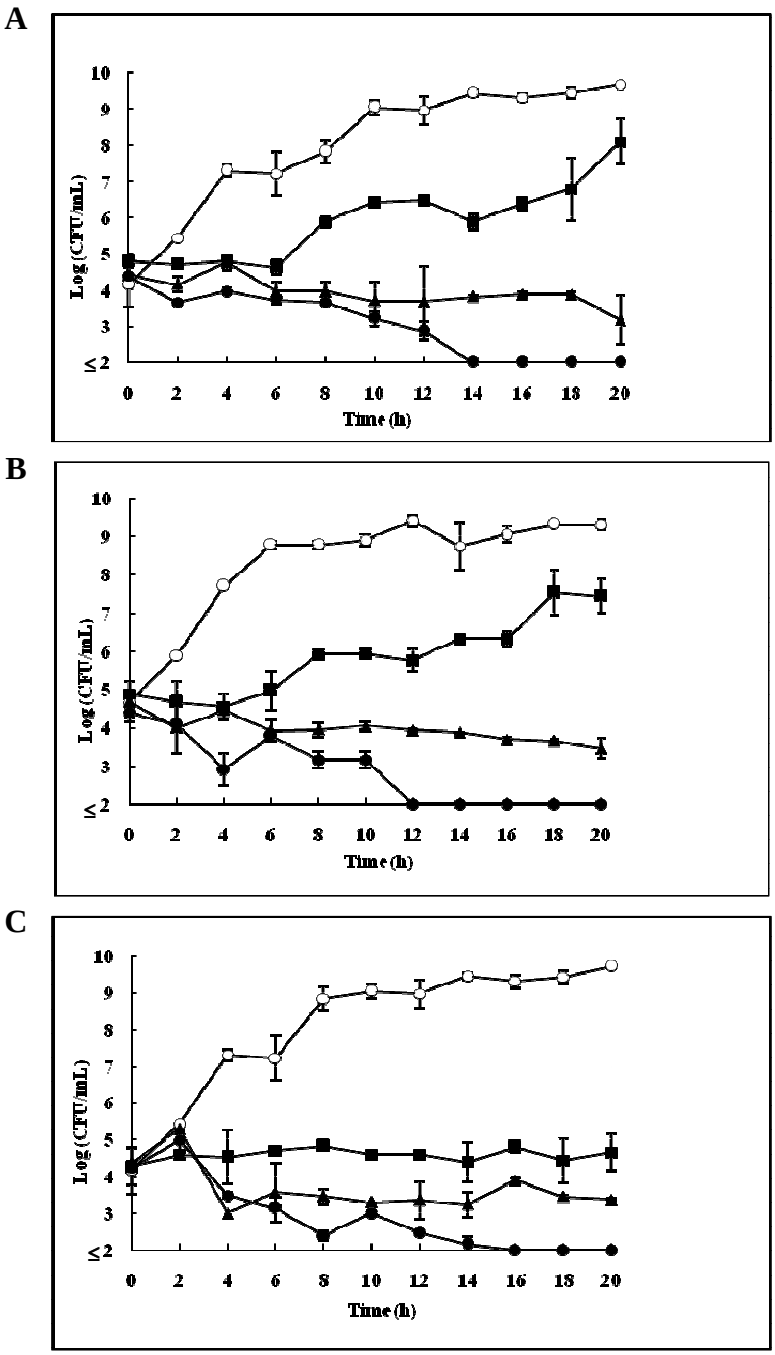

Figure 3.1 Time kill curves of methicillin-resistant *Staphylococcus aureus* NPRC R004 (A), NPRC R009 (B), and *Staphylococcus aureus* ATCC 25923 (C) after treatment with ethanol extract at MIC (■), 2MIC (▲), and 4MIC (●). 1% DMSO (o) was used as control. Means±SEs for duplicates are shown. The lowest detection threshold was 10^2 CFU/mL

4. Mechanism of actions of *Quercus infectoria* extract and its components as anti-MRSA agents

4.1 Bacteriolytic activity

Reduction of the OD at 620 nm of MRSA cell suspensions exposed to the crude extract, ethyl acetate fraction I, gallic acid, and tannic acid were shown in **Figure 3.2**. Treatment of the organisms with the plant extracts and the components at MIC, 2MIC, and 4MIC did not significantly reduce the OD at 620 nm up to 24 h. Similar results were obtained with the reference methicillin-susceptible strain, *S. aureus* ATCC 25923 as demonstrated in **Figure 3.3**. These results show that *Quercus infectoria* extract and its components failed to cause lysis in any of the bacterial strains tested.

4.2 Loss of the tolerance of MRSA cells to low osmotic pressure

In the absence of the plant extract, the fraction, and tannic acid, MRSA NPRC R001 and *S. aureus* ATCC 25923 were tolerant to low osmotic pressure. The water containing the ethanol extract and the ethyl acetate fraction I exhibited a decrease in the survival of MRSA NPRC R001 (**Figure 3.4A**) and the reference strain (**Figure 3.5A**) cells within 6 h. In the water supplemented with gallic acid and tannic acid, almost all concentrations of the compounds showed a decline in the survival of MRSA cells within 2 h (**Figure 3.4B**). The reduction in the tolerance of *S. aureus* ATCC 25923 in water containing gallic acid and tannic acid were demonstrated in **Figure 3.5B**. At the test concentrations, the clearance in viable cells was found within 6 h. At 2MIC and MIC, gallic acid and tannic acid exhibited complete eradication of the bacteria within 24 h.

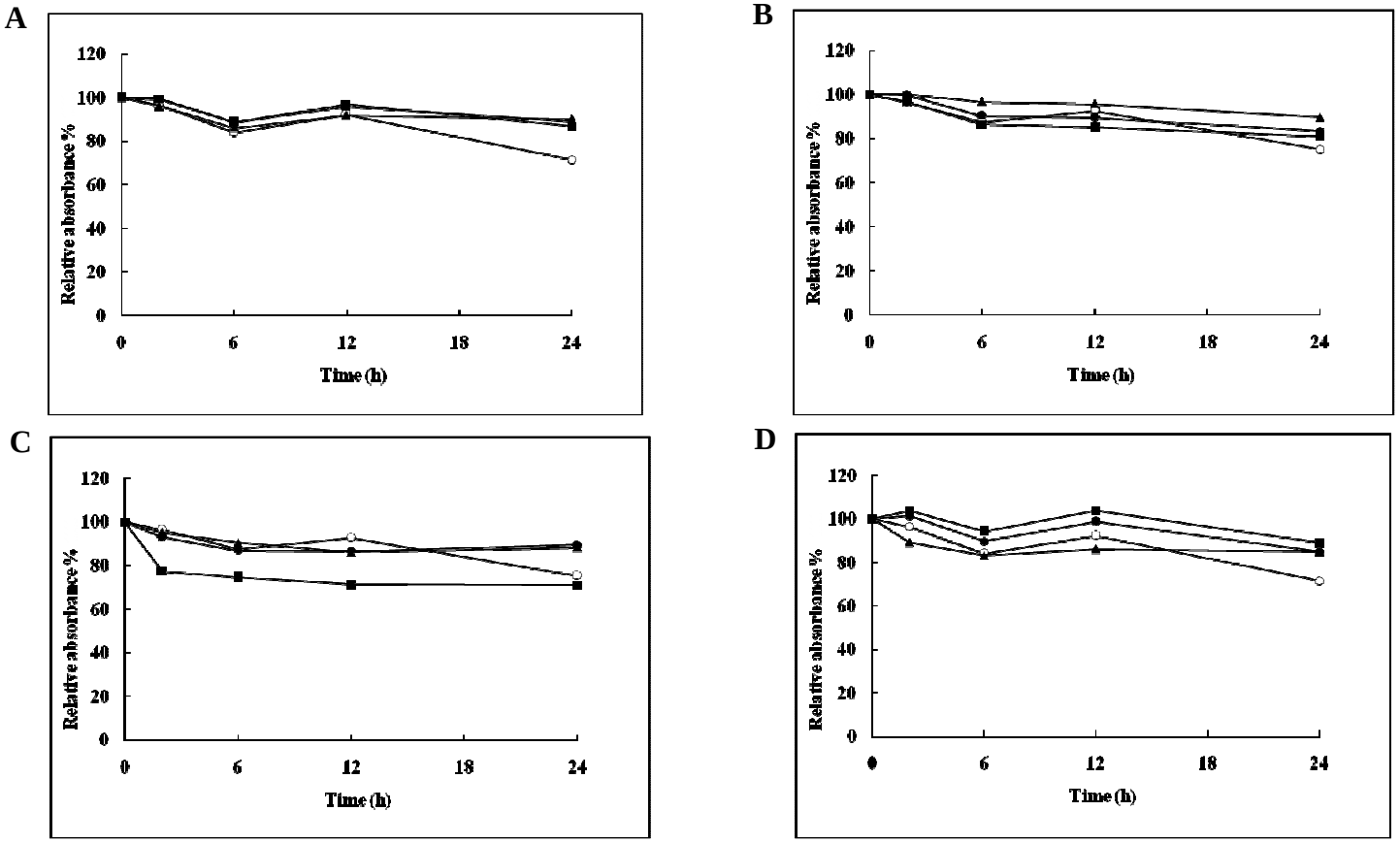

Figure 3.2 Bacteriolytic activities of *Quercus infectoria* ethanol extract (A), ethyl acetate fraction I (B), gallic acid (C), and tannic acid (D) against methicillin-resistant *Staphylococcus aureus* NPRC R001 at MIC (■), 2MIC (▲), and 4MIC (●). 1% DMSO (o) was used as control. Means±SEs for duplicates are illustrated

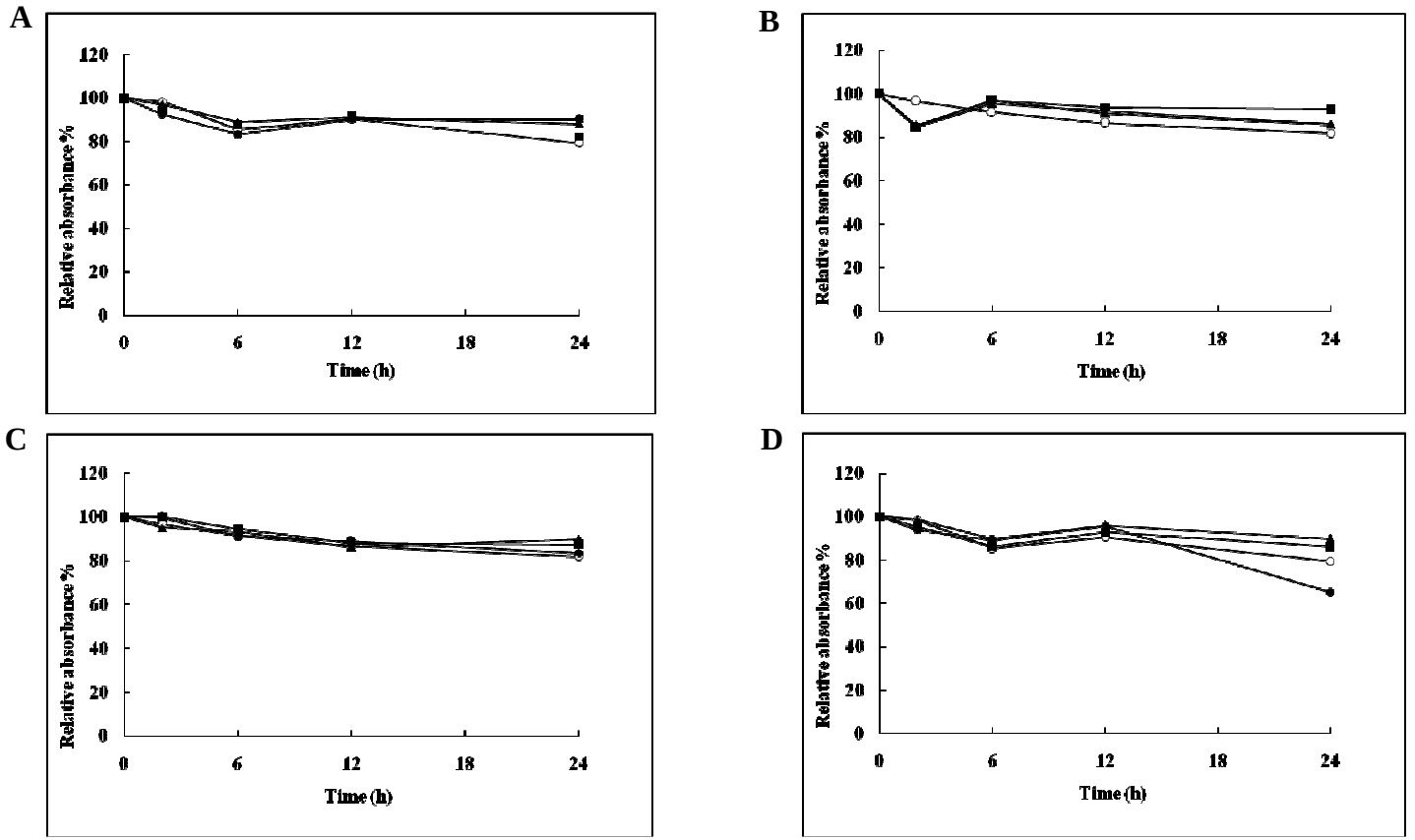

Figure 3.3 Bacteriolytic activities of *Quercus infectoria* ethanol extract (A), ethyl acetate fraction I (B), gallic acid (C), and tannic acid (D) against *Staphylococcus aureus* ATCC 25923 at MIC (■), 2MIC (▲), and 4MIC (●). 1% DMSO (o) was used as control. Means±SEs for duplicates are illustrated

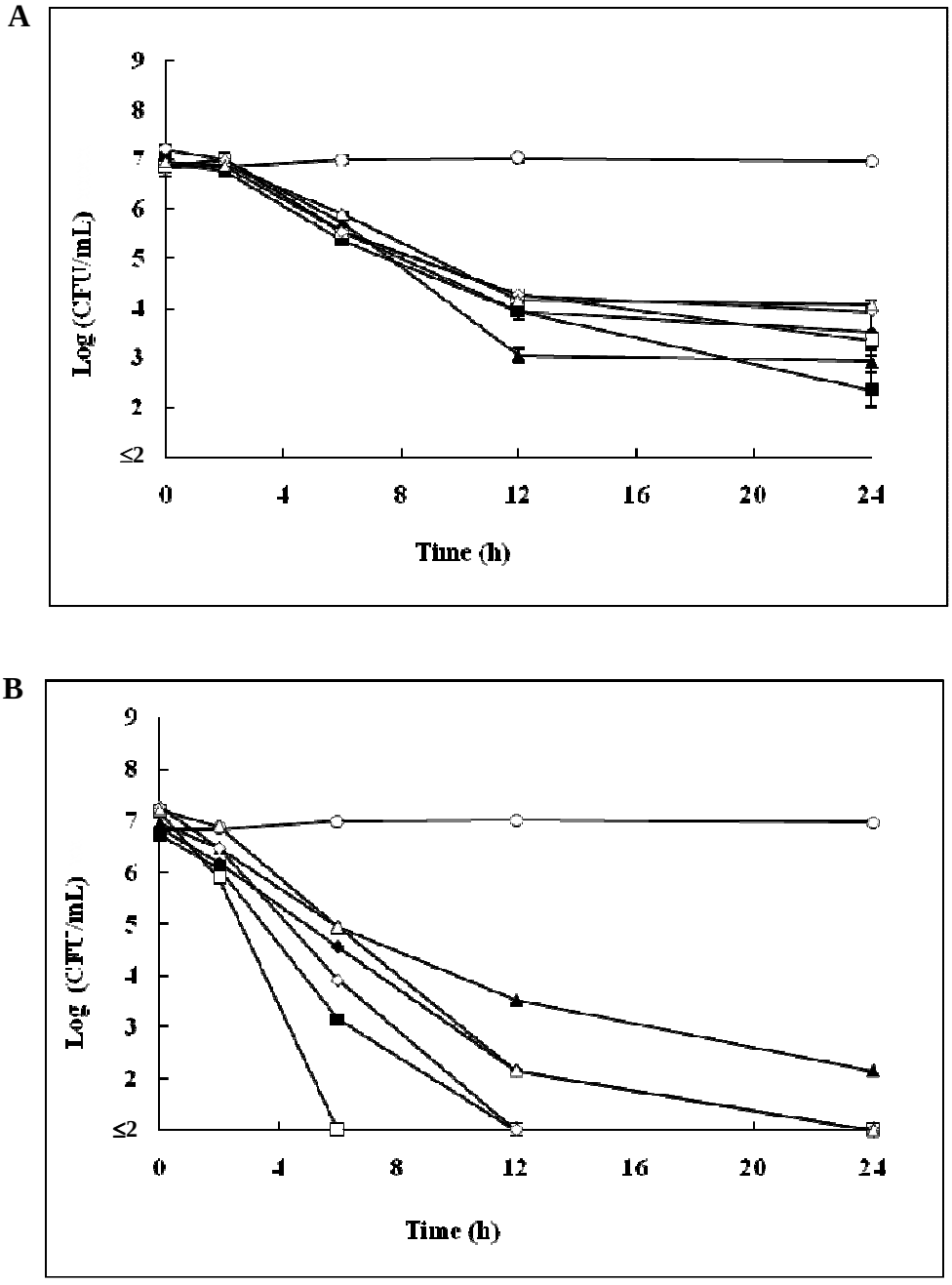

Figure 3.4 Reduced of tolerance of methicillin-resistant *Staphylococcus aureus* NPRC R001 to low osmotic pressure in the presence of *Quercus infectoria* ethanol extract (A) and tannic acid (B) at 2MIC (■) 1MIC (♦), and 1/2MIC(▲), ethyl acetate fraction I (A) and gallic acid (B) at 2MIC (□), 1MIC (◊), and 1/2MIC (∆). 1% DMSO (o) was used as control. Means±SEs for at least duplicates are illustrated. The lowest detection threshold was 10^2 CFU/mL

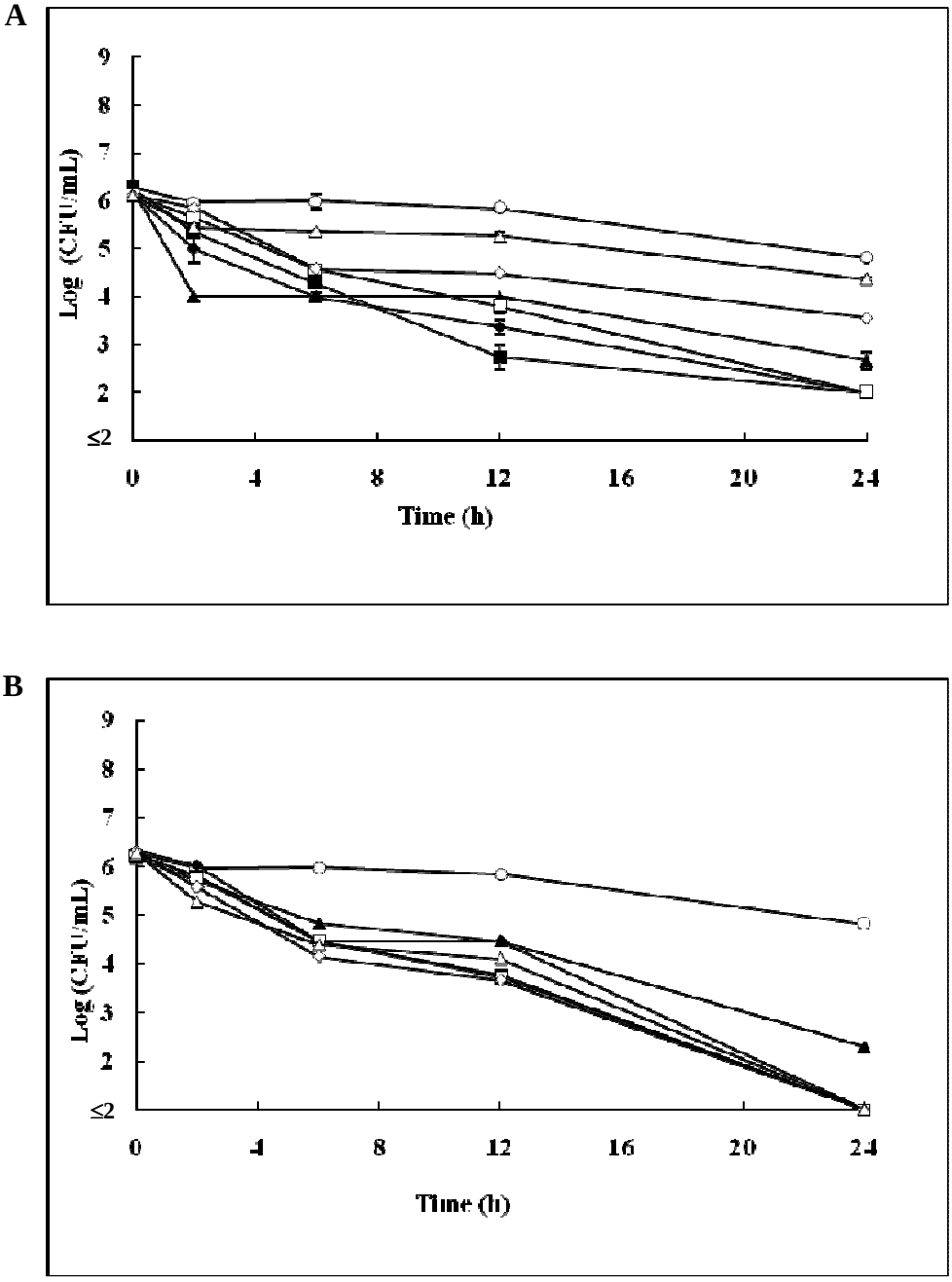

Figure 3.5 Reduced of tolerance of *Staphylococcus aureus* ATCC 25923 to low osmotic pressure in the presence of *Quercus infectoria* ethanol extract (A) and tannic acid (B) at 2MIC (■) 1MIC (♦), and 1/2MIC(▲), ethyl acetate fraction I (A) and gallic acid (B) at 2MIC (□), 1MIC (◊), and 1/2MIC (∆). 1% DMSO (o) was used as control. Means±SEs for at least duplicates are illustrated. The lowest detection threshold was 10^2 CFU/mL

4.3 Loss of salt tolerance in presence of *Quercus infectoria*

The addition of NaCl at 5% and 7.5% w/v to TSA did not affect the colony-forming ability of untreated *S. aureus* cells. After 24 h of incubation, treatment with the extract (**Figure 3.6**) significantly reduced the ability of survival cells to form colonies on TSA containing 5% NaCl with 0.7-1.5 Log(CFU/mL) and on TSA containing 7.5% NaCl with 1.4-2.3 Log(CFU/mL). The decrease of the ability to produce colonies on TSA-NaCl of viable cells treated with the fraction (**Figure 3.7**) was demonstrated at up to 2 Log(CFU/mL) fold.

As demonstrated in **Figure 3.9**, the reduction of the colony-forming ability on TSA-NaCl of cells treated with tannic acid declined 1-5 Log(CFU/mL) after 24 h incubation. Survival cells to form colonies on TSA containing 5% NaCl and 7.5% NaCl diminished slightly (0.5 Log(CFU/mL)) when the cells were treated with gallic acid (**Figure 3.8**).

4.4 Effects on the cytoplasmic membrane of MRSA by *Quercus infectoria*

Damage to the staphylococcal cytoplasmic membrane was assessed by measuring the leakage of small UV-absorbing compounds. The proportion of the OD260 nm at each time point for the MRSA isolate and the reference strain are given in **Figure 3.10** and **3.11**. The OD260 nm of suspensions did not increase significantly at any of the concentrations tested up to 20 h after treatment with the ethanol extract, its ethyl acetate fraction, or its components.

4.5 Effect of *Quercus infectoria* extract and its components on cell morphology

To visualize the effect of the *Quercus infectoria* extract and tannic acid, scanning electron microscope (SEM) and transmission electron microscope (TEM) studies of a representative MRSA were carried out. TEM micrographs of MRSA NPRC R001 after treatment with the ethanol extract, its effective partially-purified fraction, tannic acid, and gallic acid are illustrated in **Figure 3.12** and **3.13**. The results demonstrated an alteration in cell morphology of the treated cell, in comparison to the control in 1% DMSO. Untreated bacterial cells showed a uniform cellular structure with well-defined membranes. The ethanol extract and the ethyl

acetate fraction I produced pseudomulticellular MRSA cell aggregates with thickened cell walls. Tannic acid produced a similar effect while gallic acid had no observed effect.

SEM micrographs of the surface of MRSA NPRC R001 that treated with the ethanol extract, its effective partially-purified fraction (**Figure 3.14**), tannic acid, and gallic acid are shown (**Figure 3.15**) consistent with the TEM observations were additionally determined. Using SEM we found that the plant extract, the fraction, and tannic acid significantly changed the cell morphology of the test isolate, a concentration 1 mg/mL. Similar to the results from TEM determination, MRSA cells formed cell clumps with slightly rough cell walls in the presence of the plant extract and tannic acid due to incomplete separation of cells, indicating that the treatment might have affected the cell division process. These observations suggest that treatment with the *Quercus infectoria* extract as well as tannic acid might have interfered with the bacterial cell division, leading to cell deformation and some degree of incomplete division.

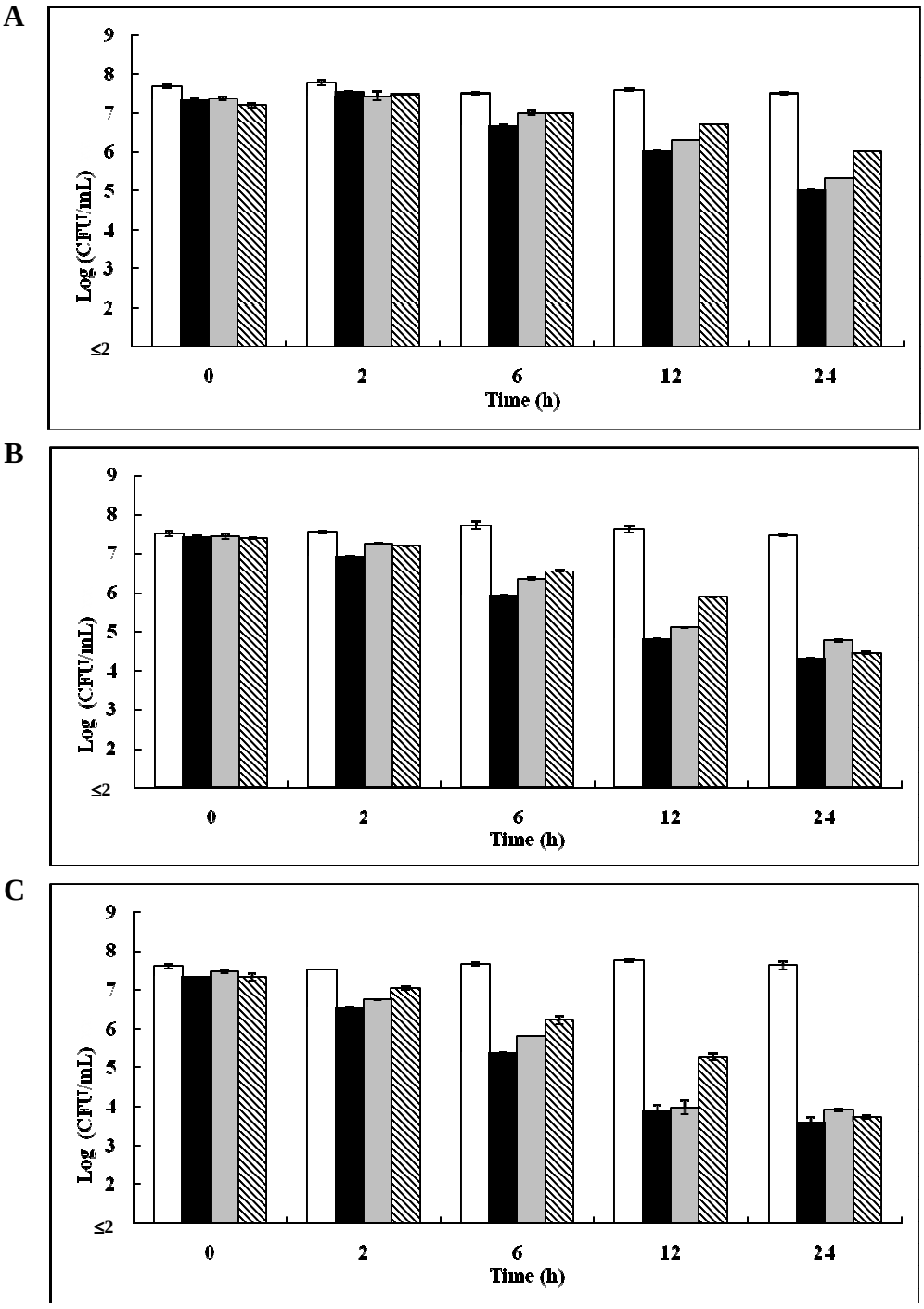

Figure 3.6 Ability of methicillin-resistant *Staphylococcus aureus* NPRC R001 cells to form colonies on TSA (A), TSA supplemented with 5% NaCl (B) and 7.5% NaCl (C) after treatment with *Quercus infectoria* ethanol extract 2MIC (■), MIC (▒), and 1/2MIC (▨). 1% DMSO (□) was used as control. Means±SEs of at least duplicates are illustrated. The lowest detection threshold was 10^2 CFU/mL

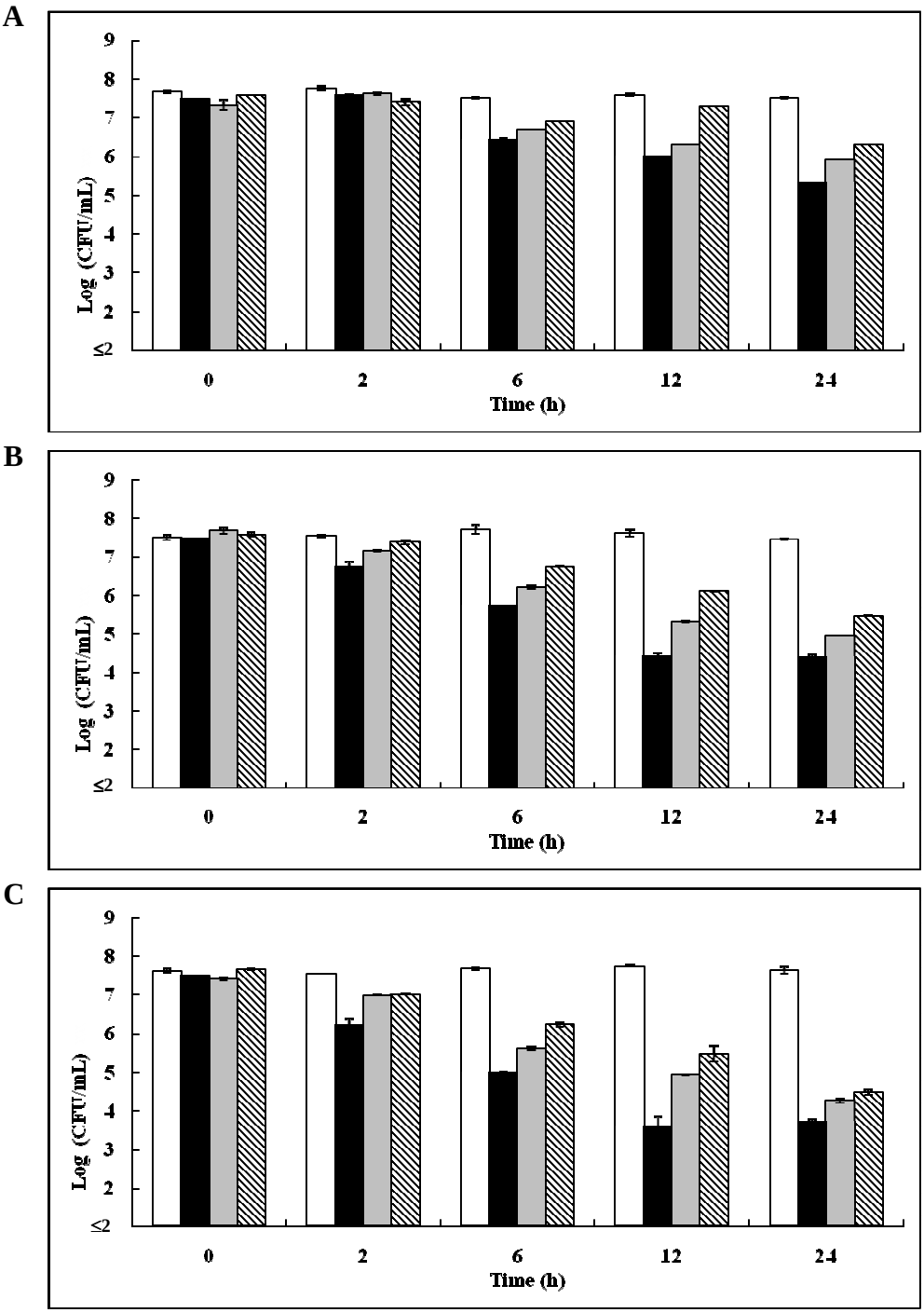

Figure 3.7 Ability of methicillin-resistant *Staphylococcus aureus* NPRC R001 cells to form colonies on TSA (A), TSA supplemented with 5% NaCl (B) and TSA 7.5% NaCl (C) after treatment with *Quercus infectoria* ethyl acetate fraction I at 2MIC (■), MIC (▒), and 1/2MIC (▨). 1% DMSO (□) was used as control. Means±SEs of at least duplicates are illustrated. The lowest detection threshold was 10^2 CFU/mL

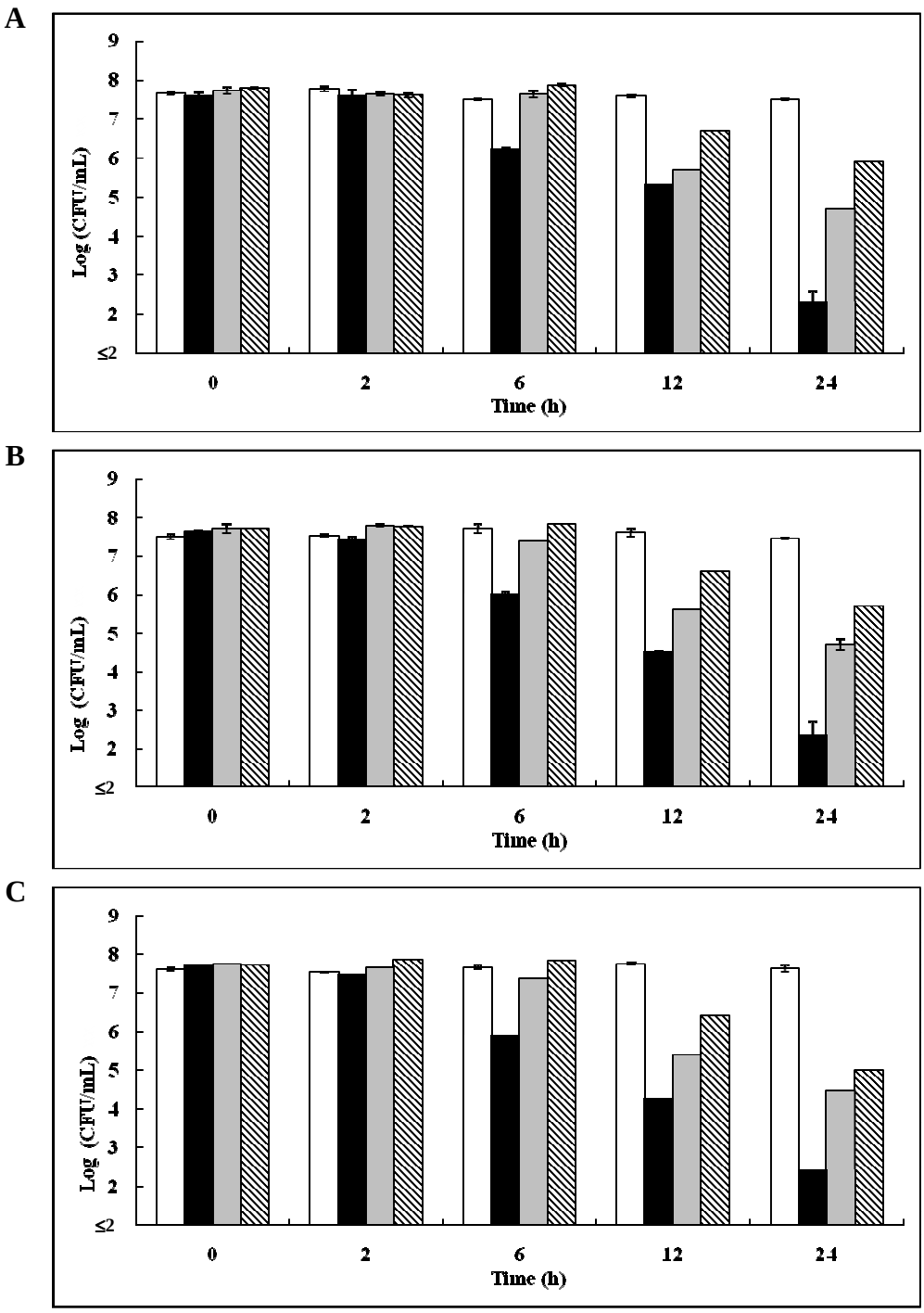

Figure 3.8 Ability of methicillin-resistant *Staphylococcus aureus* NPRC R001 cells to form colonies on TSA (A), TSA supplemented with 5% NaCl (B) and 7.5% NaCl (C) after treatment with gallic acid at 2MIC (■), MIC (▒), and 1/2MIC (▨). 1% DMSO (□) was used as control. The Means±SEs of at least duplicates are illustrated. The lowest detection threshold was 10^2 CFU/mL

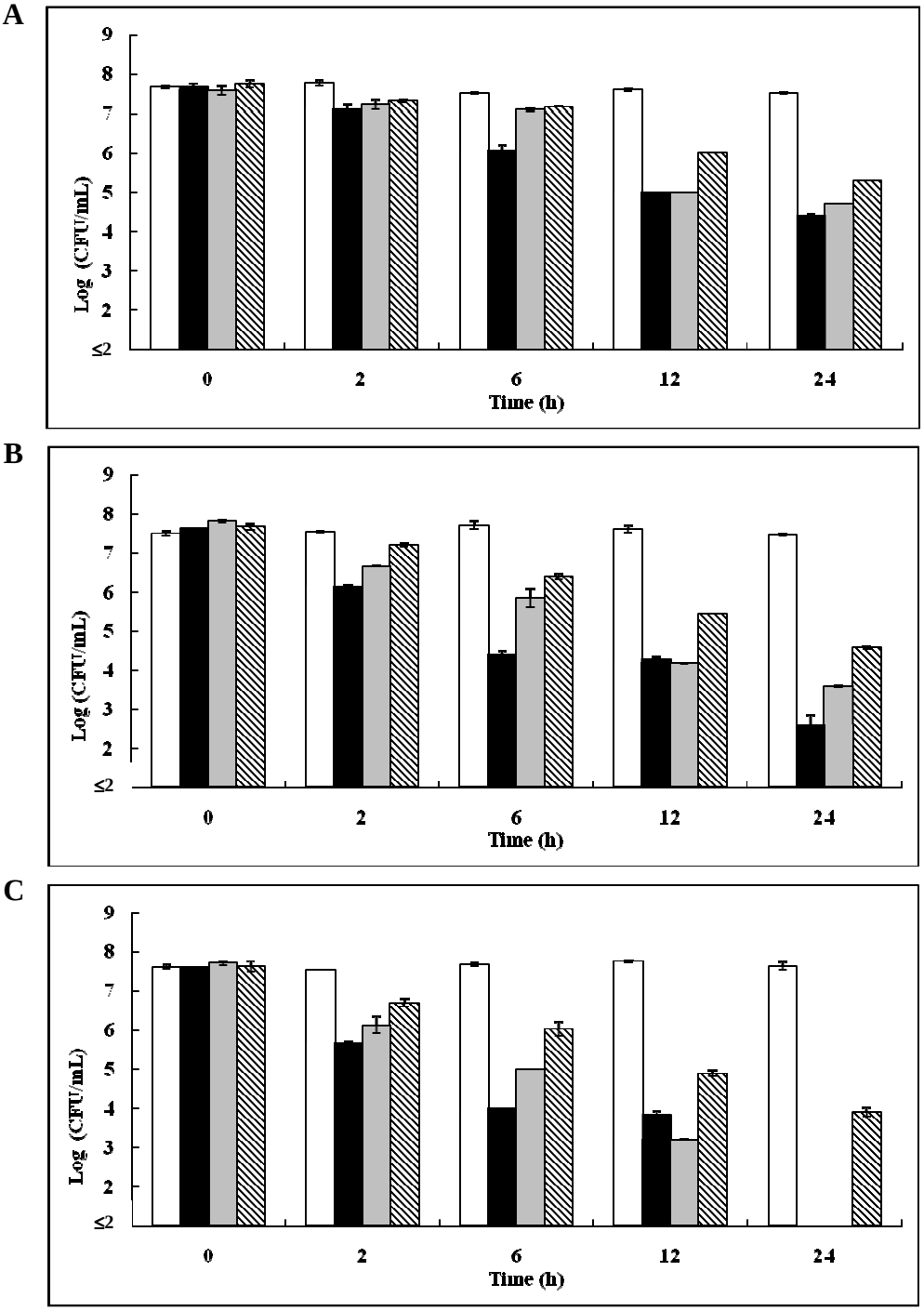

Figure 3.9 Ability of methicillin-resistant *Staphylococcus aureus* NPRC R001 cells to form colonies on TSA (A), TSA supplemented with 5% NaCl (B) and 7.5% NaCl (C) after treatment with tannic acid at 2MIC (■), MIC (▒), and 1/2MIC (▨). 1% DMSO (□) was used as control. Means±SEs of at least duplicates are illustrated. The lowest detection threshold was 10^2 CFU/mL

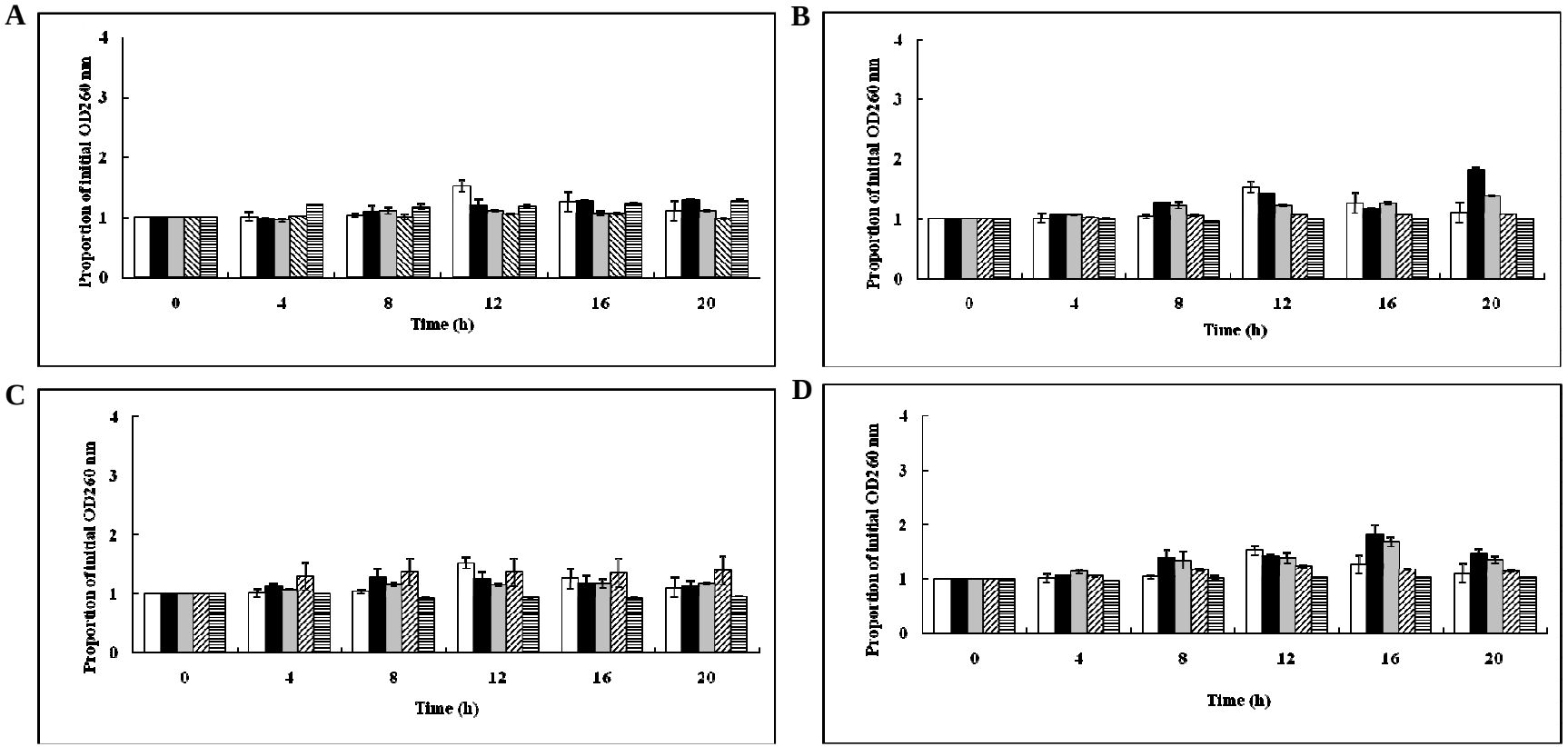

Figure 3.10 Release of the cell contents from methicillin-resistant *Staphylococcus aureus* NPRC R001 after treated with *Quercus infectoria* ethanol extract (A), ethyl acetate fraction I (B), gallic acid (C), and tannic acid (D) at 1/2MIC (■), MIC (▒), 2MIC (▨), and 4MIC (▤). 1% DMSO (□) was used as control. Means±SEs for duplicates are illustrated

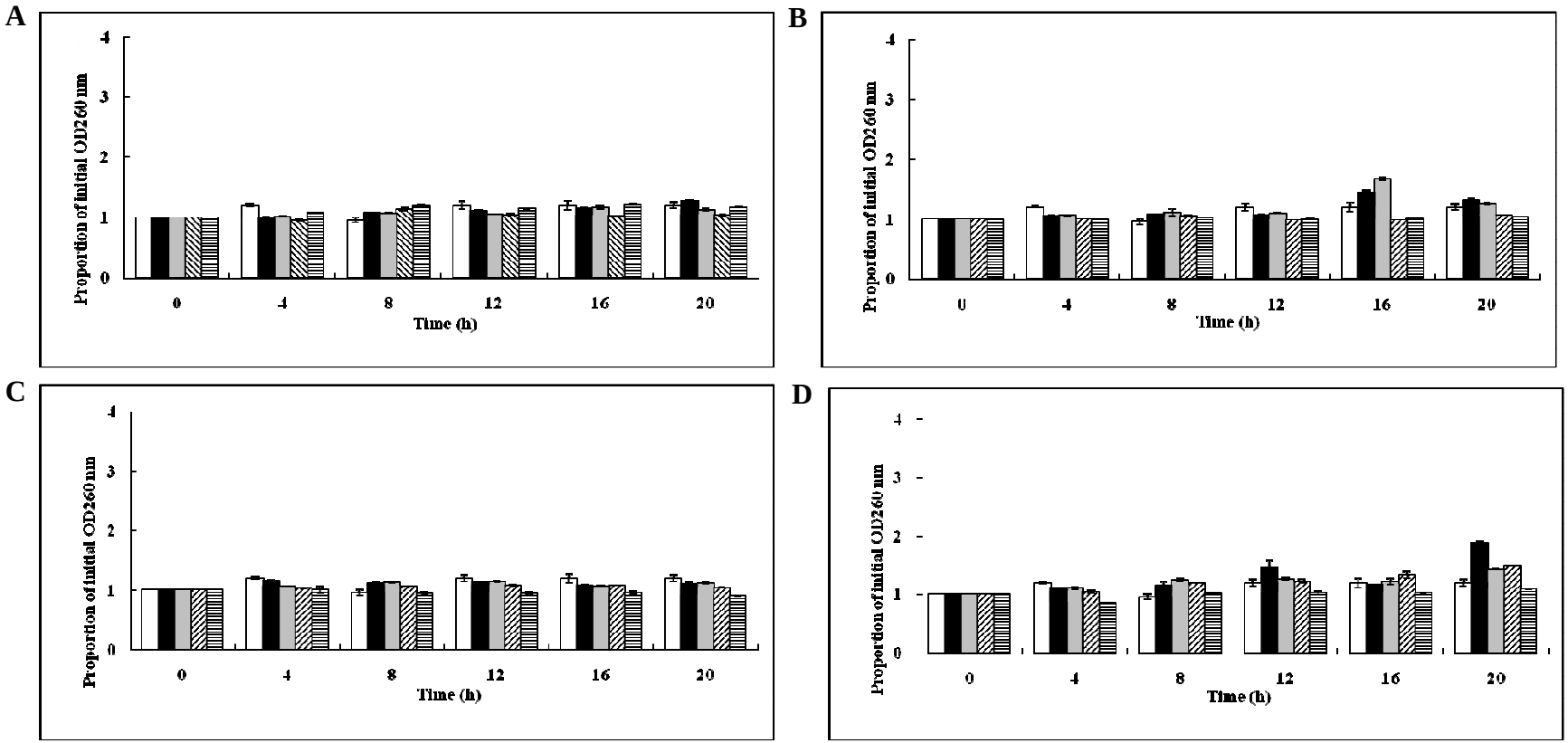

Figure 3.11 Release of the cell contents from *Staphylococcus aureus* ATCC 25923 after treated with *Quercus infectoria* ethanol extract (A), ethyl acetate fraction I (B), gallic acid (C), and tannic acid (D) at 1/2MIC (■), MIC (▒), 2MIC (▨), and 4MIC (▤). 1% DMSO (□) was used as control. Means±SEs for duplicates are illustrated

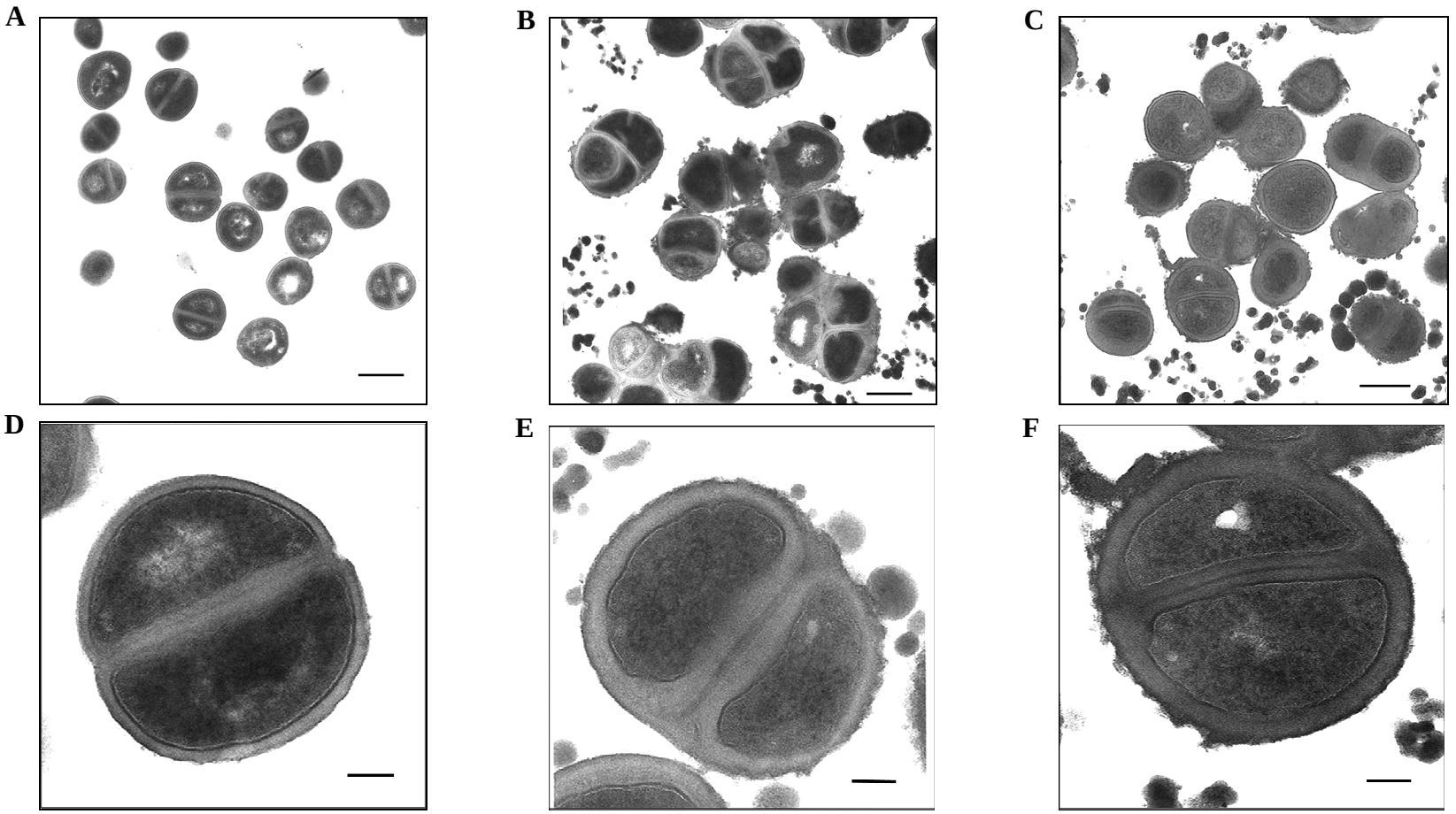

Figure 3.12 Effects of *Quercus infectoria* ethanol extract (B and E) and ethyl acetate fraction I (C and F) on the cell morphology of methicillin-resistant *S. aureus* NPRC R001 at 4MIC, for 12 h. 1% DMSO was used as control (A and D). Scale bars represent 500 nm (A, B, and C) and 100 nm (D, E, and F)

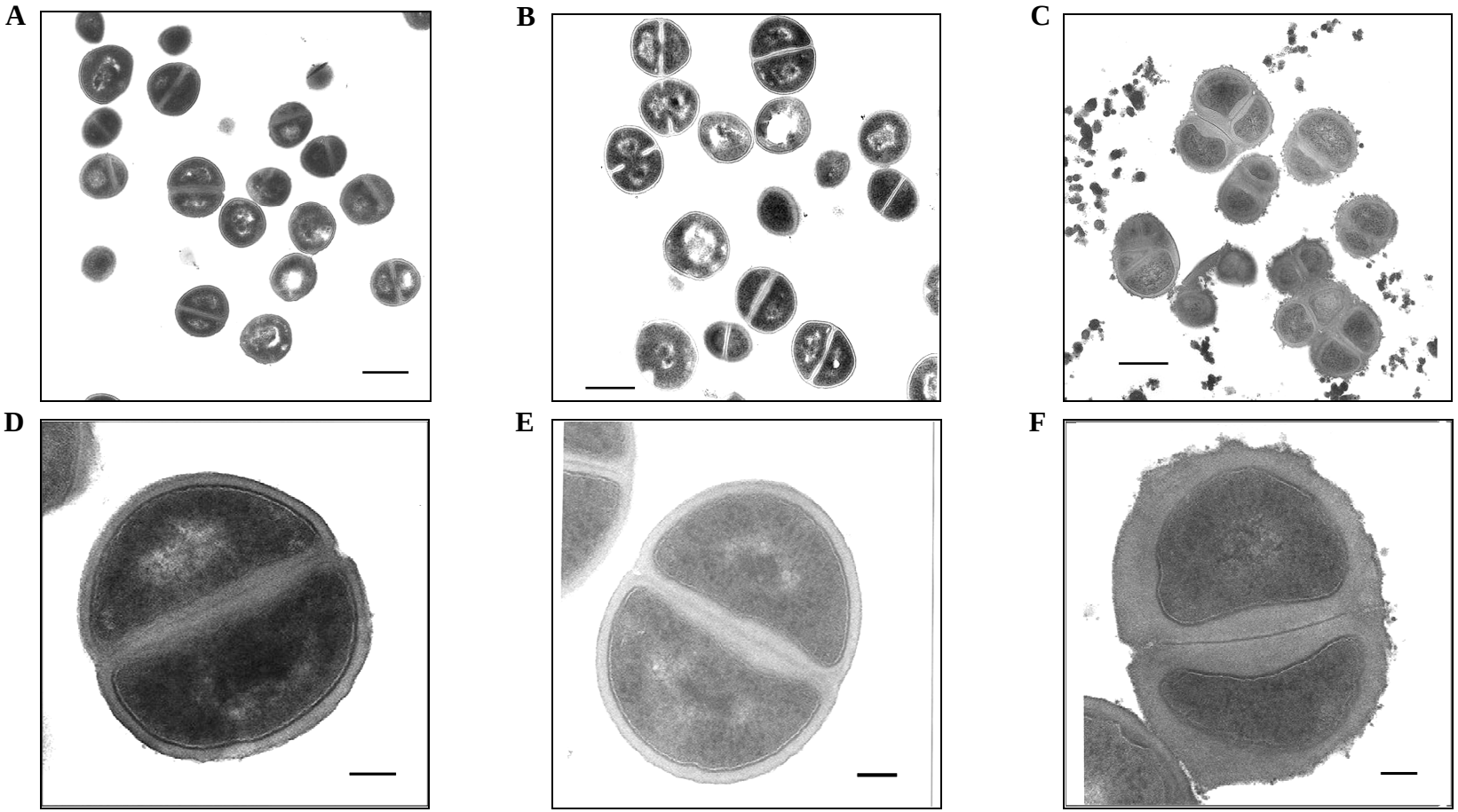

Figure 3.13 Effects of gallic acid (B and E) and tannic acid (C and F) on the cell morphology of methicillin-resistant *S. aureus* NPRC R001 at 4MIC, for 12 h. 1% DMSO was used as control (A and D). Scale bars represent 500 nm (A, B, and C) and 100 nm (D, E, and F)

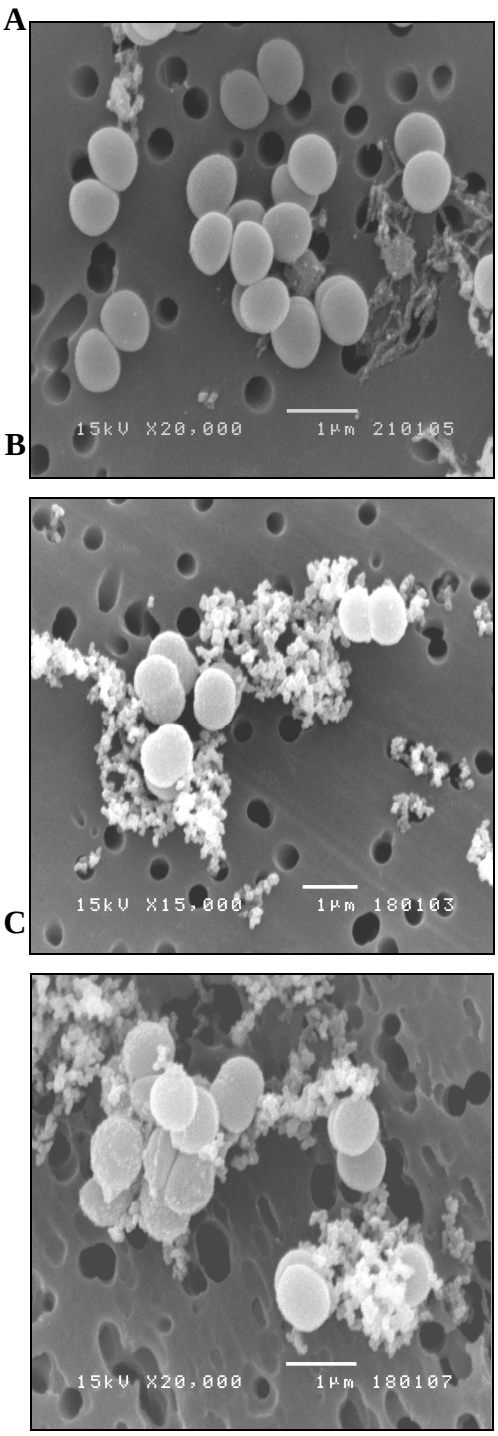

Figure 3.14 Scanning electron micrographs showing the cell surface of methicillin-resistant *Staphylococcus aureus* NPRC R001 grown in TSB containing *Quercus infectoria* ethanol extract (B) and ethyl acetate fraction I (C) at 4MIC, for 12 h, 1% DMSO was used as control (A)

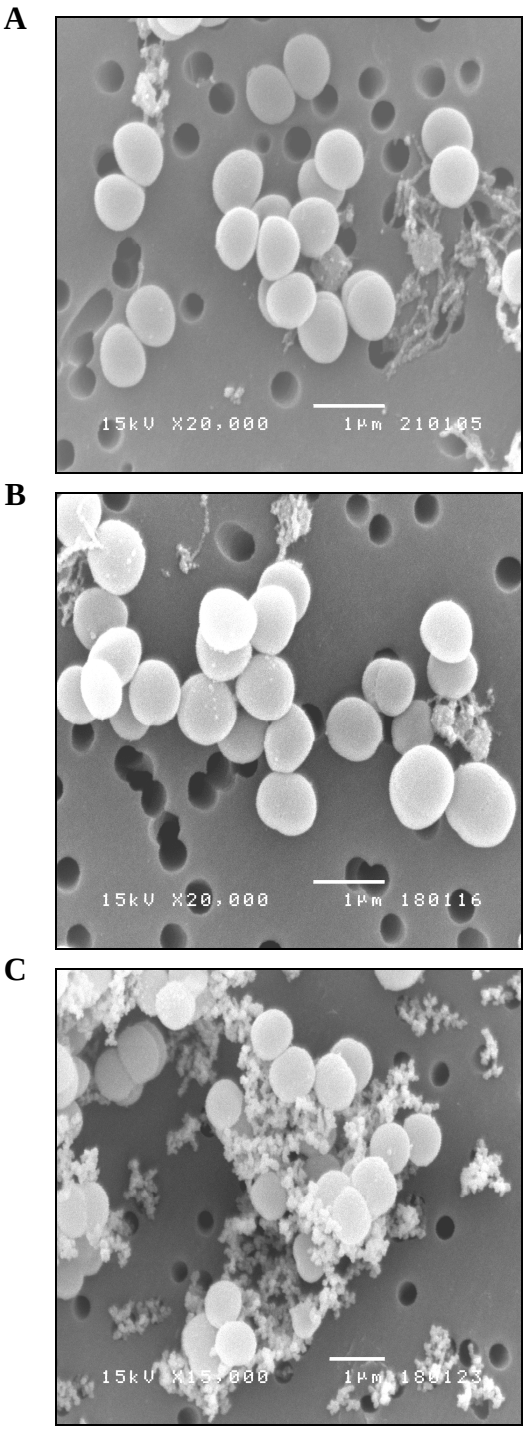

Figure 3.15 Scanning electron micrographs showing the cell surface of methicillin-resistant *Staphylococcus aureus* NPRC R001 grown in TSB containing gallic acid (B) and tannic acid (C) at 4MIC, for 12 h, 1% DMSO was used as control (A)

5. Effects of *Quercus infectoria* extract and its components on staphylococcal colonization

5.1 The effect of *Quercus infectoria* extract and tannic acid on biofilm forming ability of MRSA and MSSA

In the subsequent experiments, six representative MRSA isolates (NPRC R015, R016, R029, R033, R034, and R035), six representative MSSA isolates (NPRC S010, S024, S028, S029, S042, and S045), and *S. aureus* ATCC 25923 were used.

With the exception of MRSA NPRC R015 and R033, all test concentrations (1MIC-1/16MIC) of the ethanol extract were significantly able to reduce the biofilm formation of the test MRSA isolates ($P<0.05$). While only at MIC and some sub-MICs of tannic acid showed decreased in biofilm formation of the test MRSA isolates (**Figure 3.16**). When exposed to sub-MIC of both the ethanol extract and tannic acid, there was no reduction in the biofilm formation of the MSSA isolates. With the exception of MSSA NPRC S010, S024, and S028, the inhibition of biofilm formation of the MSSA isolates was observed after treated with MIC of the ethanol extract and tannic acid (**Figure 3.17**). The inhibition of the biofilm formation is similar for both MSSA isolates and the reference strain (**Figure 3.18**).

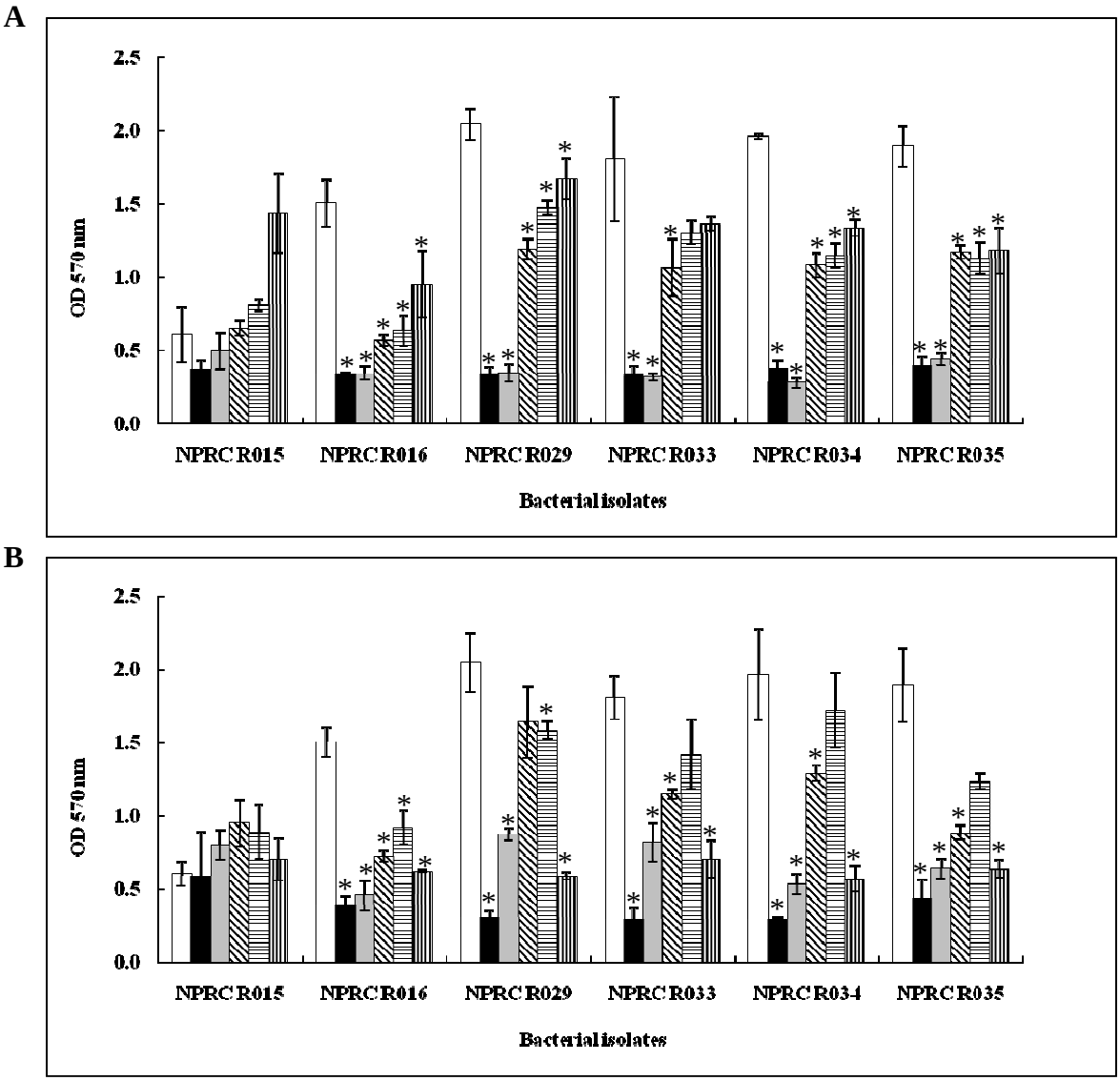

Figure 3.16 The effect of *Quercus infectoria* ethanol extract (A) and tannic acid (B) at minimum inhibitory concentration (MIC, ■), 1/2MIC (▒), 1/4MIC (▨), 1/8MIC (▤), and 1/16MIC (▥) on the biofilm formation of representative methicillin-resistant *Staphylococcus aureus* isolates, 1% DMSO (□) was used as control. The means±SDs for at least duplicates are illustrated. * The biofilm formation of the isolates after treated with the compounds are significantly lower than the control (P<0.05)

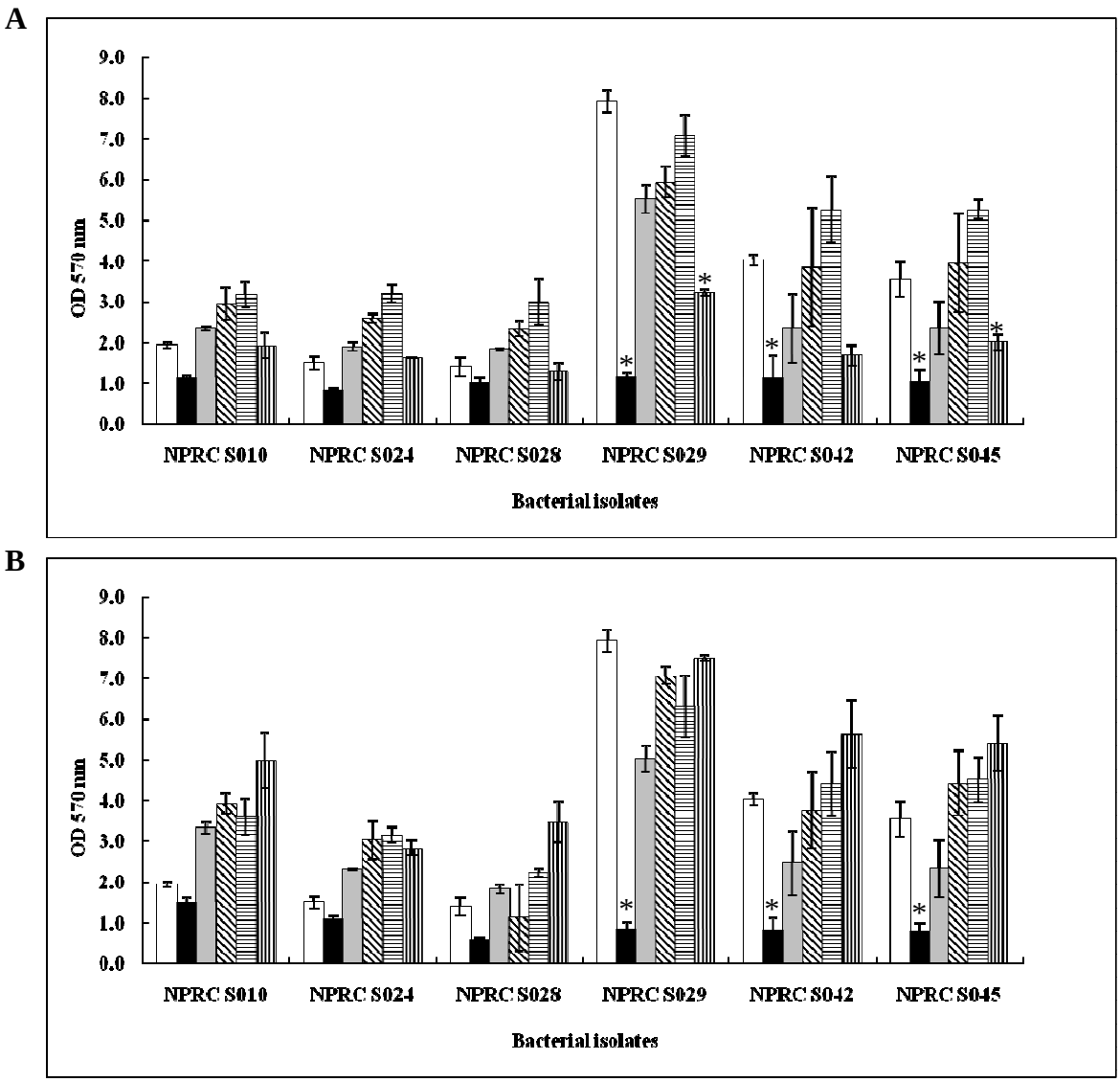

Figure 3.17 The effect of *Quercus infectoria* ethanol extract (A) and tannic acid (B) at minimum inhibitory concentration (MIC, ■), 1/2MIC (▒), 1/4MIC (▨), 1/8MIC (▤), and 1/16MIC (▥) on the biofilm formation of representative methicillin-susceptible *Staphylococcus aureus* isolates, 1% DMSO (□) was used as control. The means±SDs for at least duplicates are illustrated. * The biofilm formation of the isolates after treated with the compounds are significantly lower than the control (P<0.05)

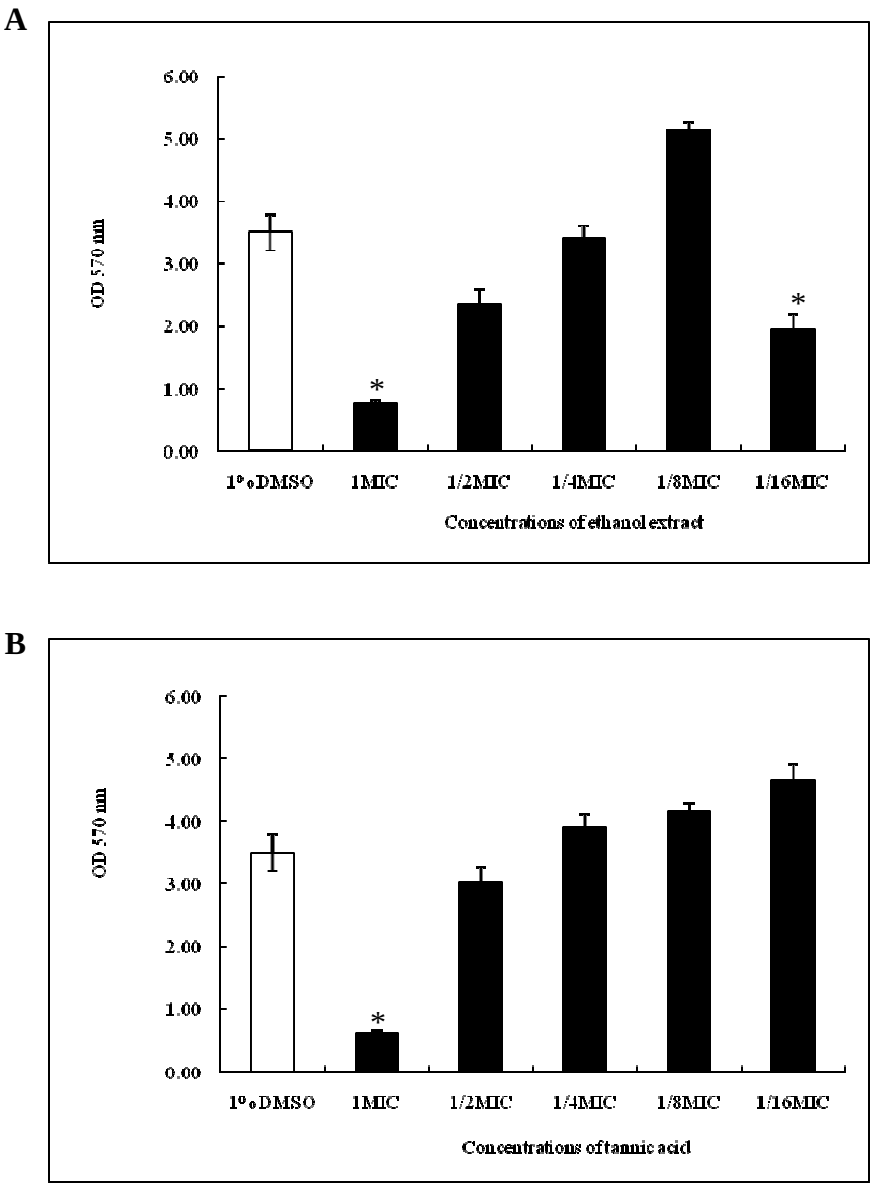

Figure 3.18 The effect of *Quercus infectoria* ethanol extract (A) and tannic acid (B) on the biofilm formation of *Staphylococcus aureus* ATCC 25923, 1% DMSO (□) was used as control. The means±SDs for at least duplicates are illustrated. * The biofilm formation of the isolates after treated with the compounds are significantly lower than the control (P<0.05)

5.2 The effect of *Quercus infectoria* extract and tannic acid on MRSA and MSSA cell surface hydrophobicity

In the following studies, six high hydrophobic strains (NPRC R004, R014, R015, S001, S019, and S023), six low hydrophobic strains (NPRC R002, R006, R011, S002, S034, and S044), and *S. aureus* ATCC 25923 were used. The HPBI of all test strains increased after treated with supra-MIC, MIC, and sub-MIC values of both the plant extract and tannic acid.

Supra-MIC values of both the plant extract and tannic acid significantly increased the HPBI of low hydrophobic MRSA and MSSA strains ($P< 0.05$) while only low hydrophobic MRSA strains were significantly affected after treatment with MIC of the ethanol extract and its constituent. Sub-MICs values of these compounds (from 31.26-125 µg/mL) demonstrated weak effect on the cell surface properties of these strains (**Figure 3.19** and **3.20**). The CSH of the high hydrophobic strains was slightly altered following the treatment of supra-MIC, MIC, and sub-MIC values of both the extract and tannic acid (**Figure 3.21** and **3.22**). For the reference strain, supra-MIC, MIC, and sub-MIC of the extract significantly increased its HPBI ($P< 0.05$). All concentrations of tannic acid increased the hydrophobic index of this strain but significant effect was observed only at 4MIC, 2MIC, and 1/4MIC (**Figure 3.23**).

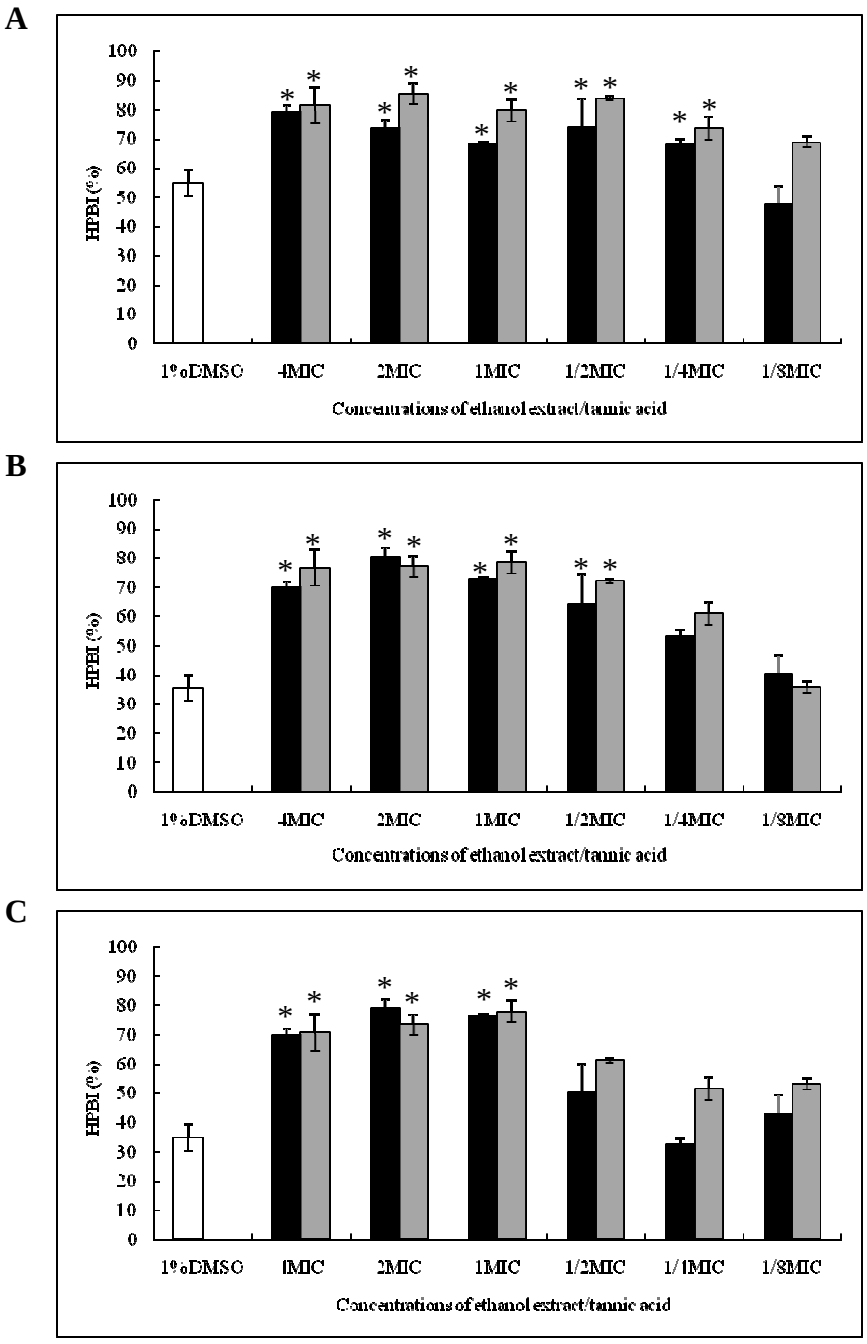

Figure 3.19 The effect of *Quercus infectoria* ethanol extract (■) and tannic acid (▒) on the cell surface hydrophobicity of representative low hydrophobic methicillin-resistant *Staphylococcus aureus* NPRC R002 (A), R006 (B), and R011 (C) isolates1% DMSO (□) was used as control. The means±SEs for duplicates are illustrated. * The hydrophobicity index of the isolates after treated with the compounds are significantly different from the control (P<0.05)

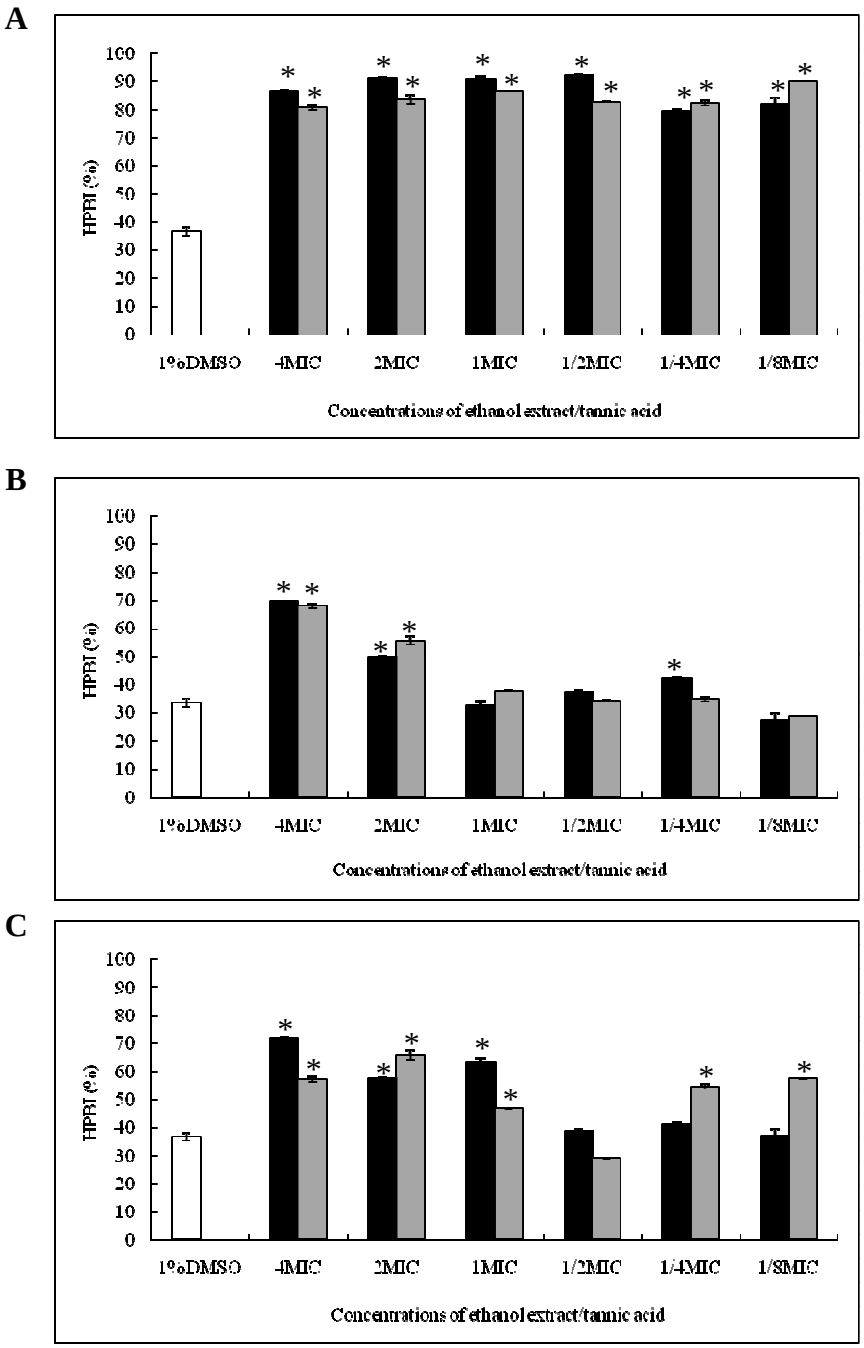

Figure 3.20 The effect of *Quercus infectoria* ethanol extract (■) and tannic acid (□) on the cell surface hydrophobicity of representative low hydrophobic methicillin-susceptible *Staphylococcus aureus* NPRC S003 (A), S034 (B), and S044 (C) isolates, 1% DMSO (□) was used as control. The means±SEs for duplicates are illustrated. * The hydrophobicity index of the isolates after treated with the compounds are significantly different from the control (P<0.05)

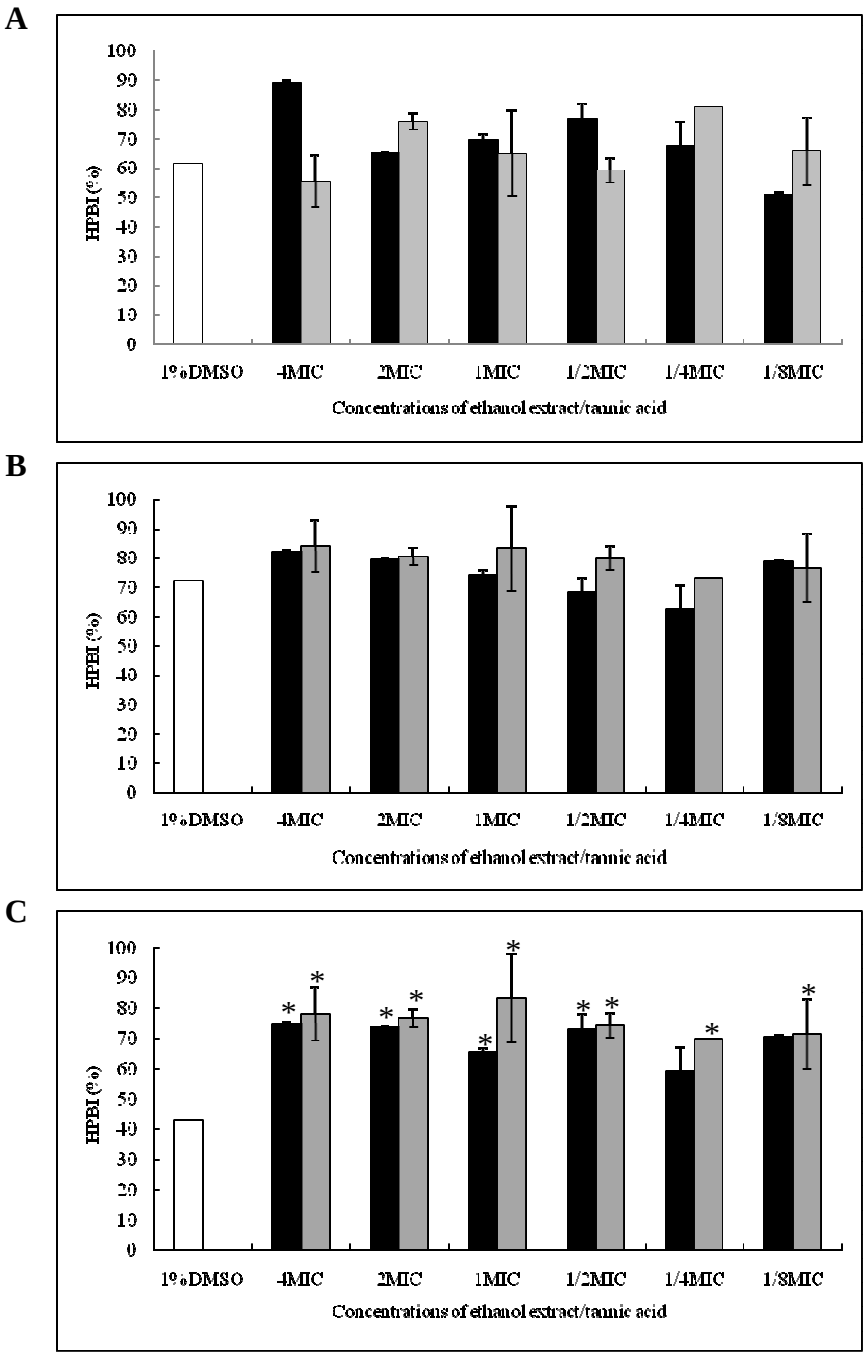

Figure 3.21 The effect of *Quercus infectoria* ethanol extract (■) and tannic acid (□) on the cell surface hydrophobicity of representative high hydrophobic methicillin-resistant *Staphylococcus aureus* NPRC R004 (A), R014 (B), and R015 (C) isolates, 1% DMSO (□) was used as control. The means±SEs for duplicates are illustrated. * The hydrophobicity index of the isolates after treated with the compounds are significantly different from the control (P<0.05)

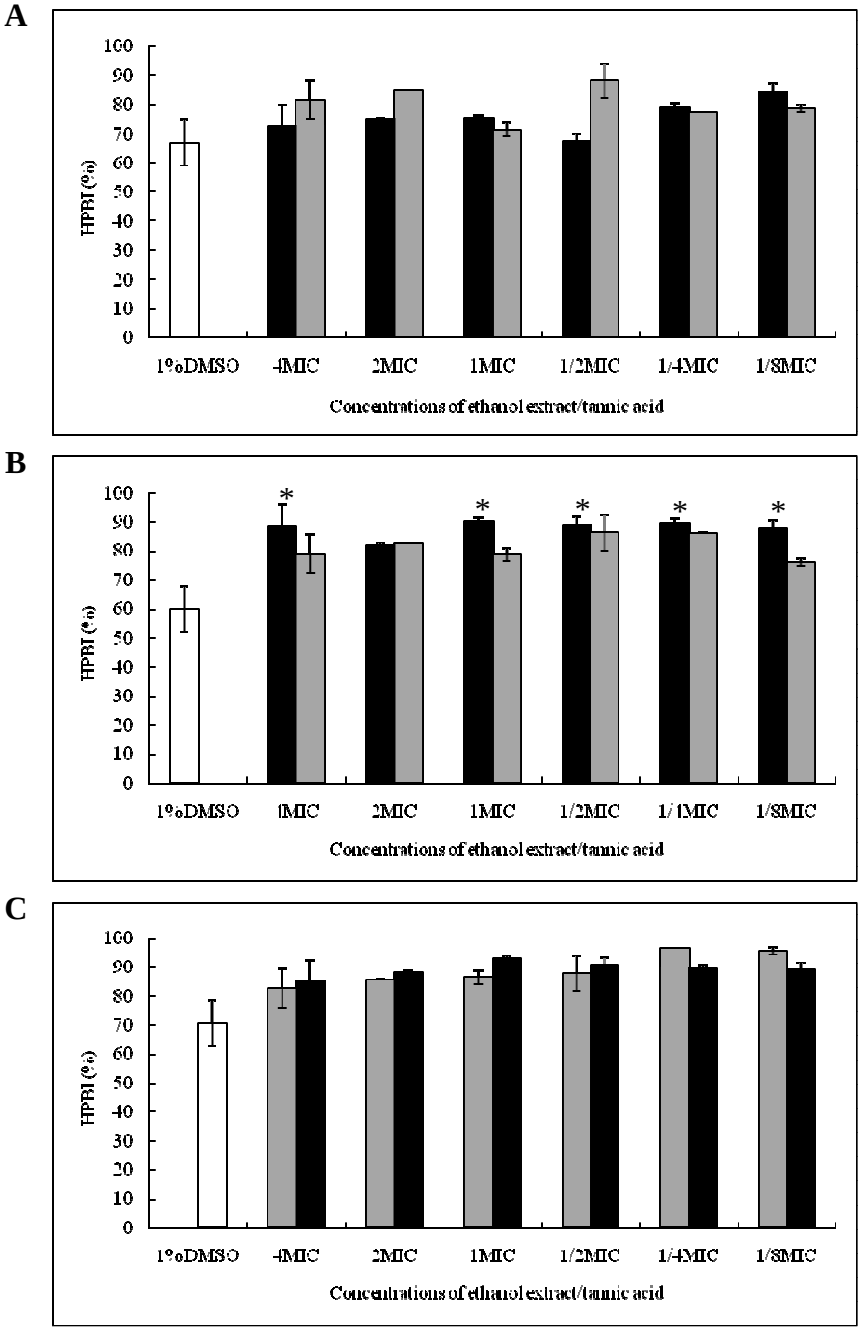

Figure 3.22 The effect of *Quercus infectoria* ethanol extract (■) and tannic acid (□) on the cell surface hydrophobicity of representative high hydrophobic methicillin-susceptible *Staphylococcus aureus* NPRC S001 (A), S019 (B), and S023 (C) isolates, 1% DMSO (□) was used as control. The means±SEs for duplicates are illustrated. * The hydrophobicity index of the isolates after treated with the compounds are significantly different from the control (P<0.05)

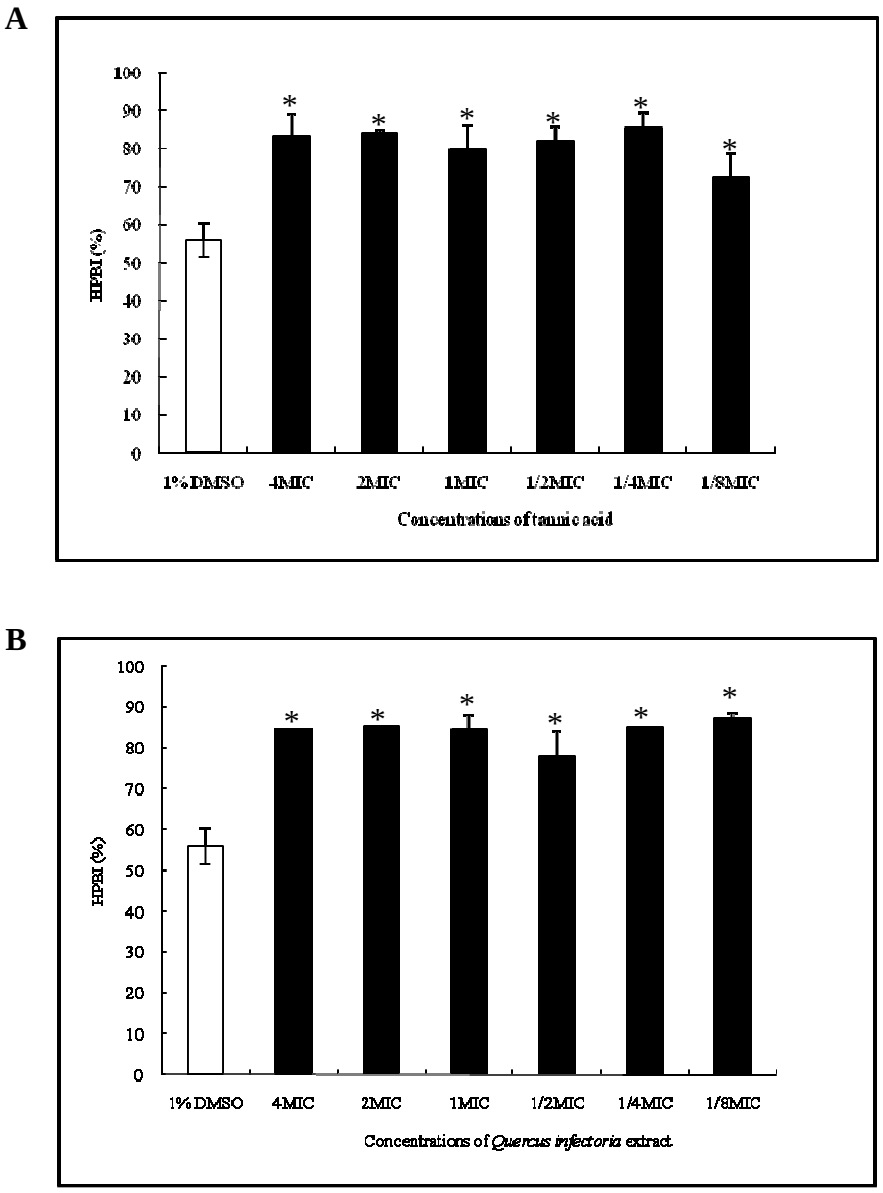

Figure 3.23 The effect of *Quercus infectoria* ethanol extract (A) and tannic acid (B) on the cell surface hydrophobicity of *Staphylococcus aureus* ATCC 25923, 1% DMSO (□) was used as control. The means±SEs for duplicates are illustrated. * The hydrophobicity index of the isolate after treated with the compounds are significantly different from the control (P<0.05)

6. Other biological activities of *Quercus infectoria* extract

In the evaluation for more information of *Quercus infectoria* extract, some additional biological activities such as anti-viral, anti-Mycobacterium, anti-malarial, anti-inflammation, and anti-cancer activities were presented in **Table 3.9**. These *in vitro* studies have provided some support for traditional uses of this plant as anti-diarrhoea, anti-inflammatory, and anti- ulceration agents. The results indicated that the extract demonstrated excellent scavenging activities and good anti-malarial activity. With the exception of anti-cancer activity against oral human epidermal carcinoma, there were no anti-cancer, anti-viral, anti-Mycobacterium, and anti-inflammatory activities.

Table 3.9 Additional biological activities of ethanol extract of *Quercus infectoria*

	IC_{50} (μg/mL)	
Test biological activities	Extract	Positive control
Anti-microbial		
Anti-Herpes simplex virus type 1	>50	1.84 (Acyclovir)
Anti-*Mycobacterium tuberculosis* [a]	>100	0.02 (Rifampicin)/1.25(Kanamycin)/
		0.05 (Isoniazid)
Anti-*Plasmodium falciparum* K1	0.89	0.001 (Dihydroartemisinine)
Anti-cancer		
Anti-KB-Oral human epidermal carcinoma	11.7	0.68 (Ellipticine)/0.052 (Doxorubicin)
Anti-MCF7-Breast cancer	>20	0.955 (Ellipticine)/0.097 (Doxorubicin)
Anti-NCI-H187	>20	0.409 (Ellipticine)/0.021 (Doxorubicin)
Free radical scavenging	10	20 (BHT)
	0.5	2530 (Tannin)
	320	85 (Trolox)
Anti-inflammation		
Anti-COX-1/Anti-COX-2	>10/>10	4-5 (Aspirin)/9-10 (Aspirin)

[a] The presented values are minimum inhibitory concentrations of *Quercus infectoria* extract, rifampicin, kanamycin, and isoniazid against *Mycobacterium tuberculosis*

7. *Quercus infectoria*; a source for resistant modifying agents for multidrug-resistant Gram-positive and Gram-negative bacteria

7.1 Effect of combination *Quercus infectoria* extract and its components with antibiotics against MRSA

The test MRSA isolates are resistant to amoxicillin, oxacillin, and penicillin G with the MIC values ranging from 35-512, 16-256, and 160-1,280 µg/mL, respectively. The results of the checkerboard titration are explained in **Table 3.10**. The majority of the FIC indices were in the range of 0.5-2.0 therefore the main interaction between the samples and the 3 antibiotics was considered to be additive. However, gallic acid was the only compound that showed any antagonistic action to the β-lactam antibiotics. Only oxacillin and penicillin G were antagonized and antagonism was demonstrated by only one of the 17 MRSA strains. With all other strains, gallic acid had an additive action with all 3 antibiotics and no synergistic effect. The effect of tannic acid and the β-lactams was very similar to that of gallic acid and the β-lactams, in that an additive effect was observed with most strains except for 3, that showed a synergistic effect with amoxicillin only. A synergistic reaction of the ethanol extract and ethyl acetate fraction with β-lactams was obtained. A synergistic response with amoxicillin was obtained with 76% and 59% of strains respectively, for oxacillin 12% and 35 % of strains and for penicillin G, 53% and 12% of strains respectively. For all other strains the responses were additive.

Table 3.10 The fractional inhibitory concentration (FIC) of β-lactam antibiotics in combination with *Quercus infectoria* extract and its components against methicillin-resistant *Staphylococcus aureus* (n=17)

Antibacterial agents	Percentage of tested isolates		
	∑ FIC index [a]		
	≤0.5	0.5-2.0	≥2.0
Amoxicillin			
Ethanol extract	76	24	0
Ethyl acetate fraction I	59	41	0
Gallic acid	0	100	0
Tannic acid	18	82	0
Oxacillin			
Ethanol extract	12	88	0
Ethyl acetate fraction I	35	65	0
Gallic acid	0	94	6
Tannic acid	0	100	0
Penicillin G			
Ethanol extract	53	47	0
Ethyl acetate fraction I	12	88	0
Gallic acid	0	94	6
Tannic acid	0	100	0

[a] Results for the FIC indices ≤0.5 are synergistic, those for FIC indices >0.5 to <2 are additive, and those for FIC indices ≥2 are antagonistic

7.2 Ellagic acid: a potentiater for multidrug-resistant *Acinetobacter baumannii*

7.2.1 Antibacterial activity and antibiotic adjuvant ability of *Quercus infectoria* components and other phenolic compounds

Multidrug-resistant (MDR) *A. baumannii* JVC 1053 exhibited a novobiocin MIC of 32 mg/L. The plant-derived compounds to be used exhibited no intrinsic antibacterial activity against *A. baumannii* JVC 1053 (MIC ≥ 320 μM). Tests of the plant compounds for their antibiotic adjuvant ability in combination with a subinhibitory concentration of novobiocin against MDR-*A. baumannii* are shown in **Table 3.11**. With the exception of ellagitannin and tannic acid, the plant polyphenols displayed weak effects on bacterial growth at 40 μM. The combination of ellagic acid and tannic acid completely inhibited bacterial growth in the presence of 1/4MIC of novobiocin.

7.2.2 Antibiotic susceptibility patterns of *A. baumannii* in the presence of antibiotic adjuvants

Table 3.12 shows the ellagic acid and tannic acid enhancement of the antibacterial activity of aminocoumarins, rifampicin, fusidic acid, and tetracycline against *A. baumannii*. Ellagic acid supplementation did not increase the susceptibility of MDR *A. baumannii* to macrolides, aminoglycosides, ampicillin, and colistin, but did improve the antibacterial activity of macrolides and aminoglycosides against the ATCC 19606 strain. The activity of imipenem was slightly reduced. The changes in susceptibility patterns of the *A. baumannii* strains in the presence of ellagic and tannic acids were compared to those of known antibiotic adjuvants including EDTA, CCCP, and reserpine.

Different concentrations of ellagic acid and tannic acid (10 to 40 μM) were further tested for their stimulation of antibacterial activities of aminocoumarins, rifampicin, fusidic acid, and tetracycline against both MDR and reference *A. baumannii* strains (**Table 3.13**). In the presence of all concentrations of ellagic acid, the MICs of the test antibiotics, with the exception of tetracycline, were reduced 2- to 4- fold. Tannic acid stimulated antibacterial activities against MDR *A. baumannii*

(the MIC values were changed 2- to 4-fold), but did not decrease the MIC of fusidic acid against the MDR strain.

Combinations of ellagic acid (10 µM or 3 µg/mL) with novobiocin, chlorobiocin, coumermycin, fusidic acid, or rifampicin (1/8 to 1/16 MIC) dramatically reduced the bacterial growth in both MDR and the reference strains (**Figure 3.24**).

Table 3.11 Effect of *Quercus infectoria* components and other plant-derived phenolics on the antibiotic activity of novobiocin against *Acinetobacter baumannii* JVC 1053

Plant phenolics	OD595 nm±SD [a] in the presence of:	
	Plant phenolics (40 μM)	Novobiocin [b] +Plant
phenolics		
Catechol	0.494±0.02	0.469±0.10
Cinnamic acid	0.552±0.10	0.424±0.10
p-Coumaric acid	0.521±0.13	0.615±0.05
Ellagic acid	0.447±0.03	0.078±0.05
Ellagitannin	0.239±0.03	0.366±0.00
Ferulic acid	0.507±0.00	0.419±0.10
Gallic acid	0.575±0.07	0.275±0.06
Quercetin	0.615±0.03	0.735±0.08
Syringic acid	0.541±0.06	0.264±0.05
Tannic acid	0.360±0.00	0.069±0.02

[a] Mean values±SD of at least triplicate results

[b] In the presence of 1% DMSO, used as positive control, and novobiocin (8 μg/mL) the OD595 nm of the *A. baumannii* were 0.824±0.02 and 0.567±0.04, respectively

Table 3.12 Effect of efflux pump inhibitors, a permeabilizer, and plant-derived compounds on the susceptibility of *Acinetobacter baumannii* JVC 1053 and *Acinetobacter baumannii* ATCC 19606

	Changes of antibiotic inhibition zones [a]					
Test antibiotics (μg/disc)	CCCP (50 μM)	Reserpine (100 μM)	EDTA (250 μM)	Ellagic acid (40μM)	Gallic acid (40 μM)	Tannic acid (40 μM)
Aminocoumarins						
Chlorobiocin (16)	(0) (0) [b]	(0) (0)	(+2) (0)	(+2) (+3)	(0) (0)	(+2) (+1)
Coumermycin (100)	(0) (0)	(0) (0)	(0) (+1)	(+2) (+3)	(0) (0)	(+2) (+1)
Novobiocin (30)	(0) (+2)	(0) (+1)	(+2) (+1)	(+2) (+3)	(0) (0)	(+2) (+1)
Aminoglycosides						
Amikacin (30)	(+1) (0)	(+1) (0)	(+1) (0)	(0) (+2)	(0) (0)	(0) (0)
Gentamicin (10)	(+1) (+1)	(0) (0)	(0) (0)	(0) (+2)	(+1) (0)	(-2) (0)
Neomycin (30)	(+1) (0)	(0) (0)	(0) (0)	(0) (+1)	(0) (0)	(-1) (0)
Tobramycin (10)	(+1) (+1)	(0) (0)	(0) (0)	(0) (+1)	(-1) (-2)	(-1) (-1)
β-lactams						
Ampicillin (10)	(0) (0)	(0) (0)	(0) (0)	(0) (0)	(0) (0)	(0) (0)
Imipenem (10)	(0) (+2)	(0) (0)	(0) (0)	(0) (-1)	(-1) (0)	(-1) (0)
Fluoroquinolones						
Ciprofloxacin (51)	(0) (+2) [b]	(-1) (0)	(+1) (+1)	(-2) (+1)	(-1) (-1)	(-1) (-1)

Table 3.12 Effect of efflux pump inhibitors, a permeabilizer, and plant-derived compounds on the susceptibility of *Acinetobacter baumannii* JVC 1053 and *Acinetobacter baumannii* ATCC 19606 *(Contd.)*

	The changes of antibiotic inhibition zones [a]					
Test antibiotics (µg/disc)	CCCP (50 µM)	Reserpine (100 µM)	EDTA (250 µM)	Ellagic acid (40µM)	Gallic acid (40 µM)	Tannic acid (40 µM)
Macrolides						
Erythromycin (15)	(0) (+1)	(-2) (0)	(0) (+2)	(0) (+2)	(+1) (+1)	(0) (+1)
Azithromycin (100)	(+2) (-2)	(+2) (-2)	(+2) (+2)	(0) (+1)	(-1) (0)	(-2) (-1)
Tetracyclines						
Tetracycline (30)	(0) (+2)	(-2) (0)	(+2) (+2)	(+2) (+2)	(0) (0)	(+2) (+1)
Other antibiotic						
Trimethoprim (100)	(+1) (+1)	(-1) (-2)	(+2) (+1)	(-1) (0)	(-1) (0)	(-1) (-1)
Rifampin (20)	(0) (+1)	(0) (+1)	(0) (+2)	(+2) (+3)	(-1) (+1)	(+2) (+2)
Colistin (10)	(+1) (+1)	(0) (0)	(0) (0)	(0) (0)	(0) (0)	(0) (0)
Fusidic acid (100)	(0) (+3)	(0) (0)	(0) (+1)	(+3) (+2)	(-1) (0)	(+2) (+2)

[a] (+) or (-) the inhibition zones of MHA plate containing test compounds have increased (+) or decreased (-) when compare to MHA plate. The scores were ±3, ±2, and ±1 if the changes in zone size are more than 8 mm, between 4-8 mm, and between 2-4 mm, respectively. (0) the inhibition zones of MHA plate containing test compounds have changed within 1 mm when compare to MHA plate , [b] The changes of antibiotic inhibition zones in the presence of antibiotic adjuvants for; (*A. baumannii* JVC 1053) (*A. baumannii* ATCC 19606)

Table 3.13 Minimum inhibitory concentrations (MIC) of selected antibiotics against *Acinetobacter baumannii* JVC 1053 and *Acinetobacter baumannii* ATCC 19606 in the presence of ellagic and tannic acids

Tested compounds (μM)	MIC values of tested antibiotics (μg/mL)					
	Aminocoumarins			Other antibiotics		
	Chlorobiocin	Coumermycin	Novobiocin	Fusidic acid	Rifampicin	Tetracycline
Without plant-derived compounds	(2) (1)	(62.5) (15.6)	(32) (8) [a]	(500) (125)	(2) (2)	(2) (1)
Ellagic acid						
40	(1) (0.25)	(15.6) (7.8)	(8) (4)	(125) (31.3)	(0.5) (0.25)	(2) (1)
20	(1) (0.5)	(15.6) (7.8)	(8) (4)	(125) (62.5)	(0.5) (0.5)	(2) (1)
10	(1) (0.5)	(15.6) (7.8)	(16) (4)	(250) (62.5)	(1) (1)	(2) (1)
Tannic acid						
40	(0.5) (0.5)	(15.6) (7.8)	(8) (2)	(250) (125)	(1) (0.5)	(2) (0.5)
20	(0.5) (0.5)	(15.6) (7.8)	(8) (2)	(250) (125)	(1) (0.5)	(1) (0.5)
10	(1) (0.5)	(15.6) (7.8)	(16) (2)	(250) (125)	(1) (1)	(1) (0.5)

[a] (*A. baumannii* JVC 1053) (*A. baumannii* ATCC 19606)

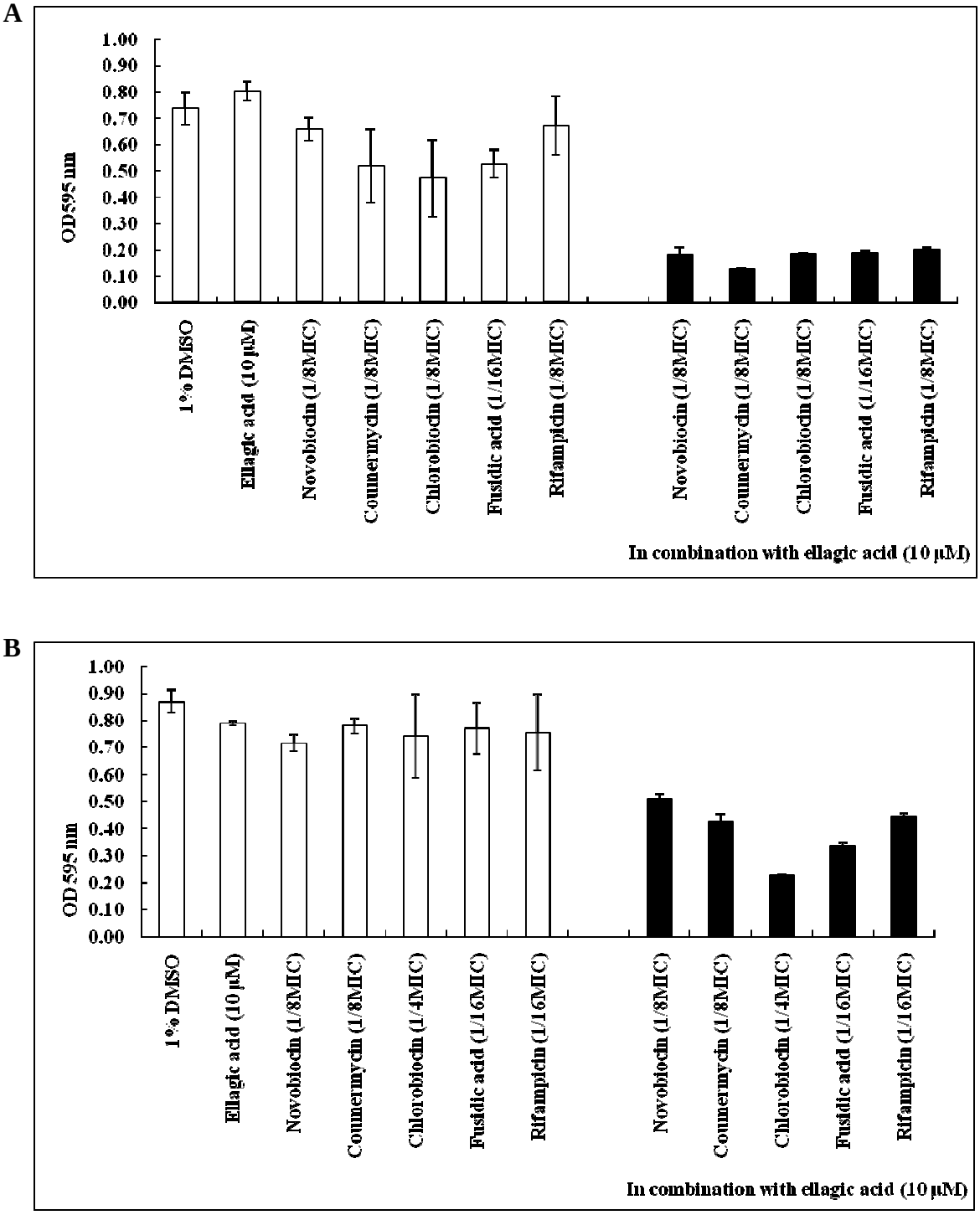

Figure 3.24 Synergistic effects of ellagic acid (10 µM) combined with sub-minimum inhibitory concentrations (MIC) of novobiocin, chlorobiocin, coumermycin, fusidic acid, and rifampin (■) against *Acinetobacter baumannii* JVC 1053 (A) and *Acinetobacter baumannii* ATCC 19606 (B), 1% DMSO was used as positive control. Means±SDs for at least triplicates are illustrated

In addition, the effect of ellagic acid in combination with the aminocoumarins including chlorobiocin (**Figure 3.25** and **3.26**), coumermycin (**Figure 3.27** and **3.28**), and novobiocin (**Figure 3.29** and **3.30**), fusidic acid (**Figure 3.31** and **3.32**), or rifampicin (**Figure 3.33** and **3.34**) on the kinetic growth of the test strains was investigated. Supplementation by all tested concentrations of ellagic acid enhanced the susceptibility of MDR *A. baumannii* cells to novobiocin, coumermycin, and fusidic acid. Susceptibility of these bacteria to chlorobiocin and rifampicin was improved only in the presence of 20 and 40 µM of this compound. In *A. baumannii* ATCC 19606, the antibacterial activities of all tested antibiotic were increased in the presence of 20 and 40 µM of ellagic acid, but slightly stimulated by the addition of 10 µM of this compound. With the exception of chlorobiocin, the growth of MDR *A. baumannii* was almost completely prevented by the combination of ellagic acid (20 and 40 µM) and the test antibiotics after 18 h. For the reference strain, the bacterial growth was totally inhibited by the combination of ellagic acid (20 and 40 µM) and novobiocin, coumermycin or rifampicin after 18 h.

7.2.3 Effect of ellagic and tannic acids on the membrane permeability and active efflux pumps of *A. baumannii*

Results of NPN uptake experiments with the permeabilizer, EDTA (1 mM and 0.25 mM), and the antibiotic adjuvants, ellagic and tannic acids (40µM) are presented in **Figure 3.35**. EDTA weakened the outer membrane of both clinically isolated *A. baumannii* and the reference strain, as indicated by an increase in the NPN uptake. Neither ellagic nor tannic acids enhanced NPN uptake.

Figure 3.36 and **3.37** illustrate the effect of ellagic acid on cell accumulation of EtBr and pyronin Y, respectively. In the presence of different concentrations of ellagic acid, ranging from 3-24 µg/mL, the accumulation of pyronin was dramatically increased while no enhancement of the accumulation of EtBr was observed.

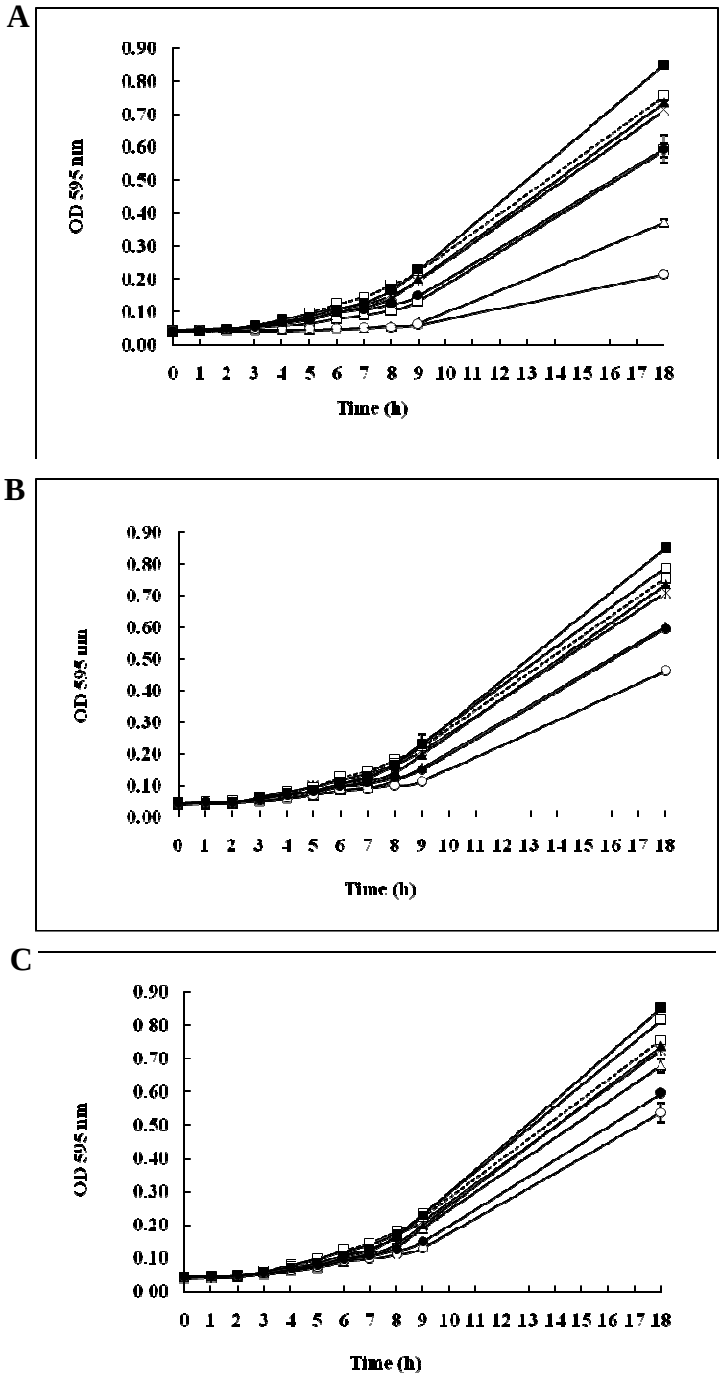

Figure 3.25 The effect of sub-MIC of chlorobiocin (*) including 1/4MIC (A), 1/8MIC (B), and 1/16MIC (C) in combination with 40 (○), 20 (Δ), and 10 μM (□) of ellagic acid on the growth of *Acinetobacter baumannii* JVC 1053. Ellagic acid at 40 (●), 20 (▲), and 10 μM (■) and 1% DMSO (--□--) were used as control. Means±SDs for at least triplicates are illustrated

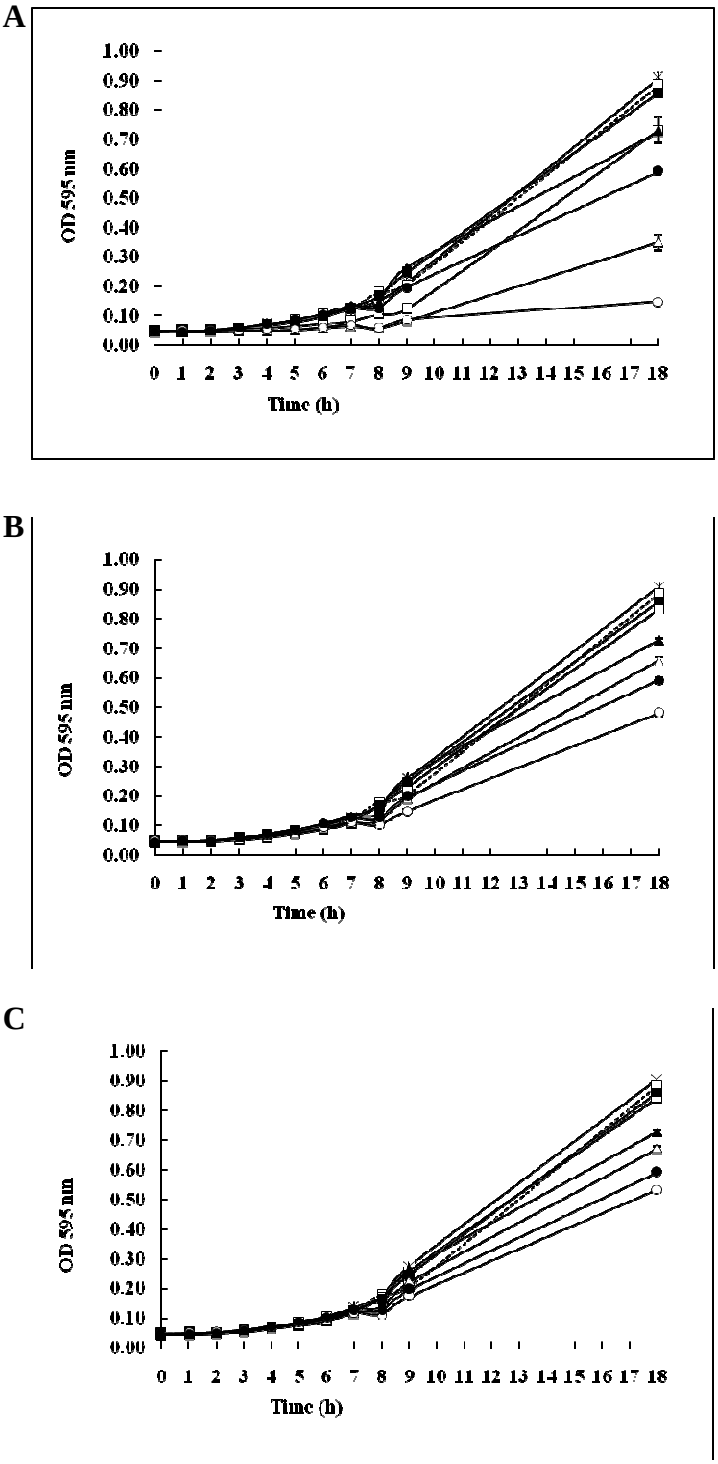

Figure 3.26 The effect of sub-MIC of chlorobiocin (*) including 1/4MIC (A), 1/8MIC (B), and 1/16MIC (C) in combination with 40 (○), 20 (Δ), and 10 μM (□) of ellagic acid on the growth of *Acinetobacter baumannii* ATCC 19606. Ellagic acid at 40 (●), 20 (▲), and 10 μM (■) and 1% DMSO (--□--) were used as control. Means±SDs for at least triplicates are illustrated

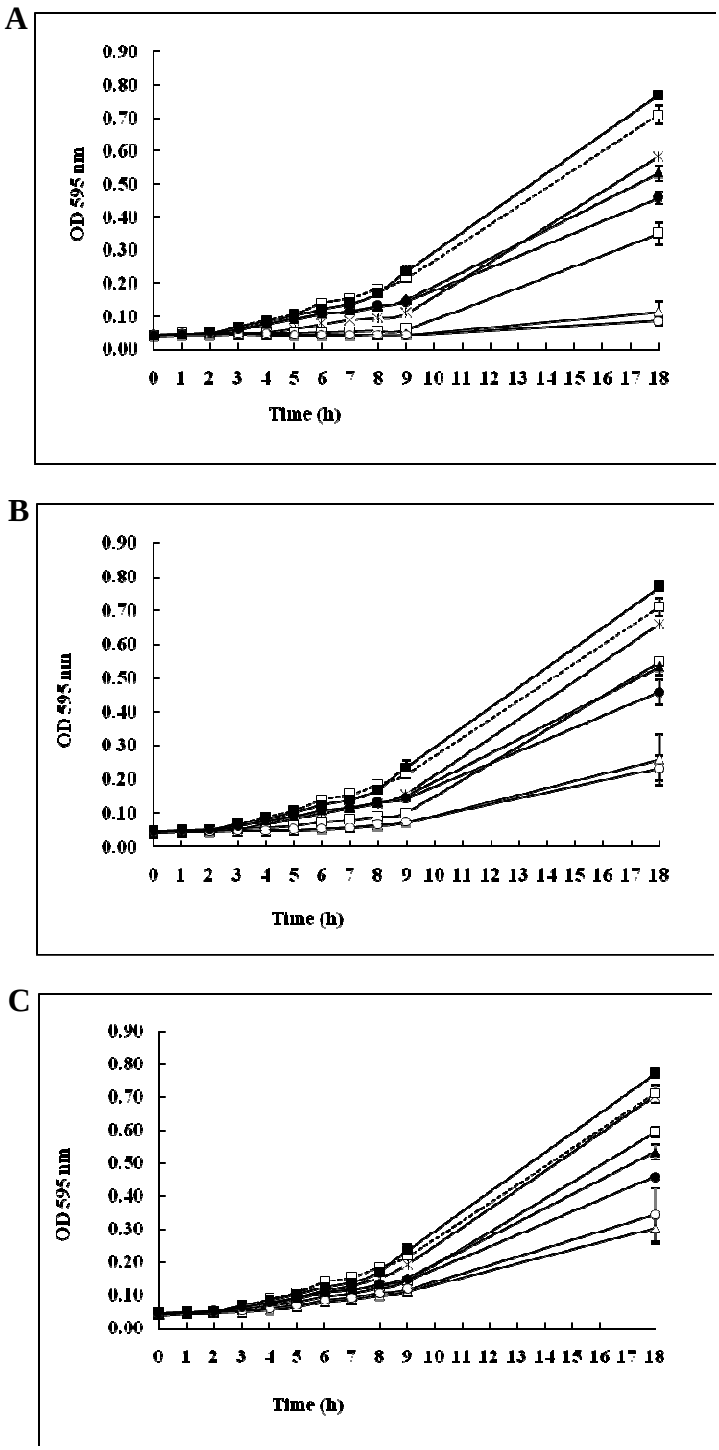

Figure 3.27 The effect of sub-MIC of coumermycin (*) including 1/4MIC (A), 1/8MIC (B), and 1/16MIC (C) in combination with 40 (○), 20 (Δ), and 10 μM (□) of ellagic acid on the growth of *Acinetobacter baumannii* JVC 1053. Ellagic acid at 40 (•), 20 (▲), and 10 μM (■) and 1% DMSO (--□--) were used as control. Means±SDs for at least triplicates are illustrated

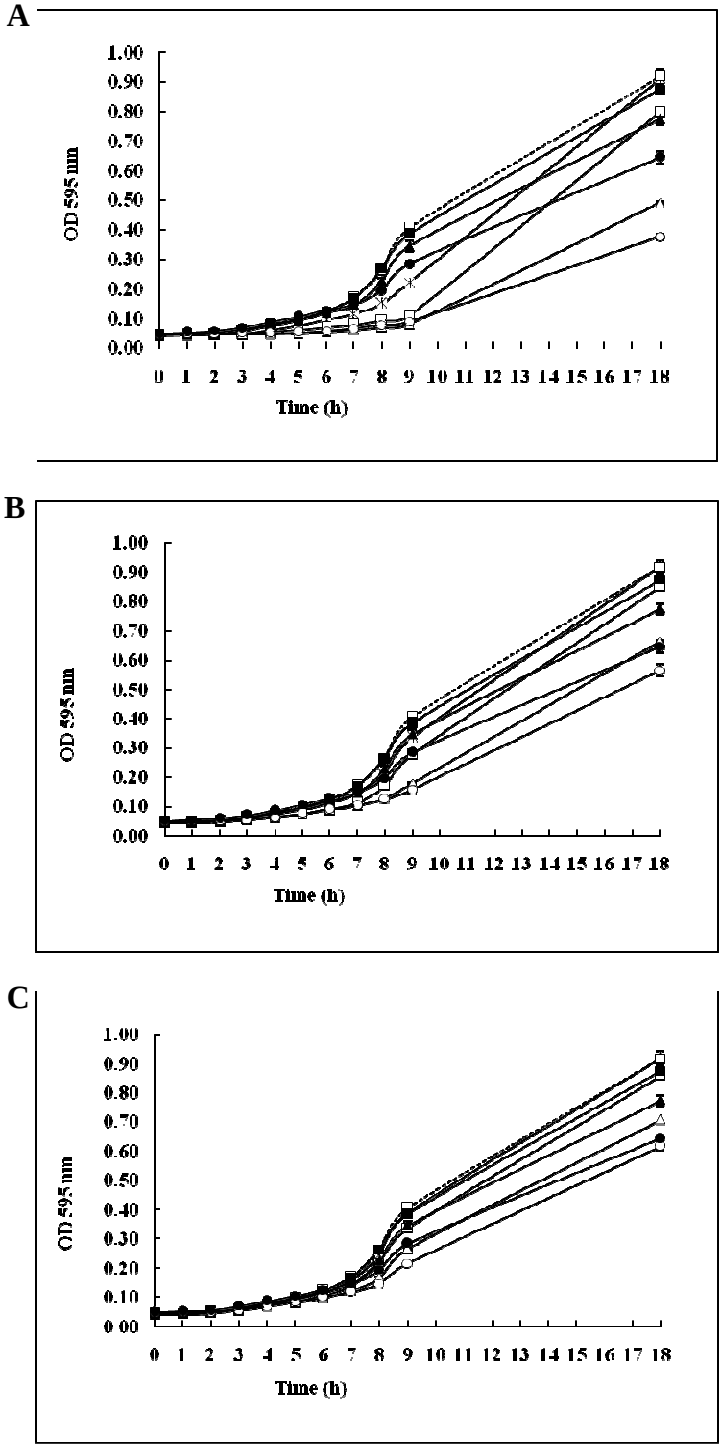

Figure 3.28 The effect of sub-MIC of coumermycin (*) including 1/4MIC (A), 1/8MIC (B), and 1/16MIC (C) in combination with 40 (○), 20 (Δ), and 10 μM (□) of ellagic acid on the growth of *Acinetobacter baumannii* ATCC 19606. Ellagic acid at 40 (●), 20 (▲), and 10 μM (■) and 1% DMSO (--□--) were used as control. Means±SDs for at least triplicates are illustrated

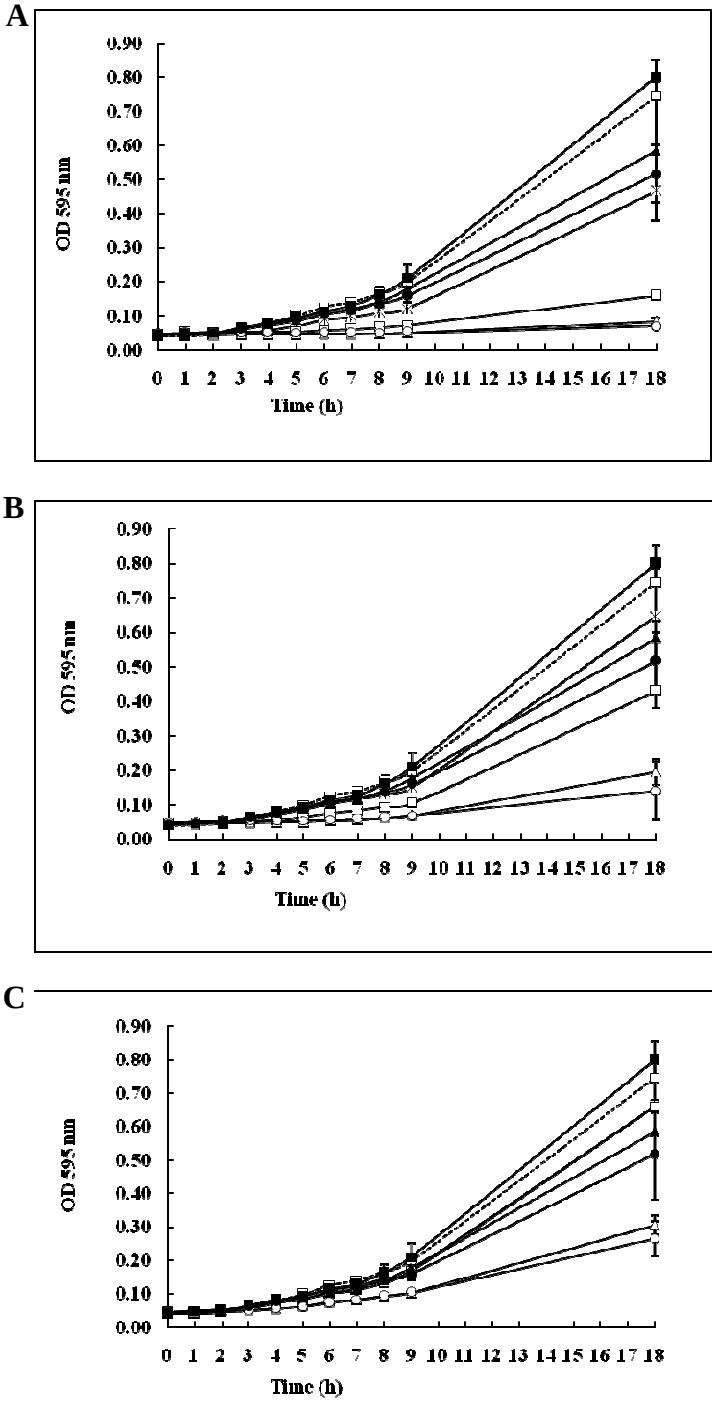

Figure 3.29 The effect of sub-MIC of novobiocin (*) including 1/4MIC (A), 1/8MIC (B), and 1/16MIC (C) in combination with 40 (○), 20 (Δ), and 10 μM (□) of ellagic acid on the growth of *Acinetobacter baumannii* JVC 1053. Ellagic acid at 40 (●), 20 (▲), and 10 μM (■) and 1% DMSO (--□--) were used as control. Means±SDs for at least triplicates are illustrated

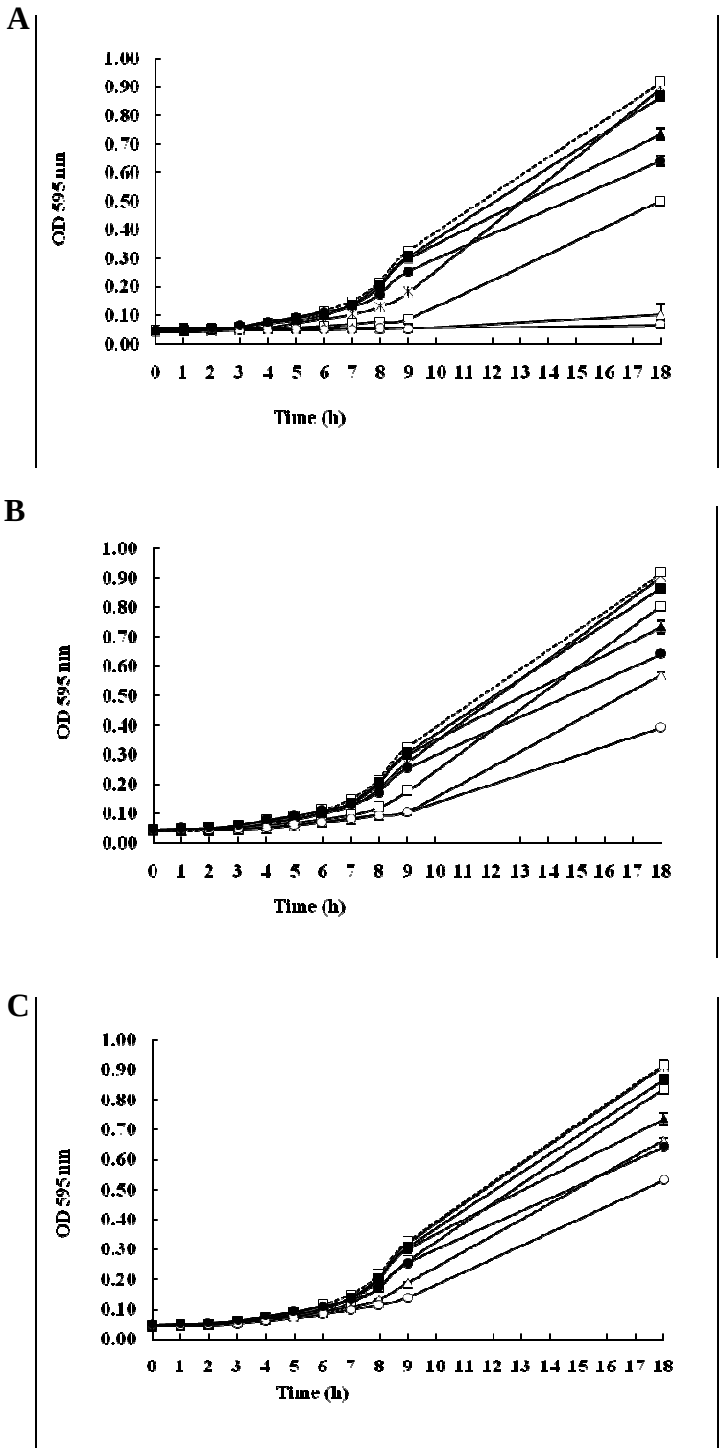

Figure 3.30 The effect of sub-MIC of novobiocin (*) including 1/4MIC (A), 1/8MIC (B), and 1/16MIC (C) in combination with 40 (○), 20 (Δ), and 10 μM (□) of ellagic acid on the growth of *Acinetobacter baumannii* ATCC 19606. Ellagic acid at 40 (●), 20 (▲), and 10 μM (■) and 1% DMSO (--□--) were used as control. Means±SDs for at least triplicates are illustrated

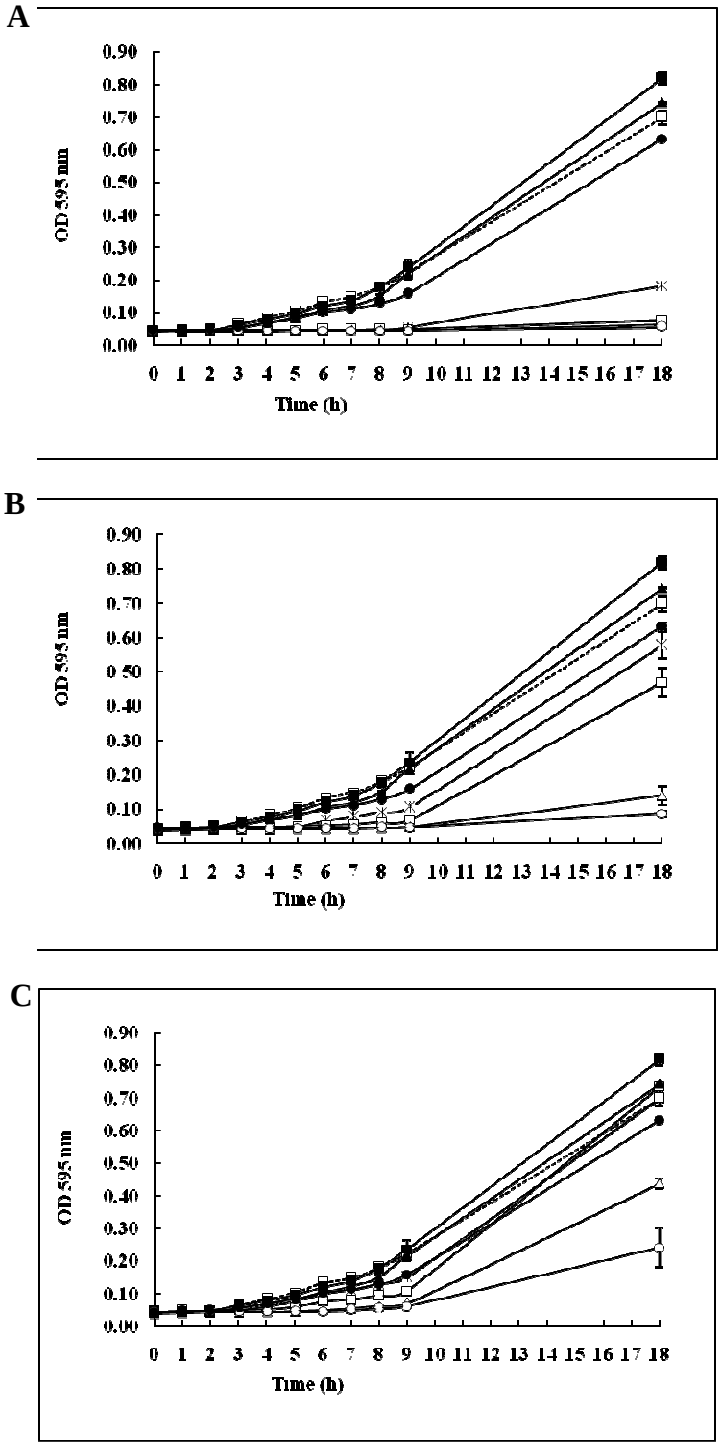

Figure 3.31 The effect of sub-MIC of fusidic acid (*) including 1/4MIC (A), 1/8MIC (B), and 1/16MIC (C) in combination with 40 (○), 20 (Δ), and 10 μM (□) of ellagic acid on the growth of *Acinetobacter baumannii* JVC 1053. Ellagic acid at 40 (•), 20 (▲), and 10 μM (■) and 1% DMSO (--□--) were used as control. Means±SDs for at least triplicates are illustrated

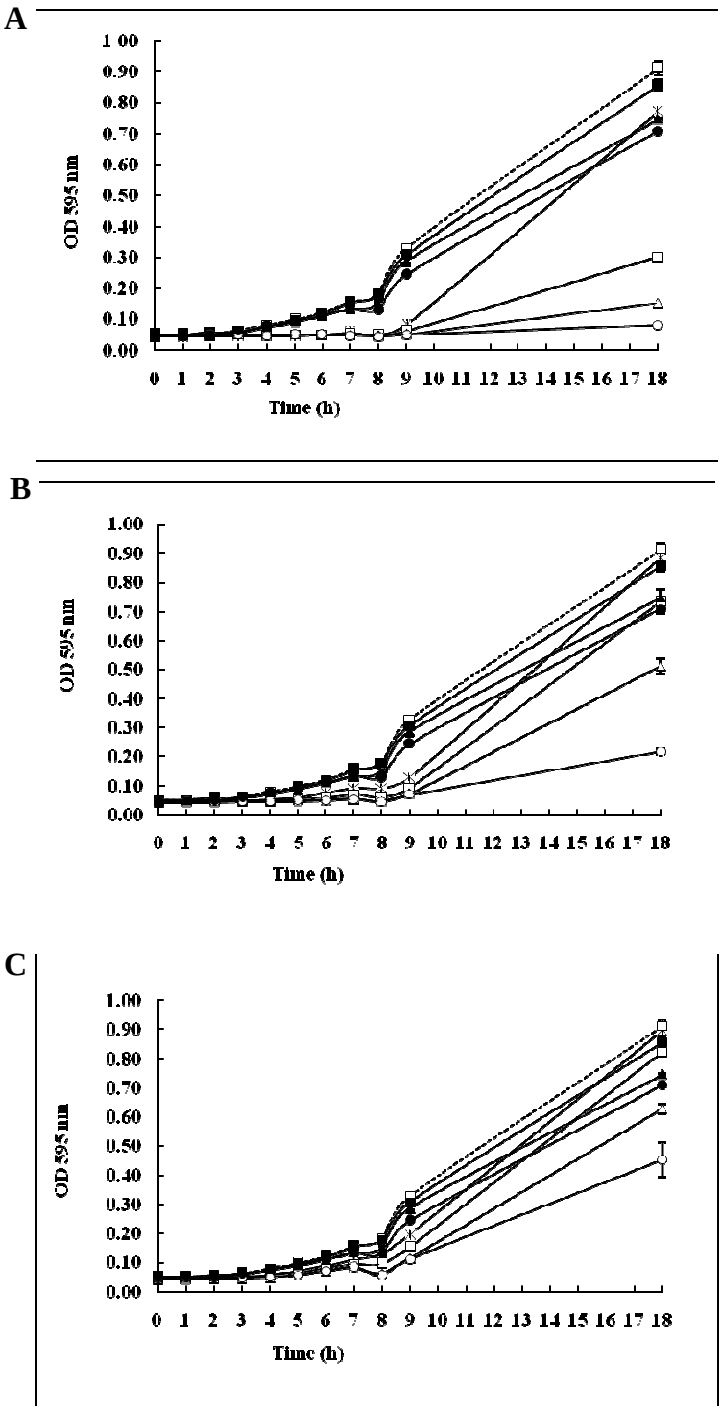

Figure 3.32 The effect of sub-MIC of fusidic acid (*) including 1/4MIC (A), 1/8MIC (B), and 1/16MIC (C) in combination with 40 (○), 20 (Δ), and 10 μM (□) of ellagic acid on the growth of *Acinetobacter baumannii* ATCC 19606. Ellagic acid at 40 (●), 20 (▲), and 10 μM (■) and 1% DMSO (--□--) were used as control. Means±SDs for at least triplicates are illustrated

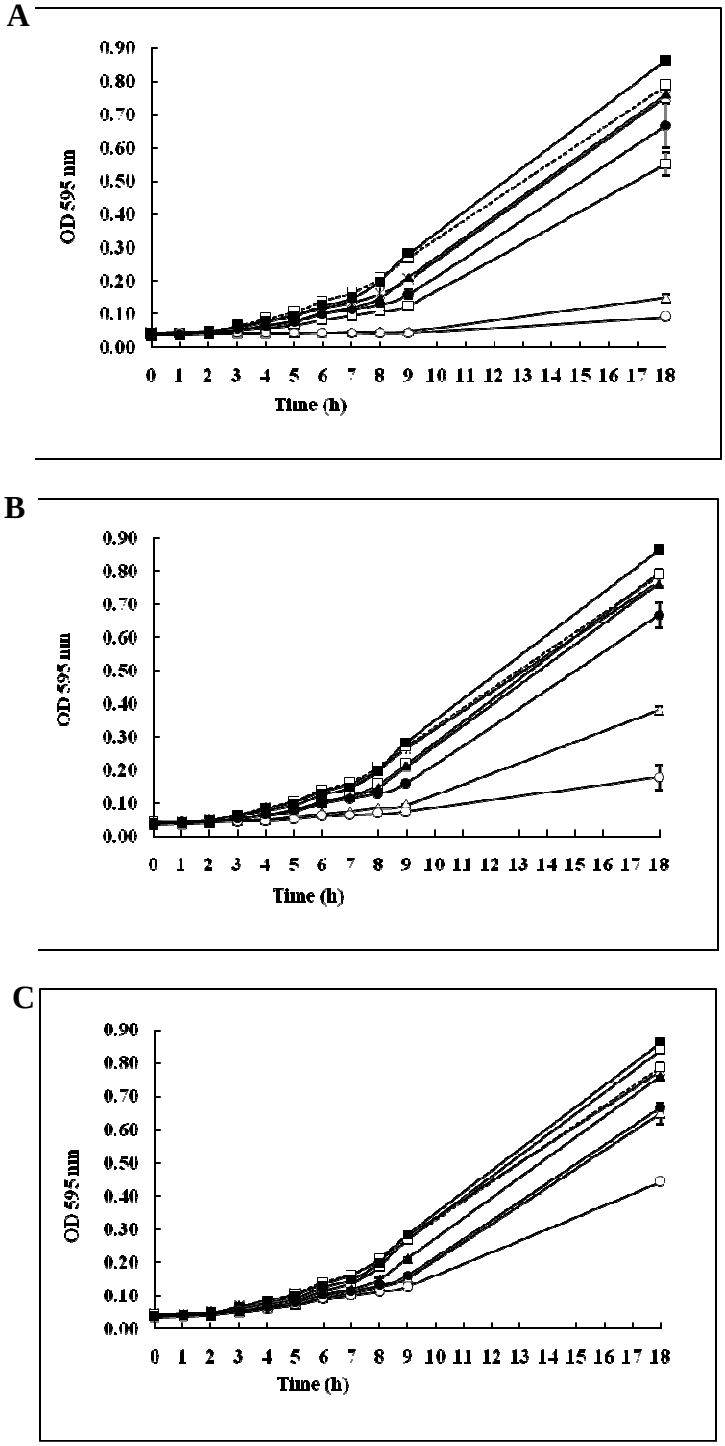

Figure 3.33 The effect of sub-MIC of rifampicin (*) including 1/4MIC (A), 1/8MIC (B), and 1/16MIC (C) in combination with 40 (○), 20 (Δ), and 10 μM (□) of ellagic acid on the growth of *Acinetobacter baumannii* JVC 1053. Ellagic acid at 40 (●), 20 (▲), and 10 μM (■) and 1% DMSO (--□--) were used as control. Means±SDs for at least triplicates are illustrated

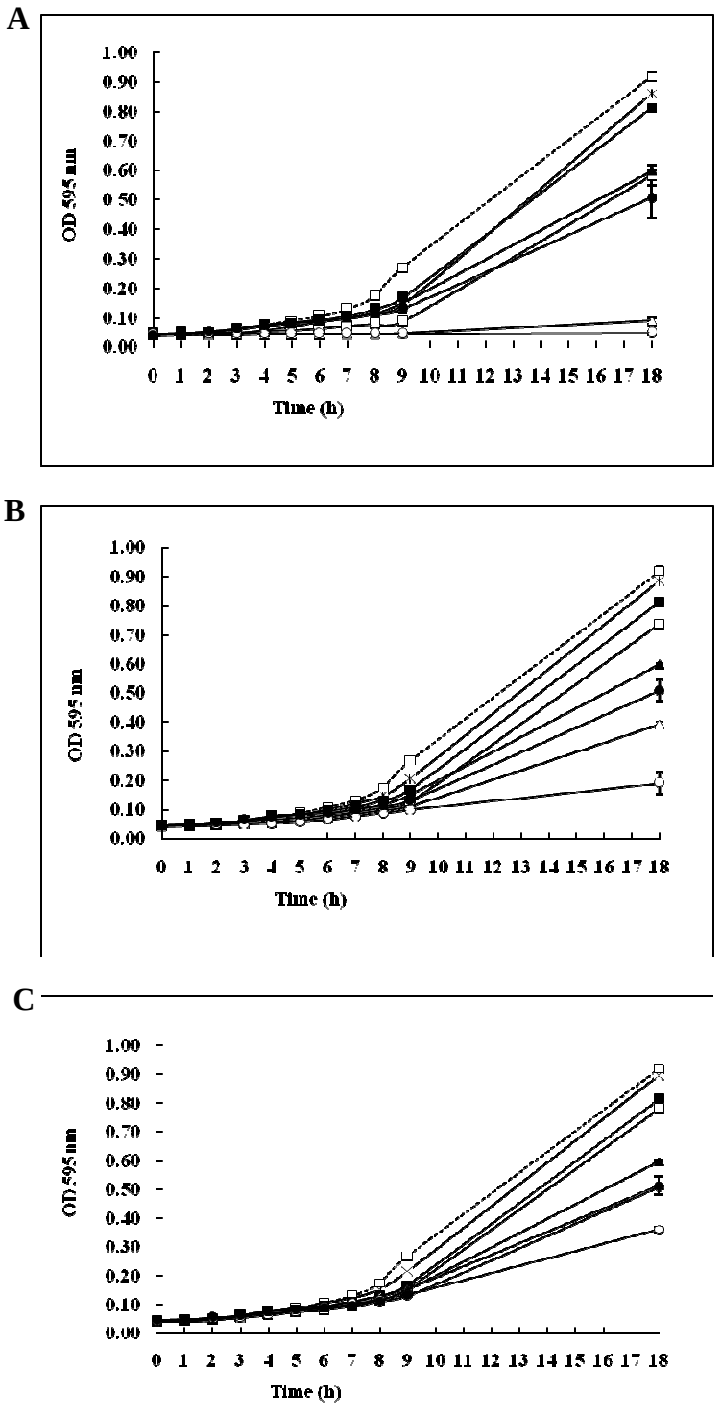

Figure 3.34 The effect of sub-MIC of rifampicin (*) including 1/4MIC (A), 1/8MIC (B), and 1/16MIC (C) in combination with 40 (○), 20 (Δ), and 10 μM (□) of ellagic acid on the growth of *Acinetobacter baumannii* ATCC 19606. Ellagic acid at 40 (•), 20 (▲), and 10 μM (■) and 1% DMSO (--□--) were used as control. Means±SDs for at least triplicates are illustrated

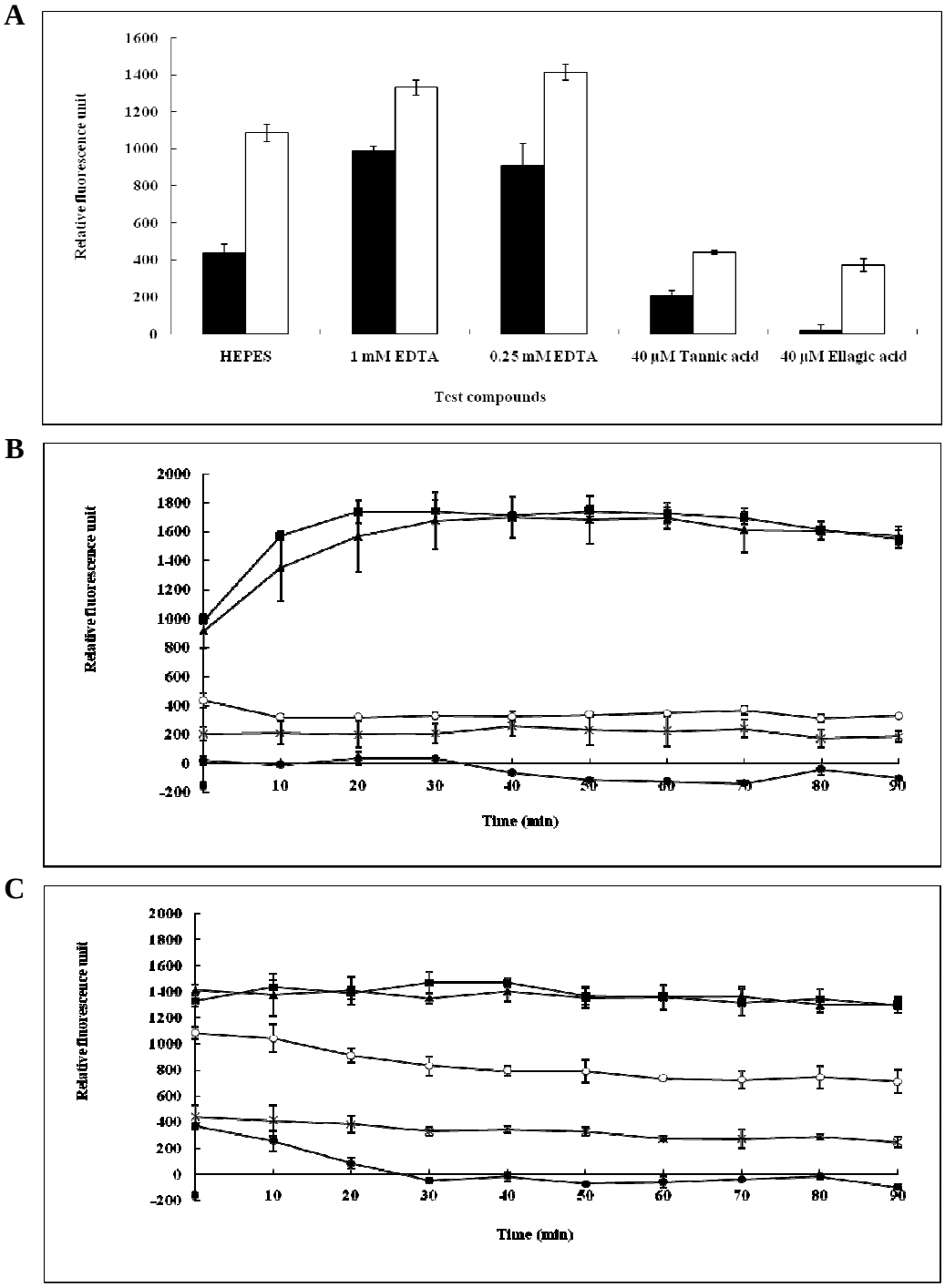

Figure 3.35 Uptake of NPN by *Acinetobacter baumannii* JVC 1053 (■) and *Acinetobacter baumannii* ATCC 19606 (□) in the presence of EDTA, ellagic acid, and tannic acid (A). Time course of membrane permeabilization by ellagic acid (•), tannic acid (*), and EDTA (1 mM; ■ and 0.25 mM;▲) of *A. baumannii* JVC 1053 (B) and *A. baumannii* ATCC 19606 (C). HEPES buffer (○) was used as negative control. Means±SDs for at least triplicates are illustrated

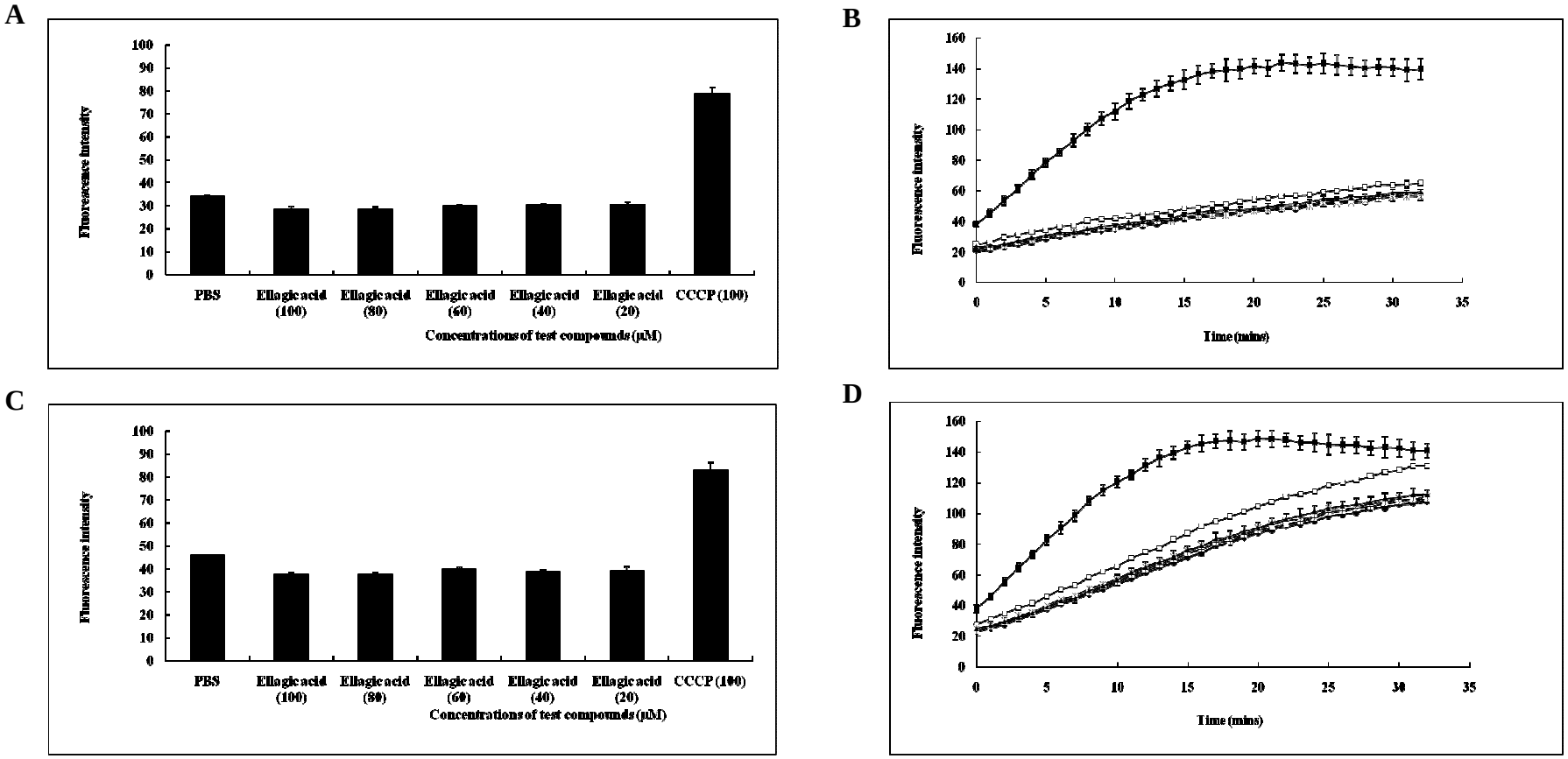

Figure 3.36 Intracellular accumulation of EtBr in ellagic acid treated *Acinetobacter baumannii* JVC 1053 (A and B) and *Acinetobacter baumannii* ATCC 19606 (C and D), the present results are the EtBr fluorescence intensity that measured at 5 min (A and C) and the time course of the fluorescence intensity (B and D). The means±SDs for at least triplicates are illustrated (PBS, □; ellagic acid at 20 μM, ▲, 40 μM, |, 60 μM, *, 80 μM,●, 100 μM, ♦; CCCP, ■)

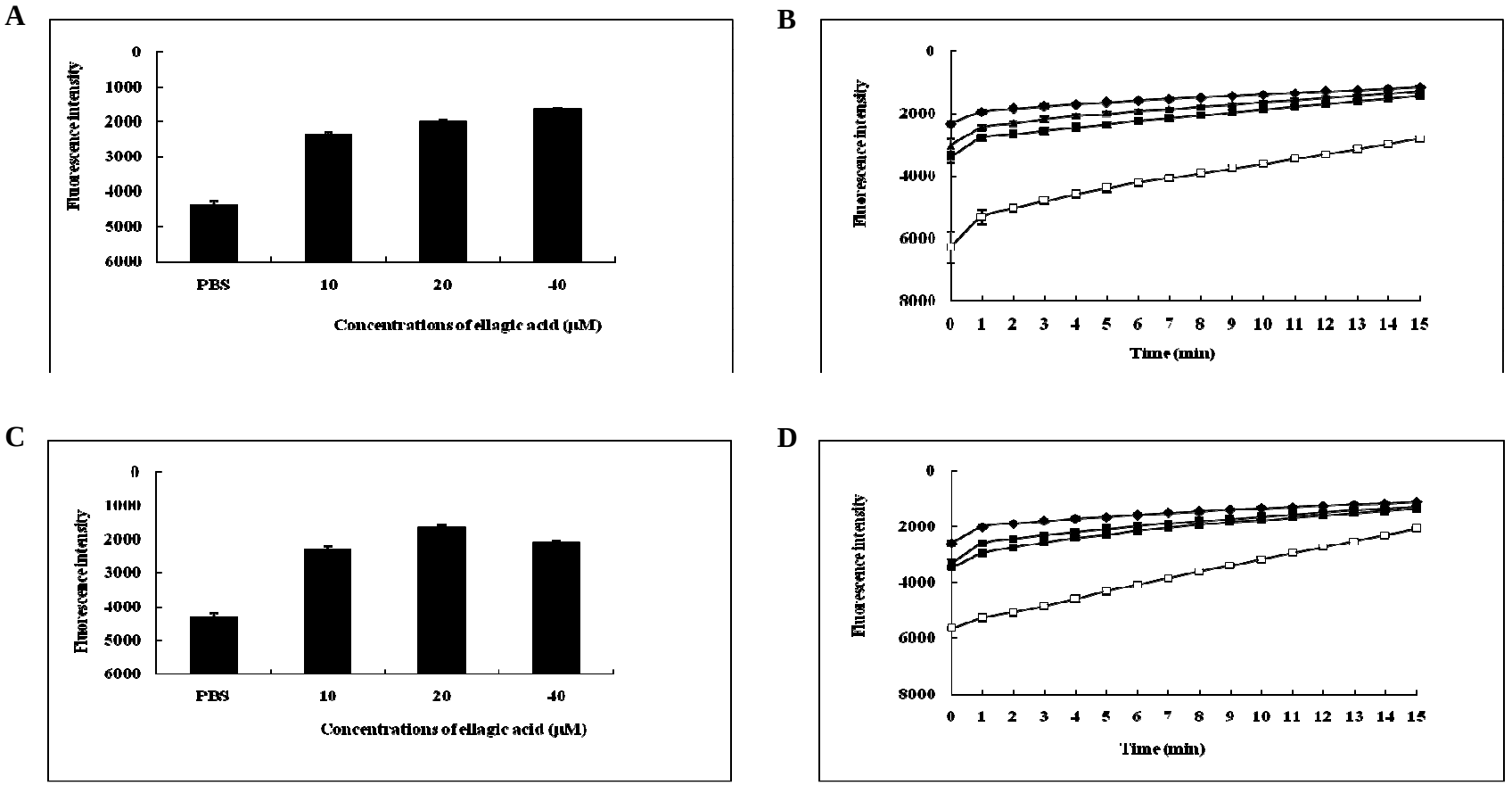

Figure 3.37 Intracellular accumulation of pyronin Y in ellagic acid treated *Acinetobacter baumannii* JVC 1053 (A and B) and *Acinetobacter baumannii* ATCC 19606 (C and D), the present results are the pyronin Y fluorescence intensity that measured at 5 min (A and C) and the time course of the fluorescence intensity (B and D). The means±SDs for at least triplicates are illustrated (PBS, □; ellagic acid at 10 μM, ■; 20 μM, ▲; 40 μM,♦)

CHAPTER 4
DISCUSSION

Staphylococcus aureus colonization of the nose is an endemic risk factor for infectious diseases such as bacteraemia and skin and soft tissue infections in many patient populations (Coates *et al.,* 2009). The rates of *S. aureus* autoinfection, where the *S. aureus* strain detected from the wound matches that swabbed form the nose, tend to lie between 70 to 80% (von Eiff *et al.*, 2001; Perl, 2003). In our study, more than 60% of the healthy volunteers are carriers of *S. aureus*; however there is no methicillin-resistant *S. aureus* (MRSA) was found. MRSA was a more common cause of infection than methicillin-susceptible *S. aureus* (MSSA) but a less frequent colonizer. In USA, more than 30% of the general adult population are colonized, but nasal colonization with MRSA is much lower than 1% of the total population (Tenover *et al.*, 2008).

We further examined adherence characteristics and virulence factors of both infective origin, MRSA and colonization origin, MSSA. Many reports have demonstrated some correlation between their capability to adhere to human cell or biological surfaces and the cell surface hydrophobicity (CSH) and the biofilm formation of microorganisms (Grivet *et al.*, 2000; Malm *et al.*, 2005; Pan *et al.*, 2006). The nature of CSH of infective and colonization origin *S. aureus* were dramatically different. While there was no significant difference in biofilm formation of MRSA and MSSA isolates. The adherence characteristic of *S. aureus* is necessary for the isolates that attached to superficial skin tissue, but not essential for the isolates that infiltrated the deep skin tissues (Akiyama *et al.*, 2000). Contrastly, in coagulase negative staphylococci (CNS) isolates the high hydrophobicity of CNS was more frequently observed in cells of infective origin than in cells of colonization origin (Akiyama *et al.*, 1998). The study of *S. aureus* form skin lesions indicated that the biofilm formation of the isolates appears to be related to the prolongation but not to development of their infection (Akiyama *et al.*, 1998). In clinical settings, biofilms are also closely related to persistent infection (Lindsay and von Holy, 2006). Although, the recently study suggested that there is a relationship between multidrug

resistance and biofilm development (Telang, 2010), we found that most of MRSA and MSSA isolates were identified as moderate adherence. Although, all test *S. aureus* isolates possess the biofilm associated genes, *icaADBC*, approximately 60% of *S. aureus* strains were able to produce biofilm (Arciola *et al.*, 2001; Rohde *et al.*, 2001). The virulence factors produced by *S. aureus* have a wide array of biological properties, including disruption of the epithelial barrier (Reviewed by Iwatsuki *et al.*, 2006). In this report we indicated that MSSA isolates significantly demonstrated higher degree of hydrolysis of haemolysin, lipase, and protease compared with MRSA isolates. Organisms that elaborate small amounts of extracellular products such as lipase, nuclease, and haemolysin may be unable to colonize the skin but may colonize mucus membranes such as anterior nares. In staphylococcal species that express large amounts of lipase, nuclease, and haemolysin may be expected to colonize the skin and cause furuncles (Boden Wastfelt and Flock, 1995). The wound isolate *S. aureus* produced more virulence enzymes and toxins as compared to the nasal isolates from the healthy volunteers, but with the exception of DNase production there was no significant (Daghistani *et al.*, 2000). *S. aureus* is also a pathogen that is frequently involved in device-centered infections and that has been shown to bind specifically to a variety of plasma proteins. Coagulase is a well recognized cell surface associated protein that has been implicated in binding to fibrinogen. Staphylo**c**oagulase binds with prothrombin to form a complex compound called staphylothrombin that can stimulate plasma clotting by converting fibrinogen into fibrin. The formation of fibrin enhances the resistance of *S. aureus* against phagocytosis (Sawai *et al.*, 1997). The inhibition of MRSA staphylocoagulase by siRNA *in vitro* significantly lowered the number of viable MRSA *in vivo* (Yanagihara *et al.*, 2006). Our result showed that all test MRSA isolates exhibited coagulase positive within 4 h, while some MSSA isolates revealed delayed coagulase activity. Almost all *S. aureus* strains carry the coagulase gene, but the levels of expression differ with different serotypes due to transcriptional and post-transcriptional modifications in coagulase negative strains (Rivera *et al.*, 2007).

While MRSA is endemic in many jurisdictions worldwide and creates challenges from infection prevention and treatment perspectives, MSSA is of comparable or greater impact and has been neglected in the vast majority of studies to

date (Niven *et al.*, 2009). Asymptomatic nasal colonization with *S. aureus* (MRSA or MSSA) is common among patients admitted to intensive care unit (ICU) and is a major risk factor for development of ICU-acquired *S. aureus* infections. In this observation, there are different in antibacterial susceptibility patterns, production of virulent factors, and CSH of the colonizing and infecting isolates. But, previous studies have identified that more than 80% (von Eiff *et al.*, 2001) in general hospital populations and 100% (Pujol *et al.*, 1996) in ICU are the same isolates in colonizing and infecting strains. Nasal colonization with *S. aureus* is a significant risk factor for *S. aureus* infections (Foster, 2009), hence eradicating or suppressing *S. aureus* colonization has remained an attractive strategy for preventing infections and transmission. Based on these assumptions, several intervention studies have been performed to eradicate the pathogen in order to reduce the rates of MRSA infections. Decolonization agents such as chlorhexidine, mupirocin, and triclosan have successfully been used to reduce substantial nasal and hand carriage of MRSA. However, emergence of resistant strains as a result of long-term and intermittent usage of the antibiotic has been often reported (Brenwald and Fraise, 2003; Noguchi *et al.*, 2006; Gadepalli *et al.*, 2007). Therefore, the discovery of new disinfectants remains an important challenge to the scientific community, and plants may supply promising materials as sources of novel antibacterial compounds with fewer side effects, wider spectrum of actions, and lower cost.

The search for new ways to control MRSA infections stimulates the investigation of natural compounds as an alternative treatment of these infections. In our ongoing research to investigate Thai medicinal plant derived compounds that exhibit anti-MRSA activity, we found that extracts of *Quercus infectoria* nutgalls had significant antibacterial activity against this pathogen. Acetone, ethyl acetate, ethanol, and aqueous extracts from the nutgalls possessed significant activity against both infective origin, MRSA and colonization origin, MSSA isolates. The ethanol extract that showed the highest extraction yield was further fractionated. The results illustrated that its partially purified fractions, ethyl acetate fraction I and II were highly active against MRSA isolates. Phytochemical constituents such as tannins, saponins, flavonoids, alkaloids, and aromatic compounds are secondary metabolites of plants that serve as defense mechanisms against predation by many microorganisms,

insects and other herbivores (Afolayan and Meyer, 1997; Bonjar, 2004; Yamashita *et al.*, 2009). This can explain the demonstration of antimicrobial activity by the nutgall of *Quercus infectoria*. Hydrolysable tannins (tannic acid) have been reported as the main constituent of the nutgalls (Kaur *et al.*, 2008). In addition, other compounds have been reported from this plant including gallic acid (Kaur *et al.*, 2008), ellagic acid (Onal *et al.*, 2005), flavonoids (Ahmad *et al.*, 1991), hexagalloyl glucose (Hwang *et al.*, 2000), methyl betulate, methyl gallate (Redwane *et al.*, 1998; Redwane *et al.*, 1998), sitosterol, and syringic acid (Dar and Ikram, 1979). Tannic acid is considered as a generally recognised as safe (GRAS) food substance and also as an official in Europe and North America (Akiyama *et al.*, 2001; Pyla *et al.*, 2010). Tannins and tannin derivatives such as gallic acid and ellagic acid are commonly found in many different plant species and foods including tea, cocoa beans, grapes, strawberry, and persimmon (Akiyama *et al.*, 2001; Labieniec and Gabryelak, 2006). Tannic acid is a water-soluble polyphenol containing sugar esters, mainly glucose, and phenol carboxylic acids, such as gallic acid, hexahydroxydiphenic acid, or its stable dilactone ellagic acid (Okuda *et al.*, 1995; Akiyama *et al.*, 2001; Labieniec and Gabryelak, 2006; Rodriguez *et al.*, 2008). Hydrolysable tannins both tannic acid and epigallocatechin gallate have been reported to have antimicrobial activity (Akiyama *et al.*, 2001). Numerous studies have shown a broad antimicrobial activity of tannic acid on *Helicobacter pylori* (Funatogawa *et al.*, 2004), *S. aureus* (Akiyama *et al.*, 2001), enterotoxigenic *Escherichia coli* (Taguri *et al.*, 2004), *Listeria monocytogenes* and *Salmonella enteritidis* (Chung *et al.*, 1990). Similarly, the nutgall extracts also have been reported to have a broad spectrum of antibacterial activity against both Gram-negative (Voravuthikunchai *et al.*, 2004) and Gram-positive bacteria (Hwang *et al.*, 2004; Voravuthikunchai and Kitpipit, 2005). We also found that gallic acid and tannic acid were significantly active against test MRSA isolates and had similar MICs and MBCs to the crude extract. Even though, gallic acid, ellagic acid, and syringic acid have been reported with antibacterial activity on *S. aureus* and *Bacillus cereus* (Aziz *et al.*, 1998; Stapleton *et al.*, 2004; Thiem and Goslinska, 2004), in this study demonstrated that either ellagic acid or syringic acid did not express anti-staphylococcal activity at the test concentrations.

Demonstration of broad spectrum of antibacterial activity by the nutgalls of *Quercus infectoria* may help to discover alternative antibacterial substances that could serve as agents for treatment and control staphylococcal infections. Hence, the anti-MRSA mechanisms are further discussed.

The antimicrobial mechanism of tannic acid could be iron deprivation which may work like a siderophore to chelate essential iron from the medium and make its iron unavailable to the microorganisms (Chung *et al.*, 1998). (Puupponen-Pimia *et al.*, 2005) have reported that polyphenol compounds that contain three hydroxyl groups in the B ring such as flavonol myricetin have more active antimicrobial activity than other flavonols which have less hydroxyl groups in the B ring. When tannic acid, a mixture of polygalloyl glucopyranose is hydrolysed, the hydrolysis ends up forming one more gallic acid and other galloyl groups on gallotanins which could enhance the antimicrobial activity of tannic acid. Although, it is well recognized that both tannic acid and gallic acid bind to and precipitate proteins and enzymes, gallic acid is known to be a less effective antimicrobial than tannic acid (Akiyama *et al.*, 2001). This mechanism could be explained by hydrogen binding of polygalloyl glucopyranose and hydrophobic interactions by gallic acid with surface proteins on bacteria cells. Yang *et al.* (2006) have shown that, tannic acid was bound to the ATP binding pockets of tyrosine kinases in cancer tissues, through unique hydrogen and hydrophobic interactions between the amino acids of tyrosine kinases and groups of tannic acid. Labieniec and Gabryelak (2006) proved that there is a direct interaction between tannic and gallic acids and DNA or bovine serum albumin (BSA) and this interaction causes the conformational changes in DNA and BSA. A sufficient number of galloyl groups are needed to form a strong binding between ligand and BSA in a polyphenol molecule (Kawamoto *et al.*, 1997). Even though gallic acid is not as effective as tannic acid (Akiyama *et al.*, 2001), an increased number of hydrolysed gallic acid and galloyl groups on PTA could enhance the antimicrobial activity by increasing their protein binding capacity (Kawamoto *et al.*, 1997). Although, the ethanol extract, the ethyl acetate fraction I, gallic acid, and tannic acid failed to lyse MRSA cells, these test materials with the exception of gallic acid could strongly reduce the tolerance of MRSA to the low osmotic pressure and high ionic strength. It has been well-documented that *S. aureus* is highly resistant to

NaCl and can grow well at low osmolarity, suggesting an efficient means of regulating cytoplasmic osmolarity. Halotolerance in *S. aureus* is determined by an ability to accumulate osmoprotective molecules such as choline, glycine betains, and L-proline (Graham and Wilkinson, 1992) and by the extrusion of Na^+ driven by a Na^+/H^+ antiporter systems (Hiramatsu *et al.*, 1998). Sublethal injury of microbial cell membrane provided by subinhibitory concentrations of antimicrobial compounds may alter their permeability and affect the ability of the membrane to osmoregulate the cell adequately or to exclude toxic material. (Carson *et al.*, 2002) found that the treatment of *S. aureus* with *Melaleuca alternifolia* essential oil or its components (terpinen-4-ol, a-terpineol, 1,8-cienol) caused a significant loss of tolerance to NaCl. Moreover, membrane damage in *S. aureus* cells that caused by *Origanum vulgare* essential oil was also indicated by the loss of salt tolerance of the treated cells (de Souza *et al.*, 2010). The plant polyphenols, proanthocyanidins, epicatechin, and epigallocatechin gallate, which display that their anti-MRSA property are associated with the inhibitory effect of the bacterial membrane, significantly decreased the viability of treated cells in low and high osmotic pressure (Kusuda *et al.*, 2006; Stapleton *et al.*, 2006). Epigallocatechin gallate caused leakage of 5, 6-carboxyfluorescein from phosphatidylcholine liposomes, but not epicatechin that demonstrated very weak bactericidal activity (Ikigai *et al.*, 1993). (Caturla *et al.*, 2003) demonstrated that the killing results from bacterial membrane damage possibly through the interaction of the catechin with phosphatidylethanolamine, phospholipids present in bacterial membrane. Since the increased susceptibility to the low osmotic pressure and high concentration of NaCl with no cell lysis were found in the treated cells, it is proposed that the anti-MRSA mechanisms of *Quercus infectoria* components could additionally be the result from bacterial membrane damage.

In addition, the ethanol extract of nutgall, the partially-purified fraction, and the components did not appear to alter membrane permeability, but the ethanol extract and the ethyl acetate fraction of the nutgalls significantly gave rise to pseudomulticellular aggregates with thickened cell walls. This has been reported previously using antibiotics such as chloramphenicol, penicillin, and other β-lactam antibiotics (Giesbrecht *et al.*, 1998). Similar pseudomulticellular aggregates with thickened internal cell walls have also been detected in MRSA treated with an active

principle of crude tea extract (*Camellia sinensis*) (Hamilton-Miller and Shah, 1999). Condensed tannins including catechin, epicatechin, epicatechin gallate, and epigallocatechin gallate are well-recognized as the main component of tea extract. Recent studies indicate that *S. aureus* cells treated with epicatechin gallate also produce thickened cell walls (Stapleton *et al.*, 2004b). In addition, epigallocatechin gallate, mixed with *S. aureus* cells at 4MIC, gave rise to a moderate increase in permeability of the staphylococcal cytoplasmic membrane (Stapleton *et al.*, 2004b). Lately, it has been reported that staphylococcal daughter cells in cultures exposed to epigallocatechin gallate, as evidenced by the pseudomulticellular appearance and by more than 2-fold increase in cell wall thickness. But the adaptation to EGCG led to a moderate increase in heat resistance of staphylococcal strains including *S. epidermis* ATCC 12228, *S. aureus* Newman, and *S. aureus* ATCC 29213, and an extremely pronounced increase for *S. aureus* ATCC 6538 and *S. aureus* RN4220 (Bikels-Goshen *et al.*, 2010). Many experimental conditions that have the ability to induce the formation of these pseudomulticellular staphylococci have been reviewed. All of the conditions for example, divalent cations, negatively charged anticoagulants, sodium dodecyl sulfate, and triton X-100 interfered both directly or indirectly with the activity of wall autolysins involved in cell separation (Giesbrecht *et al.*, 1998). It is therefore most likely that the anti-MRSA mechanism of the plant extract should involve interference with the staphylococcal autolysins.

Decolonization may be defined as treatment to eradicate *S. aureus* or MRSA carriage. Potential benefits of decolonization include decreased risk of subsequent staphylococcal infection and prevention of staphylococcal transmission to reduce endemic rates of infection or manage outbreaks (Simor and Daneman, 2009). In recent year, increasing attention has been focused on phytochemicals as medicinal decolonization agents. For example, tea tree oil, an essential oil steam distilled from the leaves of the Australian native plant *Melaleuca alternifolia*, is considered as an effective decolonization agent for MRSA both *in vitro* (LaPlante, 2007) and *in vivo* (Caelli *et al.*, 2000). Even though, the use of plants against skin disease is a common practice in the popular medicine of most cultures, the precise causation of disease and mechanism of cure is not always understood (Grierson and Afolayan, 1999; Srinivasan *et al.*, 2001). As a consequence of the well recognized activity of *Quercus*

infectoria nutgalls is anti-ulceration. Therefore, the effect of the extract from the nutgalls of *Quercus infectoria* as well as tannic acid, its major component with anti-MRSA activity, on the biofilm formation and CSH of infective origin, MRSA and colonization origin, MSSA were further discussed as an alternative decolonization agent**.**

Biofilm formation is a key factor in the establishment and persistence of staphylococcal infections in humans and animals, and biofilm formation on tissues or on medical devices is an important first step in the pathogenesis of *S. aureus* infection in humans. A recent study has demonstrated that the anti-biofilm activity of medicinal plants used for skin and soft tissue infection is significantly greater than plants without any ethnomedical applications (Quave *et al.*, 2008). The ethanol extract at MIC and sub-MIC (1/2MIC-1/16MIC), were significantly able to reduce the biofilm formation of MRSA. Even though, tannic acid is the main component in the extract, the compound is found to be a less effective anti-biofilm than the extract. Surprisingly, when exposed to sub-MIC of both the ethanol extract and tannic acid, there was no reduction in the biofilm formation of the MSSA isolates. However, the biofilm formation of some test MSSA isolates was inhibited by the ethanol extract and tannic acid at MIC. It should be stated that at the sub-MIC of the plant extract prevent bacterial adhesion and biofilm formation by MRSA only. Staphylococcal biofilm formation is known to be mediated through the production of the extracellular polysaccharide adhesin termed both polysaccharide intercellular adhesin (PIA) and poly-*N*-acetyl glucosamine (PNAG) that are synthesized by the *icaADBC* genes and also has a function in cell aggregation (Heilmann *et al.*, 1996; McKenney *et al.*, 1998; Mack *et al.*, 1999; O'Gara, 2007). An *icaB* deletion mutant of *S. aureus* exhibits deficiencies in biofilm formation, immune evasion, as well as a decreased ability to survive in the blood of infected mice (Cerca *et al.*, 2007). In addition, wall teichonic acids (WTAs), a staphylococcal cell wall associated component, which plays an important role for the bacterial interaction with human nasal epithelial cell s as well as with endothelial cells (Weidenmaier *et al.*, 2004; Weidenmaier *et al.*, 2005). WTAs has a highly variable content of D-alanine ester substitutions (Fischer, 1994; Neuhaus and Baddiley, 2003), the absence of D-alanine esters in *S. aureus* mutant also causes a deficiency in their capacity to form biofilms on polystyrene or glass surfaces (Gross *et*

al., 2001). The recent study showed that epicatechin gallate-grown MRSA produces large reductions in D-alanylation of WTAs (Bernal *et al.*, 2009). Galloyl catechins promote cell wall thickening and cell aggregation (Hamilton-Miller and Shah, 1999) also reduce slime production and inhibit the formation of staphylococcal biofilms (Blanco *et al.*, 2005; Stapleton *et al.*, 2007). WTA-deficient mutants by deletion of the *tagO* gene in *S. aureus*, showed a higher degree of cell aggregation, had reduced initial adherence to abiotic surfaces and had a reduced capacity to form biofilms under both steady-state and flow conditions (Vergara-Irigaray *et al.*, 2008). A current work has also demonstrated that *S. aureus ypfP* mutant with failure in lipoteichoic acid production displayed a significant increase in their hydrophobicity index (HPBI) but dramatically lose their adhesion ability on polystyrene microtiter plates (Fedtke *et al.*, 2007).

The adherence of microorganisms is not only mediated by a variety of specific factors like bacterial adhesins but also by nonspecific factors such as hydrophobicity and electrostatic charge (Lerebour *et al.*, 2004). CSH is one of the most important factors that govern initial bacterial adhesion to various surfaces such as medical devices (Hanlon *et al.*, 2004), teeth (Prabu *et al.*, 2006), contact lenses (Bruinsma *et al.*, 2001), and glass surface (Nostro *et al.*, 2004). Many reports have suggested that the influence of anti-infective agents on CSH of bacterial cells would be important for anti-adhesion and anti-biofilm formation of the treated organisms (Nostro *et al.*, 2004; Prabu *et al.*, 2006; Walencka *et al.*, 2007; Wojnicz and Jankowski, 2007). Although, the nature of CSH of infective and colonization origin *S. aureus* were dramatically different, we found that the plant extract increased the HPBI of both MRSA and MSSA isolates. Several researchers have reported that sub-MIC values of some antibiotics normally decrease the surface hydrophobicity and the ability of bacteria to adhere to epithelial (Furneri *et al.*, 2003; Kustos *et al.*, 2003; Wojnicz and Jankowski, 2007). Contrastly, a limited study on plant extracts revealed both enhancing and lowering of the CSH of bacteria (Annuk *et al.*, 1999; Dykes *et al.*, 2003; Nostro *et al.*, 2004; Ishida *et al.*, 2006; Prabu *et al.*, 2006; Voravuthikunchai and Limsuwan, 2006). In order to elucidate which compounds of the ethanolic extract of the nutgall interfered with CSH of *S. aureus*, we observed the effect of tannic acid, a main constituent of the nutgall extract (Kaur *et al.*, 2008), on CSH of both high

hydrophobic and low hydrophobic *S. aureus* isolates. It is noteworthy that pure tannic acid, as tested by BATH assay, showed similar results to the plant extract, suggesting that tannic acid may be the component of the herb extract with highest effect in relation to its consequence on CSH of MRSA and MSSA. Similarly, an enhancement of cell aggregation of all test isolates of *Helicobacter pylori* by salt aggregation test was observed in the presence of bearberry and cowberry extracts, the plant extracts with a high amount of tannins (Kustos *et al.*, 2003). Recently, the influence of the ethanolic extract of bearberry on the CSH of 25 food-related bacteria was determined by BATH assay. The presence of this extract caused a significant increase in HPBI of most test bacteria (14/25), while a significant decrease in HPBI was reported in four bacteria (Dykes *et al.*, 2003). Previous works have documented that the increase in bacterial surface hydrophobicity is strongly related to the increase in ability of specific bacterial pathogens to cause infection (Absolom, 1988; Andersson *et al.*, 1998). The significant effect of the extract and its main component on *S. aureus* cell surface hydrophobicity that may cause to reduced staphylococcal biofilm formation, leads the extract to be a promising plant material for further development as an alternative decolonization agent for prevention and control of the spread of MRSA infections.

It is well recognized that epicatechin gallate promotes retention of autolysins, enzymes that play a key role in cell separation and peptidoglycan turnover in the cell wall, probably in a mainly inactive form (Stapleton *et al.*, 2007). In addition, the growth of MRSA in broth supplemented with epicatechin gallate markedly decreases susceptibility to lysostaphin, a peptidoglycan hydrolase that cleaves pentaglycine cross-bridges between glycan strands within peptidoglycan (Iversen and Grov, 1973). (Stapleton *et al.*, 2007) further demonstrated that peptidoglycan-WTAs complexes extracted from cells grown in epicatechin gallate-supplemented medium are less susceptible to lysostaphin hydrolysis than complexes from bacteria grown in none supplemented medium. But after removal of WTA, rates of peptidoglycan hydrolysis by lysostaphin were essentially identical, regardless of the source of substrate. The information from that study suggests that epicatechin gallate also induces alterations in the composition, structure or conformation of WTA. The observations made in this study similar to those of a previous study, the anti-

staphylococcal mechanisms of *Quercus infectoria* extract and its main component may not only involve the staphylococcal autolysins but also affect with biophysical properties of bacterial cytoplasmic membrane functions and at least induce the changes in WTA.

It is also well known that the nutgall of *Quercus infectoria* is one of the most commonly used plants in a traditional Asian medicine to treat skin infections, gastrointestinal disorders, and vaginal infections (Dayang *et al.*, 2005; Voravuthikunchai *et al.*, 2006; Kaur *et al.*, 2008). The nutgalls are known to produce various bioactive compounds, including gallotannin with anti-tumor activity (Gali *et al.*, 1993), polyphenols with anti-venom activity (Pithayanukul *et al.*, 2005), gallic acid, methyl gallate, and ellagic acid with anti-oxidant activity (Hamid *et al.*, 2005; Krishnaraju *et al.*, 2005). In this communication, we reported that that the extract demonstrated excellent scavenging activities and good anti-malarial activity. Even though the extract has commonly used as anti-inflammatory agents, *in vitro* study of the extract, there is no anti-inflammatory activity. According to the high extracted yield and variety of biological properties, *Quercus infectoria* nutgalls may be a new, cheap and effective weapon for treating and preventing *S. aureus* infections, including MRSA.

Plants are known to produce an enormous variety of small-molecule phytoalexins containing terpenoids, glycosteroids, flavonoids, and polyphenols. Interestingly, most of these small molecules exhibit weak antibiotic activity, several orders of magnitudes less than that of common antibiotic produced by bacteria and fungi. Despite the fact that plant-derived antibacterial agents are less potent, plants fight infections successfully. Hence, it becomes apparent that plants may adopt a different paradigm synergy to fight infections. A case in study to reiterate this view is the observation on the combined action of berberine and 5′-methoxyhydnocarpin, both of which are produced by berberry plants. Berberine, a hydrophobic alkaloid that intercalates into DNA, is ineffective as an antibacterial because it is readily extruded by bacterial efflux pumps. Hence, the plant produces 5′-methoxyhydnocarpin that blocks the pumps (Stermitz *et al.*, 2000a; Stermitz *et al.*, 2000b). This combination is a potent antibacterial agent. Several studies have indicated that epicatechin gallate and epigallocatechin gallate have the capacity to

reduce oxacillin resistance in *S. aureus* (Shiota *et al.*, 1999; Hamilton-Miller and Shah, 2000; Stapleton and Taylor, 2002; Stapleton *et al.*, 2004a). The peptidoglycan binding capacity of epigallocatechin gallate was discussed as being the reason for its synergistic effect with oxacillin. However, (Zhao *et al.*, 2001) have mentioned that the binding of epigallocatechin to penicillin-binding protein 2′ was not relevant to any synergy with β-lactam antibiotics. In addition, the observed restoration of β-lactam antibiotic activity might be explained by the presence of hydrolysable tannins such as corilagin, tellimagrandin I, proanthocyanidin that suppressed the activity of β-lactamase (Shiota *et al.*, 2004; Kusuda *et al.*, 2006). It was therefore surprising that although restoration of oxacillin activity by the plant extract was not observed, most of the tested strains demonstrated a synergistic effect between the nutgall ethanol extract and the β-lactamase-susceptible penicillins, amoxicillin (76% of strains) and penicillin (53% of strains) whereas a synergistic effect was produced by only 12% of strains with oxacillin. The results of our work indicate that at least part of the synergistic activity may be caused by the effect of the active constituents of the nutgall ethanol extract on the function or production of β-lactamase.

Acinetobacter baumannii are ubiquitous, non-lactose-fermenting Gram-negative coccobacilli that have emerged in recent years as a major cause of nosocomial infections associated with significant morbidity and mortality rates (Choi *et al.*, 2005; Lee *et al.*, 2007). *Acinetobacter* infections are difficult to treat, due to both the pathogen's intrinsic resistance and its ability to readily acquire new resistance mechanisms (Magnet *et al.*, 2001; Vila *et al.*, 2007; Damier-Piolle *et al.*, 2008; Karageorgopoulos and Falagas, 2008). It has been suggested that we are closer to the end of the antibiotic era with *Acinetobacter* than with MRSA (Hanlon, 2005; Giamarellou *et al.*, 2008). There is significant interest in plant compounds which inhibit bacterial efflux pumps, such as the plant alkaloid reserpine, which is an efflux pump inhibitor (EPI) for NorA (Neyfakh *et al.*, 1993; Schmitz *et al.*, 1998) and TetK (Gibbons and Udo, 2000). Unfortunately, the concentrations of this compound required for inhibition cause toxic reactions in humans (Gibbons *et al.*, 2003). According to recent reviews (Stavri *et al.*, 2007; Hemaiswarya *et al.*, 2008), no effective natural EPIs for use with *A. baumannii* are available; therefore there is a need for antibiotic adjuvants acting EPIs clinically. *Quercus infectoria* components

display a variety of biological activities; hence we have further tested the antibiotic adjuvant activity of the plant components and other plant phenolics against MDR *A. baumannii* using novobiocin as model. We have shown that certain plant phenolic compounds enhance the activity of a variety of antibiotics against MDR *A. baumannii*. In particular, ellagic acid at 40 µM (12 µg/mL), which has no intrinsic antibacterial activity, in combination with 1/4MIC of the GyrA inhibitor novobiocin, led to a marked decrease in the bacterial growth. The antibiotic adjuvant abilities of this compound (changes in antibacterial susceptibility patterns of *A. baumannii* strains in the presence of ellagic acid) were compared to those in the presence of the permeabilizer (EDTA) and the EPIs (CCCP and reserpine). Although the antibiotic adjuvant ability of the plant phenolic compound was similar to that of the permeabilizer EDTA, in contrast to other permeabilizers (Helander and Mattila-Sandholm, 2000) neither ellagic nor tannic acids increased the bacterial uptake of NPN. This is similar to results found in *Salmonella* Typhimurium (Nohynek *et al.*, 2006).

Intrinsic and acquired multidrug resistance in Gram-negative bacteria is related to the synergistic interaction between limited outer membrane permeability and energy-dependent multidrug efflux pumps (Savage, 2001). Enhancement of novobiocin activity against other Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Stenotrophomonas morelense* by EDTA and polyethyleneimine, compounds that alter outer membrane permeability has been reported (Alakomi *et al.*, 2006; Khalil *et al.*, 2008). Similar activity was found for berry-derived phenolic compounds including 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, and 3-(3,4-dihydroxyphenyl) propionic acid (Alakomi *et al.*, 2007). Such compounds efficiently destabilize outer membrane permeability and increased the novobiocin susceptibility of *S.* Typhimurium. Although ellagic acid exhibited a synergistic effect with novobiocin as well as other aminocoumarins, the mechanisms may not be similar to those reported for other known permeabilizers.

Broad-range efflux systems are universal in microorganisms and confer resistance to many compounds, including antibiotics, by drug extrusion. To date, three resistance-nodulation-cell divisions (RND) efflux systems including AdeABC, AdeDE, and AdeIJK (Magnet *et al.*, 2001; Chau *et al.*, 2004; Damier-Piolle

et al., 2008) have been described in *Acinetobacter*. Novobiocin, fusidic acid, rifampicin, and pyronin Y were exported by the AdeIJK efflux pump while EtBr is a substrate for AdeABC. An efflux pump inhibitor, 1-(1-naphthylmethyl-) piperazine (NMP), was found to moderately reverse multidrug resistance in both an *E. coli* overexpressing an RND type efflux pump (Bohnert and Kern, 2005) and clinical isolates (Kern *et al.*, 2006). Furthermore, NMP has been shown to partially reverse MDR in *A. baumannii* but was not capable of destabilizing the outer membrane of the tested bacteria (Pannek *et al.*, 2006).

Ellagic acid is a dimeric derivative of gallic acid, which naturally exists in fruits and nuts including the nutgall. It is usually found in combination with its precursor, hexahydroxydiphenic acid or bound to glucose in the form of ellagitannins (Amakura *et al.*, 2000). Interest in ellagic acid has increased during the past few years due to its potential antimutagenic and anticarcinogenic effects (Heber, 2008). Several different mechanisms of action have been suggested for ellagic acid activity, some of which involve binding to protein or DNA (Whitley *et al.*, 2003). Ellagic acid is an approved food additive in Japan, functioning as an antioxidant and its levels of toxicity have been established (Tasaki *et al.*, 2008). Ellagic acid could improve the antibacterial activity of aminocoumarins, rifampicin, and fusidic acid against the serious Gram-negative pathogen, *A. baumannii*. It represents a promising antibiotic adjuvant lead compound especially given its low cytotoxicity.

As described above, therefore for the important nosocomial pathogens eradication, both as a disinfectant and as a resistant modifying agent *Quercus infectoria* nutgall is a promising alternative agent.

CHAPTER 5
CONCLUSIONS

This work was performed with numbers of infective origin, methicillin-resistant *Staphylococcus aureus* (MRSA) isolates and colonization origin, methicillin-susceptible *S. aureus* (MSSA) isolates that the ethanol extract of *Quercus infectoria* G. Olivier (nutgall) produced inhibitory effect on the test isolates. As expected, in our study, most of the healthy volunteers are carriers of *S. aureus*; however there is no MRSA was found. The nature of cell surface hydrophobicity and virulence factors of infective and colonization origin *S. aureus* were dramatically different. This could be suggestion that some adherence characteristic and virulence factors of *S. aureus* is necessary for the isolates that attached to superficial skin tissue, but not essential for the isolates that infiltrated the deep skin tissues. Demonstration of the high extracted yield and broad spectrum of antibacterial activity by the nutgalls of *Quercus infectoria* may help to discover alternative antibacterial substances that could serve as agents for treatment and control staphylococcal infections.

Tannins and tannin derivatives such as gallic acid and ellagic acid are commonly found in *Quercus infectoria* nutgall. Since the increased susceptibility to the low osmotic pressure and high concentration of sodium chloride with no either cell lysis or alteration of membrane permeability were found in the treated cells, it is proposed that the anti-MRSA mechanisms of *Quercus infectoria* components may possibly be the disruption of the biophysical properties of bacterial cytoplasmic membrane. Furthermore, the ethanol extract of nutgall, the partially-purified fraction, and the components significantly gave rise to pseudomulticellular aggregates with thickened cell walls. Therefore, it is suggested that the anti-MRSA mechanism of the plant extract could involve interference with the staphylococcal autolysins.

The significant effect of the extract and its main component on *S. aureus* cell surface hydrophobicity that may cause to reduced staphylococcal biofilm formation, leads the extract to be a promising plant material for further development

as an alternative decolonization agent for prevention and control of the spread of MRSA infections. The information from this study additionally proposes that the anti-staphylococcal mechanisms of *Quercus infectoria* extract may induce alterations in the composition, structure or conformation of bacterial wall teichoic acid. We reported that that the extract demonstrated anti-oxidant activity and good anti-malarial activity. According to the variety of biological properties, *Quercus infectoria* nutgalls may be a new, cheap, and effective weapon for treating and preventing *S. aureus* infections, including MRSA.

One approach to the restoration of exiting antibiotics is to administer them in conjunction with non-antibiotic compounds that depress resistance mechanisms. A synergistic effect between the nutgall ethanol extract and the β-lactamase-susceptible penicillins indicates that at least part of the synergistic activity may be caused by the effect of the active constituents of the nutgall ethanol extract on the function or production of β-lactamase. Moreover, ellagic acid, *Quercus infectoria* component with low antibacterial activity, enhanced the activity of aminocoumarins, rifampicin, and fusidic acid against *A. baumannii*. Although, there were no increases in the uptake of 1-*N*-Phenylnaphthylamine or in the accumulation of ethidium bromide after the isolates were treated with the compound, the intracellular accumulation of pyronin Y by the treated cells was significantly increased. Further studies could include the application of *Quercus infectoria* nutgall components as both an alternative disinfectant and a resistant modifying agent.

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Educational Attainment

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B. Sc. (Microbiology)	Prince of Songkla University	2005

Awards during Enrolment

2006-2010	The Royal Golden Jubilee Ph.D. Program 2006
2009	National Research Council of Thailand and Japan Society for the Promotion of Science
2005-2006	Graduate School Scholarship, Prince of Songkla University
2001-2005	Faculty of Science Scholarship, Prince of Songkla University

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