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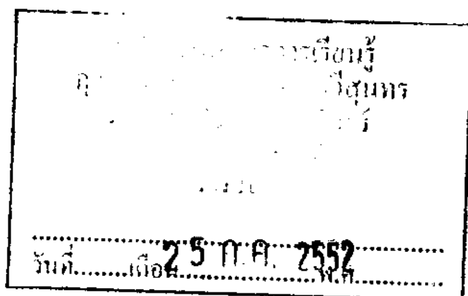

บทคัดย่อ

ได้สร้างงานวิจัยให้สอดคล้องกับนโยบายและแนวทางการวิจัยของชาติ ฉบับที่ 6 (พ.ศ. 2545-2549) แผนงานวิจัยเพื่อผลิตยา ผลิตภัณฑ์และระบบการวินิจฉัยและรักษาทางการแพทย์โดยให้มีมาตรฐานระดับสากลซึ่งเป็นการวิจัยที่จะนำประเทศไปสู่การพึ่งพาตนเอง โดยทำการศึกษาฤทธิ์ของสารสกัดพืชสมุนไพรที่ใช้ในตำราแพทย์แผนไทยในการรักษาโรคติดเชื้อกว่า 100 สารสกัด ต่อแบคทีเรียก่อโรคที่มีความสำคัญทางการแพทย์จำนวน 16 species (Book Chapter) พบว่าปูด (nutgall) ของเบญกานี (*Quercus infectoria*) (สิทธิบัตร เรื่องที่ 1) สามารถออกฤทธิ์แบบ broad spectrum ต่อ Gram-positive และ Gram-negative bacteria ซึ่งน่าสนใจมากเนื่องจากพืชสมุนไพรส่วนใหญ่ไม่มีฤทธิ์ต่อ Gram-negative bacteria (งานตีพิมพ์ที่ 4, 9) ดังนั้นจึงได้นำมาทำการศึกษาต่อในรายละเอียดเพื่อพัฒนาเป็นยาทางเลือกในการรักษา diarrhoea จาก *Escherichia coli* O157: H7 และผลต่อการสร้างสารพิษ Verocytotoxin (งานตีพิมพ์ที่ 3) ซึ่งจะเป็นแนวทางที่จะลดปัญหาการกระตุ้นการสร้าง Verocytotoxin ของเชื้อ เนื่องจากการใช้ยาปฏิชีวนะที่ subinhibitory concentration ส่วนใบกระทุ (*Rhodomyrtus tomentosa*) (สิทธิบัตร เรื่องที่ 2) และว่านหอมแดง (*Eleutherine americana*) มีฤทธิ์ต้าน Gram-positive bacteria ที่ดีมาก พบสารสำคัญ Rhodomyrtone และ derivatives ในกลุ่มของ acylphloroglucinols มีค่าความเข้มข้นต่ำสุดที่สามารถยับยั้ง (minimal inhibition concentration, MIC) และค่าความเข้มข้นต่ำสุดที่สามารถฆ่า (minimal inhibition concentration, MBC) เชื้อ *Staphylococcus aureus* ที่ 0.5 μg/มล. เปรียบเทียบกับยาปฏิชีวนะ vancomycin ที่ 1 μg/มล. พบสารสำคัญในกลุ่มของ naphthoquinones จากหัวของว่านหอมแดง (*Eleutherine americana*) มีฤทธิ์ดีในการต้าน methicillin-resistant *S. aureus* (MRSA) (งานตีพิมพ์ที่ 8) รวมทั้ง *Campylobacter* spp. ซึ่งเป็น Gram-negative bacteria

ศึกษารายละเอียดฤทธิ์ต้านแบคทีเรียของสมุนไพรต่อเชื้อก่อโรคที่มีความสำคัญทางการแพทย์ได้แก่ MRSA (Book Chapter และงานตีพิมพ์ที่ 4, 10) *Helicobacter pylori* (งานตีพิมพ์ที่ 5) และ multiple-resistant *Klebsiella pneumoniae* (MRKP) (งานตีพิมพ์ที่ 6) ได้ศึกษาเกี่ยวกับกลไกการออกฤทธิ์ เช่น ความสามารถในการเปลี่ยนแปลงภาวะของผิวเซลล์แบคทีเรีย (bacterial cell surface hydrophobicity) และ การเกาะกลุ่ม (bacterial cell aggregation) (งานตีพิมพ์ที่ 11) พบว่าสมุนไพรบางชนิดยับยั้งการสร้างไบโอฟิลม์ (biofilm) และได้นำเสนอสมมุติฐานที่สัมพันธ์กับ quorum sensing molecules หรือ bacterial chatting (งานตีพิมพ์ที่ 2) พบแนวทางการประยุกต์ใช้สารสกัดจาก *Quercus infectoria* ร่วมกับยาปฏิชีวนะกลุ่ม β-lactam ในการออกฤทธิ์ต้าน MRSA (งานตีพิมพ์ที่ 1) และได้ศึกษาฤทธิ์ต้านแบคทีเรียของพืชในวงศ์ Zingiberaceae ต่อเชื้อกลุ่มก่อโรคในอาหาร (งานตีพิมพ์ที่ 7, 12)

ABSTRACT

Our research work has been carried out implementing drug research policy and strategies established by the National Research Council of Thailand (NNRCT) (fiscal year 2002-2006). More than 100 extracts from medicinal plant species commonly employed in Thai traditional medicine against infectious diseases were studied against pathogenic bacteria of medical importance (Book chapter). *Quercus infectoria* nutgalls were shown to possess broad spectrum of activity against both Gram-positive bacteria and Gram-negative bacteria (Patent 1). This is of our interest since most medicinal plants are not active against Gram- negative bacteria (Publication 4, 9). Further studies indicated that the nutgall could effectively inhibit Verocytotoxin production in *Escherichia coli* O157: H7 (Publication 3). The results suggest an alternative mean for the treatment of the infection. *Rhodomyrtus tomentosa* (Patent 2) and *Eleutherine americana* have been demonstrated to be very active against Gram-positive bacteria. Rhodomyrtone and its derivatives belonging to acylphloroglucinol group exhibited very low minimal inhibition concentration (MIC) and minimal inhibition concentration (MBC) at 0.5 μg/ml, compared to 1 μg/ml of vancomycin. Active principles in naphthoquinone group from *Eleutherine americana* bulbs showed good anti-methicillin resistant *Staphylococcus aureus* (MRSA) activity (Publication 8) as well as *Campylobacter* spp., Gram-negative bacteria.

Detailed studies were carried out on bacteria of medical importance such as MRSA (Book Chapter, Publication 4, 10), *Helicobacter pylori* (Publication 5), and multiple-resistant *Klebsiella pneumoniae* (MRKP) (Publication 6). Antibacterial mechanisms including an effect on bacterial cell surface hydrophobicity and bacterial cell aggregation were reported (Publication 11). Effects of certain plants on biofilm production have been demonstrated and a hypothesis relating to quorum sensing molecules or bacterial chatting has been proposed (Publication 2). Restoration of β-lactam antibiotic activity was observed in the presence of *Quercus infectoria* extract (Publication 1). In addition, antibacterial activities of plants from family Zingiberaceae against common foodborne pathogenic bacteria were reported (Publication 7, 12).

EXECUTIVE SUMMARY

In developing countries where antibiotics can be unrestrictly used, the emergence of antibiotic-resistant pathogenic bacteria has caused considerable public health concern. Treatment failure due to MRSA with reduced susceptibility to vancomycin was reported. More recently, resistance to new antimicrobial agents such as linezolid, quinupristin, and daffopristin has already occurred. Multiple antibiotic resistant *K. pneumoniae*, another important pathogen, has become increasingly reported. Furthermore, problems due to subinhibitory concentration of certain antibiotics on the stimulation of Verocytoxin production in *E. coli* O157: H7 have been documented.

Recent studies conducted in our laboratories and in others have revealed that medicinal plants from various parts of the world can provide a rich source of antibiotic activities. In Thailand, many plant species have been widely used to cure many infectious diseases. They are available locally, inexpensive, and become increasingly popular. The detailed biological evaluation of these extracts and pure compounds will make it possible to assess the potential applications of these materials for the targeted infectious organisms.

The main objective was to find active compounds from certain plants with-broad spectrum activity as antimicrobial and antiparasitic agents. Further investigations on selected effective medicinal plants have been carried out according to the following aspects:

1. Phytochemical study on active compounds
2. Bacteriological study
3. Antiprotozoal assay
4. Detailed studies on antibacterial mechanisms involved
5. Toxicity tests

RESEARCH METHODOLOGY

Selected plants with the above mentioned biological activities, as alluded to previous literatures, ethno-medicine and ethno-botany, were be subjected to bioassay-guided fractionation for the isolation of pure active components. Various chromatographic techniques e.g. medium pressure chromatography (MPLC), preparative layer chromatography (PLC), radial chromatography (chromatotron) and semi-preparative high pressure chromatography (HPLC) will be employed. Bioassay systems were be developed for each targeted infectious organism.

Preparation of crude extracts of medicinal plants

Initial screenings of plants for possible antimicrobial activities typically began by using crude aqueous or alcohol extractions and followed by various organic extraction methods. Aqueous extracts were dissolved in water while other extracts were dissolved in dimethysulfoxide (DMSO) before use in bacteriological study.

Phytochemical screening for the presence of active compounds

Thin layer chromatography (TLC)

The extract was first subjected to a coarse separation by column chromatography over silica gel (500 g). Elution was conducted initially with hexane, gradually enriched with acetone and followed by increasing amount of methanol in acetone and finally with methanol. The volume of each solvent mixture was 4 l. All fractions were collected (250 ml), monitored by TLC and combined on the basis of their TLC characteristics.

Preparative thin layer chromatography (PLC)

Further purification of the active fractions was carried out using PLC.

Chemical analysis of purified compound

Compounds were elucidated on the basis of spectroscopic data and chemical transformations. Structure elucidation of the chemical constituents of pure compounds isolated was determined by standard spectroscopic techniques including infrared spectroscopy (IR), ultraviolet spectroscopy (UV), mass spectrometry (MS), and high resolution nuclear magnetic resonance spectroscopy (NMR). For the NMR techniques, both proton and carbon-13, were be the main methods used, with the additional

employment of the experiments such as correlated spectroscopy (COSY), heteronuclear chemical shift (HETCOR) and correlation spectroscopy via long-range coupling (COLOC). For pure compounds with complex structures, if the suitable crystal can be obtained, the confirmation of the structure was be done by single crystal X-ray diffraction analysis.

Tested bacterial strains

Important pathogenic bacteria encountered problems with antibiotic resistance were used. These may include methicillin-resistant *S. aureus*, multiple-resistant strains of *K. pneumoniae, H. pylori, E. coli, P. aeruginosa*. Each bacterial strain was suspended in Mueller Hinton broth and incubated at 37°C for 18 h. Mueller Hinton agar was used for testing antibacterial activity.

Antibacterial mechanisms

Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

A modified agar microdilution method waas used to determine the MIC. One microliter of an overnight culture of each bacterial strain containing approximately 10^4 CFU was applied onto MHA supplemented with different fractions of extracts at concentraton ranging from the MIC values of crude extracts. The microtiter plates were incubated at 35°C for 18 h. Results expressed as the lowest concentration of plant extracts that produced a complete suppression of colony growth, MIC. Minimal bactericidal concentration was performed using agar dilution method in petri dishes with millipore filter.

Effect on adhesion of bacterial cells

The hydrophobicity of different strains of bacteria was determined by the salt aggregation test (SAT). Culture was suspended in 0.02 M potassium phosphate buffer (pH 6.8) according to the 5 McFarland turbidity standard to a final concentration of 1.5 x 10^9 cells/ml. SAT studies were performed using ammonium sulfate solutions (0.1, 0.5, 1.0, 1.5, 3.0 M) in 0.02 M Pp buffer (pH6.8). An aliquot (0.02 ml) of ammonium sulfate solution and additionally the same amount of Pp buffer for the control were pipetted into Nunclon plate wells. To each well, 0.02 ml of standardized microbial suspension was

added so that the final concentraitons for ammonium sulfate are 0.05, 0.25, 0.5, 0.75 and 1.5 M. and for 0.75 x 10^9 cells/ml. The microtitre plates are gently rotated for 5 min. The presence of aggregation was examined by light microscopy using a dark background. The strains were tested for autoaggregation using 0.02 M Pp buffer instead of ammonium sulfate. SAT was defined as positive if bacterial aggregation is clearly visible and negative if no aggregation. The SAT titre was the lowest concentration of at which microbes still yield clearly visible aggregaion. Strains autoaggregating in Pp uffer and/or expressing SAT titres 0.05 and 0.25 were considered highly aggregative/hydrophobic, strains with titres 0.5-1.5 are considered low aggregative. A strain was considered nonaggregative if it did not express positive SAT even at a 1.5 M concentration of ammonium sulfate.

Other antibacterial mechanisms involved were be studied depending on preliminary results.

Tested protozoa

E. histolytica strain HM1: IMSS and *G. intestinalis* local Thai strain, originally described are axenically cultured in screw capped tubes at 37°C on YI medium supplement with 10% heat-inactivated calf bovine serum under anaerobic condition.

Antiprotozoal assay

Trophozoites, 2x10^5 cells/ml, were incubated, in triplicate, in the presence of serial two fold dilutions of plant extracts in 96 well tissue culture plates. Metronidazole and complete medium with added DMSO were used as negative and positive control, respectively. After 24h of incubation at 37°C under anaerobic conditions, the trophozoites from each well were examined with an inverted microscope. The appearance of trophozoites was scored and presented as score values from 1 to 4 with 1 showing the most inhibition of growth and 4 showing no inhibition, MIC was recorded.

OUTPUT

Our findings have been summarized in terms of 12 papers, one book chapter, two patents, and three presentations at international congress,reporting the results of our work which have been published as follows:

Papers:

1. Chusri S., **Voravuthikunchai S.P.** 2008. Detailed studies on *Quercus infectoria* Olivier (nutgalls) as an alternative treatment for methicillin-resistant *Staphylococcus aureus* infections. J. Appl. Microbiol. JAM-2008-0613.R1 (*In press)*. **JIF 2.206**
2. Limsuwan S., **Voravuthikunchai S.P.** 2008. *Boesenbergia pandurata* (Roxb.) Schltr., *Eleutherine americana* Merr., and *Rhodomyrtus tomentosa* (Aiton) Hassk as anti-biofilm producing and anti-quorum sensing in *Streptococcus pyogenes*. FEM Immunol. Med. Microbiol. **53**: 429-436. **JIF = 2.281**
3. **Voravuthikunchai S.P.**, Suwalak S. 2008. Antibacterial activities of semi-purified fractions of *Quercus infectoria* (nut galls) against enterohaemorrhagic *Escherichia coli* O157: H7. J. Food Protect. **71**: 1223-1227. **JIF 1.921**
4. Chusri S., **Voravuthikunchai S.P.** 2008. *Quercus infectoria*: A candidate for control of methicillin-resistant *Staphylococcus aureus* infections. Phytother. Res. **22**: 560-562. **JIF 1.144**
5. **Voravuthikunchai S.P.**, Mitchell H. 2008. Inhibitory and killing activities of medicinal plants against multiple antibiotic-resistant strains of *Helicobacter pylori*. J. Health Sci. **54**: 1-8. **JIF 0.793**
6. **Voravuthikunchai S.P.**, Chusri S., Suwalak S. 2008. *Quercus infectoria* Oliv. Pharm.Biol. **46**: 367-372. **JIF 0.397**
7. **Voravuthikunchai S.P.** 2008. Family Zingiberaceae compounds as functional antimicrobials, antioxidants, and antiradicals in foods. FOOD **1**: 227-240.
8. **Voravuthikunchai S.P.**, B.O.T Ifesan, Mahabusarakam W., Hamtasin C. 2008. Antistaphylococcal activity of semi-purified fractions from *Eleutherine americana*. Clin. Microbiol. Infect. **14 (Suppl. 7)**: S137. **JIF 3.254**

9. **Voravuthikunchai S.P.**, Limsuwan S., Subhadhirasakul S. 2007. Screening for medicinal plants with broad spectrum of antibacterial activity. Clin. Microbiol. Infect. **13 (Suppl. 1)**: S599. **JIF 3.254**
10. **Voravuthikunchai S.P.**, Chusri S., and Kleiner P. 2006. Inhibitory activity and killing activity of extracts from the gall of *Quercus infectoria* against methicillin-resistant *Staphylococcus aureus.* Clin. Microbiol. Infect. **12 (Suppl. 4)**: R1980. **JIF 3.254**
11. **Voravuthikunchai S.P.**, Limsuwan S. 2006. Medicinal plant extracts as anti-*Escherichia coli* O157: H7 agents and their effects on bacterial cell aggregation. J. Food Protect. **69**: 2336-2341. **JIF 1.921**
12. **Voravuthikunchai S.P.**, Limsuwan S., Supapol O, Subhadhirasakul S. 2006. Antibacterial activity of extracts from family Zingiberaceae against foodborne pathogens. J. Food Safety **26**: 325-334. **JIF 0.565**

Book chapter:

Voravuthikunchai S.P.,Limsuwan S., Chusri S. 2007. 'New Perspectives on Herbal Medicines for Bacterial Infections'. *In:* Govil G.N.and Singh V.K., and Siddqui T. (eds), Recent Progress in Medicinal Plants Vol.18: Natural Products II. Studium Press, LLC, USA; pp. 41-101. Invited Author.

Patents:

1. ศุภยางค์ วรวุฒิคุณชัย และ ศศิธร ชูศรี 2007 กระบวนการสกัดสารออกฤทธิ์ทางชีวภาพจาก เบญกานี (*Quercus infectoria*) Thai Patent Document No. 0701 003210.
2. **ศุภยางค์ วรวุฒิคุณชัย** สุรศักดิ์ ลิ้มสุวรรณ อัษฎาวุธ หิรัญรัตน์ และวิลาวัณย์ มหาบุษราคัม 2008 กระบวนการสกัดสารออกฤทธิ์ทางชีวภาพจากกะทุ (โทะ) (*Rhodomyrtus tomentosa*) Thai Patent Document No.

International Meetings:

1. **Voravuthikunchai S.P.**, Ifesan B.O.T., Mahabusarakam W., Hamtasin C. 2008. Antistaphylococcal activity of semi-purified fractions from *Eleutherine americana*. Programme and Abstracts, The 19th European Congress of Clinical Microbiology and Infectious Diseases, 19-22 April, 2008 at Barcelona, Spain.
2. **Voravuthikunchai S.P.**, Limsuwan S., Subhadhirasakul S. 2007. Screening for medicinal plants with broad spectrum of antibacterial activity. Programme and Abstracts, 17th European Congress of Clinical Microbiology and Infectious Diseases, 31 March-3 April, 2007 at Munich, Germany.
3. **Voravuthikunchai S.P.**, Suwalak S., Supawita T. 2006. Antibacterial activity of fractions of *Quercus infectoria* (nut galls) against enterohaemorrhagic *Escherichia coli*. Programme and Abstracts, 16th European Congress of Clinical Microbiology and Infectious Diseases, 2-4 April, 2006 at Nice, France.

Note:

1. Three abstracts from ECCMID were subsequently published in Clinical Microbiology and Infection.
2. Results from *in vitro* screening of the extracts on axenic culture of *Giardia intestinalis* have completed and being prepared as a manuscript entitled: 'Antibacterial, anti-giardial, and anti-oxidant activities of *Walsura robusta*'. (Voravuthikunchai S.P., Kanchanapoom T., Sawangjaroen, Hutadilok-Towatana N. 2008).

INTERNATIONAL PUBLICATIONS

Detailed studies of Quercus infectoria Olivier (nutgalls) as a novel tool for treating methicillin-resistant Staphylococcus aureus

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Detailed studies of *Quercus infectoria* Olivier (nutgalls) as a novel tool for treating methicillin-resistant *Staphylococcus aureus*

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Abstract

Aims: To investigate the anti-methicillin-resistant *Staphylococcus aureus* (MRSA) mechanism of *Quercus infectoria* (nutgalls) extract as well as its effective components.

Methods and Results: The ethanol extract, an ethyl acetate fraction I, and its components - gallic acid and tannic acid, all inhibited the growth of clinically-isolated MRSA strains. Clumps of partly-divided cocci with thickened cell wall were observed by transmission electron microscopy in the cultures of MRSA incubated in the presence of the ethanol extract, the ethyl acetate fraction I, and tannic acid. Since cell wall structure of the organism structures seemed to be one possible site for these actions, their effect with representative β-lactam antibiotics were determined. Synergistic effects were observed with 76% and 53% of the tested strains for the combination of ethanol extract and amoxicillin and penicillin G, respectively.

Conclusions: The appearance of pseudomulticellular bacteria in the nutgall ethanol extract treated cells and the synergistic effect of the plant extract with β-lactamase-susceptible penicillins indicated that the extract may interfere with staphylococcal enzymes including wall autolysins and β-lactamase.

Significance and Impact of the Study: Our results provide scientific data on the use of the nutgall extract for the treatment of staphylococcal infections.

Keywords *Quercus infectoria*, pseudomulticellular bacteria, β-lactam antibiotics, methicillin-resistant *Staphylococcus aureus*

Introduction

Natural products and their derivatives have continued to be 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 antiinfectious and anticancer agents were developed from natural plant compounds (Newman *et al.* 2003; Cragg and Newman 2005). Although a number of herbs have a long history of use as cures for many human ailments, often there has been very little scientific evidence to substantiate their claims. Recently, various new plant components have been isolated, chemically characterized, and their mechanisms of biological action established.

The continuing emergence and development of resistance to existing antibacterial agents by bacteria has created the need for new antibacterial compounds that exhibit activity against these resistant strains (Levy and Marshall 2004; Norrby *et al.* 2005). Methicillin-resistant *Staphylococcus aureus* (MRSA) has become increasingly widespread as a major cause of both nosocomial and community infections. This situation has placed limits on our options to treat infections by this organism. Glycopeptide derivatives such as vancomycin and teicoplanin are now considered to be agents of last resort for the treatment of MRSA infections (Ferrara 2007). In 2002, vancomycin-resistant *S. aureus* (VRSA) strains emerged in the United States (Centers for Disease Control and Prevention 2002; Chang *et al.* 2003), followed by reports of these isolates from other parts of the world (Biedenbach *et al.* 2007; Emaneini *et al.* 2007). Therefore, before therapy with vancomycin and teicoplanin fails completely, we need to find some alternative antibacterial agents against MRSA infections.

A nutgall of *Quercus infectoria* Olivier (Fagaceae) is one of the most commonly used plants in a traditional Asian medicine to treat skin infections, gastrointestinal disorders, and vaginal infections (Kaur *et al.* 2004; Dayang *et al.* 2005; Voravuthikunchai *et al.* 2007;

Kaur *et al.* 2008). The nutgall from *Quercus infectoria* contains the highest naturally occurring level of tannin, approximately 50-70% (Ikram and Nowshad 1977). Tannins have been used for hundreds of years for medical purposes and are currently indispensable in dermatology (Folster-Holst and Latussek 2007). Although, catechins, a condense tannin, have been intensively studied on their mechanisms of actions on bacterial cells. Detailed antimicrobial mechanisms from the hydrolysable group of tannins, a main component in the nutgalls, have not been addressed. Likewise, there is very little published information on the antibacterial mechanisms of the *Quercus infectoria* nutgall.

A series of experiments in our laboratory has demonstrated the extremely broad-spectrum of inhibitory activity of the nutgall extract against a wide range of important pathogenic bacteria (Voravuthikunchai *et al.* 2004; Voravuthikunchai and Kitpipit 2005; Voravuthikunchai and Mitchell 2008). Some work has also clearly indicated that various extracts from the nutgalls exhibited high activity against MRSA (Voravuthikunchai and Kitpipit 2005; Chusri and Voravuthikunchai 2008). The primary mechanisms of actions of the plant extract against other important pathogens have been previously described (Voravuthikunchai and Limsuwan 2006; Voravuthikunchai *et al.* 2006). In this present communication, we further compare the anti-MRSA activities of an ethanol extract of the nutgall, its effective fraction, and main constituents. Since the extract, the fraction, and tannic acid exhibited a significant effect on the cell wall of MRSA strain, therefore their effects with representative β-lactam antibiotics have been further explored.

Materials and methods

Tested Bacterial strains

Methicillin-resistant *Staphylococcus aureus* (NPRC 01-17) were obtained from Hat Yai hospital, Hat Yai, Thailand. *S. aureus* ATCC 25923 was used as an antibiotic sensitive control. The bacteria were maintained on Tryptic soy agar (TSA, Becton, Dickinson and

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Company, France) slant at 4 °C. For use in experiments, the bacteria were grown separately on TSA at 35 °C for 18-24 h.

Preparation of *Quercus infectoria* extract and β-lactam antibiotics

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 (100g) was extracted with 95% ethanol (500 mL) 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 (the extract yield was 57.15 %.). The ethanol extract was dissolved in 10% dimethylsulfoxide (DMSO, Merck, Germany) before use.

Representative β-lactam antibiotics including amoxicillin (Sigma-Aldrich, CH-9471 Buchs, Switzerland), oxacillin (Sigma-Aldrich, CH-9471 Buchs, India), and penicillin G (General Drugs House Co., Ltd) were used as control antibiotics in the study.

Fractionation of active constituents

Nutgall 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 (Merck $60GF_{254}$ 70-230 mesh; 500g; 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 $60GF_{254}$ TLC aluminium sheets (Merck; 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 since it had no UV absorbance and its yield was too low to assay for antibacterial activity. Since the ethyl acetate fraction I showed strong anti-MRSA activity and the highest percentage yield (22.65%), therefore its activity was tested in a further set of experiments. Tannic acid (Fluka, Switzerland) and gallic acid (Fluka, Spain), the main constituents with anti-MRSA activity, (henceforth called components) were included in the tests. For bioassay, the extract, the fraction and the components were dissolved in 10% dimethylsulfoxide (DMSO, Merck, Germany) before use.

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 analyzed according to previously described methods (Houghton and Raman, 1998; 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 (Mg-HCL) reagent for flavonoids, 5% $FeCl_3$ and Bromine water for hydrolysable and condensed tannins, and the Liebermann-Burchard test for triterpenes.

Determination of Minimum inhibitory concentrations (MIC)

A broth microdilution method (CLSI 2006) was used to determine the MICs of the *Quercus infectoria* extract, its components and the antibiotics. Serial two-fold dilutions of the plant extract and its separated components were mixed with Mueller-Hinton broth (MHB, Becton, Dickinson and Company, France) at 1: 10 (v/v) in 96-well sterile microtiter plates to give final concentrations of 0.03 -1.00 mg mL^{-1}. Twenty microlitres of a 3-5 h culture of each

bacterial strain, containing approximately 5 x 10^6 CFU mL^{-1}, were added to the MHB supplemented with the tested compounds. The microtiter plates were then incubated at 35 °C for 18 h. Minimum inhibitory concentrations were observed at least in duplicate as the lowest concentration of plant extracts that produced a complete suppression of bacterial growth.

The β- lactam antibiotics were serially diluted two-fold with 10% DMSO to obtain solutions of 512-0.06, 640-0.06, and 256-0.02 μg mL^{-1} for amoxicillin, oxacillin, and penicillin G, respectively. Their MIC values were measured as described above.

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

A bacterial suspension of 1.5x10^9 CFU mL^{-1} 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 35 °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 levels of purines, pyrimidines, and their derivatives in the supernatant were determined at each time interval by measuring the optical density (OD) at 260 nm using a UV/VIS spectrophotometer (Shimadzu UV-1601 Spectrophotometer, Japan). Each concentration of the extract and its components without the bacterial suspension were used as blanks. The OD_{260} at each time point was expressed as a proportion of the initial OD_{260}. Mean ratios for each treatment agent and time were calculated and compared to the means for the corresponding untreated samples (Carson *et al.* 2002).

Transmission electron microscopy (TEM)

MRSA was cultured in TSB at 35 °C for 5 h and then 800 μL of the bacterial suspension was transferred into TSB supplemented with the ethanol extract, its fraction, and its components at 4x their MIC concentrations (10: 1, v/v). The suspensions were incubated at 35 °C for 12 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. Cells were washed three times with 0.1 mol L^{-1} phosphate buffer solution, pH 7.2 and post-fixed with 1% (w/v) osmium tetroxide 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 sectioned with an ultramicrotome (MTXL, RMC, USA) and sections observed using a transmission electron microscope (JEOL, JEM 100 CX II, Japan).

Checkerboard study

The combined effects of the ethanol extract, the ethyl acetate fraction I, gallic acid, and tannic acid with β-lactam antibiotics (amoxicillin, oxacillin, and penicillin G) 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 two-fold along the *x*-axis, and rows in which each well contains the same amount of the plant extract and its components diluted two-fold 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).

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Results

Anti-MRSA activity and phytochemical composition of *Quercus infectoria* extract

As indicated in Table 1, the initial ethanol extract, the ethyl acetate fraction I, and components gallic acid and tannic acid demonstrated anti-MRSA activity. Comparison of the MIC values of these showed that there were no significant differences (63-250 μg mL^{-1}). The MRSA strains however were all resistant to amoxicillin, oxacillin, and penicillin G with the MIC values ranging from 35-512, 16-256, and 160-1,280 μg mL^{-1}, respectively compared to values of < 0.06 μg mL^{-1} for the sensitive control strain.

Preliminary phytochemical analysis of the ethanol extract revealed the presence of flavonoids, tannins, and steroidal compounds. Tannins were detected in the ethyl acetate fraction I.

Effects on the cytoplasmic membrane of MRSA

Damage to the staphylococcal cytoplasmic membrane was assessed by measuring the leakage of small UV-absorbing compounds. The proportion of the OD_{260} at each time point is given in Fig. 1. The OD_{260} of suspensions was not increased significantly at any of the concentrations tested up to 20 h after treatment with the ethanol extract, its ethyl acetate fraction, and its components.

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

The ethanol extract and the ethyl acetate fraction I produced pseudomulticellular MRSA cell aggregates with thickened cell walls, (Fig. 2A and 2B). Tannic acid produced a similar effect (Fig. 2D) but gallic acid had no observed effect (Fig. 2C).

Effects on resistance to β-lactam antibiotics

The results of the checkerboard titration are presented in Table 2. 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 action. 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.

Discussion

In our continuing work to investigate Thai medicinal compounds derived from plants that exhibit anti-MRSA activity, we found that the ethanol extract of the nutgalls had the ability to inhibit and eradicate all clinical isolates of MRSA. The high amounts of tannins found in the nutgalls implied that tannins could be the active compounds responsible for the anti-MRSA activity. The antibacterial, antifungal, and antiviral properties of plant-derived tannins are well-known, but there are very few reports on their antimicrobial mechanisms (Singh *et al.* 2003). Thus, the effects of the nutgall extract as well as the main constituents on MRSA cell has been investigated and discussed in this report.

Although, the ethanol extract of nutgall, the partially-purified fraction, and the components did not appear to alter membrane permeability, the ethanol extract and the ethyl acetate fraction of the nutgalls significantly gave rise to pseudomulticellular aggregates with thickened cell walls. 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. 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.* 2004). 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.* 2004).

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.* 2004). 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 (corilagin and tellimagrandin I) and a polymeric proanthocyanidin (ZP-CT-A) 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.

In conclusion, the appearance of pseudomulticellular bacteria in the nutgall ethanol extract treated cells and the synergistic effect of the plant extract with β-lactamase-susceptible penicillins indicated that the extract may interfere with staphylococcal enzymes including wall autolysins and β-lactamase. However, further studies are required to directly determine the site and mechanism of action of the plant extract on the staphylococcal autolysins as well as β-lactamase activity.

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Table 1 Minimal inhibitory concentration (MIC) of the ethanolic extract of *Quercus infectoria*, the ethyl acetate fraction I, gallic acid, tannic acid, β-lactam antibiotics against methicillin- resistant *S. aureus*

Quercus infectoria extract, its components, and antibiotics	MIC (μg mL^{-1})	
	MRSA (n=17)	*S. aureus* ATCC 25923
Ethanolic extract	125-250	250
Ethyl acetate fraction I	125-250	250
Gallic acid	63-125	125
Tannic acid	125-250	250
Amoxicillin	32-512	<0.0625
Oxacillin	160-1,280	<0.0625
Penicillin G	16-256	<0.015

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Table 2 Activities of β-lactam antibiotics in combination with *Quercus infectoria* extract and its components against methicillin-resistant *S. aureus*

	Number of tested strains (%)		
	∑ FIC index *		
	≤ 0.5	0.5-2.0	≥ 2.0
Amoxicillin			
Ethanolic extract	13 (76%)	4 (24%)	0
Ethyl acetate fraction I	10 (59%)	7 (41%)	0
Gallic acid	0	17 (100%)	0
Tannic acid	3 (18%)	14 (82%)	0
Oxacillin			
Ethanolic extract	2 (12%)	15 (88%)	0
Ethyl acetate fraction I	6 (35%)	11 (65%)	0
Gallic acid	0	16 (94%)	1 (6%)
Tannic acid	0	17 (100%)	0
Penicillin G			
Ethanolic extract	9 (53%)	8 (47%)	0
Ethyl acetate fraction I	2 (12%)	15 (88%)	0
Gallic acid	0	16 (94%)	1 (6%)
Tannic acid	0	17 (100%)	0

* Results for the fractional inhibitory concentration (FIC) indices ≤ 0.5 are synergistic, those for FIC indices > 0.5 to < 2 are additive, and those for FIC indices ≥ 2 are antagonistic.

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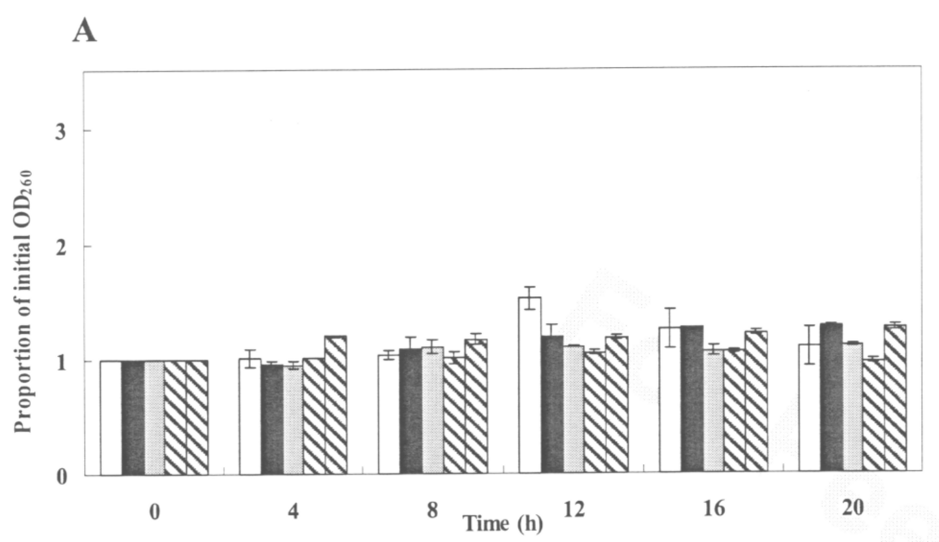

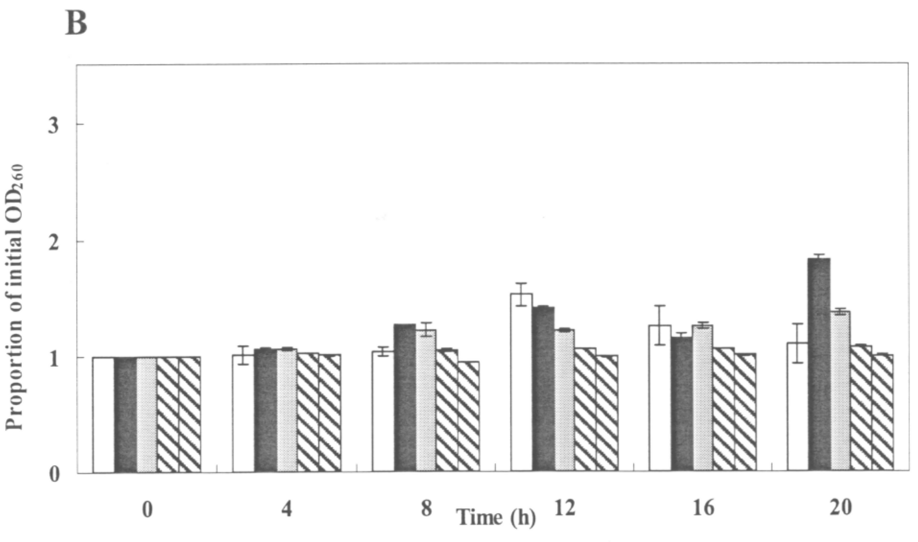

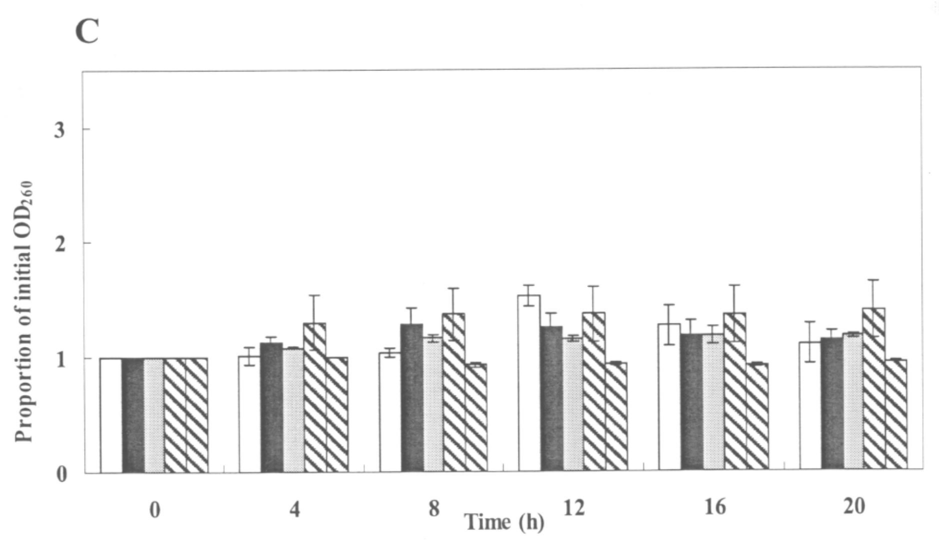

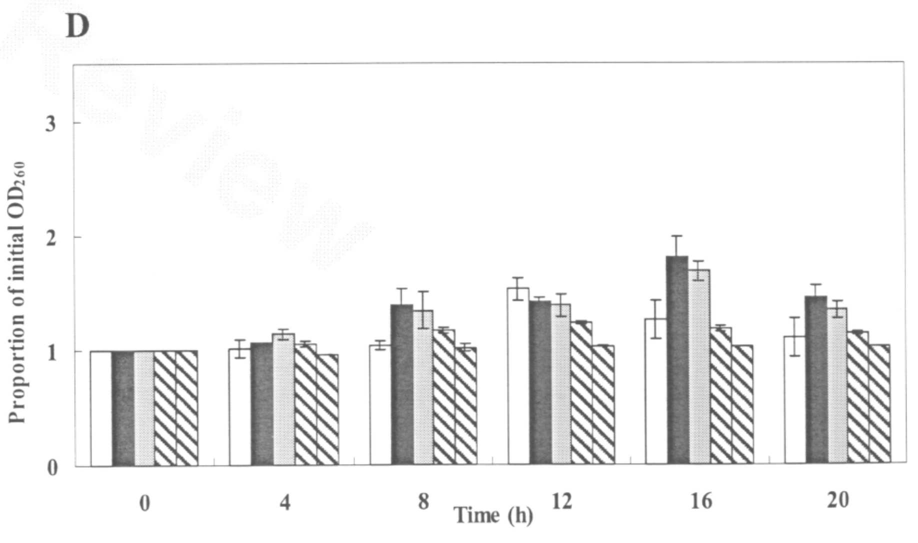

Figure 1 Absorbance of the cell material contents at 260 nm releasing from MRSA cells after treated with the ethanolic extract (A), the ethyl acetate fraction I (B), gallic acid (C), and tannic acid (D) at 1/2MIC (black bars), MIC (gray bars), 2MIC (bars with diagonal stripes), and 4MIC (bars with horizontal stripes). One per cent of DMSO (white bars) was used as control.

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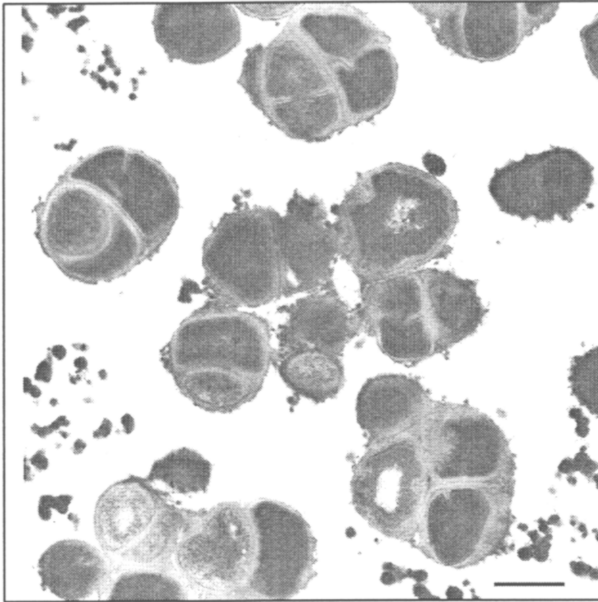

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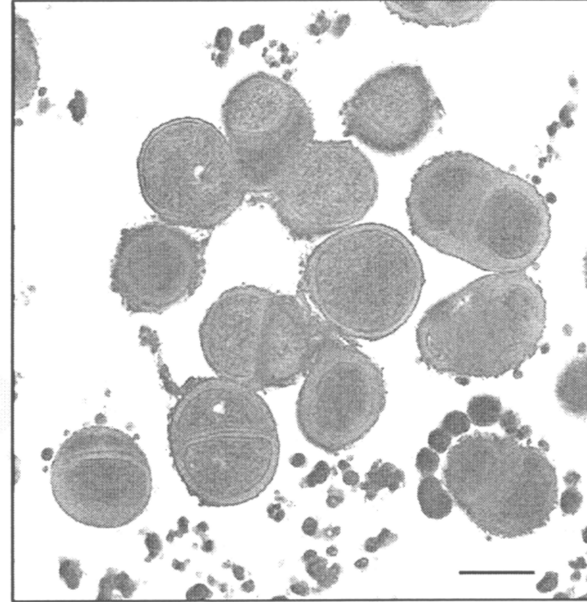

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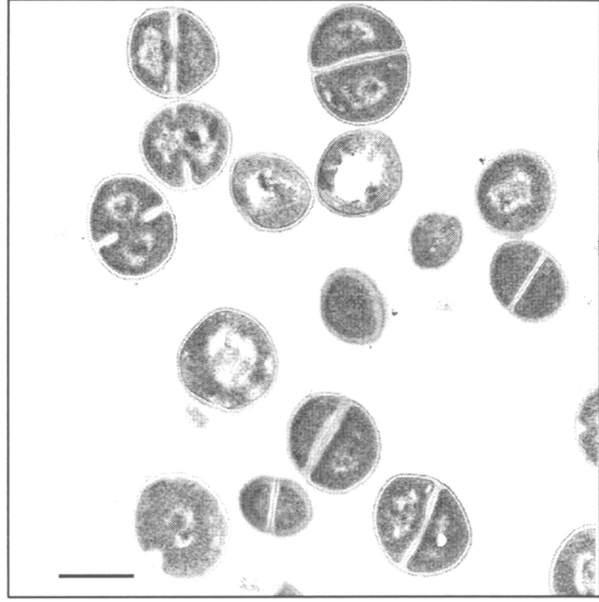

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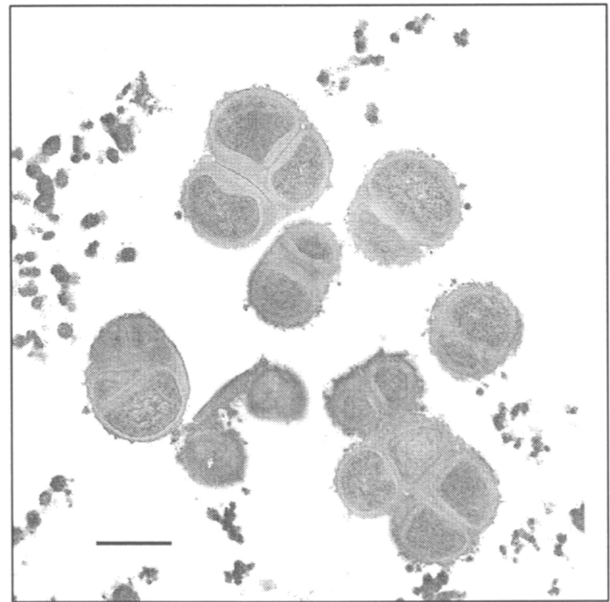

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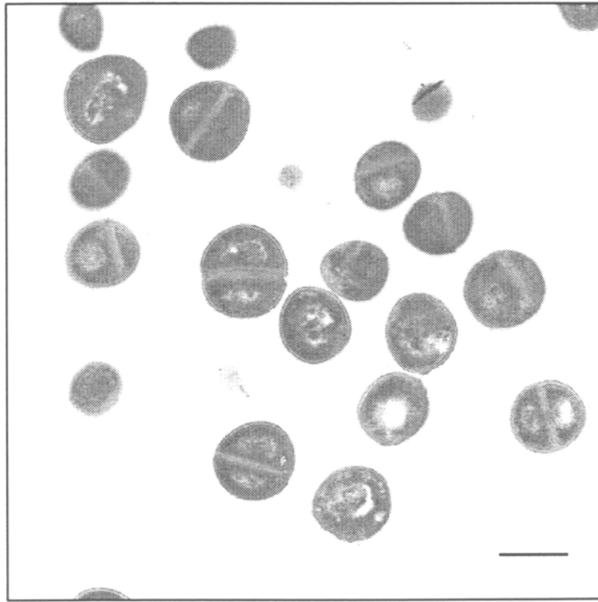

Figure 2 Effects of the ethanolic extract (A), the ethyl acetate fraction I (B), gallic acid (C), and tannic acid (D) on the cell morphology of methicillin-resistant *S. aureus* at 4MIC concentration for 12 h. One per cent of DMSO was used as control (E). Scale bars represent 500 nm.

RESEARCH ARTICLE

Boesenbergia pandurata (Roxb.) Schltr., *Eleutherine americana* Merr., and *Rhodomyrtus tomentosa* (Aiton) Hassk. as antibiofilm producing and antiquorum sensing in *Streptococcus pyogenes*

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Keywords

antibiofilm; antiquorum sensing; herbal extract; *Streptococcus pyogenes*; hydrophobicity; *Boesenbergia pandurata*; *Eleutherine americana*; *Rhodomyrtus tomentosa*.

Abstract

Biofilm formation has been demonstrated as one of potentially important mechanisms contributing to antibiotic treatment failure on *Streptococcus pyogenes*. It could play a significant role in recurrent and chronic infections and should be investigated. *Boesenbergia pandurata* (Roxb.) Schltr., *Eleutherine americana* Merr., and *Rhodomyrtus tomentosa* (Aiton) Hassk. have been previously reported from our laboratory as effective agents against *S. pyogenes*. Therefore, in the present study, we observed the effect of these plants on biofilm formation. The bacterial biofilms were quantified by safranin staining and measuring $A_{492\,nm}$. The results clearly demonstrated that all subinhibitory concentrations [1/32–1/2 minimal inhibitory concentration (MIC)] of *E. americana* (7.81–125 $\mu g\,mL^{-1}$) and *R. tomentosa* (0.24–7.81 $\mu g\,mL^{-1}$) extracts significantly prevented biofilm formation while 1/2MIC (7.81 $\mu g\,mL^{-1}$) of *B. pandurata* extract produced this effect. A modern issue on antiquorum sensing of this pathogenic bacteria has been further explored. A correlation between antiquorum-sensing and antibiofilm-producing activities was demonstrated. Strong inhibition on quorum sensing was displayed with the extract of *R. tomentosa*. *Eleutherine americana* extract showed partial inhibition, while *B. pandurata* did not show this activity. Contrastly, a microbial adhesion to hydrocarbon assay revealed no changes in the cell-surface hydrophobicity of treated organisms. Active principles with abilities to inhibit quorum sensing and biofilm formation are worth studying to provide complimentary medicine for biofilm-associated infections.

Introduction

Many pathogenic bacteria with the ability to form biofilms are responsible for acute and chronic infections. Examples of biofilm-associated diseases are dental caries, gingivitis, periodontitis, endocarditis, and prostatitis (Hall-Stoodley *et al.*, 2004). In addition, implanted medical devices including intravenous catheters, artificial joints, and cardiac pacemakers can become rapidly coated with human extracellular matrix and plasma proteins which are prime targets for bacterial biofilm formation (Costerton *et al.*, 1999; Donlan & Costerton, 2002; Parsek & Singh, 2003). Because of the sharply decreased susceptibility of biofilm-forming bacteria to host defenses and antibiotic treatments, biofilms on implanted devices remain a major medical problem (Hoyle & Costerton, 1991; Costerton *et al.*, 1999; Pajkos *et al.*, 2004; Vickery *et al.*, 2004).

Streptococcus pyogenes, one of the most important human pathogens associated with extensive human morbidity worldwide. It causes primary infections of skin, throat, and mucosal surfaces. Even though the infections are normally self-limited, antibiotic treatment is usually employed to relief discomfort, minimize transmission, and reduce complications (Bisno *et al.*, 1997). It has to be considered that the newly emerging *S. pyogenes* virulence trait potentially renders this important pathogen more resistant to antibiotic therapy and to innate as well as adaptive immune responses. Biofilm is known to allow bacteria to become more resistant

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to antibiotic treatment, and significantly impairs antimicrobial therapy even in those cases caused by strains that are not resistant to the relevant antibiotics (Macris *et al.*, 1998; Kuhn *et al.*, 2001). Many forms of streptococcal infections, especially recurrent and chronic ones, are associated with the formation of bacterial biofilm (Lembke *et al.*, 2006).

Penicillin is the antibiotic of choice for *S. pyogenes* infections according to its narrow spectrum of effect, efficacy, safety, and low cost (Bisno *et al.*, 1997, 2002). However, penicillin treatment failure of *S. pyogenes* infection has been demonstrated to associate with biofilm formation (Conley *et al.*, 2003). In patients with known or suspected allergy to penicillin, erythromycin and other macrolides are considered as alternative treatment (Hooton, 1991; Stein *et al.*, 1991; Adam & Scholz, 1996). Recently, macrolide resistance in *S. pyogenes* has been reported in several countries (Eisner *et al.*, 2006; Hsueh *et al.*, 2006; Littauer *et al.*, 2006). Because of the increasing resistance of *S. pyogenes* to antibiotics, much effort is being exerted to identify novel compounds with antibacterial activity and to analyse their mechanisms of action. Specifically, there is a critical need to identify therapeutic strategies which are directed towards the inhibition of biofilm formation and effective treatment of biofilms once they have formed. Quorum sensing molecules have been shown to be essential for biofilm formation (Chen *et al.*, 2004; Hornby & Nickerson, 2004). Quorum sensing is a strategy of cell–cell communication favouring the biofilm community by regulating unnecessary overpopulation and nutrient competition with important implications for the infectious process (Davey & O'Toole, 2000; Douglas, 2003).

Many plants have been reported to demonstrate anti-quorum-sensing activity (Rasmussen *et al.*, 2005; Adonizio *et al.*, 2006; Choo *et al.*, 2006). Our preliminary screening on a wide range of Thai plant species demonstrated that *Boesenbergia pandurata* (Roxb.) Schltr., *Eleutherine americana* Merr., and *Rhodomyrtus tomentosa* (Aiton) Hassk. had good antibacterial activities on *S. pyogenes* (Voravuthikunchai *et al.*, 2007). The aim of the present study was to investigate the effect of these effective plants on biofilm development and on quorum-sensing activity. Aspects on their effects on cell-surface hydrophobicity (CSH) of *S. pyogenes* was also examined.

Materials and methods

Medicinal plants

Three medicinal plants were used in this study. Classified reference voucher specimens were deposited at the Herbarium of Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla, Thailand. All of the plant materials were dried at 60 °C overnight. They were crushed and soaked with extractive solvents for 7 days. The solvent was then distilled under reduced pressure in a rotary evaporator until it became completely dry. The extracts were dissolved in dimethyl sulfoxide (DMSO, Merck, Germany) before use.

Bacterial strains

Eleven clinical isolates of *S. pyogenes* (NPRC 101–111) were isolated from patients with tonsillitis. Throat swab of each patient was individually plated onto Columbia blood agar base (Oxoid) containing 5% sheep red blood cells. β-Haemolytic streptococcal-like colonies were subjected to appropriate biochemical testing as described previously (Forbes *et al.*, 2002). All isolates were negative for catalase using 3% H_2O_2, susceptible to bacitracin disc (0.04 U), positive for pyrrolidonyl aminopeptidase reagent and produced no growth on 6.5% NaCl agar and bile esculin hydrolysis agar. The isolates were stored in brain heart infusion (BHI) broth (Difco Laboratories, Detroit) containing 50% glycerol at −70 °C until use. *Chromobacterium violaceum* DMST 21761 was purchased from the National Institute of Health (NIH), Department of Medical Sciences, Ministry of Public Health, Thailand. All strains were routinely grown in BHI broth (Difco, France) or trypticase soy agar (TSA, Difco, France) plates.

Determination of minimal inhibitory concentration (MIC)

A modified broth microdilution method outlined by the Clinical and Laboratory Standards Institute (CLSI, 2006) was performed. The bacterial suspensions (10^5 CFU mL^{-1}) were added into BHI broth supplemented with the plant extracts serially diluted twofold to give final concentrations ranging from 0.5 to 1000 μg mL^{-1} and incubated at 37 °C for 20 h. MIC was recorded as the lowest concentration that produced complete suppression of visible growth.

Biofilm assays

All *S. pyogenes* isolates were screened for biofilm production according to a method of Lembke *et al.* (2006). Glass test tubes containing BHI broth were added with bacterial suspensions (10^5 CFU mL^{-1}), and the remaining planktonic bacteria were removed by aspiration of the liquid after desired incubation time points. The test tubes with biofilms were washed four times with 0.85% normal saline solution (NSS) and stained in a 0.1% safranin solution for 30 min. The stained biofilms were washed four times with NSS and allowed to dry. The stained biofilms were removed from the tube surface by adding ethanol with vigorous vortexing, and

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quantified in a spectrophotometer (Shimadzu UV-1601 Spectrophotometer, Japan) at 492 nm. Tubes containing BHI were used as controls.

The effect of the plant extracts on biofilm formation of *S. pyogenes* was investigated by adding the extracts at subinhibitory concentrations into glass test tubes containing BHI broth. Subsequently, the tubes were added with the bacterial suspension. After incubation, biofilm staining was performed as described above. The tubes contained the media and the extracts were tested as control. In parallel experiments, unstained biofilm and planktonic bacteria were mixed by vigorous vortexing, and bacterial growth was quantified in a spectrophotometer at 660 nm.

For visualisation of biofilms, they were allowed to grow on glass pieces in glass test tubes supplemented with the extracts. Following incubation, the glass pieces were washed four times with NSS and stained with 0.1% safranin solution. Stained glass pieces were observed at a magnification of 40 using light microscope (Olympus CX-31).

Antiquorum-sensing activity

The paper disc agar diffusion method was used to detect antiquorum-sensing activity of the extracts (Adonizio *et al.*, 2006). The extracts were dissolved in DMSO, 10 μL (250 mg mL^{-1}) of the crude extracts were applied to sterile filter paper discs (Whatman no. 1; 6 mm in diameter) so that each disc was saturated with 2.5 mg of the extract. Dry discs (dried at 37 °C overnight) were applied onto the surface of TSA seeded with 3–5 h TSB culture of *C. violaceum*. The plates were incubated overnight at 37 °C and examined for violacein production. Quorum-sensing inhibition was detected by a colourless, opaque halo around the disc. Dimethyl sulfoxide was used as control.

CSH testing

The effect on CSH of *S. pyogenes* was measured by microbial adhesion to hydrocarbon (Rozenberg *et al.*, 1980). Briefly, the bacterial cells grown in BHI broth with subinhibitory concentrations of the plant extracts were washed twice and suspended in NSS so that their $OD_{600\,nm}$ was 0.3. The cell suspension (3 mL) was placed in tubes and 0.25 mL of toluene was added. The tubes were agitated uniformly in a vortex mixer for 2 min and allowed to equilibrate at room temperature for 10 min. After toluene phase was separated from the aqueous phase, the OD of the aqueous phase was determined spectrophotometrically. Controls consisted of cells incubated with 1% DMSO. The hydrophobicity index (HPBI) was calculated as: OD initial − OD final/OD initial × 100%. *Streptococcus pyogenes* with a hydrophobic index > 70% was arbitrarily classified as hydrophobic (Martin *et al.*, 1989; Nostro *et al.*, 2004).

Statistical analysis

Statistical analysis was performed using SPSS. Values were expressed as mean ± SD. Duncan-ANOVA test was used to compare the parameters between the groups. Dunnett-ANOVA test was used to compare between the tests and control.

Results

Screening for biofilm formation

Eleven isolates of *S. pyogenes* were preliminarily screened for biofilm production (Fig. 1). Most strains produced high level of biofilm during 24–48 h. *Streptococcus pyogenes* NPRC 109, NPRC 110, and NPRC 111 were among efficient strains for biofilm production (Duncan test, $P < 0.05$). *Streptococcus pyogenes* NPRC 109 was the most efficient strain in terms of biofilm production at 24 and 48 h, whereas *S. pyogenes* NPRC 110 and 111 showed this efficacy at 24 h. However, we decided to select 48 h for further study because the organisms have recovered from inhibitory effect. Differences in biofilm levels indicated that there was a spectrum of ability of different *S. pyogenes* isolates to form biofilms.

Biofilm assay with subinhibitory concentrations of plant extracts

The MICs of the plant extracts on *S. pyogenes* are shown in Table 1. *Boesenbergia pandurata* and *R. tomentosa* demonstrated antibacterial activity with MIC values (7.81 μg mL^{-1}) while *E. americana* inhibited the growth of this strain with the MIC value of 250 μg mL^{-1}. The plant extracts at subinhibitory concentrations (1/2, 1/4, 1/8, 1/16, and 1/32MIC) were used for biofilm assay. Their effects on biofilm production are shown in Fig. 2. *Eleutherine americana* and

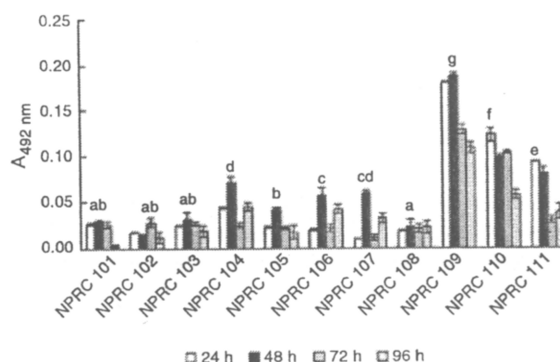

Fig. 1. Biofilm quantification of different strains of *Streptococcus pyogenes* (NPRC 101–111) to glass surfaces by standard safranin staining of potential biofilms and measuring $A_{492\,nm}$. The mean values of duplicate independent experiments and deviations are shown. Duncan test demonstrates significant difference in biofilm production ($P < 0.05$).

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Table 1. MIC of crude plant extracts on *Streptococcus pyogenes* NPRC 109

Specimen No.	Botanical species	Plant part tested	% Extract yield	MIC (μg mL^{-1})
NPRC0007	*Boesenbergia pandurata* (Roxb.) Schltr. (*Zingiberaceae*)	Rhizome	1.58 (chloroform)	7.81
NPRC0044	*Eleutherine americana* Merr. (*Iridaceae*)	Bulb	4.80 (ethanol)	250.00
NPRC0057	*Rhodomyrtus tomentosa* (Aiton) Hassk. (*Myrtaceae*)	Leaf	7.40 (ethanol)	7.81

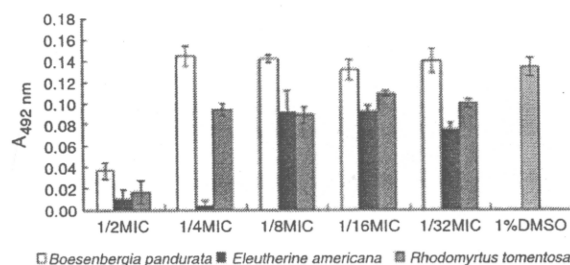

Fig. 2. Effects of subinhibitory concentrations (1/2–1/32MIC) of the plant extracts on the biofilm production by *Streptococcus pyogenes* NPRC 109 at 48 h, quantified by safranin staining and subsequently by measuring $A_{492\,nm}$. The mean values of duplicate independent experiments and SDs are shown. Dunnett test demonstrates significant difference between the tests and the control (*$P < 0.05$).

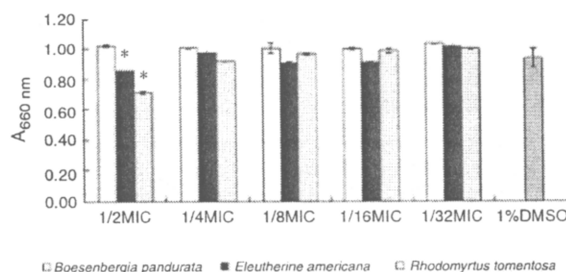

Fig. 3. Subinhibitory (1/2–1/32MIC) effects of the plant extracts on the growth of *Streptococcus pyogenes* NPRC 109. Bacterial growth was quantified by measuring $A_{660\,nm}$ at 48 h. The mean values of duplicate independent experiments and SDs are shown. Dunnett test demonstrates significant difference between the tests and the control (*$P < 0.05$).

R. tomentosa extracts at all concentrations significantly inhibited biofilm formation of *S. pyogenes* (Dunnett-ANOVA test, $P < 0.05$). In contrast, *B. pandurata* extract demonstrated this effect only at 1/2MIC. At almost all subinhibitory concentrations, growth of *S. pyogenes* was at the same level as that of the control (Fig. 3). This result suggested that inhibition of biofilm formation did not result from inhibition of cell growth.

Evaluation and detection of biofilm formation by microscopy

Streptococcus pyogenes NPRC 109 did not form dense biofilm layers when treated with subinhibitory concentrations (1/2 and 1/4MIC) of the extracts (Fig. 4a–f). In contrast, the control (1% DMSO) revealed a strong and dense adherence after 48 h incubation (Fig. 4g).

Antiquorum sensing

Chromobacterium violaceum synthesizes the violet pigment violaceine as a result of quorum sensing (Lichstein & Van De Sand, 1946). Loss of purple pigment violaceine in *C. violaceum* is an indicative of quorum-sensing inhibition by the plant extracts (Fig. 5). The yellowish zone of inhibition observed was opaque and not transparent, indicating that the halo around the disc was caused by inhibition of quorum sensing, not inhibition of cell growth. The antiquorum-sensing activity is dose-dependent (data not shown). Strong quorum-sensing inhibition was observed in the extract of *R. tomentosa* (Fig. 5c). *Eleutherine americana* showed partial inhibition with an incomplete zone (Fig. 5b). On the other hand, *B. pandurata* did not demonstrate this activity (Fig. 5a).

CSH

The effect of subinhibitory concentrations of the plant extracts on CSH of *S. pyogenes* was investigated (results not shown). The growth of *S. pyogenes* NPRC 109 cells in the presence of subinhibitory concentrations of all plant extracts resulted in high CSH (HPBIs were 95.8–98.8%). However, no significant differences in HPBIs were observed between the tests and control (HPBI was 97.3%).

Discussion

Very few *S. pyogenes* are nonbiofilm formers, up to 90% (289 strains) (Baldassarri *et al.*, 2006) and 100% (99 strains) (Conley *et al.*, 2003) were able to form biofilm. Biofilm production by this organism is a new issue and has not been well documented. Lembke *et al.* (2006) reported that serotype M2 strain was connected by thread-like structures of an as-yet-unknown chemical composition. There was no other report on extracellular polymeric substance or exopolysaccharide production.

Many factors influence the biofilm production including the capability of adherence with the substratum (Donlan, 2002), quorum sensing (Li *et al.*, 2001), growth rate (Sissons *et al.*, 1995; Rozen *et al.*, 2001), and anaerobiosis (Baldassarri *et al.*, 2006). Antibiofilm activities have been demonstrated in a number of medicinal plants (Limsong *et al.*, 2004; Duarte

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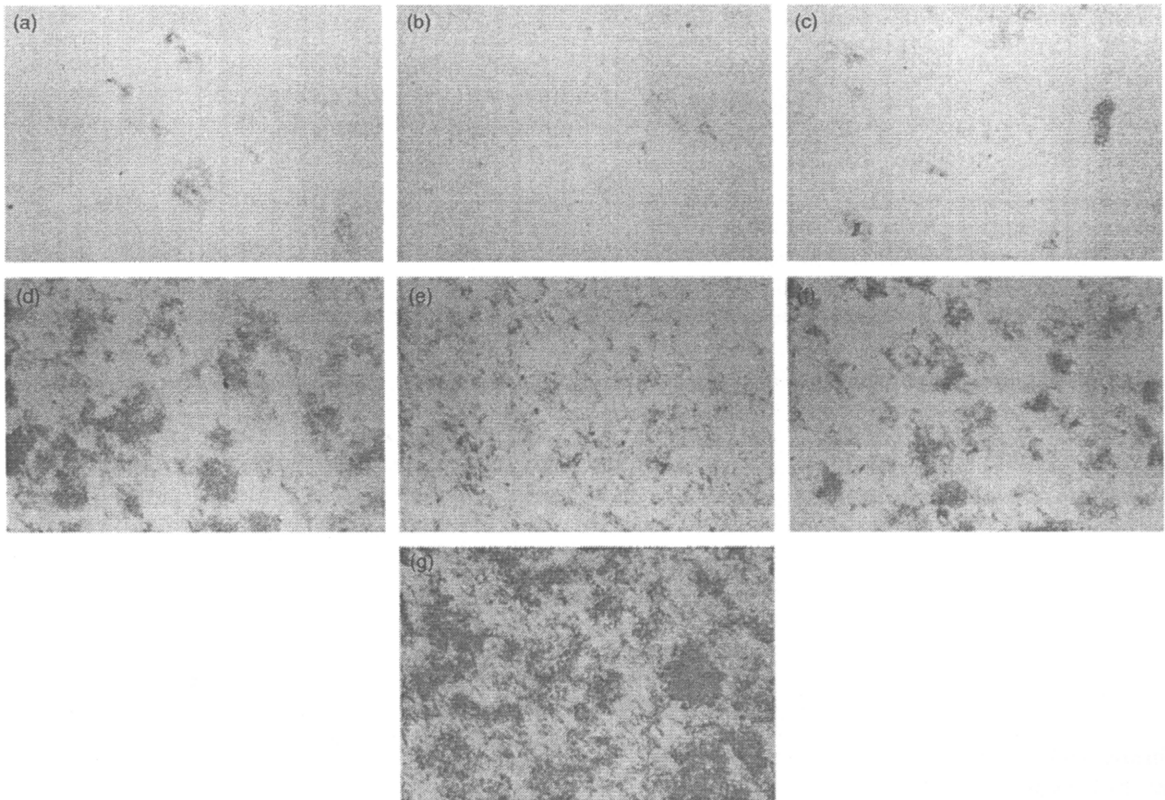

Fig. 4. Inspection of biofilm development of *Streptococcus pyogenes* NPRC 109 on glass surfaces by light microscopy at a magnification × 40. Bacterial biofilms grown in (a–c) 1/2MIC and (d–f) 1/4MIC of the plant extracts at 48 h. (a and d) *Boesenbergia pandurata*, (b and e) *Eleutherine americana*, (c and f) *Rhodomyrtus tomentosa*, and (g) 1% DMSO.

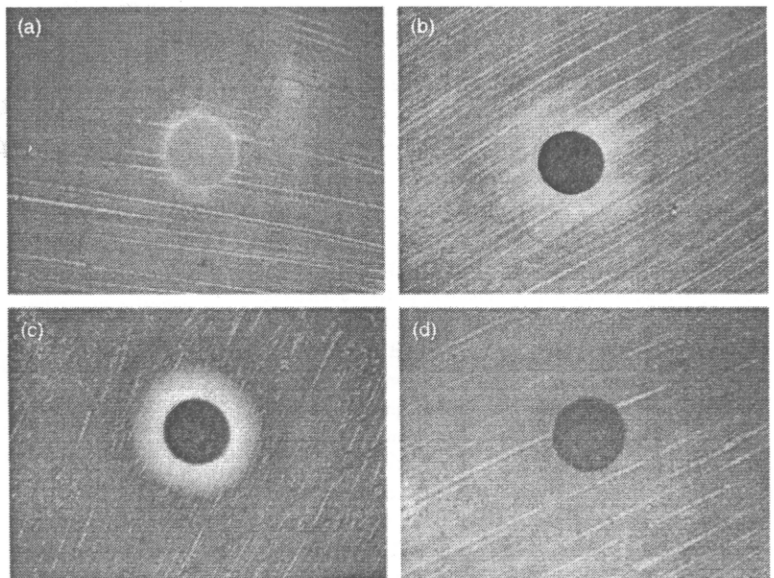

Fig. 5. Inhibition of violacein production by (a) *Boesenbergia pandurata*, (b) *Eleutherine americana*, and (c) *Rhodomyrtus tomentosa* extracts using *Chromobacterium violaceum* DMST 21761 biomonitor strain and agar disc diffusion method. The inhibition was detected by a colourless, opaque halo around the discs. (d) Dimethyl sulfoxide was used as control.

Colour

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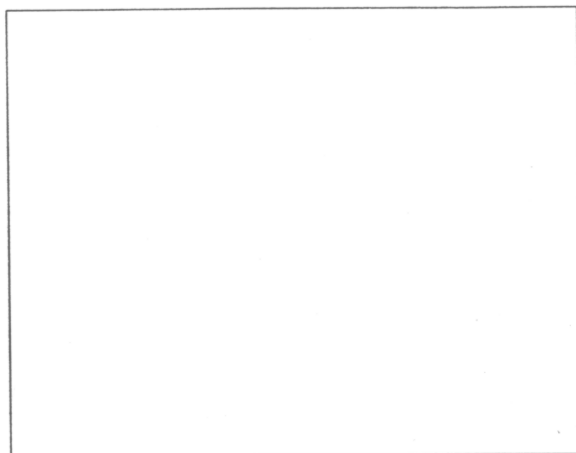

Fig. 6. The effect of subinhibitory concentrations (1/2–1/32MIC) of the plant extracts on CSH of *Streptococcus pyogenes* NPRC 109 by microbial adhesion to hydrocarbon (MATH) method. The hydrophobicity index (HPBI) was calculated as: OD initial − OD final/OD initial × 100%. *S. pyogenes* with a hydrophobic index > 70% was arbitrarily classified as hydrophobic.

et al., 2006; Cartagena *et al.*, 2007; Kuzma *et al.*, 2007). Berberine sulphate, an alkaloid extracted from the roots and bark of various plants, had been reported to interfere with the adherence of *S. pyogenes* by releasing the adhesin lipoteichoic acid from the streptococcal cell surface and directly preventing or dissolving lipoteichoic acid–fibronectin complexes (Sun *et al.*, 1988). In this study, the plant extracts possibly interfered at any step of *S. pyogenes* biofilm formation, but obviously did not inhibit growth at all subinhibitory concentrations tested. Observation on the architecture of *S. pyogenes* biofilms by light microscopy further confirmed that the bacteria did not form dense biofilm layers when treated with the subinhibitory concentrations of the extracts.

Quorum sensing is composed of the release and the reception of signalling molecules produced by bacteria within a given population. This exchange of signals subsequently leads to the induction of more signalling molecules and finally in the acquisition of defined cellular characteristics. The signalling molecules involved in quorum sensing are called 'autoinducers'. Gram-negative bacteria use derivatives of homoserine lactones as autoinducers while Gram-positive bacteria use secreted peptides. The concentration of the signalling molecules produced by the bacteria depends on the population density (Obst, 2007). Many streptococci use quorum-sensing systems to regulate several physiological properties, including the ability to incorporate foreign DNA, tolerate acid, form biofilms, and become virulent (Cvitkovitch *et al.*, 2003). In the present study, antiquorum-sensing activity was observed with the extracts of *E. americana* and *R. tomentosa*. With equal numbers of bacterial populations, *S. pyogenes* NPRC 109 showed a decrease in biofilm level when treated with the extracts. The extracts may have an effect on the level or the activity of quorum-sensing signalling molecules.

Cell surface charge and CSH are known to play an important role in bacterium–host cell interaction (Magnusson, 1989; Swiatlo *et al.*, 2002). Previous studies on plant extracts have demonstrated that many plant extracts can affect CSH of Gram-negative bacteria including *Escherichia coli* (Turi *et al.*, 1997; Voravuthikunchai & Limsuwan, 2006), *Acinetobacter baumannii* (Turi *et al.*, 1997), *Helicobacter pylori* (Annuk *et al.*, 1999), and *Salmonella typhimurium* (Das & Devaraj, 2006), and Gram-positive bacteria such as *Streptococcus mutans* (Nostro *et al.*, 2004; Prabu *et al.*, 2006; Rahim & Khan, 2006) *Streptococcus sanguinis*, *Streptococcus mitis*, and *Actinomyces* sp. (Razak *et al.*, 2006). Changes in bacterial hydrophobicity resulted in a significant decrease in adhesion ability (Fonseca *et al.*, 2004; Das & Devaraj, 2006; Razak *et al.*, 2006) and may be associated with biofilm formation. However, our results showed no effect from all plant extracts on the CHS of *S. pyogenes*, irrespective of the significant decrease in biofilm production.

Conclusions

Our study described the inhibition of biofilm formation of *S. pyogenes* by *B. pandurata*, *E. americana*, and *R. tomentosa*. A correlation between antiquorum-sensing and antibiofilm-producing activities was demonstrated. Active principles from these effective plant species are worth studying in order to solve problems with biofilm-associated infections.

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Research Note

Antibacterial Activities of Semipurified Fractions of *Quercus infectoria* against Enterohemorrhagic *Escherichia coli* O157:H7 and Its Verocytotoxin Production

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ABSTRACT

Escherichia coli O157:H7 is one of the most important foodborne pathogens, causing nonbloody and bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome. Use of antibiotics has been demonstrated to result in increased levels of verocytotoxin (VT) production as well as antibiotic resistance. *Quercus infectoria* was investigated for its antibacterial activity against *E. coli* O157:H7 and other VT-producing enterohemorrhagic *E. coli* (VTEC). The MIC was determined by a broth microdilution method, and the MBC was assessed by subculturing the bacteria from the wells that showed no apparent growth onto Mueller-Hinton agar. The fractions Q_i2, Q_i3, and Q_i4 of *Q. infectoria* were demonstrated to possess good antibacterial activity, with MICs and MBCs ranging from 250 to 500 μg/ml. The effect of the effective fraction, Q_i4, on the production of VT was determined using a reversed passive latex agglutination. The results indicate that at 20 h, fraction Q_i4 markedly inhibits the release of VT1 and VT2 from VTEC cells at both inhibitory and subinhibitory concentrations. Furthermore, verotoxicity assay demonstrated that bacterial cultures treated with fraction Q_i4 exerted less toxic effect on Vero cells. These in vitro results clearly indicate that the fraction Q_i4 might constitute a promising natural food additive for the control of food poisoning by *E. coli* O157:H7 as well as other VTEC strains.

Verocytotoxin-producing enterohemorrhagic *Escherichia coli* (VTEC) O157:H7 is one of the most important foodborne pathogens. *E. coli* O157:H7 is a prototype of VTEC, a subgroup of *E. coli* causing nonbloody and bloody diarrhea, hemorrhagic colitis (HC), and hemolytic uremic syndrome (HUS). The pathogenicity of *E. coli* O157:H7 is associated with virulence factors including verocytotoxin (VT), intimin, enterohemolysin, and lipopolysaccharide. The major virulence factor of VTEC is VT, of which two major subtypes, VT1 and VT2, have been reported. VT is the major pathogenicity factor responsible for multiple damaging effects on eukaryotic cells. It usually involves life-threatening complications of HC and HUS, which makes VTEC infection a public health problem of serious concern. It may complicate 14% of cases of *E. coli* O157:H7 infection *(41)*. The organisms can carry one or both VT subtypes, while strains associated with human disease and in particular HUS more commonly carry VT2 rather than VT1 alone *(14, 22)*.

Up to the present time, a number of *E. coli* O157:H7 outbreaks have been reported from many countries including Great Britain *(30)*, Japan *(16)*, and Mexico *(36)*. Argentina has a high global incidence of HUS, 12.2 cases per 100,000 children *(26)*. Healthy cattle are the reservoir host of the pathogen. Three principal routes of transmission are contaminated food and water, person-to-person spread, and animal contact. Because *E. coli* O157:H7 can survive in the environment for more than 10 months, humans may be at risk of infection *(37)*. This is especially true since the infective dose for *E. coli* O157:H7 in humans has been estimated to be fewer than 50 organisms *(35)*.

Many reports pointed out that antimicrobial agents actually worsen the clinical course of *E. coli* O157:H7 infection and in some cases result in HUS *(6, 12, 17, 19, 33, 42–44)*. Although various antibiotics have the potential to kill *E. coli* O157:H7 present in the intestines of patients, they can stimulate VT production during the course of the infection and bacteriolysis causes the release of VT from the bacterial cells, thereby exerting a disastrous effect on the patient *(6, 12, 17–19, 42–44)*. In addition, a multidrug-resistant phenotype and the identification of a β-lactamase gene in *E. coli* O157:H7 have been recently reported *(1)*.

The antimicrobial activities of a wide range of many plant extracts against *E. coli* O157:H7 have been studied including those of *Camellia sinensis (28)*, *Geranium purpureum (24)*, *Origanum vulgare (4)*, and *Quercus infectoria (3)*. The extracts that contain catechol groups have been well demonstrated to inhibit extracellular release of Vero toxin from *E. coli* O157:H7 *(31)*. However, there are still limited studies on those with pyrogallol groups *(32)*. The nut galls of *Quercus infectoria* (Fagaceae) are an excretory

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product caused by infection by the larvae of *Cynips gallae tinctoriae*. They have been traditionally used as astringents, antidiabetics, local anesthetics, anti-Parkinson's disease agents, hypoglycemics, anti-inflammatory agents, and antidiarrheal drugs *(29, 39, 40)*. The present communication was aimed at finding an alternative additive for food preservation. The nut gall is worth studying, as it could be applied as a direct human food ingredient to prevent contamination by *E. coli* O157:H7. It has been reviewed by the U.S. Food and Drug Administration and determined to be generally recognized as safe.

Previous work with crude ethanolic extract of nut galls demonstrated their effect on *E. coli* O157:H7 *(38)*. However, active compounds of this plant and its antibacterial mechanisms are still unclear. In this study, a detailed investigation of the activity of semipurified fractions of *Q. infectoria* against three strains of *E. coli* O157:H7 and other VTEC strains, including VTEC O26:H11, VTEC O111:NM, and VTEC O22, was carried out to examine their inhibitory effects on VT production.

MATERIALS AND METHODS

Plant materials. The air-dried nut galls of *Q. infectoria* were collected and identified based on physical characteristics. The nut galls were crushed to small pieces by using pestle and mortar and pulverized in an electric grinder.

Preparation of crude extracts of *Q. infectoria*. Dry powder of nut galls was extracted at least three times with 50% ethanol (vol/vol) at 60°C for 30 min. The solvent was then distilled under reduced pressure in a rotary evaporator until it became completely dry. Ethanolic extracts were dissolved in dimethyl sulfoxide (Merck, Darmstadt, Germany) before use in bacteriological study.

Fractionation of ethanolic extract of *Q. infectoria*. The 50% ethanolic extract was first subjected to separation by quick column chromatography using silica gel $60GF_{254}$ (Merck) and then eluted stepwise with chloroform, methanol, and a linear gradient of chloroform-methanol (100:0 to 0:100, vol/vol). The 250-ml volume of eluent was collected in a flask. An aliquot of quick column fractions was spotted on thin-layer chromatography on silica gel $60GF_{254}$ plates. The plates were developed with different proportions of chloroform-methanol-H_2O (6.0:3.7:0.3, vol/vol) as mobile phase. After air drying, the spots on the plate were located by exposure to UV light. Different fractions (Q_i1, Q_i2, Q_i3, and Q_i4) were concentrated by vacuum evaporator and assayed for antibacterial activity.

Bacterial strains. Enterohemorrhagic *E. coli* O157:H7 RIMD 05091078 and RIMD 05091083 were isolated in the 1996 outbreak in Japan. RIMD 05091078 produces both VT1 and VT2, while RIMD 05091083 produces only VT2. Another enterohemorrhagic *E. coli* O157:H7 strain, EDL 933, isolated in the 1983 outbreak in the United States, produces both VT1 and VT2. Other Shiga-like toxin–producing strains were enterohemorrhagic *E. coli* O26:H11 RIMD 05091055 (VT1), enterohemorrhagic *E. coli* O111:NM RIMD 05091056 (VT1), and enterohemorrhagic *E. coli* O22 RIMD 05091556 (VT2). *E. coli* ATCC 25922 was used as a reference strain. Each bacterial strain was suspended in Mueller-Hinton broth (MHB; Difco, Becton Dickinson, Sparks, Md.) and incubated at 37°C for 18 h. Mueller-Hinton agar (MHA; Difco, Becton Dickinson) was used to determine antibacterial activity.

Determination of MIC and MBC. A modified broth microdilution assay using the method outlined by the Clinical and Laboratory Standards Institute *(5)* was performed. Fractions of 0.01 g were dissolved in 1 ml of 10% dimethyl sulfoxide (10 mg/ml). The resulting solution was diluted twofold, in concentrations ranging from 1 to 0.0005 mg/ml. Twenty microliters of an overnight culture of each bacterial strain, containing approximately 10^4 CFU, was inoculated in 160 μl of MHB supplemented with 20-μl fractions. The microtiter plates were incubated at 37°C for 18 h. Experiments were performed in triplicate, and the MIC was recorded as the lowest concentration of plant extracts that showed no turbidity. The MBC was determined by subculturing onto fresh MHA the bacteria from the wells that demonstrated no apparent growth. The lowest concentration that produced a complete kill of bacteria was taken as MBC. Norfloxacin was included as a control.

Determination of VT production by VTEC O157:H7. Fraction Q_i4 (2× MIC) was diluted twofold at the following concentrations: 2× MIC, 1× MIC, 0.5× MIC, 0.25× MIC, 0.12× MIC, 0.06× MIC, and 0.03× MIC. Ten microliters of *E. coli* O157:H7 culture in Trypticase soy broth (TSB; Difco, Becton Dickinson) (cultured at 37°C for 18 h) was inoculated into 90 μl of fresh TSB and incubated at 37°C for 20 h. The culture was centrifuged at 5,000 × *g* for 5 min to separate the supernatant and cell pellet. VT in the periplasmic space was obtained by subjecting the cell pellets to the above centrifugation after treating the pellets with 100 μl of polymyxin B (5,000 IU/ml; Merck) at 37°C for 30 min *(13)*. VT in the culture supernatant (mainly VT2) and cell-associated VT (periplasmic, VT1) were separately determined using a reversed passive latex agglutination test kit (*E. coli* Vero toxin detection kit; Denka Seiken Co; Tokyo, Japan), which can differentiate VT1 and VT2 by using anti-VT1 antibody-sensitized latex and anti-VT2 antibody-sensitized latex, respectively.

Verotoxicity assay. Culture supernatants and periplasmic extracts were filtered through 0.22-μm-pore-size membrane filters. The filtrates were tested for cytotoxic activity in a Vero cell assay system according to a method described earlier *(42)*. The cell survival was examined by a colorimetric (MTT) assay (Cell Counting Kit, Wako Chemicals, Osaka, Japan) *(9, 11)*. The plates were read on a reader (Titertek Multiskan MCC/340, Labsystems, Finland) using a test wavelength of 595 nm.

RESULTS

Fractions Q_i2, Q_i3, and Q_i4, but not Q_i1, were shown to have antibacterial activity on enterohemorrhagic *E. coli* O157:H7, other enterohemorrhagic *E. coli*, and the reference strain. Significant antibacterial effects, expressed as MICs and MBCs of these fractions against enterohemorrhagic *Escherichia coli*, are presented in Table 1. The MICs and MBCs of Q_i2, Q_i3, and Q_i4 against all test strains were from 250 to 500 μg/ml. Due to low yield (0.28%), only a preliminary test was done with fraction Q_i1 against enterohemorrhagic *E. coli* O157:H7 RIMD 0501078 and *E. coli* ATCC 25922. It was demonstrated to have too high of a MIC (>1,000 μg/ml).

Since fraction Q_i4 gave the highest yield (79.37%), it was used for further experiments. Enterohemorrhagic *E. coli* O157:H7 RIMD 05091078 was cultured with fraction Q_i4 at 2× MIC, 1× MIC, and subinhibitory concentrations (0.5× MIC, 0.25× MIC, 0.12× MIC, 0.06× MIC, and 0.03× MIC) for 20 h. Its effect on VT production was

TABLE 1. *MIC and MBC of semipurified fractions of* Q. infectoria *against enterohemorrhagic* E. coli *by broth microdilution method*

Strains	MIC/MBC (μg/ml)[a]			
	Q_i1	Q_i2	Q_i3	Q_i4
E. coli O157:H7 (RIMD 05091078)	>1,000/NA	500/500	500/500	250/250
E. coli O157:H7 (RIMD 05091083)	ND	250/250	500/500	250/500
E. coli O157:H7 (EDL933)	ND	250/250	250/500	500/500
E. coli O26:H11 (RIMD 05091055)	ND	500/500	500/500	250/250
E. coli O111:NM (RIMD 05091056)	ND	500/500	250/1000	500/500
E. coli O22 (RIMD 05091556)	ND	250/250	250/500	500/500
E. coli ATCC 25922	>1,000/NA	250/250	250/500	500/500

[a] NA, not applicable; ND, not done.

detected immunologically. It was clear that the effective fraction Q_i4 markedly inhibited the production of VT both in the periplasmic space (VT1) and in the cell supernatant (VT2) (Table 2). At the highest concentration (2× MIC) to 0.12× MIC, VT1 production was decreased at least 64-fold compared with the control values. At 0.06× MIC, VT1 level decreased 16-fold. Similar patterns were obtained with extracellular VT2 production. At the lowest concentration (0.03× MIC) of Q_i4, both VT1 and VT2 productions were lowered fourfold.

The biological activities of the culture supernatant and polymyxin B–treated pellets in 20-h cultures with Q_i4 at different concentrations (0.03× MIC to 2× MIC) were analyzed (Fig. 1). The cell pellets treated with Q_i4 at 2× MIC were significantly less toxic to Vero cells than the control ($P < 0.05$). Similarly, the 20-h culture supernatant treated with the fraction Q_i4 at 0.5× MIC, 1× MIC, and 2× MIC exerted less toxic effect on Vero cells than the control ($P < 0.05$).

DISCUSSION

Most medicinal plants can produce antibacterial effect against gram-positive bacteria, whereas very few are active against gram-negative bacteria. Interestingly, our current finding shows a remarkable antimicrobial activity of *Q. infectoria* against enterohemorrhagic *E. coli* isolates. The antimicrobial properties of polyphenols from medicinal plants against bacteria causing foodborne diseases have been documented. Green tea, one of the most common medicinal plants studied, contains mostly condensed tannin such as epigallocatechin and epigallocatechin-3-*O*-gallate *(32)*. In contrast, little is known about the role of the hydrolyzable group. Regarding the purified compounds from this plant species (data not shown), Q_i1 and Q_i2 were found to contain aliphatic hydrocarbons with long alkyl chains (Q_i1) and two phenolic components (Q_i2). Q_i3 appeared to be a phenolic compound. Further purification of Q_i4, which contained active components, is currently under investigation. The ethanolic extract of *Q. infectoria* has been previously reported to contain mainly hydrolyzable tannin of the gallic acid type *(23)*. The reaction of both condensed and hydrolyzable tannins on proteins depends on the spatial configuration of the molecules and availability of the reactive phenolic groups. There are several hypotheses on the antibacterial activity of polyphenols. For example, Ikigai et al. *(8)* demonstrated that bactericidal catechins primarily act on and damage bacterial cell membranes. Arakawa et al. *(2)* suggested that oxidative polyphenols generate hydrogen peroxide, which may mediate antibacterial activity.

In addition to the antibacterial activity of semipurified fraction Q_i4, its inhibitory effect on VT1 and VT2 production by *E. coli* O157:H7 at 20 h was clearly demonstrated. This is of interest as it is well documented that VT can cause vascular endothelial damage in HC and HUS patients *(25)*. The verocytotoxicity assay further confirmed that fraction Q_i4 primarily inhibited the release of VT, resulting in significantly higher cell recovery than that of the control ($P < 0.05$). In contrast, the administration of certain antimicrobial agents for the treatment of VTEC infection has been previously reported to result in the increased level of VT production *(42, 43)*. Verocytotoxins are holotoxins composed of a single enzymatic A subunit of approximately 32 kDa in association with a pentamer of receptor-binding B subunit of 7.7 kDa *(7)*. The expression of the A- and B-subunit genes differently regulated the production of VT, which is negatively regulated at the transcriptional level by an iron-sulfur protein corepressor complex *(21)*. Plant extracts might act either directly or indirectly by interfering with the transcription and/or translational steps and reduce the production of toxins *(27)*. The biological activities of VT1 and VT2 have been reported to be different. It has

TABLE 2. *Inhibitory effect of semipurified fraction of* Q_i4 *on VT production by enterohemorrhagic* E. coli *O157:H7 (RIMD 05091078) after 20 h*

Concn of semipurified fraction, Q_i4	Concn of VT in culture (titer of reversed passive latex agglutination)	
	VT1 titer	VT2 titer
2× MIC[a]	<2	<2
1× MIC	<2	<2
0.5× MIC	<2	<2
0.25× MIC	<2	4
0.12× MIC	2	4
0.06× MIC	8	32
0.03× MIC	32	32
Control with no Q_i4	128	128

[a] MIC, 250 μg/ml.

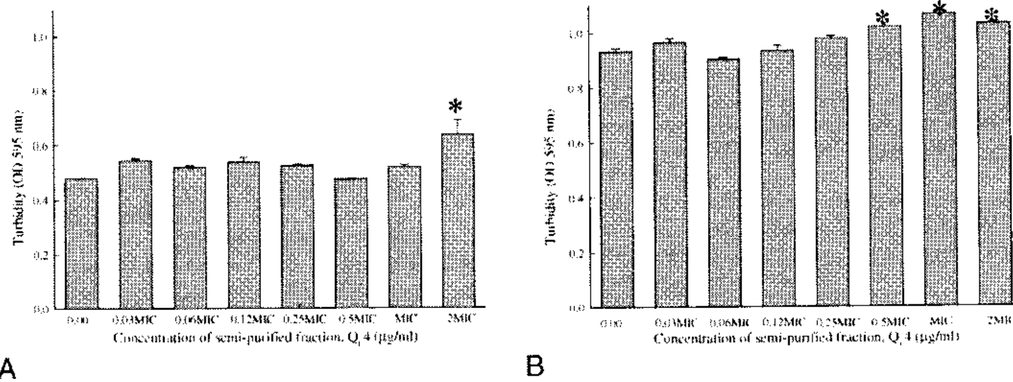

FIGURE 1. *Effects of semipurified fraction Q_i4 on the survival of* E. coli*–infected Vero cells at 20 h: VT1 (A), VT2 (B). The data are presented as means ± standard errors. An asterisk (*) indicates values significantly higher than those of the controls (*P *< 0.05 in Dunnett's test).*

been proven that VT1 and VT2 are apparently different in both the secretion systems and the gene regulation systems *(20)*. VT2 was 1,000-fold more active on human renal endothelial cells than VT1 *(15)*, and VT2 also had a 50% lethal dose that was 400 times lower than that of VT1 when injected intravenously or intraperitoneally into mice *(34)*. Many other factors such as growth conditions, including composition of medium, addition of antibiotic, and aeration affected the production level of the toxins or release of the toxins outside the cells *(10)*.

In conclusion, this study demonstrates that semipurified fractions of *Q. infectoria* possess good antibacterial activity against enterohemorrhagic *E. coli* O157:H7. In contrast to many antibiotics, the effective semipurified fraction Q_i4 inhibited the production of VT both in the periplasmic space (VT1) and the cell supernatant (VT2) at subinhibitory concentrations. The findings described here suggest that *Q. infectoria* can effectively prevent both the growth of *E. coli* O157:H7 and the production of VT. Whether or not the presence of VT in food is a problem needs more investigation. However, since both VT1 and VT2 can result in serious complications, it would be of great advantage to find some safe and effective food additive which could inhibit both the growth of the organisms and the release of the toxins.

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SHORT COMMUNICATION

Quercus infectoria: A Candidate for the Control of Methicillin-resistant *Staphylococcus aureus* Infections

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Acetone, ethyl acetate, 95% ethanol and aqueous extracts of *Quercus infectoria* (*Q. infectoria*) demonstrated significant antibacterial activities against all strains of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *Staphylococcus aureus* (MSSA). Inhibition zones were in the range 11.75–16.82 mm. Both MRSA and MSSA strains exhibited minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values at 0.13 and 0.13–1.00 mg/mL, respectively. At 2 MIC, the growth of two representative MRSA strains was continually inhibited for at least 20 h. Surviving MRSA cells were not detected within 12–14 h after treatment with the extract at 4 MIC concentration. *Staphylococcus aureus* ATCC 25923 demonstrated similar results. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: Quercus infectoria; medicinal plant; methicillin-resistant *Staphylococcus aureus*; antibiotic-resistant bacteria; staphylococcal infections.

INTRODUCTION

During the past decade, community-acquired MRSA (CA-MRSA) infections among young persons without healthcare-associated risk factors have emerged in several areas (Maltezou and Giamarellou, 2006). The majority of CA-MRSA infections are mild skin and soft tissue infections but the consequences of the infections are severe, especially when there is no effective antibacterial treatment.

To overcome the problem of antibiotic resistance in bacteria, many medicinal plants have been studied extensively as an alternative way to treat and prevent the infection. A preliminary study from this laboratory demonstrated that the ethanol extract from *Q. infectoria* possessed significant activity against MRSA isolates (Voravuthikunchai and Kitpipit, 2005). Therefore, the aim of this current communication was further to investigate closely the bacteriostatic and bactericidal activities of different extracts from *Q. infectoria* nutgalls. Time-kill curves were observed to determine the bactericidal activities of the effective extracts for MRSA at different concentrations.

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MATERIALS AND METHODS

Preparation of plant extracts. A voucher specimen of *Q. infectoria* nutgalls was deposited at the Herbarium of Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. The dry plant materials were extracted with solvents of increasing polarity at room temperature for 7 days. The extracts were completely dried and dissolved in 10% dimethylsulfoxide (DMSO, Merck, Germany) before use.

Bacterial strains and culture conditions. Seventeen strains of clinical isolated MRSA were kindly provided from Hat Yai hospital. Thirty-three strains of methicillin-susceptible *S. aureus* (MSSA) were isolated from carriers. *S. aureus* ATCC 25923 was used as control.

Antibacterial activities. The paper disc agar diffusion method (Clinical and Laboratory Standards Institute: CLSI, 2006b) was used for preliminary screening. A modified broth microdilution method was performed according to CLSI Guidelines (2006a).

Time-kill assay. The bactericidal activity of the ethanol extract was studied using the time-kill assay. The bacterial culture (5×10^5 cfu/mL) was added to MHB containing the plant extract at MIC, 2 MIC and 4 MIC and incubated at 35 °C with shaking. The samples were collected every 2 h interval until 20 h, a control tube with 1% DMSO was used as control. Surviving bacteria were cultured on TSA.

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Table 1. Antibacterial activity of the crude extracts from *Quercus infectoria* on methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *Staphylococcus aureus* (MSSA)

Quercus infectoria extracts (2.5 mg/disc)	Inhibition zone (mm)		
	MRSA (*n* = 17)	MSSA (*n* = 33)	*S. aureus* ATCC 25923
Acetone	16.59 ± 0.23[a]	14.61 ± 1.09	15.50 ± 0.50[b]
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 ± 1.8	11.75 ± 0.75

[a] Mean values ± standard error from 17 MRSA and 33 MSSA strains.
[b] Mean values ± standard error of duplicate results.

Table 2. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) 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

RESULTS AND DISCUSSION

Extracts from *Q. infectoria* have been reported to have a broad spectrum of activity against both Gram-negative (Voravuthikunchai *et al.*, 2004) and Gram-positive bacteria (Hwang *et al.*, 2004). In this study, acetone, ethyl acetate, ethanol and aqueous extracts from *Q. infectoria* demonstrated significant antibacterial activities against all MRSA and MSSA strains (Table 1). The results showed similar activities among all strains of MRSA, MSSA, as well as *S. aureus* ATCC 25923, with inhibition zones in the range 11.75–16.82 mm.

Significant antibacterial effects, expressed as the MIC and MBC of the crude extracts against MRSA, MSSA strains and *S. aureus* ATCC 25923, were at concentrations of 0.13–1.00 mg/mL (Table 2). Comparisons of the MIC and MBC values of the acetone, ethyl acetate and ethanol extracts showed no difference.

Time-kill curves are examples of bactericidal activity. Figure 1 shows the results of the time-kill curves for two representative MRSA strains (NPRU 04 and NPRU 09), and a reference strain (ATCC 25923). At the MIC concentration, the number of viable MRSA and the reference strain declined significantly (3 log cfu/mL) after 6 h. The clinical isolates were followed by bacterial re-growth after 8 h, while *S. aureus* ATCC 25923 was still inhibited until 20 h. At 2 MIC, all strains decreased approximately 3–4 log cfu/mL and their levels were suppressed until 20 h. At 4 MIC, the ethanol extract exhibited complete eradication of NPRU 04 and 09 within 12–14 h, and 16 h for *S. aureus* ATCC 25923. The time-kill curves of the extract showed a relationship between the concentration of *Q. infectoria* and the extent of inhibition.

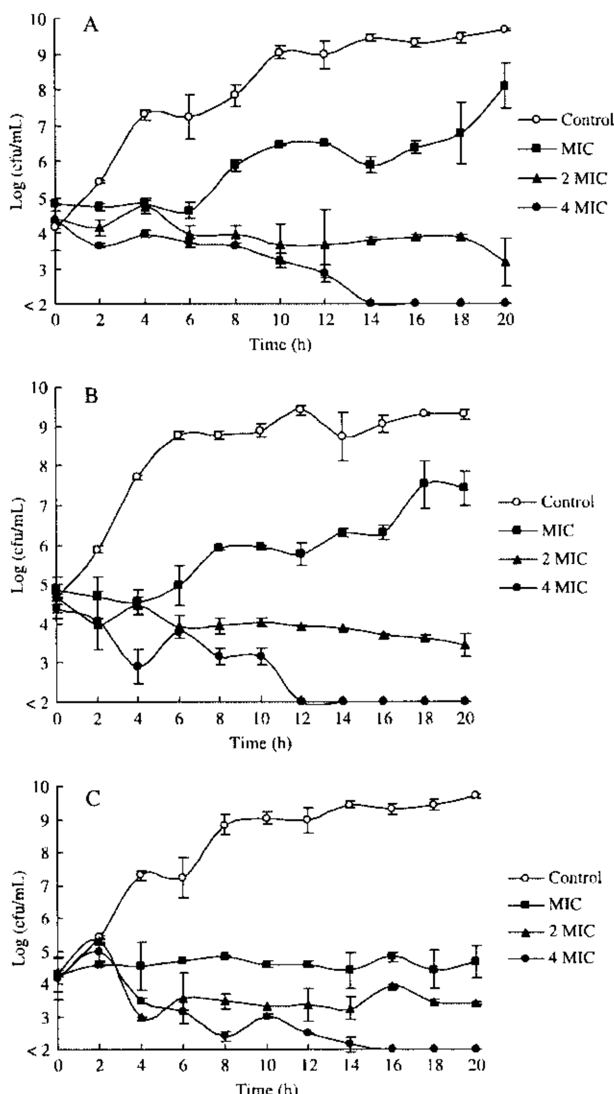

Figure 1. Time-kill curves of MRSA NPRU 04 (A), MRSA NPRU 09 (B) and *S. aureus* ATCC 25923 (C) in control suspensions (○) and after treatment with the ethanol extract at the MIC (■), two times MIC (▲) and four times MIC (●). Each symbol indicates the mean ± SD for at least duplicates. The lowest detection threshold was 10^2 cfu/mL.

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The nutgalls of *Q. infectoria* are known to produce various bioactive compounds, including gallotannin with antitumor activity (Gali *et al.*, 1993), polyphenols with antivenom activity (Pithayanukul *et al.*, 2005), gallic acid, methyl gallate, and ellagic acid with antioxidant activity (Hamid *et al.*, 2005; Krishnaraju *et al.*, 2005). Our preliminary data demonstrated that gallic acid and tannic acid had similar MICs and MBCs to the crude extracts. Research is ongoing to investigate other compounds responsible for antibacterial mechanisms.

The MIC, MBC values depicted in Table 2 and the time-kill curves described above confirm the potency of the ethanol extract from nutgalls against *S. aureus* and the MRSA strains. The isolated bioactive compounds may be used as therapeutic agents for the control of staphylococcal infections. Testing their mechanisms of action on MRSA and their toxicity in humans are warranted for further studies.

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Inhibitory and Killing Activities of Medicinal Plants against Multiple Antibiotic-resistant *Helicobacter pylori*

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Multiple antibiotic-resistant *Helicobacter pylori* (*H. pylori*), one of the major causes of gastric cancer, is now increasingly reported. The aim of this study was to screen medicinal plants widely used in Thailand as possible sources of medicines that can be used to treat *H. pylori* infection. Twenty-four extracts from 13 kinds of Thai herbs were tested for their antibacterial activity against 20 strains of antibiotic-resistant *H. pylori*. Inhibition of growth was tested by the paper disc agar diffusion method. Most strains of *H. pylori* examined were proved to be susceptible to seven medicinal plants; *i.e.*, *Peltophorum pterocarpum*, *Piper betle*, *Punica granatum* (*P. granatum*), *Quercus infectoria* (*Q. infectoria*), *Tamarindus indica*, *Uncaria gambir*, and *Walsura robusta*. Among these extracts, *P. granatum* and *Q. infectoria* exhibited the greatest inhibitory potencies. Minimal inhibitory concentrations (MIC) were determined by the agar dilution method in Petri dishes with a Millipore filter membrane, and minimal bactericidal concentrations (MBC) were assessed with the extract that gave a significant MIC value against each bacterial strain by placing the Millipore filter membrane onto a fresh Isosensitest agar plate. Ethanolic extracts of *P. granatum* and *Q. infectoria* significantly reduced the growth of all strains of *H. pylori*, with the best MIC values at 0.8 and 3.1 mg/ml, and the best MBC values at 3.1 and 6.2 mg/ml, respectively. Effective fractions partially purified from both plant species yielded MICs and MBCs that were at least 10-fold less compared with the crude extracts. From the data obtained, it is hoped that *P. granatum* and *Q. infectoria* will become useful sources with which to develop new therapeutic agents for *H. pylori* infection.

Key words —— *Helicobacter pylori*, *Punica granatum*, *Quercus infectoria*, antibacterial activity, medicinal plant

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative spirally shaped bacterium that has been implicated to cause not only gastritis and peptic ulcer disease but also gastric carcinoma and lymphoma.[1–3] Unless specifically treated, infection with the gastric pathogen *H. pylori* is lifelong. Infection with this bacterium induces the development of an active chronic gastritis. While chronic inflammation is the major outcome of infection, this disorder often develops into a number of more serious conditions such as peptic ulcer disease (PUD), gastric cancer and B cell mucosa-associated lymphoid tissue (MALT) lymphoma. For example, approximately 20% of infected persons develop PUD during their lifetime.[4,5]

Currently, antimicrobial therapy represents the sole approach for the eradication of *H. pylori* infection. The eradication of *H. pylori* with antibiotics significantly decreased the recurrent rates of gastric and duodenal ulcers in both adults and children.[6] A wide variety of antimicrobial regimens have been used for the treatment of *H. pylori* infection with varying degrees of success.[7]

It is obvious that the appearance of antibiotic-resistant strains decreases the efficacy of eradication therapy. Alternative approaches on the use of plant extracts to cure *H. pylori* infection have become increasingly reported. Previously, we have reported that aqueous and ethanolic extracts of certain medicinal plants have antibacterial activity against a number of pathogenic bacteria.[8–10] This includes enterohaemorrhagic *Escherichia coli*,[8] methicillin-resistant *Staphylococcus aureus*,[9] and opportunistic pathogens in AIDS patients.[10] The aim of this study was to investigate the effects of some selected plants, the ingredients of which may be useful for treating *H. pylori*. We have attempted to find some bioactive compounds that are simple, affordable, and have minimal side effects.

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MATERIALS AND METHODS

Plant Collection —— Thirteen well-recognized traditional Thai medicinal plants used to cure gastrointestinal diseases were collected in Thailand in September 2003 on the basis of traditional practices by Thai herbalists. They were air-dried, and their botanical identification was kindly made by Associate Professor T. Supavita, Department of Pharmacognosy, Faculty of Pharmacy, Prince of Songkla University, Thailand. A classified reference voucher specimen was deposited at the Herbarium of Prince of Songkla University. The parts of the plants, their uses in traditional medicine,[11] as well as the percentage yield for each extract are summarized in Table 1.

Plant Extraction —— The plant parts were washed with distilled water, dried at 60°C overnight, cut into small pieces, and crushed in a mechanical mortar. Powdered samples (100 g) were soaked either in water or 95% ethanol (500 ml, w/v) at room temperature for 7 days and then filtered through Whatman No. 2 filter paper. The filter was extracted three times, and the combined filtrate was evaporated under reduced pressure at 60°C until they became completely dry. The aqueous extract was dissolved in 250 mg/ml water, and the ethanolic extract was dissolved in dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany) before use.

Fractionation of Active Compounds —— The ethanolic exudates from *Punica granatum* (*P. granatum*, 26.5 g) and *Quercus infectoria* (*Q. infectoria*, 41.2 g) were partially purified as follows. The exudate was dissolved in 95% ethanol a concentration of 10% (w/v), and applied onto a silica gel column (Merck $60GF_{254}$ 70–230 mesh; 500 g; column *i.d.*, 5 cm). In the purification of *P. granatum* exudate, the column was eluted by a linear gradient from hexane-ethyl acetate (100 : 0 to 0 : 100, v/v; total volume, 8.5 l) to 5% methanol-ethyl acetate (100 : 1900, v/v; total volume, 2 l) and 100% methanol (total volume, 4 l). In the case of *Q. infectoria*, the column was developed by a gradient from chloroform to methanol. Each fraction (250 ml) was monitored by thin layer chromatography (TLC) on silica gel $60GF_{254}$ TLC aluminium sheets (Merck; layer thicknesss 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 plate were visualized under UV light (200–400 nm). Desired fractions were then concentrated to complete dryness. *P. granatum* and *Q. infectoria* yielded 20 and 100 fractions, respectively. Fractions with similar TLC patterns were pooled as depicted in Fig. 1. Some fractions from *P. granatum* exudate were further purified to yield Pg1, 2 and 3 (Fig. 1A). Fraction IV containing a white solid in yellowish oil gave Pg1 by washing with hexane. Fractions VII (white solid) and XV (dark red hexagonal planar solid) were recrystallized from methanol : chloroform (8 : 2, v/v) to Pg2 and Pg3, respectively. Fractions 1–9 (i) of the exudate from *Q. infectoria* contained only trace amounts of extracts. Fractions 10 and 11 (ii: Qi1), frac-

Table 1. List of Medicinal Plants Used in the Antimicrobial Assay

Botanical species	Family	Plant part	Anticancer[a]	Anti-ulcerogenic[a]	Extract yield (%)[b]	
					Aqueous	Ethanolic
Andrographis paniculata (Burm.f) Nees.	*Acanthaceae*	leaf	+	−	ND	11.2
Centella asiatica (L.) Urb.	*Apiaceae*	leaf	+	+	ND	6.8
Curcuma longa L.	*Zingiberaceae*	rhizome	+	+	ND	15.9
Garcinia mangostana L.	*Clusiaceae*	pericarp	+	−	ND	ND
Peltophorum pterocarpum (DC.) Backer ex K. Heyne	*Fabaceae*	bark	−	−	8.6	7.1
Piper betle L.	*Piperaceae*	leaf	−	−	ND	12.4
Psidium guajava L.	*Myrtaceae*	leaf	+	+	2.8	8.0
Punica granatum L.	*Punicaceae*	pericarp	+	−	8.0	13.0
Quercus infectoria Oliv.	*Fagaceae*	fruit	−	−	37.8	32.4
Tamarindus indica L.	*Fabaceae*	leaf	−	−	37.1	4.8
Uncaria gambir Hunter Roxb.	*Rubiaceae*	leaf, stem	−	−	59.8	65.4
Walsura robusta Roxb.	*Meliaceae*	wood	−	−	2.3	4.3
Zingiber cassumanar Roxb.	*Zingiberaceae*	rhizome	+	+	ND	ND

a) See reference 11 for these effects. *b*) Each value represents the percentage (w/w) on the basis of the weight of dried plant. ND = not done.

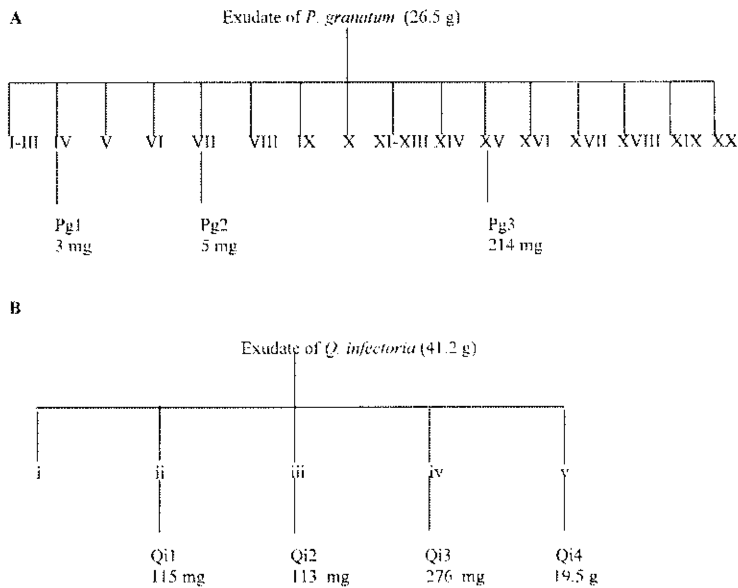

Fig. 1. Fractionation of the Exudate of Medicinal Plants
Schematic representations of the fractionation of exudates from *Punica granatum* pericarp (A) and *Quercus infectoria* nutgall (B) are shown.

tions 12–17 (iii: Qi2), fractions 18–26 (iv: Qi3), and fractions 27–100 (v: Qi4) were pooled, and each combined fraction was designated as indicated (Fig. 1B). When the 1H-nuclear magnetic resonance (NMR) spectrum was measured for structural elucidation, it was taken in CDCl3, using a 500 MHz Varian Unity Inova (Merck, Darmstadt, Germany). Fractions Pg3, Qi2, Qi3, and Qi4 were used for further study. The others were not further investigated since they have no UV absorbance or their yields were too low to assay for antibacterial activities.

Bacterial Strains Tested —— Reference guidelines for culturing and antibiotic sensitivity testing were used.[12] Biopsy specimens sampled from the gastric antrum and body of the stomach were received in Amies or Stuart's transport media and maintained between 4°C and 7°C. Following the Gram stain, the biopsy specimens were cultured without prolonged delay on Campylobacter-selective agar (CSA)[13] to enable the growth and detection of *H. pylori*. The plates were incubated with a lid uppermost at 37°C in 10% CO_2 and 95% relative humidity. Some strains required up to 5–7 days incubation. These plates were examined at 3 day intervals for 12 days. All strains were maintained frozen in Brain Heart Infusion broth (Oxoid, Basingstoke, Hampshire, U.K.) containing 31% (w/v) glycerol in liquid nitrogen. They were thawed just before use and inoculated directly onto CSA. After 48–72 hr incubation, the cultures were checked for purity by phase-contrast microscopy. Biochemical tests including rapid urease, oxidase, and catalase reactions were performed to verify each culture.

Paper Disc Agar Diffusion Method —— A sterile filter paper disc (6 mm) was soaked with 10 µl of plant extract (250 mg/ml extraction solvent) so that each disc was impregnated with 2.5 mg of a substance whose antimicrobial activity was to be examined. Bacterial suspensions were adjusted to a McFarland turbidity of 3.0 (approximately 9.0×10^8 cfu/ml) and cultured on Isosensitest agar plates (Oxoid) enriched with 5% horse blood (Oxoid) by dipping a sterile swab into the suspension and swabbing over the entire plate surface in three directions. Both a wet disc and a dry disc (dried at 37°C overnight) were applied to the surface of the Isosensitest agar (pH adjusted to 4–6) seeded with the test bacteria culture and then the cultures were incubated anaerobically. Antibiotic susceptibility discs (10–30 µg) were used as controls. The antibacterial activity was evaluated by measuring the annular radius of the inhibition zone. The urease test was performed to elicidate a clear zone.[14] The experiments were performed in triplicate and the mean of the diameter of the inhibition zones (annular radii) was calculated.

Determination of Minimal Inhibition Concentration (MIC) and Minimal Bactericidal Concentration (MBC) —— The MIC was determined according to an established method.[15] Briefly, one mi-

croliter of each bacterial strain containing approximately 10^4 colony forming unit (CFU) was seeded on an Isosensitest agar plate supplemented with different fractions of the effective extracts at concentrations starting from the MIC values of crude extracts. The plates were incubated anaerobically at 37°C for 48–72 hr. Observations were performed at least in triplicate and the results expressed as the lowest concentration of a plant extract that produced complete suppression of colony growth (MIC). The MBC was determined with the extract that gave MIC values against each bacterial strain by placing a Millipore filter membrane onto an Isosensitest agar plate.

RESULTS AND DISCUSSION

The plants were initially screened for their antibacterial activity against 20 different clinical strains of *H. pylori* with multiple resistance to amoxicillin, clarithromycin, and metronidazole. Two reference strains, ATCC 43504 and ATCC 43579, were used as controls. Among 26 crude aqueous and ethanolic extracts tested, only 12 extracts (46.15%) of 7 plant species were demonstrated to have antibacterial activity against these strains (Table 2). The extracts from *P. granatum*, *Q. infectoria*, *Uncaria gambir*, and *Walsura robusta* (*W. robusta*) produced inhibition zones against all strains of *H. pylori* tested. The inhibition zones ranged from 4.95 to 16.5 mm. Both aqueous and ethanolic extracts of *P. granatum* and *Q. infectoria*, and the ethanolic extract of *W. robusta*, exhibited high activity against all strains tested. The maximum zone (16.5 mm) of antibacterial effect against *H. pylori* was demonstrated with the ethanolic extracts from *P. granatum*.

The antibacterial effects, expressed as MIC and MBC, of both aqueous and ethanolic extracts of each medicinal plant against each *H. pylori* strain are illustrated in Figs. 2 and 3. The ethanolic extracts of both *P. granatum* and *Q. infectoria* were among the most active, showing very strong activity against all *H. pylori* strains, with the best MIC and MBC values being 0.8, 3.1 and 3.1, 6.2 mg/ml, respectively. Partially purified fractions of both plant species yielded MICs and MBCs that were at least 10-fold less compared with the crude values (Table 3). Thus, we purified or partially purified crude extracts from the above two plants as described in the Experimental Section. Although the components involved in the samples obtained should be further clarified, preliminary experiments suggest the following (data not shown). Of three fractions from *P. granatum*, Pg1 did not contain any ingredient showing UV absorption. Based on data such as the TLC profile and 1H-NMR spectra,

Table 2. Antibacterial Activity of Aqueous and Ethanolic Extracts of Medicinal Plants (2.5 mg/disc) against *H. pylori*

H. pylori strain	Mean values of radii of inhibition zone in wet disc/dry disc (mm)[a]						
	Peltophorum ptercarpum	*Piper betle*	*Punica granatum*	*Quercus infectoria*	*Tamarindus indica*	*Uncaria gambir*	*Walsura robusta*
Aqueous extract:							
H. pylori (20 strains)	4.5/4.3 (4)	2.6/3.0 (5)	13.7/12.9 (20)	11.7/12.0 (20)	ND	4.9/5.1 (20)	ND
H. pylori ATCC 43504	3/2	3/2	13/15	13/12	ND	5/5	ND
H. pylori ATCC 43579	–/–	–/–	12/12	10/10	ND	6/5	ND
Ethanolic extract:							
H. pylori (20 isolates)	4.4/4.0 (8)	3.8/3.9 (9)	16.1/15.9 (20)	13.7/13.1 (20)	3.6/3.6 (14)	5.5/5.3 (20)	8.7/8.5 (20)
H. pylori ATCC 43504	3/4	1/1	14/12	10/10	5/4	2/4	10/9
H. pylori ATCC 43579	–/–	–/–	15/14	11/11	5/4	7/7	8/8

Andrographis paniculata, *Centella asiatica*, *Curcuma longa*, *Garcinia mangostana*, and *Zingiber cassumanar* produced no inhibition zones for all strains tested. *a*) Each value represents the mean of inhibition zones of susceptible strains isolated from human gastrointestinal tract or the mean of three assays for reference strains (ATCC 43504 and ATCC 43579). Numbers of susceptible strains among 20 strains are shown in parentheses. – = no inhibition zone; ND = not done.

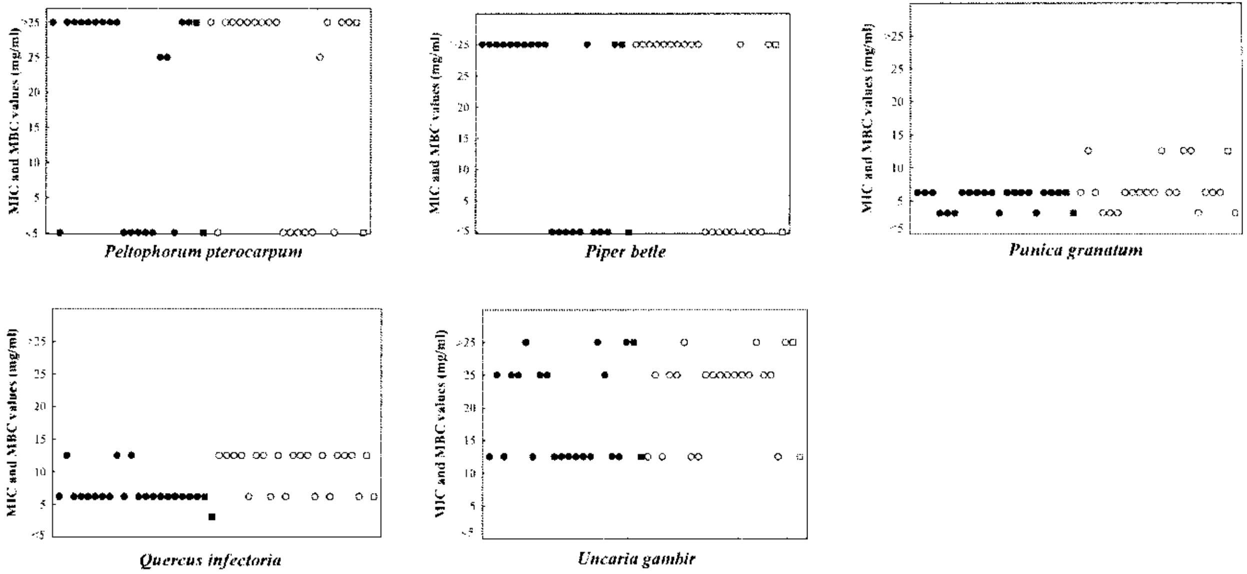

Fig. 2. The MIC and MBC of Aqueous Extracts from Medicinal Plants against *H. pylori*

The closed (●) and open (○) circles indicate the MIC and MBC, respectively, against 20 strains of *H. pylori* isolated from human gastrointestinal tracts. The closed (■) and open (□) squares indicate the MIC and MBC, respectively, against 2 reference strains (ATCC 43504 and ATCC 43579). Each plot represents the value against different strains of *H. pylori*.

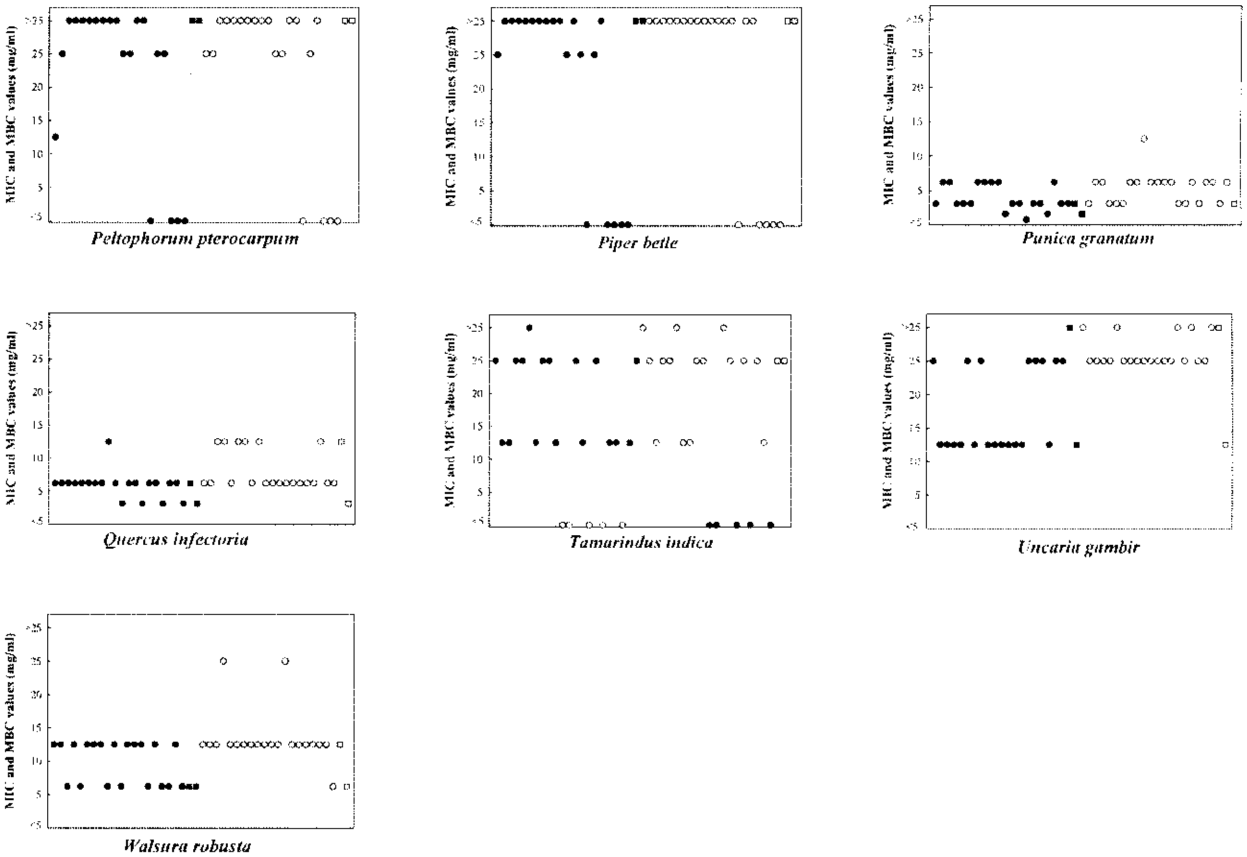

Fig. 3. The MIC and MBC of Ethanolic Extracts from Medicinal Plants against *H. pylori*

The closed (●) and open (○) circles indicate the MIC and MBC, respectively, against 20 strains of *H. pylori* isolated from human gastrointestinal tracts. The closed (■) and open (□) squares indicate the MIC and MBC, respectively, against 2 reference strains (ATCC 43504 and ATCC 43579). Each plot represents the value against different strains of *H. pylori*.

Table 3. MICs and MBCs of Partially-Purified Fractions of *P. granatum* and *Q. infectoria* against *H. pylori*

Effective fraction	MIC/MBC (mg/ml) on *H. pylori* strain				
	PSU HP 4	PSU HP5	UNSW 03-03-21-002	UNSW 03-03-25-001	ATCC 43504
P. granatum Fraction Pg3	0.32/0.32	0.32/0.32	0.16/0.16	0.16/0.16	0.32/0.32
Q. infectoria Fraction Qi2	0.16/0.16	0.16/0.16	0.16/0.16	0.16/0.16	> 2.5 /NA
Q. infectoria Fraction Qi3	0.32/0.32	0.16/0.16	0.32/0.32	0.16/0.16	0.32/0.32
Q. infectoria Fraction Qi4	0.32/0.32	0.32/0.32	0.32/0.32	0.32/0.32	> 2.5 /NA

NA = not applicable.

while Pg2 seemed to be a mixture of stigmasterol and fisitosterol (1 : 1), Pg3 is thought to be a pure compound belonging to the tannin group. Regarding the purified samples from *Q. infectoria*, Qi1 and Qi2 were suggested to contain aliphatic hydrocarbons with long alkyl chains and two phenolic components, respectively. Qi3 appeared to be a phenolic compound. Qi4, which contained active components, was obtained in a large quantity, but its further purification is currently underway. In this study, Pg3, Qi2, Qi3 and Qi4 were further examined for their anti-*H. pylori* activities.

Although the majority of individuals infected by *H. pylori* are asymptomatic, a proportion of them develop peptic ulcers. This organism is the major cause of gastric cancer and has been classified as a Class I carcinogen by the World Health Organization (WHO). It is well-established that an asymptomatic *H. pylori* infection could be a risk factor for gastric cancer. Due to the increasing rate of antibiotic-resistant organisms, many workers have attempted to eradicate the organism with natural products. Plants contain a number of organic components including alkaloids, flavones, phenols, quinines, terpenoids, and tannins, all of which are known to have antibacterial activity.[16] Among the plants that exhibited an antibacterial effect, *P. granatum* and *Q. infectoria* were the most efficient, probably due to the production of novel metabolites capable of inhibiting *H. pylori* growth. *P. granatum* has been extensively studied. This medicinal plant possesses a high amount of tannin (25%). The antimicrobial properties of this substance are well-established.[17] Recently, the antimicrobial properties of polar fractions, which contain ellagitannin and punicalagin, of *P. granatum*, were reported by other workers.[18] In contrast, very limited studies have been done on *Q. infectoria*, and thus this plant species is being brought into focus in this laboratory. Preliminary results from our laboratory indicate the activity is due to hydrolysable tannins.

Plants also contain a number of water-soluble proteins, lectins, and carbohydrates which may bind specifically to sugar residues, polysaccharides, glycoproteins or glycolipids such as adhesins present on the cell surface of *H. pylori*. Lengsfeld *et al.*[19] have demonstrated that adhesion of *H. pylori* to stomach sections is almost completely inhibited by pre-incubating with a fresh juice preparation of the fruit of the *Abelmoschus esculentus* (L.) Moench (okra plant). Many other plant extracts including turmeric, borage, and parsley were also reported to possess similar ability.[20] We have previously described that both *P. granatum* and *Q. infectoria* can increase the cell hydrophobicity of *H. pylori*.[21] Modulation of cell surface hydrophobicity by the plants may synergistically facilitate the elimination of the bacterial cells from the human body.

Our finding tentatively suggests important therapeutic implications for some herbal preparations with antibacterial properties for patients with *H. pylori*-induced PUD or gastric cancer. The high activity of both *P. granatum* and *Q. infectoria* against all strains of *H. pylori* could allow their use in the treatment of an *H. pylori* infection. The partially-purified fractions of *P. granatum* and *Q. infectoria* were the most effective against *H. pylori*, and had the same MIC and MBC values (0.16 mg/ml). In addition, many plants also have anti-ulcerogenic or anti-cancer effects. They may enable a treatment that is simple and relatively inexpensive by incorporation into the normal diet of the patient since the plants are already known to be safe and are commonly employed in traditional Thai medicine with

no toxicity having been reported. Alternatively, they could be used in combination with antibiotics, possibly increasing the success of eradication, as has been demonstrated earlier with cranberry juice.[22] However, many more studies are needed to confirm the *in vivo* effects of plant ingredients. Such information would be more important if administration of the pure forms of these substances to patients is desired. *Q. infectoria* is rather interesting since it is inexpensive and can be recovered at very high extract yields. This medicinal plant should be further analyzed as it might provide a new effective compound against *H. pylori* infections. Further studies are in progress in this laboratory to determine more precisely the effects of different fractions of the plant in order to provide an alternative treatment of *H. pylori* infection.

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Quercus infectoria Oliv.

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Abstract

Bacterial resistance to antibiotics is enhanced by unrestricted use of such drugs. In addition, stimulating effects of certain antibiotics on the production of verocytotoxin by enterohemorrhagic *Escherichia coli* have been reported. The aim of this communication was to report a medicinal plant species that is effective against a broad range of bacteria of clinical importance including *Acinetobacter baumannii, Bacillus cereus, Enterobacter faecalis, Escherichia coli, Helicobacter pylori, Klebsiella pneumoniae, Listeria monocytogenes, Pseudomonas aeruginosa, Salmonella* spp., *Shigella flexneri, Staphylococcus aureus, Streptococcus mutans*, and *Streptococcus pyogenes*. Ethanol extracts of *Quercus infectoria* Oliv. (Fagaceae) demonstrate a broad spectrum of activity against all bacterial species tested. Detailed studies were carried out on clinical isolates of pathogens commonly presenting problems with the use of antibiotics, including enterohemorrhagic *Escherichia coli* (EHEC), methicillin-resistant *Staphylococcus aureus* (MRSA), multidrug-resistant *Klebsiella pneumoniae*, multidrug-resistant *Helicobacter pylori*, and *Salmonella* spp. The extracts of *Quercus infectoria* displayed remarkable activity against MRSA with MICs ranging from 0.02 to 0.4 mg/mL, and MBCs ranging from 0.4 to 1.6 mg/mL. More importantly, this plant species could exhibit strong antibacterial activity against all Gram-Negative organisms. Its significant activity was shown with EHEC, with MICs of 0.05 to 0.1 mg/mL and MBCs of 0.8 to 1.6 mg/mL. The results from this study indicate that *Quercus infectoria* is potentially a good source of antibacterial substances with broad spectrum of activities against antibiotic-resistant bacteria.

Keywords: *Escherichia coli* O157: H7, *Helicobacter pylori*, methicillin-resistant *Staphylococcus aureus*, multidrug-resistant *Klebsiella pneumoniae, Quercus infectoria, Salmonella.*

Introduction

In developing countries where antibiotics can be used on an unrestrict basis, the emergence of antibiotic-resistant pathogenic bacteria has caused considerable public health concern. Treatment failure due to methicillin-resistant *Staphylococcus aureus* (MRSA) with reduced susceptibility to vancomycin has been reported (Ward et al., 2001). More recently, resistance to new antimicrobial agents such as linezolid, quinupristin, and daffopristin has already occurred (Leclercq, 2002; Berns, 2003). Multiple-antibiotic resistance of *Klebsiella pneumoniae*, *Salmonella* spp., and other important pathogens has become increasingly reported (Guan et al., 2006; Martinez-Martinex et al., 1999).

Helicobacter pylori has been implicated as a major causative agent of gastritis and peptic ulcer disease and as a carcinogen responsible for gastric carcinoma and lymphoma (Dixon, 1997; Kuipers, 1997; Marshall & Armstrong, 1995; Wotherspoon et al., 1993). A wide variety of antimicrobial regimens have been used for the treatment of *H. pylori* infection with varying degrees of success (Megraud, 1997). However, the use of antibiotics induces the appearance of resistant strains (Megraud, 2001). Over recent years, there has been a global increase in the prevalence of antibiotic resistance to metronidazole and clarythromycin in *H. pylori* strains (Graham, 1998; Tompkins et al., 1997).

Enterohemorrhagic *Escherichia coli* (EHEC), in particular serotype O157: H7 (Bower, 1999; Paton & Paton, 1998; Wells et al., 1995) have increasingly emerged as pathogens that cause significant human diseases including diarrhea (Pai et al., 1988) enterohemorrhagic colitis (HC) (Riley, 1987), and occasionally complications such as

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hemolytic-uremic syndrome (HUS) and thrombocytopenic purpura (TTP) (Griffin & Tauxe, 1991; Scotland et al., 1988). The largest outbreak reported to date occurred in Japan in 1996. The issue of the risk involved in the treatment of the organism with antimicrobial agents has been reported. Fosfomycin, the most frequently prescribed antibiotic in Japan for the treatment of this organism, was reported to stimulate the production and the release of verocytotoxin 1 (VT1) (Yoh et al., 1997). Furthermore, work from the same laboratory clearly demonstrated that subinhibitory concentrations of quinolones including norfloxacin, sparofloxacin, and grepafloxacin markedly stimulated the production of both VT1 and VT2 (Yoh et al., 1999).

The investigation of traditionally used plants as a guide to biologically active extracts has been well-documented. Complications of the use of antibiotics in the treatment of bacterial infections encourage us to find an effective medicinal plant as an alternative treatment. Recent studies conducted in our laboratories (Voravuthikunchai et al., 2004, 2006; Voravuthikunchai & Kitpipit, 2005) and in others (Cowan, 1999) have revealed that medicinal plants from various parts of the world can provide a rich source of antibiotic activities. In Thailand, many plant species have been widely used to cure many infectious diseases. They are available locally, inexpensive, and are very popular. As part of a collaborative research program among our groups, we have investigated almost 100 medicinal plant species. The purpose was to find some attributed antibiotic properties of plant extracts that have been used in traditional medicine to cure infections.

Quercus infectoria Oliv. (Fagaceae) is an evergreen shrub growing to 1.8 m, valued for excrescences formed upon the young branches, known in the markets as galls or nut-galls. They are the result of a puncture made in the bark by an insect, *Diplolepis gallae tinctoriae* or *Cynips quercufolii*, for the purpose of depositing its egg. 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. Any galls produced on the tree are strongly astringent and have been employed in traditional medicine in the treatment of chronic diarrhea, dysentery, hemorrhages, leucorrhea, and sore throat. In addition, the bark and acorns are astringent and used in the treatment of intertrigo, impetigo, and eczema. Although the nut-gall is routinely prescribed for the treatment of diarrhea in Thai herbal medicine, scientific data supporting the use of this plant as an herbal medicine against bacteria is scarce. The purpose of this investigation was to observe the antibacterial effects of this plant species against a wide range of pathogenic bacteria according to traditional practices. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of both extracts against selected important pathogens were established.

Materials and Methods

Medicinal plant materials

The air-dried nut-galls of *Quercus infectoria* were purchased from traditional Thai herbalists. Botanical identification of the plants materials was authenticated by Dr. Tanomjit Supavita, Reader in the Department of Pharmacognosy, Faculty of Pharmacy, Prince of Songkla University. A classified reference voucher specimen was deposited at the Herbarium of Prince of Songkla University.

Preparation of plant extracts

The extracts used in this investigation have been previously described (Voravuthikunchai et al., 2004). Aqueous extracts were dissolved in water and ethanol extract was dissolved in dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) before use.

Tested bacterial strains

Methicillin-resistant *S. aureus* (MRSA) and multidrug-resistant *K. pneumoniae* (MRKP) isolates were obtained from Hatyai Hospital in Thailand. Clinical isolates of *H. pylori* were from The Australian Helicobacter Reference Laboratory and Chulalongkorn Hospital, Thailand. *E. coli* O157: H7 RIMD 0509952, RIMD 05091078, and RIMD 05091083 were isolated in the 1996 outbreak in Japan. RIMD 0509952 and RIMD 05091078 produced both VT1 and VT2 ($VT1^+$, $VT2^+$), whereas RIMD 05091083 produced only VT2 ($VT2^+$). Other Shiga-like toxin producing strains included *E. coli* O26: H11 RIMD 05091055 ($VT1^+$), *E. coli* O111: NM RIMD 05091056 ($VT1^+$), and *E. coli* O22 RIMD 05091556 ($VT2^+$). All of the *E. coli* strains were kindly provided by the Research Institute for Microbial Diseases, Osaka University. Other pathogens included *Acinetobacter baumannii*, *Bacillus cereus*, *Enterobacter faecalis*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Shigella flexneri*, *Streptococcus mutans*, and *Streptococcus pyogenes*. Each bacterial strain was suspended in Mueller-Hinton broth (MHB; Becton, Dickinson and Company, Sparks, MD, USA), incubated at 35°C for 3 to 5 h, and turbidity adjusted to McFarland no. 0.5. Mueller-Hinton agar (MHA; Difco) was used for testing antibacterial activity.

Paper disk diffusion method

Sterile filter paper disks (Whatman no. 1; 5 mm in diameter) were soaked with 10 μL of extract residue diluted in the corresponding extractive solvent (250 mg/mL), so that each disk was impregnated with 2.5 mg of residue. Dry disks (dried at 37°C overnight) were applied on the

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Table 1. Antibacterial susceptibility patterns.

Bacterial strains	Antibiotics susceptibility patterns (%)															
	Amikacin	Ampicillin	Cefamandole	Cefoxitin	Cephalothin	Chloramphenicol	Ciprofloxacin	Clarithromycin	Erythromycin	Gentamicin	Kanamycin	Metronidazole	Norfloxacin	Oxacillin	Tetracycline	Trimethoprim-sulfamethoxazole	Vancomycin
MRSA						17.3 (R)			100 (R)	94.2 (R)				100 (R)			0 (R)
(n = 52)						82.7 (S)			0 (S)	5.8 (S)				0 (S)			100 (S)
MRKP			95.2 (R)	33.3 (R)	100 (R)		38.1 (R)			90.4 (R)	95.2 (R)		28.5 (R)			90.4 (R)	
(n = 26)			4.8 (I)	66.7 (S)	0 (S)		19.1 (I)			4.8 (I)	4.8 (S)		4.8 (I)			9.6 (S)	
			0 (S)				42.8 (S)			4.8 (S)			66.7 (S)				
EHEC	0 (R)	100 (R)	0 (R)			0 (R)				0 (R)	20 (R)		0 (R)		40 (R)		
(n = 5)	100 (S)	0 (S)	100 (S)			100 (S)				100 (S)	80 (S)		100 (S)		60 (S)		
H. pylori								27.3 (R)				63.6 (R)					
(n = 11)								72.7 (S)				36.4 (S)					

I, intermediate; R, resistant; S, sensitive.

Note:

E. coli ATCC 25922: Susceptible to amikacin, ampicillin, gentamycin, kanamycin, tetracycline.

H. pylori ATCC 43504: Susceptible to amoxicillin, clarithromycin, tetracycline.

H. pylori ATCC 43579: Susceptible to amoxicillin, clarithromycin, tetracycline.

K. pneumoniae ATCC 10273: Susceptible to cefamandole, cefoxitin, cephalothin, ciprofloxacin, gentamicin, kanamycin, norfloxacin, trimethoprim-sulfamethoxazole.

S. aureus ATCC 25923: Susceptible to chloramphenical, erythromycin, gentamicin, oxacillin, vancomycin.

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Table 2. Antibacterial activity of the ethanol extracts of *Quercus infectoria* (concentration 2.5 mg/disk) against important pathogenic bacteria.

Bacterial strains	Mean of inhibition zone (mm)	
	Aqueous extract	Ethanol extract
Acinetobacter baumannii	14.00	14.00
Bacillus cereus	15.00	14.75
Enterobacter faecalis	12.00	13.50
Escherichia coli ATCC 25922	11.50	12.50
Helicobacter pylori ATCC 43504	23.50	24.00
Helicobacter pylori ATCC 43579	21.00	20.50
Klebsiella pneumoniae ATCC 10273	11.00	11.00
Listeria monocytogenes	12.00	12.00
Pseudomonas aeruginosa ATCC 27853	11.00	10.00
Salmonella typhi	9.50	9.00
Salmonella typhimurium	8.75	9.06
Salmonella weltevreden	9.00	8.75
Shigella flexneri	17.00	17.00
Staphylococcus aureus ATCC 25923	16.25	17.00
Streptococcus mutans	15.50	15.25
Streptococcus pyogenes	22.50	23.00

surface of MHA plates seeded with a 24-h broth culture of the tested strains in trypticase soy broth (TSB; Oxoid, Hampshire, England). The inoculum size was adjusted to approximately 10^8 colony-forming units (CFU)/per milliliter. The plates were then incubated at 35°C for 18 h. Antibiotic susceptibility disks including chloramphenicol, erythromycin, gentamicin, kanamycin, oxacillin, tetracycline, and vancomycin (10–30 μg) were used as controls (Table 1). The activity was evaluated by measuring the diameter of the inhibition zone. The experiment was performed in duplicate and the mean of the diameter of each inhibition zone was calculated.

Determination of MIC and MBC

A modified agar microdilution method was used to determine the MIC of aqueous and ethanol extracts of medicinal plants that produced inhibition zones. Chloramphenicol, erythromycin, gentamicin, kanamycin, oxacillin, tetracycline, and vancomycin (10–32 μg/mL) were used as reference standards. The culture of each bacterial species (1 μL) containing approximately 10^4 CFU was applied onto MHA supplemented with the medicinal plant extracts at concentrations ranging from 0.12 to 250 mg/mL. The microtiter plates were incubated at 35°C for 18 h. Observations were performed in triplicate and results expressed as the lowest concentration of plant extracts that produced a complete suppression of colony growth (MIC). Minimal bactericidal concentration was performed with the extracts that gave significant MIC values against each bacterial isolate by culture on fresh MHA.

Results and Discussion

Preliminary test for antibacterial activity of the ethanol extracts of this plant material against a wide range of important pathogenic bacteria is reported in Table 2. The plant extracts produced inhibition zones against all bacterial species, ranging from 8.75 to 23.00 mm. Both aqueous and ethanol extracts were effective against all clinical isolates of selected pathogens tested; in general, the ethanol extracts produced larger zones (Table 3). Significant antibacterial effects, expressed as MICs and MBCs of the crude extracts of *Quercus infectoria*, were observed (Table 4). The MICs were from 0.05 to 6.2 mg/mL, and the MBCs ranged from 0.4 to 12.5 mg/mL. Both aqueous and ethanol extracts displayed similar antimicrobial activity. Although the extracts demonstrated some inhibitory effect against *H. pylori*, the MICs and MBCs were much higher than those of the other organisms.

This is the first report of broad antibacterial activity of extracts from *Quercus infectoria* against a wide range of important pathogens. It was demonstrated to have significant

Table 3. Antibacterial activities of aqueous and ethanol extracts of *Quercus infectoria* (concentration 2.5 mg/disk) against clinical isolates.

Clinical bacterial isolates	Inhibition zone (mm) (Mean ± standard error)	
	Aqueous extract	Ethanol extract
Escherichia coli O157: H7 (RIMD 0509952)	8.00	11.00
Escherichia coli O157: H7 (RIMD 05091078)	9.00	12.50
Escherichia coli O157: H7 (RIMD 05091083)	8.00	14.00
Escherichia coli O26: H11(RIMD 05091055)	9.00	12.00
Escherichia coli O111: NM (RIMD 05091056)	8.00	15.00
Escherichia coli O22 (RIMD 05091556)	9.00	11.00
Helicobacter pylori (20 isolates)	24.10 ± 0.70	26.30 ± 0.73
Multidrug-resistant *Klebsiella pneumoniae* (21 isolates)	11.57 ± 0.17	9.28 ± 0.74
Salmonella spp. (6 isolates)	ND	8.25 ± 0.15
Methicillin-resistant *Staphylococcus aureus* (52 isolates)	18.5 ± 0.57	17.97 ± 0.24

ND, not determined.

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Table 4. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of crude *Quercus infectoria* extracts against bacterial strains.

Bacterial strains	Range of MIC/MBC values (mg/mL)	
	Aqueous extract	Ethanol extract
Escherichia coli O157: H7 (RIMD 0509952)	0.1[a]/0.8[b]	0.1/0.8
Escherichia coli O157: H7 (RIMD 05091078)	0.1/0.8	0.1/0.8
Escherichia coli O157: H7 (RIMD 05091083)	0.1/3.1	0.1/1.6
Escherichia coli O26: H11(RIMD 05091055)	0.1/1.6	0.1/0.8
Escherichia coli O111: NM (RIMD 05091056)	0.1/0.8	0.1/0.8
Escherichia coli O22 (RIMD 05091556)	0.1/1.6	0.05/1.6
Escherichia coli ATCC 25922	0.4/0.4	0.8/0.8
Helicobacter pylori (21 isolates)	6.2–12.5/6.2–12.5	3.1–12.5/6.2–12.5
Helicobacter pylori ATCC 43504	6.2/12.5	6.2/12.5
Helicobacter pylori ATCC 43579	3.1/6.2	3.1/3.1
Klebsiella pneumoniae ATCC 10273	0.2/0.4	0.1/0.8
Multidrug-resistant *Klebsiella pneumoniae* (20 isolates)	0.1–0.2/0.4–12.5	0.1–0.8/1.6–12.5
Salmonella spp. (6 isolates)	ND	0.02–0.04/6.2–12.5
Staphylococcus aureus ATCC 25923	0.2/1.6	0.1/1.6
Methicillin-resistant *Staphylococcus aureus* (52 isolates)	0.2–0.4/0.4–0.6	0.2–0.4/0.4–1.6

ND, not determined.
[a]MIC.
[b]MBC.

antibacterial activity against pathogenic bacteria, irrespective of their antibiotic-resistant patterns. In general, a much greater number of medicinal plants have been found to be more active against Gram-positive bacteria than against Gram-negative bacteria (McCutcheon et al., 1992). The basis for this difference might be the differences in plant cell wall composition (Grosvenor et al., 1995). The antibacterial activity may be indicative of the presence of some metabolic toxins or broad-spectrum antibiotic compounds. The galls of *Quercus infectoria* are a potentially good source of antimicrobial substances with broad spectrum of significant antibacterial activity. The main constituents are tannin (50–70%), a small amount of free gallic acid, and ellagic acid (Wiart et al., 2001). The high activity of *Quercus infectoria* against all bacteria tested could allow its application in the treatment of many types of infections. The use of such a biologically active compound from a natural source is another approach to solve the problem of drug-resistant pathogens. The results presented here suggest that this plant extract should be further analyzed as it might provide a new compound that is effective against multiresistant infections. This simple and inexpensive alternative to conventional treatment of a number of infections may be worth further rigorous investigations. Our research group is carrying out extensive studies on this plant species as we found that it has a broad spectrum of activity against all bacterial species of medical importance tested. In addition, it is inexpensive, and very high yield up to 38% (Voravuthikunchai et al., 2006) was recovered.

Conclusions

This is to date the most comprehensive report on antibacterial activities of *Quercus infectoria*. This plant species was demonstrated to have significant antibacterial activity against many important pathogens, irrespective of their antibiotic patterns. Detailed studies of its constituents and mechanisms of action, toxicology study, and clinical trials may lead to a solution for the problem of unrestricted use of antibiotics.

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Family Zingiberaceae Compounds as Functional Antimicrobials, Antioxidants, and Antiradicals

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ABSTRACT

Increasing numbers of reported cases of food-associated infections and health problems associated with synthetic additives have led to a growing interest by consumers in ingredients from natural sources. Some members of the family Zingiberaceae have been extensively used as a condiment for flavoring as well as traditional medicines. These include *Alpinia galanga* (galanga), *Boesenbergia pandurata* (krachai), *Curcuma amada* (mango ginger), *Curcuma longa* (turmeric), *Curcuma zedoria* (zedoary), *Kampferia galanga* (proh hom), *Zingiber officinale* (ginger), and *Zingiber zerumbet* (zerumbet ginger). Their antimicrobial activities against important foodborne pathogens including *Bacillus cereus*, *Campylobacter jejuni*, *Clostridium botulinum*, *Clostridium perfringens*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp., *Shigella* spp., *Staphylococcus aureus*, *Vibrio* spp., *Yersinia enterocolitica*, Hepatitis A Norwalk virus, *Entamoeba histolytica*, and *Giardia lamblia* are outlined. In addition to the antimicrobial activities against a wide range of microorganisms, their antioxidant activities have been documented. The potential uses of these plant species as food preservatives are discussed.

Keywords: *Alpinia galanga*, antimicrobial activity, antioxidant activity, *Boesenbergia pandurata*, *Curcuma amada*, *Curcuma longa*, *Curcuma zedoria*, food poisoning, food preservation, food spoilage, galangal, ginger, *Kampferia galanga*, krachai, mango ginger, proh hom, turmeric, zedoary, zerumbet ginger, zingiberaceae, *Zingiber officinale*, *Zingiber zerumbet*

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INTRODUCTION

At present, food safety is a fundamental concern to both consumers and food industries as there are increasing numbers of reported cases of food-associated infections. Food-borne illness remains a major problem even in industrialised countries (Gould *et al.* 1995). In addition, lipid oxidation is another issue affecting quality loss in muscle foods. There has been a growing interest in new and effective techniques to reduce the cases of food-borne illness (Otshudi *et al.* 2000). Consumers prefer high quality, nutritious, and long shelf-life food products with no preservative agents. Food preservation, therefore, is the basis of most modern food industries in the world.

A number of botanical supplements have been used for centuries in the ancient Indian system of medicine known as *Ayurveda*. Almost every nation has traditional folk medicines or folk remediation with medicinal plants. The use of herbs and their extracts as functional ingredients in foods is expanding rapidly both for the growing interest of consumers in ingredients from natural sources and also increasing concern about potential health problems associated with synthetic additives (Reische *et al.* 1998). Antimicrobials from natural sources have been used for food safety since antiquity (Alzoreky and Nakahara 2003). There is an increasing interest in the use of plant-derived antimicrobial compounds as natural food preservatives. Natural antimicrobials found in medicinal plants can protect us from infectious diseases caused by bacteria, fungi, and viruses including HIV, the virus that produces AIDS. Interestingly, a new emerging food threat, bird flu virus H5N1, has been claimed to be effectively eliminated using plant extracts such as hypercine

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Invited Review

(www.scidev.net/News/index), sambucol (www.nutraingredients.com/news). The secondary metabolites of plants provide humans with numerous biologically active products, which have been used extensively as food additives, flavors, colors, insecticides, drugs, fragrances, and other fine chemicals. These plant secondary metabolites including several classes such as terpenoids, flavonoids, and alkaloids comprise of diverse chemicals and biological activities. In addition, plant derivatives have unique structural diversity. This has led to a renewed interest in bioactive compounds.

The public is using natural products for a wide range of health-related problems. A common need is availability of natural extracts with a pleasant taste or smell combined with a preservative action to avoid both microbial contamination and lipid deterioration. Those undesired phenomena are not an exclusive concern of the food industry but a common risk wherever a pathogen is present. Spoilage microorganisms, lipid oxidation, protein oxidation, and enzymatic oxidation severely affect the shelf-life of many foods in addition to the development of undesirable off-flavours (Farag *et al.* 1990; Hirasa and Takemasa 1998).

In recent years, much attention has been focused on extracts from herbs and spices which have been used traditionally for centuries to improve the sensory characteristics and to extend the shelf-life of foods. Spices and their essential oils have been widely used as natural food preservatives to make processed foodstuff safe for consumers. They are gaining increasing interest because they impart desirable flavors but they may fulfil more than one function to the food when they are added (Nasar-Abbas and Halkman 2004). Spices have been extensively studied by various groups of scientists because of their relatively safe status, their wide acceptance by consumers, and their exploitation for potential multi-purpose functional use (Sawamura 2000; Ormancey *et al.* 2001). Plants produce an array of defensive molecules including antimicrobial proteins and peptides (Xu 1990; Ng and Wang 2000; Wang *et al.* 2000; Ye *et al.* 2000). We reported earlier antibacterial activities of a number of Thai medicinal plants against a wide range of bacteria (Voravuthikunchai *et al.* 2002; Voravuthikunchai and Kitpipit 2003; Voravuthikunchai *et al.* 2004a, 2004b, 2004c; Voravuthikunchai and Kitpipit 2005; Voravuthikunchai *et al.* 2005a, 2005b, 2005c, 2006a, 2006b, 2006c, 2006d; Voravuthikunchai and Limsuwan 2006; Voravuthikunchai *et al.* 2007). In addition, their antimicrobial, antioxidant and radical-scavenging properties by spices and essential oils have been reported (Hirasa and Takemasa 1998) and in some cases, a direct food-related application has been tested. Several antioxidants were used to extend food shelf life. It was anticipated that they might inhibit the oxidation reaction involved in enzymatic browning (Madsen and Bertelson 1995).

Rhizomes of the family Zingiberaceae contain some important aromatic and color-producing spices such as turmeric, ginger, galanga, krachai, cardamom, and grains of paradise. Currently, there is an increasing demand for new ethnic foods. The foods also include the emerging cuisines such as Thai, Vietnamese, Indian, and Moroccan, which have strong flavors and aromas. Some of the popular ingredients for developing these foods include tamarind, cardamon, lemon grass, basil, and galanga (Cousminer and Hartman 1996; Uhl and Mermelstein 1996). Many studies have demonstrated that they contain bioactive compounds that have excellent antimicrobial activities against a diverse group of pathogens. Therefore, they are potential candidates for a preservative substance in food. *Tom-yum*, a well-known Thai traditional seasoning containing galanga and many other herbs, has been shown to possess both antioxidation and antimicrobial effects. *Tom-yum* mix was demonstrated to have a potential as a natural preservative agent for ensuring safe marinated food products (Siripongvutikorn *et al.* 2005).

In tropical countries, many kinds of gingers are cultivated and used not only for spices but also as traditional medicines. This review attempts to gather important scientific information on the family Zingiberaceae in relation to health care concepts as food supplements and preservatives. Particularly, an overview of recent progress reports on the antimicrobial and antioxidant activities of common species of this plant family is substantially highlighted.

FACTORS AFFECTING FOOD SAFETY

More than 200 known diseases are transmitted through food. Food-borne illnesses result from ingesting food contaminated with bacteria or toxic substances they produce, yeast, fungi, viruses, prions, parasites, chemicals, and metals. Reactions and the duration of the illness vary according to the type of organism or toxic substance consumed. The symptoms may be mild gastroenteritis and last only a few hours. These usually include diarrhea, malaise, dizziness, nausea, vomiting, headache, and fever. On the other hand, there are more serious, life-threatening infections which last much longer, and require intensive medical treatment, for example, botulism caused by *Clostridium botulinum*, hepatitis A from Hepatitis A virus, and renal syndromes from *Escherichia coli* O157: H7. In specific groups such as children and the elderly, death may encounter.

Food spoilage microorganisms

All food, unless just cooked or sterilised, contains some bacteria. The numbers present will depend on conditions in which the food has been handled and stored. If allowed to grow, some of these bacteria may cause spoilage. Most common organisms include various yeast species such as *Candida albicans*, *Rhodotolura glutinis*, *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*, and *Yarrowia lypolitica* (Sacchetti *et al.* 2005). Generally, these spoilage organisms are harmless and do not cause illness. However, if spoilage is noticeable, the food should not be consumed.

Food-borne pathogens and food poisoning

Food-borne pathogens continue to cause major public health problems world-wide. These organisms are the leading causes of illness and death in less developed countries, killing approximately 1.8 million people annually (Fratamico *et al.* 2005). Even in developed countries, food-borne pathogens are responsible for millions of cases of infectious gastrointestinal diseases each year, costing billions of dollars in medical care and decreasing productivity. Furthermore, new food-borne diseases are likely to emerge driven by factors such as pathogen evolution, changes in agricultural and food manufacturing practices, and changes to the human host status.

Harmful organisms often do not alter the appearance, taste or smell of food. Because of this, it is impossible to visually determine whether or not food is contaminated. Only a laboratory analysis can verify the presence of these pathogenic microorganisms. Food-borne pathogens cover diverse groups of microorganisms including bacteria, yeast, fungi, enteric viruses, and protozoan parasites. Most common contamination encounters pathogenic bacteria such as *Bacillus cereus*, *Campylobacter jejuni*, *C. botulinum*, *Clostridium perfringens*, *E. coli*, *Listeria monocytogenes*, *Salmonella* spp., *Shigella* spp., *Staphylococcus aureus*, *Vibrio* spp., and *Yersinia enterocolitica*. In addition to bacteria, food may also become contaminated with viruses. Unlike bacteria, viruses cannot multiply in food and do not cause spoilage. They do not cause any change in the appearance, taste or smell of food and cannot be detected by ordinary laboratory tests. Once they get into the human body, however, they can multiply and cause disease. Fortunately, most viruses are destroyed by adequate cooking. Cooking eggs at 160°F (71°C) can kill the avian flu virus (Wiwanitkit 2007). The diseases produced by parasites are varied, and in some countries, they are more important than bacterial food-borne illnesses. Many infected individuals do not show signs of infection, but the symptoms, when they occur, are similar to

Table 1 Major food-borne infections.

Infection	Incubation period	Symptoms	Sources of contamination
Bacterial			
Bacillus cereus	30 min-15 h	abdominal cramps, diarrhea nausea, **vomiting**	cheese, fish, meat, milk, pasta, potatoes, rice, vegetables
Campylobacter jejuni	1-7 d	abdominal cramps, headache, **inflammatory diarrhea**, nausea	raw beef, cake, eggs, unpasteurised milk, poultry, water
Clostridium botulinum	12-72 h	diarrhea, double vision, dry mouth, fatigue, headache, **muscle paralysis**, nausea, respiratory failure, vomiting	bottled garlic, fermented fish, herb-infused oils, low-acid canned foods, meats, sausage
Clostridium perfringens	8-22 h	abdominal cramps, some with dehydration, nausea, **watery diarrhea**	gravy, meat, poultry
Clostridium parvum	2-28 d	watery diarrhea	fruit, unpasteurized milk, vegetables, water
Enterotoxigenic *Escherichia coli*	1-3 d	watery diarrhea	fecal contaminated food or water
Escherichia coli 0157: H7	1-8 d	abdominal cramps, bloody diarrhea, **hemorrhagic colitis**, hemolytic uremic syndrome	egg, ground beef, meat, unpasteurised milk, milk products, poultry
Listeria monocytogenes	2 d to 6 wks	diarrhea, fever, muscle aches, meningitis, nausea, septicemia, miscarriage	cheese, hot dogs, meat, milk, seafood, vegetables
Salmonella spp.	12-72 h	abdominal pain, chills, dehydration, diarrhea, fever, headache, **inflammatory diarrhea**, nausea, prostration	dairy products, poultry, raw vegetables, salads
Shigella spp.	12-72 h	abdominal pain, cramps, fever, inflammatory diarrhea, vomiting	fecal contaminated food, salads, water
Staphylococcus aureus	1-6 h	abdominal cramps, diarrhea, **severe vomiting**	cream-filled baked goods, cream sauces, custard, diary, dressing, eggs, gravy, ham, meat, poultry, salads, sandwich fillings
Vibrio spp.	4 h-7 d	abdominal cramps, chills, nausea, diarrhea, fever, headache, nausea, vomiting	infected fish and shellfish
Vibrio parahemolyticus	2-48 h	inflammatory diarrhea	raw shellfish
Yersinia enterocolitica	1-3 d	enterocolitis (may mimic acute appendicitis)	chocolate milk, raw milk, pork, water, raw meats
Fungal			
Cryptosporidium parvum	2-15 d	loss of appetite, mild stomach cramps, nausea, **watery diarrhea**	food, milk, water
Viral			
Hepatitis A virus	15-50 d	abdominal discomfort, fever, malaise, nausea, **hepatitis, jaundice**, liver failure	iced drinks, fruits, salads, shellfish, vegetables, water
Norwalk virus	12-48 h	abdominal cramps, diarrhea, nausea, **vomiting**	frosting, fruit, ice, raw oysters, salads, sandwiches, shellfish, water
Enteric virus	10-72 h	watery diarrhea	fecal contaminated food or water
Parasitic			
Cyclospora cayetanensis	1-11 d	watery diarrhea	imported berries, basil
Giardia lamblia	1-2 wks	infection of the small intestine, diarrhea, **loose or watery stool**, stomach cramps	fecal contaminated food or water
Toxoplasma	5-23 d	no symptoms or mild illness (swollen lymph glands, fever, headache, and muscle aches) severe infection for unborn babies, immunocompromised host	raw or undercooked food

Modified from Centers for Disease Control 2006; bold words are the most pronounced symptoms.

those produced by bacteria. Diarrhea is usually the most common symptom. As with viruses, parasites need a host in which to multiply and contaminated food and water act only to transport the parasite from one host to the next. Infections by common pathogens including incubation period, symptoms, and possible causes of contamination are summarised in **Table 1** (http://en.wikipedia.org/wiki/Centers_for_Disease_Control_and_Prevention).

Campylobacter is a pathogen that causes fever, diarrhea, and abdominal cramps. It is the most commonly identified bacterial cause of diarrheal illness in the world. Campylobacter enteritis occurs throughout the world, particularly in the temperate areas during the warmer months. The bacteria may be responsible for some 'traveler's diarrhea'. These bacteria live in the intestines of healthy birds, therefore most raw poultry meat has *Campylobacter* on it. Eating undercooked chicken or other food that has been contaminated with juices dripping from raw chicken is the most frequent source of this infection. Campylobacter enteritis is self limiting and of short duration, with the symptoms lasting from one to four days (Pebody *et al.* 1997; Altekruse *et al.* 1999).

Clostridium perfringens food poisoning is characterised by a sudden onset of abdominal pain and diarrhea. Nausea is common but vomiting and fever are usually absent. This type of food poisoning is mild and usually lasts only one day or less (Eley 1992b).

Escherichia coli O157:H7 is a pathogen that has a reservoir in cattle and other similar animals. Human illness typically follows the consumption of food or water that has been contaminated with cow feces. The illness it causes is often a severe and bloody diarrhea and painful abdominal cramps, without much fever. Hemorrhagic colitis, commonly referred to as 'hamburger disease' or 'barbecue season syndrome', is a recognised type of emerging foodborne illness. The bacteria can produce verocytotoxin which damages the lining of the intestine resulting in diarrhea and pain. While most people recover from this disease within two weeks, in three to five percent of cases, a complication called 'hemolytic uremic syndrome' (HUS) can occur several weeks after the initial symptoms. This illness affects the kidneys and blood. Severe complications include temporary anemia, profuse bleeding, and kidney failure. It is especially dangerous to young children and the elderly. Death can result from either HUS or the intestinal disease (Canada Communicable Disease Report 2000; O'Connor 2002).

Listeriosis is an illness caused by the *Listeria* spp. present in soil and water. Animals such as cattle and sheep can carry it without appearing ill and can contaminate foods of animal origin such as meats and dairy products. About ten per cent of healthy persons may also harmlessly carry this organism in their bowel. Symptoms can be similar to the flu, with fever, muscle aches, and often gastrointestinal symptoms such as nausea or diarrhea. Listeriosis can be deadly if it encounters meningitis, an infection of the fluid around the brain, causing headache, stiff neck, confusion, loss of balance, or seizures (Eley 1992c).

Salmonellosis Salmonella is widespread in the intestines of birds, reptiles, and mammals. It is also found in food such as raw eggs and egg products, meat and meat products, and poultry. The organism can spread to humans via a variety of different foods of animal origin. Illness may occur after individuals eat food or drink water contaminated with faeces. The bacteria multiply in the small intestine and invade the intestinal lining. The illness caused by *Salmonella* typically includes fever, diarrhea, and abdominal cramps. Dehydration, especially among infants, may be severe. In persons with poor underlying health or weakened immune systems, it can invade the bloodstream and cause life-threatening infections (Eley 1992a).

Shigellosis is commonly known as 'bacillary dysentery'. Shigellosis occurs throughout the world and is most often associated with children under ten years. If the disease is not properly treated, it can be fatal. Symptoms such as diarrhea, fever, nausea, vomiting, and cramps are most common. Blood may also be found in the feces (Eley 1992c).

Staphylococcal food poisoning or food intoxication syndrome was first studied in 1894 (Jay 2000). *Staphylococcal gastroenteritis* is caused by the ingestion of enterotoxins produced by some strains of *Staphylococcus aureus* (Vanderzant and Splittstoesser 1992). The toxin is not destroyed by cooking. Although the illness may be of short duration, usually less than two days, it can become very severe. In processed foods in which *S. aureus* should have been destroyed by processing, the reappearance of this particular bacterium can cause damages to food industries as it is a vector of food poisoning. It may be inferred that sanitation or temperature control or both are inadequate. There is no guarantee that foodstuff is safe enough for consumption, although only a trace amount of *S. aureus* is present. Natural preservatives such as spices and plant essential oils can be used as additives instead of chemical preservatives because food remains safe for consumers while *S. aureus* is eliminated (Oonmetta-aree *et al.* 2006).

Hepatitis A is caused by the Hepatitis A virus. Many adults and most children may be infected but have no or very mild symptoms. These symptoms may be followed by jaundice which is the yellowing of the skin and the whites of the eyes. People with symptoms may be ill for a few days, but most people do not feel fully recovered for quite a few weeks. In some rare cases, people are severely ill for several months with liver failure and death occasionally occur (http://www.health.gov.ab.ca/about/about.html).

Norwalk virus is an extremely common cause of foodborne illness, though it is rarely diagnosed, because the laboratory test is not widely available. It causes an acute gastrointestinal illness, usually with more vomiting than diarrhea, that resolves within two days. Outbreaks of Norwalk virus gastroenteritis are often associated with consumption of contaminated oysters (Tian *et al.* 2006). The viruses spread primarily from one infected person to another. Infected kitchen workers can contaminate a salad or sandwich as they prepare it, if they have the virus on their hands. Infected fishermen have contaminated oysters as they harvested them.

Amoebiasis is an intestinal disease caused by the parasite '*Entamoeba histolytica*'. The disease is commonly known as '*amoebic dysentery*' and results when the parasite invades the wall of the large intestine, forming ulcers in the process. Community outbreaks usually involve water supplies contaminated with the cysts of the parasite. Invasive amoebiasis is a potentially fatal condition. It ranks third on a global scale after malaria and schistosomiasis as a cause of death among people with parasitic infections. Infection with has been reported to be an important cause of acute and chronic diarrhea in HIV patients (Arenas-Pinto *et al.* 2003).

Giardiasis caused by the parasite *Giardia lamblia* (syn. *Giardia intestinalis, Giardia duodenalis*). The disease occurs world-wide although it is more common in areas with poor sanitation. Children appear to be infected more frequently than adults. The parasite produces cysts which are responsible for the spread of the disease. Feces containing these cysts can contaminate both water and food. Species within this genus cause human giardiasis, which probably constitute the most common causes of protozoal diarrhoea worldwide, leading to significant morbidity and mortality in both developing and developed countries (Cacciò *et al.* 2005).

Lipid oxidation

It is now widely accepted that apart from microbial spoilage, lipid oxidation is the primary process by which quality loss of muscle foods occurs (Buckley *et al.* 1995). Lipid oxidation in muscle foods is initiated in the highly unsaturated phospholipid fraction in subcellular biomembranes (Gray and Pearson 1987). Lipid hydroperoxides formed during the propagation phase of the peroxidation process are unstable and are reductively cleaved in the presence of trace elements to give a range of new free-radicals and other non-radical compounds including alkoxyl and alkyl radicals, aldehydes, ketones, and a range of carboxyl compounds which adversely affect nutritive value, texture, color, flavor, and more seriously, the safety of muscle food (Buckley *et al.* 1995). Oxidative deterioration of fat components in foods is responsible for the rancid odors and flavors which decrease nutritional quality. Undesirable flavors in precooked meats are caused by volatile compounds such as hexanal, pentanal, 2,4-decadienal, 2,3-octanedione, and 2-octenal (St. Angelo *et al.* 1987; Trout and Dale 1990; Kerler and Grosch 1996).

The addition of antioxidants is required to preserve food quality. Many plants can extend shelf life by slowing oxidation. Rancidity development is an oxidative process that can be blocked by antioxidants, which block formation of free radicals by donating electrons or hydrogen ions to halt the oxidative process. Oxidative damage is thought to be a factor in cardiovascular disease, cancer, neurological disorders, arthritis, and other aging-related degenerative diseases. The benefits of antioxidant are not just limited to food preservation. In the human body, free radicals are initiated by a number of processes such as heat, UV light, radiation, alcohol, and tobacco. Antioxidants prevent damage from reactive oxygen species to tissues throughout the body. Free-radical damage to cells can limit the ability of cells to fight cancer or to limit aging. Numerous studies have indicated that lipid oxidation may be controlled through the use of antioxidants (Gray *et al.* 1996; El-Alim *et al.* 1999; McCarthy *et al.* 2001; Ahn *et al.* 2002; Sanchez-Escalante *et al.* 2003) Synthetic antioxidants from phenolic compounds such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tertiary butylhydroquinone (TBHQ), and propyl gallate (PG) have long been used in the food industry, but their use has recently come into dispute to a suspected carcinogenic potential (Chen *et al.* 1992) and the general rejection of synthetic food additives by consumers. BHA was shown to be carcinogenic in animal experiments. At high doses, BHT may cause internal and external hemorrhagic, which contributes to death in some strain of mice and guinea pigs. This effect is due to the ability of BHT to reduce vitamin K-depending blood-clotting factor (Ito *et al.* 1986). Therefore, the importance of replacing synthetic antioxidants by natural ingredients is obvious according to health implications.

Many plants have high antioxidant activity and are used in many food applications (Hirasa and Takemasa 1998).

Table 2 Studies on antimicrobial activities of well-known Zingiberaceae species.

Microorganisms	Common Zingiberaceae spp.				
	Alpinia galanga	***Boesenbergia pandurata***	***Curcuma longa***	***Zingiber officinale***	***Zingiber zerumbet***
Aspergillus niger				Konning *et al.* 2004	
Bacillus cereus			Jagannath and Radhika 2006	Alzoreky and Nakahara 2003	
Bacillus subtilis				Konning *et al.* 2004	
Candida albicans	Haraguchi *et al.* 1996; Jantan *et al.* 2003	Jantan *et al.* 2003	Sacchetti *et al.* 2005	Konning *et al.* 2004; Sacchetti *et al.* 2005	
Cryptococcus neoformans	Jantan *et al.* 2003	Jantan *et al.* 2003			
Dengue virus		Kiat *et al.* 2006			
Entamoeba histolytica	Sawangjaroen *et al.* 2006	Sawangjaroen *et al.* 2006		Sohni and Bhatt 1996	Sawangjaroen *et al.* 2006
Escherichia coli			Jagannath and Radhika 2006	Alzoreky and Nakahara 2003, (-)	
Escherichia coli O157: H7				Konning *et al.* 2004; Samy 2005, (-)	
Giardia intestinalis	Sawangjaroen *et al.* 2005	Sawangjaroen *et al.* 2005			Sawangjaroen *et al.* 2005
Haemophilus influenza				Akoachere *et al.* 2002	
Helicobacter pylori			Mahady *et al.* 2005	Mahady *et al.* 2005	
Listeria monocytogenes		Thongson *et al.* 2004, 2005	Leal *et al.* 2003; Thongson *et al.* 2004, 2005	Alzoreky and Nakahara. 2003, (-); Leal *et al.* 2003; Thongson *et al.* 2004, 2005	
Mycobacterium tuberculosis	Phongpaichit *et al.* 2006	Phongpaichit *et al.* 2006			
Pseudomonas aeruginosa				Konning *et al.* 2004; Samy 2005, (-)	
Salmonella spp.	Thongson *et al.* 2004, 2005	Thongson *et al.* 2004, 2005	Thongson *et al.* 2004; Jagannath and Radhika 2006	Alzoreky and Nakahara 2003	
Staphylococcus aureus	Oonmetta-aree *et al.* 2006; Voravuthikunchai *et al.* 2005b, 2006d	Voravuthikunchai *et al.* 2005b, 2006d	Jagannath and Radhika 2006	Akoachere *et al.* 2002; Alzoreky and Nakahara 2003, (-); Konning *et al.* 2004; Samy 2005; Voravuthikunchai *et al.* 2005b, 2006d	Voravuthi-kunchai *et al.* 2005b, 2006d
Streptococcus mutans		Hwang *et al.* 2004			
Streptococcus pneumoniae				Akoachere *et al.* 2002	
Streptococcus pyogenes				Akoachere *et al.* 2002	
Trichophyton mentagrophytes		Janssen and Scheffer 1985			

Natural antioxidants have been isolated from various kinds of plant materials such as oilseeds, leaves, roots, spices, herbs, cereal crop, vegetables, and fruits (Ramarathnam *et al.* 1995). A number of studies deal with the antioxidant activity of extracts from herbs and spices (Economou *et al.* 1991; Kikuzaki and Nakatani 1993; Cuvelier *et al.* 1994; Lu and Foo 2001). Among natural antioxidants, plant-derived phenolic compounds are in the forefront as they are widely distributed in the plant kingdom. This may be applicable to such diverse areas as human health and the preservation of food lipids. The antioxidative potential in herbs is related to their redox properties of phenolic compounds. The antioxidant action is similar to synthetic phenolic antioxidants which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers (Caragay 1992; Rice-Evans *et al.* 1997).

POPULAR MEMBERS OF FAMILY ZINGIBERACEAE USED IN FOOD PRESERVATION

It is a perennial herb with a modified fleshy stem termed the rhizome, which occurs below ground. Some common members of family Zingiberaceae have been extensively used as condiment for flavoring. Many species are frequently prescribed by practitioners of traditional Thai medicine for treating stomach-ache, carminative, diarrhea, and dysentery. Important studies of the antimicrobial activities of important species are presented in **Table 2**. However, results from different laboratories may be varied since antimicrobial properties depends on several factors such as type, composition and concentration of spices, extraction method, and numbers of contaminating microorganisms. In addition to their antimicrobial activities, it has been reported that all tropical ginger extracts have antioxidant activities (Jitoe *et al.* 1992). Moreover, several plants in this family have been used in Thai traditional treatment of allergy and allergic-related diseases (Tewtrakul and Subhadhirasakul 2006). In this communication, the species that provide most of the known benefits to human beings will be reviewed in detail. These include *Alpinia galanga* (galanga), *Boesenbergia pandurata* (krachai), *Curcuma amada* (mango ginger), *Curcuma longa* (turmeric), *Curcuma zedoria* (zedoary), *Kampferia galanga* (proh hom), *Zingiber officinale* (ginger), and *Zingiber zerumbet* (zerumbet ginger). Their potential uses as food preservatives are discussed. Some other species with fewer applications will only be briefly mentioned.

Alpinia galanga (L.) Willd.

Syn. *Alpinia galanga* (Linn); *Languas galanga*. Common names: da liang jiang, el adkham, el galanga, galanga, galanga de l'inde, galanga maior, galanga majeur, galangal, galgant, grand galanga, greater galanga, grober galgant, hang dou kou laos, herbe indienne, java galanga, khaa, lenkuas, naukyo, riêng, siamese galanga, siamese ginger, stor kalanga, ulanjan (**Plate 1**).

A tropical plant, a member of the ginger family, is native to Southern China, South East Asia, and West Africa. Galanga is a perennial growing up to seven feet tall. The leaves are lanceolate while the flowers are small greenish-white and the fruit is orange-red. Galanga has characteristic fragrance as well as pungency. The rhizome is a hot, sweet, spicy aromatic root-stock like ginger with slightly sour and peppery notes. It is commonly used in stir-fries, curries and soups in the Eastern-Caribbean, and Southeast Asia kitchen. Galanga is commomly used as a flavoring especially in the

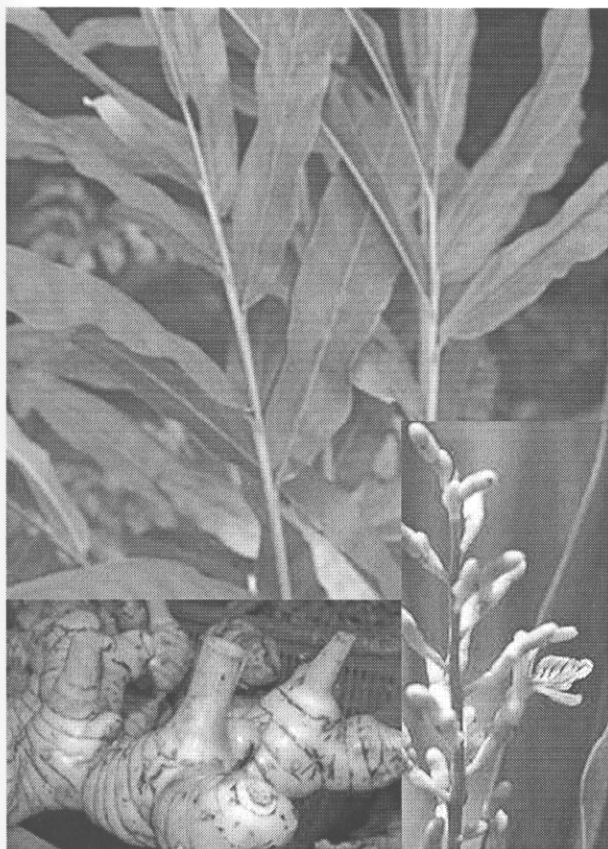

Plate 1 ***Alpinia galanga*** **(L.) Willd.**

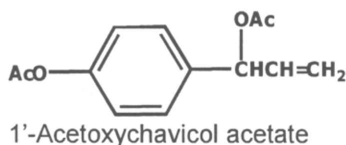

Fig. 1 1'-Acetoxychavicol acetate isolated from the rhizomes of ***Alpinia galanga*** **(L.) Willd.**

preparation of fresh Thai curry paste and Thai soup (Uhl and Mermelstein 1996; Oonmetta-aree *et al.* 2006). The rhizome is used as a medicine for curing allergy, bad breath, bronchial catarrh, dyspepsia, fever, rheumatism, stomachache, throat infections, toothache, ulcers, and whooping cough in children (Yang and Eilerman 1999; Yoshikawa *et al.* 2004).

With regard to biological activities, it has been shown that essential oils from both fresh and dried rhizomes of galanga exhibit antimicrobial activities against Gram-positive bacteria, fungi, yeast, and parasite (Farnsworth and Bunyapraphatsara 1992). Essential oil from the rhizomes comprised 1,8-cineole, β-pinene, α-terpineol, fenchyl acetate, α-pinene, camphene, guaiol, camphor and β-elemene (Raina *et al.* 2002). In dried galanga, the essential oil has quantitatively different composition than in fresh one. Whereas α-pinene, 1,8-cineol, α-bergamotene, *trans*-β-farnesene and β-bisabolene seem to contribute to the taste of fresh galanga equally, the dried rhizome shows lesser variety in aroma components (cineol and farnesene). The chemical constituents, cineole, camphor, δ-pinene, methyl cinnamate, and volatile essential oil, were reported to be effective against dermatophytes, filamentous fungi, and yeast-like fungi including *C. albicans* and *Cryptococcus neoformans* (Jantan *et al.* 2003). It has been reported that terpinen-4-ol, one of the monoterpenes in the essential oil from fresh galanga rhizomes, contains an antimicrobial activity against *Trichophyton mentagrophytes* (Janssen and Scheffer 1985).

It is well-documented that 1'-acetoxychavicol acetate (ACA) (**Fig. 1**) (Voravuthikunchai *et al.* 2007), the major constituent isolated from an ethanolic extract of dried galanga rhizomes possess antimicrobial activities. This substance is present in some other plants in the Zingiberaceae family. It has been demonstrated to be very active against *S. aureus* (Voravuthikunchai *et al.* 2005b; Oonmetta-aree *et al.* 2006; Voravuthikunchai *et al.* 2006d), *Mycobacterium tuberculosis* (Palittapongarnpim *et al.* 2002), many dermatophyte species (Janssen and Scheffer 1985), *E. histolytica* (Sawangjaroen *et al.* 2006), and *Giarrdia intestinalis* (Sawangjaroen *et al.* 2005). The galanga extract had the greatest inhibitory effect against *S. aureus*, compared to ginger, turmeric, and krachai (Oonmetta-aree *et al.* 2006). As have been mentioned earlier that different results may occur from different laboratories. Khattak *et al.* (2005) have reported a weak inhibition activity of ethanolic extracts of *Bosenbergia pandurata* against *S. aureus* while we found better activity from chloroformic extracts of this plant (Voravuthikunchai *et al.* 2005b, 2006d). The antimicrobial effect of the extract depends on many factors such as extractive solvents, the exposure time and the bacterial cell concentration. It has been demonstrated that the methyl ester penetrated to the hydrophobic regions of the membranes and the carboxyl groups pass through the cell membrane, perturbed internal pH and denatured proteins inside the cell which resulted in coagulation of cell contents (Marquis *et al.* 2003; Oonmetta-aree *et al.* 2006). Furthermore, it disrupted the cytoplasmic membrane function of *S. aureus* cells which resulted in a loss of cytoplasmic constituents and ions. In contrast, the extract could not inhibit the growth of *E. coli* because the extract could not penetrate through the outer membrane which was composed of a lipopolysaccharide monolayer surrounding the cell wall that restricts diffusion of hydrophobic compounds (Burt 2004).

Galanga extract may be a possible additive for meat and meat products. The potent antioxidant activity of curcuminoids isolated from *A. galanga* was reported (Barik *et al.* 1987; Cheah and Abu Hasim 2000; Siripongvutikorn *et al.* 2005). Two phenolic compounds, *p*-hydroxycinnamaldehyde and di-*p*-hydroxy-*cis*-styryl methane, were isolated from the chloroform extract of the rhizomes (Barik *et al.* 1987). Cheah and Abu Hasim (2000) reported the antioxidative effect of galanga in raw and cooked minced beef during storage at 4°C. It was found to delay the induction period of lipid oxidation and affect microbial growth in cooked beef. The application of dried galanga powder and its ethanolic extracts has been demonstrated to enhance oxidative stability of meat. Furthermore, its free radical scavenging activity, superoxide anion radical scavenging activity, Fe^{2+} chelating activity, lipoxygenase inhibitory activity, and reducing power have been documented (Juntachote and Berghofer 2005). Some components found in galagal root are effective in inhibiting tumors in the digestive tract (Murakami *et al.* 1993, 1995). The ability of ACA to act as an antiulcer, antitumor agents as well as an inhibitor of chemically induced carcinogenesis is event (Murakami *et al.* 2000).

Boesenbergia pandurata (Roxb.) Schltr.

Syn. *Boesenbergia pandurata* Holtt; *Boesenbergia pandurata* (Roxb.) Holtt.; *Boesenbergia rotunda* (L.) Mansf.; *Kaempferia pandurata* Roxb. Common names: Chinese ginger, finger root, krachai, temu kunci (**Plate 2**).

B. pandurata, the yellow variety, is a perennial herb found in Southern China and Southeast Asia. A tall ginger has the long tubers sprouting in the same direction from the middle of the rhizome with large beautiful pink-purple flowers. There are culinary applications of its rhizome as a spice in Thai and Indonesian kitchen. 'Thai ginger' or 'Thai krachai' is used for similar purposes as ginger in Thai cuisine. It is one of the plants in the primary health care project of Thailand for medical purposes such as treatment of diarrhea, dyspepsia, inflammation, and wounds.

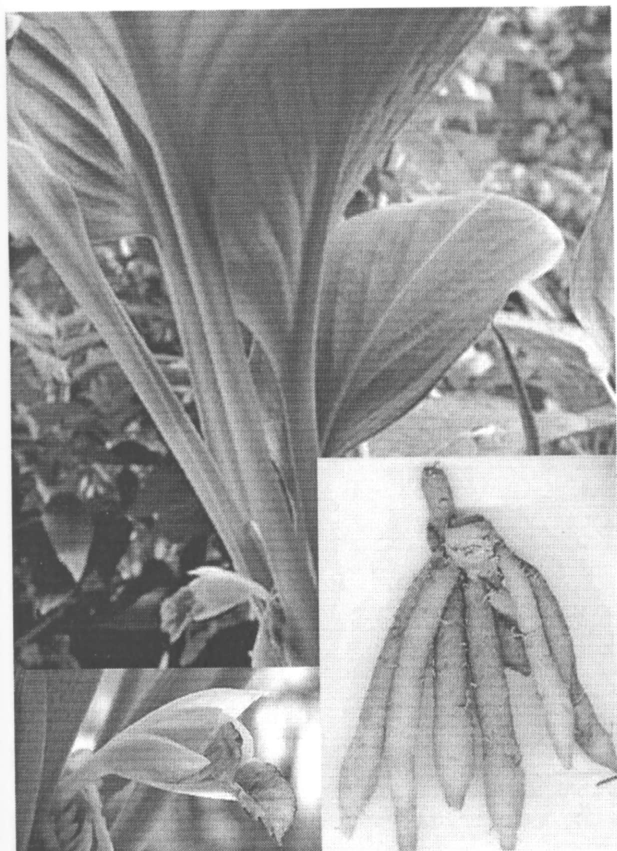

Plate 2 ***Boesenbergia pandurata*** **(Roxb.) Schltr.**

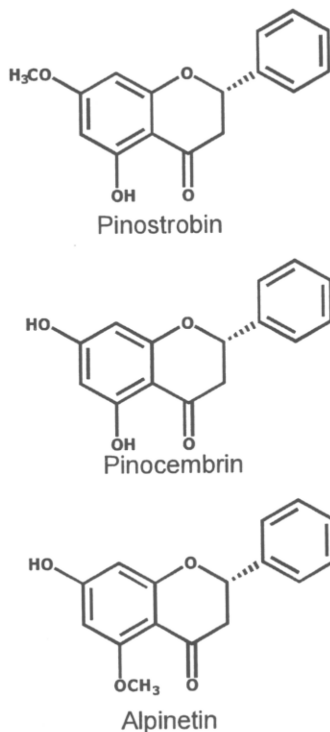

Fig. 2 Main chemical constituents isolated from the rhizomes of ***Boesenbergia pandurata*** **Holtt.**

Regarding the chemical constituents of *B. pandurata*, there are many reports on chalcones (Trakoontivakorn *et al.* 2001), flavonols (Jaipetch *et al.* 1983), flavones (Jaipetch *et al.* 1982), and essential oil (Pandji *et al.* 1993). Flavonoids such as boesenbergin A, boesenbergin B, panduratin A, panduratin B, cardomin, cardamonin, pinostrobin, pinocembrin, alpinitin, 5-hydroxy-7-dimethoxyflavanone (Jaipetch *et al.* 1982; Jaipetch *et al.* 1983; Pancharoen *et al.* 1987; Pandji *et al.* 1993), and 1,8-cineole are recognised as the bioactive compounds (Pancharoen *et al.* 1987). Main chemical constituents isolated from the rhizomes of *B. pandurata* are presented in **Fig. 2** (Voravuthikunchai *et al.* 2007).

A broad range of biological activities have been attributed to *B. pandurata*. These include antibacterial (Palittapongarnpim *et al.* 2002; Voravuthikunchai *et al.* 2005b, 2006d) and anti-giardial (Sawangjaroen *et al.* 2005) activities. Finger root contains 1-3% of essential oil. Several aroma components from its rhizomes contained high levels of 1-8 cineol, camphor, δ-borneol, methyl cinnamate, geraniol, and camphene being the most important. Trace components are δ-pinene, zingiberene, zingiberone, curcumin, and zedoarin. The oil of *Boesenbergia pandurata* rhizomes has been reported to be effective against dermatophytes, filamentous fungi and yeast-like fungi including *C. albicans* and *C. neoformans* (Jantan *et al.* 2003). Its activities against *S. mutans* (Hwang *et al.* 2004), *L. monocytogenes* and *S.* Typhimurium have been reported (Thongson *et al.* 2005). In our series of studies, we found that among the three flavonoids, alpinetin, pinocembrin, and pinostrobin, isolated from methanolic extract of *B. pandurata*, pinocembrin was the most potent antimicrobial compound. It exhibited activity against *S. aureus* (MIC 256 μg/ml) (Voravuthikunchai *et al.* 2006d), *E. histolytica* (MIC 125 μg/ml) (Sawangjaroen *et al.* 2006) *M. tuberculosis* (MIC 25 μg/ml (Phongpaichit *et al.* 2006), and *M. gypseum* (MIC 32 μg/ml) (Phongpaichit *et al.* 2005). However, it showed no effect on *C. albicans* (Phongpaichit *et al.* 2005).

Moreover, this plant also displayed antimutagenic (Trakoontivakorn *et al.* 2001), antitumor (Murakami *et al.* 1993, 1995), anti-hepatocarcinogenic (Tiwawech *et al.* 2000), anti-inflammatory, analgesic, and antipyretic activities (Pathong *et al.* 1989). Both natural and synthetic chalcones are known to exhibit immunostimulatory activities (Barfod *et al.* 2002), anti-inflammatory (Tuchinda *et al.* 2002), anticancer (Saydam *et al.* 2003), and anti-tuberculosis (Lin *et al.* 2002). Panduratin A, sakuranetin, pinostrobin, pinocembrin, and dihydro-5,6-dehydrokawain from chloroform extracts of the rhizomes were reported to be responsible for the anti-inflammatory effect (Tuchinda *et al.* 2002). In addition, the chloroform and methanol extracts of *B. pandurata* have been reported to have HIV-1 protease inhibitory activity (Trakoontivakorn *et al.* 2001).

It is obvious that this plant may have a high potency to be used as a food additive since it possesses appreciable antibacterial activities. Its safety is also supported by a previous report on the low toxicity and lack of mortality in rats after 7 days of treatment (Pathong *et al.* 1989).

Curcuma amada Roxb.

Common names: amada, amba haldi, mango ginger (**Plate 3**).

The main use of *C. amada*, or mango ginger rhizome is in the manufacture of pickles. It has a morphological and phylogenic resemblance with ginger but imparts a mango (*Mangifera indica*) flavor. Themango flavor is mainly attributed to car-3-ene and *cis*-ocimene among the 68 volatile aroma components present in the essential oil of mango ginger rhizome (Singh *et al.* 2002, 2003). The mango ginger rhizome has been extensively used as an appetizer, alexteric, antipyretic, aphrodisiac, and laxative. In Ayurveda, it has been applied to cure biliousness, itching, skin diseases, bronchitis, asthma, hiccough and inflammation as a result of injuries (Warrier *et al.* 1994). High antibacterial activity of difurocumenonol, a new antimicrobial compound from mango ginger against a wide range of bacteria has been recently demonstrated (Policegoudra *et al.* 2006). Difurocumenonol possesses four-hydroxyl, six-methyl and one-carbonyl groups along with two furan rings. Difurocumenonol by virtue of possessing two furan rings, which are aromatic in nature, thus possesses units, which are capable of exhibiting

Plate 3 ***Curcuma amada*** **Roxb.**

Plate 4 ***Curcuma longa*** **L.**

delocalization of electrons, a feature that has been proposed to be responsible for increased antibacterial activity (Ultee *et al.* 2002). These may account for the enhanced activity of difurocumenonol compared with its source extract. The bioactivity of difurocumenonol may be similar to several other compounds like curcumin, capsaicin, caffeic acid, carvacrol, eugenol and menthol (Apisariyakul *et al.* 1995; Cichewicz and Thorpe 1996; Ali-Shtayeh *et al.* 1997; Cowan 1999). In addition, the presence of hydroxyl groups in plant derivatives has been associated with many biological activities (Phillipson 1995; Halliwell *et al.* 1995; Tess *et al.* 1999; Laurence *et al.* 2001; Tegos *et al.* 2002; Adewole *et al.* 2004; Burt 2004). The hydroxyl group may be actively responsible for depletion of ATP-dependent metabolic functions, ultimately leading to cell death (Ultee *et al.* 2002). Further, the presence of oxygen function in the framework of the compound increases the antibacterial properties (Naigre *et al.* 1996).

Curcuma longa L.

Common names: curcuma, cucurmin, geelwortel, huldi, gelbwurz haldi, Indian safran, kakoenji, koenir, koenjet, koenjit, kondin, kurkuma, kunir, kunyit, oendre, rame, renet, safrandes indes, temu, temu kuning, tius tumeric, turmeric, ukon goeratji (**Plate 4**).

This perennial plant is native to Indonesia, India, South and Southeast Asia. When the roots of *Curcuma longa* are dried and ground, the result is a yellowish-orange powder called 'turmeric' (Indian saffron). Turmeric is an ancient spice and a traditional remedy that has been used as a medicine, condiment and flavoring. There is also a vegetable which has all the properties of the true saffron, as well as the color, and yet it is not really saffron. From thousand of years turmeric has been used with no side effects. Curcumin is the active ingredient in turmeric which has been shown to have a wide range of therapeutic effects and can be used as natural preservative. Powdered turmeric, or its extract, is found in numerous commercially available botanical supplements. Studies have also shown that curcumin even in large quantities does not produce any known side effects in humans. The FDA classifies turmeric as GRAS (General Recognized as Safe).

The presence of carotenoids is responsible for its lemon yellow color. It has a bitterish, slightly acrid taste and a peculiar fragrant odor. It is one of the principle ingredients of curry powder. It is also used in pickles, relishes, and mustards as a coloring and flavoring agent. Turmeric has found application in canned beverages, baked products, dairy products, ice cream, yogurts, yellow cakes, biscuits, popcorn-color, sweets, cake icings, cereals, sauces, gelatines, direct compression tablets, etc. In combination with Annatto (E160b) it has been used to color cheeses, dry mixes, salad dressings, winter butter, and margarine. Interestingly, γ–irradiation showed no effect on the color of turmeric (Chatterjee *et al.* 1998).

In Ayurvedic medicine, turmeric, the powdered rhizome of the herb has traditionally been used as a treatment for epilepsy, bleeding disorders, skin diseases, fevers, diarrhea, urinary disorders, poisoning, cough, lactation problems as well as inflammation, wounds and tumors (Ammon and Wahl 1991). The rhizome of *C. longa* has long been used in Thai traditional medicine for treatment of itching and other skin diseases (Tewtrakul and Subhadhirasakul 2006). The Chinese use turmeric to improve digestion, reduce gas, and to stimulate bile production in the liver. The rhizome are crushed fresh and the juice was mixed with water and used as a treatment for ear infections, cleaning the nasal passages. Herbalists recommend it for many health disorders like digestive disorders, irritable bowel syndrome, colitis, Crohn's disease, diarrhea, and post-salmonella infection, skin diseases, wound healing, eye disorder, atherosclerosis, and liver problems. It improves beneficial intestinal microbiota, while inhibiting certain harmful bacteria.

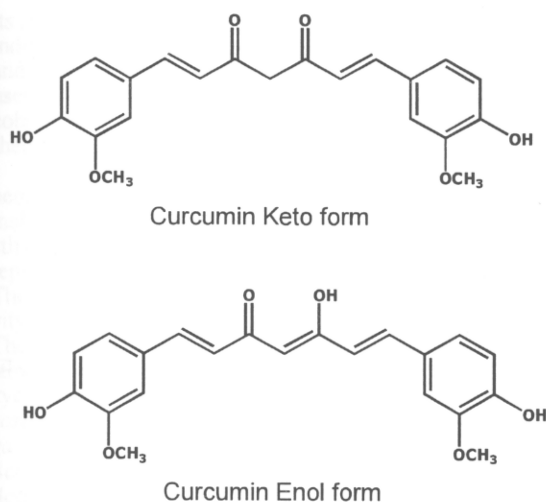

Fig. 3 Curcumin from the rhizomes of *Curcuma longa* L.

Curcumin (**Fig. 3**) (http://en.wikipedia.org/wiki) is known for its antimicrobial (Martins *et al.* 2001), anti-inflammatory, antioxidant (Nakatani 2000), anticancer (Surh 1999), and anti-allergic (Yano *et al.* 2000) properties. The active components of turmeric are the curcuminoids (Xu *et al.* 2006). Interestingly, the rhizome has liver protection properties. This juice is taken one spoon for children and one to two for adults, once a day for 10 to 15 consecutive days for hepatitis. In preclinical animal studies, turmeric has shown anti-inflammatory (Araújo and Leon 2001), cancer-chemopreventive and antineoplastic properties (Kelloff *et al.* 1996). Curcumin appears to be able to act at multiple sites to reduce inflammation (Aggarwal *et al.* 2003; Lantz *et al.* 2005). Turmeric has proven to decrease blood lipid peroxides in humans (Ramirez-Bosca *et al.* 1995, 1997) and prevent ulcers (Prucksunand *et al.* 2001). It also protects the liver from chemical injury (Sohni and Bhatt 1996; Song *et al.* 2001), and alleviate pain from arthritis (Kulkarni *et al.* 1991). A recent study showed that turmeric dramatically lowers blood fibrinogen levels (Dean 2000). Fibrinogen is a substance in the blood that is responsible for the final step in the blood clotting cascade. The formation of blood clots may cause heart attacks or strokes (Olajide 1999). High fibrinogen levels have been shown to be an even more significant risk factor for heart disease and stroke than cholesterol.

Curcuma zedoaria (Christm.) Roscoe

Common name: white turmeric, zedoary, zedoary root (**Plate 5**).

It is found in the East Indies and Cochin-China. There are two kinds of zedoary, the long and the round, distinguished by the names of *radix zedoaria longae* (Curcuma Zerumbet, the Long Zedoary of the shops) and *radix zedoaria rotundae*. The long is in slices, or oval fingers; the round in transverse, rounded sections, twisted and wrinkled, greyish-brown in color, hairy, rough, and with few root scars. The odor is camphoraceous, and the taste warm, aromatic, and slightly bitter, resembling ginger. The powder is colored brown-red by alkalis and boric acid. The zerumbet has been erroneously confused with the round zedoary. The main chemical components are curzerenone 22.3%, 1-8 cineole 15.9%, germacrone 9% (Purkayastha *et al.* 2006). *Curcuma zedoaria* has been used as a substitute for *Curcuma longa*. It is used in flatulent colic and debility of the digestive organs. It is used as an ingredient in antiperiodic pills and antiperiodic tincture. It has recently been reported to show anti-allergic activity (Matsuda *et al.* 2004).

Plate 5 *Curcuma zedoaria* (Christm.) Roscoe.

Kaempferia galanga

Common name: Proh hom (**Plate 6**).

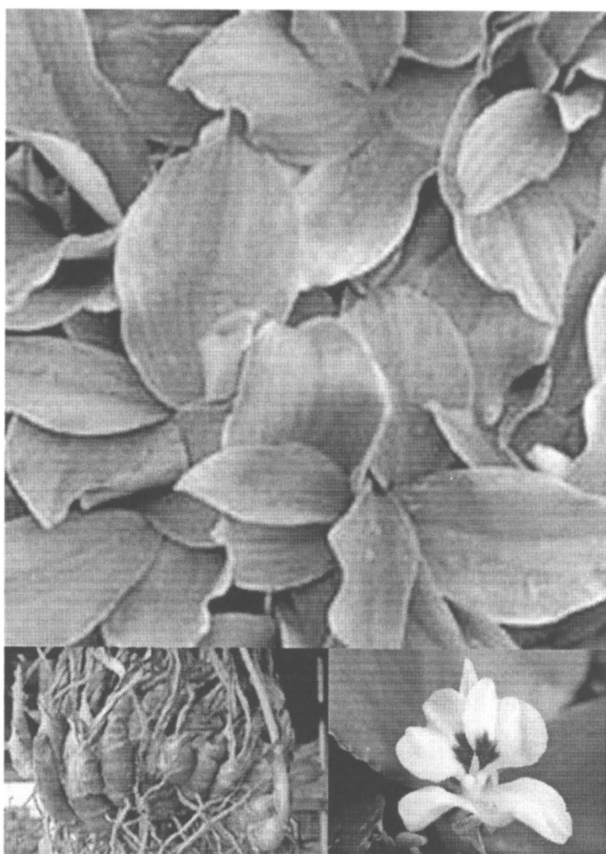

Plate 6 *Kaempferia galanga*.

It is an acaulescent perennial that grows in Southern China, Indochina, Malaysia and India. Essential oils from

its rhizomes have been used in Thai traditional medicine for indigestion, cold, pectoral and abdominal pains, headache and toothache urticaria and allergy. The rhizomes have been used in Chinese medicine as an aromatic stomachic. Its alcoholic maceration has also been applied as liniment for rheumatism (Keys 1976).

The constituents of this rhizome consist of cineol, borneol, 3-carene, camphene, kaempferol, kaempferide, cinnamaldehyde, *p*-methoxycinnamic acid, ethyl cinnamate, and ethyl *p*-methoxycinnamate. Ethyl *p*-methoxycinnamate was reported to inhibit monoamine oxidase (Noro *et al.* 1983). The rhizome extract of *K. galanga* exhibited inhibitory activity against Epstein-Barr virus (EBV) (Vimala *et al.* 1999). The methanolic extract of *K. galanga*, which identified as ethyl cinnamate, ethyl *p*-methoxycinnamate and *p*-methoxycinnamic acid, showed larvicidal activity against *Toxocara canis* (dog roundworm) (Kiuchi *et al.* 1988). *K. galanga* extract possessed effective amoebicidal activities for *Acanthamoeba culbertsoni*, *Acanthamoeba castellanii*, and *Acanthamoeba polyphaga*, the causative agents of granulomatous amoebic encephalitis and amoebic keratitis (Chu *et al.* 1998). Pitasawat *et al.* (1998) demonstrated significant larvicidal activity of this plant species against *Culex quinquefasciatus*.

Zingiber officinale

The genus *Zingiber* has about 85 species of aromatic herbs mostly distributed in East Asia and tropical Australia (Mabberley 1990). The term '*Zingiber*' is derived from the Sanskrit word '*shringavera*', owing to their 'horn-shaped' rhizomes (Sabulal *et al.* 2006). *Zingiber* species are rich in volatile oils and are used in traditional medicine and as spices. Ginger is on the GRAS list from FDA, however, like other herbs, ginger may be harmful because it may interact with other medications, such as warfarin.

Even though ginger is native to Southeast Asia, it is widely used in both western and oriental dishes. Oleoresin from ginger roots can be found in ginger ale, gingerbread, gingersnap cookies, ginger tea, ginger wine, cordials and candies, as well as a number of great Chinese, Indian, and Jamaican dishes. It has been used in Indian traditional medicine for relief from arthritis, rheumatism, sprains, muscular aches and pains, congestion, coughs, sinusitis, sore throats, diarrhoea, cramps, indigestion, loss of appetite, motion sickness, fever, flu, chills, etc. (Varier 1996). In addition to its aromatic contribution to a food, ginger tea is often used to improve circulation, aid digestion, and treat nausea from motion sickness, pregnancy or chemotherapy. Medical research has shown that ginger root is an effective treatment for nausea caused by motion sickness or other illness (Ernst and Pittler 2000).

Organic compounds present in ginger include zingiberol, zingiberene (**Fig. 4**), bisabolene, α-curcumene, linalool, cineole, gingerol, and gingerone (Xu 1990). Volatile oils from the rhizomes of *Z. officinale* (**Plate 7**) have been characterised (Pino *et al.* 2004). The volatile oil of ginger contains zingiberene, α-curcumene and farnesene, while the pungent taste is due to gingeroles and zingerone. Zingiberene and α-curcumene, the major constituents in most of the rhizome oils of *Z. officinale*, are known for insecticidal, repellent and insect feeding deterrent activities (Sakamura *et al.* 1986; Millar 1998; Pino *et al.* 2004).

The ethnomedical and pharmacological activities of *Z. officinale* have been reviewed by various authors (Afzal *et al.* 2001). In addition to its antioxidant (Nakatani 2000) and antimicrobial activities (Martins *et al.* 2001; Wang and Ng. 2005), ginger is most noted for its actions to safely relieve nausea from many causes including morning sickness, labyrinthitis, and motion sickness (Ernst and Pittler 2000) improve digestion (Gupta and Sharma 2001) lower cholesterol (Bhandari *et al.* 1998) and prevent seizures (Minami *et al.* 2000). It has been used as anti-asthmatic agent in Thai traditional medicine (Wutthithamavet 1997). It can prevent cancer (Surh 1999) and the formation of blood clots which may cause heart attacks or strokes (Olajide 1999; Koo *et al.* 2001). It also protects the liver from chemical injury (Sohni and Bhatt 1996; Song *et al.* 2001) and alleviate pain from arthritis (Kulkarni *et al.* 1991; Altman and Marcussen 2001).

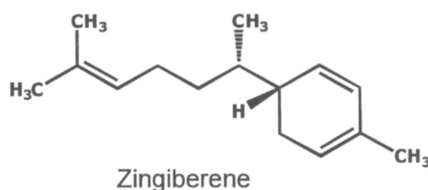

Fig. 4 Zingiberene from the rhizomes of *Zingiber officinale*.

Plate 7 *Zingiber officinale*.

Zingiber zerumbet (L.) Roscoe ex Sm.

Common names: broad-leaved ginger, pinecone ginger, pine-cone ginger, shampoo ginger, wild ginger, zerumbet ginger (**Plate 8**).

Z. zerumbet is native to Southeast Asia but has been widely cultivated in tropical and subtropical areas around the world. It grows to about seven feet tall with long narrow leaves arranged oppositely along the stem. In mid to late summer, separate stalks grow out of the ground with green cone-shaped bracts that resemble pinecones. The green cone turns red over a couple of weeks and then small creamy yellow flowers appear on the cone. In some locales, this plant is known as the 'pinecone ginger', but it is most widely known as the 'shampoo ginger' for the creamy liquid substance in the cones.

The rhizomes are mashed with salt and used to treat headaches. It has been used as against tooth and stomachache, antiflatulant, and anti-inflammatory agent (Wutthithamavet 1997). Chemical composition of the volatile oils from different parts of *Z. zerumbet* have been characterised (Chane-Ming *et al.* 2003). Zerumbone is the major component in rhizome oils of *Z. zerumbet* (Chane-Ming *et al.*

Plate 8 ***Zingiber zerumbet*** **(L.) Roscoe ex Sm.**

2003; Nakamura *et al.* 2004). It shows potential insecticidal (Chane-Ming *et al.* 2003) antibacterial (Kitayama *et al.* 2001) and chemopreventive (Murakami *et al.* 2002; Kirana *et al.* 2003) activities.

The ginger family also houses many other members which are less common such as *Kaempferia parviflora*. Its rhizome is used for the treatment of allergy and gastrointestinal disorders as well as an aphrodisiac (Pengcharoen 2002).

Zingiber nimmonii (J. Graham) Dalzell, an endemic species from the Western Ghats in South India, grows both at low and high altitudes, in moist areas under the shades of trees (Sabu. 2003). Its rhizomes are fleshy with a yellowish cross-section and an occasional purple tinge. The antibacterial and antifungal activities of the rhizome oil of *Z. nimmonii* have been reported (Sabulal *et al.* 2006).

Zingiber mioga Roscoe (Myoga) appears in Japanese cuisine; the flower buds are the part eaten.

Zingiber montanum (Koening) Link ex Dietr. (Syn. *Zingiber cassumunar* Roxb.) (Phlai) It is used for the treatment of inflammation and skin disease (Wutthithamavet 1997). Cardamom, whose sweet, aromatic seeds contain about 8% essential oil and a number of the previously mentioned compounds. In the past, cardamom was used as an aromatic in pomanders, and as an aphrodisiac. It is an essential part of Arabic coffee, and is also used in meat and rice dishes.

Properties of rhizome oils from many other Zingiber species such as *Zingiber cassumunar* (Bordoloi *et al.* 1999; Tewtrakul and Subhadhirasakul 2006), *Zingiber ottensii* (Thubthimthed *et al.* 2005), *Zingiber wrayi* var. *halabala* (Chairgulprasert *et al.* 2005) have been studied.

CONCLUDING REMARKS

The literature outlines different approaches within this trend and both the biological screening of new natural products from family Zingiberaceae and the evaluation of new properties. For manufacturing processes of food products, quality, safety, long-term adverse effects, and toxicity are primary concerns. To establish food product safety and efficacy, extensive safety studies including toxicity studies, supplementary studies in animals, and clinical trials in humans are necessary. The safety assessment of chemical preservatives in food and food supplements is complicated. Detailed scientific studies on the members of family Zingiberaceae should lead to effective application of the plant extracts as natural food preservative agents to control spoilage organisms and food-borne pathogens in food industry.

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Useful websites

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Medicinal Plant Extracts as Anti-*Escherichia coli* O157:H7 Agents and Their Effects on Bacterial Cell Aggregation

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ABSTRACT

Ethanolic extracts of eight Thai medicinal plants (representing five families) that are used as traditional remedies for treating diarrhea were examined with a salt aggregation test for their ability to modulate cell surface hydrophobicity of enterohemorrhagic *Escherichia coli* strains, including *E. coli* O157:H7. Four of these medicinal plants, *Acacia catechu, Peltophorum pterocarpum, Punica granatum,* and *Quercus infectoria,* have high bacteriostatic and bactericidal activities. The ethanolic extract of *Q. infectoria* was the most effective against all strains of *E. coli,* with MICs of 0.12 to 0.98 mg/ml and MBCs of 0.98 to 3.91 mg/ml. The ethanolic extract of *P. granatum* had MICs of 0.49 to 1.95 mg/ml and MBCs of 1.95 to 3.91 mg/ml. Ethanolic extracts of *Q. infectoria, P. pterocarpum,* and *P. granatum* were among the most effective extracts against the two strains of *E. coli* O157:H7. The other four plants, *Andrographis paniculata, Pluchia indica, Tamarindus indica,* and *Walsura robusta,* did not have high bacteriostatic and bactericidal activities but were able to affect hydrophobicity characteristics on their outermost surface. All plants except *Q. infectoria* had some ability to increase cell surface hydrophobicity. There appears to be no correlation between antibacterial activity and cell aggregative properties.

Enterohemorrhagic *Escherichia coli* (EHEC) serotype O157:H7 is an important pathogen that causes significant human diseases, especially in developed countries. These diseases include diarrhea *(18)*, hemorrhagic colitis *(19)*, and occasionally complications such as hemolytic uremic syndrome and thrombocytopenic purpura *(6, 10)*. Two types of verocytotoxin (VT), VT1 and VT2, have been implicated as important factors in these diseases *(21)*. Many antibiotics stimulate the production and release of VT *(29, 30)*. Antibiotics are effective in curing many infectious diseases, but they may enhance selection of antibiotic-resistant bacteria. Some pathogens rapidly become resistant to many of the original effective drugs *(3)*. Recently, isolates of EHEC from food and animal feces were reported to be resistant to cephalothin, tetracycline, and cefazolin *(12)*.

Medicinal plants have been used by the world population for basic health care needs and to combat many kinds of infectious bacteria. Some plants may have novel antibiotics that may be effective for treating diarrhea or can be used as food additives. Researchers have reported the antibacterial activities of various species of plants against *E. coli* O157:H7 *(11, 20)*. In a study of 38 Thai medicinal plants, the extract of *Punica granatum* and *Quercus infectoria* were reported to have high activity against *E. coli* O157:H7 *(27)*. Even though use of medicinal plants has become increasingly popular in many parts of the world, there is little information published on the antibacterial mechanisms of these plants. One hypothesis is that compounds in these plants have an effect on cell surface hydrophobicity (CSH) of microbial cells. Hydrophobic interactions seem to be commonly involved in prokaryotic and eukaryotic cell interactions *(5)*. Microbial adhesion to eukaryotic cells is often the first stage in many infections *(14)*. Adhesion onto host cells is important in intestinal infections with many pathogenic gram-negative bacteria and can be influenced by the surface hydrophobicity of the microbial cell. In a previous study of aqueous extracts from plants, these extracts affected the CSH of gram-negative bacteria including *E. coli, Acinetobacter baumannii (25),* and *Helicobacter pylori (1)*.

The present study was aimed at finding some new additives for preserving foods. Thai medicinal plants were selected from traditional remedies used for treating diarrhea. The effects of the plant extracts on CSH were investigated, and the relationship between CSH and antibacterial effects was established.

MATERIALS AND METHODS

Tested bacterial strains. *Escherichia coli* O157:H7 (RIMD 05091078 and RIMD 05091083) and three other enterohemorrhagic *E. coli* strains (RIMD 05091055 O26:H11, RIMD 05091056 O111:NM, and RIMD 05091556 O22) were kindly donated by the Research Institute for Microbial Diseases, Osaka University, Osaka, Japan. *E. coli* ATCC 25922 was used as the reference strain. Each bacterial strain was precultured in nutrient agar (Difco, Becton Dickinson, Sparks, Md.) at 35°C overnight and then inoculated into Mueller-Hinton broth (Difco, Becton Dickinson) and incubated at 35°C for 3 to 5 h. Mueller-Hinton agar (MHA; Becton Dickinson) was used for testing antibacterial activity.

Preparation of crude extracts. Eight Thai medicinal plants belonging to five families were studied. Parts of plants collected

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TABLE 1. *Medicinal plants used in the antimicrobial assay*

Botanical species	Family	Plant part tested	Ethanolic extract yield (%)[a]
Acacia catechu (L.F.) Willd.	Fabaceae	Wood	5.6
Andrographis paniculata (Burm. E.) Nees	Acanthaceae	Leaf	Not done
Peltophorum pterocarpum (DC.) Backer ex K.Heyne	Fabaceae	Bark	7.1
Pluchea indica (L.) Less.	Asteraceae	Leaf	17.8
Punica granatum L.	Punicaceae	Fruit shell	13.0
Quercus infectoria Oliv.	Fagaceae	Nutgall	18.5
Tamarindus indica L.	Fabaceae	Leaf	4.8
Walsura robusta Roxb.	Meliaceae	Wood	4.3

[a] Weight of extract/total dry weight × 100.

were chosen based on practices used by traditional Thai herbalists. Botanical identification of the plant materials was verified, and authenticated voucher specimens were deposited at the herbarium at the Department of Pharmacognosy and Pharmaceutical Sciences, Faculty of Pharmacology, Prince of Songkla University. Identification was made by comparison with authentic specimens and in some cases with the assistance of specialists. The plants were cut into small pieces, dried at 60°C overnight, and crushed in a mechanical mortar, and extracts were made by soaking in 95% ethanol at room temperature for 7 days. The solvent was then distilled under reduced pressure in a rotary evaporator until it became completely dry. The ethanolic extracts were dissolved in dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany) before use.

Salt aggregation test. The hydrophobicity of different strains of *E. coli* was determined using a modification of the standard salt aggregation test (SAT) *(1, 25)*. Cultures of *E. coli* strains on nutrient agar were suspended in 0.04 M phosphate buffer (Merck) at pH 6.8 and adjusted to the 5 McFarland turbidity standard to a final concentration of 1.5×10^{10} CFU/ml. SATs were performed with 0.1, 0.5, 1.0, 1.5, and 3.0 M ammonium sulfate solutions (Merck) in buffer. One hundred microliters of an ammonium sulfate solution was added into U-shaped microtiter plate wells, and 100 μl of standardized microbial suspension was then added to each well to produce final concentrations of 0.05, 0.25, 0.5, 0.75, and 1.5 M ammonium sulfate. The microtiter plates were then gently rotated for 5 min and incubated at 35°C for 30 min. The presence of aggregation was determined with light microscopy. The strains were tested for autoaggregation using 0.04 M phosphate buffer instead of ammonium sulfate. The SAT result was defined as positive when bacterial aggregation was clearly visible and negative when no aggregation was observed. The SAT titer was defined as the lowest concentration at which microbes still yielded clearly visible aggregation. Strains autoaggregating in potassium phosphate buffer and/or expressing SAT titers of 0.05 and 0.25 were considered highly aggregative or hydrophobic, strains with titers of 0.5 to 1.5 were considered low aggregative. Strains were considered nonaggregative if they did not produce a positive SAT result even at a 1.5 M concentration of ammonium sulfate *(25)*.

The effect of ethanolic extracts on cell surface hydrophobicity of *E. coli* was investigated by mixing 50 μl of ethanolic extracts (0.12 to 250 mg/ml) with 50 μl of bacterial suspension (10 McFarland turbidity standard). After 15 min, 100 μl of ammonium sulfate was added to give final concentrations of 0.03 to 62.5 mg/ml. DMSO, 0.04 M phosphate buffer, and water were included as controls. Solutions were incubated at 35°C for 30 min before bacterial aggregation was estimated.

Determination of MIC and MBC. A modified agar microdilution method *(15)* was used to determine the MIC. Each ethanolic extract was dissolved in DMSO before use. Serial twofold dilutions of the ethanolic extracts were made to achieve solutions of 0.31 to 625 mg/ml. Each concentration was mixed with melted MHA at 1:10 in 96-well sterile microtiter plates to give final concentrations of 0.03 to 62.5 mg/ml. Ten microliters of a 3- to 8-h culture of bacterial strains containing approximately 10^4 CFU/ml was applied to MHA supplemented with the medicinal plant extracts. The microtiter plates were then incubated at 35°C for 18 h. Observations were made in at least triplicate, and MIC was defined as the lowest concentration of extracts that produced a complete suppression of bacterial growth. The cultures of bacterial strains in microtiter plate wells at or above the MIC were inoculated onto MHA and incubated at 35°C overnight, and the lowest concentration of fractions that produced a complete kill of bacteria was defined as the MBC.

RESULTS

The extract yield for each of the medicinal plants is shown in Table 1. *Q. infectoria* has the highest yield (18.5%), followed by *Pluchea indica* (17.8%) and *P. granatum* (13.0%).

The CSH of five clinical isolates of *E. coli* was determined with the SAT (Figs. 1 and 2). All isolates were nonaggregative (SAT, >1.50). *E. coli* ATCC 25922 (the reference strain) was low aggregative (SAT, 1.5). At high concentrations (7.81 to 62.5 mg/ml), the ethanolic extracts from five plant species were aggregated with 0.05 M ammonium sulfate (highly aggregative) (Fig. 1). *P. granatum* was highly aggregative at 62.5 mg/ml and low to nonaggregative at other concentrations. *Acacia catechu* was low to nonaggregative, and *Q. infectoria* was nonaggregative at every concentration (Fig. 2).

High concentrations (7.81 to 62.50 mg/ml) of five plant species (62.5%) resulted in high aggregation when mixed with individual bacterial strains and 0.05 M ammonium sulfate in the SAT (Fig. 1). Some plant extracts at concentrations of 0.98 to 3.91 mg/ml were able to enhance aggregation of some *E. coli* strains. The ethanolic extracts of some plants such as *P. granatum* and *A. catechu* clearly enhanced bacterial cell aggregation at high concentrations. At concentrations lower than 7.81 mg/ml, all test isolates were nonaggregative and *E. coli* ATCC 25922 was low aggregative. *Q. infectoria* was the only plant that had no aggregative effect on all bacterial strains. At 0.03 to 0.49 mg/

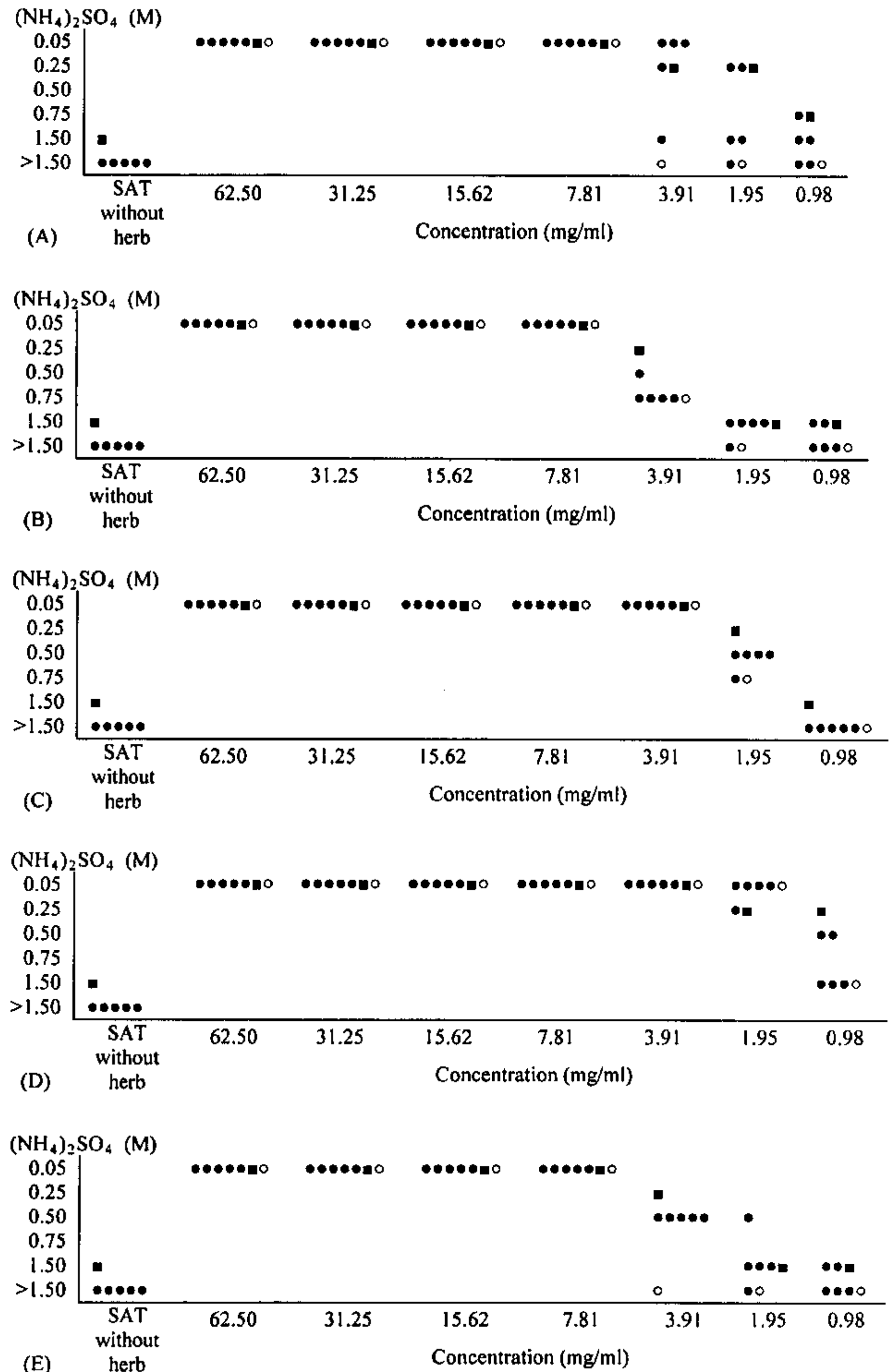

FIGURE 1. *Salt aggregation test (SAT) of* Escherichia coli *strains with ethanolic extracts of highly hydrophobic medicinal plants.* ●, E. coli *test strains;* ■, E. coli *ATCC 25922 reference strain;* ◇, *SAT results of plants:* Andrographis paniculata *(A),* Peltophorum pterocarpum *(B),* Pluchea indica *(C);* Tamarindus indica *(D), and* Walsura robusta *(E).*

ml (data not shown), no plant extract had any effect on the CSH of the *E. coli* strains.

Antibacterial activities of the medicinal plant ethanolic extracts against the six *E. coli* strains are shown in Table 2. All ethanolic extracts had activity against all strains of *E. coli*. Four plants, *A. catechu, Peltophorum pterocarpum, P. granatum,* and *Q. infectoria,* had high bacteriostatic and bactericidal activities. The ethanolic extract of *Q. infectoria* was the most effective agent against all strains of *E. coli,* with MICs of 0.12 to 0.98 mg/ml, followed by the extract of *P. granatum,* with MICs of 0.49 to 1.95 mg/ml. The highest bactericidal activities were found in the ethanolic extract of *Q. infectoria,* with MBCs of 0.98 to 3.91 mg/ml, followed by the extract of *P. granatum,* with MBCs of 1.95 to 3.91 mg/ml. The ethanolic extract of *Q. infectoria* had activity against two strains of *E. coli* O157:H7, with MICs of 0.12 and 0.98 mg/ml and MBCs of 0.98 and 1.95 mg/ml, followed by the extract of *P. pterocarpum,* with MICs of 0.24 and 0.98 mg/ml and MBCs of 0.49 and 1.95 mg/ml. No correlations between MIC, MBC, and cell aggregation were found in a detailed investigation (Table 3).

DISCUSSION

Four Thai medicinal plant extracts possess strong antibacterial activities and at high concentrations are enough to kill enterohemorrhagic *E. coli.* Ethanolic extracts of *Q. infectoria* and *P. granatum* were the most effective extracts against all *E. coli* strains tested. *Q. infectoria* has been re-

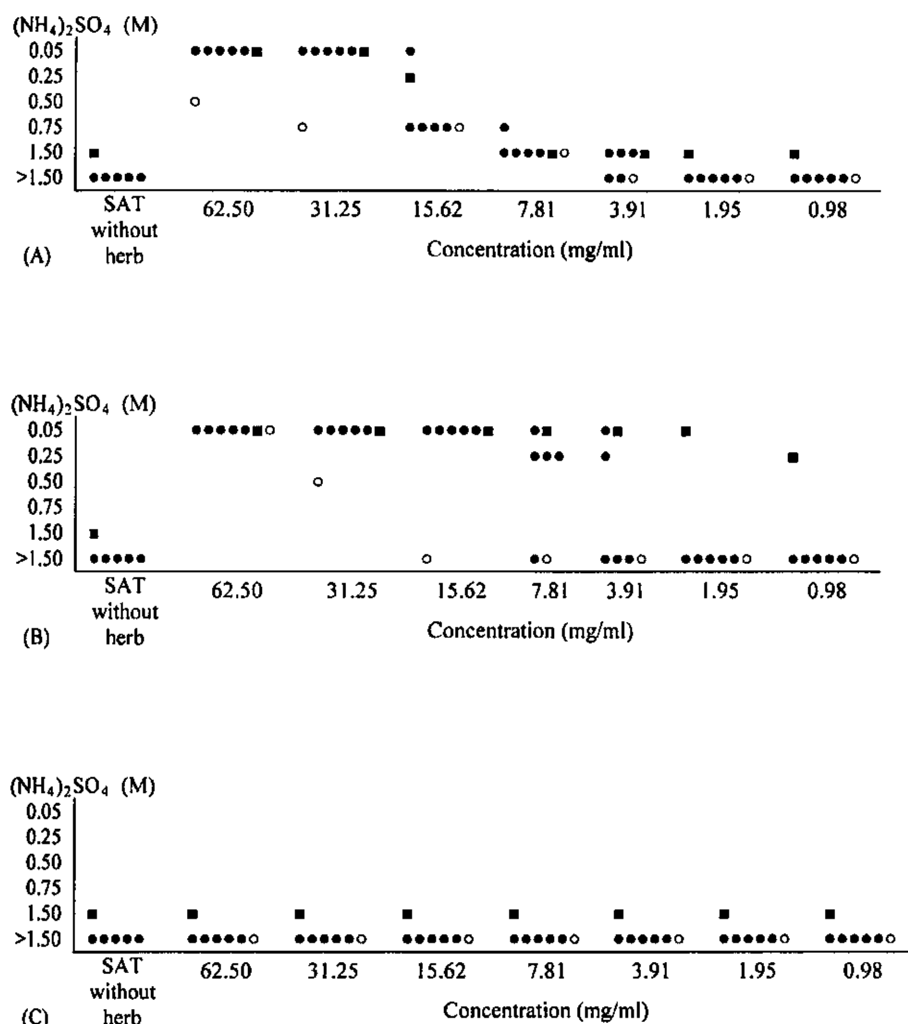

FIGURE 2. *Salt aggregation test (SAT) of* Escherichia coli *strains with ethanolic extracts of low hydrophobic and nonhydrophobic medicinal plants.* ●, E. coli *test strains;* ■, E. coli *ATCC 25922 reference strain;* ◇, *SAT results of plants:* Acacia catechu, *(A),* Punica granatum *(B), and* Quercus infectoria *(C).*

ported to have high antimicrobial activity against other pathogenic bacteria such as *Streptococcus mutans (9),* methicillin-resistant *Staphylococcus aureus (26), Bacillus subtilis, Klebsiella pneumoniae, Proteus vulgaris,* and *Pseudomonas aeruginosa (22).* All plants in this study have been investigated for their antibacterial activities against *E. coli* strains *(27),* but the effect of the plants on CSH has not been reported. The effects of various plant extracts on CSH of bacteria have been determined with the SAT *(1, 25),* but in neither study was the SAT of plants alone investigated (without bacterial suspensions). We found that at high concentrations, nearly all herb extracts were highly aggregative. Therefore, it cannot be claimed that any plant extract at high concentrations can produce the effect on CSH of bacterial cells unless the SAT of the plant itself has been defined.

Pathogenic bacteria adhere to eukaryotic cells by various mechanisms. The main action is the specific binding of bacterial adhesins to the host cell surface. Specific interactions, cell surface charge, and CSH are known to play

TABLE 2. *MICs and MBCs of crude ethanolic extracts of medicinal plants tested by the agar microdilution method against strains of* Escherichia coli

	MIC/MBC against *E. coli* strains (mg/ml):					
Medicinal plants	RIMD 05091055 O26:H11	RIMD 05091056 O111:NM	RIMD 05091078 O157:H7	RIMD 05091083 O157:H7	RIMD 05091556 O22	ATCC 25922
Acacia catechu	1.95/3.91	1.95/3.91	0.98/3.91	1.95/3.91	1.95/3.91	1.95/3.91
Andrographis paniculata	7.81/62.50	15.62/31.25	15.62/31.25	3.91/62.50	7.81/62.50	15.62/62.50
Peltophorum pterocarpum	0.98/7.81	1.95/1.95	0.98/1.95	0.24/0.49	1.95/3.91	3.91/7.81
Pluchea indica	31.25/>62.50	31.25/>62.50	31.25/>62.50	62.50/>62.50	62.50/>62.50	62.50/>62.50
Punica granatum	1.95/3.91	1.95/3.91	0.49/3.91	1.95/3.91	1.95/3.91	0.49/1.95
Quercus infectoria	0.98/3.91	0.98/3.91	0.12/0.98	0.98/1.95	0.49/3.91	0.98/1.95
Tamarindus indica	15.62/>62.50	15.62/62.50	15.62/>62.50	15.62/>62.50	15.62/>62.50	15.62/62.50
Walsura robusta	7.81/>62.50	7.81/7.81	1.95/>62.50	1.95/>62.50	15.62/>62.50	7.81/>62.50

TABLE 3. *Results of the salt aggregation test (SAT) of* Escherichia coli *strains and crude medicinal plant extracts at the MIC and MBC*[a]

	SAT of *E. coli* strains:					
Medicinal plants	RIMD 05091055 O26:H11	RIMD 05091056 O111:NM	RIMD 05091078 O157:H7	RIMD 05091083 O157:H7	RIMD 05091556 O22	ATCC 25922
Acacia catechu	N/L	N/N	N/N	N/L	N/L	L/L
Andrographis paniculata	H/H	H/H	H/H	H/H	H/H	H/H
Peltophorum pterocarpum	L/H	L/L	N/L	N/N	L/L	H/H
Pluchea indica	H/H	H/H	H/H	H/H	H/H	H/H
Punica granatum	N/N	N/L	N/H	N/N	N/N	L/H
Quercus infectoria	N/N	N/N	N/N	N/N	N/N	L/L
Tamarindus indica	H/H	H/H	H/H	H/H	H/H	H/H
Walsura robusta	H/H	H/H	L/H	L/H	H/H	H/H
Dimethyl sulfoxide	N/N	N/N	N/N	N/N	N/N	N/N
Phosphate buffer	N/N	N/N	N/N	N/N	N/N	N/N
Water	N/N	N/N	N/N	N/N	N/N	N/N

[a] Values are for the MIC/MBC. H, high aggregative; L, low aggregative; N, nonaggregative.

important roles in bacterium–host cell interactions *(16, 24)*. CSH is an important factor in the ability of the opportunistic pathogenic yeast *Candida albicans* to adhere to surfaces. Hydrophobic yeast cells adhere more readily to host tissue and are more resistant to phagocytic killing than are hydrophilic cells *(8, 17, 23)*. The surface hydrophobicity of *Klebsiella aerogenes* is influenced by the presence of capsular (K) and lipopolysaccharide (O) antigens. Loss of both K and O antigens, but not loss of the K antigen alone, increased surface hydrophobicity and susceptibility to phagocytosis *(28)*. Changes in bacterial morphology and hydrophobicity resulted in a significant decrease in adhesion in clinical isolates of *P. aeruginosa (4)*. In a study of 32 strains of enteropathogenic *E. coli* isolated from rabbits, relatively high CSH was found *(2)*. Two other strains of a verotoxigenic *E. coli* O157:H7 were reported as hydrophobic as determined with the SAT *(13)*. Preexposure of bacteria and HeLa cells to various concentrations of plant extracts affected the adhesion between non-EHEC and HeLa cells *(7)*. Our results revealed that EHEC cells did not possess high CSH. However, modification of these cells with certain medicinal plants can increase their CSH.

Because there were no correlations between MIC, MBC, and cell aggregation in this study, we hypothesize that enhancement of bacterial cell aggregation is not related to antibacterial effects. *Q. infectoria* had high antibacterial activity but produced no hydrophobic effect on bacterial cells. *Andrographis paniculata, P. indica, Tamarindus indica,* and *Walsura robusta* did not have high bacteriostatic and bactericidal activities but may be able to modify bacterial morphology and hydrophobicity characteristics, which may interfere with some bacterial functions such as mechanisms used to adhere to host cells.

Because bacteriostatic and bactericidal actions are independent of CSH in bacterial cell, the plants used in this study may provide some novel antibacterial substances or may be able to directly affect bacterial CSH. Plants with low antibacterial activity also may be useful in the treatment of bacterial contamination in food, possibly by enhancement of bacterial CSH. Because these plants are generally regarded as safe for human consumption, their crude ethanolic extracts are good candidates for development as food additives. However, pure active compounds require further study, including investigation of toxicological impacts, before these materials can be used as additives in foods.

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ANTIBACTERIAL ACTIVITY OF EXTRACTS FROM FAMILY ZINGIBERACEAE AGAINST FOODBORNE PATHOGENS

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ABSTRACT

Chloroformic extracts of selected Thai medicinal plants commonly employed to treat infections were investigated for their antibacterial activity against important foodborne pathogenic bacteria. These included Bacillus cereus, Staphylococcus aureus, *methicillin-resistant* S. aureus *(MRSA),* Escherichia coli *O157:H7,* Salmonella *Typhi and* Shigella *sp. Among 33 extracts tested, only chloroformic extracts of five plant species exhibited antibacterial properties.* Alpinia galanga, Boesenbergia rotunda, Zingiber zerumbet *and* Piper betel *were active against* S. aureus. Barleria lupulina *was active against* B. cereus. *Only the extract from* P. betel *leaves possessed activity against gram-negative bacteria. As extracts from the three plant species belonging to family Zingiberaceae displayed strong activity against* S. aureus, *they were further tested against 17 clinical isolates. Minimum inhibitory concentration (MIC) values of* B. rotunda, A. galanga *and* Z. zerumbet *extracts against most clinical* S. aureus *isolates were 0.01, 0.19 and 0.79 mg/mL and the minimum bactericidal concentration (MBC) values were 0.19, 1.57 and >12.5 mg/mL, respectively. Significant growth inhibition of MRSA was observed in the cultures incubated in the presence of the* B. rotunda *extract,* A. galanga *and* Z. zerumbet. B. rotunda *exhibited the greatest activity among the three plant species against* S. aureus *at MIC, 2MIC and MBC within 2 h.*

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INTRODUCTION

Foodborne infections have been one of the major public health concerns worldwide and account for considerably high cases of illnesses. Recent studies report that *Bacillus cereus*, *Campylobacter jejuni*, *Escherichia coli*, *Salmonella*, *Shigella* and *Staphylococcus aureus* are considered to be the most frequent pathogens (Gaulin *et al.* 2002; Prado *et al.* 2002; Di Pietro *et al.* 2004; Branham *et al.* 2005; Graham *et al.* 2005; Hall *et al.* 2005; Jimenez *et al.* 2005; Padungtod and Kaneene 2005).

Recently, a number of new drugs were discovered from ethnobotanical leads such as benzoin isolated from *Styrax tonkinensis* used for oral disinfectant and emetine from *Psychotria ipecacuanha* for amoebic dysentery (Cox 1994). Treatment failures arising from antibiotic-resistant bacteria (Boyce 1992; Berns 2003), stimulation of toxin production (Yoh *et al.* 1999), together with the recent upturn in consumer mistrust of synthetic additives, it is therefore necessary to search for natural compounds from plants to replace antibiotics or artificial antimicrobials.

Thai native herbs are becoming more widely used at a commercial scale in the food industry, mainly for their flavoring properties. Even though certain plants have been demonstrated of their effects against pathogenic bacteria (Voravuthikunchai *et al.* 2004a,b), a number of them have not yet been investigated for their antibacterial activities. Hence, it is essential to establish the scientific basis for their therapeutic actions as these may serve as the source for the development of effective drugs.

The present study was undertaken to assess the potential of medicinal plants as antimicrobial agents against common foodborne pathogenic bacteria. A total of 33 extracts from 11 plant species belonging to six families commonly used in Thailand to cure bacterial infections were used. These included *Acanthus ebracteatus* (saltbush), *Alpinia galanga* (kha), *Barleria lupulina* (hophead Philippine violet), *Boesenbergia rotunda* (fingerroot or krachai), *Coccinia grandis* (ivy gourd), *Eclipta prostrata* (swamp daisy), *Gynura pseudochina* (waan maha karn), *Murraya paniculata* (orange jasmine), *Piper betel* (betel vine), *Piper chaba* (long pepper) and *Zingiber zerumbet* (pinecone ginger). They were primarily screened for attributed antibiotic properties against selected important group of both gram-positive and gram-negative bacteria. Only chloroformic extracts of five plant species exhibited antibacterial properties. Both the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts from effective medicinal plant species were established. As the extracts from the three plant species belonging to family Zingiberaceae displayed significant activity against *S. aureus*, they were further tested against 17 clinical isolates and closely investigated by time-course growth assays.

MATERIALS AND METHODS

Medicinal Plant Materials

Eleven medicinal plant species used in this study have been previously described (Voravuthikunchai *et al.* 2005). Plant materials were dried at 60C overnight, powdered and extracted by percolation with solvents of increasing polarity beginning with chloroform, methanol and boiling water. The solvents were removed under reduced pressure in a rotary evaporator until they became completely dry. The extracts were diluted in dimethyl sulfoxide for antibacterial assays.

Tested Bacterial Strains

The following bacteria were used as tested organisms: *B. cereus* (ATCC 1778), *S. aureus* (ATCC 25923), methicillin-resistant *S. aureus* (MRSA, PSU 0205), enterohemorrhagic *E. coli* O157:H7 (RIMD 0509952), *Salmonella* Typhi (PSSCMI 0034) and *Shigella* sp. (PSSCMI 0032). In further series of experiments, 17 clinical isolates of *S. aureus* with multiple drug resistance were collected from Hatyai hospital, Songkla, Thailand. All bacterial strains were subcultured on tryptic soya agar (Oxoid, Hampshire, England) and incubated at 37C for 18 h. Mueller–Hinton agar (MHA, Oxoid) and Mueller–Hinton broth (Oxoid) were used for testing antibacterial activity.

Determination of MICs and MBCs

A modified agar microdilution method (Lorian 1996) was used to determine the MIC of extracts of the medicinal plants that produced inhibition zones. Tetracycline and vancomycin were used as reference standards. One microliter of an overnight culture of each bacterial strain containing approximately 10^4 cfu was applied onto MHA supplemented with the medicinal plant extracts. The microtiter plates were incubated at 35C for 18 h. Observations were performed in triplicate and results expressed as the mean values of the lowest concentration of plant extracts that produced a complete suppression of colony growth, MIC. MBC was determined with the extracts that gave significant MIC values by subculturing on fresh MHA.

Time-Course Growth Assay

Fifty microliters of overnight nutrient broth (NB, Oxoid) culture was added to 400 μL of NB containing 50 μL of plant extracts at MIC, 2MIC and MBC in sterile tubes. The tubes were incubated at 35C, with shaking at 100 rpm, and viable count after 0, 2, 4, 6, up to 24 h was performed using standard loop

technique (Schaedler *et al.* 1965). Control tubes without extract were incubated under the same condition. All assays were carried out in duplicate.

RESULTS

Preliminary evaluation of antibacterial activity using paper disk agar diffusion assay showed that among 33 extracts tested, only chloroformic extracts of five plant species (belonging to family Zingiberaceae [three plants], family Acanthaceae and family Piperaceae) exhibited antibacterial properties. *A. galanga*, *B. rotunda*, *P. betel* and *Z. zerumbet* were active against *S. aureus*. *B. lupulina* was active against *B. cereus*. Only the extract from *P. betel* leaves produced activity against gram-negative bacteria.

Significant antibacterial effects, expressed as MIC, of *B. rotunda*, *A. galanga*, *P. betel* and *Z. zerumbet* extracts against the pathogens tested and the MBC values are presented in Table 1. As extracts from the three plant species belonging to family Zingiberaceae displayed strong activity against *S. aureus*, they were further tested against 17 clinical isolates. MIC values of *B. rotunda*, *A. galanga* and *Z. zerumbet* extracts against most clinical *S. aureus* isolates were 0.01, 0.19 and 0.79 mg/mL and the MBC values were 0.19, 1.57 and >12.5 mg/mL, respectively (Table 2).

The antibacterial activity of the three plant species from family Zingiberaceae was further investigated by time-course growth assays using MRSA PSU039 as a representative strain. The concentration of the extracts performed in these assays was at MIC, 2MIC and MBC. Significant growth inhibition of the bacterial strain was observed in all cultures incubated in the presence of the *B. rotunda*, *Z. zerumbet* and *A. galanga* (Fig. 1). *B. rotunda* exhibited the greatest activity at MIC, 2MIC and MBC within 2 h, indicating rapid antibacterial action. At MIC, the differences among *A. galanga*, *Z. zerumbet* and control with no extracts were statistically significant (*P*, 0.05; paired *t*-test) after 8 h. At 2MIC, *A. galanga* produced similar effect as at MIC while no bacterial cells were recovered when treated with *Z. zerumbet* after 18 h. Significant bactericidal activity was demonstrated for all extracts tested.

DISCUSSION

A. galanga, *B. rotunda*, *P. chaba*, *Spilanthes acmella* and *Z. zerumbet* are medicinal plants commonly used to treat cases of diarrhea in Thai traditional medicine (Farnsworth and Bunyapraphatsara 1992). This communication demonstrated high activity of certain plants in family Zingiberaceae against *S. aureus* including multiple resistant strains. Khattak *et al.* (2005) have reported

TABLE 1.
MINIMUM INHIBITORY CONCENTRATION (MIC) AND MINIMUM BACTERICIDAL CONCENTRATION (MBC) OF CHLOROFORMIC EXTRACTS OF EFFECTIVE MEDICINAL PLANTS AGAINST FOODBORNE PATHOGENS

Medicinal plant extracts/Antibiotics	Values of MIC and MBC (mg/mL)									
	Bacillus cereus ATCC 1778		*Staphylococcus aureus* ATCC25923		MRSA PSU039		*Escherichia coli* O157:H7 RIMD 0509952		*Salmonella typhi* PSSCMI 0034	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Medicinal plants (mg/mL)										
Alpinia galanga	0.79	1.57	0.39	1.57	0.39	0.79	NA	NA	NA	NA
Boesenbergia rotunda	0.01	0.02	0.01	6.25	0.01	3.13	NA	NA	NA	NA
Piper betel	NA	NA	NA	NA	NA	NA	1.57	3.13	0.79	1.57
Zingiber zerumbet	0.39	1.57	0.39	3.13	0.39	3.13	NA	NA	NA	NA
Antibiotics (μg/mL)										
Tetracycline									1	32
Vancomycin			0.6	1.25	1.25	1.25				

NA, not applicable; MRSA, methicillin-resistant *S. aureus*.

TABLE 2.
MINIMUM INHIBITORY CONCENTRATION (MIC) AND MINIMUM BACTERICIDAL CONCENTRATION (MBC) OF CHLOROFORMIC EXTRACTS OF EFFECTIVE MEDICINAL PLANTS AGAINST CLINICAL ISOLATES OF *STAPHYLOCOCCUS AUREUS*

Bacterial strains	Values* of MIC and MBC (mg/mL)					
	Alpinia galanga		*Boesenbergia rotunda*		*Zingiber zerumbet*	
	MIC	MBC	MIC	MBC	MIC	MBC
PSU 036	0.79	1.57	0.02	0.39	0.79	>12.50
PSU 037	0.09	1.57	0.01	0.01	0.79	12.50
PSU 038	0.79	1.57	0.01	0.19	1.57	>12.50
PSU 039	0.19	1.57	0.02	0.19	0.79	3.13
PSU 040	0.39	1.57	0.01	0.04	0.79	6.25
PSU 041	0.19	1.57	0.01	0.09	0.79	>12.50
PSU 042	0.19	3.13	0.02	0.19	0.39	>12.50
PSU 043	0.39	3.13	0.02	0.39	1.57	>12.50
PSU 044	1.57	3.13	0.01	0.39	0.79	>12.50
PSU 045	0.19	1.57	0.01	0.09	0.79	>12.50
PSU 046	0.79	3.13	0.02	0.79	0.39	>12.50
PSU 047	0.39	1.57	0.01	0.19	0.79	>12.50
PSU 048	0.39	1.57	0.02	0.79	0.79	>12.50
PSU 049	0.19	1.57	0.01	0.19	0.79	>12.50
PSU 050	0.19	3.13	0.02	0.19	0.79	>12.50
PSU 051	0.79	3.13	0.01	0.39	0.39	12.50
PSU 052	0.19	1.57	0.02	0.19	1.57	>12.50

* Values from triplicate.

a weak inhibition activity of ethanolic extracts of *A. galanga* against *S. aureus*. Presumably, chloroform extracted more inhibitory principles from the plants than the others. It was clearly established that many plants contain microbial inhibitors. Naturally occurring combinations of these compounds can be synergistic and often result in crude extracts having greater antimicrobial activity than the purified individual constituents (Delaquis *et al.* 2002). Both *A. galanga* and *Boesenbergia pandurata* are used in many Thai dishes. This finding further supported that a number of edible plants are obviously a potential source for inhibitory substances for some important foodborne pathogenic bacteria. Alzoreky and Nakahara (2003) reported antibacterial activity against *B. cereus* from *Zingiber officinale*, another plant from Zingiberaceae. Flavonoids are the main bioactive constituents of these rhizomes. An active compound, 1′-acetoxychavicol acetate, was previously identified from *A. galanga*. *B. rotunda* as its primary components: alpinitin (7-hydroxy-5-methoxyflavanone), pinocembrin (5,7-dihydroxyflavanone) and pinostrobin (5-hydroxy-7-methoxy-flavanone) (Voravuthikunchai *et al.* 2005). Soluble flavonoids were reported to be more active than their glycosidic forms naturally

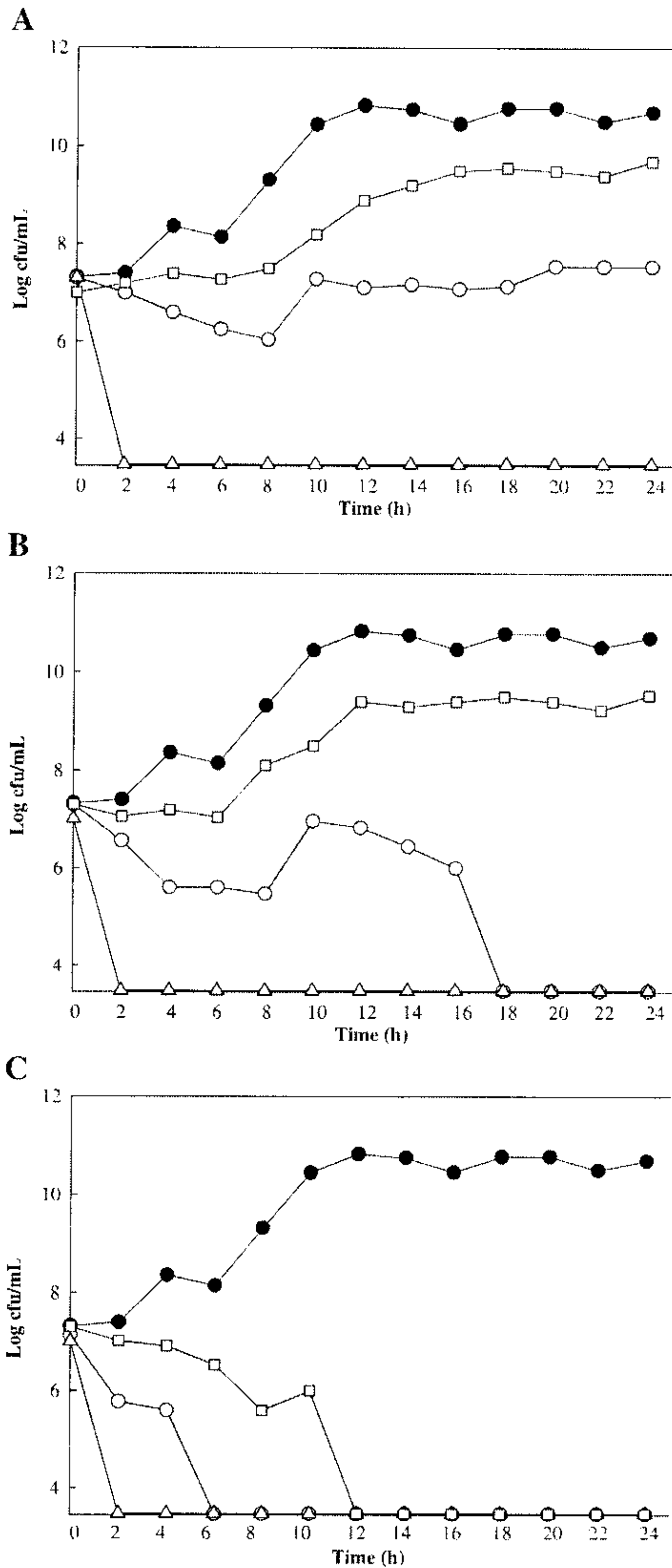

FIG. 1. DETECTION OF CELL SURVIVAL OF *STAPHYLOCOCCUS AUREUS* (PSU 039) FOLLOWING TREATMENT WITH CHLOROFORM EXTRACTS OF MEDICINAL PLANTS FROM FAMILY ZINGIBERACEAE

(A) Minimum inhibitory concentration (MIC), (B) 2MIC and (C) minimum bactericidal concentration. For all graphs, ● represents control with no extract, □, △ and ○ represent tested cultures with extracts of *Alpinia galanga*, *Boesenbergia rotunda* and *Zingiber zerumbet* cultures, respectively.

present in plants (Otshudi *et al.* 2000; Rauha *et al.* 2000). The antimicrobial compounds from plants may inhibit bacterial growth by different mechanisms than those presently used antimicrobials and may provide a significant clinical value in treatment of resistant pathogenic bacteria. β-Lactamase stable penicillins are now the drugs of choice in the treatment of *S. aureus*. However, because increasing numbers of *S. aureus* strains now demonstrate methicillin resistance, alternative antibiotics such as vancomycin are used more frequently. The MIC values of vancomycin for drug-resistant strains range from 0.6 to 1.25 μg/mL. Chloroformic extract from *B. rotunda* had MIC values of 6 to 24 μg/mL. Therefore, it appears to hold promise as an antimicrobial agent in the treatment of *S. aureus* including MRSA. The activities of these extracts provide preliminary scientific validation for the traditional medicinal use of these plants. Whether the active compounds present in these extracts can inhibit the organisms in food have yet to be characterized. However, previous study has demonstrated undetectable level of *Salmonella* Typhimurium after addition of fingerroot essential oils to apple juice (Thongson *et al.* 2005). Furthermore, both toxicity and safety issues of any purified compounds should be addressed before the application to medicine or food.

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(DA), levofloxacine (LEV), and tetracycline (TE) by disc diffusion method.
Results: All of the isolates were found susceptible to PG and 6 (12.2%) strains resistant to E by E-test method. All of the wound isolates were susceptible to LEV, 2 resistant to DA and 4 resistant to TE by disc diffusion method.
Conclusion: Penicillin is still the first choice of the treatment of GABHS infections. Although erythromycin is an alternative in patients who have penicillin hypersensitivity, there is an increasing resistance against it. Levofloxacin and clindamycin could be the other therapy options. Therefore antibiotic susceptibility testing and resistance surveillance of GABHS should be done.

Table1: Penicillin G and Erythromycine MIC ranges, MIC50, MIC90 values of the Group A Beta-haemolytic streptococci

Antibiotic	MIC range (µg/ml)		MIC50 (µg/ml)		MIC90 (µg/ml)	
	Throat	Wound	Throat	Wound	Throat	Wound
Penicillin G	0.003-0.047	0.003-0.064	0.006	0.008	0.012	0.012
Erythromycine	0.016-8	0.023-3	0.047	0.094	0.094	2

R1979

In vitro activity of cefepime against clinical isolates from patients in Tehran, Iran

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Objective: Infections caused by resistant bacterial pathogens, including Gram positive and Gram-negative bacteria, have become an increasing problem with respect to therapy in many of the Irananian medical center. Cefepime has been recently introduced in Iran, which is highly effective against these organisms. The purpose of this study was to determine in vitro activity of this antibiotic against isolated organism from patients, before introducing to the market, in Iran.
Methods: In vitro activity of Cefepime were tested against 304 clinical isolates samples including *Escherichia coli* (n = 167), *Klebsiella* (n = 49), *Staphylococcus* (n = 52), *Pseudomonas* (n = 18) and other isolates (n = 18), obtained during January–May 2005 from patients of Hazrat Rasoul hospital in Tehran, Iran. Minimum Inhibitory Concentration (MIC) of antibiotics was adjusted using an agar dilution method, described by NCCLS.
Results: The overall susceptibility rate for Cefepime against all isolates was 75%. Against *Pseudomonas aeroginosa* and *Staphylococcus* spp strains Cefepime was less active (40% resistance) and against *Enterobacteriaceae* Cefepime was excellent activity (more than 80% susceptibility).
Conclusion: Cefepime could be a valuable alternative for the treatment of infections due to multiply resistant organisms in Iran. Hence, it seems this drug could be suitable for empiric coverage of serious nosocomial infections.

R1980

Inhibitory activity and killing activity of extracts from the gall of *Quercus infectoria* against methicillin-resistant *Staphylococcus aureus*

S. Voravuthikunchai, S. Chusri, P. Kleiner *(Hatyai, Songkla, TH; Munich, DE)*

Objectives: Methicillin-resistant *S. aureus* has been well-documented as a major cause of hospital-acquired infection. Medicinal plants have been increasingly used to reduce the problem of antibiotic-resistant bacteria. *Quercus infectoria* was previously reported from this laboratory to produce high antibacterial activity. The aims of this study were to closely investigate the antibacterial activities of the extracts from *Q. infectoria* galls and to determine the effects of these extracts on the growth of clinical MRSA strains.
Methods: Fifty-one clinical isolates of MRSA were collected from Hat-Yai hospital. All isolates were multidrug-resistant. Galls of *Q. infectoria* were extracted with acetone, ethyl acetate, 95% ethanol, and water. Paper disc agar diffusion method was used to determine the antibacterial activity of these extracts. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were evaluated by modified broth microdilution method according to National Committee for Clinical Laboratory Standards. Growth curves demonstrating bacteriostatic and bactericidal activities of *Q. infectoria* against MRSA strains were documented for 24 h.
Results: All extracts of *Q. infectoria* show antibacterial activities against all strains of MRSA. The inhibition zones ranged from 11–23 mm. The ethanolic extract demonstrated the largest inhibition zone. Most of the MRSA strains treated by the ethanolic extract of *Q. infectoria* exhibited the MIC and MBC values at 0.4 and at 1.6 mg/ml, respectively. At the MIC concentration, the growth of a representative was inhibited and gradually decreased after 16 h incubation. The survival cells of the MRSA were not detected within 2 h after treated with the extract at its MBC concentration. *Staphylococcus aureus* ATCC 25923, a reference strain showed similar results.
Conclusion: The ethanolic extract of the galls of *Q. infectoria* have a high potential as antibacterial agent against MRSA. More detailed studies on the extract may provide an alternative way to treat infections caused by MRSA. Use of the active compounds could be developed as antibacterial agent in order to reduce problems with antibiotic-resistant bacteria in the hospitals.
Acknowledgement: This work was supported by Thailand Research Fund, Fiscal year 2005–2008.

New antimicrobials

R1981

Plant extracts as new anti-tuberculous agents, evaluation by MRA

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Objectives: Tuberculosis (TB) is a disease of antiquity, which is thought to have evolved sometime between the seventh and sixth millennia BC. Current estimates suggest that one third of world's population are infected resulting in some 2 million deaths per year. Pulmonary TB, the most common type of the disease, is usually acquired by inhalation of the bacillus and causes irreversible lung destruction, although other organs are sometimes involved. 50 years ago the introduction of the first drugs for TB treatment (streptomycin, para-aminosalicylic acid, isoniazid) led to optimism that the disease could be controlled if not eradicated. However, since the late 1980s the disease has

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P2074 In vitro and in vivo antibacterial activity of novel nitrile-containing fluoroquinolones

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Objectives: The continuing emergence of resistance in Gram-positive bacterial species including multi-drug resistant *Streptococcus pneumoniae*, vancomycin-R Enterococci (VRE), ciprofloxacin-R MRSA (CRMRSA), and Vancomycin-intermediate *Staphylococcus aureus* (VISA) has created the need for new antibacterial compounds. Two new nitrile-containing fluoroquinolones (#1966 and #9402) were developed to treat infections caused by susceptible and multi-drug resistant Gram-positive and fastidious Gram-negative bacterial strains. This study investigated the in vitro antimicrobial activity of 1966, 9402, moxifloxacin, and conventional antibacterials against 1204 geographically diverse recent bacterial clinical isolates. Compound efficacy was also evaluated by in vivo testing (PD50s).

Methods: Microbroth dilution MICs followed CLSI guidelines. In vivo testing was performed in CD1 female mice using protocols approved by the Pfizer Animal Use Committee in compliance with NIH guidelines for proper care and use of laboratory animals.

Results: See the table. Both of the nitrile fluoroquinolones were highly active against Gram-positive (MIC_{90}s 0.5–4 µg/mL) and fastidious Gram-negative strains (MIC_{90}s 0.03–0.06 ug/mL). This potent activity carried over to efficacy in *Streptococcus pneumoniae* animal infection models. Acute systemic PD50s (oral dosing) were 3.2 and 1.1 mg/kg, respectively, and pneumonia model PD50s (oral dosing) were 5.93 and 5.48 mg/kg.

Organism (# strains)	MIC_{90} (µg/mL)		
	#1966	#9402	Moxifloxacin
S. aureus CRMRSA (34)	1	1	8
S. aureus VISA[a] (4)	2	0.5	4
S. epidermidis ORSE (23)	2	1	32
S. pneumoniae FQ-R (26)	0.5	0.5	4
E. faecalis (13)	0.5	0.5	8
E. faecalis Van A (14)	1	0.5	16
E. faecalis Van B (22)	1	0.5	16
E. faecium Van A (45)	4	4	32
Moraxella catarrhalis (30)	0.06	0.06	0.06
Haemophilus influenzae (67)	0.03	0.06	0.03

[a]MIC_{50} (µg/mL).

Conclusion: This study confirms both the in vitro antibacterial potency and in vivo efficacy of 1966 and 9402 against clinically relevant Gram-positive and fastidious Gram-negative organisms.

P2075 Screening for medicinal plants with broad spectrum of antibacterial activity

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Objectives: Interest in the study of medicinal plants as a source of pharmacologically active compounds has increased worldwide. The objective of this study was to screen for effective plants from 31 plant species commonly used in Thai traditional medicine for bacterial infections.

Methods: Preliminary screening was performed using agar disc diffusion method. Minimal inhibitory concentration and Minimal bactericidal concentration were subsequently carried out on effective plants.

Results: Agar disc diffusion method showed that most plants were more active against Gram-positive than Gram-negative bacteria. *Streptococcus pyogenes* was the most sensitive organism inhibited by nearly all of the extracts (97.6%), followed by *Staphylococcus aureus* (61.0%), and *Bacillus cereus* (63.4%). We report two plants with very broad spectrum of activity. The ethanolic extracts from Quercus infectoria demonstrated significant activity against all important pathogens including *Acinetobacter baumannii*, *Bacillus cereus*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Shigella flexneri*, *Staphylococcus aureus*, *Streptococcus* mutans, and *Streptococcus pyogenes*. It inhibited the growth of all pathogens with the MIC values of 62.5 to 1000 ug/mL and the MBC values of 125 to >1000 µg/mL. The ethanolic extracts from Piper betle showed antibacterial activity against almost all species, except *Enterococcus faecalis*. It showed antibacterial activity with the same MIC and MBC values (125–500 µg/mL). The leaf extract of Rhodomyrtus tomentosa showed extremely good antibacterial activity on most Gram-positive bacteria with the MICs and MBCs ranging from 3.9 to 15.6 and 7.8 to 125 µg/mL.

Conclusion: Further studies on these plant species may result in discovery of novel natural medicine against pathogenic bacteria.

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P2076 Linezolid versus a glycopeptide or β-lactam for treatment of Gram-positive bacterial infections: a meta-analysis of randomised controlled trials

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Objectives: During the last decade, several new antibiotics have been released to the market for the treatment of patients with infections due to Gram-positive cocci resistant to traditionally used antibiotics, including glycopeptides. Among these antibiotics, linezolid has been reported to have excellent pharmacokinetics and effectiveness. We performed a meta-analysis of randomised controlled trials (RCTs) to further clarify the therapeutic role of linezolid.

Methods: Our data sources for relevant RCTS were PubMed, Current Contents, and Cochrane Central. A total of 12 RCTs comparing linezolid with vancomycin (6 RCTs), teicoplanin (2 RCTs), and β-lactams (amoxicillin/clavulanic acid, cephadroxil, and ceftriaxone in 2, 1, and 1 RCT, respectively) that studied 6,093 patients were included in our analysis.

Results: Two reviewers independently extracted data from published RCTs. Overall linezolid, as empirical treatment, was more effective regarding treatment success than the comparator antibiotics (OR= 1.41, 95% CI 1.11–1.81). Mortality was similar between the compared antibiotics (OR= 0.97, 95% CI 0.79–1.19). Linezolid was more effective in the subset of patients with skin and soft tissue infections (OR= 1.67, 95% CI 1.31–2.12) and bacteraemia (OR= 2.18, 95% CI 1.10–4.29). However, there was no difference in treatment success for patients with pneumonia (OR= 1.03, 95% CI 0.75–1.42). Although treatment with linezolid was not associated with more adverse effects in general (OR= 1.40, 95% CI 0.95–2.05), more episodes of thrombocytopenia were recorded in patients receiving this antibiotic (OR= 9.25, 95% CI 3.52–25.76).

Conclusion: Linezolid was more effective than a glycopeptide or a β-lactam for the empirical treatment of patients with skin and soft tissue infections and bacteraemia due to Gram-positive cocci. However, the lack of any benefit in the treatment of patients with pneumonia, the same all-cause mortality, and the higher probability of thrombocytopenia are major limitations of the antibiotic that should be taken under consideration and limit the use of linezolid to specific patient populations or infections that are difficult to treat with other antibiotics.

Methods: Consecutive, non-duplicate isolates (17,206) from bloodstream, skin and skin structure and respiratory tract infections were collected from medical centres in Europe (23), Turkey (2) and Israel (1) participating in the BPR Surveillance Program during 2005–2006. Identifications were confirmed by the central monitoring laboratory and all isolates were susceptibility (S) tested using CLSI methods against BPR and comparator agents.

Results: Results are in the Table. Among SA (27% OXA-R) and CoNS (75% OXA-R) isolates tested, BPR inhibited 100% at ⩽4 and ⩽8 mg/L, respectively. While BPR MIC90 values for OXA-R strains were elevated over those of OXA-S strains (8-fold), MIC90 values for other cephalosporins correspondingly increased ⩾32-fold. BPR was 4-fold more potent when testing beta-haemolytic streptococci (BHS) and SPN compared with CRO or FEP; all BHS were inhibited at ⩽0.25 mg/L and >99% of SPN by 0.5 mg/L. BPR was similar in potency to CAZ and FEP (MIC50 values, ⩽1 mg/L) against tested Enterobacteriaceae; coverage against EC was nearly identical for the three agents (94–95% inhibited at ⩽4 mg/L). FEP provided enhanced coverage against KSP (90% at ⩽8 mg/L vs. 78–84% for BPR and CAZ), although BPR and FEP had lower MIC values than CAZ against ESP. Cephalosporins were largely inactive against ESBL-producing EC and KSP. BPR was equal in potency to CAZ (MIC50, 2 mg/L) against PSA and two-fold more potent than FEP, although % inhibited for these agents at ⩽2/4/8 mg/L were similar. None of these agents inhibited >49% of ASP at 8 mg/L.

Species (no. tested)	MIC_{90} (% at ⩽2/4/8 mg/L)		
	BPR	CRO[a] or CAZ[b]	FEP
S. aureus (SA; 4,028)	1 (>99/100/–)	>32 (39/72/75)[a]	>16 (66/76/81)
Coagulase-negative staphylococcus (CoNS; 1,840)	2 (93/>99/100)	>32 (22/33/47)[a]	>16 (41/62/77)
S. pneumoniae (SPN; 1,528)	0.25 (100/–/–)	1 (>99/>99/100)[a]	1 (>99/>99/>99)
E. coli (EC; 2,779)	0.12 (93/94/94)	⩽1 (93/94/95)[b]	0.25 (94/95/96)
Klebsiella spp. (KSP; 883)	>8 (77/78/78)	>16 (80/81/84)[b]	16 (84/87/90)
Enterobacter spp. (ESP; 571)	>8 (81/84/87)	>16 (66/68/70)[b]	4 (88/92/96)
P. aeruginosa (PSA; 984)	>8 (54/65/79)	>16 (56/69/76)[b]	16 (49/66/80)
Acinetobacter spp. (ASP; 320)	>8 (41/41/42)	>16 (15/32/39)[b]	>16 (26/37/49)

Conclusions: BPR displays prominent activity against European staphylococci, including OXA-R strains. The compound also displayed activity against Enterobacteriaceae, similar to that of extended-spectrum cephalosporins, as well as against some non-fermentative bacilli. Given the breadth of its spectrum, BPR may be useful in those European institutions/ regions where MRSA and PSA are both prevalent.

P579 Antimycobacterial activity of two *Berberis* species used as traditional medicine in Iran

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Objectives: Tuberculosis (TB) is the leading cause of mortality worldwide, infecting about 9 million people and killing approximately 2 million people annually. In recent years, emerging multiple drug resistance (MDR) has become a major threat, thus there is an urgent need to search natural products for developing new, effective and affordable anti-TB drugs. Berberidaceae, especially berberis species are well known medicinal plants and are used in traditional medicine, foods and dies in Iran. Chemical composition of Berberidaceae species show that their anti-inflammatory and anti-tuberculosis activity is mainly due to alkaloid constituents. This study was conducted to compare antimycobacterial activity of extracted alkaloids from root bark and fruit of *Berberis vulgaris* and *B. integgerima* from the natural flora of Khorasan ecological region.

Methods: Root bark and fruit from two species were collected, shade air dried, lyophilised and powdered. Chloroform extracts containing alkaloid fractions were obtained. Fresh solutions of each extract were dissolved in 2% DMSO. The Chloroform extracts were incorporated into the Lowenstein-Johnson medium after solidification with final concentrations of 500, 250, 125, 62.5 and 31 µg/ml. Bacterial suspension test was adjusted to 3×10^5 CFU per ml *Mycobacterium bovis* BCG (Strain 1173 P2, Institut Pasteur, Paris, France) and incubated in 37 degrees of Celsius for 21 days. The minimum inhibitory concentration (MIC) amounts were evaluated after 21, 28 and 35 days of incubation. *Mycobacterium bovis* BCG was cultured in presence and absence of reference drug (Kanamycin) as controls. All tests were carried out five times.

Results: All four root bark and fruit chloroform extracts showed significant ($P < 0.05$) MIC concentrations (31–500 µg/ml), therefore 31 µg/ml was considered to be the best MIC for all four extracts.

Conclusion: Medicinal plants are an important resource to find original active drugs or new therapeutic agents especially against TB. The results indicate that root bark and fruit of *Berberis vulgaris* and *B. integgerima* can be used as antimycobacterial agents because of their considerable MIC values. Our investigations show that these remarkable results are due to alkaloids such as berberine, palmatine, oxyacantine and jatrorrhizine. Further investigations are required to assess the activities of these compounds against MDR *M. tuberculosis*.

P580 Antistaphylococcal activity of semi-purified fractions from Eleutherine americana

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Introduction: Meticillin-resistant *Staphylococcus aureus* (MRSA) infections are a global health concern due to the severity of the illnesses they may cause, ranging from mild to very severe infections. The numbers of *S. aureus* strains that exhibit antimicrobial resistance properties have increased and there is a potential risk of transmitting antibiotic resistance genes to the human microbiota through foods. Interest in plants with antibacterial properties has revived as a consequence of current problems associated with the use of antibiotics. Preliminary research from our laboratory revealed that the crude extract from Eleutherine americana produced good antibacterial effect on *S. aureus* from foods. The aim of the present study was to examine the activity of semi-purified fractions from this plant extract against meticillin-resistant strains isolated from foods and enterotoxin-producing reference strains.

Methods: Twenty-two MRSA strains isolated from food together with two enterotoxin-producing strains (ATCC 23235 and ATCC 27664) were used in this study. The Minimum inhibitory concentration (MIC) was determined by the broth dilution method and Minimum bactericidal concentration (MBC) was performed with the extracts that gave significant MIC values using a sterile loop streaking on fresh MHA (CLSI standard methods). Time-kill assay in the presence of these fractions were carried out. The crude hexane extract was chromatographed on column chromatography and fractions were obtained on the basis of their TLC characteristics.

Results: Fraction Ea 6.3 had MIC90 of 0.25 mg/ml while Ea 9.0 gave MIC50 of 0.125 mg/ml with MBC values ranging from 0.25 ⩾ 1.00 mg/ml for the two fractions on all the strains tested. Kill-curve in the presence of Ea 6.3 at 4MIC resulted in total killing of the cells at 20 h for reference strains and 24h for MRSA while fraction Ea 9.0 reduced the inoculum size by 7 log cycle.

Conclusion: Both fractions were very effective against all food isolated MRSA as well as enterotoxin-producing strains. These semi-purified fractions are being investigated in our laboratory to provide alternate treatment against MRSA.

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P581 Approach to identify novel antimicrobials against staphylococci

I. Abaev, O. Korobova, E. Pecherskikh, P. Kopylov, N. Kiseleva, D. Donovan (Obolensk, RU; Beltsville, US)

Objectives: Bacteriophage endolysins degrade the host cell wall peptidoglycan with near-specie specificity, and thus are of interest as

BOOK CHAPTER

Recent Progress in Medicinal Plants

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New Perspectives on Herbal Medicines for Bacterial Infections

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Abstract

Medicinal plants have been used for generations to treat bacterial infections. Scientific evidence is accumulating that a number of these herbs do have antibacterial activity that can alleviate symptoms or even prevent infectious diseases. The challenge of discovering new, urgent needed antibacterial drugs from medicinal plants requires a truly interdisciplinary research. The present communication discusses data from important findings with specific focus on recent literature on the antibacterial activity of some plant species with specific reference to important pathogenic bacteria. The following aspects are covered: (i) antibacterial activities of medicinal plants, (ii) plants with promising efficacious antibacterial activity, (iii) antibacterial mechanisms involved, (iv) structural aspects of antibacterial phytochemicals in medicinal plants, (v) research methodologies, and (vi) problems encountered with researches on medicinal plants.

Key words : Antibacterial activity, Antibacterial mechanism, *Escherichia coli* O157: H7, Herb, Medicinal plant, Methicillin-resistant *Staphylococcus aureus*, *Quercus infectoria*, *Staphylococcus aureus*

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Introduction

The word 'herb' comes from the Latin 'herba' meaning grass, green stalks or blades. In addition to its nutritional values, its therapeutic roles have been recognized in many parts of the world for generations. Originally, the Chinese used a number of forms of phytotherapeutic agent to develop drugs. This was also with the Egyptians, Greeks, and Romans. Countries in Africa, Asia, and Latin America use traditional medicine to achieve some of their primary health care needs. In Africa, up to 80 % of the population uses traditional medicine for primary health care (World Health Organization, 2003). In Ethiopia, traditional remedies represent not only part of the struggle of the people to fulfil their essential drug needs but also they are integral components of the cultural beliefs and attitudes (Abebe, 1996). Medicinal components from plants also form an important part of the health-care system in the western medicine (Phillipson and Anderson, 1989). For example, the use of *Arctostaphylos uva-ursi* (L.) Spreg. (bearberry) and *Vaccinium macrocarpon* Aiton (cranberry) juice to treat urinary tract infections has been reported in different manuals of phytotherapy. *Melaleuca alternifolia* (tea tree) is well-known as a common therapeutic tool to treat acne and many other skin infections (Vanaclocha and Caniguеral, 2003).

There are about 2,500,000 species of higher plant species worldwide. It has been estimated that only 14-28 % of higher plant species are used medicinally (Farnsworth and Soejarto 1991). The majority of these have not yet been examined in details for their pharmacological activities. Until natural products have been approved as new antibacterial drugs, there is an urgent need to identify novel substances active towards pathogenic organisms.

Infectious disease is the number one cause of death in tropical countries. It is remarkable that mortality rates are actually increasing in developed countries. The increases are attributed to increases in respiratory tract infections and HIV/AIDS. Other contributing factors are an increase in antibiotic-resistance in both community-acquired infections and nosocomial infections.

Drug resistance to human pathogenic bacteria has been commonly reported. Hospitals throughout the world have been faced with patients failing to respond to antibiotics. Bacteria have become resistant to drugs as a result of over prescribing antibiotics. In addition, patients' own immune systems have become compromised as a result of overuse of antibiotics. More seriously, the drug-resistant bacteria have further complicated the treatment of infectious diseases in immunocompromised, AIDS, and cancer patients (Diamond 1993; Rinaldi 1991; Senda *et al.,* 1996). In light of emerging bacteria resistant to multiple antimicrobial drugs as well as the appearance of undesirable side effects of certain antibiotics, it is critically important to develop new antibacterial compounds for these organisms before we enter the post-antibiotic era.

Traditional medicine has made use of many different plant extracts for the treatment of bacterial infections. In most developing countries, plants are the main medicinal source to treat infections. New pharmaceutically important compounds inhibiting microorganisms such as benzoin and emetine (Cox, 1994) as well as a number of top-selling drugs of modern time, for example, atropine, artemisinin, berberine, camptothecin, codeine, morphine, quinine, taxol, topotecan, vinblastine, and vincristine have been isolated from plants. To our knowledge, herb-resistant bacterial strains have never been reported. No correlation was observed between susceptibility of test strains with plant extracts and antibiotic resistance patterns (Ahmad and Beg, 2001; Voravuthikunchai and Kitpipit, 2003, 2005; Voravuthikunchai *et al.*, 2006a). The substances that can inhibit or kill the pathogens and have no or least toxicity to host cells are considered candidates for developing new antibacterial drugs. Both fresh and dried plant materials may provide clinical value in combating such resistant strains.

Over the past 20 years, there has been a lot of interest in the investigation of medicinal plants as sources of new antibacterial agents. Recent research has taken into consideration all aspects related to the use of medicinal remedies towards the development of modern medicines. During the past few years, there have been a substantial increase in the numbers of publications that authors screened plants for their antibacterial activities. We have documented here results from recent researches on selected antibacterial herbs, with specific references to important pathogenic bacteria.

Antibacterial activities of medicinal plants

In vitro antibacterial screening permits the selection of crude plant extracts with potentially useful properties to be used for further chemical and pharmacological studies. Considerable literatures involve studies on plants with antibiotic properties against a wide range of pathogenic bacteria (Al-Dabbas *et al.*, 2006; Bonjar, 2004; Boonkaew *et al.*, 2005; De Boer *et al.*, 2005; Dijipa *et al.*, 2000; Eldeen *et al.*, 2006; Elgayyar *et al.*, 2001; EL-Kamali *et al.*, 2005; Getie *et al.*, 2003; Holetz *et al.*, 2002; Kabouche *et al.*, 2006; Kone *et al.*, 2004; Magwa *et al.*, 2006; Mandal *et al.*, 2005; Matasyoh *et al.*, 2006; Messager *et al.*, 2005; Moon *et al.*, 2006; Natarajan *et al.*, 2006; Ojala *et al.*, 2000; Okeke *et al.*, 2001; Oussalah *et al.*, 2006; Ozkan *et al.*, 2006; Palombo and Semple, 2001; Ramesh *et al.*, 2001; Rojas *et al.*, 2001; Samy 2005; Singh *et al.*, 2005; Srinivasan *et al.*, 2001; Steenkamp *et al.*, 2004; Voravuthikunchai *et al.*, 2004c; Wannissorn *et al.*, 2005; Yasunaka *et al.*, 2005). Some specific researches have been focused on activity of plants against concrete pathogens including *Bacillus cereus* (Chaibi *et al.*, 1997; Delgado *et al.*, 2004; Kwona *et al.*, 2003; Smirnoff 1968; Valero and Giner 2006; Valero and Salmeron 2003), *Escherichia coli* O157: H7 (Cutter 2000;

Isogai *et al.*, 2000; Okubo *et al.*, 1998; Sakagami *et al.*, 2001; Takahashi *et al.*, 1999; Voravuthikunchai and Limsuwanm, 2006; Voravuthikunchai *et al.*, 2002, 2004b, 2005a, 2005c, 2006d), *Helicobacter pylori* (Annuk *et al.*, 1999; Fukai *et al.*, 2002; Nir *et al.*, 2000; O'Gara *et al.*, 2000; O'Mahony *et al.*, 2005; Voravuthikunchai *et al.*, 2004a, 2006b; Yesilada *et al.*, 1999), *Listeria monocytogenes* (Fyfe *et al.*, 1998; Gill and Holley 2006; Mytle *et al.*, 2006; Nairs *et al.*, 2005; Owen and Palombo, 2006; Rosooli *et al.*, 2006; Singh *et al.*, 2003; Yamazaki *et al.*, 2004), *Mycobacterium tuberculosis* (Cantrell *et al.*, 2001; Copp, 2003; Lall and Meyer 1999; Molina-Salinas *et al.*, 2006; Okunade *et al.*, 2004; Pauli *et al.*, 2005; Tosun *et al.*, 2004; van der Kooy *et al.*, 2006), *Neisseria gonorrhoeae* (Caceres *et al.*, 1995; Shokeen *et al.*,, 2005; Silva *et al.*, 2002; Swart *et al.*, 2002; Van Puyvelde *et al.*, 1983), *Pseudomonas aeruginosa* (Bonjar *et al.*, 2003; Pereira *et al.*, 2004; Senda *et al.*, 1996), *Salmonella* spp. (Evans *et al.*, 2002; Fyfe *et al.*, 1998; Owais *et al.*, 2005; Perez and Anesini, 1994; Waihenya *et al.*, 2002), *Staphylococcus aureus* (Edwards-Jones *et al.*, 2005; Hatano *et al.*, 2005; Machado *et al.*, 2003; Voravuthikunchai and Kitpipit, 2003, 2005). *Streptococcus mutans* (Hwang *et al.*, 2004; Jagtap and Karkera 2000; Rosalen *et al.*, 2000; Taweechaisupapong *et al.*, 2000), *Streptococcus pyogenes* (Ferro *et al.*, 2003). Most of the papers on preliminary screening of plant activities against bacteria have been frequently published in the *Journal of Ethnopharmacology*, followed by those appearing in *Fitoterapia*, and *Pharmaceutical Biology*.

Antibiotic-resistant bacteria are becoming more and more common in the clinical setting. *Staphylococcus aureus* is one of the major causes of hospital infections. Serious infections with this organism include wound infections, food poisoning, bacteraemia and sepsis. They are sometimes associated with a high mortality rate. Staphylococcal resistance to wide spectrum β–lactam antibiotics such as methicillin, oxacillin, and flucloxacillin emerged soon after the introduction of the first drug in this class. Since methicillin-resistant *S. aureus* (MRSA) has acquired stable resistance against almost all clinically available antibiotics, therapeutic option for MRSA infections is limited to glycopeptide-type drugs such as vancomycin (Patterson, 2000). However, treatment failure due to MRSA with reduced susceptibility to vancomycin was reported (Ward *et al.*, 2001). More recently, resistance to new antimicrobial agents such as linezolid, quinupristin, and daffopristin has already occurred (Berns, 2003; Leclercq, 2002). Extensive studies on the use of medicinal plants against *S. aureus*, in particular, MRSA have been established (Braga *et al.*, 2005; Caelli *et al.*, 2000; Carson *et al.*, 2002; Edwards-Jones *et al.*, 2005; Hatano *et al.*, 2005; Machado *et al.*, 2003; Voravuthikunchai and Kitpipit, 2003, 2005). Other workers have alternatively investigated another approach to the treatment of the infections based on agents that have no intrinsic antibacterial activity but are able to sensitize the pathogen to a previously ineffective antibiotic (Anderson *et al.*, 2005; Sato *et al.*, 2004; Stapleton *et al.*, 2004). Combination

of plant oils and derivatives of benzoic acid was demonstrated to inhibit *L. monocytogenes* and *Salmonella enteritidis* (Fyfe *et al.*, 1998). Further studies of the synergistic interaction of active phytocompounds with antibiotics or synthetic chemicals are required in order to exploit medicinal plant extracts in the combination therapy of infectious diseases caused by the multidrug-resistant bacteria.

Multiple antibiotic-resistant *Klebsiella pneumoniae* (Martinez-Martinex *et al.*, 1999; Hermandez-Alles *et al.*, 1999), vancomycin-resistant *Enterococcus faecalis* (VRE) (Nelson *et al.*, 2000), multidrug-resistant *Salmonella* (Akinyemi *et al.*, 2005; Chitnis *et al.*, 1999; Hoque *et al.*, 1994; Rao *et al.*, 1992; Threlfall *et al.*, 1996; Tuyet *et al.*, 1998; Ward *et al.*, 2005) have become increasingly reported. A significant number of work on medicinal plants have been carried out on these organisms (Fukai *et al.*, 2004; Kubo *et al.*, 2004; Owais *et al.*, 2005; Owais *et al.*, 2006; Rani and Khullar 2004).

Enterohaemorrhagic *Escherichia coli* (EHEC), in particular serotype O157: H7 (Paton and Paton, 1998) have increasingly emerged as pathogens that cause significant human diseases, including diarrhoea (Pai *et al.*, 1988), enterohaemorrhagic colitis (HC) (Riley, 1987), and occasionally complications such as haemolytic-uremic syndrome (HUS) and thrombocytopenic purpura (TTP) (Griffin and Tauxe, 1991; Scotland *et al.*, 1988). The issue of the risk involved in treatment of the organism with antimicrobial agents has been well-documented. Fosfomycin, the most frequently prescribed antibiotic in Japan for the treatment of this organism, was reported to stimulate the production and release of Verocytotoxin 1 (VT1) (Yoh *et al.*, 1997). Furthermore, work from the same laboratory clearly demonstrated that subinhibitory concentrations of quinolones including norfloxacin, sparofloxacin, and grepafloxacin markedly stimulated the productions of both VT1 and VT2 (Yoh *et al.*, 1999). Interest in plants with anti O157 properties has revived as a consequence of the problems associated with the use of antibiotics. There are a number of scientists working on the use of medicinal plants against this organism (Cutter, 2000; Isogai *et al.*, 2000; Okubo *et al.*, 1998; Sakagami *et al.*, 2001; Takahashi *et al.*, 1999; Voravuthikunchai and Limsuwan, 2006; Voravuthikunchai *et al.*, 2002, 2004b, 2005a, 2005c, 2006d).

Several other reports indicate the use of medicinal plants against groups of infectious pathogens including acne-inducing bacteria (Chomnawang *et al.*, 2005; Orafidiya *et al.*, 2002), foodborne pathogens (Cutter, 2000; Oussalah *et al.*, 2006; Owen and Palombo, 2006; Voravuthikunchai *et al.*, 2006c), gastrointestinal disorders (Alanis *et al.*, 2005; Dupont *et al.*, 2005; Mathabe *et al.*, 2006; Oussalah *et al.*, 2006; Uddin *et al.*, 2005), impetigo contagiosa (Sharquie *et al.*, 2000), opportunistic infections in AIDS patients (Miller *et al.*, 2003; Voravuthikunchai *et al.*, 2005b) and immunocompromised hosts (Senda *et al.*, 1996; Yukawa *et al.*, 1996), oral microorganisms (Al-hebshi *et al.*, 2006; Bakri and Douglas, 2005;

Koo *et al.*, 2000; Sato *et al.*, 1996), respiratory tract pathogens (Rojas, 2001; Skocibusic *et al.*, 2000), sextually-transmitted diseases (Kambizi and Afolayan, 2001; Ndubani and Hojer 1999; Okoli and Iroegbu 2004; Tshikalange *et al.*, 2005; Vermani and Garg 2002), urinary tract infection (Okoli and Iroegbu 2004; Pereira *et al.*, 2004), zoonotic enteropathogens (Wannissorn *et al.*, 2005).

During the past few years, in our laboratory, we have screened a number of medicinal plants selected from their properties described in ethnobotanical data. Table 1 lists the plants studied in our research group since 2002 with information on their traditional applications. Many of them have been used to treat infections as well as other disorders. The antibacterial activity of these medicinal plants against important pathogenic bacteria is summarized in Table 2 and Table 3.

Plants with promising antibacterial activity

In our laboratory, the major emphasis has been on plants with inhibitory activity less than 1 mg/ml for crude extracts and 0.1 mg/ml for fractions. A number of these medicinal plants have been found to be active against a wide variety of pathogenic bacteria. Many of them have been investigated for their chemical components and some of the isolated compounds have been shown to possess interesting activity (Voravuthikunchai *et al.*, 2005b, 2005c). Some of these herbs are discussed below. All of these selected plants presented here show very promising activity, warranting further investigation.

Alpinia galanga (L.) Willd. (Zingiberaceae)

Alpinia galanga is a tropical plant, originally from south-east Asia and southern China, with creamy white rhizome. It is commonly used as a flavouring agent in many Thai, Eastern and Caribbean dishes. The ginger-flavour rhizomes contain a volatile essential oil quite similar to that of the ginger. The rhizome is used in herbal remedies against rheumatism, bronchial catarrh, toothache, stomachache, bad breath, ulcers, whooping cough in children, throat infections, fever and dyspepsia. The essential oils, responsible for the characteristic odour as well as for their reported use in folk medicine, are active against Gram-positive and Gram- negative bacteria. Key constituents of this oil are methyl cinnamate, cincole, camphor, and d-pinene. We have reported some active compounds such as 1-acetoxychavicol acetate that active against MRSA from this laboratory (Voravuthikunchai *et al.*, 2005b).

Boesenbergia pandurata (Roxb.) Schltr. (Zingiberaceae)

This plant is found in south-east Asia and southern China. There are culinairy applications as a spice in the Thai and Indonesian kitchen. The

Table 1. Medicinal plants and their active phytochemicals.

Botanical name (Family name)	Common name	Plant part	Active phytochemicals	Herbal remedies	% yield
Acacia catechu (L.f.) Willd. (Fabaceae)	baval, black catechu, cachu, cutch, dark catechu, kaderi, kagli, kher, kheriya, khoira, koir babul, kugli, sandra	core	catechu tannic acid, tannin	asthma, body pain, cancer, cough, eczema, diarrhoea, pile, leprosy, sore mouth, sore throat, ulceration	6.0a 5.6e
Acanthus ebracteatus Vahl (Acanthaceae)	acanthus, saltbush	leaf	flavonoids, saponin, triterpenoids	analgesic, arthritis, kidney stones, neurotonic, pain, pruritic dermatitis, skin disease	9.0a 2.2c 5.4m
Adhatoda vasica Nees (Acanthaceae)	adosa, malabar nut, malabar nut tree, vasa, vasaka	leaf	alkaloid, vasicine	cough, inflammatory, rheumatism, scabies, skin disease	-
Aegle marmelos (L.) Corr. (Rutaceae)	bengal quince, golden apple, stone apple	fruit	aegeline, ascorbic acid, ethereal oil, mucilage, pectin, β-sitosterol, tannin	diarrhoea, dysentery, stomachache	5.37e
Alpinia galanga (L.)Willd. (Zingiberaceae)	da liang jiang, el galangal, el adkham, galgant, galanga maior, galanga majeur, grand galanga, greater galangal, grober galgant, galanga de l'inde, hang dou kou laos, java galangal, herbe indienne, lenkuas, naukyo, riêng, siamese galanga, siamese ginger, stor kalanga, ulanjan	rhizome	cineole, camphor, d-pinene, methylcinnamate, volatile essential oil	bad breath, dyspepsia, fever, rheumatism, sore throat, stomachache, toothache, ulceration, whooping cough	7.48a 5.63c 12.05m

Table 1. *(Contd.)*

Botanical name (Family name)	Common name	Plant part	Active phytochemicals	Herbal remedies	% yield
Alstonia scholaris (L.) R. Br. (Apocynaceae)	devil tree, Indian pulai, milky pine, white cheese wood	wood	echitamine	cough, fever, inflammatory, malaria, rheumatism, skin lesion, snake bite, sore throat, toothache	1.3a 2.5e
Andrographis paniculata (Burm. f.) Nees (Acanthaceae)	king of bitter, the creat	leaf	andrographolide, andrographiside, deoxyandrographolide, dehydroandrographolide, neoandrographolide, 14-deoxy-11,12-didehydroandrographolide	acne, bacterial infection, inflammatory, intestinal worm infection, promote digestion, protect liver and gallbladder, support intestine	
Ardisia colorata Roxb. (Myrsinaceae)	-	fruit	rapanone, syringic acid	diarrhoea, fever, haematinic	5.6a 4.4e
Asclepias curassavica L. (Asclepiadaceae)	bloodflower, butterfly weed, cancerillo, cotton bush, ipecacuanha, koningsbloem, milkweed, pleurisy root, redhead, scarlet milkweed, silkweed, sunset flower tropical, tropical butterfly flower	wood	alkaloid, glycosides, resinoid	asthma, cough, pain, typhus fever	0.98e
Barleria lupulina Lindl. (Acanthaceae)	barleria, hophead Philippine violet	leaf, stem	acetylbarlerin, 6-acetylshanzhiside methyl ester, 8-O-acetylshanzhiside,	inflammatory from centipede bite and insect bite, herpes	10.09a (leaf) 10.95c (leaf) 23.01m (leaf)

Table 1. *(Contd.)*

Botanical name (Family name)	Common name	Plant part	Active phytochemicals	Herbal remedies	% yield
			shanzhiside methyl ester	infection	5.75a (stem) 1.46c (stem) 7.20m (stem)
Blumea balsamifera var. *microcephala* Kitam. (Asteraceae)	blumea camphor, elumea, ngaicamphor, ngai camphor tree, sambong	leaf	cryptomeridiol	diarrhoea, gastralgia, headache, rheumatism, stomachache	8.9e
Boesenbergia pandurata (Roxb.) Schltr (Zingiberaceae)	finger root, kra chai	rhizome	camphor, 1-8 cineol, d-borneol, methylcinnamate	diarrhoea, inflammatory wounds	8.38a 1.58c 10.44m
Brucea javanica (L.) Merr. (Simaroubaceae)	bitter-barked tree	fruit	bruceine, bruceosides, glycoside	cancer, dysentery, malaria, verruca	6.8a 6.8e
Camellia sinensis (L.) Kuntze (Theaceae)	green tea, tea	leaf	catechin, polyphenols	detoxification, depression, headache, immune enhancement, pain, promote digestion	0.3e
Cassia alata L. (Fabaceae)	akapulco, akapulko, candle bush, candlestick senna, cassia flor, del Secreto, fleur darter, fleur palmiste, gelenggang, guajava, ketepeng badak,	leaf	anthraquinone compound (e.g. dianthrone glycosides), flavonoids	fungal infection, microbial infection, ringworm infection, skin infection	4.2a 5.6e

Table 1. *(Contd.)*

Botanical name (Family name)	Common name	Plant part	Active phytochemicals	Herbal remedies	% yield
	man-slabriki, ringworm, tarantana, wild senna				
Centella asiatica (L.)Urb. (Apiaceae)	American coinwort, asiatic coinwort, asiatic pennywort, gotu kola, Indian pennywort, tiger herbal	leaf	asiatic acid, asiaticoside, madecassic acid	fever	6.0e
Cinnamomum bejolghota (Buch.-Ham.) Sweet (Lauraceae)	-	bark, wood	-	-	14.68e (bark) 2.29e (wood)
Cinnamomum porrectum (Roxb.) Kosterm. (Lauraceae)	hardy cinnamon	bark, wood	-	-	7.09e (bark) 11.23e (wood)
Cleome gynandra L. (Cleomaceae)	African cabbage, African spiderflower wild, spider flower	whole plant	glucocapparin	anthelmintic, fever, rheumatism, rubefacient, scorpion sting, snake bite	6.76e
Coccinia grandis (L.) Voigh (Cucurbitaceae)	ivy gourd, scarlet-fruited gourd	leaf	asiaticoside, madecassoside, tannin	diabetic, fever, inflammatory	13.07a 6.25c 9.60m
Coriandrum sativum L. (Apiaceae)	cilantro, coriander	fruit	malic acid, tannin	antispasmodic, aphrodisiac, appetizer, carminative,	4.0e

Table 1. *(Contd.)*

Botanical name (Family name)	Common name	Plant part	Active phytochemicals	Herbal remedies	% yield
				diaphoretic, diuretic, fever, stimulant, stomachache, tonic, vomit	
Curcuma longa L. (Zingiberaceae)	curcuma, cucurmin, Indian safran, geelwortel, huldi, gelbwurz haldi, ukon goeratji, kakoenji, koenir, koenjet, koenjit, kondin, kurkuma, kunir, kunyit, oendre, rame, renet, safrandes indes, temu, temu kuning, tius tumeric, turmeric	rhizome	aromatic oil, curcumin, turmeric oil	arthritis, inflammatory, skin disease	13.9e
Curcuma zedoaria (Christm.) Roscoe (Zingiberaceae)	zedoary	rhizome	curcumin, curzerenone, 1-8 cineole, germacrone	asthma, cough, leueorrhoea, tonsillitis	9.6e
Dracaena loureoiri Gangnep. (Agavaceae)	-	core	retrodihydrochalcones	detoxification, heart tonic, tonic	16.9e
Dryopteris syrmatica O. Kze. (Polypodiaceae)	-	wood	-	-	4.5a 4.5e
Eclipta prostrata (L.) L. (Asteraceae)	-	whole plant	alkaloids, steroidal	hepatotoxic, inflammatory, insect bite, skin disease, uterine haemorrhage	17.04a 5.68c 7.49m

Table 1. *(Contd.)*

Botanical name (Family name)	Common name	Plant part	Active phytochemicals	Herbal remedies	% yield
Eleutherine americana Merr. (Iridaceae)	-	bulb	naphthoquinone	cold, diuretic, gastralgia	4.8e
Euphorbia thymifolia L. (Euphobiaceae)	chickenweed, dwarf spurge, garden spurge, gulf sandmat, red caustic creeper, thyme-leafed spurge	whole plant	kanzvil, limonene-2-sesquiterpenes, salicylic acid, taraxerol	diarrhoea, dysentery, enteritis	1.3e
Garcinia mangostana L. (Cluceaceae)	manggis, mangostan, mangosteen, mangostao, mangotanier	rind	citric acid, malic acid, pectin, tannin	chronic diarrhoea, cystitis, diarrhoea, eczema, gonorrhoea	-
Gynura pseudochina (L.) DC. (Asteraceae)	-	whole plant	-	burning pain, inflammatory, herpes infection	-
Gymnopetalum cochinchinensis (Lour.) Kurz (Cucurbitaceae)	-	fruit	-	tonic	7.66e
Holarrhena antidysenterica (L.) Wall. ex A. DC. (Apocynaceae)	kurchi	bark	conessine	amoebic dysentery, diarrhoea, fever, stomachache, tonic	2.6a 2.1e

Table 1. *(Contd.)*

Botanical name (Family name)	Common name	Plant part	Active phytochemicals	Herbal remedies	% yield
Impatiens balsamina L. (Balsaminaceae)	balsam, garden balsam, rose balsam, spotted snapweed, touch-me-not	leaf	lawsone, 2-methoxy-1,4 naphthoquinone, 1,4-naphthoquinone sodium salts	wart	-
Manilkara achras (Mill.) Fosberg (Sapotaceae)	sapodilla	fruit	tannin	diarrhoea, pulmonary complaint	26.77e
Millingtonia hortensis L.f. (Bignoniaceae)	cork tree, Indian cork tree, tree jasmine	flower	-	asthma	25.41e
Mimosa pudica L. (Mimosaceae)	sensitive plant, sleeping grass	whole plant	calcium oxalate crystals, mimosine, tannin	asthma, biliousness, blood disease, burning sensation, dysentery, fatigue, inflammatory, leprosy, vaginal and uterine complaint	4.91e
Mitragyna speciosa Korth (Rubiaceae)	kratom	leaf	7-hydroxymitragynine, mitragynine	diarrhoea	5.96e
Momordica charantia L. (Cucurbitaceae)	balsam apple, balsum pear, bitter cucumber, bittergourd,	vine	alkaloids, charantin,	aid in childbirth, cough,	10.6a

Table 1. *(Contd.)*

Botanical name (Family name)	Common name	Plant part	Active phytochemicals	Herbal remedies	% yield
	bitter melon, karela, k'u kua, kurela, kor-kuey, ku gua, melao de sao caetano, pare, pava-aki, papailla, peria laut, peria, salsamino, sorci, sorossi, sorossie, sorossies		insulin-like peptides, α-momorcharin, β-momorcharin, saponin	diabetes, fever, headache, hepatitis, hypertension, infection, malaria, measles, menstrual disorder, skin disease, stomachache	3.0e
Morinda citrifolia L. (Rubiaceae)	mulberry, noni	fruit	alizarin, anthraquinones, caproic, caprylic acids	asthma, dysentery, fever, wound, stomachache, tonic	7.36e
Murdannia loriformis (Hassk.) R.S. Rao & Kammathy (Commilinaceae)	angel grass, beijing grass	whole plant	flavonoids, glycosides	cough, diabetes	7.67e
Murraya paniculata (L.) Jack (Rutaceae)	andaman satinwood, Chinese box, cosmetic bark tree, Hawaiian mock orange, jasmine orange, mock orange, orange jasmine, orange jessamine, satinwood	leaf	-	bleeding wound, diarrhoea, dysentery, tapeworm infestation	8.38a 11.27c 17.31m
Oroxylum indicum (L.) Kurz (Bignoniaceae)	Indian trumpet flower	bark	chrysin, flavonoid, sitosterol, tannic acid	astringent, diarrhoea, dysentery, rheumatism, tonic	3.71e

Table 1. *(Contd.)*

Botanical name (Family name)	Common name	Plant part	Active phytochemicals	Herbal remedies	% yield
Peltophorum pterocarpum (DC.) Backer ex K. Heyne (Fabaceae)	copperpod, copper pod tree, golden flamboyant, yellow flamboyant, yellow flame tree, yellow poinciana	bark	hirsutidin, propelargonidin, rhamnetin	bruises tonic, disorder, inflammatory, sprain	8.6a 7.1e
Phyllanthus niruri L. (Euphorbiaceae)	stonebreaker	whole plant	alkaloids, hypophyllanthin, phyllanthine, quercetin	anemia, anuria, asthma, biliousness, bronchitis, diuretic, leprosy, urinary discharge	7.82e
Piper betle L. (Piperaceae)	betle vine	leaf	chavicol, cineol, eugenol,	antiseptic, astringent, carminative, cough, dental care, nose bleeding, mouth odour, stomachache, tonic	7.26a 16.36c 12.4e 14.68m
Piper chaba Hunter (Piperaceae)	Indian long pepper, Javanese long pepper, long pepper	fruit	alkaloid, cymene, piperine, terpinolene, thujene, zingiberene	carminative, diaphoretic, diuretic, skin liniment	10.49a 9.19c 7.0e 5.07m
Piper nigrum L. (Piperaceae)	black pepper, green peppercorns, pepper, white pepper	fruit	piperine, piplartine	cough, diarrhoea, dyspepsia, headache, pharyngitis	4.2e
Piper sarmentosum Roxb. (Piperaceae)	wildbetal, leafbrush	leaf	oxalate	asthma, cough, diabetes, fever, pleurisy, toothache	1.7e

Table 1. *(Contd.)*

Botanical name (Family name)	Common name	Plant part	Active phytochemicals	Herbal remedies	% yield
Pluchea indica (L.) Less. (Asteraceae)	Indian marsh fleabane	leaf	-	diabetes, diuretic, haemorrhoid	17.8e
Psidium guajava L. (Myrtaceae)	common guava, guava	leaf	carotenoids, flavonoids, lectins, phenols, saponins, tannins, triterpenes	bad breath, diarrhoea, dysentery	2.8a 8.0e
Punica granatum L. (Punicaceae)	pomegranate	rind	Alkaloid, ascorbic acid, tannin (punica tannic acid, gallic acid, mannite)	anthelmintic, diarrhoea, dyspepsia	8.0a 13.0e
Quercus infectoria G.Olivier (Fagaceae)	downy oak, gall nut, oak gall tree	fruit	tannic acid	biliousness, chronic dysentery, diarrhoea, fever	37.8a
Quisqualis indica L. (Combretaceae)	rangoon creeper	flower	quisqualic acid	diarrhoea, dysentery, toothache	11.08e
Rhizophora mucronata Lam. (Rhizophoraceae)	asiatic mangrove, red mangrove	bark, fruit	catechol, tannin	-	11.67e (bark) 10.75e (fruit)
Rhodomyrtus tomentosa (Aiton) Hassk (Myrtaceae)	downy myrtle, hill gooseberry, Isenberg bush, rose myrtle	leaf, wood	-	-	7.40e (leaf) 7.17e (stem)
Sandoricum indicum Cav. (Meliaceae)	red santol, santol, yellow sentol	root	-	astringent, diarrhoea, dysentery, fever, skin disease, stomachache	5.6a 4.0e

Table 1. *(Contd.)*

Botanical name (Family name)	Common name	Plant part	Active phytochemicals	Herbal remedies	% yield
Spilanthes acmella Murray (L.) (Asteraceae)	brazil cress, paper cress, para cress, spot flower, toothache plant,	whole plant	spilanthol, stigmasterol	diuretic, lithotriptic, skin disease, toothache	15.87a 23.32c 12.29m
Tamarindus indica L. (Fabaceae)	celagi, tangal asam, tamarind, tamarinde	leaf	citric acid, malic acid, tartaric acid	fever, anthelmintic, dysentery	37.1a 4.8e
Terminalia sp. (Combretaceae)	-	fruit	-	diarrhoea, fever	23.90e
Terminalia chebula Retz. (Combretaceae)	myrobolan wood	fruit	chebulagic, chebulinic acid, corilagin, tannin	bacterial infection	8.0a 8.2e
Terminalia bellirica (Gaertn.) Roxb. (Combretaceae)	belliric myrobalan	fruit	chebulagic, ellagic acid, ethyl gallate, gallic acid, galloyl, glucose, β- sitosterol	-	14.88e
Theobroma cacao L. (Sterculiaceae)	cacao, cacaoyer, chocolate, criollo, kakao	pericarp, seed	caffeine, theobromine	antiseptic, diuretic, emmenagogue, parasiticide, vulnerary	3.67e (pericarp) 5.93e (seed)
Uncaria gambir (Hanter) Roxb. (Rubiaceae)	cat's claw	leaf, branch	isomitraphylline, isopteropodine, isorhynchophylline, oxindole alkaloids, pteropodine, rhynchophylline	inflammatory, enhancement immune	65.4a 65.4e 65.4e

Table 1. *(Contd.)*

Botanical name (Family name)	Common name	Plant part	Active phytochemicals	Herbal remedies	% yield
Vitex trifolia L. (Verbenaceae)	beach vitex, chaste berry, costal vitex, namulega, simpleleaf chastetree, three leaved chaste tree, variegated vitex	leaf	acetate, camphene, diterpene alcohol, pinene, terpinyl	fibrocystic breast disease, infertility, menopause, menorrhagia, menstrual difficulties, premenstrual syndromes	-
Walsura robusta Roxb. (Meliaceae)	-	bark	-	diarrhoea, dysentery	2.4a 4.3e
Wrightia tomentosa (Roxb.) Roem. & Schult. (Apocynaceae)	darabela, ivory, sweet indrajao, white indrajau	stem	conessine	bitter tonic, dysentery	3.9e
Xylocarpus granatum J. Koeg. (Meliaceae)	-	pericarp, seed	tannin	diarrhoea, dysentery	2.68e (pericarp) 6.77e (seed)
Zingiber zerumbet (L.) Roscoe ex Sm. (Zingiberaceae)	broad-leaved ginger, pinecone ginger, pine-cone ginger, shampoo ginger, wild ginger, zerumbet ginger	rhizome	α -humulene, camphene, zer-umbone	headache, stomachache, toothache	14.12a 1.87c 4.26m

a = aqueous; e = ethanolic; c = chloroform; m = methanol, - = not known.

Table 2. Medicinal plants and their inhibitory effects on pathogenic bacteria.

Medicinal plants	Pathogenic bacteria																
	A. baumannii	*B. cereus*	*E. faecalis*	*E. coli* (6 strains)	*K. pneumoniae*	*L. monocytogenes*	MRSA	*P. aeruginosa*	*Salmonella* spp. (6 strains)	*S. typhi*	*S. typhimurium* (2 strains)	*S. welteureden*	*S. flexneri*	*S. sonnei*	*S. aureus*	*S. mutans*	*S. pyogenes*
Acacia catechu (Wood)																	
Ethanol extract	ND	ND	ND	+	ND	ND	+	ND	ND	ND	ND	ND	ND	ND	+	ND	ND
Water extract	ND	ND	ND	+	ND	ND	+	ND	ND	ND	ND	ND	ND	ND	+	ND	ND
Acanthus ebracteatus (Leaf)																	
Chloroform extract	ND	ND	ND	ND	ND	ND	-	ND	ND	-	ND	ND	ND	ND	-	-	ND
Methanol extract	ND	ND	ND	ND	ND	ND	-	ND	ND	-	ND	ND	ND	ND	-	-	ND
Water extract	ND	ND	ND	ND	ND	ND	-	ND	ND	-	ND	ND	ND	ND	-	-	ND
Adhatoda vasica (Leaf) Ethanol extract	ND	ND	ND	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Aegle marmelos (Fruit)																	
Ethanol extract	-	-	-	-	-	-	ND	-	-	-	-	-	-	ND	+	+	+

Table 2. *(Contd.)*

Medicinal plants	Pathogenic bacteria																
	A. baumannii	*B. cereus*	*E. faecalis*	*E. coli* (6 strains)	*K. pneumoniae*	*L. monocytogenes*	MRSA	*P. aeruginosa*	*Salmonella* spp. (6 strains)	*S. typhi*	*S. typhimurium* (2 strains)	*S. welteveden*	*S. flexneri*	*S. sonnei*	*S. aureus*	*S. mutans*	*S. pyogenes*
Alpinia galanga (Rhizome)																	
Chloroform extract	ND	+	ND	ND	ND	ND	+	ND	ND	-	ND	ND	ND	ND	+	+	ND
Methanol extract	ND	ND	ND	ND	ND	ND	-	ND	ND	-	ND	ND	ND	ND	-	-	ND
Water extract	ND	ND	ND	ND	ND	ND	-	ND	ND	-	ND	ND	ND	ND	-	-	ND
Alstonia scholaris (Wood)																	
Ethanol extract	ND	ND	ND	-	ND	ND	+	-	ND	-	ND	ND	ND	-	-	ND	ND
Water extract	ND	ND	ND	-	ND	ND	-	-	ND	-	ND	ND	ND	-	+	ND	ND
Andrographis paniculata (Leaf)																	
Ethanol extract	ND	ND	ND	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ardisia colorata (Fruit)																	
Ethanol extract	ND	ND	ND	-	ND	ND	+	-	ND	-	ND	ND	ND	-	+	ND	ND

Table 2. *(Contd.)*

Medicinal plants	Pathogenic bacteria																
	A. baumannii	*B. cereus*	*E. faecalis*	*E. coli* (6 strains)	*K. pneumoniae*	*L. monocytogenes*	MRSA	*P. aeruginosa*	*Salmonella* spp. (6 strains)	*S. typhi*	*S. typhimurium* (2 strains)	*S. welteureden*	*S. flexneri*	*S. sonnei*	*S. aureus*	*S. mutans*	*S. pyogenes*
Water extract	ND	ND	ND	-	ND	ND	+	-	ND	-	ND	ND	ND	-	+	ND	ND
Asclepias curassavica (Wood)																	
Ethanol extract	-	-	-	-	-	-	ND	-	-	-	-	-	-	ND	+	-	+
Barleria lupulina (Leaf/stem)																	
Chloroform extract	ND	ND	ND	ND	ND	ND	-/-*	ND	ND	-/-	ND	ND	ND	ND	-/-	-/-	ND
Methanol extract	ND	ND	ND	ND	ND	ND	-/-	ND	ND	-/-	ND	ND	ND	ND	-/-	-/-	ND
Water extract	ND	ND	ND	ND	ND	ND	-/-	ND	ND	-/-	ND	ND	ND	ND	-/-	-/-	ND
Blumea balsamifera (Leaf)																	
Ethanol extract	ND	ND	ND	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Boesenbergia pandurata (Rhizome)																	
Chloroform extract	-	+	+	-	-	-	+	-	-	-	-	-	-	ND	+	+	+
Ethanol extract	ND	ND	ND	-	ND	ND	+	-	ND	-	ND	ND	ND	-	-	ND	ND

Table 2. *(Contd.)*

Medicinal plants	Pathogenic bacteria																
	A. baumannii	*B. cereus*	*E. faecalis*	*E. coli* (6 strains)	*K. pneumoniae*	*L. monocytogenes*	MRSA	*P. aeruginosa*	*Salmonella* spp. (6 strains)	*S. typhi*	*S. typhimurium* (2 strains)	*S. weltevreden*	*S. flexneri*	*S. sonnei*	*S. aureus*	*S. mutans*	*S. pyogenes*
Methanol extract	ND	ND	ND	ND	ND	ND	+	ND	ND	+	ND	ND	ND	ND	+	+	ND
Brucea javanica (Seed)																	
Ethanol extract	ND	ND	ND	-	ND	ND	+	-	ND	-	ND	ND	ND	-	-	ND	ND
Water extract	ND	ND	ND	-	ND	ND	-	-	ND	-	ND	ND	ND	-	-	ND	ND
Ethanol extract	ND	ND	ND	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Cassia alata (Stem)																	
Ethanol extract	ND	ND	ND	-	ND	ND	+	-	ND	+	ND	ND	ND	-	+	ND	ND
Water extract	ND	ND	ND	-	ND	ND	+	-	ND	-	ND	ND	ND	-	+	ND	ND
Centella asiatica (Leaf)																	
Ethanol extract	ND	ND	ND	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 2. *(Contd.)*

Medicinal plants	Pathogenic bacteria																
	A. baumannii	*B. cereus*	*E. faecalis*	*E. coli* (6 strains)	*K. pneumoniae*	*L. monocytogenes*	MRSA	*P. aeruginosa*	*Salmonella* spp. (6 strains)	*S. typhi*	*S. typhimurium* (2 strains)	*S. weltevreden*	*S. flexneri*	*S. sonnei*	*S. aureus*	*S. mutans*	*S. pyogenes*
Cinnamomum bejolghota (Bark/wood)																	
Ethanol extract	-/-	+/+	-/-	-/-	-/-	-/-	ND	-/-	-/-	-/-	-/-	-/-	-/-	ND	+/+	-/-	+/+
Cinnamomum porrectum (Bark/wood																	
Ethanol extract	-/-	+/+	-/-	-/-	-/-	-/-	ND	-/-	-/-	-/-	-/-	-/-	-/-	ND	+/+	-/-	+/+
Cleome gynandra (Whole plant)																	
Ethanol extract	-	-	-	-	-	-	ND	-	-	-	-	-	-	ND	-	-	+
Coccinia grandis (Leaf)																	
Chloroform extract	ND	ND	ND	ND	ND	ND	-	ND	ND	-	ND	ND	ND	ND	-	-	ND
Methanol extract	ND	ND	ND	ND	ND	ND	-	ND	ND	-	ND	ND	ND	ND	-	-	ND
Water extract	ND	ND	ND	ND	ND	ND	-	ND	ND	-	ND	ND	ND	ND	-	-	ND
Coriandrum sativum (Fruit)																	
Ethanol extract	ND	ND	ND	-	ND	ND	+	-	ND	-	ND	ND	ND	-	-	ND	ND

Table 2. *(Contd.)*

Medicinal plants	Pathogenic bacteria																
	A. baumannii	*B. cereus*	*E. faecalis*	*E. coli* (6 strains)	*K. pneumoniae*	*L. monocytogenes*	MRSA	*P. aeruginosa*	*Salmonella* spp. (6 strains)	*S. typhi*	*S. typhimurium* (2 strains)	*S. welteureden*	*S. flexneri*	*S. sonnei*	*S. aureus*	*S. mutans*	*S. pyogenes*
Water extract	ND	ND	ND	-	ND	ND	-	-	ND	-	ND	ND	ND	-	-	ND	ND
Curcuma longa (Rhizome)																	
Ethanol extract	ND	ND	ND	-	ND	ND	+	-	ND	-	ND	ND	ND	-	-	ND	ND
Curcuma zedoaria (Rhizome)																	
Ethanol extract	ND	ND	ND	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Curcuma zedoaria (Rhizome)																	
Ethanol extract	ND	ND	ND	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Derris scandens (Stem)																	
Ethanol extract	ND	ND	ND	-	ND	ND	+	-	ND	-	ND	ND	ND	-	+	ND	ND
Water extract	ND	ND	ND	-	ND	ND	+	-	ND	-	ND	ND	ND	-	+	ND	ND
Dracaena loureiroi (Wood)																	
Ethanol extract	ND	ND	ND	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 2. *(Contd.)*

Medicinal plants	Pathogenic bacteria																
	A. baumannii	*B. cereus*	*E. faecalis*	*E. coli* (6 strains)	*K. pneumoniae*	*L. monocytogenes*	MRSA	*P. aeruginosa*	*Salmonella* spp. (6 strains)	*S. typhi*	*S. typhimurium* (2 strains)	*S. weltevreden*	*S. flexneri*	*S. sonnei*	*S. aureus*	*S. mutans*	*S. pyogenes*
Dryopteris syrmatica (Stem)																	
Ethanol extract	ND	ND	ND	-	ND	ND	+	-	ND	-	ND	ND	ND	-	+	ND	ND
Water extract	ND	ND	ND	-	ND	ND	+	-	ND	-	ND.	ND	ND	-	+	ND	ND
Eclipta prostrata (Whole plant)																	
Chloroform extract	ND	ND	ND	ND	ND	ND	-	ND	ND	-	ND	ND	ND	ND	-	-	ND
Methanol extract	ND	ND	ND	ND	ND	ND	-	ND	ND	-	ND	ND	ND	ND	-	-	ND
Water extract	ND	ND	ND	ND	ND	ND	-	ND	ND	-	ND	ND	ND	ND	-	-	ND
Eleutherine americana (Bulb)																	
Ethanol extract	-	+	+	-	-	+	ND	-	-	-	-	-	-	ND	+	+	+
Ethanol extract	ND	ND	ND	-	ND	ND	+	-	ND	-	ND	ND	ND	-	+	ND	ND

Table 2. *(Contd.)*

Medicinal plants	Pathogenic bacteria																
	A. baumannii	*B. cereus*	*E. faecalis*	*E. coli* (6 strains)	*K. pneumoniae*	*L. monocytogenes*	MRSA	*P. aeruginosa*	*Salmonella* spp. (6 strains)	*S. typhi*	*S. typhimurium* (2 strains)	*S. weltevreden*	*S. flexneri*	*S. sonnei*	*S. aureus*	*S. mutans*	*S. pyogenes*
Garcinia mangostana (Pericarp)																	
Ethanol extract	ND	ND	ND	-	ND	ND	+	-	ND	-	ND	ND	ND	-	+	ND	ND
Gymnopetalum cochinchinensis (Fruit)																	
Ethanol extract	-	-	-	-	-	-	ND	-	-	-	-	-	-	ND	-	-	+
Holarrhena antidysenterica (Bark)																	
Ethanol extract	ND	ND	ND	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Water extract	ND	ND	ND	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Impatiens balsamina (Leaf)																	
Ethanol extract	ND	ND	ND	-	ND	ND	+	ND	ND	ND	ND	ND	ND	ND	+	ND	ND
Manilkara achras (Fruit)																	
Ethanol extract	-	+	-	-	-	-	ND	-	-	-	-	-	-	ND	+	-	+
Millingtonia hortensis (Flower)																	
Ethanol extract	-	-	-	-	-	-	ND	-	-	-	-	-	-	ND	-	-	+

Table 2. *(Contd.)*

Medicinal plants	Pathogenic bacteria																
	A. baumannii	*B. cereus*	*E. faecalis*	*E. coli* (6 strains)	*K. pneumoniae*	*L. monocytogenes*	MRSA	*P. aeruginosa*	*Salmonella* spp. (6 strains)	*S. typhi*	*S. typhimurium* (2 strains)	*S. welteureden*	*S. flexneri*	*S. sonnei*	*S. aureus*	*S. mutans*	*S. pyogenes*
Mimosa pudica (Whole plant)																	
Ethanol extract	-	-	-	-	-	-	ND	-	-	-	-	-	-	ND	-	-	+
Mitragyna speciosa (Leaf)																	
Ethanol extract	-	-	-	-	-	-	ND	-	-	-	-	-	-	ND	-	-	+
Ethanol extract	ND	ND	ND	-	ND	ND	+	-	ND	-	ND	ND	ND	-	+	ND	ND
Water extract	ND	ND	ND	-	ND	ND	+	-	ND	-	ND	ND	ND	-	-	ND	ND
Morinda citrifolia (Fruit)																	
Ethanol extract	-	-	-	-	-	-	ND	-	-	-	-	-	-	ND	-	-	+
Murdannia loriformis (Whole plant)																	
Ethanol extract	-	-	-	-	-	-	ND	-	-	-	-	-	-	ND	-	-	-
Murraya paniculata (Leaf)																	
Chloroform extract	ND	ND	ND	ND	ND	ND	-	ND	ND	-	ND	ND	ND	ND	-	-	ND
Methanol extract	ND	ND	ND	ND	ND	ND	-	ND	ND	-	ND	ND	ND	ND	-	-	ND

Table 2. *(Contd.)*

Medicinal plants	Pathogenic bacteria																
	A. baumannii	*B. cereus*	*E. faecalis*	*E. coli* (6 strains)	*K. pneumoniae*	*L. monocytogenes*	MRSA	*P. aeruginosa*	*Salmonella* spp. (6 strains)	*S. typhi*	*S. typhimurium* (2 strains)	*S. weltevreden*	*S. flexneri*	*S. sonnei*	*S. aureus*	*S. mutans*	*S. pyogenes*
Water extract	ND	ND	ND	ND	ND	ND	-	ND	ND	-	ND	ND	ND	ND	-	-	ND
Oroxylum indicum (Bark)																	
Ethanol extract	-	+	-	-	-	+	ND	-	-	-	-	-	-	ND	+	-	+
Peltophorum pterocarpum (Bark)																	
Dichloromethane extract		-	+	-	-	-	-	ND	-	-	-	-	-	-	ND	-	- +
Ethanol extract	ND	ND	ND	+	ND	ND	+	-	ND	-	ND	ND	ND	-	+	ND	ND
Hexane extract	-	+	-	-	-	-	ND	-	-	-	-	-	-	ND	-	-	+
Methanol extract	-	+	-	-	-	-	ND	+	-	-	-	-	-	ND	+	+	+
Water extract	ND	ND	ND	+	ND	ND	+	-	ND	-	ND	ND	ND	-	+	ND	ND
Phyllanthus amarus (Whole plant)																	
Ethanol extract	-	+	-	-	-	-	ND	-	-	-	-	-	-	ND	+	-	+
Piper betle (Leaf)																	
Ethanol extract	+	+	-	+	+	+	ND	+	+	+	+	+	+	ND	+	+	+

Table 2. *(Contd.)*

Medicinal plants	Pathogenic bacteria																
	A. baumannii	*B. cereus*	*E. faecalis*	*E. coli* (6 strains)	*K. pneumoniae*	*L. monocytogenes*	MRSA	*P. aeruginosa*	*Salmonella* spp. (6 strains)	*S. typhi*	*S. typhimurium* (2 strains)	*S. welteureden*	*S. flexneri*	*S. sonnei*	*S. aureus*	*S. mutans*	*S. pyogenes*
Piper chaba (Fruit)																	
Ethanol extract	-	-	-	-	-	-	ND	-	-	-	-	-	-	ND	-	-	+
Piper nigrum (Fruit)																	
Ethanol extract	-	-	-	-	-	-	ND	-	-	-	-	-	-	ND	-	-	+
Piper sarmentosum (Leaf)																	
Ethanol extract	-	-	-	-	-	-	ND	-	-	-	-	-	-	ND	-	-	+
Pluchea indica (Leaf)																	
Ethanol extract	ND	ND	ND	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Psidium guajava (Leaf)																	
Ethanol extract	ND	ND	ND	+	ND	ND	+	ND	ND	ND	ND	ND	ND	ND	+	ND	ND
Water extract	ND	ND	ND	+	ND	ND	+	ND	ND	ND	ND	ND	ND	ND	+	ND	ND
Punica granatum (Pericarp)																	
Ethanol extract	ND	ND	ND	+	ND	ND	+	+	ND	+	ND	ND	ND	+	+	ND	ND

Table 2. *(Contd.)*

Medicinal plants	Pathogenic bacteria																
	A. baumannii	*B. cereus*	*E. faecalis*	*E. coli* (6 strains)	*K. pneumoniae*	*L. monocytogenes*	MRSA	*P. aeruginosa*	*Salmonella* spp. (6 strains)	*S. typhi*	*S. typhimurium* (2 strains)	*S. weltevreden*	*S. flexneri*	*S. sonnei*	*S. aureus*	*S. mutans*	*S. pyogenes*
Water extract	ND	ND	ND	+	ND	ND	+	+	ND	+	ND	ND	ND	+	+	ND	ND
Quercus infectoria (Nut gall)																	
Ethanol extract	+	+	+	+	+	+	+	+	+	+	+	+	+	ND	+	+	+
Water extract	ND	ND	ND	+	ND	ND	+	ND	ND	ND	ND	ND	ND	ND	+	ND	ND
Quisqualis indica (Flower)																	
Ethanol extract	-	+	-	-	-	+	ND	-	-	-	-	-	+	ND	+	-	+
Rhizophora mucronata (Bark/Fruit)																	
Ethanol extract	+/-	+/+	+/+	-/-	-/-	+/+	ND	-/-	-/-	-/-	-/-	-/-	-/-	ND	+/+	+/+	+/+
Rhodomyrtus tomentosa (Leaf/wood)																	
Ethanol extract	-/-	+/+	+/+	-/-	-/-	+/+	ND	-/-	-/-	-/-	-/-	-/-	+/+	ND	+/+	+/-	+/+
Sandoricum indicum (Root)																	
Ethanol extract	ND	ND	ND	-	ND	ND	+	-	ND	-	ND	ND	ND	+	+	ND	ND

Table 2. *(Contd.)*

Medicinal plants	Pathogenic bacteria																
	A. baumannii	*B. cereus*	*E. faecalis*	*E. coli* (6 strains)	*K. pneumoniae*	*L. monocytogenes*	MRSA	*P. aeruginosa*	*Salmonella* spp. (6 strains)	*S. typhi*	*S. typhimurium* (2 strains)	*S. weltevreden*	*S. flexneri*	*S. sonnei*	*S. aureus*	*S. mutans*	*S. pyogenes*
Water extract	ND	ND	ND	-	ND	ND	+	-	ND	-	ND	ND	ND	+	-	ND	ND
Spilanthes acmella (Whole plant)																	
Chloroform extract	ND	ND	ND	ND	ND	ND	-	ND	ND	-	ND	ND	ND	ND	-	+	ND
Methanol extract	ND	ND	ND	ND	ND	ND	-	ND	ND	-	ND	ND	ND	ND	-	-	ND
Water extract	ND	ND	ND	ND	ND	ND	-	ND	ND	-	ND	ND	ND	ND	-	-	ND
Tamarindus indica (Leaf)																	
Ethanol extract	ND	ND	ND	-	ND	ND	-	ND	ND	ND	ND	ND	ND	ND	-	ND	ND
Water extract	ND	ND	ND	-	ND	ND	-	ND	ND	ND	ND	ND	ND	ND	-	ND	ND
Terminalia bellirica (Fruit)																	
Ethanol extract	+	+	+	-	-	-	ND	-	-	-	-	-	-	ND	+	-	+
Terminalia chebula (Fruit)																	
Ethanol extract	-	+	+	-	-	-	+	+	-	+	-	-	-	+	+	-	+

Table 2. *(Contd.)*

Medicinal plants	Pathogenic bacteria																
	A. baumannii	*B. cereus*	*E. faecalis*	*E. coli* (6 strains)	*K. pneumoniae*	*L. monocytogenes*	MRSA	*P. aeruginosa*	*Salmonella* spp. (6 strains)	*S. typhi*	*S. typhimurium* (2 strains)	*S. weltevreden*	*S. flexneri*	*S. sonnei*	*S. aureus*	*S. mutans*	*S. pyogenes*
Water extract	ND	ND	ND	-	ND	ND	+	-	ND	-	ND	ND	ND	-	+	ND	ND
Terminalia sp. (Fruit)																	
Ethanol extract	+	+	+	-	-	-	ND	+	-	-	-	-	+	ND	+	-	+
Theobroma cacao (Pericarp)																	
Ethanol extract	-	-	-	-	-	-	ND	-	-	-	-	-	-	ND	-	-	+
Uncaria gambir (Leaf, stem)																	
Ethanol extract	ND	ND	ND	+	ND	ND	+	-	ND	+	ND	ND	ND	+	+	ND	ND
Water extract	ND	ND	ND	-	ND	ND	+	-	ND	+	ND	ND	ND	+	+	ND	ND
Vitex trifolia (Leaf)																	
Ethanol extract	ND	ND	ND	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Walsura robusta (Branches)																	
n-Butanol	-	+	-	-	-	-	ND	-	-	-	-	-	-	ND	+	+	+

Table 2. *(Contd.)*

Medicinal plants	Pathogenic bacteria																
	A. baumannii	*B. cereus*	*E. faecalis*	*E. coli* (6 strains)	*K. pneumoniae*	*L. monocytogenes*	MRSA	*P. aeruginosa*	*Salmonella* spp. (6 strains)	*S. typhi*	*S. typhimurium* (2 strains)	*S. weltevreden*	*S. flexneri*	*S. sonnei*	*S. aureus*	*S. mutans*	*S. pyogenes*
Ethyl-acetate	-	-	-	-	-	-	ND	-	-	-	-	-	-	ND	-	-	+
Ethanol extract	ND	ND	ND	+	ND	ND	+	-	ND	+	ND	ND	ND	+	+	ND	ND
Methanol extract	-	+	-	-	-	-	ND	-	-	-	-	-	-	ND	-	+	+
Water extract	ND	ND	ND	+	ND	ND	+	+	ND	+	ND	ND	ND	+	+	ND	ND
Wrightia tomentosa (Stem)																	
Ethanol extract	ND	ND	ND	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Xylocarpus granatum (Pericarp/seed)																	
Ethanol extract	-/-	+/+	-/+	-/-	-/-	-/-	ND	-/-	-/-	-/-	-/-	-/-	-/-	ND	+/+	-/-	+/+
Zingiber zerumbet (Rhizome)																	
Chloroform extract	ND	ND	ND	ND	ND	ND	+	ND	ND	-	ND	ND	ND	ND	+	+	ND
Methanol extract	ND	ND	ND	ND	ND	ND	-	ND	ND	-	ND	ND	ND	ND	-	-	ND
Water extract	ND	ND	ND	ND	ND	ND	-	ND	ND	-	ND	ND	ND	ND	-	-	ND

+ = active; - = inactive; * = 2 different parts of plants tested.

Table 3. Percentage of effective Thai medicinal plants against pathogenic bacteria.

Pathogenic bacteria	% Active	
	Plant species	Plant extracts
A. baumannii	12.9 (31)	12.5 (40)
B. cereus	59.4 (32)	63.4 (41)
E. faecalis	29.0 (31)	27.5 (40)
E. coli (6 strains)	14.8 (61)	16.3 (92)
K. pneumoniae	6.5 (31)	5.0 (40)
L. monocytogenes	22.6 (31)	22.5 (40)
MRSA	77.4 (31)	56.5 (70)
P. aeruginosa	15.6 (45)	11.3 (71)
Salmonella spp. (6 strains)	6.5 (31)	5.0 (40)
S. typhi	15.1 (53)	11.1 (99)
S. typhimurium (2 strains)	6.5 (31)	5.0 (40)
S. weltevreden	6.5 (31)	5.0 (40)
S. flexneri	16.1 (31)	15.0 (40)
S. sonnei	27.8 (18)	28.1 (32)
S. aureus	63.2 (57)	57.7 (97)
S. mutans	30.8 (39)	23.4 (64)
S. pyogenes	96.8 (31)	97.5 (40)

rhizome is used to treat colic disorders, wound infections and inflammation. 32It contains 1 to 3% of an essential oil. Several aroma components have been identified, 1-8 cineol, camphor, d-borneol and methyl cinnamate being the most important. Trace components are d-pinene, zingiberene, zingiberone, curcumin, zedoarin, and others. Among the non-volatile constituents, flavones and flavonoids (pinostrobin, alpinetin, pinocembrin), chalcones (cardamonin), and dihydrochalcones (boesenbergin A) have been identified. This plant species is found to be effective against all Gram-positive bacteria tested (Table 2), with high activity against MRSA (Voravuthikunchai *et al.,* 2005b, 2006c).

Garcinia mangostana L. (Clusiaceae)

The mangosteen tree is very slow-growing, erect, with a pyramidal crown. The sliced and dried rind is powdered and administered to treat dysentery. It is also applied as an ointment on eczema and other skin disorders. A portion of the rind is steeped in water overnight and the infusion given as a remedy for chronic diarrhoea, cystitis and gonorrhoea. The rind of partially ripe fruits yields a polyhydroxy-xanthone derivative termed β-mangostin.

That of fully ripe fruits contains the xanthones, gartanin, 8-disoxygartanin, and normangostin. A decoction of the leaves and bark is employed by the Filipinos to treat thrush, diarrhoea, dysentery, and urinary disorders. In Malasia, an infusion of the leaves, combined with unripe banana and a little benzoin is applied to the wound of circumcision. It is well-documented that this plant species has significant antibacterial activity. We have reported good activity against MRSA (Voravuthikunchai *et al.*, 2003, 2005).

Peltophorum pterocarpum (DC.) Backer ex K. Heyne (Fabaceae)

Yellow poincianas are usually planted in tropical Asia and South Pacific as specimen trees or as shade trees. Significant antibacterial activity of this medicinal plant against two important pathogenic bacteria, MRSA (Voravuthikunchai and Kitpipit, 2003) and *E. coli* O157: H7 (Voravuthikunchai *et al.*, 2002, 2004b) have been reported from this laboratory. Important chemical constituents of bark are bergenin, hirsutidin, propelargonidin, quercetin-3-O-β-D-diglucoside, rhamnetin and rhamnetin-3-O-β-D-glucoside.

Piper betle L. (Piperaceae)

Piper betle is native to India and throughout the balmy Asian tropics. The plant grows as a vigorous vine which is usually supported by a trellis of bamboo poles. The heart-shaped leaves are marvelously pungent and spicy. Leaves have long been used in Indonesia as traditional medicine. The essential oil is produced by steam distillation from the leaves is yellow to brown with an distinctly phenolic, almost tar-like or smoky. The essential oils of the plant contained phenolic compounds. These leaves have antimicrobial activity towards bacteria in the mouth. They are employed as stomatitis, antitussive, astringent, antiseptic, carminative, stimulant, stomachic, tonic, expectorant, nerve stimulant, for mouth odour, dental cares and inhibit nose bleeding. In addition, betel leaves have immune boosting properties as well as anti-cancer properties. The results from our research group show that it is one of the very interesting plant species with broad spectrum of activity against nearly all bacterial species tested (Table 2).

Punica granatum L. (Punicaceae)

The pomegranate is a shrub, usually with multiple stems, that commonly grows 1.8 to 4.6 m tall. It is found in Asia, the Middle East to the Himalayas. The fruit is technically a berry. It is filled with crunchy seeds each of which is encased in a juicy, somewhat acidic pulp that is itself enclosed in a membranous skin. Pomegranate bark contains about 20% of tannin, consisting of two astringent principles, gallotannic acid and punicotannic. The flowers and rind of the fruit are astringent and have been used for arresting chronic mucous discharges, passive haemorrhages, disorders of

the mouth, night sweats and diarrhoea. Many workers have demonstrated significant activity of *Punica granatum* against MRSA (Braga *et al.*, 2005; Holetz *et al.*, 2002; Machado *et al.*, 2003; Voravuthikunchai and Kitpipit, 2003, 2005). Contrastly, very few reports indicate its activity against Gram-negative bacteria. The methanolic extract of its pericarp showed good activity against *Proteus vulgaris* (Prashanth *et al.*, 2001). In addition to its antibacterial activity, the active fractions of pericarp have been reported to inhibit Verocytotoxin production by *E. coli* O157: H7 (Voravuthikunchai *et al.*, 2005c).

Quercus infectoria Oliv. (Fagaceae)

An evergreen shrub growing to 1.8 m, valued for excrescences formed upon the young branches, known in market as galls or nut-galls. They are the result of a puncture made in the bark by an insect, *Diplolepis gallse tinctoriae* or *Cynips quercufolii* for the purpose of depositing its egg. 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.

The main constituents found in the galls of *Quercus infectoria* are tannin (50-70 %) and small amount of free gallic acid and ellagic acid. (Evans, 1996; Ikram and Nowshad, 1977; Wiart and Kumar, 2001). Any galls produced on the tree are strongly astringent and can be used in the treatment of haemorrhages, chronic diarrhoea and dysentery. They may be used as a wash and gargle in sore throat and as an injection in bad leucorrhoea; in which cases they arrest putrefactive tendencies, and may be combined with suitable stimulants. By coagulating the blood, they frequently will arrest haemorrhage from small vessels and sometimes are used for bleeding piles, both as ointment and suppository.

Although nut gall is routinely prescribed for the treatment of diarrhoea in Thai herbal drug, scientific data supporting the use of this plant as a herbal medicine is scarce. At present, there have been very limited studies that demonstrate an effect of this plant against bacteria (Basri and Fan, 2005; Voravuthikunchai and Kitpipit, 2003, 2005; Voravuthikunchai *et al.*, 2002, 2004b, 2006a, 2006b). Our research group have been extensively studied on this plant species since we found that it has a broad spectrum of activity against all bacterial species tested (Table 2). In addition, the bark and acorns are astringent. They are used in the treatment of intertrigo, impetigo and eczema.

Uncaria gambir (Hunter) Roxb. (Rubiaceae)

It is a small creeping herb. The leaves are oval-shaped with taper ends. Important chemical constituents are catechutannic acid 22 % to 25 %, pyrocatechol 30 %, catechin 33 %, gambir-fluorescein, catechu red, and quercetin. Pyrocatechin and tannin are thought to be the main components.

Very limited information on its antibacterial property has been reported. Results from our laboratories indicate high activity of this medicinal plant against two important pathogenic bacteria, MRSA (Voravuthikunchai and Kitpipit, 2003) and *E. coli* O157: H7 (Voravuthikunchai *et al.*, 2002, 2004b).

Walsura robusta Roxb. (Meliaceae)

It is a perennial plant with fresh green leaves. Its bark is the most commonly used in Thai medicine. Very limited information has been reported on this medicinal plant. However, antibacterial activity against two important pathogenic bacteria, MRSA (Voravuthikunchai and Kitpipit, 2003, 2005) and enterohaemorrhagic *E. coli* O157: H7 have been reported from our laboratory (Voravuthikunchai *et al.*, 2002, 2004b). Chemical constituents of this medicinal plant have not yet been reported.

Antibacterial mechanisms

Inhibition of cell growth and killing ability

As have been earlier pointed out, most of the preliminary data on the efficacy of medicinal plants against pathogenic bacteria reported inhibition zones, minimum bacteriostatic concentration (MIC), and minimum bactericidal concentration (MBC). Some studies have shown that the antibacterial activity of some medicinal plants is too low to kill them or to inhibit their growth in human tissues, therefore their actions must be based on other mechanisms (Annuk *et al.*, 1999; Braga *et al.*, 2005; Turi *et al.*, 1997; Voravuthikunchai and Limsuwan, 2006). It is expected that plant extracts showing target sites other than those used by antibiotics will be active against drug-resistant organisms. In addition to direct inhibition of growth and some physiological functions as well as killing ability, many other different antibacterial mechanisms operated by medicinal plants than the presently used antibiotics have been proposed. Some of them will be discussed as follows:

Inhibition of virulence factors

Several lesions associated with *S. aureus* manifest as result of toxin production and release. It has been reported that some of the MRSA strains were equipped with genes for several enterotoxins (Naimi *et al.*, 2003). The effects of some medicinal plant extracts on Staphylococcal enterotoxin production have been investigated (Braga *et al.*, 2005; Nostro *et al.*, 2002). Recently, it has been shown that methanolic extract of *Punica granatum* (pomegranate) is effective at reducing staphylococcal enterotoxin secretion (Braga *et al.*, 2005). The ability of *Punica granatum* to inhibit such virulence factors, in addition to its ability to inhibit or kill bacteria, may be exceedingly effective at controlling resistant organisms. The inhibitory effect of several

plant extracts such as on the production of Verocytotoxin have been demonstrated by a number of scientists. These plants include *Camellia sinensis* (tea) (Sugita-Konishi *et al.*, 1999), *Punica granatum* (Voravuthikunchai *et al.*, 2005c), *Quercus infectoria* (Voravuthikunchai *et al.*, 2006d), *Cupressus lustianica* Mill. (Mexican cypress), *Jusiaea peruviana* L. (Peruvian primrose), *Limonium californicum* (Boiss.) A. Heller (sea lavender, marsh rosemary), and *Salvia urica* Epling (blue bush sage) (Sakagami *et al.*, 2001).

Antiadhesive properties

Microbial adhesion to host cells is often the first step in many infections (Law, 1994). Pathogenic bacteria attach commonly to target host tissues by specific adhesion-receptor mechanisms. Extract of *Julans regia* L. (walnut) has been demonstrated *in vitro* to inhibit adherence of *S. mutans* to glass and tooth surface (Jagtap and Karkera 2000). Many studies reported the antiadhesive effect of medicinal plants on *H. pylori* (Burger *et al.*, 2000; Lengsfeld *et al.*, 2004a, 2004b; O'Mahony *et al.*, 2005; Shmuely *et al.*, 2004). This bacterium is the major cause of gastric cancer and has been classified as Class I carcinogen by WHO. Successful inhibition of adhesion has been shown *in vitro* with cranberry juice against *H. pylori* (Burger *et al.*, 2000; Shmuely *et al.*, 2004). The adhesion of this bacterium to human stomach sections was inhibited by *Abelmoschus esculentus* (L.) Moench (okra) and *Ribes nigrum* L. (blackcurrant) seeds have been demonstrated (Lengsfeld *et al.*, 2004a, 2004b). More recently, a study of O'Mahony *et al.*, (2005) suggested a potent alternative therapy for *H. pylori* by ingestion of the plants with antiadhesive properties.

Ability to increase and / or decrease cell surface hydrophobicity

Microbial cell surface hydrophobicity (CSH) is often associated with binding to the specific cell and tissue receptors of mucosal surfaces in the infected host (Rozgonyi *et al.*, 1990). It has been demonstrated that *Arctostaphylos uva-ursi* and *Vaccinium vitis-idaea* L. (cowberry) can enhance cell aggregation of *E. coli*, *Acinetobacter baumannii* (Turi *et al.*, 1997), and *H. pylori* (Annuk *et al.*, 1999). Recently, detailed studies on CSH demonstrated that ability of medicinal plants to increase and/or decrease CSH of *E. coli* O157: H7 is not correlated with bacteriostatic or bacteriocidal effect of the medicinal plants tested (Voravuthikunchai and Limsuwan 2006).

Effects of medicinal plants on normal microbiota

While many studies on the antibacterial effects of medicinal plants on pathogenic bacteria have been extensively carried out, there have been very limited researches on their effects on indigenous microbiota. It would be expected that the activity of the herbs would be similar. A case report by

Logan *et al.*, (2002) suggest an antimicrobial activity of *Mentha piperita* L. (peppermint) on small intestinal bacterial overgrowth. Irritable bowel syndrome symptoms were improved after treatment with enteric-coated peppermint oil. Contrastly, it has been demonstrated that herbal decoctions exhibited a positive effect on the growth of indigenous oral microbiota rather than produced a detrimental effect (Woodward 1999). One possible mechanism proposed is probably due more to aiding the normal microbiota as opposed to hindering pathogenic bacteria. Possibly, the phytochemicals in medicinal plants are closed to those present in our regular diet and as such would not inhibit the growth of indigenous organisms due to their continued exposure to such compounds. Disease alleviation through the use of herbs would be a classic matter of bacterial competition as opposed to a drug-pathogen interaction. Many antagonistic mechanisms played by the resident microorganisms against pathogenic bacteria have been well-described (Fujimori *et al.*, 1996; Vieira *et al.*, 1998; Voravuthikunchai and Lee 1987).

Why the antibacterial effects on overpopulated indigenous normal microbiota (Logan *et al.*, 2002) and those under normal equilibrium (Woodward 1999) are so different? It would be very interesting to find out whether the same plant species will produce similar results when a single resident bacterial species is tested. As have been demonstrated by many other researchers that various kinds of plants produce antibacterial activity against *S. mutans* (Hwang *et al.*, 2004; Jagtap and Karkera 2000; Rosalen *et al.*, 2000; Taweechaisupapong *et al.*, 2000). We thought that a lot more studies on the effect of herbs on human microbiota are required before this can be concluded. If the phenomenon reported by Woodward (1998) can be repeatable, this is another big advantage of the use of medicinal plants against infection. In general, antibiotics produce the same effect on normal microbiota as on pathogenic bacteria (Haahr *et al.*, 1997).

Immunomodulation

The immune system does a great job to keep us healthy and prevent infections from overwhelming the body. Unhealthy lifestyle practices, stress, environmental pollution, and many other factors have rendered our immune systems weak. In addition to antibacterial properties, many studies have illustrated immunomodulatory activities of various kinds of medicinal plants (Prince *et al.*, 2004; Rege *et al.*, 1989; Thatte *et al.*, 1992). Treatment with *Tinospora cordifolia* (Willd.) Miers (guduchi) was associated with significantly improved bacterial clearance as well as improved bacteria-fighting capacities of cells of the immune system (Thatte *et al.*, 1992). By virtue of its potent antioxidant activity, *Tinospora cordifolia* is not only powerful scavengers of bacteria but also help suppress the associated infection and inflammation. It effectively suppresses oxidative stress and thereby helps the body fight infections (Prince *et al.*, 2004). Glycyrrhizin, a

principle constituent from *Glycyrrhia glabra* L. (licorice or yashimadhu) has been studied for its immunomodulating effects in many researches and found to have a powerful stimulant action on the functioning of various cells of the immune system (Sohni *et al.,* 1996). *Eugenia malaccensis* L. (Malay apple) was found to inhibit the classical pathway of complement activation suggesting an immunological basis for its *in vivo* activity (Locher *et al.,* 1995). It has been shown that *Camellia sinensis* (L.) Kuntze, containing L-theanine, a precursor of the non-peptide antigen ethylamine, could prime peripheral blood T cells to mediate a memory response on re-exposure to ethylamine, and secreted interferon-γ in response to bacteria. Such priming may enhance innate immunity to bacteria (Kamath *et al.,* 1999). *Phyllanthus emblica* L. (Indian gooseberry, amla, or amalaki) has been proved to have potent immunostimulating activity in addition to its antibacterial property (Haque *et al.,* 2001; Sai Ram and Neetu, 2003). *Balsamodendron mukul* (Indian bedellium or guggulu) is another herb with infection-fighting and immunomodulatory potential (Kimura *et al.,* 2001; Sharma *et al.,* 2005).

Many plants have tropisms to specific organs or human body systems. Phytomedicines usually have multiple effects on our body. Their actions often act beyond the symptomatic treatment of disease. *Hydrastis canadensis* L. (golden seal) not only possesses antimicrobial activity, but also increases blood supply to the spleen promoting optimal activity of the spleen to release mediating compounds (Murray, 1995).

Structural Aspects of Antibacterial Phytochemicals in Medicinal Plants

Antibacterial phytochemicals of herbs are secondary metabolites of plants which are structurally heterogeneous. Relatively structural complexity is very high and includes the occurrence of multiple stereoisomers, for example, most triterpenes contain 10 or more chiral centers (Pauli *et al.,* 2005). The major phytochemicals of medicinal plants include multiple organic components such as catechins, curcumin, phenols, quinines, flavones, tannins, terpenoids, and alkaloids, all of which are known to have antibacterial effects (Cowan, 1999). The larger the variety of compounds that are extracted, the better the chance that biologically active components will also be extracted if a specific class of chemical component is not targeted. In general, phenolics are the predominant active chemical in many plants, with Gram-positive bacteria being more sensitive.

Most phytochemicals contain substituted phenolic ring. The -OH groups in phenolic compounds are thought to be related to their inhibitory action (Gelssman, 1963). There is some evidence that increased numbers of –OH groups in the compounds exhibit more toxicity to microorganisms (Cowan, 1999). Flavonoids are hydrolyxated phenolic compounds linked to additional aromatic rings. Quinones are known to complex with amino acid residues

to inactivate microbial proteins (Stern *et al.*, 1996). *Allium sativum* L. (garlic) contains non-aromatic sulfur compounds, thiosulfinates that acts as antibacterial substance.

Detailed studies on some active compounds from our laboratories (Fig. 1) illustrate that pinocembrin is the most potent antibacterial compound among the three flavonoids isolated from *Boesenbergia pandurata*. Acetoxychavicol acetate from *Alpinia galanga* is very active against *S. aureus* and MRSA (Voravuthikunchai *et al.*, 2005a). Purified compounds from other active plant species, *Punica granatum*, demonstrated various groups of antibacterial compounds which are similar to those earlier reported (Machado *et al.*, 2003). The antibacterial activity of active compounds from *Walsura robusta* from this study group are new and will have to be reported.

Research methodologies

Plant selection

Scientific criteria should be used in the selection of plant materials. The selection of plants should be made from an ethnopharmacological perspective. All the plant species tested should be described and identified. The use of commercial samples should be avoided. Even though both fresh and dried materials can be used, most workers have tended to use dried materials for the following reasons: (i) most plants are used in the dried form or as aqueous extract by traditional healers; (ii) there are fewer problems with the large scale extraction; (iii) the secondary metabolites to be employed as antibacterial agent should be stable; (iv) differences in water content in fresh material may affect solubility or subsequent separation by liquid-liquid extraction (Eloff 1998).

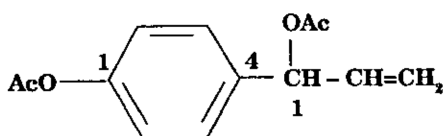
Extraction Method

The first generation of plant medicines were usually simple botanicals used in their crude form. Many effective drugs were employed in their natural state based on empirical evidence of their clinical practice by traditional practitioners, for example, *Aloe vera* (L.) Burm.f. (aloe vera), *Atropa belladona* (deadly nightshade), *Cinchona officinalis* (L.) (cinchona or quinine), and *Papaver somniferum* L. (opium). Various solvents have been used to extract plant metabolites. Importantly that the extractant should not inhibit the bioassay procedures. Scientists use different extractants varying from chloroform, ether, ethanol (50 % to 90 %), methanol, petroleum and water. Many researchers obtain Soxhlet extraction of dried plant material using solvent with increasing polarity, for example, ether, petroleum ether, chloroform, ethyl acetate, ethanol, and water. This procedure works well with compounds that can withstand the temperature of the boiling solvent, but cannot be used for thermolabile compounds. Eloff (1998) suggest acetone as an ideal extractant due to its votality, miscibility with both polar and non-polar solvents, and relatively low toxicity to test

organisms. As aqueous (decoction) and ethanolic (infusion) are routinely employed to treat infectious diseases, they are among the most common in many studies.

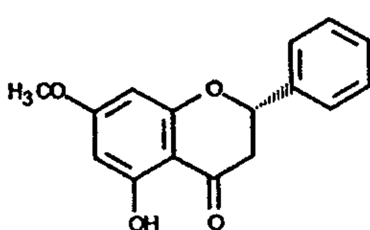
There are a variety of techniques that could be employed to prepare extracts such as steam-distillation, dichlolomethane extraction (Laenger *et al.*, 1996), maceration, sonication, Soxhlet and supercritical fluid extraction with hexane (Janete *et al.*, 1997). These types of preparations are normally unavailable to persons in a domestic setting for purposes of self-medication.

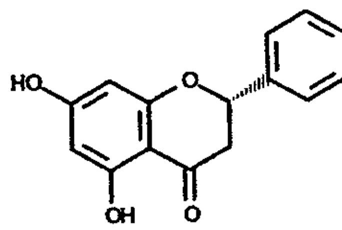
The efficacy of herbal remedy may be varied depending on both the solvents and extraction methods. The ideal approach would be that in which the extract were the same as that used in phytotherapy or folk medicine. Plant extracts obtained by chemical processing would be impractical in many



1'-Acetoxychavicol acetate

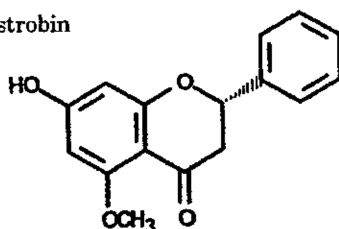
Alpinia galanga





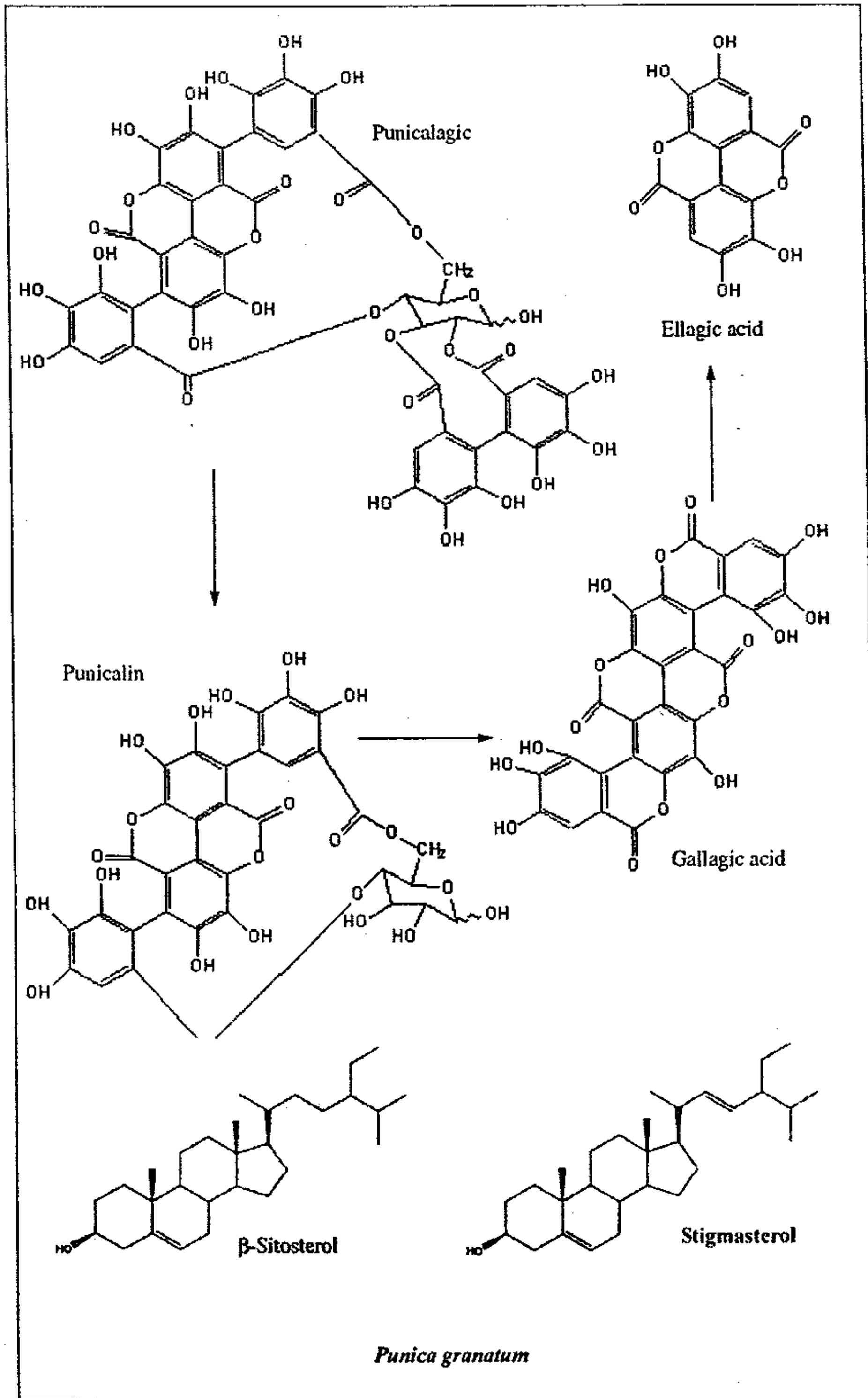
Pinocembrin

Pinostrobin



Alpinetin

Boesenbergia pandurata



Punica granatum

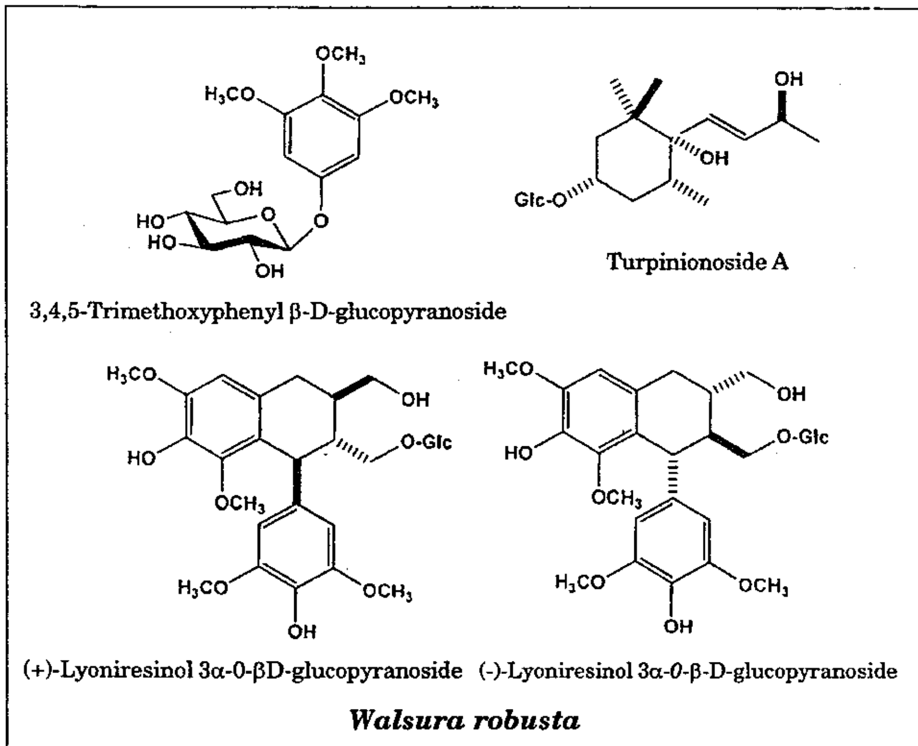


Fig. 1. Representative structures of effective antibacterial phytochemicals (Voravuthikunchai *et al.*, 2005b, and some of unpublished data).

developing countries. Inevitably that the composition and concentrations of active compounds within different types of preparations would be different (Laenger *et al.*, 1996; Lienert *et al.*, 1998).

Isolation and identification of active compounds

When crude extracts of medicinal plants are used, it is thought that the effects are the combined effects of multiple bioactive phytochemicals interacting synergically. However, towards developing modern drugs, a scientist has to identify major bioactive compound. Some of the pure phytopharmaceutical agents were more pharmacologically active that their synthetic counterparts. Well-known examples are quinine, reserpine from *Rauwolfia serpentine* (L.) Benth. ex Kurz, and more recently taxol from *Taxus* species. These compounds differ from the synthetic therapeutic agents only in their origin.

Antibacterial assays

For standard antibacterial assays, most of preliminary works on screening antibacterial activity tend to use agar well diffusion bioassay (NCCLS 2003)

and measuring their inhibition zones. This assay should be used to obtain preliminary data. Our experiences indicate that it does not always correlate well with the MIC or MBC values. Especially, for non-polar extracts, the use of this technique seems to be inadequate. More assays should be further set up in order to confirm the antibacterial activity. Many extracts give very dark background which make it rather difficult to read the result from broth microdilution technique. In these cases, agar dilution method may be substituted.

The use of tetrazolium salts in bioautographic procedures has been described (Begue and Kline 1972). Chromatography plates are dried overnight and sprayed with a concentrated suspension of actively growing cells, incubated at 38 °C in a chamber at 100% relative humidity. Plates are then sprayed with a 2 mg/ml solution of *p*-iodonitrotetrazolium violet. Inhibition of growth was indicated by clear zones on the chromatogram after incubating for 1 hr. Toxicity of solvents are determined by serial dilution with medium to the level where growth is not inhibited.

Problems encountered with researches on medicinal plants

There are several practical aspects to be considered when trying to explain difficulties that are associated with medicinal plant research. Results of different reports on the use of same plant species against same pathogen may be varied. Differences in results obtained from different laboratories may be due to the stage of plant collection, extraction methods used in each study protocol, or different sensitivity patterns of test bacterial strains. Many studies have illustrated the differences in composition and concentrations of phytochemicals among plants of the same species that are collected at different times of the year or in different geographic, soil, and climatic conditions (Laenger *et al.*, 1996; Marotti *et al.*, 1994; McGimpsey *et al.*, 1994; Piccaglia and Marotti 1993; Saez, 1995; Svoboda and Deans 1992). Critically, some plants may accumulate minerals and heavy metals from the soil. Contamination by arsenic, cadmium, lead, and mercury has been reported (De Smet *et al.*, 1996; Ko, 1998). In addition, the efficacy of an herbal remedy may be different depending on the preparation methods employed (Laenger *et al.*, 1996; Lienert *et al.*, 1998).

Among substantial plant species studied, we found that *Quercus infectoria* exhibited the greatest activity against *E. coli* O157: H7. Work from other laboratory support this finding (Nimri *et al.*, 1999), however, other reported different results (Alkofahi and Atta, 1999). *Camellia sinensis* showed no activity against *E. coli* O157: H7 (Voravuthikunchai *et al.*, 2004b) while epigallocatechin gallate and gallocatechin gallate in green tea catechins has been earlier reported by other workers to inhibit extracellular release of Verocytotoxin from *E. coli* O157: H7 (Sugita-Konishi *et al.*, 1999).

Panax ginseng C.A. Mey. (gingseng) has been shown to inhibit *H. pylori* adhesion to human gastric adenocarcinoma cells (Lee *et al.*, 2004). However, it failed to inhibit *H. pylori* adhesion to human stomach sections (O'Mahony *et al.*, 2005). The discrepancy is thought to be due to the differences between cell-lines and whole tissue.

Many researchers reported antibacterial activity of medicinal plants though concentrations of plant extracts used in their studies were relatively high (Bonjar *et al.*, 2003; O'Mahoni *et al.*, 2005). Information on the minimum concentration needed for pharmacological activity has not been standardized. Experiments with quantities higher than 1000 µg/ml for extracts or 100 mg/ml isolated compounds should be avoided, whereas the presence of activity is very challenging in the case of concentration below 100 µg/ml for extracts and 10 µg/ml for isolated compounds (Rios and Recio 2005).

Conclusions

Some general considerations must be established for the study of the antibacterial activity of medicinal plants such as selection of plant materials, extraction methods, and test bacterial strains.

In general, a much greater numbers of medicinal plants were found to be more active against Gram-positive bacteria than Gram-negative bacteria (McCutcheon *et al.*, 1992). The basis for their differences in susceptibility might be due to the differences in their cell wall composition (Grosvenor *et al.*, 1995). Work from this laboratory further support this. *Streptococcus pyogenes* was demonstrated to be sensitive to almost all plant species tested (96.8%). Methicillin-resistant *Staphylococcus aureus*, an extremely antibiotic-resistant pathogen responded to a wide range of the medicinal plants tested (77.4%). Contrastly, we found that only 14.8% of plants studied have activity against *E. coli*, a representative of Gram-negative bacteria.

The primary benefits of using plant derived medicines are that they are relatively safer than synthetic alternatives, offering profound therapeutic advantages and more affordable treatments. Decades of use of a particular plant may point to its non-toxicity, however, it is important to note that herbs are not necessarily harmless. Many plants are toxic and may be extremely dangerous. In general, when a herb is used as food, it may be harmless, but it may produce toxicity when used as medicine as a relatively higher doses administered or interacted with other pharmaceutical medications (Argento *et al.*, 2000; Earns, 2003). There have been a number of reports of human poisoning due to the ingestion of decoctions made from commonly used medicinal herbs (Hamouda *et al.*, 2000; Onen *et al.*, 2002). The chemical composition and safety of the extracts should always be determined before formulation into dosage forms. On the other hand, due to inadequate usage, medicinal plants may not yield desired

effects. Safety on herbs, contra-indications, side effects, other herbal classifications of commercial uses, and general information, are available in a published handbook (McGuffin *et al.*, 1997). A reference database on Natural medicines (Jeff, 1999) that documented reference resource, based on scientific data is available. In addition, WHO has developed a strategy to intensify research into the safety and effectiveness of traditional medicines between 2000 and 2005.

It is obvious that continued and further exploration of plant antibacterial drugs needs to occur. Plants based antimicrobials have enormous therapeutic potential. They are effective in the treatment of many bacterial infections while simultaneously mitigating many of the undesirable side effects that are commonly associated with synthetic antimicrobials. It is to be noted that follow-up researches are mostly lacking, since most efforts are not part of focused drug development programmes and simply lack the opportunity for synthetic follow-up of promising leads. The isolated active principles rarely exhibit potent activity themselves, but further follow-up improvement is required in order to be attractive.

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PATENTS

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15 ถนนกาญจนวณิชย์
อ.หาดใหญ่ จ.สงขลา 90110

19 มิถุนายน 2550

สำนักสิทธิบัตร รับที่ 01656 วันที่ 25 มิ.ย. 2550 เวลา.........น.

กรมทรัพย์สินทางปัญญา รับที่ 9999 วันที่ 22 มิ.ย. 2550 เวลา 14.00

เรื่อง ขอจดสิทธิบัตรผลงานการประดิษฐ์

เรียน อธิบดีกรมทรัพย์สินทางปัญญา

สิ่งที่ส่งมาด้วย
1. คำขอรับสิทธิบัตร จำนวน 1 ชุด
2. คำบรรยายการประดิษฐ์ จำนวน 1 ชุด
3. หนังสือสัญญาโอนสิทธิ จำนวน 1 แผ่น
4. สำเนาคำสั่งมอบหมายให้รองอธิการบดีปฏิบัติราชการแทนอธิการบดี จำนวน 1 ชุด
5. สำเนาบัตรประจำตัวรองอธิการบดีฝ่ายวิจัยและบัณฑิตศึกษา จำนวน 1 แผ่น

ด้วยมหาวิทยาลัยสงขลานครินทร์ โดย รองศาสตราจารย์ ดร.ศุภยางค์ วรวุฒิคุณชัย และคณะ ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยสงขลานครินทร์ ได้ประดิษฐ์ กระบวนการสกัดสารออกฤทธิ์ทางชีวภาพจากเบญกานี (*Quercus infectoria*)

ในการนี้ มหาวิทยาลัยสงขลานครินทร์จึงขอยื่นจดสิทธิบัตรผลงานการประดิษฐ์ดังกล่าวต่อกรมทรัพย์สินทางปัญญา ทั้งนี้ โดยขอยกเว้นการเรียกเก็บค่าธรรมเนียมสำหรับการขอรับสิทธิบัตรหรืออนุสิทธิบัตร ตามความในข้อ 3 แห่งกฎกระทรวงว่าด้วยอัตราค่าธรรมเนียมและการยกเว้นค่าธรรมเนียมสำหรับสิทธิบัตรหรืออนุสิทธิบัตร พ.ศ. 2547

จึงเรียนมาเพื่อโปรดพิจารณาดำเนินการต่อไปด้วย จักขอบคุณยิ่ง

ขอแสดงความนับถือ

(รองศาสตราจารย์ ดร.สุรพล อารีย์กุล)
รองอธิการบดีฝ่ายวิจัยและบัณฑิตศึกษา ปฏิบัติราชการแทน
อธิการบดีมหาวิทยาลัยสงขลานครินทร์

เรียน อธิบดี
เพื่อโปรดพิจารณา

ส่งสำนัก สบ.

สำนักวิจัยและพัฒนา
โทร. 0-7428-6946
โทรสาร 0-7421-2839
E-mail : panpimon.h@psu.ac.th

คำขอรับสิทธิบัตร/อนุสิทธิบัตร

☑ การประดิษฐ์

☐ การออกแบบผลิตภัณฑ์

☐ อนุสิทธิบัตร

ข้าพเจ้าผู้ลงลายมือชื่อในคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ ขอรับสิทธิบัตร/อนุสิทธิบัตร ตามพระราชบัญญัติสิทธิบัตร พ.ศ.2522 แก้ไขเพิ่มเติมโดยพระราชบัญญัติสิทธิบัตร (ฉบับที่ 2) พ.ศ.2535 และพระราชบัญญัติสิทธิบัตร (ฉบับที่ 3) พ.ศ.2542

สำหรับเจ้าหน้าที่	
วันรับคำขอ 27 มิ.ย. 2550	เลขที่คำขอ 0701003210
วันยื่นคำขอ	
สัญลักษณ์จำแนกการประดิษฐ์ระหว่างประเทศ	
ใช้กับแบบผลิตภัณฑ์ ประเภทผลิตภัณฑ์	
วันประกาศโฆษณา	เลขที่ประกาศโฆษณา
วันออกสิทธิบัตร/อนุสิทธิบัตร	เลขที่สิทธิบัตร/อนุสิทธิบัตร
ลายมือชื่อเจ้าหน้าที่	

1. ชื่อที่แสดงถึงการประดิษฐ์/การออกแบบผลิตภัณฑ์
กระบวนการสกัดสารออกฤทธิ์ทางชีวภาพจากเบญกานี (*Quercus infectoria*)

2. คำขอรับสิทธิบัตรการออกแบบผลิตภัณฑ์นี้เป็นคำขอสำหรับแบบผลิตภัณฑ์อย่างเดียวกันและเป็นคำขอลำดับที่ 1
ในจำนวน 1 คำขอ ที่ยื่นในคราวเดียวกัน

3. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร และที่อยู่ (เลขที่ ถนน ประเทศ)
มหาวิทยาลัยสงขลานครินทร์
15 ถนนกาญจนวณิชย์
อำเภอหาดใหญ่ จังหวัดสงขลา 90110

3.1 สัญชาติ ไทย
3.2 โทรศัพท์ 0-7428-7409
3.3 โทรสาร 0-7428-7412
3.4 อีเมล์ natchayamon-t@psu.ac.th

4. สิทธิในการขอรับสิทธิบัตร/อนุสิทธิบัตร
☐ ผู้ประดิษฐ์/ผู้ออกแบบ ☑ ผู้รับโอน ☐ ผู้ขอรับสิทธิโดยเหตุอื่น

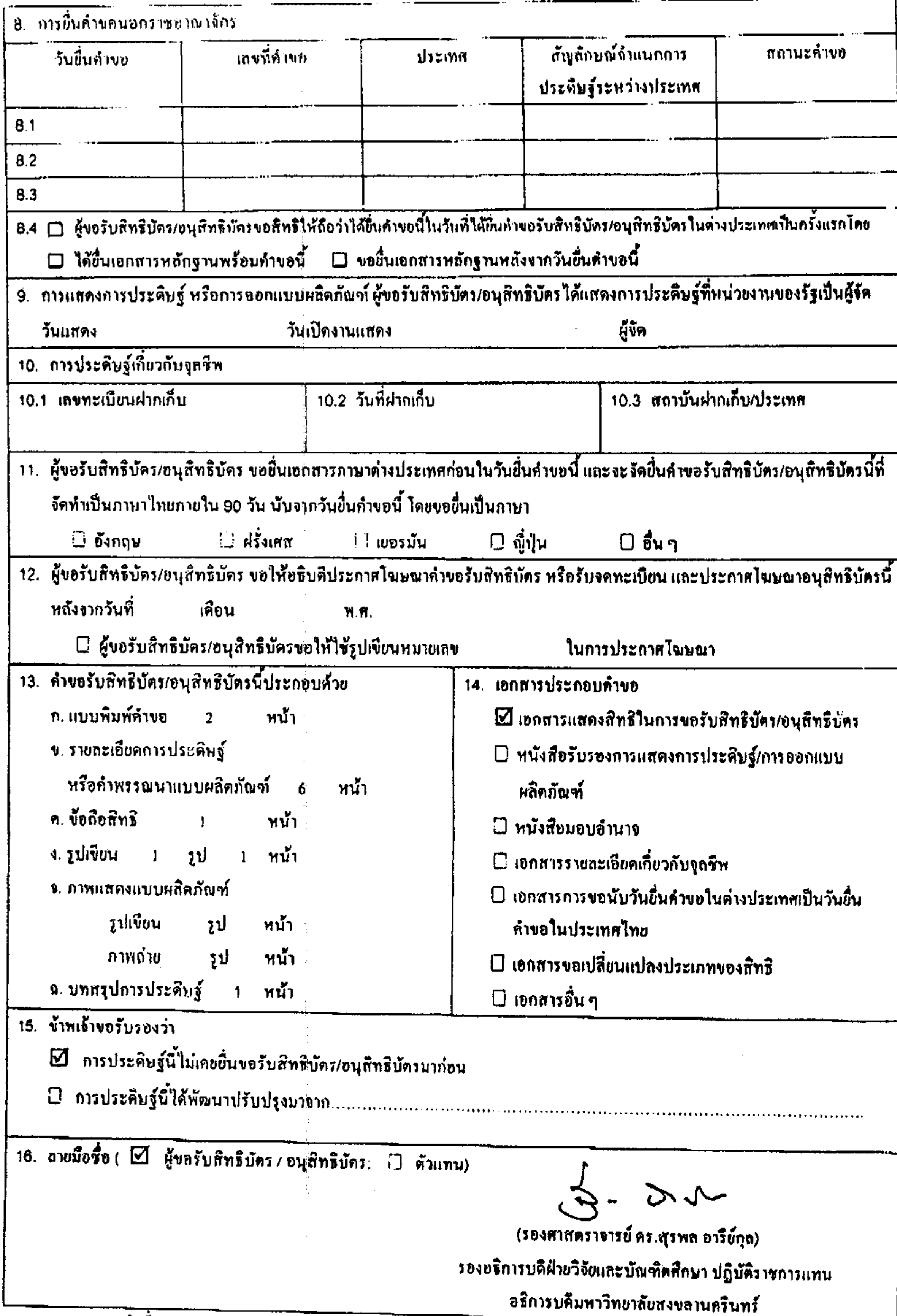
5. ตัวแทน (ถ้ามี) / ที่อยู่ (เลขที่ ถนน จังหวัด ประเทศ รหัสไปรษณีย์)

5.1 ตัวแทนเลขที่
5.2 โทรศัพท์
5.3 โทรสาร
5.4 อีเมล์

6. ผู้ประดิษฐ์/ผู้ออกแบบผลิตภัณฑ์ และที่อยู่ (เลขที่ ถนน ประเทศ)
รองศาสตราจารย์ ดร. ศุภยางค์ วรวุฒิคุณชัย
ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยสงขลานครินทร์ อ. หาดใหญ่ จ. สงขลา 90110 ประเทศไทย
นางสาวศศิธร ชูศรี
ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยสงขลานครินทร์ อ. หาดใหญ่ จ. สงขลา 90110 ประเทศไทย

7. คำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้แยกจากหรือเกี่ยวข้องกับคำขอเดิม
ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรขอให้ถือว่าได้ยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ในวันเดียวกับคำขอรับสิทธิบัตรเลขที่
วันยื่น เพราะคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้แยกจากหรือเกี่ยวข้องกับคำขอเดิมเพราะ
☐ คำขอเดิมมีการประดิษฐ์หลายอย่าง ☐ ถูกคัดค้านเนื่องจากผู้ขอไม่มีสิทธิ ☐ ขอเปลี่ยนแปลงประเภทของสิทธิ

หมายเหตุ ในกรณีที่ไม่อาจระบุรายละเอียดได้ครบถ้วน ให้จัดทำเป็นเอกสารแนบท้ายแบบพิมพ์นี้ โดยระบุหมายเลขกำกับข้อและหัวข้อที่แสดงรายละเอียดเพิ่มเติมดังกล่าวด้วย



8. การยื่นคำขอนอกราชอาณาจักร

วันยื่นคำขอ	เลขที่คำขอ	ประเทศ	สัญลักษณ์จำแนกการประดิษฐ์ระหว่างประเทศ	สถานะคำขอ
8.1				
8.2				
8.3				

8.4 ☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรขอสิทธิให้ถือว่าได้ยื่นคำขอนี้ในวันที่ได้ยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรในต่างประเทศเป็นครั้งแรกโดย
☐ ได้ยื่นเอกสารหลักฐานพร้อมคำขอนี้ ☐ ขอยื่นเอกสารหลักฐานหลังจากวันยื่นคำขอนี้

9. การแสดงการประดิษฐ์ หรือการออกแบบผลิตภัณฑ์ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรได้แสดงการประดิษฐ์ที่หน่วยงานของรัฐเป็นผู้จัด
วันแสดง วันเปิดงานแสดง ผู้จัด

10. การประดิษฐ์เกี่ยวกับจุลชีพ

10.1 เลขทะเบียนฝากเก็บ	10.2 วันที่ฝากเก็บ	10.3 สถาบันฝากเก็บ/ประเทศ

11. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอยื่นเอกสารภาษาต่างประเทศก่อนในวันยื่นคำขอนี้ และจะจัดยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ที่จัดทำเป็นภาษาไทยภายใน 90 วัน นับจากวันยื่นคำขอนี้ โดยขอยื่นเป็นภาษา
☐ อังกฤษ ☐ ฝรั่งเศส ☐ เยอรมัน ☐ ญี่ปุ่น ☐ อื่น ๆ

12. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอให้อธิบดีประกาศโฆษณาคำขอรับสิทธิบัตร หรือรับจดทะเบียน และประกาศโฆษณาอนุสิทธิบัตรนี้หลังจากวันที่ เดือน พ.ศ.
☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรขอให้ใช้รูปเขียนหมายเลข ในการประกาศโฆษณา

13. คำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ประกอบด้วย
ก. แบบพิมพ์คำขอ 2 หน้า
ข. รายละเอียดการประดิษฐ์
หรือคำพรรณนาแบบผลิตภัณฑ์ 6 หน้า
ค. ข้อถือสิทธิ 1 หน้า
ง. รูปเขียน 1 รูป 1 หน้า
จ. ภาพแสดงแบบผลิตภัณฑ์
รูปเขียน รูป หน้า
ภาพถ่าย รูป หน้า
ฉ. บทสรุปการประดิษฐ์ 1 หน้า

14. เอกสารประกอบคำขอ
☑ เอกสารแสดงสิทธิในการขอรับสิทธิบัตร/อนุสิทธิบัตร
☐ หนังสือรับรองการแสดงการประดิษฐ์/การออกแบบผลิตภัณฑ์
☐ หนังสือมอบอำนาจ
☐ เอกสารรายละเอียดเกี่ยวกับจุลชีพ
☐ เอกสารการขอนับวันยื่นคำขอในต่างประเทศเป็นวันยื่นคำขอในประเทศไทย
☐ เอกสารขอเปลี่ยนแปลงประเภทของสิทธิ
☐ เอกสารอื่น ๆ

15. ข้าพเจ้าขอรับรองว่า
☑ การประดิษฐ์นี้ไม่เคยยื่นขอรับสิทธิบัตร/อนุสิทธิบัตรมาก่อน
☐ การประดิษฐ์นี้ได้พัฒนาปรับปรุงมาจาก...

16. ลายมือชื่อ (☑ ผู้ขอรับสิทธิบัตร / อนุสิทธิบัตร ☐ ตัวแทน)

(รองศาสตราจารย์ ดร.สุรพล อารีย์กุล)
รองอธิการบดีฝ่ายวิจัยและบัณฑิตศึกษา ปฏิบัติราชการแทน
อธิการบดีมหาวิทยาลัยสงขลานครินทร์

หมายเหตุ บุคคลใดยื่นคำขอรับสิทธิบัตรการประดิษฐ์หรือการออกแบบผลิตภัณฑ์ หรืออนุสิทธิบัตร โดยการแสดงข้อความอันเป็นเท็จแก่พนักงานเจ้าหน้าที่ เพื่อให้ได้ไปซึ่งสิทธิบัตรหรืออนุสิทธิบัตร ต้องระวางโทษจำคุกไม่เกินหกเดือน หรือปรับไม่เกินห้าพันบาท หรือทั้งจำทั้งปรับ

หนังสือสัญญาโอนสิทธิขอรับสิทธิบัตร/อนุสิทธิบัตร

เขียนที่ มหาวิทยาลัยสงขลานครินทร์
15 ถนนกาญจนวณิชย์
อ.หาดใหญ่ จ.สงขลา 90110

วันที่ 22 เดือนพฤษภาคม พ.ศ 2550

สัญญาระหว่างผู้โอน คือ รองศาสตราจารย์ ดร. ศุภยางค์ วรวุฒิคุณชัย และ นางสาวศศิธร ชูศรี ที่อยู่ ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยสงขลานคริทร์ และผู้รับโอน คือ มหาวิทยาลัยสงขลานครินทร์ โดยรองศาสตราจารย์ ดร.สุรพล อารีย์กุล ตำแหน่ง รองอธิการบดีฝ่ายวิจัยและบัณฑิตศึกษา อยู่บ้านเลขที่ 15 ถนนกาญจนวณิชย์ อ.หาดใหญ่ จ.สงขลา 90110

โดยสัญญานี้ ผู้โอนซึ่งเป็นผู้ประดิษฐ์ กระบวนการสกัดสารออกฤทธิ์ทางชีวภาพจากเบญกานี (*Quercus infectoria*) โอนสิทธิในการประดิษฐ์ดังกล่าว ซึ่งรวมถึงสิทธิขอรับสิทธิบัตร/อนุสิทธิบัตรและสิทธิอื่นๆ ที่เกี่ยวข้องให้แก่ ผู้รับโอน โดยผู้รับโอนได้จ่ายค่าตอบแทนที่เหมาะสมให้แก่ผู้โอน

เพื่อเป็นพยานหลักฐานแห่งการนี้ ผู้โอนและผู้รับโอนได้ลงลายมือชื่อไว้ข้างล่างนี้

(ลงชื่อ)..................................ผู้โอน 1
(รองศาสตราจารย์ ดร.ศุภยางค์ วรวุฒิคุณชัย)

(ลงชื่อ)..................................ผู้โอน 2
(นางสาวศศิธร ชูศรี)

(ลงชื่อ)..................................ผู้รับโอน
(รองศาสตราจารย์ ดร.สุรพล อารีย์กุล)

(ลงชื่อ)..................................พยาน
(ดร.อัครวิทย์ กาญจนโอภาษ)

(ลงชื่อ)..................................พยาน
(นางสาวณัฐชยามน ถาวระ)

รายละเอียดการประดิษฐ์

ชื่อที่แสดงถึงการประดิษฐ์

กระบวนการสกัดสารออกฤทธิ์ทางชีวภาพจากเบญกานี (*Quercus infectoria*)

สาขาวิทยาการที่เกี่ยวข้องกับการประดิษฐ์

5 สาขาวิทยาศาสตร์สุขภาพในส่วนที่เกี่ยวข้องกับกระบวนการสกัดสารออกฤทธิ์ทางชีวภาพจากเบญกานี

ภูมิหลังของศิลปะหรือวิทยาการที่เกี่ยวข้อง

ได้มีการนำยาปฏิชีวนะมาใช้ในการรักษาโรคติดเชื้อเป็นระยะเวลานาน แต่ในช่วงทศวรรษที่ผ่านมามีการเพิ่มจำนวนของเชื้อดื้อยาอย่างต่อเนื่อง จนได้กลายเป็น 10 ปัญหาสำคัญของการเกิดโรคติดเชื้อทั้งในโรงพยาบาลและในชุมชน นอกจากนี้การใช้ยาปฏิชีวนะในการรักษาโรคส่งผลต่อข้างเคียงต่าง ๆ ต่อร่างกายผู้ป่วย รวมทั้งต้องสิ้นเปลืองค่าใช้จ่ายในการนำเข้ายาจากต่างประเทศ การหาแนวทางใหม่ในการรักษาโรคติดเชื้อจึงมีความจำเป็นอย่างยิ่ง การใช้สมุนไพรในการรักษาและป้องกันโรคเป็นอีกหนึ่งทางเลือกที่ได้รับความนิยมเป็นอย่างมาก สำหรับประเทศไทยมีการสนับสนุนให้มีการ 15 ใช้ความรู้ทางด้านภูมิปัญญาไทยและสมุนไพรในการรักษาโรคซึ่งสอดคล้องกับแผนพัฒนาสุขภาพแห่งชาติของประเทศไทย (The Ninth Five-Year National Health Development Plan 2002-2006, 2001)

ปูด (gall, nutgall, gallnut) ของเบญกานี (*Quercus infectoria*) ใช้ตามตำราแพทย์แผนไทยในลักษณะของสารฆ่าแบคทีเรีย โดยใช้ฝนกับน้ำเพื่อฆ่าเชื้อบริเวณที่เป็น 20 บาดแผล หรือใช้ต้มกับน้ำสำหรับกินแก้อาการท้องร่วง ข้อเสียของการใช้ดังกล่าวคือ ไม่มีการควบคุมวิธีและสภาวะที่เหมาะสมในการสกัด ทำให้ได้สารสกัดที่มีคุณภาพไม่แน่นอน ในปัจจุบันมีการประยุกต์ใช้สารสกัดจากเบญกานีในหลายรูปแบบ ตัวอย่างเช่น คำขอสิทธิบัตรเกาหลี KR 2003073441 มีการทดสอบความสามารถในการยับยั้งแบคทีเรียแกรมบวก ได้แก่ โปรพิโอนิแบคทีเรียม แอคเน่ (*Propionibacterium* 25 *acne*), สแตฟฟีโลคอคคัส ออเรียส (*Staphylococcus aureus*), สแตฟฟีโลคอคคัส อีพิเดอร์มิดิส (*Staphylococcus epidermidis*) ของสารสกัดจากเบญกานี คำขอสิทธิบัตร

เกาหลี KR 2006036168 มีการผสมสารสกัดด้วยเอทานอลจากเบญกานีกับสารสกัดอื่น ๆ เพื่อใช้เป็นน้ำยาทำความสะอาดช่องคลอด

มีรายงานฤทธิ์ทางชีวภาพอื่น ๆ ของสารสกัดจากเบญกานี อาทิเช่น ฤทธิ์ต้านอนุมูลอิสระ (Choi และ Hwang, Fitoterapia. 2005, 76, 194) ต้านการอักเสบ (Kaur และ
5 คณะ, J Ethnopharmacol. 2004, 90, 285) ต้านพิษงู (Pithayanukul และคณะ, J Ethnopharmacol. 2005, 97, 527) และยับยั้งปรสิต (Sawangjaroen และคณะ, J Ethnopharmacol. 2005, 91, 357) คณะผู้ประดิษฐ์ได้ทำการทดสอบฤทธิ์ของสารสกัดจากเบญกานีในการยับยั้งเชื้อแบคทีเรีย พบว่าสามารถยับยั้งการเจริญและฆ่าแบคทีเรียที่ทดสอบได้ดีทั้งแบคทีเรียแกรมบวกและแกรมลบ

10 จากการทดลองพบว่าสารสกัดจากปูดของเบญกานีให้ผลิตผลในปริมาณที่สูงตั้งแต่ 5% ถึง 60% ทั้งนี้ขึ้นอยู่กับวิธีในการสกัด ดังนั้นคณะผู้ประดิษฐ์จึงได้คิดค้นพัฒนาวิธีการที่ดีที่สุดในการสกัดสารออกฤทธิ์ทางชีวภาพจากส่วนปูดแก่ของเบญกานีเพื่อเพิ่มประสิทธิภาพและความบริสุทธิ์ของสารสกัดจากเบญกานีเพื่อให้มีฤทธิ์ทางชีวภาพต่าง ๆ ได้ดียิ่งขึ้น และสามารถนำไปพัฒนาโดยการใช้สารสกัดจากเบญกานีเป็นส่วนผสมที่มี
15 ประสิทธิภาพที่ดีและมีราคาถูกในผลิตภัณฑ์ต่าง ๆ ทางอุตสาหกรรม

ลักษณะและความมุ่งหมายของการประดิษฐ์

การประดิษฐ์นี้เกี่ยวข้องกับกระบวนการสกัดสารออกฤทธิ์ทางชีวภาพจากเบญกานี

ความมุ่งหมายของการประดิษฐ์นี้คือ เพื่อพัฒนาวิธีการสกัดสารออกฤทธิ์ทาง
20 ชีวภาพจากเบญกานีให้มีประสิทธิภาพดียิ่งขึ้น และสามารถนำสารสกัดจากเบญกานีมาใช้เป็นส่วนผสมและ/หรือแทนสารสังเคราะห์

การเปิดเผยการประดิษฐ์โดยสมบูรณ์

1. กระบวนการเตรียมสารสกัดจากเบญกานีมีขั้นตอนดังนี้

1.1 คัดเลือกปูดแก่ของเบญกานีที่มีเส้นผ่านศูนย์กลางตั้งแต่ 3 ซม. ล้างด้วยน้ำ
25 กลั่น 2 ครั้ง อบให้แห้งโดยใช้อุณหภูมิ 60°ซ ถึง 75 °ซ จากนั้นนำไปบดหยาบ

1.2 สกัดผงของเบญกานีด้วยเอทานอล 50% ถึง 95% เป็นเวลา 7 วัน อย่างน้อย 3 ครั้ง นำสารละลายที่ได้มาระเหยให้แห้งโดยใช้โรตารีอีแวบโปเรเตอร์ (rotary evaporator) ที่อุณหภูมิ 40 °ซ ถึง 60°ซ ในการสกัดสารออกฤทธิ์ทางชีวภาพจากปูดของเบญกานีเป็นสารสกัดหยาบ สามารถใช้น้ำหรือตัวทำละลายอินทรีย์อื่น เช่น อะซิโตน และ เอทิลอะซิเตทได้ ซึ่งสารที่ได้มีความสามารถในการยับยั้งแบคทีเรียใกล้เคียงกัน แต่สารสกัดจากกระบวนการดังกล่าวได้ผลิตผลในปริมาณต่ำ (ตารางที่ 1)

2 กระบวนการแยกสารสกัดจากจากเบญกานีเป็นสารสกัดกึ่งบริสุทธิ์มีขั้นตอนดังนี้

2.1 ละลายสารสกัดหยาบในข้อ 1.2 ด้วย 95% เอทานอล ในอัตราส่วน 1: 10 โดยน้ำหนักของสารสกัดหยาบต่อเอทานอล จากนั้นผสมกับซิลิกาเจล 60 GF_{254} คนจนเข้ากันพักไว้จนกระทั่งแห้ง

2.2 บรรจุสารสกัดหยาบในข้อ 2.1 ลงในควิกคอลัมน์โครมาโตกราฟี (quick column chromatography) ที่ใช้ซิลิกาเจล 60 GF_{254} เป็นเฟสอยู่กับที่ โดยมีความหนาไม่เกิน 5 ซม.

2.3 ใช้คลอโรฟอร์ม 100%, เอทิลอะซิเตท 100%, เอทานอล 95%, เอทานอล 20% ถึง 40% เป็นตัวชะสารสกัดกึ่งบริสุทธิ์ออกจากคอลัมน์ตามลำดับ

2.4 ใช้คลอโรฟอร์ม เมทานอล และน้ำในอัตราส่วน 6 ต่อ3.8 ต่อ 0.3 เป็นเฟสเคลื่อนที่ ทำการเก็บสารสกัดกึ่งบริสุทธิ์โดยใช้ทินเลเยอร์โครมาโตกราฟี (thin layer chromatography)

ตารางที่ 1 แสดงปริมาณผลิตผลที่ได้จากการสกัดปูดของเบญกานีด้วยตัวทำละลายชนิดต่าง ๆ

ชนิดของตัวทำละลาย	ปริมาณผลิตผล (%)
เอทานอล 50%	51.70
เอทานอล 95%	57.15
อะซิโตน	18.19
เอทิลอะซิเตท	8
น้ำ	24.1

<u>ตัวอย่างที่ 1</u> การทดสอบความสามารถในการยับยั้งและฆ่าเชื้อแบคทีเรียแกรมบวกคือ *Staphylococcus aureus* ที่ดื้อต่อยาเมทธิซิลิน (methicillin-resistant *Staphylococcus aureus*, MRSA) และแบคทีเรียแกรมลบคือ เอสเชอริเชีย โคไล (*Escherichia coli* O157: H7) ของสารสกัดชนิดต่าง ๆ จากปูดของเบญกานี โดยใช้วิธีบรอทไมโครไดลูชั่น (broth
5 microdilution) เตรียมเชื้อแบคทีเรียที่ต้องการทดสอบ โดยเลี้ยงเชื้อบนอาหารนิวเทรียนท์เอการ์ (nutrient agar, NA) ที่ 35 °ซ เป็นเวลา 18 ถึง 24 ชั่วโมง เขี่ยเชื้อลงในอาหารมูลเลอร์ฮิลตันบรอท (Mueller Hinton broth, MHB) และบ่มต่อที่ 35 °ซ เป็นเวลา 3 ถึง 8 ชั่วโมงจากนั้นปรับให้ได้ความขุ่นเท่ากับ0.5 แมคฟาร์แลนด์ (McFarland standard) ในน้ำเกลือปราศจากเชื้อ (มีเชื้อประมาณ 1.5 x 10^8 โคโลนีต่อมล.) ละลายสารสกัดที่
10 ต้องการทดสอบด้วยไดเมทิลซัลฟอร์กไซด์แล้วนำไปผสมกับ MHB ในอัตราส่วน 1 ต่อ 10 โดยปริมาตร และทำการเจือจางตามลำดับส่วน ถ่ายเชื้อลงในแต่ละหลุม ($5x10^5$โคโลนีต่อหลุม) บ่มที่ 35 °ซ เป็นเวลา 16 ถึง 18 ชั่วโมง อ่านผลจากการเจริญของเชื้อ หลุมที่มีความเข้มข้นต่ำที่สุดที่เชื้อไม่เจริญคือหลุมที่เป็นสามารถอ่านค่าความเข้มข้นต่ำสุดที่สามารถยับยั้งเชื้อได้ (Minimal inhibitory concentration, MIC) ใช้ห่วงถ่ายเชื้อ
15 (loop) จุ่มเชื้อจากหลุมที่ไม่มีการเจริญทุกหลุมมาลากบนอาหารมูลเลอร์ฮิลตันเอการ์ (Mueller Hinton agar, MHA) แล้วบ่มที่ 35 °ซ อ่านค่าความเข้มข้นต่ำสุดที่เชื้อไม่เจริญเป็นค่าความเข้มข้นต่ำสุดที่ฆ่าเชื้อได้ (Minimal bactericidal concentration, MBC)

ตารางที่ 2 ค่าความเข้มข้นต่ำสุดของสารสกัดด้วยตัวทำละลายชนิดต่าง ๆ จากปูดขอเบญกานีที่สามารถยับยั้ง (MIC) และฆ่า (MBC) methicillin-resistant *Staphylococcus*
20 *aureus*

สารสกัดชนิดต่าง ๆ จากปูดของเบญกานี	MIC/MBC (มก./มล.)		
	MRSA (n=17)	*S. aureus* ATCC 25923	*E. coli* O157: H7 RIMD 05091078
อะซีโตน	0.13/0.13-1.00	0.13/0.50	0.71/1.42
เอทิล อะซิเตท	0.13/0.13-1.00	0.13/0.50	0.71/11.36
เอทานอล 95%	0.13/0.13-0.50	0.13/0.50	0.35/0.71

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ตัวอย่างที่ 2 การทดสอบความไวในการยับยั้งและฆ่า MRSA ของสารสกัดชนิดต่าง ๆ จากปูดของเบญกานี เตรียมแบคทีเรียทดสอบโดยเลี้ยงเชื้อบนอาหาร NA ที่ 35 °ซ เป็นเวลา 18 ถึง 24 ชั่วโมง ปรับความขุ่นของเชื้อให้เท่ากับ McFarland standard หมายเลข 0.5 ในน้ำเกลือปราศจากเชื้อ เจือจางสารสกัดที่ทดสอบด้วยไดเมทิลซัลฟอร์กไซด์แล้ว
5 นำไปผสมกับ MHB ในอัตราส่วน 1 ต่อ 10 บ่มที่ 35 °ซ นับจำนวนแบคทีเรียที่รอดชีวิตทุก ๆ 2 ชั่วโมงจนครบ 20 ชั่วโมง บนอาหาร tryptic soy agar (ดังแสดงผลในรูปที่ 1)

ตัวอย่างที่ 3 การทดสอบความสามารถในการยับยั้งและฆ่า MRSA ของสารสกัดกึ่งบริสุทธิ์ชนิดต่าง ๆ จากปูดของเบญกานี โดยใช้วิธี broth microdilution เตรียมเชื้อแบคทีเรียที่ต้องการทดสอบ โดยเลี้ยงเชื้อบนอาหาร NA ที่ 35 °ซ เป็นเวลา 18 ถึง 24
10 ชั่วโมง เขี่ยเชื้อลงใน MHB และบ่มต่อที่ 35 °ซ เป็นเวลา 3 ถึง 8 ชั่วโมงจากนั้นปรับให้ได้ความขุ่นเท่ากับ McFarland standard หมายเลข 0.5 ในน้ำเกลือปราศจากเชื้อ นำสารสกัดกึ่งบริสุทธิ์ที่ต้องการทดสอบมาเจือจางด้วยไดเมทิลซัลฟอร์กไซด์แล้วนำไปผสมกับ MHB ในอัตราส่วน 1 ต่อ 10 ทำการถ่ายเชื้อลงในแต่ละหลุม ($5x10^5$โคโลนีต่อหลุม) บ่มที่ 35 °ซ เป็นเวลา 16 ถึง 18 ชั่วโมง อ่านผลจากการเจริญของเชื้อหลุมที่มีความเข้มข้น
15 ต่ำที่สุดที่เชื้อไม่เจริญคือหลุมที่เป็นสามารถอ่านค่า ความเข้มข้นต่ำสุดที่สามารถยับยั้งเชื้อได้ ใช้ห่วงถ่ายเชื้อจุ่มเชื้อจากหลุมที่ไม่มีการเจริญทุกหลุมมาลากบนอาหาร MHA แล้วบ่มที่ 35 °ซ อ่านค่าความเข้มข้นต่ำสุดที่เชื้อไม่เจริญเป็นค่าความเข้มข้นต่ำสุดที่ฆ่าเชื้อได้

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ตารางที่ 3 ค่าความเข้มข้นต่ำสุดของสารสกัดกึ่งบริสุทธิ์จากปูดของเบญกานีที่สามารถยับยั้ง (MIC) และฆ่า (MBC) methicillin-resistant *Staphylococcus aureus*

สารสกัดกึ่งบริสุทธิ์	MIC/MBC (มก./มล.)	
	MRSA (n = 17)	*S. aureus* ATCC 25923
เอทิลอะซิเตท ส่วนที่ 1	0.13/0.50	0.13/0.25
เอทิลอะซิเตท ส่วนที่ 2	0.06/0.25	0.13/0.50
เอทานอล 95%	0.25/0.50	0.50/0.50
เอทานอล 30%	0.50/1.00	0.50/>1.00

คำอธิบายของรูปเขียน

รูปที่ 1 แสดงการลดลงของจำนวน methicillin-resistant *Staphylococcus aureus* ที่อยู่ในสารสกัดด้วยเอทานอล 95% จากปูดของเบญกานี

ก ได้แก่ MRSA NPRU 04

ข ได้แก่ MRSA NPRU 09

ค ได้แก่ *S. aureus* ATCC 25923

ในอาหารที่ผสม 1% ไดเมทิลซักฟอร์กไซด์ (o), หรือสารสกัดด้วยเอทานอล 95%ที่ความเข้มข้นเท่ากับค่า MIC (■), 2 เท่าของค่า MIC (▲), และ4 เท่าของค่า MIC (●)

วิธีการในการประดิษฐ์ที่ดีที่สุด

เหมือนกับที่กล่าวไว้ข้างต้นซึ่งได้เปิดเผยไว้ในหัวข้อการการเปิดเผยการประดิษฐ์โดยสมบูรณ์

ข้อถือสิทธิ

1. กระบวนการสกัดสารออกฤทธิ์ทางชีวภาพจากเบญกานี (*Quercus infectoria*) ที่ประกอบด้วยขั้นตอนต่อไปนี้ตามลำดับ

   1.1 สกัดผงของเบญกานีที่ผ่านการอบแห้งหรือไม่ก็ได้ด้วยตัวทำละลายอินทรีย์ ได้แก่ แอลกอฮอล์ขนาดเล็กที่มีคาร์บอน 1 ถึง 6, อะซิโตน, เอทิลอะซิเตท

   1.2 นำสารสกัดหยาบในข้อ 1.1 ไปแยกให้เป็นสารสกัดกึ่งบริสุทธิ์ด้วยวิธีควิกคอลัมน์โครมาโตกราฟี (quick column chromatography) โดยใช้ตัวชะได้แก่ คลอโรฟอร์ม, เอทิลอะซิเตท, เอทานอล, เอทานอล 20% ถึง 40% ตามลำดับ

   1.3 ทำการแยกส่วนของสารสกัดกึ่งบริสุทธิ์โดยใช้ทินเลเยอร์โครมาโตกราฟี (thin layer chromatography) โดยใช้ซิลิกาเจล (silica gel) เป็นเฟสอยู่กับที่ (stationary phase) และสารผสมระหว่างคลอโรฟอร์ม, เมทานอล, น้ำ เป็นเฟสเคลื่อนที่ (mobile phase)

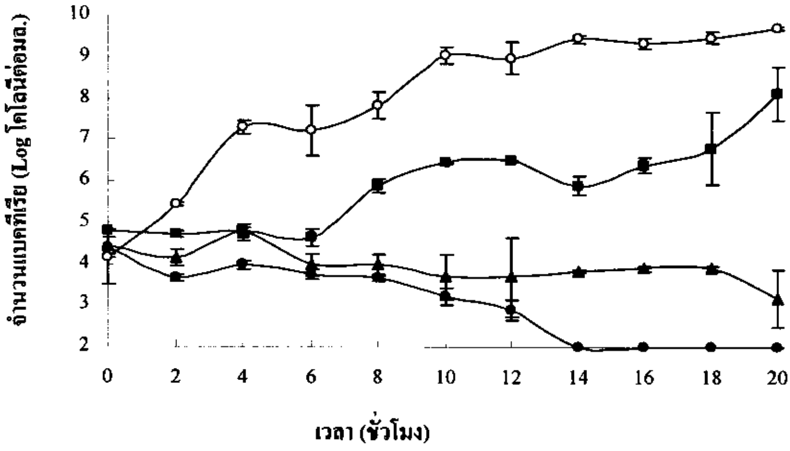
2. กระบวนการตามข้อถือสิทธิข้อที่ 1, ที่ซึ่งส่วนของเบญกานีที่นำมาสกัดเป็นปูด (nutgall) ที่แก่ ซึ่งมีขนาดเส้นผ่านศูนย์กลางตั้งแต่ 3 ซม. ขึ้นไป และนำมาบดหยาบก่อนเติมตัวทำละลาย

3. กระบวนการตามข้อถือสิทธิข้อที่ 1 หรือ 2 ที่ซึ่งตัวทำละลายอินทรีย์เป็นเอทิลแอลกอฮอล์ 50% ถึง 95%

4. กระบวนการตามข้อถือสิทธิข้อที่ 1 ถึง 3 ข้อใดข้อหนึ่ง ที่ซึ่งใช้ซิลิกาเจล 60 GF_{254} เป็นเฟสอยู่กับที่และเฟสเคลื่อนที่เป็นสารผสมระหว่างคลอโรฟอร์ม, เมทานอล, น้ำ ในอัตราส่วน 6: 3.7: 0.3

5. ผลิตภัณฑ์ที่มีส่วนผสมของสารสกัดในกระบวนการตามที่ขอถือสิทธิในข้อใดข้อหนึ่งของข้อถือสิทธิที่ 1 ถึง 4

ก



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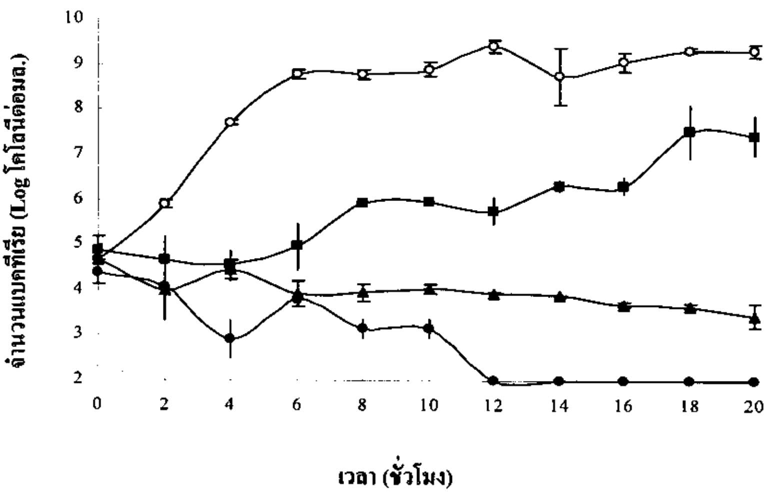
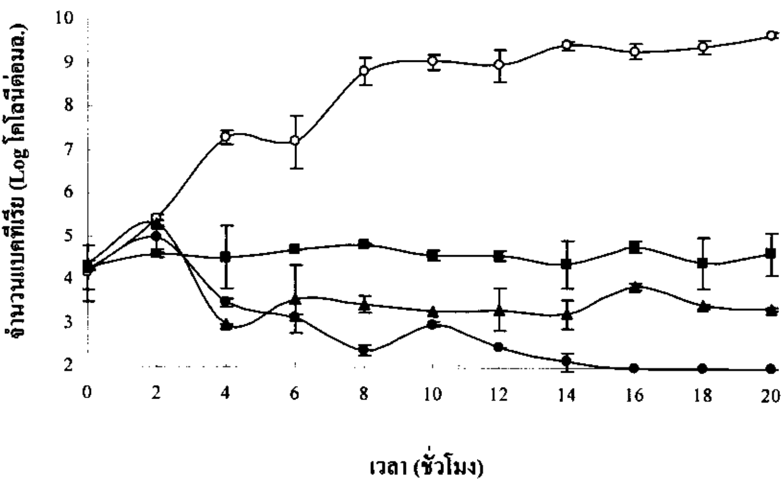


ค



รูปที่ 1

บทสรุปการประดิษฐ์

ได้บรรยายกระบวนการสกัดสารจากเบญกานี ให้ได้สารที่มีผลิตผลปริมาณสูง และมีฤทธิ์ทางชีวภาพดี โดยสกัดจากส่วนปูดแก่ ด้วยเอทานอล 50% ถึง 95% เป็นเวลา 7 ถึง 10 วัน และทำการแยกเป็นสารสกัดกึ่งบริสุทธิ์ด้วยวิธีควิกคอลัมน์โครมาโตกราฟีที่ใช้

5 ซิลิกาเจล 60 GF_{254} เป็นเฟสอยู่กับที่ และใช้คลอโรฟอร์ม, เอทิลอะซิเตท, เอทานอล, เอทานอล 20% ถึง 40% ตามลำดับ เป็นตัวชะสารสกัดกึ่งบริสุทธิ์ออกจากคอลัมน์ ทำการเก็บรวบรวมสารสกัดกึ่งบริสุทธิ์โดยใช้ทินเลเยอร์โครมาโตกราฟี และใช้คลอโรฟอร์ม เมทานอล และน้ำในอัตราส่วน 6 ต่อ 3.8 ต่อ 0.3 เป็นเฟสเคลื่อนที่

รายละเอียดการประดิษฐ์

ชื่อที่แสดงถึงการประดิษฐ์

กระบวนการสกัดสารออกฤทธิ์ทางชีวภาพจากกระทุ (โทะ) (*Rhodomyrtus tomentosa* (Ait.) Hassk.)

สาขาวิทยาการที่เกี่ยวข้องกับการประดิษฐ์

5 วิทยาศาสตร์สุขภาพในส่วนที่เกี่ยวข้องกับกระบวนการสกัดสารออกฤทธิ์ทางชีวภาพจากกระทุ

ภูมิหลังของศิลปะหรือวิทยาการที่เกี่ยวข้อง

ยาปฏิชีวนะมีบทบาทสำคัญในการใช้รักษาโรคติดเชื้อมาเป็นระยะเวลานาน แต่ปัจจุบันพบว่ามีรายงานการดื้อยาของเชื้อเพิ่มขึ้นอย่างต่อเนื่อง ซึ่งปัญหาเชื้อดื้อยาปฏิชีวนะเป็นปัญหาที่เกิดขึ้นโดยเฉพาะในกลุ่มประเทศที่กำลังพัฒนา เนื่องจากไม่มีมาตรการเข้มงวดในการใช้ยาปฏิชีวนะ นอกจากนี้การใช้ยา
10 ปฏิชีวนะในการรักษาโรคยังส่งผลข้างเคียงต่างๆ ต่อร่างกายผู้ป่วย ทั้งยังต้องสิ้นเปลืองค่าใช้จ่ายในการนำเข้ายาจากต่างประเทศ ดังนั้นการหาแนวทางใหม่ในการรักษาโรคติดเชื้อจึงมีความจำเป็นอย่างยิ่ง ซึ่งในปัจจุบันการใช้สมุนไพรในการรักษาและป้องกันโรคก็เป็นอีกหนึ่งทางเลือกที่ได้รับความนิยมเป็นอย่างมาก สำหรับประเทศไทยเองนั้นก็มีการสนับสนุนให้ใช้ความรู้ทางด้านภูมิปัญญาท้องถิ่นไทยและสมุนไพรไทยในการรักษาโรค ซึ่งวัตถุประสงค์นี้มีความสอดคล้องกับแผนพัฒนาสุขภาพแห่งชาติของประเทศไทย (The Ninth
15 Five-Year National Health Development Plan 2002-2006, 2001) ด้วย

กระทุ (หรือที่บางท้องถิ่นเรียกว่า “โทะ”) (*Rhodomyrtus tomentosa*) เป็นพืชป่าที่ขึ้นเองตามธรรมชาติและสามารถทำการเพาะปลูกได้ง่าย ผลของกระทุสามารถใช้รับประทานได้ และยังมีสรรพคุณทางยาอีกด้วย ตัวอย่างเช่น ในประเทศมาเลเซียมีการนำผลกระทุมาใช้รักษาโรคบิดและโรคท้องร่วง, ในประเทศอินโดนีเซียมีการใช้รากและใบต้มแก้ท้องร่วงและปวดท้อง นอกจากนี้ยังมีการใช้กับสตรีหลังคลอดบุตรเพื่อ
20 ช่วยบรรเทาอาการปวดเกร็งเนื่องจากการคลอดบุตรอีกด้วย (http://www.plantgenetic-rspg.org/dr_punee/drpn_series.htm) แต่สำหรับในประเทศไทยยังไม่เคยพบข้อมูลของการใช้กระทุในตำราแพทย์แผนไทย

ในปัจจุบันมีการประยุกต์ใช้สารสกัดจากกระทุในหลายรูปแบบ ตัวอย่างเช่น คำขอสิทธิบัตรญี่ปุ่น JP 2066199678 มีการใช้สารสกัดจากกระทุในเครื่องสำอางและอาหารเสริม, คำขอสิทธิบัตรจีน CN 1853687
25 มีการใช้กระทุผสมกับสมุนไพรหลายชนิดเพื่อรักษาโรคติดเชื้อทางเดินปัสสาวะ ซึ่งสำหรับคำขอสิทธิบัตรจีนหลายสิทธิบัตร ตัวอย่างเช่น CN 1868513, CN 1850189, CN 1850188 และ CN 1850187 มีการผสมรากกระทุกับสมุนไพรอื่นๆ เพื่อใช้เป็นยารักษาโรคทางนรีเวช นอกจากนี้ยังมีรายงานฤทธิ์ทางชีวภาพอื่นๆ ของสารสกัดจากกระทุ เช่น มีฤทธิ์ในการต้านอนุมูลอิสระ (Miyake and Nojima, Jpn. Kokai Tokkyo Koho, 2006) เป็นต้น

การสกัดสารจากกระทุ (โทะ) ในปัจจุบันนั้นมีการใช้กรรมวิธีที่หลากหลายแตกต่างกันออกไป ทั้งนี้ยังไม่ปรากฏว่ามีรายงานใดที่เปรียบเทียบหรือบ่งชี้ให้เห็นถึงกรรมวิธีหรือกระบวนการที่ดีที่สุดในการสกัดสารออกฤทธิ์ทางชีวภาพจากกระทุ (โทะ) เพื่อให้สารสกัดที่ได้มีความบริสุทธิ์ และมีฤทธิ์ทางชีวภาพสูง

ลักษณะและความมุ่งหมายของการประดิษฐ์

5 การประดิษฐ์นี้เกี่ยวข้องกับกระบวนการสกัดสารออกฤทธิ์ทางชีวภาพจากใบกระทุ

ความมุ่งหมายของการประดิษฐ์นี้ คือ เพื่อคิดค้นพัฒนาหาวิธีการที่ดีที่สุดในการสกัดสารออกฤทธิ์ทางชีวภาพจากกระทุ ซึ่งเป็นพืชที่ขึ้นอยู่เป็นจำนวนมากในแถบดินปนทราย ตามชายฝั่งทะเลในภาคใต้ของประเทศไทย และมีคุณค่าทางอาหารและสรรพคุณทางยาที่ดี แต่เป็นพืชที่ยังไม่มีรายงานคุณค่าทางเศรษฐกิจ โดยเฉพาะใบกระทุนั้นสามารถเก็บเกี่ยวได้ทุกฤดูกาล ดังนั้นเพื่อเพิ่มประสิทธิภาพและความบริสุทธิ์ของสาร 10 สกัดจากกระทุให้มีฤทธิ์ทางชีวภาพได้ดียิ่งขึ้น คณะผู้ประดิษฐ์จึงได้ตัดสินใจพัฒนาวิธีการสกัดสารออกฤทธิ์ทางชีวภาพจากกระทุให้มีประสิทธิภาพดียิ่งขึ้น เพื่อให้สามารถใช้สารสกัดจากกระทุ (โทะ) ที่ได้จากกรรมวิธีนี้เป็นส่วนผสมที่มีประสิทธิภาพดี และมีราคาถูกในผลิตภัณฑ์ต่างๆ ทางอุตสาหกรรมยา เครื่องสำอางอาหาร และอื่นๆ ต่อไป โดยในเบื้องต้นคณะผู้ประดิษฐ์จึงได้ทำการทดสอบฤทธิ์ของสารสกัดจากส่วนต่างๆ ของกระทุ เช่น ใบ ลำต้น ดอก ผล และราก ในการส่งผลยับยั้งเชื้อแบคทีเรียก่อโรคที่เป็น 15 ปัญหาสำคัญทางการแพทย์ ซึ่งพบว่าสารสกัดจากส่วนต่าง ๆ ดังกล่าว ล้วนก็สามารถให้ผลในการยับยั้งการเจริญและฆ่าแบคทีเรียแกรมบวกที่ทดสอบได้ดี โดยผลที่ได้ไม่แตกต่างกันมากนัก

การเปิดเผยการประดิษฐ์โดยสมบูรณ์

กระบวนการสกัดสารออกฤทธิ์ทางชีวภาพจากกระทุตามการประดิษฐ์นี้ ประกอบด้วย 2 กระบวนการหลัก ๆ คือ

20 1. กระบวนการเตรียมสารสกัดจากกระทุ โดยมีขั้นตอนดังนี้

1.1 คัดเลือกส่วนของใบ/ลำต้น/ผล/ดอก/ราก ของกระทุที่มีความสมบูรณ์และไม่เป็นโรค ล้างทำความสะอาดด้วยน้ำ อบให้แห้งที่อุณหภูมิ 60-75 °ซ จากนั้นนำไปบดหยาบ

1.2 สกัดผงกระทุในข้อ 1.1 โดยการแช่ในไดคลอโรมีเทน (CH_2Cl_2) เป็นเวลา 3-7 วัน อย่างน้อย 3 ครั้ง กรองและแยกเอากากออก แล้วนำสารละลายที่ได้มาระเหยให้แห้งโดยใช้โรตารีอีแวบโปเรเตอร์ 25 (Rotary evaporator) ที่อุณหภูมิ 40 - 60 °ซ

ทั้งนี้สามารถใช้ตัวทำละลายอินทรีย์อื่นๆ เช่น เอทานอล (CH_3CH_2OH)/เมทานอล (MeOH)/เฮกเซน (hexane, $CH_3(CH_2)_4CH_3$) / อะซีโตน ($MeCo_2$) ในการสกัดสารออกฤทธิ์ทางชีวภาพจากกระทุเป็นสารสกัดหยาบได้ แต่สารสกัดที่ได้มีฤทธิ์ในการยับยั้งแบคทีเรียได้น้อยกว่าการใช้ไดคลอโรมีเทนเป็นตัวทำละลาย

30

2 กระบวนการแยกสารสกัดจากจากกระทุเป็นสารสกัดกึ่งบริสุทธิ์ โดยมีขั้นตอนดังนี้

2.1 ละลายสารสกัดในข้อ 1.2 ด้วยเมทานอล แล้วทำการแยกส่วนที่ไม่ละลายออกโดยการกรอง นำส่วนที่เป็นสารละลายมาระเหยให้แห้งโดยใช้โรตารีอีแวบโปเรเตอร์ ที่อุณหภูมิ 40-60 °ซ

2.2 นำสารสกัดในข้อ 2.1 มาละลายด้วยไดคลอโรมีเทนในปริมาณที่น้อยที่สุดที่สามารถละลายสาร

5 สกัดได้หมด

2.3 ทำการแยกส่วนสารสกัดด้วยวิธีคอลัมน์โครมาโตกราฟี (Column chromatography) โดยบรรจุซิลิกาเจล (Silica gel) $60GF_{254}$ ลงในคอลัมน์ที่มีเส้นผ่านศูนย์กลางประมาณ 8 ซม. ให้ได้ ความสูงประมาณ 32 ซม. ใช้ไดคลอโรมีเทนทำให้ซิลิกาเจลเปียก แล้วค่อย ๆ บรรจุลงในคอลัมน์

2.4 นำสารละลายในข้อ 2.2 ถ่ายลงไปในคอลัมน์โดยใช้หลอดหยดค่อยๆ หยดลงในคอลัมน์อย่างช้าๆ

10 เพื่อให้ผิวหน้าของซิลิกาเจลเรียบอยู่เสมอ

2.5 ใช้ไดคลอโรมีเทน 100 เปอร์เซ็นต์ (%) และอะซีโตน 10-100% เป็นตัวชะสารสกัดกึ่งบริสุทธิ์ออกจากคอลัมน์ตามลำดับ

2.6 ใช้ 100% ไดคลอโรมีเทนเป็นเฟสเคลื่อนที่ (Mobile phase) ทำการตรวจสอบสารละลายที่ถูกชะออกมาด้วยทินเลเยอร์โครมาโตกราฟี (Thin layer chromatography; TLC) ที่มีซิลิกาเจลเป็นเฟสอยู่

15 กับที่ (Stationary phase) และทำการรวมสารละลายที่มีลักษณะของจุดสารบนทินเลเยอร์โครมาโตกราฟีที่คล้ายกันเข้าด้วยกัน

2.7 ใช้การทดสอบฤทธิ์ทางชีวภาพทำการคัดแยกสาร (Bioassay-guided fractionation) จากสารสกัดไดคลอโรมีเทนให้ได้สารกึ่งบริสุทธิ์ (Semi-purified fraction) และ/หรือสารบริสุทธิ์ (Pure compound)

20 2.8 ใช้วิธีการเอกซ์เทนซีฟ 1 (Extensive 1-) และ 2ดี เอ็นเอ็มอาร์ สเปคโทรสโคปี (2D NMR spectroscopy) และแมส สเปกโทรสโครปี (Mass spectroscopy) ทำการวิเคราะห์หาสูตรโครงสร้างของสารบริสุทธิ์ที่มีฤทธิ์ทางชีวภาพที่ดี ได้สารสำคัญในกลุ่มของเอซิลโฟโรกลูซินอล (Acylphloroglucinol) อาทิเช่น โรโดไมรโทน (Rhodomyrtone) และสารอนุพันธ์ (Derivatives) ที่มีโครงสร้างคล้ายคลึงกัน

25 <u>ตัวอย่างที่ 1</u> การหาค่าความเข้มข้นต่ำสุดที่สามารถยับยั้งการเจริญ (Minimal inhibitory concentration; MIC) และการหาค่าความเข้มข้นต่ำสุดที่สามารถฆ่าเชื้อแบคทีเรีย (Minimal bactericidal concentration; MBC) โดยใช้วิธีบรอทไมโครไดลูชัน (Broth microdilution)

เตรียมเชื้อแบคทีเรียที่ต้องการทดสอบโดยเลี้ยงเชื้อบนอาหารทริปติกซอยเอการ์ (Tryptic soy agar; TSA) ที่อุณหภูมิ 37 °ซ เป็นเวลา 18-24 ชั่วโมง จากนั้นเขี่ยเชื้อลงในน้ำเกลือที่ปราศจากเชื้อ (Sterile normal

30 saline solution, NSS) ปรับความขุ่นให้ได้ 0.5 แมคฟาร์แลนด์ (McFarland) และทำการเจือจางเชื้อด้วย NSS ให้ได้เชื้อประมาณ 10^5 โคโลนี (Colony) ต่อมล. ละลายสารสกัดที่ต้องการทดสอบด้วย 10% ไดเมทิลซัลฟอร์กไซด์ (Dimethyl sulfoxide; DMSO) แล้วนำไปผสมกับเบรนฮาร์ทอินฟิวชันบรอท (Brain heart infusion

broth; BHI) ในอัตราส่วน 1:10 โดยปริมาตรและทำการเจือจางตามลำดับส่วน จากนั้นทำการถ่ายเชื้อลงในแต่ละหลุม แล้วบ่มที่อุณหภูมิ 37 °ซ เป็นเวลา 18-24 ชั่วโมง อ่านค่า MIC จากหลุมที่มีความเข้มข้นต่ำที่สุดที่เชื้อไม่เจริญ ใช้ห่วงถ่ายเชื้อ (Loop) ถ่ายเชื้อจากหลุมที่ไม่มีการเจริญทุกหลุมมาลากบนอาหาร TSA แล้วบ่มที่อุณหภูมิ 37 °ซ เป็นเวลา 18-24 ชั่วโมง อ่านค่าความเข้มข้นต่ำสุดที่เชื้อไม่เจริญเป็นค่า MBC (ตารางที่ 1 และ 2)

ตารางที่ 1 ค่าความเข้มข้นต่ำสุดของสารสกัดกึ่งบริสุทธิ์จากกระทุที่สามารถยับยั้ง (MIC) และฆ่า (MBC) เชื้อเสตรปโตคอคคัส ไพโอจีเนส (*Streptococcus pyogenes*)

สารสกัดกึ่งบริสุทธิ์	MIC (ไมโครกรัม/มล.)	MBC (ไมโครกรัม/มล.)
R1	-	-
R2	-	-
R3	-	-
R4	0.78	12.50
R5	0.39	1.56
R6	0.39	1.56
R7	0.78	1.56
R8	1.56	3.12
R9	3.12	6.25
R10	3.12	12.50
R11	6.25	12.50
R12	25.00	50.00
R13	25.00	50.00
R14	100.00	100.00
R15	100.00	200.00
R16	50.00	100.00
R17	25.00	100.00
R18	12.50	50.00
R19	6.25	12.50
R20	200.00	200.00

หมายเหตุ – หมายถึง ไม่ได้ทำการทดสอบ เนื่องจากสารไม่ละลายใน DMSO

ตารางที่ 2 ค่าความเข้มข้นต่ำสุดของสารสกัดกึ่งบริสุทธิ์จากกระทุ (โทะ) ที่สามารถยับยั้ง (MIC) และฆ่า (MBC) เชื้อแบคทีเรียก่อโรคชนิดต่างๆ รวมทั้งเชื้อที่ดื้อต่อยาปฏิชีวนะ

ชนิดของเชื้อแบคทีเรีย	**โค้ดของเชื้อแบคทีเรียแต่ละชนิด**	**ค่า MIC/MBC ของสารสกัดกึ่งบริสุทธิ์ (ไมโครกรัม/มล.)**			
		A 14.33-66S	**A 15.1-283S**	**A 12.44**	**A 14.22B**
สแตฟฟีโลคอคคัส ออเรียส (*Staphylococcus aureus*)	(ATCC 25923)	0.39/0.39	0.39/0.39	>50/>50	>50/>50
บาซิลลัส ซีเรียส (*Bacillus cereus*)	(ATCC 11778)	0.39/0.39	0.39/0.39	>50/>50	>50/>50
สเตรปโตคอคคัส ไพโอจีเนส (*Streptococcus pyogenes*)	(DMST 17020)	0.39/1.56	0.39/1.56	>50/>50	>50/>50
สเตรปโตคอคคัส ไพโอจีเนส (*Streptococcus pyogenes*)	(NPRC 101)	0.39/0.78	0.39/0.78	>50/>50	>50/>50
สตาไฟโลคอคคัส ออเรียส (*Staphylococcus aureus*)	(NPRU R01*)	0.39/0.39	0.39/0.39	>50/>50	>50/>50

* เชื้อ *Staphylococcus aureus* ที่ดื้อต่อยาเมทธิซิลิน (Methicillin-resistant *Staphylococcus aureus*)

<u>ตัวอย่างที่ 2</u> การศึกษาฤทธิ์ต้านอนุมูลอิสระ (Antioxidant activity) ทำการทดสอบฤทธิ์ต้านอนุมูลอิสระของสารสกัดกระทุ (โทะ) โดยใช้วิธีมาตรฐาน และหาค่าความเข้มข้นของสารที่ออกฤทธิ์จับอนุมูลอิสระได้ 50% (IC_{50}) (ตารางที่ 3)

(1) การทดสอบฤทธิ์ต้านอนุมูลดีพีพีเอช (1,1-diphenyl-2-picrylhydrazyl; DPPH) ของสารสกัดกระทุ (โทะ)

นำสารสกัดจากใบกระทุมาทดสอบฤทธิ์ต้านอนุมูลอิสระเบื้องต้น ซึ่งอาศัยหลักการทดสอบความสามารถของสารสกัดใบกระทุในการจับอนุมูล DPPH ตามวิธีการที่ระบุ (Yen และ Hsieh, <u>Biosci. Biotech. Biochem</u>. 1997, <u>61</u>, 1646) โดยเจือจางสารสกัดในเมทานอล 95% ให้มีความเข้มข้นต่างๆ กัน อย่างน้อย 5 ความเข้มข้นๆ ละ 3 ซ้ำ ดูดสารปริมาณ 0.5 มล. นำมาผสมกับสารละลาย DPPH 0.2 mM ในเมทานอลปริมาณ 1 มล. ตั้งทิ้งไว้ในที่มืด ณ อุณหภูมิห้อง นาน 30 นาที ทำการวัดค่าการดูดกลืนแสง (OD) ที่ความยาวคลื่น 523 นาโนเมตร (Nanometer; nm) เปรียบเทียบกับชุดควบคุมที่ใช้เมทานอลแทนสารสกัด นำค่าเฉลี่ยที่วัดได้ในแต่ละความเข้มข้นของสารสกัดไปหา % ต้านอนุมูลอิสระ (% scavenging) จากสูตร

$$\%\ \text{ต้านอนุมูลอิสระ} = [(\text{ค่า } OD_{\text{ชุดควบคุม}} - \text{ค่า } OD_{\text{สาร}})/\text{ค่า } OD_{\text{ชุดควบคุม}}] \times 100$$

จากนั้นคำนวณหาค่า IC_{50} ของสารสกัดที่สามารถดักจับอนุมูล DPPH ได้ 50% จากการวิเคราะห์ค่าการถดถอย (Linear regression analysis) ของกราฟระหว่าง % ต้านอนุมูลอิสระกับความเข้มข้นของสารสกัด

(2) การทดสอบฤทธิ์ต้านอนุมูลไฮดรอกซิล (Hydroxyl; OH) ของสารสกัดกระทุ (โทะ)

ทำการทดสอบความสามารถของสารสกัดกระทุ (โทะ) ในการกำจัดอนุมูล OH จากการดัดแปลงวิธีการที่ได้ระบุไว้ (Murcia และคณะ, J. Food Prot. 2001, 64, 2037) โดยนำสารสกัดที่ต้องการทดสอบมาละลายในเมทานอล 95% ให้มีความเข้มข้นต่างๆ กัน อย่างน้อย 5 ความเข้มข้นๆ ละ 3 ซ้ำ ดูดสารปริมาตร 67
5 ไมโครลิตร (microliter; µl) วางทิ้งไว้ให้ระเหยจนแห้ง จากนั้นเติมสารละลายของโพแทสเซียม ไดไฮโดรเจนฟอสเฟต (KH_2PO_4) 30 มิลลิโมล (mM)/โพแทสเซียมไฮดรอกไซด์ (KOH) ซึ่งมีค่าความกรดเป็นด่าง (pH) 7.4 ปริมาตร 134 µl, ดิออกซิไรโบส (Deoxyribose) 17 mM ปริมาตร 67 µl, ไฮโดรเจนเปอร์ออกไซด์ (H_2O_2) 34 mM ปริมาตร 33 µl, อีดีทีเอ (EDTA) 1.2 mM ปริมาตร 33 µl, $FeCl_2$ 300 µM ปริมาตร 67 µl และ กรดแอสคอร์บิก (ascorbic acid) 600 ไมโครโมล (Micromole, µM) ปริมาตร 67 µl ตามลำดับ หลังจากบ่มที่
10 อุณหภูมิ 37 ^{0}ซ เป็นเวลา 1 ชั่วโมง เติมกรดไทโอบาร์บิทิวริก (Thiobarbituric acid) ในโซเดียมไฮดรอกไซด์ (Sodium hydroxide; NaOH) 50 mM 1% (w/v) ปริมาตร 333 µl และกรดไตรคลอโรอะซีติก (Trichloroacetic acid) 2.8% ของน้ำหนักโดยปริมาตร (w/v) ปริมาตร 333 µl ทำการอุ่นที่อุณหภูมิ 80^{0}ซ นาน 20 นาที ทิ้งไว้ให้เย็นและนำไปหมุนเหวี่ยงเพื่อแยกเฉพาะส่วนใส วัดค่า OD ของสารสกัดที่ 532 nm เปรียบเทียบกับชุดควบคุมที่ใช้เมทานอลแทนสารสกัด และชุดแบลงค์ (blank) ซึ่งประกอบด้วยสารสกัดกับชุดทดสอบข้างต้นที่
15 ไม่มีดิออกซิไรโบส แล้วนำค่าเฉลี่ยที่วัดได้ในแต่ละความเข้มข้นของสารสกัดไปหา % การยับยั้ง จากสูตร

$$\% \text{ ต้านอนุมูลอิสระ} = [OD_{\text{ชุดควบคุม}} - (OD_{\text{สาร}} - OD_{\text{สาร-blank}})/OD_{\text{ชุดควบคุม}}] \times 100$$

จากนั้นคำนวณหาค่า IC_{50} ของสารสกัดที่สามารถกำจัดอนุมูล OH ได้ 50%

(3) การทดสอบฤทธิ์ต้านซุปเปอร์ออกไซด์แอนอิออน (Super oxide anion) ของสารสกัดกระทุ (โทะ)

ทำการทดสอบความสามารถดักจับซุปเปอร์ออกไซด์แอนอิออนของสารสกัดตามวิธีการที่ระบุไว้
20 (Beyer และ Fridovich, Anal. Biochem. 1987, 161, 559) โดยนำสารสกัดมาละลายในเมทานอล 95% ให้มีความเข้มข้นต่างๆ กัน อย่างน้อย 5 ความเข้มข้นๆ ละ 3 ซ้ำ ดูดสารปริมาตร 20 µl ผสมกับสารละลายซึ่งประกอบด้วยแอล-เมทิโอไนน์ (L-methionine) 9.9 mM, ไนโตรบลูเตทตระโซเลียม (Nitrobluetetrazolium; NBT) 1.72 mM, ไทตันเอ็กซ์ (Teiton X) -100 และไรโบฟลาวิน (Riboflavin) 117 µM ในโพแทสเซียม ไดไฮโดรเจน ฟอสเฟต (K_2HPO_4) 50 mM (1% w/v) ที่ pH 7.8 ปริมาณ 1 มล. จากนั้นนำไปส่องด้วยแสงจาก
25 หลอดฟลูออเรสเซนต์ขนาด 40 วัตต์ (Watt) นาน 7 นาที แล้ววัดค่าดูดกลืนแสงที่ 560 nm ของสารประกอบเชิงซ้อนระหว่าง NBT กับซุปเปอร์ออกไซด์แอนอิออนที่เกิดขึ้นในแต่ละชุดการทดลองเทียบกับชุดควบคุมที่ใช้เมทานอลแทนสารสกัด และชุด blank ซึ่งประกอบด้วยสารสกัดกับชุดทดสอบข้างต้นที่ไม่มีไรโบฟลาวิน นำค่าเฉลี่ยที่วัดได้ในแต่ละความเข้มข้นของสารสกัดไปหา % ต้านอนุมูลอิสระ จากสูตรในข้อ (2) จากนั้นคำนวณหาค่า IC_{50} ของสารสกัดที่สามารถกำจัดอนุมูลซุปเปอร์ออกไซด์แอนอิออนได้ 50%

30

ตารางที่ 3 ค่าฤทธิ์ต้านอนุมูลอิสระของกระทุ (โทะ)

สารที่ทดสอบ	ค่าฤทธิ์ต้านอนุมูลอิสระ (IC_{50} มก./มล.)		
	ดีพีพีเอช (DPPH)	ไฮดรอกซิล (Hydroxyl)	ซุปเปอร์ออกไซด์ (Super oxide)
สารมาตรฐาน BHT	0.02	-	-
สารมาตรฐานแทนนิน (Tannin)	-	2.53	-
สารมาตรฐานโทรลอกซ์ (Trolox)	-	-	0.85
สารสกัดกระทุ (โทะ)	0.04^{+++}	0.001^{++++}	1.08^{++}

5

หมายเหตุ ++++ หมายถึง ฤทธิ์ดีมาก

10 +++ หมายถึง ฤทธิ์ดี

++ หมายถึง ฤทธิ์ปานกลาง

\- หมายถึง ไม่มีฤทธิ์

<u>วิธีการในการประดิษฐ์ที่ดีที่สุด</u>

เหมือนกับที่ได้กล่าวไว้แล้วข้างต้นในหัวข้อการเปิดเผยการประดิษฐ์โดยสมบูรณ์

15

ข้อถือสิทธิ

1. กระบวนการสกัดสารออกฤทธิ์ทางชีวภาพจากกระทุ (โทะ) ที่ซึ่งประกอบด้วยขั้นตอนดังต่อไปนี้
   1.1 สกัดส่วนต่างๆ ของกระทุที่ผ่านการอบแห้งหรือไม่ก็ได้ ด้วยตัวทำละลายอินทรีย์ ซึ่งเป็นแอลกอฮอล์ที่มีโมเลกุลขนาดเล็กและมีคาร์บอนเป็นองค์ประกอบ 1-6 อะตอม อันได้แก่ เฮกเซน
5 หรือ เอทานอล หรือ เมทานอล หรือ ไดคลอโรมีเทน หรือ อะซีโตน หรือ เอทิลอะซีเตท หรือตัวทำละลายอื่น ๆ ที่มีโมเลกุลขนาดเล็กที่มีจำนวนคาร์บอนไม่เกิน 6 อะตอม หรือส่วนผลมของตัวทำละลายดังกล่าวตั้งแต่สองชนิดขึ้นไป
   1.2 นำสารสกัดหยาบที่ได้จากขั้นตอนในข้อ 1.1 ไปแยกให้เป็นสารสกัดกึ่งบริสุทธิ์ด้วยวิธีคอลัมน์โครมาโตกราฟี (Column chromatography) โดยใช้ซิลิกาเจล (Silica gel) $60GF_{254}$ เป็นเฟสอยู่กับที่
10 (Stationary phase) และใช้ไดคลอโรมีเทน 100% และอะซีโตน 10-100% เป็นตัวชะตามลำดับ
   1.3 ทำการแยกส่วนของสารสกัดกึ่งบริสุทธิ์โดยใช้ทินเลเยอร์โครมาโตกราฟี (Thin-layer chromatography) ซึ่งใช้ซิลิกาเจล (Silica gel) เป็นเฟสอยู่กับที่ (Stationary phase) และใช้ไดคลอโรมีเทนเป็นเฟสเคลื่อนที่ (Mobile phase)

2. กระบวนการสกัดสารออกฤทธิ์ทางชีวภาพจากกระทุสดหรือตากแห้งตามข้อถือสิทธิที่ 1 ที่ซึ่งส่วนต่าง ๆ
15 ของกระทุ (โทะ) ที่นำมาสกัด คือ ส่วนของ ใบ หรือ ลำต้น หรือ ผล หรือ ดอก หรือราก ก็ได้ โดยนำมาบดหยาบก่อนเติมตัวทำละลาย

3. สารสกัดหยาบที่ได้จากการสกัดตามวิธีการในข้อถือสิทธิที่ 1 และ 2 ที่มีฤทธิ์ในการยับยั้งการเจริญของแบคทีเรีย

4. ผลิตภัณฑ์ที่มีส่วนผสมของสารสกัดที่ได้จากกระบวนการสกัดสารออกฤทธิ์ทางชีวภาพจากกระทุ
20 ตามข้อถือสิทธิที่ 1–3 ข้อใดข้อหนึ่ง

บทสรุปการประดิษฐ์

ได้บรรยายกระบวนการสกัดสารออกฤทธิ์ทางชีวภาพจากกระทุ (โทะ) ที่สามารถทำให้สารสกัดที่ได้มีความบริสุทธิ์สูงและมีฤทธิ์ทางชีวภาพดี โดยการสกัดส่วนต่างๆ ของกระทุด้วยการนำไปแช่ในไดคลอโรมีเทนเป็นเวลา 3-7 วัน และทำการแยกเป็นสารสกัดกึ่งบริสุทธิ์ใช้ไดคลอโรมีเทน 100% และ อะซีโตน 10-
5 100% เป็นตัวชะสารสกัดกึ่งบริสุทธิ์ออกจากคอลัมน์ตามลำดับ และทำการตรวจสอบสารละลายที่ถูกชะออกมาด้วยทินเลเยอร์โครมาโตกราฟี ด้วยวิธีคอลัมน์โครมาโตกราฟีที่ใช้ซิลิกาเจล $60GF_{254}$ เป็นเฟสอยู่กับที่และใช้ไดคลอโรมีเทน 100% เป็นเฟสเคลื่อนที่ แล้วทำการรวมสารละลายที่มีลักษณะของจุดสารบนทินเลเยอร์โครมาโตกราฟีที่คล้ายกันเข้าด้วยกัน แล้วจึงใช้การทดสอบฤทธิ์ทางชีวภาพทำการคัดแยกสารให้ได้สารกึ่งบริสุทธิ์ และ/หรือสารบริสุทธิ์ และใช้วิธีการ Extensive 1- และ 2D NMR spectroscopy และ Mass
10 spectroscopy ทำการวิเคราะห์หาสูตรโครงสร้างของสารบริสุทธิ์ที่มีฤทธิ์ทางชีวภาพที่ดี เพื่อให้ได้สารสำคัญในกลุ่มของเอซิลโฟโรกลูซินอล (Acylphloroglucinol) อาทิเช่น โรโดไมรโทน (Rhodomyrtone) และสารอนุพันธ์ (Derivatives) ที่มีโครงสร้างคล้ายคลึงกัน