CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1) Chemicals

Chemicals	Company
Absolute ethanol	Merck
Acetic acid glacial (AR)	BDH
Acetone (AR)	Merck
Acetone deuterated	Merck
Ammonium hydroxide 25% (AR)	BDH
Bactoagar	Difco
Barium chloride (Purum)	Fluka
n-Butanol (AR)	LAB SCAN
β-Carotene	Sigma
Chloroform (AR)	LAB SCAN
Dichloromethane (AR)	Fisher
Dimethylsulfoxide (AR)	LAB SCAN
Ethyl acetate (AR)	J. T. Baker
Folin-Ciocalteu's reagent	Carlo Erba
Gallic acid (Extra pure)	Merck
Hexane (AR)	J. T. Baker

Chemicals	Company
Linoleic acid 70% (LAB)	BDH
Methanol (AR)	Fisher
Methanol (HPLC)	Fisher
Mueller-Hinton agar	Difco
Nutrient broth	Difco
Nystatin disc	Oxoid
Potato dextrose agar	Difco
Sabouraud dextrose agar	Difco
Sephadex LH-20	Amersham Biosciences
Sodium carbonate (GR)	BDH
Sodium hydrogen carbonate (GR)	Merck
Sodium sulfate anhydrous (AR)	Fluka
Streptomycin disc	Oxoid
Sulfuric acid 96% (GR)	Merck
Tannic acid (Purum)	Fluka
Tetramethylsilane	Merck
Tween 40	Fluka

2.1.2) Materials and instruments

Materials and instruments	Company
Analytical balance	Sartorius
Autoclave	Sanyo

Materials and instruments	Company
Centrifuge JA20-MC	BECKMAN
Fraction collector	Pharmacia Biotech
FT-IR spectrometer	Nicolet
Haemacytometer	Hausser Scientific
Heating mantle	Electrothermal
HP gas chromatograph 5890 series II	Hewlett-Packard
HP GC/MS system 6890 series	Hewlett-Packard
HP 1100 series liquid chromatograph	Hewlett-Packard
Hot air blowing oven	-
Incubator	Edu Systems
Lambda 25 UV-Vis spectrometer	Perkin Elmer
Laminar flow	TELSTAR
LCMS-MS spectrometry	Micromass Instrument
Microscope	Olympus
NMR spectrometer	Bruker Avance
Peristaltic pump	Eyela
Rotary evaporator	Buchi
Shaker (orbital incubator)	GALLENKAMP
TLC plate	Merck
Ultraviolet lamp	FLUOTEST
Water bath	Heto

2.1.3) Microorganisms

The microorganisms used in the study (Table 2.1) consisted of 8 strains of human pathogenic bacteria, 1 yeast, and 5 filamentous fungi (including 2 dermatophytic fungi). All the bacterial strains, yeast, and dermatophytic fungi were obtained from the Department of Clinical Microbiology, Faculty of Associated Medical Sciences, Chiang Mai University. The plant pathogenic fungi were obtained from the Department of Plant Pathology, Faculty of Agriculture, Chiang Mai University. The bacteria were grown and maintained on nutrient agar slants, while the fungi on potato dextrose agar slants. The inoculated slants were incubated at 37 °C (overnight) for bacteria and yeast, and at 30 °C (3 days) for filamentous fungi.

Table 2.1 Microorganisms used in this study

Bacteria	Yeast	Filamentous fungi
Gram negative bacteria	*Candida albicans*	*Aspergillus* sp.
Escherichia coli		*Fusarium* sp.
Klebsiella pneumoniae		*Microsporum gypseum**
Pseudomonas aeruginosa		*Penicillium* sp.
Salmonella typhi		*Trichophyton rubrum**
Vibrio cholerae		
Gram positive bacteria		
Enterococcus faecalis		
Staphylococcus aureus		
S. epidermidis		

* Dermatophytic fungi

2.2 GENERAL METHODS

2.2.1) Preparation of plant materials

Five plant species (*Caesalpinia mimosoides* : Phak-pu-ya; *Coccinia grandis* : Tam-lueng or ivy gourd; *Gymnema inodorum* : Phak-sieng-da; *Pimpinella anisum* : Tian-pom or anise; and *Polygonum odoratum* : Phak-phai) were collected in June-July, 2002 from local markets in Chiang Mai, Thailand. They, except anise, were cleaned and cut into small pieces before drying in a hot air blowing oven at 50 °C. After drying, all dried plant materials had water content less than 10%, and they were kept at 4 °C at the Department of Chemistry, Faculty of Science, Chiang Mai University.

2.2.2) Extraction for screening of antimicrobial activity

The dried plant materials (50 g) were extracted with distilled water, 95% ethanol, chloroform, and acetone by soaking at room temperature for 3 days, followed by filtration. The solvent from each extract was removed under reduced pressure using a rotary evaporator. The extracts containing 250 mg/ml were prepared in the same solvent of extraction and kept at 4 °C until used. These crude extracts were used for screening antimicrobial activity.

2.2.3) Preparation of nutrient agar slant

A 100-ml agar medium comprising of 0.8 g nutrient broth and 2 g Bactoagar was melted by heating in boiling water for 1 min. The medium (approximate 5 ml) was poured into test tubes before sterilization at 121 °C for 15 min. Then the sterile agar medium was solidified slantingly by slanting laying.

2.2.4) Preparation of potato dextrose agar slant

Potato dextrose agar (3.9 g) was melted by heating in 100 ml of boiling water for 1 min. The medium (approximate 5 ml) was poured into test tubes before sterilization at 121 °C for 15 min. Then the sterile agar medium was solidified slantingly by slanting laying.

2.2.5) Preparation of Mueller-Hinton agar plate

Mueller-Hinton agar (3.8 g) was melted by heating in 100 ml of boiling water for 1 min. It was then sterilized at 121 °C for 15 min. Twenty-five ml of the sterile medium were poured into each Petri dish.

2.2.6) Preparation of Sabouraud dextrose agar plate

Sabouraud dextrose agar (6.5 g) was melted by heating in 100 ml of boiling water for 1 min. It was then sterilized at 121 °C for 15 min. Twenty-five ml of the sterile medium were poured into each Petri dish.

2.2.7) Preparation of cell suspension (Barry and Thornsberry, 1991)

A few colonies of bacteria or yeast were aseptically transferred into screw-capped vials containing sterile distilled water. The turbidity of the cell suspensions was then adjusted with the sterile distilled water to obtain a turbidity visually comparable to that of a turbidity standard prepared by adding 0.5 ml of 0.048 M barium chloride (1.75% w/v $BaCl_2 \cdot 2H_2O$) to 99.5 ml of 0.36 N sulfuric acid (1% v/v H_2SO_4). This turbidity is half the density of a McFarland No. 1 standard, and is often referred to as a McFarland 0.5 standard.

2.2.8) Preparation of fungal spore suspension (Sakorn, 2002)

Spores from the cultures grown in potato dextrose agar slants were aseptically transferred into screw-capped vials containing sterile distilled water. They were vigorously shaken for 3-5 min or until no cluster of spores was observed. The spores in each suspension were counted by using a haemacytometer under a microscope. The suspensions were adjusted to desired concentration. Normally, inoculum was made from 2-weeks old culture and the concentration was $1x10^6$ spores/ml, unless stated otherwise.

2.2.9) Packing and equilibrating Sephadex LH-20 column

Two hundred grams of Sephadex LH-20 resin were allowed to swell in 95% ethanol (diluted from distilled absolute ethanol) at room temperature overnight. The gel slurry was then poured carefully into a 4x60cm column, connected to a peristaltic pump operating at a flow rate of 1 ml/min. The column was equilibrated with 95% ethanol using 5-10 times of the total column volume.

2.2.10) Preparation of essential oil of star anise

Preparation of the essential oil of star anise (*Illicium verum* Hook.) was carried out by steam-distillation for 3 h. The water in the oil was then removed by addition of anhydrous sodium sulfate.

2.3 SCREENING OF TOTAL PHENOLIC CONTENT, ANTIOXIDANT, AND ANTIMICROBIAL ACTIVITIES

2.3.1) Determination of total phenolic content (Naczk *et al*., 1992; Waterman and Mole, 1994)

One gram of dried plant material was extracted three time with 20 ml of acetone-methanol-water (7:7:6 v/v/v) at room temperature. The extract was centrifuged at 6000 rpm for 10 min. After centrifugation, the combined supernatant was analyzed for total phenolic content. The procedure consisted of combining 1 ml of test sample with 60-75 ml of deionized water and 5 ml of Folin-Ciocalteu's reagent. After 1 min, 15 ml of saturated sodium carbonate solution were added. The time was recorded as time zero, and the volume of the mixture was adjusted to 100 ml exactly with deionized water. After 2 h, the absorbance of the reactant was read at 760 nm. The total phenolic content was then determined by referring to the calibration graph, using tannic acid solution as a standard solution.

2.3.2) Determination of antioxidant activity (Hammerschmidt and Pratt, 1978)

Dried plant material was soaked overnight in methanol at room temperature. Each flask consisted of 0.5 g sample in 10 ml of methanol. The extract was filtered through Whatman No.42 filter paper, and the residue was washed with hot methanol. The insoluble residue was discarded. The filtrate was then concentrated in a water bath at 40 °C to a final volume of 1 ml. Antioxidant activity was determined by measuring the coupled oxidation of carotene and linoleic acid. One ml of β-carotene solution in chloroform (1 mg/10 ml) was pipetted into a flask, which contained linoleic acid and Tween 40. After removal of the chloroform on a water bath at 50 °C,

50 ml of distilled water (which was bubbled by an air pump for 1 h) were added to the flask with vigorous swirling. Five ml aliquots of this emulsion were placed in test tubes which contained 0.2 ml of the extracts. Samples were read against a blank containing the emulsion minus the carotene. A reading at 470 nm was taken immediately (t=0) and then at 15 min intervals for 105 min. The bleaching rate of β-carotene was determined by the difference in the spectral absorbance reading between the initial and last reading of bleaching, (which remained essentially linear), divided by time. The antioxidant index was the ratio of the bleaching rate of the control (system with no added test compound) to the bleaching rate when a test compound was in the system.

2.3.3) Determination of antimicrobial activity (Bauer *et al*., 1966)

The antimicrobial activity of plant extracts was determined by the disc diffusion method, using bacterial or yeast cell suspensions which equilibrated their concentration to a 0.5 McFarland standard, or $1x10^6$ spores/ml of fungal spore suspension. Each bacterial suspension (100 μl) was spread on Mueller-Hinton agar plates, while yeast or fungal suspension was spread on Sabouraud dextrose agar plates. Sterile paper discs, 6 mm diameter, were impregnated with 20 μl of each test sample. The discs were allowed to dry and then placed on the agar surface of each Petri dish which had previously been inoculated with the test microorganisms. Discs with the solvents used for extraction were used as negative controls, while two standard antibiotics (10 μg streptomycin discs and 100 units nystatin discs) were used as positive controls. The plates were incubated at 37 °C (overnight) for bacteria and yeast, or at 30 °C (3 days) for filamentous fungi. After incubation, zones of inhibition

appearing around the discs were measured and recorded. The values were the average of three measurements per disc, taken at three different directions in order to minimize errors. The inhibition zones were expressed as the mean of three separation experiments.

2.4 SEPARATION AND PARTIAL PURIFICATION OF ANTIMICROBIAL COMPOUNDS FROM *Caesalpinia mimosoides* AND *Pimpinella anisum*

From the result of the screening of antimicrobial activity, it was found that the aqueous and the ethanolic extracts of *Caesalpinia mimosoides*, and the ethanolic extract of *Pimpinella anisum* (anise) possessed potent antimicrobial activities. Therefore, the phenolic compounds of these extracts were separated, partially purified, and their antimicrobial activities were examined.

2.4.1) Separation and partial purification of phenolic compounds by paper chromatography (Raymond *et al*., 1984)

The separation of phenolic compounds from the aqueous and the ethanolic extracts of *C. mimosoides*, and the ethanolic extract of *P. anisum* (anise) was carried out on two-dimensional paper chromatography. The chromatograms were developed with n-butanol-acetic acid-water (BAW) (6:1:2 v/v/v) in one direction and 2% aqueous acetic acid in the other direction. The air-dried chromatograms were examined under ultraviolet light (254 and 365 nm). The phenolic compounds on the chromatograms were visualized with Folin-Ciocalteu's reagent before fumigation with ammonia solution. The spots on the chromatograms were cut out and eluted with ethanol for UV-visible analysis in the range of 200-800 nm.

2.4.2) HPLC analysis of partial purified phenolic compounds

A Hewlett-Packard 1100 series liquid chromatograph system comprising vacuum degasser, binary pump, auto-sampler, and variable wavelength detector was used for analytical analysis of the partial purified phenolics. The column used was a C18 reversed phase Hypersil ODS (4.0x125mm, 5μm). The mobile phase eventually adopted for this study was (A) water-acetic acid (97:3 v/v) and (B) methanol (A:B = 80:20), and the flow rate was 0.5 ml/min. Absorption wavelength was selected at 280 nm.

2.4.3) Determination of antimicrobial activity by bioautography (Betina, 1973)

For bioautography, the spots on the chromatograms developed as described in the section 2.4.1 were tested for their antimicrobial activity by using the diffusion technique on solid media. The bacteria *Escherichia coli* and *Staphylococcus aureus*, and the dermatophytic fungi *Microsporum gypseum* and *Trichophyton rubrum* were used as test microbial strains. Individual compounds on the chromatograms mentioned above were cut out, and placed on plates of Mueller-Hinton agar or Sabouraud dextrose agar which had been surface spread with 100 μl of microbial suspensions. The plates were incubated at 37 °C (overnight) for bacteria, or 30 °C (3 days) for fungi. Zones of inhibition of the microbial growth could be seen around the active compounds.

2.5 PRELIMINARY IDENTIFICATION OF ANTIMICROBIAL COMPOUNDS FROM *Pimpinella anisum*

2.5.1) GC analysis of antimicrobial compounds (Santos *et al*., 1998)

Gas chromatographic analysis was performed on a Hewlett-Packard-5890 series II gas chromatograph (Hewlett-Packard) equipped with a flame ionization detector (FID) and an INNOWAX column (30m x 0.25mm ID, film thickness 0.25μm), connected to the injector port. The carrier gas (helium) was operated constantly at a flow rate of 0.5 ml/min. The oven temperature was programmed from 45-75 °C at 3 °C/min, subsequently at 15 °C/min up to 240 °C, and then held isothermally for 10 min. The temperature of the injector and detector were maintained at 220 and 240 °C, respectively. Samples were injected using the split-sampling technique with a ratio of 10:1, while an essential oil of star anise was injected with a split ratio of 50:1. The identification of the compounds was based on comparison of the retention times with those of compounds in the essential oil of star anise (*e.g.* anethole, methyl chavical, anisaldehyde)

2.5.2) GC-MS analysis of antimicrobial compounds

GC-MS (gas chromatography-mass spectrometry) analysis was performed on a Hewlett-Packard-6890 series GC-system equipped with a AT-1MS column (30m x 0.25mm ID, film thickness 0.25μm), connected to the Agilent 7683 series injector port. The carrier gas (helium) was operated constantly at a flow rate of 1.0 ml/min. The temperature of the column was 240 °C at 70-80 °C/min. Each compound was analyzed for its spectral pattern by a Hewlett-Packard 5973 mass selective detector (electron impact).

2.6 SEPARATION AND PURIFICATION OF ANTIMICROBIAL SUBSTANCE FROM *Caesalpinia mimosoides*

From the result of the screening of antimicrobial activity, the various extracts of the plant *C. mimosoides* exhibited different levels of antimicrobial activity. The strong activity was observed in the aqueous and the ethanolic extracts. Therefore, both of extracts were considered for determination of active compounds in this plant, particularly phenolic compounds. Preliminary investigation of the compounds in these extracts was carried out by TLC analysis. The chromatograms were developed using hexane-ethyl acetate-acetic acid (2:1:0.3 v/v/v) as a mobile phase. Two specific spray reagents, Dragendorff's and Folin-Ciocalteu's reagents, were used to indicate the appearance of alkaloids and phenolic compounds, respectively. Spots on the chromatogram of the aqueous extract of this plant gave a positive test (orange-brown spots) with the Dragendorff's reagent, indicated the presence of the alkaloids. Whereas, spots on the chromatogram of the ethanolic extract showed blue color after spraying with the Folin-Ciocalteu's reagent, indicated the appearance of the phenolics. Therefore, the ethanolic extract of this plant was used for further study.

2.6.1) Preparation of ethanolic extract

The dried plant material (100 g) was extracted three times with 1 l of absolute ethanol by shaking at 200 rpm for 30 min at room temperature. After filtration, the supernatants were pooled, and then evaporated for elimination of the solvent by the rotary evaporator to afford a yellow-green solid as crude extract.

2.6.2) Separation and purification of phenolic compounds by column chromatography (Amarowicz and Shahidi, 1996)

Ten ml of crude ethanolic extract dissolved in ethanol (50 mg/ml) were applied onto a column packed with Sephadex LH-20 and eluted with 95% ethanol. The column dimension was 4x60 cm, and the flow rate was 1 ml/min. Ethanolic fractions (10 ml each) were collected in test tubes placed in a RadiFrac fraction collector, and their absorbances were read at 280 nm. Eluates were then pooled into fraction 1-8 (code: F1-F8). The solvent of each separated fraction was evaporated under reduced pressure at 45 °C. Each fraction was then dissolved in ethanol at the concentration of 10 mg/ml.

2.6.3) TLC and HPLC analyses of the fractions

For TLC analysis, column chromatographic fractions (F1-F8) were spotted on an analytical TLC plate (Silica gel 60 F_{254} precoated alumina sheet). The chromatogram was developed using hexane-ethyl acetate-acetic acid (2:1:0.3 v/v/v) as the mobile phase. After drying, spots on the chromatogram were visualized under UV light (254 and 365 nm). Folin-Ciocalteu's spray reagent was also used to indicate phenolic compounds.

For HPLC analysis, the fractions were assayed on a Hewlett-Packard 1100 series liquid chromatograph system comprising vacuum degasser, binary pump, auto-sampler, and variable wavelength detector as described in the section 2.4.2.

2.6.4) Determination of antimicrobial activity

The antimicrobial activities of the crude ethanolic extract and the column chromatographic fractions in ethanol (10 mg/ml) were investigated by using the disc diffusion method as described in the section 2.3.3. Minimum inhibitory concentrations (MICs) were also investigated for the microbial strains which were determined as sensitive to the test sample in the disc diffusion assay. In this study, broth macrodilution was used (Nakamura *et al*., 1999). Serial 2-fold dilutions of the samples were prepared in 10% dimethylsulfoxide (DMSO), and 30 μl of each dilution was added to 3 ml of Mueller-Hinton or Sabouraud dextrose broth. These were inoculated with 30 μl of the cultures of microbial strains. After incubation of the cultures at 37 °C (bacteria and yeast) and 30 °C (filamentous fungi) for 48 h, the MICs values were determined as the lowest concentration of the test compounds that demonstrated no visible growth.

2.7 IDENTIFICATION OF ANTIMICROBIAL SUBSTANCE FROM *Caesalpinia mimosoides*

The antimicrobial substance of the separated fraction which exhibited antimicrobial activity was identified by spectroscopic methods.

2.7.1) Ultraviolet-visible spectroscopy

UV-vis absorption spectrum (200-800 nm) was measured using a Lambda 25 UV-vis spectrometer.

2.7.2) Infrared spectroscopy (IR)

Infrared spectrum was recorded as KBr disc on a Nicolet 510 FT-IR spectrometer.

2.7.3) Nuclear magnetic resonance spectroscopy (NMR)

Proton (^{1}H) and carbon (^{13}C) NMR spectra were recorded on a Bruker Avance-400 spectrometer (400 MHz), using deuterated acetone as a solvent and tetramethylsilane (TMS) as an internal standard, and the chemical shifts reported in δ (ppm) units relative to TMS signal and coupling constants (J) in Hz. A complete attribution was performed on the basis of two-dimensional NMR (^{1}H-^{13}C COSY or HMQC and long range ^{1}H-^{13}C COSY or HMBC).

2.7.4) Liquid chromatography-tandem mass spectrometry (LCMS/MS)

High resolution-mass spectrometry (electrospray) or HRMS (ESI) spectrum was recorded on a Micromass Instrument type QTOF 2 spectrometer.

2.8 STRUCTURAL MODIFICATION OF ANTIMICROBIAL SUBSTANCE FROM *Caesalpinia mimosoides*

Since the antimicrobial substance (fraction 4 or F4) separated from *C. mimosoides* was identified as gallic acid (3,4,5-trihydroxybenzoic acid), and it possessed a low level of antimicrobial activity, therefore, it would be interesting to modify its structure in order to enhance the activity. The structural modification was carried out by acid-catalyzed esterification with ethanol to yield ethyl gallate (ethyl 3,4,5-trihydroxybenzoate). The substance F4 (0.5 g) was dissolved in ethanol in the

presence of concentrated sulfuric acid (1 ml). The mixture was heated under reflux for 3 h, and allowed to cool at room temperature. The reaction mixture was evaporated under reduced pressure until dry. The residue was subsequently separated by partitioning between dichloromethane and saturated sodium hydrogen carbonate solution. The organic layer was then evaporated to yield the desired ester. The modified ethyl gallate was then used for structural elucidation and antimicrobial assay.

2.9 CHARACTERIZATION AND STRUCTURAL ELUCIDATION OF CHEMICALLY MODIFIED ETHYL GALLATE

The characteristic of modified ethyl gallate was evaluated by TLC and HPLC analyses as described in the section 2.6.3, and compared with those of its parent structure, gallic acid.

The structure of modified ethyl gallate was elucidated by spectroscopic method including UV-vis spectroscopy, IR spectroscopy, NMR spectroscopy, and liquid chromatography-tandem mass spectrometry as described in the section 2.7.1-2.7.4, respectively.

2.10 DETERMINATION OF ANTIMICROBIAL ACTIVITY OF CHEMICALLY MODIFIED ETHYL GALLATE

The antimicrobial activity of modified ethyl gallate in ethanol (10 mg/ml) was examined by using the disc diffusion method as described in the section 2.3.3. The MIC values were also determined by using the broth macrodilution method as described in the section 2.6.4.