CHAPTER II

EXPERIMENTAL

Materials

Materials used in all experiments of this study are:

1. Plant Materials:

The sources of plant materials mainly are from medicinal herb shops and in Chulalongkorn campus and are authenticated by the staff of the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2. Microorganisms Used:

The microorganisms used are listed in Table 3 p. 30.

Items no. 1,2 and 5 were obtained from the Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok.

Item no. 3 was obtained from the Department of Medical Sciences, Ministry of Public Health, Bangkok.

Item no. 4 was obtained from the Division of Plant Pathology, Vegetable and Ornamental Plant Pathology Branch, Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok.

Table 3

Microorganisms Used for Antimicrobial Activity

Number	Microorganism	Identification Number	Classification
1	*Staphylococcus aureus* Rosenbach	*1 ATCC 10536	Gram positive bacteria
2	*Escherichia coli* (Migula) Castellani et Chalmers	ATCC 6633	Gram negative bacteria
3	*Bacillus subtilis* (Ehrenberg) Cohn	ATCC 6538P	Spore-forming bacilli
4	*Aspergillus niger* van Tieghem	*2 K 535	Seed fungi
5	*Penicillium chrysogenum* Thom	*3 USIS 53-70	Seed fungi

*1 ATCC American Type Culture Collection.

*2 K Kasetsart University, Bangkok.

*3 USIS United States Information Service, Bangkok.

Cultures of no. 1 and 2 were maintained on Difco Antibiotic Medium I agar slant at 5°C in refrigerator.

Bacterial spore suspension (Item no. 3) was prepared by the method conforming to the British Pharmacopoeia 1963 pp. 1104 to 1105.

The seed fungi (Items no. 4 and 5) were cultured on Sabouraud's agar slants and incubated at room temperature (approximately 28°C to 30°C) for 7 days.

3. Solvents and Chemicals:

3.1	Diethyl ether (AnalaR)	BDH Chemical Ltd. Poole England.
3.2	Acetone*	- do.-
3.3	Ethyl alcohol USP.	Government Pharmaceutical Organization, Bangkok.
3.4	Distilled Water	- do.-
3.5	Antibiotic Medium I	Difco Laboratories Detroit Michigan, U.S.A.
3.6	Bacto-Beef Extract	- do.-
3.7	Bacto-Peptone	- do.-
3.8	Bacto-Dextrose	- do.-
3.9	Bacto-Agar	- do.-
3.10	Benzoic acid BP.	Monsanto Chemicals Ltd. Victoria Street. London S.W.I.

*Maximum limits of impurities acidity 0.2 ml N/1 %, Non-volatile matter 0.005 %.

4. Culture Media:

1. Antibiotic Medium I (Difco)

Bacto-Beef Extract	1.5	g
Bacto-Yeast Extract	3.0	g
Bacto-Casitone	4.0	g
Bacto-Peptone	6.0	g
Bacto-Dextrose	1.0	g

Use 30.5 g of dehydrated medium/1,000 ml distilled water, final pH 6.6 $\pm$ 0.1. Sterilized at 15 lbs/in^2 pressure for 15 minutes.

2. Nutrient broth

Bacto-Beef Extract	3.0	g
Bacto-Peptone	10.0	g
Distilled Water	1,000.0	ml

Adjust to pH 7.4 with 1 % HCl acid or 1 % NaOH solution. Sterilized at 15 lbs/in^2 pressure for 15 minutes.

3. Sabouraud's agar

Bacto-Dextrose	40.0	g
Bacto-Peptone	10.0	g
Bacto-Agar	30.0	g
Distilled Water	1,000.0	ml

Adjust to pH 5.5 with 1 % HCl acid or 1 % NaOH solution. Sterilized at 15 lbs/in^2 pressure for 15 minutes.

5. Other Materials:

5.1 Bacteriological loop (0.4 cm diameter).

5.2 Glass petri-dishes (Pyrex U.S.A.) 9 cm diameter.

5.3 "Millipore" Swinny Stainless Steel Filter Holder 13 mm diameter (Millipore Corporation Bedford, Massachusetts).

5.4 "Millipore" Prefilter, 13 mm diameter.

5.5 Glass syringe 2 ml for millipore holder.

5.6 Blotting paper discs 9 mm diameter and 0.2 mm thick.

6. Antibacterial Standards:

6.1 Amoxycillin 10 mcg/disc (Amoxil, Bencard).

6.2 Ampicillin trihydrate equivalent to ampicillin 10 mcg/disc (Amcillin, Dumex).

6.3 Chloramphenicol 30 mcg/disc (Chloramex, Dumex).

6.4 Cloxacillin 5 mcg/disc (Orbenin, Beecham).

6.5 Cotrimoxazole 25 mcg/disc (Septrin, Wellcome).

6.6 Gentamicin 10 mcg/disc (Garamycin, Schering U.S.A.).

6.7 Kanamycin sulfate 30 mcg/disc (Kanoxin, Dumex).

6.8 Penicillin G sodium 10 units/disc (Pegemex, Dumex).

6.9 Streptomycin sulfate 10 mcg/disc (Dumex).

6.10 Tetracycline hydrochloride 30 mcg/disc (Dumocycline, Dumex).

7. Antifungal Standard:

7.1 Benzoic acid 0.01 mg/disc.

Methods

1. Preparation of Plant Extracts:

The procedure employed for this preparation was derived from MacDonald and Bishop (1953).

1.1 Plant samples are fresh or dried in hot air oven at 50°C.

1.2 The dried specimens were mechanically ground to a powder and 20 grams of powder was macerated with 50 ml of distilled water for 1 hour or if the fresh specimens, 30 grams of samples were blended with 50 ml of distilled water in electric blender for 30 seconds. The product was then transferred into an Erlenmeyer flask and allowed to stand for 1 hour at room temperature (approximately 28°C to 30°C).

1.3 Strain through cheesecloth and squeeze draining. The aqueous filtrate was sterilized by passing through the millipore prefilter paper syringe.*

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* The complete set of millipore syringe is composed of 2 layer-filters, one millipore membrane and the other millipore prefilter paper.

1.4 The drained residue was then divided into three equal parts, each of which was placed in 3 separate mortars and ground for 3 to 5 minutes with 10 ml each of ethanol , acetone, and diethyl ether. The filtrate from each extract was drained through gauze and then through the steriled millipore prefilter paper syringe.

2. Preparation of Discs:

2.1 Blotting paper discs of 0.2 mm thick and 9 mm in diameter were sterilized by dry heat at 160°C for 1 hour.

2.2 Ten paper discs were aseptically absorbed with 1 ml, to make approximately 0.1 ml per disc, of each of the prepared filtrates and allowed to dry at 5°C in the refrigerator.

2.3 Control paper discs absorbed with solvent only were prepared by the same method mentioned in 2.2.

2.4 Paper discs of known chemical and antibiotic concentrations mentioned in materials (Antibacterial and Antifungal Standards) used in this experiment were also included as positive control of the tests (Table 6 p. 114).

3. Preparation of Plate Culture:

3.1 The incubation temperature of all bacterial cultures used in this experiment was at 37°C for 18 to 24 hours. Fresh culture from the stock cultures of *Staphylococcus aureus* and *Escherichia coli* was grown on Difco Antibiotic Medium I agar slants a day before the test. Tranferred one loopful fresh

culture from the slant into each of 5 ml nutrient broth and incubated for 6 hours. Prepared seed agar poured-plates at the day of the test by using 1 % of 6 hour-culture suspension in melted agar medium at 50°C and mix well to insure uniform distribution before pouring. Allow these agar poured-plates to harden at room temperature and they were ready for use.

3.2 *Bacillus subtilis* spore suspension prepared by the method mentioned earlier on page 31 was used for preparing the spore suspension agar poured-plates in the same manner as the preparation of bacterial culture agar poured-plates mentioned in 3.1.

3.3 The seed fungi, *Aspergillus niger* and *Penicillium chrysogenum* were prepared separately by inoculating onto Sabouraud's agar slants, incubated at room temperature (approximately 28°C to 30°C) for 7 days, and then the surface growth of those were aseptically scraped off by using sterile bacteriological loop, to make a spore suspension with 15 ml sterile distilled water. Transferred the suspension into sterile Erlenmeyer flask and shook thoroughly before counting the number of spores per millilitre under light microscope. Two hundred spores per plate in Sabouraud's agar poured-plates were used for each fungus.

4. Testing:

The dried discs of plant extracts, each of which consisted of four different solvents, were placed onto the surface of seeded

agar poured-plates which had been already prepared. The blank control discs of the different solvents were run along in each experiment. The antibiotic standard discs were also included for the positive controls.

Zones of inhibition were measured after incubation at $37^{o}C$ overnight (about 16 to 18 hours), except that of fungi which were observed after $37^{o}C$ overnight and left at room temperature for at least 3 more days, till the fungus-growth was confluent over the agar surface and the clear zones of inhibition were sharp. The zones diameter were measured in millimeters by a ruler, the length measured included that of the surrounding zone.