CHAPTER 2

MATERIALS, APPARATUS AND METHOD

I. Materials

Materials used in all experiments of the study are :

Solvents and Chemicals :

Petroleum ether (B.P 30-50°)	Carlo Erba Co., Milan, Italy.
Diethyl ether	E. Merck, Darmstadt, Germany.
Ethanol	Government Pharmaceutical Organization, Bangkok, Thailand.
Distilled Water	Vidhayasom Co. Ltd. Bangkok, Thailand
Bacto Beef Extract	Difco Laboratories Inc., Michigan, U.S.A.
Bacto Peptone	- do -
Bacto Agar	- do -
Bacto Dextrose	- do -
Tomato Juice Agar	- do -

Plant Materials :

The sources of plants are from the Department of Pharmacognosy, Chulalongkorn University Faculty of Pharmaceutical Sciences, and local medicinal herb shops. All specimens are authenticated by Department of Pharmacognosy, Chulalongkorn University Faculty of Pharmaceutical Sciences.

Other Materials :

1. Bacteriological loop (about 2 mm diameter)

2. Glass petri dishes (9 cm diameter)

3. Blotting paper discs (9 mm diameter) oftained from Department of Microbiology, Chulakorn University Faculty of Pharmaceutical Sciences.

4. Cefalotin standard (30 mcg/disc, diameter 6 mm). Eli-Lilly and Co., Indiana, U.S.A.

5. Streptomycin (Dumex; 30 mcg/disc), Tetracycline (Hoechst; 30 mcg/disc), Chloramphenicol (Hoechst; 30 mcg/disc), Colimycin (Atlantic; 1000 unit/disc), Neomycin (The Government Pharmaceutical Organization; 30 mcg/disc), Penicillin (The Government Pharmaceutical Organization; 10 unit/disc), Ampicillin (Beecham; 10 mcg/disc), and Kanamycin (Dumex; 30 mcg/disc), obtained from the Medical Sciences Department, Ministry of Public Health, Bangkok. (diameter disc 8 mm).

Culture Media :

1. Nutrient agar

Bacto Beef Extract	3 g
Bacto Peptone	10 g
Bacto Agar	20 g
Distilled Water	1000 g

Adjust to pH 7.4

2. Nutrient broth

All ingredients in 1 are used excluding Bacto Agar.

3. Sabouraud's agar

Bacto Dextrose	40 g
Bacto Peptone	10 g
Bacto Agar	30 g
Distilled Water	1000 ml

Adjust to pH 5.5

4. Sabouraud's broth

All ingredients in 3 are used excluding Bacto Agar.

5. Tomato Juice Agar

To rehydrate the medium, suspend 51 g of Bacto-Tomato Juice Agar in 1000 ml of cold distilled water and heat to

boiling to dissolve the medium completely. Sterilize in the autoclave for 15 minutes at 15 lb (121°C). The final reaction of the medium will be pH 6.1.

Microorganisms Used :

1. Bacillus subtilis ATCC 6633
2. Escherichia coli ATCC 10536
3. Lactobacillus fermentum ATCC 9338
4. Pseudomonas aeruginosa
5. Salmonella typhi
6. Shigella dysenteriae 115 118 1SS
7. Staphylococcus aureus ATCC 1538-P
8. Streptococcus faecalis

Items No. 1, 2 and 3 were obtained from the Department of Microbiology, Chulalongkorn University Faculty of Pharmaceutical Sciences.

Items No. 4 and 5 were obtained from the Department of Clinical Microbiology, Mahidol University Faculty of Medical Technology.

Items No. 6 and 7 were obtained from the Department of Microbiology, Chulalongkorn University Faculty of Pharmaceutical Sciences.

Item No. 8 was obtained from the Department of Clinical Microbiology, Mahidol University Faculty of Medical Technology.

All microorganisms were maintained in the laboratory by cultivating on nutrient agar slant medium excepted Lactobacillus fermentum on tomato juice agar deep medium and all were subcultured once a week.

After growth, all of these cultures were kept at 4°C in refrigerator.

II. APPARATUS

Heating Mantle	Karl Kolb Scientific Technical Supplies, Frankfurt, Germany.
Rotavapor 'R'	Buchi Co., Flawill, Switzerland
Incubator (R B 500)	Heraeus GMBH, Hanau, Germany.
Electric Blender	Karl Kolb Scientific Technical Supplies, Frankfurt, Germany.
Large Hot Air Oven	KSL, Bangkok, Thailand.

III. METHODS

1. Preparation of Extracts

1.1 Wholeplants or Plantparts (leaf, root, rhizome, stem, flower, seed) used in this study were cleaned, chopped and dried in hot air oven (50°C).

1.2 The dried specimens were blended in electric blender to coarse powder.

1.3 Fifty grams of ground materials were extracted by Soxhlet's extractor (52) with petropeum ether until exhaustion. The redisues were air dried, and reextracted with ethyl ether, then with ethanol and finally with water. The extracts were kept separately.

1.4 Each extract was evaporated under reduced pressure not higher than 50°C until dryness.

1.5 The dried extracts were weighed and dissolved in their former solvents (10% solution) in sterile container. The aliquots were used to test for antibacterial activity and some phytochemical screening. The extracts were stored at 4°C in refrigerator throughout the study.

2. Preparation of Discs for Testing

Blotting paper discs were sterilised by dry heat (160°C) for one hour and used as carriers of the solution to be tested.

2.1 0.05 ml of 10% plant extracts were aseptically introduced to discs. These discs were in presterilized petri dishes.

2.2 Discs introduced with 0.05 ml of petroleum ether, ether, ethanol and distilled water were used as control solvents. Empty-disc was used as blank control.

2.3 All discs were dried in oven (40°C) for overnight.

2.4 Antibiotic discs mentioned in material sections will then be used as antibiotic controls.

3. Antibacterial Susceptibility Testing

The procedure employed for this test was derived from Blaire et al. (1970)(8).

3.1 Preparation of inoculum

3.1.1 Inoculated the organisms to be tested on the nutrient agar slant, incubated at 37°C for overnight, and transfered one loop to a nutrient broth (10 ml/tube), incubated at 37°C for 5 hours.

3.1.2 For Lactobacillus fermentum inoculated in tomato juice agar deep, incubated at 37°C for overnight and transfered one loop to Sabourand's broth (5 ml/tube), incubated at 37°C for 5 hours.

3.1.3 Determination of the amount of bacterial cells (5 hours culture) was performed by modified technique of Miles and Misra (1938)(55).

3.2 Plate preparation

3.2.1 Added 15 ml of melting agar medium to each plate. The depth of agar was approximately 3 mm. Plates were allowed to dry for at least 30 min. before being inoculated.

3.2.2 Pipetted 0.1 ml of 5 hour culture into the prepared plate. Smeared the entire surface of the plate evenly with the sterile triangular glass rod. Allowed the plate to dry at room temperature. Discs introduced with extracts were applied with sterile flamed forceps and must be gently pressed to the surface of the agar. Each experiment was done duplicately to obtain the average value.

Control solvents and antibiotic standard discs were also performed in the similar manner.

3.2.3 The plate were incubated, not inverted, at 37 C for overnight (16-18 hours).

3.2.4 The end point of complete inhibition of growth was observed by naked eyes. The entire inhibition zone including the disc diameter was measured in mm with a ruler.

Determination of MIC was carried out for the plant extracts which gave inhibition zones to the test microorganisms by lowering the amount of 0.01 ml at a time until no inhibition zone was noticed.

4. Some Phytochemical Tests for Antibacterial Plant Extracts

Farnsworth (1966) noted that the ultimate goal in screening medicinal plants should be the isolation of responsible constituents for biological or medicinal activity. Some investigators select initially only alkaloid-containing plants for study on the premises that

a. alkaloids normally exert some types of pharmacological activity, usually on the central nervous system, but not always so;

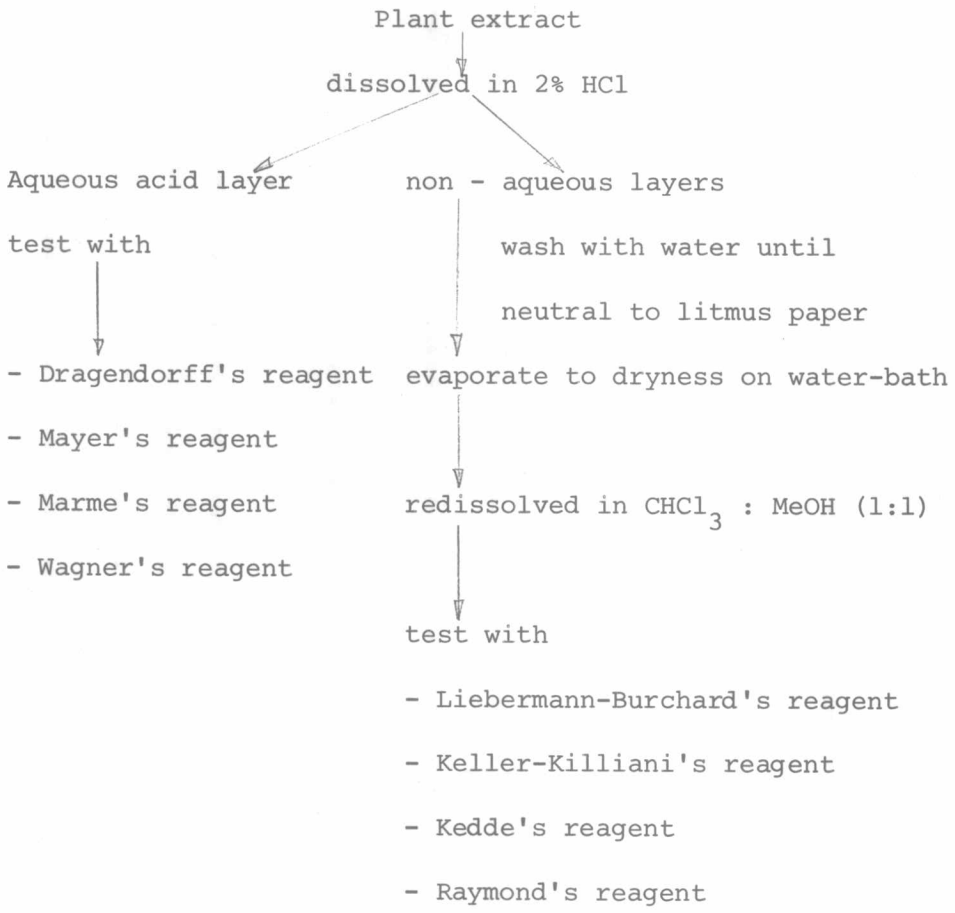
b. the greatest majority of natural products used in medicine today are alkaloidal in nature;

c. tests for the presence of these compounds in plants are

simple, can be conducted rapidly, and are reasonably reliable, and

d. because of their chemical nature, alkaloids are more easily manipulated making extraction and isolation less of a problem (29).

The procedure for chemical tests will be shown in the following schematic chart (65).



For detection of alkaloids in phytochemical screening, two types of reagents are available, i.e., alkaloidal precipitants and spray or dip reagents. Because of the variable sensitivities of alkaloidal reagents and nonspecificity for alkaloids, many investigators utilize 4 or 5 reagents in their screening of plant extracts, and only sample yielding precipitates with all reagents are considered to contain alkaloids (29).

The reagents tested in this study are Mayer's reagent, Wagner's reagent, Marme's reagent and Dragendorff's reagents as described by Stewart and Stolman (1961) in the followings.

a. Marme's reagent (Potassium Cadmium Iodide)

CdI_2 (1 g), KI (2 g) in H_2O (100 ml)

This reagent gives white or yellowish precipitates with alkaloids in dilute solution of acid. The precipitates are amorphous at first but become crystalline on standing. The precipitates are soluble in excess of the reagent and in ethyl alcohol.

b. Mayer's reagent (Potassium Mercuric Iodide)

$HgCl_2$ (1.5 g), KI (5 g) in H_2O (100 ml)

This reagent is the most generally useful of the alkaloid reagents and gives precipitates with the hydrochloride of most alkaloids in very dilute solution. The reagent should be added to solutions rendered distinctly acid with dilute hydrochloric or sulfuric acid. The solution should not contain acetic acid and more than a small amount of ethanol in

which the alkaloid precipitates are soluble. A few drops only of the reagent should be added as the precipitates with some alkaloids are soluble in excess of the reagent.

c. Wagner's reagent (Potassium Tri-Iodide)

Dissolve Iodine (1 g) and KI (1.75 g) in water (2 ml), then add 98 ml of water. This reagent gives brown flocculent precipitates with most of the alkaloids.

d. Dragendorff's reagent (Potassium Bismuth Iodide)

Solution A : 850 mg Bismuth subnitrate + 40 cc H_2O
+ 10 ml Glacial acetic acid

Solution B : 8 g KI + 20 ml H_2O

Solution A & B : are mixed and 10 ml of the mixed solution are diluted with 20 ml Glacial acetic acid + 100 ml H_2O

This reagent produce orange to reddish orange colors with most alkaloids (85).

The following tests were also applied.

e. Liebermann-Burchard's (LB)

To the dried plant extract (0.1 g) on a white tile was added acetic anhydride (0.5 ml) and then 2 drops of concentrated sulfuric acid. The LB test was regarded as positive if green, blue (formed with steroidal nucleus), red, pink or purple (formed with triterpenoids) colors were produced (29).

f. Keller-Kiliani's reagent

Place 1 ml of extract in a test-tube, added 3 ml of 3.5% Ferric Chloride in glacial acetic acid and then few drops of concentrated sulfuric acid. If the upper layer is pale green and the conjunction is brown then it is the positive test. This reagent reacts with the doexysugar moiety of cardiac glycosides (29)

g. Kedde's reagent

This reagent is 3,5 dinitrobenzoic (2%) acid and 2.5 N alcoholic-potash which react with active methylene groups as found in the C_{17}-unsaturated lactone moiety. The color of positive test is purple (29).

h. Raymond's reagent

This reagent is m-dinitrobenzene (2%) and 2.5 N alcoholic-potash which react with active methylene groups as found in the C_{17}-unsaturated lactone moiety. The color of positive test is blue (29).

Farnsworth (1966) pointed out that in a phytochemical screening program for cardiac glycosides, initial positive tests on plant extracts using any one reagent should be confirmed with reagents specific for the 2 additional reactive sites. An initial positive Keller-Kiliani reaction is indicative only of the presence of a doexy-sugar. This should be followed by a second test which might be the Lieberman-Burchard reaction for the steroid nucleus. Kedde

test which denotes an unsaturated lactone at C_{17}. Positive tests with all 3 reagents offer reasonable assurance of the presence of cardioactive glycosides (29).