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ANTIFUNGAL ACTIVITY OF CALOTROPIS GIGANTEA EXTRACTS

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
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จาก
Faculty of Graduate studies

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FACULTY OF GRADUATE STUDIES
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1989

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Thesis

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was submitted to the Faculty of Graduate Studies, mahidol University

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Finally, the author would like to express her thanks to all of those whose names have not been memtioned and to those who helped her to make this thesis reality.

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ชื่อวิทยานิพนธ์ ฤทธิ์ต้านเชื้อราของสารสกัดจากต้นรัก
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บทคัดย่อ

ในการทดสอบฤทธิ์ต้านเชื้อราของสารสกัดจากส่วนของต้น ราก ใบ และดอกของต้นรัก (Calotropis gigantea) ด้วยเอทธานอล โดยการสกัดพืชสดด้วยวิธีการหมัก และสกัดพืชแห้งด้วยวิธีการสกัดต่อเนื่อง ทดสอบกับเชื้อราที่ทำให้เกิดโรคผิวหนังในคน 8 ชนิด ด้วยวิธี Agar diffusion พบว่าสารสกัดจาก ใบ ต้น และรากของต้นรัก ทั้งสด และแห้ง ขนาดน้ำหนักของสารสกัด 5 มิลลิกรัม มีผลยับยั้งการเจริญเติบโตของเชื้อ Trichophyton mentagrophyte และTrichophyton rubrumขนาดต่ำสุดของสารสกัดที่มีผลยับยั้งเชื้อรามีขนาด 1-2.5 มิลลิกรัม

ในการทดสอบความเป็นพิษเบื้องต้นของสารสกัดจากใบรักแห้ง ด้วยเอทธานอล ต่อผิวหนังของหนูขาว เพื่อทดสอบความระคายเคือง และการทำลายผิวหนังในสัตว์ทดลอง ในระยะ 28 วันพบว่าไม่มีการทำลายผิวหนังบริเวณที่ทำการทดสอบ และน้ำหนักของสัตว์ทดลองไม่ผิดปกติไป

เมื่อตรวจสอบคุณสมบัติทางเคมีเบื้องต้นของสารสกัดจากใบรักแห้ง พบว่า สารสกัดมี cardiac glycosides, coumarins และ triterpenoids เป็นส่วนประกอบ แต่ไม่พบ alkaloid และ saponin.

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Thesis Title Antifungal activity of Calotropis gigantea extracts

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Abstract

Antifungal activities were found in the ethanolic extracts of the leave, stem and root of Calotropis gigantea againsts Trichophyton mentagrophyte and Trichophyton rubrum by agar diffusion method. The result showed that the mininum inhibitory doses of the extract of Calotropis gigantea were 1 - 2.5 milligram.

The skin test of the ethanolic extract from the dried leaves on rats was non toxic and the weight of rats were normal.

A preliminary phytochemical screening on the leaves of Calotropis gigantea indicated the presence of cardiac glycosids, coumarins and triterpenes while alkaloids and saponins were not detected.

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Chapter I

Introduction

It is well known that fungi are one of the major causes of skin diseases in man and animal. According to statistics, fungal infection account for 14 % of all skin diseases where as dermatitis eczema accounts for 36 % (The Institnte of Dermatology, 1980). Although dermatophytosis is not an important cause of death in man but the skin lesion in troduce poor personality and occasionally allergic reaction to human. The treatment of fungal infections is costly and time cousuming. At present, the treatment of fungal infections by polyene antibiotics such as amphotericin, nystatin, which have been shown to cause hemolysis *in vitro*. Therefore hemolytic anemia is a possible side effect (The Institute of Dermatology, 1978). It is the attempts of the present study to serch for novel plants in various localities of Thailand which has the possibilities of fungal treatment and no side effects. Moreover, the methodlogy of treatment have to be low and simple for the possibilities and skills of cantribute to the economic progress of the rural household.

A literature survey revealed that *Calotropis gigantea*, R. Br., known as "Ruk" or "Ruk dok", has been widely used to treat skin diseases e.g. tenea, elephantiasis, syphilistic ulceration in Thai folkloric and Indian folkloric medicine in the past (Pupipatpong, 1982)

C. gigantea, an erect perenial shrub growing chiefly in arid land, is found throughout Thailand. It is known mostly for its flowers which are often strung into garlands but no report of this plant which is commonly compounded in preparation of local remedies are reasonably believed to be active against fungus, so the study of activity of *C. gigantea* extracts to inhibit dermatophyte is very interesting.

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Literatures Survey

1. Background of Calotropis gigantea R.Br.

Calotropis gigantea R.Br., found throughout Thailand and in arid parts of Asia and Africa (Bentley and Trimen 1880), was locally known in Thailand as Ruk or Ruk dok (Pupipatpong, 1982) and in English as gigantea swallow-wort or murdar or madar (Dutta, 1974). Calotropis is a small genus of fleshy shrubs of the family Asclepiadaceae (Dutta, 1975).

The plant is a medium-sized shrub, 2-3 meters in height, the young parts are covered with appressed white hairs. The bark is pale, with thick twisted branches.

The leaves are obovate or oblong, 10-20 centrimeters long, 3-8 centrimeters wide, broadly ovate the upper ones narrower, acute at apex, rounded or cardate at base, thick usually smooth on both surfaces when mature, when young covered especially underneath with fine white dower, pale glaucous green, with a tuft or ring of setaceous hairs obove at the insertion.

The flowers are rather large, on pedicels 1-2 centri-meters long arranged in umbellate corymbs, an erect cylindrical stout peduncles about half the length of the leaves and coming off from between the leaf-bases on alternate sides of the stem bracts lanceolate, soon falling; peduncles and pedicels covered with fine white wool. Calyx is very deeply cut into 5 lanceolate, acute, spreading segments, with a ring of bristly hairs and the base inside. Corolla is shallowly cup-shaped, three times the length of the calyx, deeply cut about 3/4 down into 5 ovate, acuminate, blunt segments, valvate in the globular buds, thick, quite smooth, pink, shaded and dotted with purple. Stamens 5 are inserted near the base of the

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corolla and alternating with its lobes, filaments combined into a solid fleshy tube surrounding the pistil and united outside with the "corona" which consists of five fleshy compressed masses, rounded on the outside at the tops which are somewhat bifid, and produced at the bases into short blunt turned-up spurs or horns (Bentley, 1880).

The fruit (a follicle) is recurved, and 7.5-10 centrimeters long. The seeds are ovale, 5-6 millimeters long, and furnish with a bright, silky-white coma (Quisrembing, 1951).

Calotropis procera Act. f. is another species which has similar morphology to C. gigantea, but the former is more nearly a tree, with some what larger, softer leaves (Burkill , 1966) , and slightly different flowers with its flat corollas and conical flower buds (Bently,1880), however, both are used medicinally in the same way (Burkill , 1966).

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2.2 Medicinal Properties and Uses of C. gigantea

The medicinal properties of C. gigantea were known in India from the earliest time (Chopra, 1958). The dried root-barks of this plant were official in the Pharmacopeia of India (Quisumbing , 1951).

Stem :- The extract from the stem-bark seemed useful in wide cases of dysentery like ipecacuanha, but could not be regarded as a specific in all cases had a tendency to produce vomiting and depression (Burkill , 1966).

Root :- In India, The Hidu physicians considered the root bark as a valuable remedy in skin diseases, enlargement of abdominal viscera, intestinal worms, cough, ascites, etc. (Chopra, 1958). The usual dose of the powdered bark as an alterative is 3-10 gm, twice a day. In a dose of 30-60 gm, the root bark acted as an emetic and had been used as a substitute for ipecacuanha (Bentley and Trimen, 1880). The root bark in the form of a paste, was applied in elephantiasis. The fresh or dried juice of root bark, forcing the juice the throat was a common method of infanticide or applied locally to procure abortion (Quisumbing , 1951 and Chopra , 1958).

Flower :- The flowers and leaves might be boiled, and the decoction was drunk as a stimulant (Burkill, 1966). Powdered flowers, in small doses, were useful in the treatment of colds, coughs, asthma and indigestion (Quisumbing , 1951).

Leaf :- The dried juice, a fluid from the leaves, was used like the bark and flowers. The diseases treated were intermittent fever, dysentery, leprosy, elephantiasis sysphilis, and rheumatism. In small dose it was alterative, in larger doses emetic, and in still larger doses poisonous. The

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leaf was applied for poulticing sores. It was burned, and the smoke inhaled, for ulceration of the nose (Burkill, 1966). Extracts gave strongly positive antibiotic tests (Watt and Breyer , 1962).

Latex :- The latex was a strong irritant to the skin and mucous membranes. The milky juice was regarded as a drastic purgative: it was dropped into wounds and into hollow aching teeth, recommended for ringworm of the scalp (Chopra ,1958). In India, this latex was used externally for skin infections and as a depilatory (Quisumbing, 1951). In the Dutch Indies and the Malay Penninsular, the latex was used for similarly to syphilitic sores, wounds, and as an abortifacient and an infanticide (Watt and Breyer , 1962). An extract of the latex caused the slowing of the heart and acute gastroenterities when injected into the lymph sac of frogs; it was highly toxic to dogs and donkeys when it was given orally or subcutaneously (Chopra , 1958). It acted similarly to histamine on smooth muscle, cardiac muscle, and increase blood pressure of albino rat, gunea pig, rabbit and dog (Saha , 1963).

In Thailand, C. gigantea has been used in indigenous medicine in the treatments of many diseases, as follows:

latex for killing earth-worm,

flower for killing tenea,

leaves for the treatment of leprosy and fistula,

root for retieving intermitten fever and a cure for bloody stool.

stem as an emetic, and

fruit for treating dandruff and as a stimulant (Sopis and Mougthira , 1981)

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In Thai folk medicine, the latex of C. gigantea was applied to the fish which was then grilled and eaten in the bellef that this could kill the earth-worm in the gastrointestinal tract (Pupipatpang, 1982). The extract from the air-dried bark of C. gigantea had been tested on earth-worms for its anthemintic property. It could kill all the earth - worms in two hours (Rasanondha , 1948).

3. The chemical composition of C. gigantea

3.1 Chemical composition of the stem

The stem bark was found to contain α and β - calotropeols, β - amyrin, giganteol, a colourless wax, small amounts of tetracyclic terpenes, and traces of sterols (Muti and Seshadri, 1943 ; Muti and Seshardri, 1945).

In 1948, Raviwan studied the constituents of stem extraction of C. gigantea and found that glycosides and proteolytic enzymes were the main components.

3.2. Chemical composition of root

The root bark was found to contains β - amyrin, 2 -isomeric crystalline alcohols, giganteol (m.p. 223°c - 224°c) and isogiganteol (m.p. 117 °c - 178°c), which were probably dihydric, giving colour reactions similar to those of calotropeols (Muti and Seshardri ,1945).

3.3 Chemical composition of flower

Esters of β - calotropeopls, β - amyrin, volatile and long chain fatty acids and esters of waxy acids and alcohols were reported in the flower (Muti and Seshardri , 1945)

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3.4 Chemical composition of latex

The latex contained 86.0-95.5% of water and soluble matter and 0.6-1.0% of caoutchouc. The coagulum consisted of 5.1-18.6 % , 73.6-87.8% and 4.5-13.8% of caoutchouc, resins and insoluble matter respectively. Two isomeric resinols of latex are α - colotropeol, $C_{30}H_{50}O$ (m.p. 204°c - 205°c), and β - calotropeol (m.p. 216°c-217°c) mainly in ester combination with acetic and isovaloeric acids, and β - amyrin. In 1943, Murti and Seshadri found gigantin which was the chemical substance in latex. The main compositions of gigantin were nitrogen and sulphur which depressed heart and acted as a poison to fish. In 1938, Ganapati and Sastri found that the latex contained trace amounts of glutathione and proteolytic enzyme similar to papain.

4. Pathogenic fungi

There are many species of fungi which cause infectious diseases in man (about 100 species are known). It is useful to divide the mycoses into four groups, differing in the levels of the infected tissue :-

1) systemic or deep mycoses
2) subcutaneous mycoses
3) cutaneous mycoses
4) superficial mycoses

The dermatophytes which cause cutaneous mycoses have a striking predilection for growth in the epidermis, hair and nail. Three genera of fungi which are dermatophytes are

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Trichophyton, Microsporum and Epidermophyton.

Many dermatophytes are found in mammalian skin and only two or three of the pathogenic species has been found in soil and are acquired by man from contaminated soil or from animal tend to produce relatively intense. The skin lesion, in human skin, is usually roughly circular tend to expand equally in all directions, and has raised serpiginous borders. It is called ringworm, or tinea or dermatophytosis. The names are usually qualified by the area of the the skin involved eg. ringworm of the scalp is called tinea capitis, of the body tinea corporis, of the feet tinea pedis and of the intertriginous areas tinea cruris.

These infections are usually eradicated by griseofulvin, but the response varies with the thickness of the keratin (a fibrous and very insoluble protein which dermatophytes depended on) and the rate of its replacement. Infections of the scalp and smooth skin are usually cured after several weeks of therapy, but infections of the feet, especially of toenails, require many months of continuous treatment.

Though most dermatophytes are epidermal parasites in nature, most species grow well in simple media, with ammonium salts and glucose as sole sources of nitrogen, carbon and energy. Growth is more vigorous, however, in media enriched with protein or amino acids. Sabouraud's agar is the most widely used medium for cultivating of the fungi.

Candidiasis is an acute or secondary infection of the skin or mucous membrane caused by a yeast, Candida albican and other species of Candida. The skin lesions appear in isolated vesicles surrounded by redness, in granulomatous infections; it might become

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crusted and hypertrophic at scalp, face, body, and extremities.

The candidiasis is usually treated with nystatin, amphotericin - B, miconozole and clotrimazole (Rippon, 1982)

The method of antifungal testing

The three methods which are most widely used to determine the susceptibility of microorganisms to antimicrobial agents are the agar diffusion method, the agar plate dilution method, and the broth test tube dilution method. The choice of technique to be employed in a susceptibility test depended upon the application of the results. The principle of any diffusion method as stated by Blair et al. (1970), was dependent upon the inhibition of the growth of a microorganism on the surface of, or in, an inoculated agar plate, by the antimicrobial agent that diffused into the surrounding medium from a depot on the surface of, or in, the medium. The resulting area of inhibition or zone was dependent both upon the diffusibility of the agent in the medium and the degree of susceptibility of the microorganism (Disyaboot , 1977).

Objectives

1. To study antifungal activities of the ethanolic extracts from various parts of C. gigantea , against eight fungi which causes skin diseases.
2. To investigate the toxicity of these extracts on the skin
3. To study the chemical composition of the leaves of the plant

Scope of the study

The four parts of C. gigantea used in the study are the leave, stem, root and flower. These were extracted with ethanol using a continuous extraction method for dried plant sample and maceration for fresh plant sample.

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The method of antifungal testing is agar diffusion method (Blair et al. 1970) with griseofulvin as standard antifungal agent. Eight microorganisms which are the main causes of skin diseases used in the experiment are as follows :

Trichophyton mentagrophyte
Trichophyton rubrum
Trichophyton tonsurans
Microsporum gypseum
Microsporum audounii
Microsporum canis
Epidermophyton floccosum
and Candida albicans

The rats used in the primary irritation test are Wistar strain

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Chapter II

Experimental

The experiments are divided into three parts.

Part 1 Plant extraction and antifungal testing of plant extracts

2 Test of primary irritation on rats

3 Preliminary phytochemical screening on the leaves of C. gigantea

Part 1 : Plant extraction and antifungal testing

1.1 Materials

Solvents and Chemicals

Ethanol	Goverment Pharmaceutical Organization Bangkok, Thailand.
Distilled Water	Vidhayasom Co.Ltd Bangkok Thailand
Griseofulvin	General Drugs Thouse Co.,Ltd. Bangkok, Thailand.

Culture Media

Sabouraud dextrose agar (S.D.A.) Difco Laboratories, Detroit, Michigan, U.S.A.

Instruments

Autoclave	Hirayama Manufacturing Corporation, Japan
Hot Air Over	Elektro Helios Type 282 08, Sweden (KSL, Bangkok Thailand)
Electric Blender	Karl Kolb Scientific Technical Supplies, Frankfurt, Germany.
Soxhlet apparatus	
Incubator	Memmert, West Germany
Rotary evaporator	Buchi Co., Flawil Germany

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Organisms Used :

Trichophyton	mentagrophyte
Trichophyton	rubrum
Trichophyton	tonsurans
Microsporum	gypseum
Microsporum	audounii
Microsporum	canis
Epidermophyton	floccosum
Candida	albicans

The microorganisms were obtained from the Department of Microbiology, Faculty of Medical Sciences, Chulalongkorn University, Bangkok. All microorganisms were maintained in the laboratory by cultivating on Sabouraud 's dextrose agar slant and all were subcultured for two weeks at room temperature.

Plant Materials

C. gigantea was mainly collected from the Faculty of Environment and Resources Studies, Mahidol University at Salaya Campus.

1.2 Method

1.2.1 Preparation of Plant Extracts

The extracts were prepared from fresh and dried plant parts namely the leaves, stems, roots and flowers. The specimens were cleaned and chopped

-: The extracts from fresh sample

Each part of plant was finely chopped and homogenized in a blender for three mimutes. The plant material was then macerated with ethanol for three day at 15° c. The solution was filtered through four layers of gauzes followed by whatman no.1 filter paper. The filtrate was evaporated in the vacuum until dry.

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The dried residue was dissolved in ethanol in the concentrations of 25, 50, 100 and 150 milligrams per millilitre. The extracts were stored at 4° C throughout the study and used for antifungal test.

-: The extracts from dried sample.

Plant parts used in this study were cleaned, chopped and dried in hot air oven about 45° C

The dried specimens were blanded in electric blender to a coarse powder. Ten grams of ground materials were extracted in a soxhlet extractor with ethanol for 24 hours.

Each extract was evaporated to dryness under reduced pressure at a temperature not higher than 50° C. The dried extracts were dissolved in ethanol in sterile container. The extracts were stored at 4° C throughout the study.

1.2.2 Antifungal testing

The agar diffusion method employed in this study was derived from Blaire et.al.1970

Preparation of agar slant for testing

Sixty-five grams of sabouraud dextrose agar was suspend in 1000 milliliter of distilled water, mixed throughly to give a uniform suspension and heated to boiling with frequent agitation until the medium was completely dissolved.

This solution was sterillized by autoclave at 121°c, 15 lbs pressure, for 15 minutes. Eight milliliter of steriled medium were dispensed in screw-capped tube, cooled to room temperature in slanted position.

The fungus to be tested was inoculated on the nutrient agar slant at 30 + 1°c for two weeks.

Plate preparation

The sabouraud dextrose agar was prepared at the same ratio as agar slant by aseptic method. Twenty milliliter of the mixture were poured into ten centrimeter diameter plates. The depth of agar

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was approximately three millimeter. The plates were cooled to room temperature to let it set for total of five sets.

The fungus was transfered from agar slant with the minimum amount agar material in order to have maximem amout of fungus. The fungus was tranfered to the steriled and dried hand homoginizes which contained 0.9 % sodium chloride solution and two drops of tween 80 and mixed well.

This solution was added into 90 milliliter of steriled sabouraud dextrose agar at temperature 45° - 50°c. This mixture was poured in to those five plates about 15 milliliter each and allowed to set. After the mixture was setting, made a hole about 5 millimeter in diameter and drop 0.1 millileter of C. gigantea extracts and then these plates were incubated in Ultraviolet incubator at 30 + 1 °c temperature for 48 hours. Solvent control and griseofulvin were applied to the agar plates similar manner.

The end point of complete inhibition of growth was observed by eye.

The inhibition zones were measured by using the diameter of the zone in 8 directions and value used was an average of these measurement.

Part II Test of Primary Irritation on Rat

2.1 Preparation of the extract.

The dried extract of dried leave was weighed and dissolved in ethanol (20 % solution) in sterile container. The extract was stored at 4° C throughout the study.

2.2 Preparation of the rats.

The rats used in this test were Wistar strain rats obtained from National Laboratory Animal Centre, Mahidol University. There were 24 males and 24 females, at the age of 4 weeks. In the test, the rat were divided into 4 groups and each group comprises of 4 males and 4 females. Eight of them were applied by the extract of dried leave of

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C. gigantea, two of them were applied by ethanol (solvent control) and two were control. The first, second, third and fouth groups were applied by the extract for 1, 2, 3 and 4 weeks respectively as follows:

Treatment / Duration	The extract		Control			
	Number of		Solvent control		Control	
			Number of		Number of	
	Male	Female	Male	Female	Male	Female
1 week	4	4	1	1	1	1
2 week	4	4	1	1	1	1
3 week	4	4	1	1	1	1
4 week	4	4	1	1	1	1

Twenty four hours before beginning of the test, an area of about 2.5 x 2.5 cm. of hair of the rat was cut at the neck (near the head) by scissors, except the control rats

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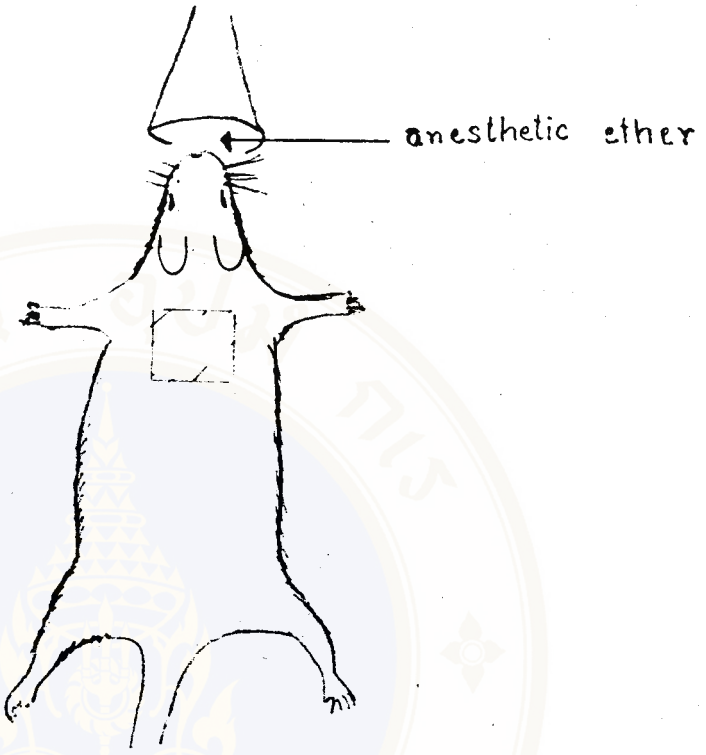

Preparation of rat for skin test

2.2 Method

0.1 ml of the extract of C. gigantea was applied to the skin after which this area was covered with gauze. The response of the rat's skin to the extract was observed and the extent of inflamation and hair growth on the skin recorded every day.

Part III Preliminary Phytochemical Screening of the Leaves of Calotropis gigantea

3.1 Extraction

One hundred grams of dried powdered leaves of C. gigantea was placed into a 500 ml Erlenmeyer flask and macerated with 300 ml of 80% ethanol, refluxed on hot bath for 1 hour, the extract filtered and concentrated to 100 ml.

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3.2 Phytochemical Screening Procedure (Farnsworth , 1966)

3.2.1 Screening for Alkaloids

A. Preliminary Alkaloid Test

The extract (25 ml), was evaporated to a syrupy consistency over a water-bath. A 15 ml aliquot of 2 N hydrochloric acid was added to the syrupy residue and stirred while heating on a water-bath for 10 minutes. The concentrated extract was the filtered and diluted to 18 ml with 2 N. HCl. The filtrate was divided into two portions, portion A (3 ml) and B (15 ml). Portion A was divided into 6 aliquotes and to each was added the following reagents : Valser's, Wagner's, Dragendorff's, and Marme's reagent (positive indication : white, brown, orange, and white precipitate, respectively). The results were recorded as (+) for slight turbidity or opacity, (++) for difinite turbidity but no flocculation, (+++) for heavy precipitate or heavy flocculation.

B. Confirmed Alkaloidal Test

To portion B (15 ml), 28% W/V ammonium hydroxide was added to render the solution alkaline to litmus paper. The alkaloids were extracted from the alkaline solution with 2X10 ml portions of chloroform. The alkaline residue was later tested for quarternary and amine oxide bases. All the chloroform extracts were combined and concentrated on awater-bath. The extract was tested for the presence of alkaloids by spotting the extract onto a piece of filter paper. After air-drying, the spot was visualized with Dragendorff's spray reagent. The residue was again dissolved in 3 ml of 2 N. HCl, filtered and tested for the presence of alkaloids with four alkaloidal reagents as above.

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C. Test for Quarternary and / or Amine oxide Bases

The alkaline extract remaining in the separatory funnel in step B was tranfered to a small beaker and 2 N. HCl was sufficiently added to render the solution acid to litmus paper and then filtered to obtain a clear solution. The clarified extract was then tested for alkaloidal reagents as in step A and B.

3.2.2 Screening for Cardiac Glycosides

To 10 ml. of 80% ethanolic extract, 40 ml. of 10% lead acetate solution were added. The mixture was gently boiled for 15 minutes, cooled and filtered. The filtrate was extracted with 3 successive 30, 20, 20 ml portions of chloroform. The chloroform extracts were combined and dried with a small amount of anhydrous sodium sulfate. After the anhydrous sodium sulfate was filtered off, the filtrate was concentrated to 7 ml and cooled.

A. Detection of the Steroidal Moiety

Liebermann-Burchard Test : About 2 ml of concentrated filtrate were evaporated almost to dryness on a water-bath. To the cooled residue 3 drops of acetic anhydride was added and mixed well, then 1 drop of concentrated sulfuric acid was added onto the side wall of the dish, and the two solutions allowed to mix gently. The color change was observed immediately and over a period of 1 hour. The colors produced vary slightly from compound to compound.

B. Detection of Unsaturated Lactone of C 17 (butenolide)

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Two milliliters of the concentrated filtrate were evaporated to almost dryness on a water-bath, and cooled. Kedde's

reagent (1 ml) was then added to the residue, together with a few drops of 1 N. NaOH. The appearence of a blue or violet color is indicative of the presence of unsaturated lactones.

C. Detection of Deoxy Sugar

Keller-Kiliani Test : Two milliliters of the concentrated filtrate were tranfered into a test tube, 3 ml of ferric chloride reagent was added, the solution mixed well and allowed to stand for a few minutes. With test tube held at 45° angle, 1-2 ml of concentrated sulfuric acid was carefully added so that it ran down the wall of the tube forming a separate layer at the bottom. On standing a brown color at the interfere (due to deoxy sugar) and pale green color in the upper layer (due to the steroid nucleus) is the indicative of the presence of cardiac glycosides.

3.2.3 Screening for Saponins

The Froth Test About 100 mg. of dried leaves of plant was placed in the test tube together with 5 ml of water, the suspension was then heated on a water-bath for 5 minutes. The mixture was immediately filtered. After the filtrate was cooled. It was vigorously shaken for one minute. The appearence of a characteristic honey-comb froth, which persists for at least 30 minutes is a presumptive evidence for the presence of saponins.

The Hemolysis Test : About 1.0 g. of dried powder was warmed with normal saline solution on a water-bath, the mixture filtered and the filtrate allowed to cool. A 2 ml portion of 2-3 % solution of washed red blood cells in normal saline solution was placed into each of the two test tubes. To one, 5 ml of normal saline solution was added and served as a control. To the other, 5 ml of the

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seed extract in normal saline was added. The tubes were gently shaken. The presence of saponins will cause the lysis of the red blood cells and the release of the red pigment into the solution thus giving a clear red solution while the control tube remain relatively opaque.

Liebermann-Burchard Test : To 5 ml of 80% ethanolic extract, 10 ml of diluted sulphuric acid were added and the mixture boiled for 15 minutes. After cooling, the hydrolysate was transferred into a separatory funnel and extracted with 15 ml of chloroform. A small amount of anhydrous sodium sulphate was added to the chloroform extract and the mixture filtered through dry filter paper. The filtrate was evaporate to dryness on a water-bath. The Liebermann-Burchard test was performed as described in cardiac glycosides section. In general a blue or blue green color is formed with steroidal saponins and red, pink or purple color results if triterpenoids are present. However, there is great variation in the colors produced.

3.2.4 Screening for coumarins

Three grams of the powder were placed in a 100 ml flask and moistened with water. The mouth of the flask was covered with a disk of filter paper moistened with 5 N. sodium hydroxide solution. The flask was then covered firmly with a piece of aluminium foil and then placed on a water-bath for 30 minutes. The filter paper was exposed to ultraviolet light. If coumarin is present, a yellow-green fluorescence appears within a few minutes.

3.2.5 Triterpenoid screening

- The experiment was carried out the same as Liebermann-Burchard Test on page 18

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Chapter III

Results

Part I The results of *C. gigantea* extraction and antifungal testing of plant extracts.

The colors of the crude extracts varied with the parts of the plant. It gave a deep green when extracted from leaves, green from stems, clear - violet from flower and yellowish brown from roots. After being concentrated under reduced pressure the residue was sticky and the color changed from green and yellowish brown to dark brown, except for the extracts from flowers where a clear - violet color resulted when evaporated and residue was not sticky.

The weight of dried crude ethanolic extracts from 100 grams of fresh samples were 0.1611 - 0.4330 grams (see Table 1) and from 10 grams of dried samples were 0.4324 - 1.4100 gram as shown in Table 2)

Table 1 The weight of dried crude extracts from 100 grams of fresh samples.

Part of *C. gigantea*	Weight of extract (gm)	% yield
Leave	0.2262	0.23 %
Stem	0.3455	0.35 %
Root	0.1611	0.16 %
Flower	0.4330	0.43 %

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Table 2 The dry weight of crude extracts from 10 grams of dried samples

Part of C. gigantea	weight of extract (gm)	% yield
Leave	1.4100	14.1 %
Stem	0.4324	4.3 %
Root	0.7256	7.2 %
Flower	1.0938	10.9 %

The results of antifungal testing

The concentration of the crude extracts used was 50 milligram / milliliter (5 milligram/well) and that of griseofulvin 200 and 300 microgram/milliliter (20 and 30 microgram/well)

The antifungal activity of ethanolic extracts from fresh and dried parts of C. gigantea against eight fungi are presented in Table 3

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Table 3 Antifungal activity of ethanolic extracts from the fresh and dried parts of C. gigantea

Extracts		Intibition Zone (cm.)							
(50 mg/ml)		T.ment	T.rubrum	T.ton	M.gyp	M.canis	M.aud	E.floc	C.alb
Fresh	Leave	0.7	0.9	-	-	-	-	-	-
	Stem	0.7	0.8	-	-	-	-	-	-
	Root	0.6	0.8	-	-	-	-	-	-
	Flower	-	-	-	-	-	-	-	-
Dried	Leave	0.8	1.0	-	-	-	-	-	-
	Stem	0.7	0.8	-	-	-	-	-	-
	Root	0.7	0.7	-	-	-	-	-	-
	Flower	-	-	-	-	-	-	-	-
Solvent control		-	-	-	-	-	-	-	-
griseofulvin 30		1.8	2.2	1.1	1.8	1.8	2.0	1.8	-
(µg/well) 20		0.8	1.0	0.9	0.9	0.9	1.1	1.2	-

(- No zone)

T. ment	-	Trichophyton	mentagrophyte
T. rubrum	-	Trichophyton	rubrum
T. tan	-	Trichophyton	tonsuran
M. gyp	-	Microsporum	gypseum
M. canis	-	Microsporum	canis
M. aud	-	Microsporum	audounii
E. floc	-	Epidermophyton	floccosum
C. alb	-	Candida	albicans

The data shown in Table 3 indicated that the ethanolic extracts from the fresh and dried leave, stem and root of C. gigantea were effective against T. mentagrophyte and T. rubrum at the concentration of 50 milligram/milliliter. However, none of the extracts showed inhibitory effect on the other 6 fungi, namely T. tonsuran, M. gypseum, M. canis, M. audounii ,E. floccosum and C. albicans, at the concentration used. The extract from the flower appeared to possess

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no antifungal activity.

From the preliminary results in Table 3, it can be concluded that antifungal agent may be present in the leave, stem and root but not in the flower.

To study the relationship between the concentrations of the extracts and the inhibition zones, tests were carried out using different concentrations of extracts and the results are shown in Table 4 and 5

Table 4 Antifungal activity of various concontration of the extract against T. mentagrophyte

Concentration of the extracts (mg/ml)	Inhibition zone (cm.)					
	Fresh			Dried		
	Leave	Stem	Root	Leave	Stem	Root
150	0.7	0.8	0.8	0.8	0.8	0.7
100	0.7	0.7	0.7	0.7	0.7	0.7
50	0.7	0.7	0.6	0.7	0.7	0.7
25	0.5	0.5	0.5	0.6	0.5	0.5
10	0.5	0.5	0.5	0.5	0.5	0.5
control	0.5	0.5	0.5	0.5	0.5	0.5

Table 5 Antifungal activity of various concentration of the extracts against T. rubrum

Concentration of the extracts (mg/ml)	Inhibition zone (cm.)					
	Fresh			Dried		
	Leave	Stem	Root	Leave	Stem	Root
150	0.9	0.9	1.0	1.0	0.8	0.8
100	0.9	0.9	0.9	0.9	0.8	0.7
50	0.9	0.8	0.7	1.0	0.8	0.7
25	0.5	0.5	0.7	0.7	0.8	0.5
10	0.5	0.5	0.5	0.5	0.5	0.5
control	0.5	0.5	0.5	0.5	0.5	0.5

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As can be seen in Table 4 and 5, the increase in the concentration of the extracts did not appear to result in the increase in the diameter of the inhibition zones.

Part II The result of primary skin test

The ethanolic extract in a dose of 0.2 ml was applied on skin of rats in four groups every day for 1 to 4 weeks.

The extract had no inflamatory effect on the skin of rats. The body weight appeared to increase at the normal rate (The final weight of every rat was between 190-220 grams) and the hairs grew normally.

In conclusion, the extract from dried leave had no adverse effect on the skin of rats.

Part III The results of Preliminary phytochemical screening of the leaves of C. gigantea

Crude ethanolic extracts of the leaves of C. gigantea was screened for various chemical constituents and the results are presented in Table 6

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Table 6 Phytochemical Screening of the leaves of C. gigantea

Test	Results
Alkaloid	
Marme's reagent	-ve
Dragendarff's reagent	-ve
Valser's reagent	-ve
Wagner's reagent	-ve
Cardiac glycosides	
Liebermann-Burchard test	+ve
Kedde's test	+ve
Keller-Kiliani reaction	+ve
Saponins	
Froth Test	-ve
Hemolysis Test	-ve
Liebermann-Burchard test	+ve
Coumarins	
Fluorescence Test	+ve
Triterpenes	
Liebermann-Burchard Test	+ve

From the preliminary results in Table 6, it can be concluded that cardiac glycosides, coumarins and triterpenes may be present in the leaves of C. gigantea. There were no alkaloid nor saponins in this plant.

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Chapter IV

Discussion

A survey of the literature showed that no investigation on the antifungal activity of calotropis gigantea has been reported, thus this is the first report of such finding.

The studies of antimicrobial activities of the plants varied from one worker to another. For example, Disyaboot et. al. (1976) and Chumsri et. al. (1977) reported possitive results for Acacia auriculoefacformis A. Cunn. against Staphylococus aureus but negative result was obtained by Lohakachornpan (1978). The study of antifungal activity of C. gigantea had been rarely tested. However, Lokakachornpan found that the extracts of the leaves of C. gigantea extracted with ethanal, acetone, diethyl ether and distilled water showed no activity against Aspergillus niger and Penicillium chrysogenum. In the present study, it is indicated the extracts from fresh and dried leave, stem and root of C. gigantea had activities against T. mentagrophyte and T. rubrum. In many cases, it might happen that plant showed antimicrobial activity by one worker but the other might get the opposite result.

The discrepensis in the results of the antimicrobial testing could be caused by the following factors:

-: Condition of the test plant.

-: The method of the extraction.

-: Antimicrobial substance within the plant.

-: The effects of the techique used in testing.

-: Condition of the test plant.

Although, many plant antimicrobial substances disappeared from the tissue as the plant dry out (Osborn, 1943). for example allicin in garlic (Cavallits, Bailey and Buch, 1945), but other antimicrobial substances may also develop in the tissue on drying (Skinner, 1955) In this study, the extracts from dried plant were more effective than fresh plant.

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-: The method of the extraction

The method of the extraction had to be simple and could get the active substance from plant, but if the amount of active constituent in plant was very low, a positive result could hardly get by simple method.

It was stated that hot extracts might be more toxic to microorganism than cold extracts (Shinner, 1955). But heating may tend to destroy enzyme and lead to the production of only inactive extracts such as allicin in garlic (Cavallito, Bailey and Buck ,1945).

Antimicrobial substances had been demonstrated in the aqneous extracts of a great number of plants. Nevertheless, not all antimicrobial substances in plant tissue were soluble in, or directly extractable with water. For example, the study of Acharalith in 1973 showed that the choloroform extract from Kaempferia pandurata was active against E. floccosum while the water extract had no activity.

-: Antimicrobial substances in plant.

Antimicrobial substances very greatly as to their potency and distribution within plants (Cavallito, Bailey and Kirchner , 1945 and Osborn ,1943). For example it was found that extracts of the flowers of Datura metel L. inhibited the growth of test microorganisms but the green leaves and fruits showed negative test (Lohachornpan ,1978). In this study, it was found that the extracts from leaves, stem and roots inhibit the growth of fungus more than the extract from flowers (Table 3).

The possible reason for the divergence of some results between different workers may be due the seasonal variation in the antimicrobial substances content of certain plants (Hayes , 1946)

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Other contributing factors are the difference in the ceye of the plant samples, location of collections and variation in soil compositions.

-: The effects of the technique used in testing of antimicrobial activity.

The detection of antimicrobial substances are usually carried out by means of standard in vitro methods because they provid simple and rapid methods for detection and do no necessitate the use of large quantity of extracts. Therefore in this study, the agar diffusion method was employed. However, the agar diffusion method has sertain disadventages in that the inhibition is wholely dependent upon the rate of diffusion of the antimicrobial substance through the agar (Disayaboot 1977). Therefore, the antimicrobial substance that can not diffuse throug agar, will give a negative result. In general, the diffusion method is less sensitive than the dilution method and the disc diffusion less sensitive than the well method.

From the results of the antifungal test, it can be seen that the extracts of C. gigantea showed activity against T. mentagrophyte, and T. rubrum in the concentration range of 50-150 milligram/milliliter (Table 3) which was equivalent to 20 microgram of griseofulvin. However the inhibition zones did not increase with the increase in extract concentration. This could be due to the slow diffusion rate of the antifungal substance. Therefore, the activity should be confirmed using other methods, e.g. broth dilution method.

In comparison to previous reports on the antifungal activity of other plants, the extracts of C. gigantea can not be regaeded as highly active. For instance, was found antifungal activities of 1 milligram of the extract from tea seed to be equal to be equal to 33 microgram of griseofulvin (Laonapibul and Tosuknowong, 1983) and the extracts from 10 gram of Alpinia siamensis and Kaempferia pandurata

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had activity equivalent to 0.01 gram of griseofulvin against T. mentagrophyte (Acharalith 1983). For this reason, the antifungal substance that presented in C. gigantea had less than the other plants.

In the result of primary rat 's skin of rat test was shown that ethanolic extract from dried leaves of C. gigantea was applied daily for 4 weeks had no toxic to rat's skin. It may be does of the extract was no more effect. The last weights of all rat were increased in normal rate, it may be cardiac glycoside was not absorbed into dermal. Hair of them was normal growth that indicated the extract had no toxic substance

The results of the phytochemical screening indicated the presence of cardiac glycaside and triterpenoid - type compounds in the ethanolic extract of the leave of C. gigantea which is in agreement with previous findings (Chen, Brown and Wooth 1938; Rasanondha and Suvagoudha, 1949 ; Bhaskara and Seshadri, 1943). The presence of coumarins was based on the result of the fluorescent test only and, thus, requires confirmation using more specific tests.

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Chapter V

Conclusion and Recommendation

There were many plants in various localities that have been used for medicinal purposes. C. gigantea was a one of plant that has been reported to be useful for the treatment of skin disease, cold, coughs, asthma (Burkill, 1966). In this study the ethanolic extracts of the stems, leaves, flowers, and roots were tested with 8 fungi using the agar diffusion method.

The results showned the ethanolic extracts of the leave, stem and root of C. gigantea to be effective against two of the eight organisms tested. These were T. mentagrophyte, and T. rubrum, both showing similar degrce of sensitivity toward C. gigantea extracts. Extracts from the flowrs, on the other hand, appeared to possess no antifungal activity.

In a preliminary toxicity test, the ethanolic extract from dried leaves of C. gigantea showed no effect on rat 's skin over a 4 week peroid.

The chemical screening of C. gigantea showed that cardiac glycosides, coumarins, and triterpenes were present in the extract while alkaloid and saponin were not detected.

In conclusion, it would be appropriate to mention that the results obtained in this study may have some contribution to medical science. However, more precise and confirmative data are needed such as purification of the active constituents and in vivo study. Hopefully this screening would stimulate further attempt to use this plant for other remedial purposes.

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Appendix

Culture Media

Sabouroud Dextrose Agar

Bacto - Destrose	40.0	gram
Bacto - Peptone	10.0	gram
Bacto - Agar	30.0	gram
Distilled Water	1000	ml.

1. Suspend 65 gram of powdered medium in 1000 milliter of distilled water and mix throughly to give a unifrom suspension.

2. Heat to boiling with frequent agitation until medium is completely dissolved.

3. Slants
   a. Dispense 8 ml. in screw - capped tube
   b. Sterilize by autoclaving at 121°C 15 lbs pressure, for 15 miuntes
   c. Cool to room temperature in slanted position.

4. Petri dishes
   a. Sterilize by autoclaving at 121 °C, 15 lbs pressure, for 15 minutes
   b. Dispense 20 ml. in sterile petri dishes (base layer) (and dispense 15 ml on base layer was a seed layer)

5. Final pH 5.6 at room temperature

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Reagent for basic chemical composition test

Wagner's Reagent

I crystal	1.27	gr.
Potassium iodide	2	gr.
Water to make	100	ml.

Marme's Reagent

Cadmium iodide	10.0	gr.
Potassium iodide	5.0	gr.
Distilled Water	100	ml.

Kedd's Reagent

2% of 3,5 dinitrobenzoic acid solution in methanol

Dragendorff's Reagent

Bismuth nitrate	8.0	gr.
Nitric acid (30% w/v)	12.0	ml.
Potassium iodide	272	ml.
Distilled Water	100	ml.

Valser's Reagent

Potassium iodide	10	gr.
Red Mercuric iodide	2	gr.
Water	100	ml.

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