การคัดเลือกพืชสมุนไพรที่มีฤทธิ์ยับยั้งโซเดียมโพแทสเซียมอะดีโนซีนไทรฟอสฟาเทส

นางสาวศรีนวล มานะกิจ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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SCREENING OF HERBS WITH Na^+-K^+- ADENOSINE TRIPHOPHATASE INHIBITORY ACTIVITY

Miss Srinual Manakit

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Biotechnology
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ศรีนวล มานะกิจ : การคัดเลือกพืชสมุนไพรที่มีฤทธิ์ยับยั้งโซเดียมโพแทสเซียมอะดีโนซีนไทรฟอสฟาเทส (SREENING OF HERBS WITH Na^+-K^+-ADENOSINE TRIPHOSPHATASE INHIBITORY ACTIVITY) อ. ที่ปรึกษา : ผศ. ดร. นาตยา งามโรจนวณิชย์, อ.ที่ปรึกษาร่วม : รศ. ดร. อมร เพชรสม; 79 หน้า ISBN 974-17-4637-7

ได้นำสารสกัดจากสมุนไพร 10 ชนิด ได้แก่ กระเจี๊ยบแดง, ขลู่, ชุมเห็ดเทศ, โด่ไม่รู้ล้ม, ว่านน้ำ, สับปะรด, หญ้าคา, หญ้าหนวดแมว, แห้วหมูและอ้อยแดง มาคัดกรองฤทธิ์การยับยั้งโซเดียมโพแทสเซียมอะดีโนซีนไทรฟอสฟาเทส ผลการทดสอบฤทธิ์ทางชีวภาพพบว่าส่วนสกัดของพืชสมุนไพร ได้แก่ ว่านน้ำ (ส่วนสกัดคลอโรฟอร์ม), สับปะรด (ส่วนสกัดเอทานอล, เฮกเซน, คลอโรฟอร์ม), แห้วหมู (ส่วนสกัดเอทานอล, เฮกเซน), โด่ไม่รู้ล้ม (ส่วนสกัดเอทานอล), หญ้าหนวดแมว (ส่วนสกัดเฮกเซน) และขลู่ (ส่วนสกัดเฮกเซน) ที่ความเข้มข้นของส่วนสกัด 250 ไมโครกรัมต่อมิลลิลิตรแสดงฤทธิ์การยับยั้งโซเดียมโพแทสเซียมอะดีโนซีนไทรฟอสฟาเทสสูงกว่า 50% โดยส่วนสกัดเฮกเซนของแห้วหมูมีค่าการยับยั้งโซเดียมโพแทสเซียมอะดีโนซีนไทรฟอสฟาเทสสูงสุด เมื่อทำการสกัดแยกสารจากส่วนสกัดเฮกเซนของแห้วหมูและทำให้บริสุทธิ์ จะได้สาร 2 ชนิด ซึ่งพิสูจน์เอกลักษณ์ด้วยสมบัติทางกายภาพและทางสเปกโตรสโกปี พบว่าประกอบด้วย ของผสมของ stigmasterol และ β-sitosterol และของผสมของกรดคาร์บอกซิลิกโซ่ตรง (C_{22}, C_{24}, C_{26}, C_{28} and C_{30}) ตามลำดับ สารดังกล่าวสามารถยับยั้งการทำงานของเอนไซม์ Na^+-K^+-ATPase ได้ 38.13 และ 44.0 เปอร์เซ็นต์ ที่ความเข้มข้น 1000 ส่วนในล้านส่วน ตามลำดับ

ภาควิชา.....-..... ลายมือชื่อนิสิต.....

สาขาวิชา……..เทคโนโลยีชีวภาพ..... ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา.....2546.....ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

4372537023 : MAJOR BIOTECHNOLOGY

KEY WORD: diuretic, Na^+-K^+-ATPase, brain microsome, *Hibiscus sabdariffa*, *Pluchea indica*, *Cassia alata*, *Elephantopus scaber*, *Acorus calamus*, *Ananas comosus*, *Imperara cylindrica*, *Orthosiphon aristatus*, *Cyperus rotundus*, and *Saccharum sinense*

SRINUAL MANAKIT : SREENING OF HERBS WITH Na^+-K^+-ADENOSINE TRIPHOSPHATASE INHIBITORY ACTIVITY. THESIS ADVISOR : ASSIST. PROF. NATTAYA NGAMROJNAVANICH, Ph.D. THESIS COADVISOR : ASSOC. PROF. AMORN PETSOM, Ph.D. 79 pp. ISBN 974-17-4637-7

Crude extract of the following ten herbs were screened for Na^+-K^+-ATPase inhibitory activity: *Hibiscus sabdariffa* Linn., *Pluchea indica* Linn. Less., *Cassia alata* Linn., *Elephantopus scaber* Linn., *Acorus calamus* Linn., *Ananas comosus* Linn. Merr., *Imperara cylindrica* Beauv., *Orthosiphon aristatus* Miq., *Cyperus rotundus* Linn., and *Saccharum sinense* Roxb.. The bioassay results indicated that at 250 μg/ml of each extract from *A. calamus* ($CHCl_3$), *A. comosus* (hexane, $CHCl_3$, EtOH), *C. rotundus* (hexane, EtOH), *E. scaber* (EtOH), *O. aristatus* (hexane) and *P. indica* (hexane) showed more than 50% inhibitory activity on Na^+-K^+-ATPase. The *C. rotundus* crude extract showed the highest inhibitory activity. Separation of the crude hexane extract of *C. rotundus* gave two mixtures. Using of physical and spectroscopic properties the mixtures were identified to be a mixture of long chain carboxylic acids (C_{22}, C_{24}, C_{26}, C_{28} and C_{30}) and a mixture of stigmasterol and β-sitosterol. At 1000 ppm, they exhibited Na^+-K^+-ATPase inhibitory activity at 38.13 and 44.0 %, respectively.

Department……..-……Student's signature…….
Field of study….BIOTECHNOLOGY...Advisor's signature……..
Academic year……2003…… Co-advisor's signature…….

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CONTENTS

Page

CONTENTS (continued)

Page

CONTENTS (continued)

Page

LIST OF FIGURES

Figures **Page**

LIST OF TABLES

Tables Page

LIST OF SCHEMES

Schemes **Page**

LIST OF ABBREVIATIONS AND SYMBOLS

ATP	Adenosine triphosphatase
^{13}C NMR	Carbon-13 Nuclear Magnetic Resonance
°C	Degree Celsius
DMSO	Dimethyl sulfoxide
EDTA	ethylenediamine tetraacetic acid
g	Gram (s)
^{1}H NMR	Proton-1 Nuclear Magnetic Resonance
Hz	Hertz
IR	Infrared
Kg	Kilogarm (S)
M	Molar
Min	Minute
m.p.	Melting point
mmol	Milimol
ml	Mililiter (s)
mg	Miligram (s)
MW	Molecular weight
MHz	mega hertz
No.	Number
OD	optical density
ppm	Part per million
R_f	Retarding factor in chromatogram
rpm	round per minute
TCA	trichloroacetic acid
TLC	Thin Layer Chromatography
µg	Microgram (s)
µl	Microliter (s)

CHAPTER I

INTRODUCTION

Recently, there is a rapid growing movement back to the nature in the search for health and longevity. Traditional medicines, herbal-based medicines, and organic food have long been a part of worldwide culture for many centuries. Their importance to the world today is spreading due to increasing awareness of the limited horizon of synthetic pharmaceutical products to control major diseases and the need to discover new molecular structures as lead compounds from plant kingdom. Therefore, today's herbal pharmacopoeia can frequently offer relief and prevention while avoiding the potential harmful side effects from many great pharmaceutical drugs.

It has been a long time that plants are used as diuretic remedies. A diuretic is defined as a chemical that increases the rate of urine formation which excretes water and toxic wastes from the body (Delgado, 1998). One way to screen for this kind of biological activity of medicinal plants is the inhibition of enzyme – Na^+-K^+-ATPase. Inhibition of Na^+-K^+-ATPase leads to a higher level of Na^+-inside the cell (Stryer, 1995) and due to inhibition of renal with the re-absorption of the electrolytes Na^+, Cl^- and/or K^+ and water is retained in the tubular fluid to "balance out" the increase in salt content of the developing urine (http://www.muhealth.org/~pharm204/diuretics.htm, Figure 1.1). The diuretic drugs are mainly used in patient afflicted with various edematous disorders e.g. urinary stones, nephritis, cystitis, urinary retention and incontinence---with severe edema associated with dropsy, ascites, lymphatic disease and hypertension (Delgado, 1998, Mills, 2000 and Daravan, 2525 in Thai).

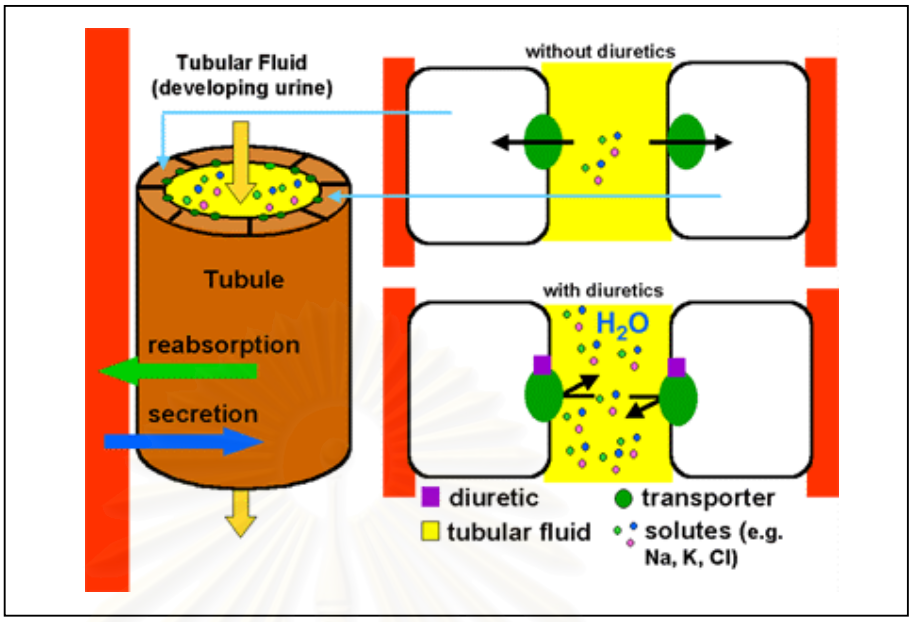


Figure 1.1 Mechanism of diuretic action in tubular fluid (adapted from http://www.zerobio.com/central/na-k.htm.)

Animal experiment using Thai herbs such as *Ananas comosus* and *Carica papaya* were found to increase urine output and electrolyte excretion in rat (Sripanidkulchai, 2001). However, screening for this biological activity using Thai herbs on the inhibition of enzyme---Na^+-K^+-ATPase has were been reported. This thesis is aimed to investigate the plants using in traditional medicines for the treatment of dysuria by screening for their inhibitory activity on Na^+-K^+-ATPase.

1.2 The purposes of the research

The goal of this research could be summarized as follows:

1. Preliminary screening of herb with Na^+-K^+-ATPase inhibitory activity.
2. To extract and isolate the organic constituents from the plant with high activity.
3. Determination of the structure of isolated compounds and their inhibition of Na^+-K^+-ATPase activity.

CHAPTER II

LITERATURES REVIEW

2.1 Structure-function of the Na^+-K^+-ATPase

The Na^+-K^+-ATPase or sodium pump is an integral membranes protein found in the cell of all higher eukaryotes, which transfers chemical energy of hydrolysis of ATP to potential energy of electrochemical ion gradients for Na^+ and K^+ across the cell membrane. The maintenance of the ion gradients with low intracellular [Na^+] and high [K^+] is achieved by the operation of Na^+-K^+-ATPase (Alberts, 1997 and Vasilets, 1993), which uses the energy of the hydrolysis of 1 mole ATP to ADP and Pi to export 3 moles of Na^+ out of the cell and to import 2 moles of K^+ into cell (Lingrel, 1994). The pump works in a cycle, as illustrated schematically in Figure 1.1. Na^+ binds to the pump at sites exposed intracellular (stage 1), activating the ATPase activity. ATP is split, with the release of ADP and the transfer of a phosphate group into a high-energy linkage to the pump itself-which means, the pump phosphorylates itself (stage 2). Phosphorylation causes the pump to switch its conformation so as to release Na^+ at the exterior surface of the cell and at the same time, to expose a binding site for K^+ at the same surface (stage 3). The binding of extracellular K^+ triggers the removal of the phosphate group (dephosphorylation) (stages 4 and 5), causing the pump to switch back to its original conformation, discharging the K^+ into the cell interior (stage6) and then, the pump is ready to go again (Alberts, 1997). Physiologically, Na^+-K^+-ATPase present in organs such as the intestines and the kidney regulates fluid re-absorption and electrolyte movement by establishing an ionic gradient across epithelial membranes (Lingrel, 1994). In the kidneys, Na^+ and water are absorbed in tubules, especially Henle's loop and distal tubules, where Na^+-K^+-ATPase is abundantly localized (Satoh, 1992).

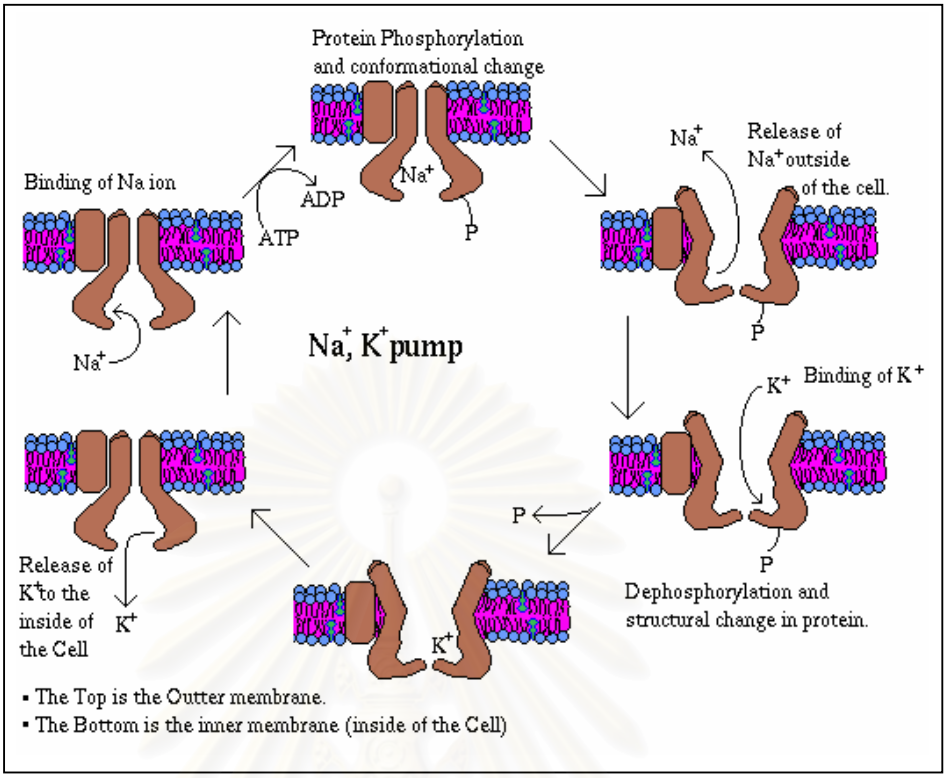


Figure 2.1 Model of the pumping cycle of the Na^+-K^+-ATPase (adapted from http://www.cbc.umn.edu/~mwd/cell_www/chapter2/Na-Kpump.html)

There are poisons or toxins that also interfere with the pump. One is called "ouabain", an arrow poison. Ouabain works by attaching to the pump and blocking its action. This can cause serious loss of nerve function and even death (http://www.zerobio.com/central/na-k.htm).

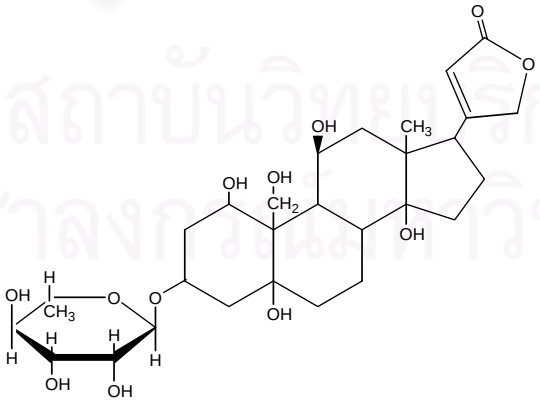


Figure 2.2 Structure of ouabain (adapted from http://www.uni-giessen.de/fb18/biochem/schomer/schonereng.htm)

2.2 Literature review of chemical nature of action of Na^+-K^+-ATPase

Plants can produce secondary metabolites which not only benefit for growth but it can also possess medicinal and pharmacological activity. The idea of using natural products in place of synthetic chemicals has become more and were important for modern scientists at present. Various types of chemicals are known to inhibit Na^+-K^+-ATPase activity. Wu, *et al.* (1984) studied marine organisms for physiologically active substances, and they found that the extract of the orange colored Okinawa sea sponge *Agelas* sp. contains, agelasine-A(1), -B(2), -C(3) and –D(4) with inhibitory effects on enzymic reaction of Na^+-K^+-ATPase. Later on, they isolated agelasine- E(5) and -F(6) from *A. nakamurai* Hishino which also showed inhibitory effects on Na^+-K^+-ATPase.

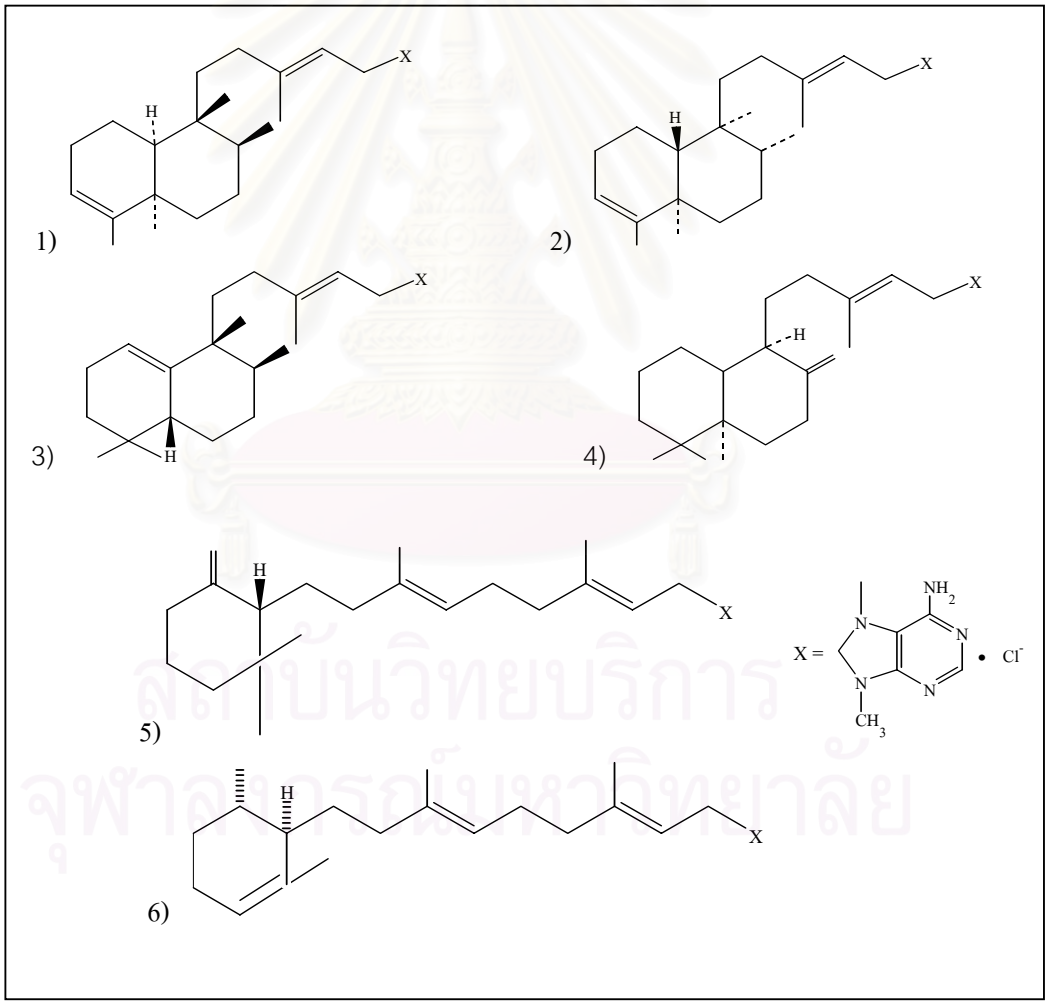


Figure 2.3 Structure of compounds isolated from *Agelas nakamurai* Hoshino

Satoh *et al*. (1991) studied the folk-medicine which used as diuretics. Twenty three kinds of diuretic drugs were chosen and examined for their effects on the horse kidney Na^+-K^+-ATPase, which is an intrinsic enzyme of the plasma membrane and responsible for the active transport of Na^+ and K^+ across the membrane. They found that Atractylodis Lanceae Rhizoma, Atractylodis Rhizoma, Plantaginis Semen, Plantaginis Herba and Alismatis Rhizome have strong inhibitory effects on the kidney Na^+-K^+-ATPase activity. In 1992, they study on the physiologically active constituents of Atractylodis Lanceae Rhizoma, and reported that high concentration of β-eudesmol interact with enzyme in the $Na\bullet E_1$ state. In 1996, they separated atractylon, a major component of the crude drug rhizomes of *Atractylodes japonica* (Byaku-jutsu), which shows strong inhibition activity Na^+-K^+-ATPase by interaction with enzyme in the E_2 state. In 1997, they reported that the inhibition of Na^+-K^+-ATPase activity is caused by interaction of 1,2,3,4,6-Penta-O-galloyl-β-D-glucose (PGG) with the enzyme in the E_2 state. In 2003, they studied the inhibition of Na^+-K^+-ATPase with the extract of *Stephania cephararantha* Hayata and found bisbenzylisoquinoline alkaloids, such as cycleanine, cepharanthine, isotetrandrine, berbamine, homoaromoline and cepharanoline. However, only cycleanine markedly inhibited Na^+-K^+-ATPase activity.

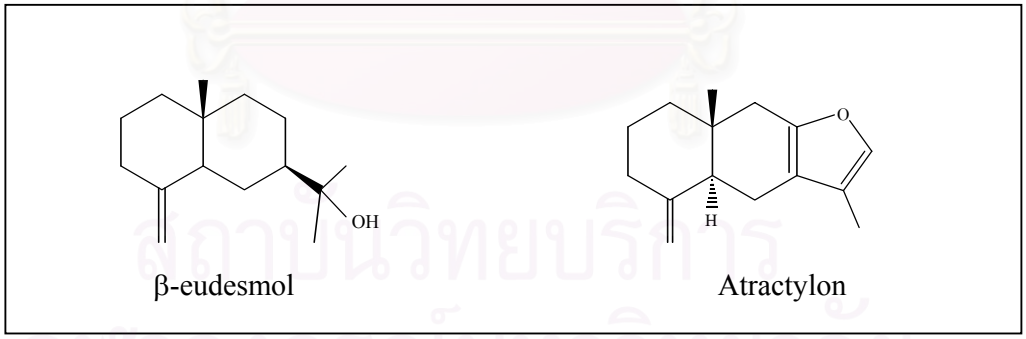
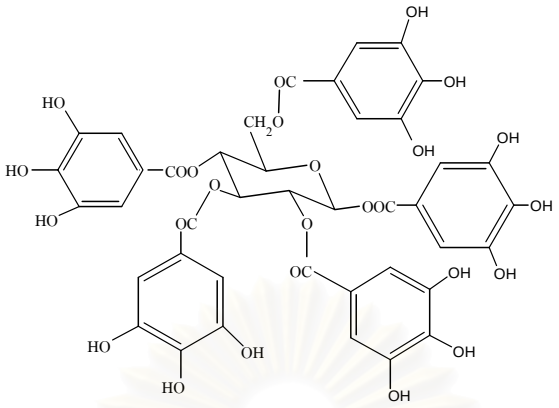
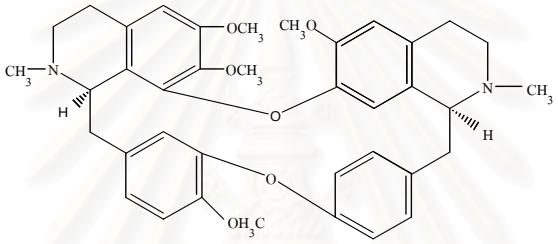


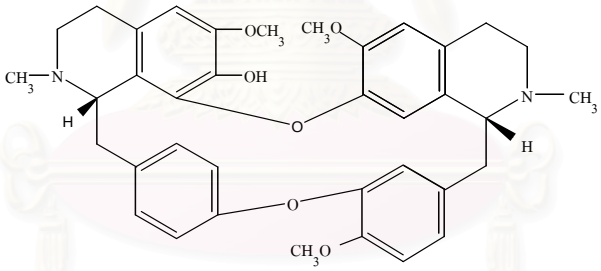
Figure 2.4 Structure of compounds from natural product with Na^+-K^+-ATPase inhibitory activity



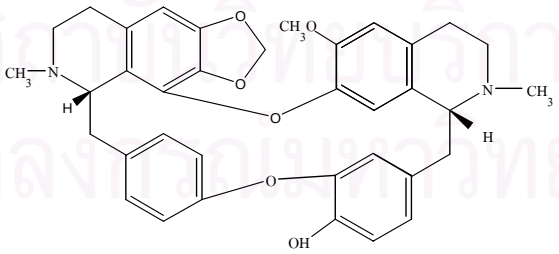
1,2,3,4,6-Penta-O-galloyl-β-D-glucose (PGG)



Isotetrandrine



Homoaromoline



Cepharanoline

Figure 2.4 Structure of compounds from natural product with Na^{+}-K^{+}-ATPase inhibitory activity (Continue)

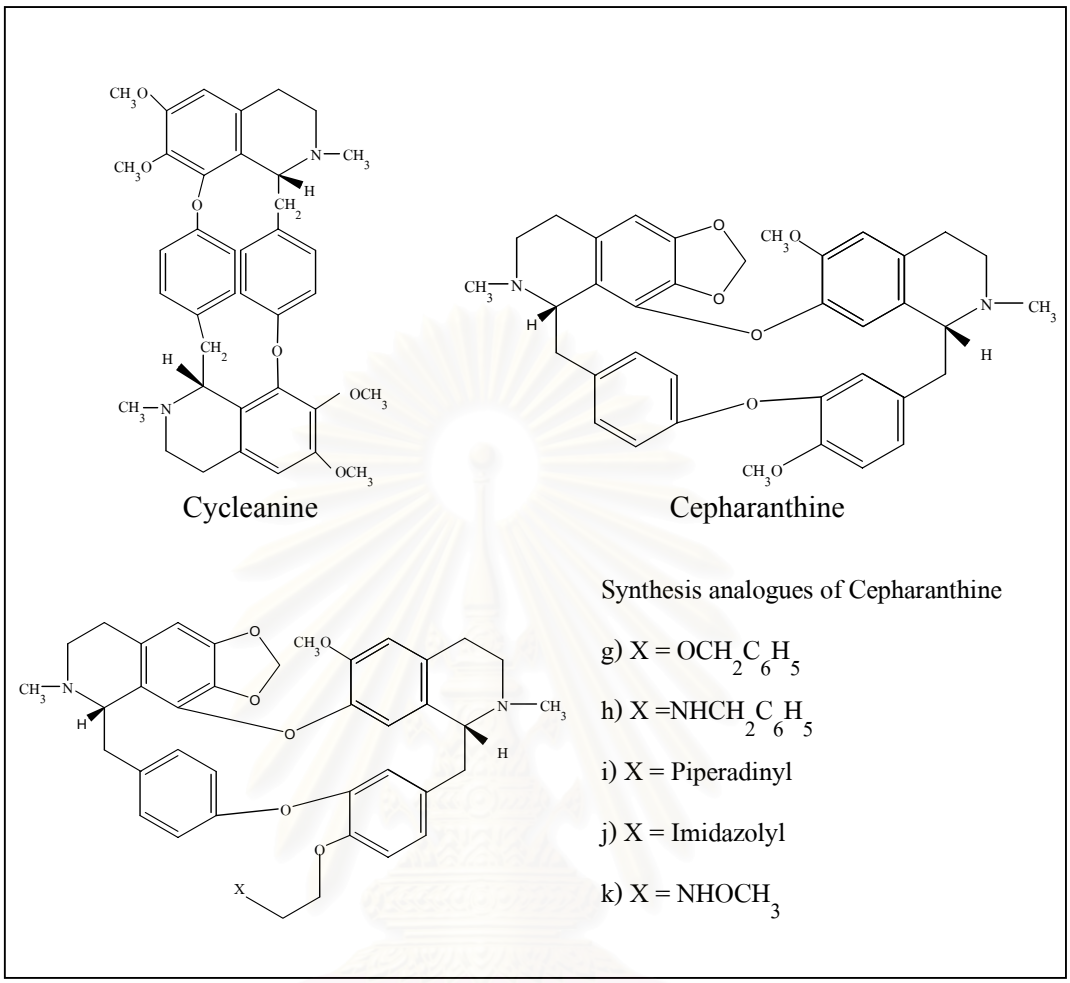


Figure 2.4 Structure of compounds from natural product with Na^+-K^+-ATPase inhibitory activity (Continue)

Furthermore, Okamoto *et al.* reported the components of marine sponge, *Ianthella* sp., such as Iantheran -A and –B and Ianthesines -A, -B, -C and - D as a Na^+-K^+-ATPase inhibitors.

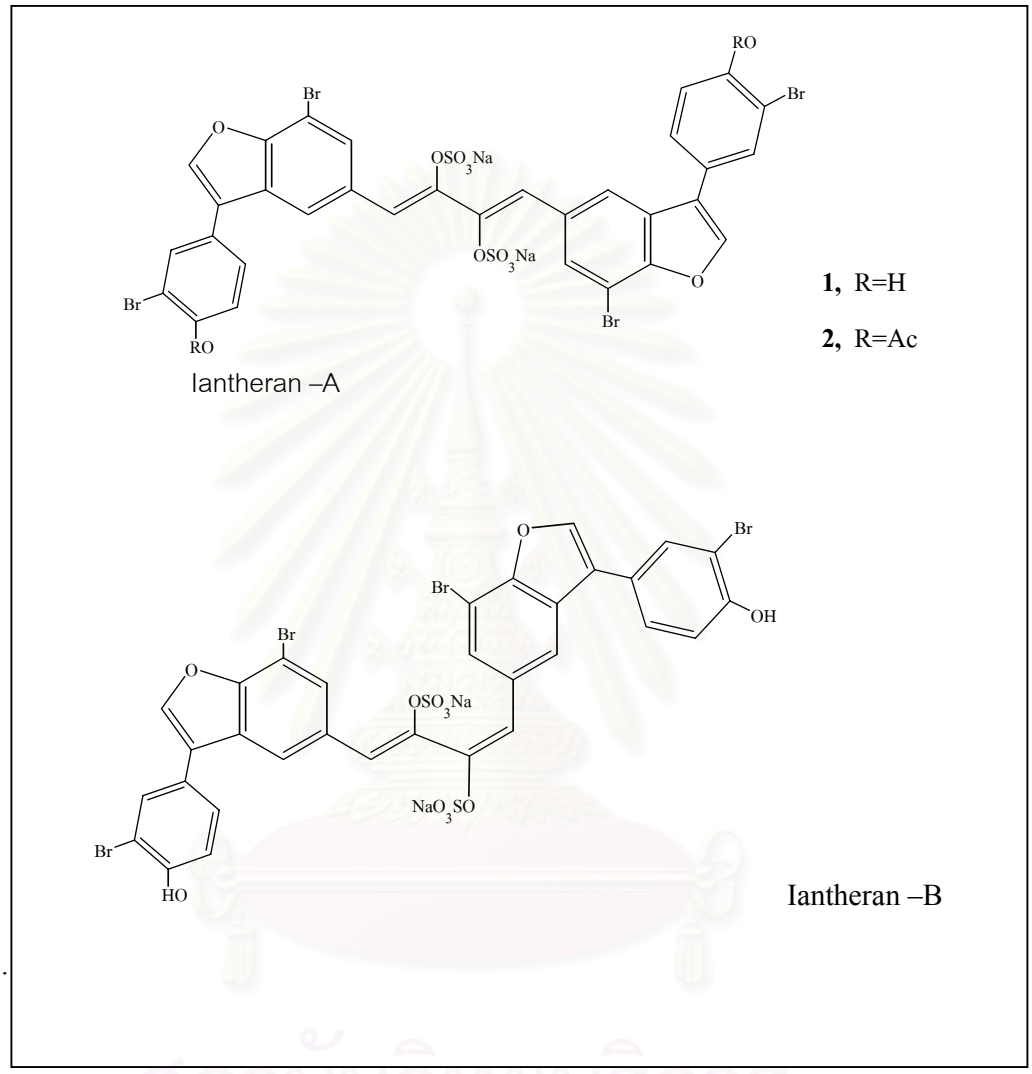


Figure 2.5 Structure of compounds isolated from a marine sponge, *Ianthella* sp.

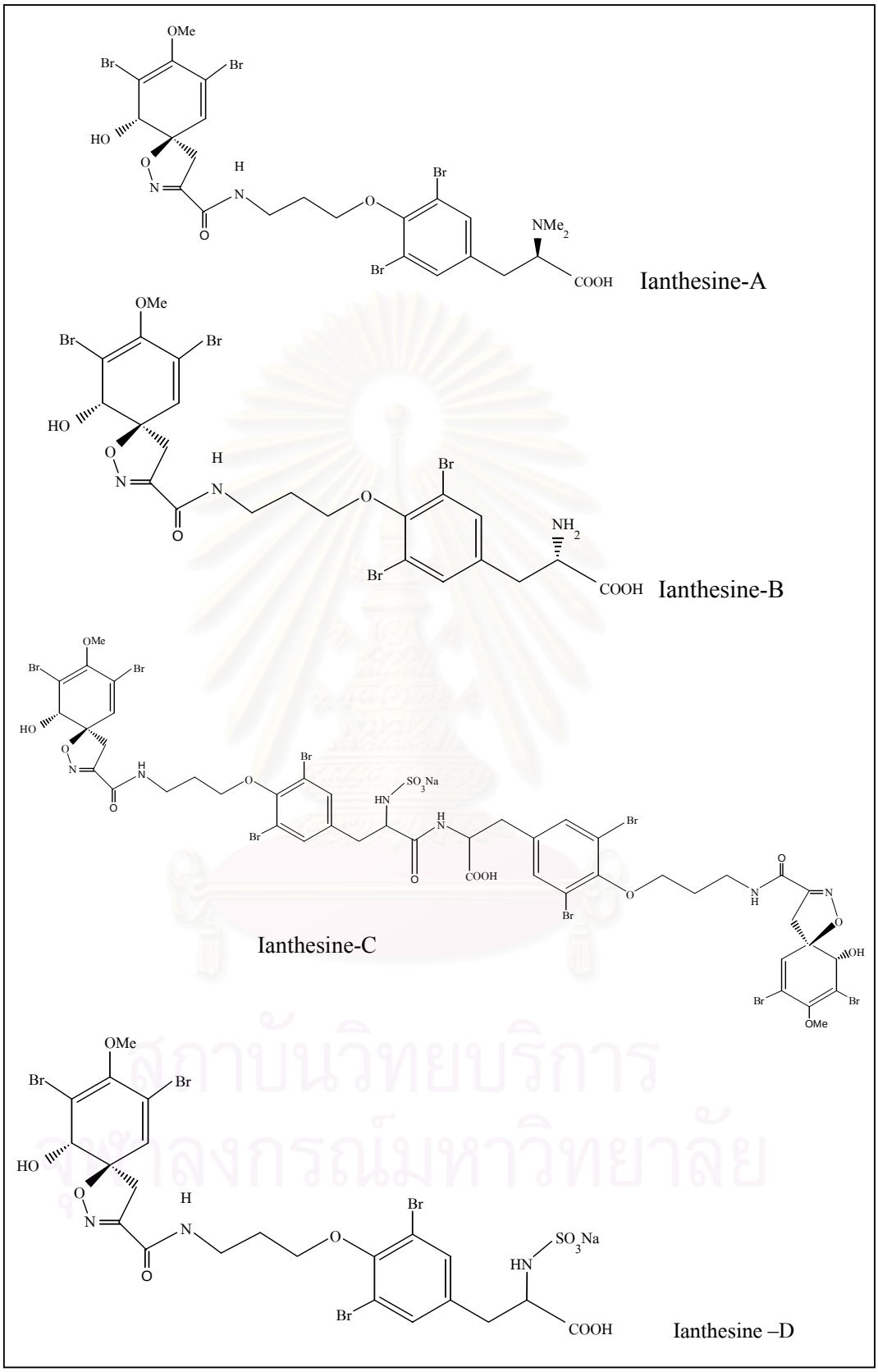
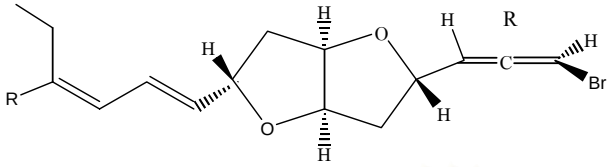


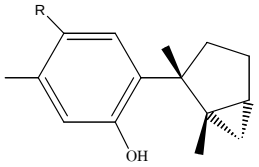
Figure 2.5 Structure of compounds isolated from a marine sponge, *Ianthella* sp. (Continue)

In 2001, Okamoto found that an organic extract of Japanese sea hare, *Aplysia kurodai* showed inhibitory activity on Na^+-K^+-ATPase.



1, R=Me

4, R=Br



2, R=Br

3, R=H

Figure 2.6 Structures of Aplysiallene (1), Laurinterol (2), Debromolaurinterol (3) and Bromoallene (4)

2.3 Relationship studies of herbs on Na^+-K^+-ATPase inhibitory activity

Some selected plants known to possess diuretic activity used in the screening for Na^+-K^+-ATPase inhibitory activity are the following: *Hibiscus sabdariffa* Linn. (กระเจี๊ยบแดง), *Pluchea indica* (Linn.) Less. (ขลู่), *Cassia alata* Linn. (ชุมเห็ดเทศ), *Elephantopus scaber* Linn. (โด่ไม่รู้ล้ม), *Acorus calamus* Linn. (ว่านน้ำ), *Ananas comosus* (Linn.) Merr.(สับปะรด), *Imperara cylindrica* Beauv. (หญ้าคา), *Orthosiphon aristatus* Miq. (หญ้าหนวดแมว), *Cyperus rotundus* Linn. (แห้วหมู), and *Saccharum sinense* Roxb. (อ้อยแดง). These plants are shown in Figure 2.7.

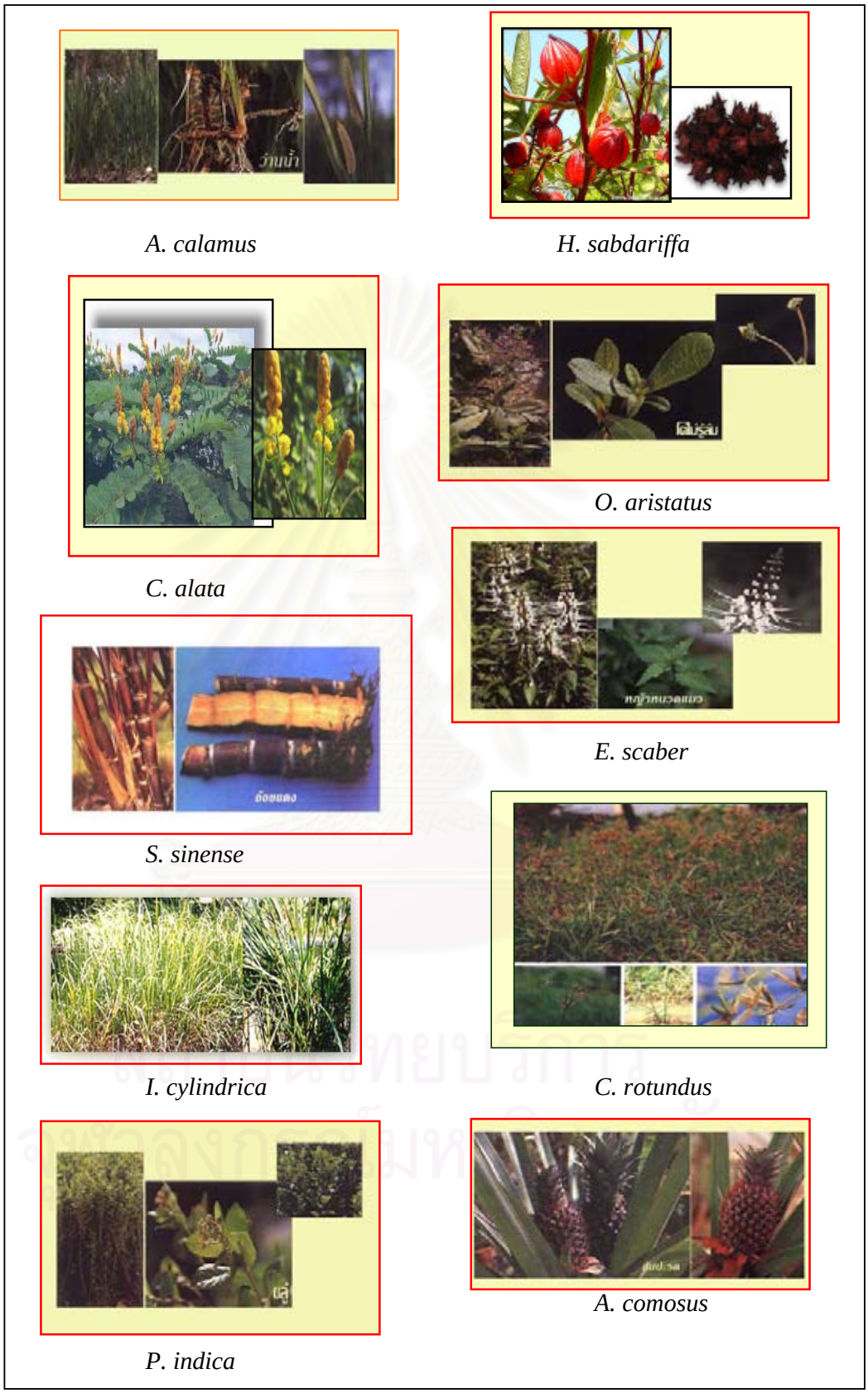


Figure 2.7 Selected plants species used in this study

2.4 Botanical aspect and distribution

2.4.1 *Cyperus rotundus* Linn.

Cyperus rotundus Linn. is cosmopolitan sedge belonging to the family of the Cyperaceae. It is a plant consisting of stems which are tuberous at base, rising singly from a creeping, underground root-stock, about 10-25 cm tall. Leaves are linear, broadly grooved on the upper surface, dark green. Flowers are in rather small inflorescence with 2-4(-6) bracts, the longest bracts are usually longer than the inflorescence but some are shorter (Farnsworth, 1992). The nut is three-angled, oblong-ovate, yellow in color and black when ripe. Major chemical constituents is essential oil consisting mainly of sesquiterpene hydrocarbons, epoxides and ketones and monoterpene and aliphatic alcohol (Williamson, 2002). It is a traditional medicinal plant appearing among India, Chinese and Japanese natural drugs used against aspasms and stomach disorder (Sonwa, 2001). The claimed efficacious in Thai traditional textbooks are diuretic, antipyretic, cardiotonic and many other (Farnsworth, 1992). Furthermore, the report about the tuber and rhizome are used to treat abdominal problems, particularly peptic ulcer, diarrhoea and dyspepsia and as a carminative, demulcent, analgesic and diuretics as well as for amenorrhoea and dysmenorrhoea. It has anthelmintic, antibacterial and fugicidal activities and has been used for many other complaints.

Common name : Nutgrass, sedge weed, nutsedge, chido, Yaa haew muu

2.4.1.1 Chemical constituents of *C. rotundus* L.

Literature surveys of chemical constituents of *C. rotundus* L. revealed that there have been variety of organic substances isolated.

Kapadia *et al.* (1965) isolated sesquiterpene ketone from *C. rotundus* L., a new ketone has been isolated and identified as mustakone and copaene.

Hikino *et al*. (1967) found a ketone isolated from *C. rotundus* L. of Japanese origin and have been identified as cyperotundone 2,4-dinitrophenylhydrazone. In 1971, they reported that *C. rotundus* L. used as a Chinese medicine for the treatment of women's diseases contains two novel sesquiterpenic keto-alcohol, α-rotunol and β-rotunol.

Neville *et al.* (1968) identified a ketone in *Cyperus* spp. by NMR and mass spectral data of the 2,4-dinitrophenylhydrazone derivation as cyperenone 2,4-dinitrophenylhydrazone.

Hiniko *et al.* (1976) isolated the essential oil from dried rhizomes of *C.rotundus* L. and identified as 4α,5α-oxidoeudesm-11-en-3α-ol which is a novel sesquiterpenoid.

Singh (1979) isolated a new saponin from mature tubers of *C. rotundus* L. yielded oleanolic acid, sitosterol and sapogenin. Thereafter, Gupta reported activities of β-sitosterol, isolated from *C. rotundus* L. as the anti-inflammatory and antipyretic. Furthermore, antimalarial activity was reported by Thebtaranonth (1995) that patchoulenone, caryphyllene α-oxide, 10,12-peroxycalamenene and 4,7- dimethyl-1-tetraloneshowed high potency in the *in vivo* test against *Plasmodium falciparum*.

Ohira *et al.* (1998) reported the sesquiterpenoids from *C. rotundus* L. which are 2α-(5-oxopentyl)-2β-methyl-5β-isopropenylcyclo-hexanone, 2β-(5-oxopentyl)-2β-methyl-5β-isopropenylcyclo-hexanone and six previously known terpenoids: cyperolone, mustakone and four eudesman-type sesquiterpenoids.

Morimoto *et al.* (1999) studied the chemical constituents and antifeedants from *Cyperus* spp. They found Cyperaceae species from Thailand with antifeedant activity were *C. cyperinus, C. diffusus,*

Jeong *et al.* (2000) reported new alkaloids, rotundines A-C from *C. rotundus*.

Sonwa *et al*. (2001) found that the essential oil of *C. rotundus* L. contains cyprotene, cypera-2,4-diene, α-copaene, cyperene, α-selinene, rotundene, valencene, ylanga-2,4-diene, γ-gujunene, *trans*-calamenene, δ-cadinene, γ-calacorene, *epi*-α-selinene, α-muurolene, γ-muurolene, cadalene, nootkatene, cyperotundone, mustakone, cyperol, isocyperol, α-cyperone, isorotundene, cypera-2,4(15)-diene, nor-rotundene and cyperadione.

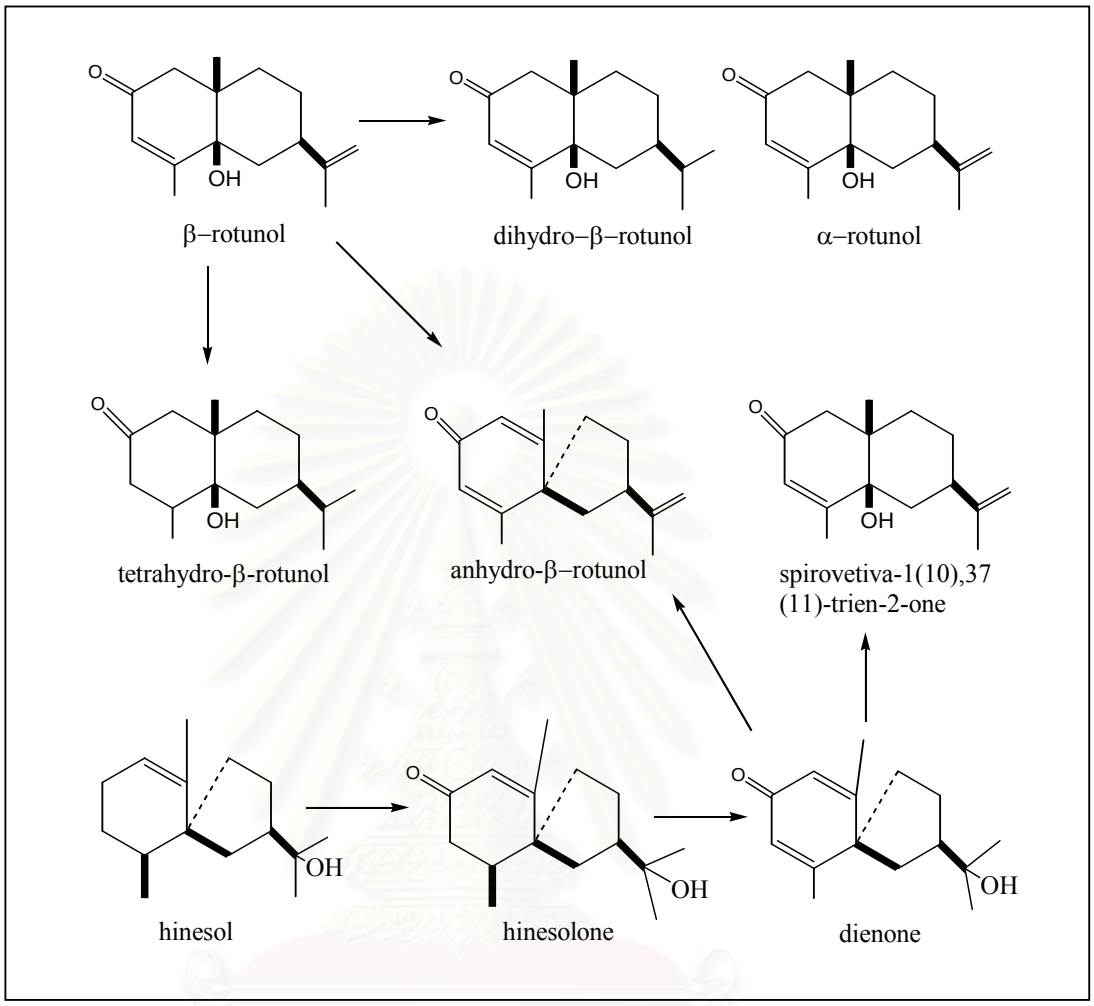
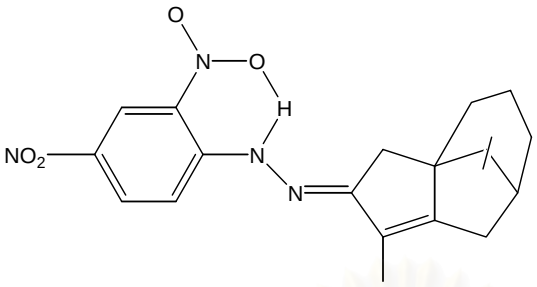
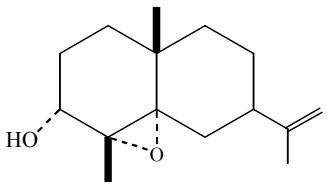


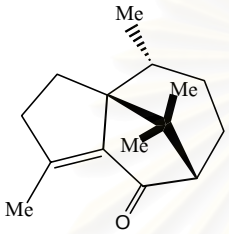
Figure 2.8 Derivatives of β-rotunnol from *C. rotundus*



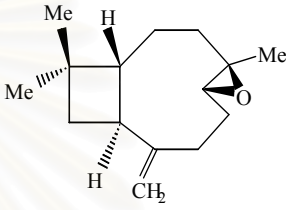
cyperenone 2,4-dinitrophenylhydrazone



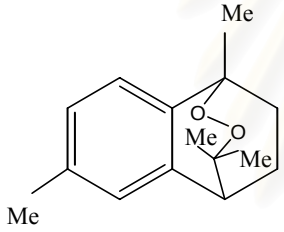
4α,5α-oxidoeudesm-11-en-3α-ol



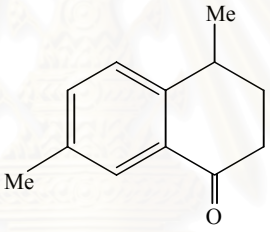
Patchoulenone



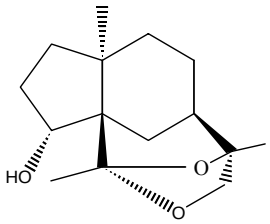
Caryophyllene α-oxide



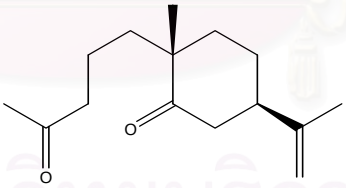
10,12-peroxycalamenene



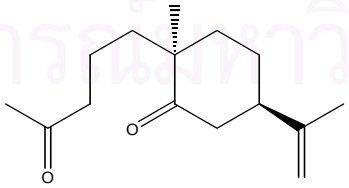
4,7-dimethyl-1-tetralone



cyperolone skeleton



2α-(5-oxopentyl)-2β-methyl-5β-isopropenylcyclo-hexanone



2β-(5-oxopentyl)-2β-methyl-5β-isopropenylcyclo-hexanone

Figure 2.9 Some isolated compounds of *C. rotundus*

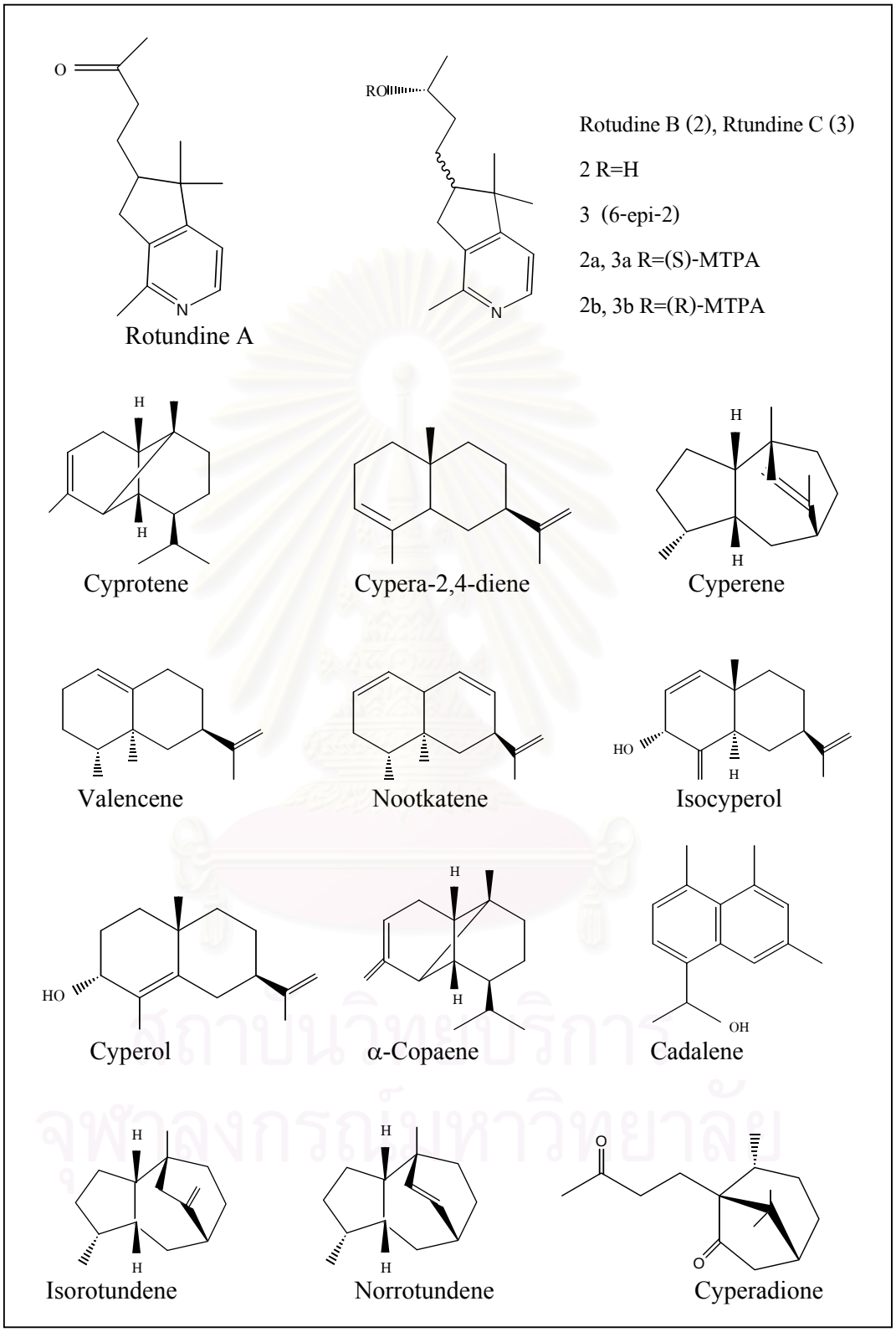


Figure 2.9 Some isolated compounds of *C. rotundus* (continue)

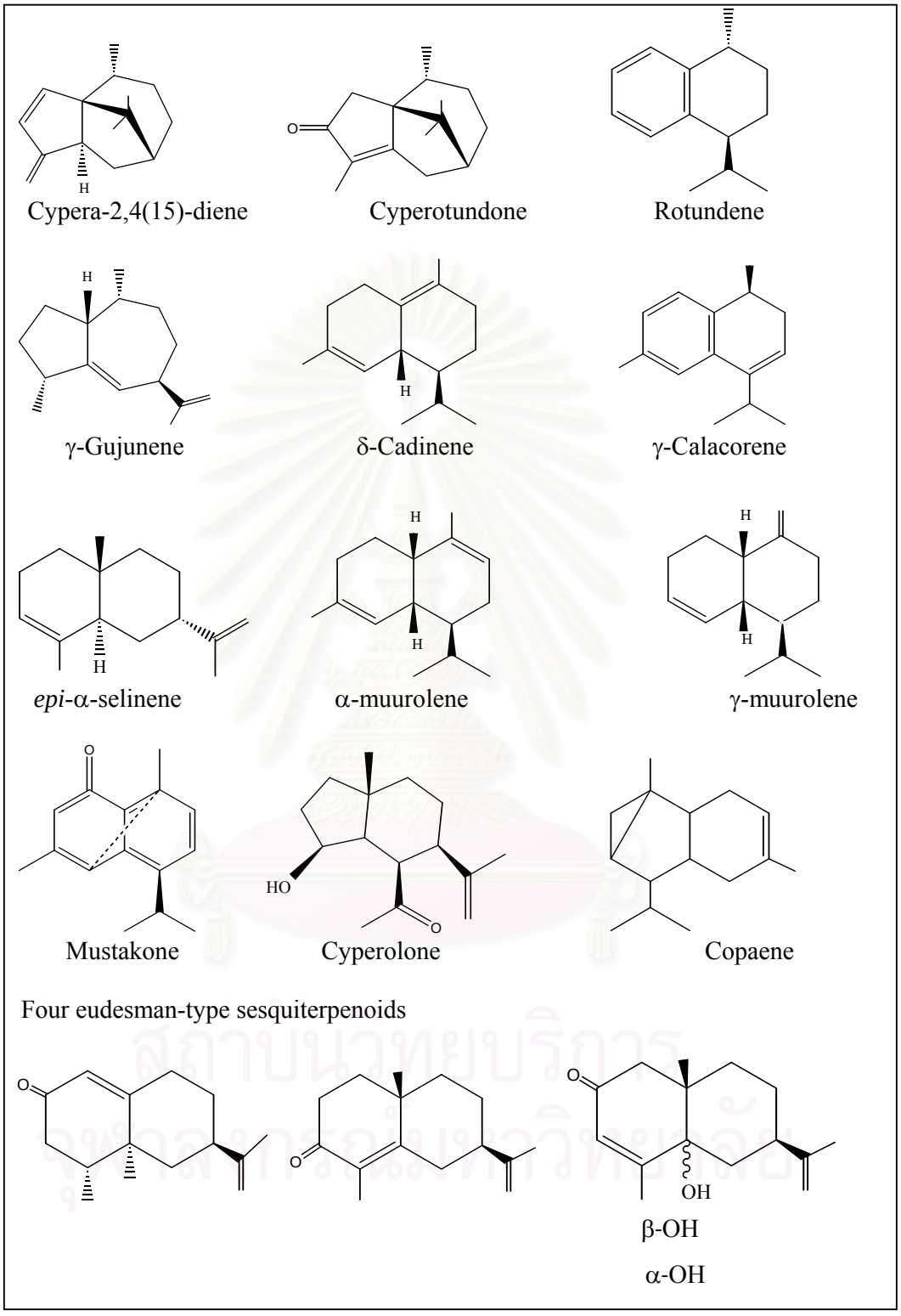


Figure 2.9 Some isolated compounds of *C. rotundus* (Continue)

2.4.2 *Elephantopus scaber* Linn.

E. scaber is used as a diuretic and as antiferbrile, antiviral and antibacterial agent, as well as in the treatment of hepatitis, bronchitis, the cough associated with pneumonia, and arthralgia (But *et al*., 1997)

2.4.2.1 Chemical constituent of *E. scaber* Linn.

Literature survey on chemical constituents of this species have been reported as shown below.

A methanolic extract of powdered air-dried plant was found to contain lupeol, stigmasterol and new germacranolide dilactone 11,13-dihydrodeoxyelephantopin (Silva, 1982). In 1992, Hisham isolated guaianolide glucosides from this plant which name deacylcyanaropicrin and glucozaluzanin-C. Moreover, But *et al.* (1997) reported the known deoxyelephantopin and isodeoxyelephantopin and scabertopin.

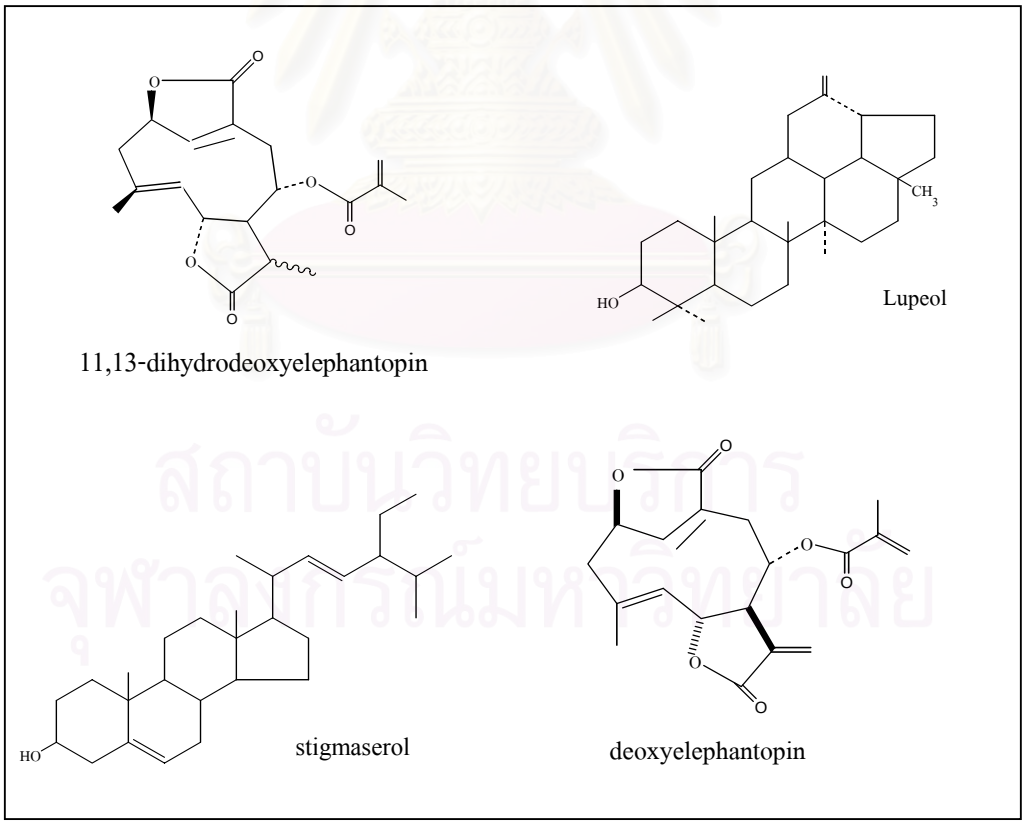


Figure 2.10 Some isolated compounds of *E. scaber*

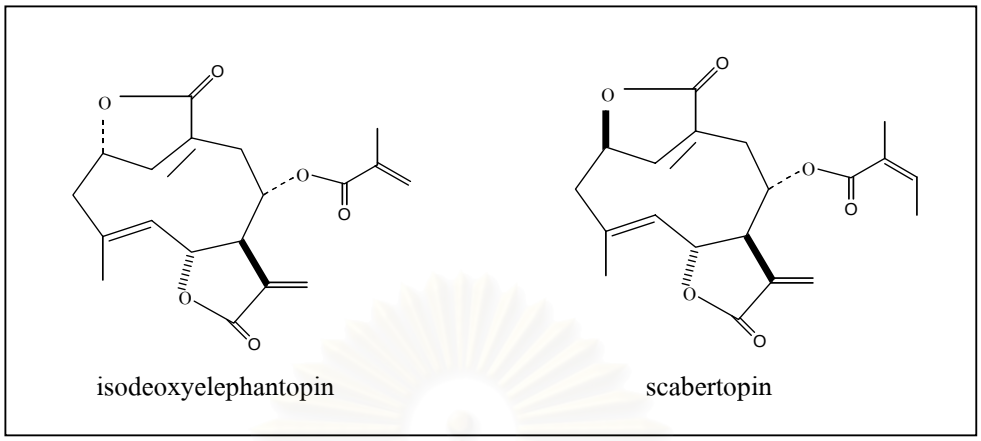


Figure 2.10 Some isolated compounds of *E. scaber* (Continue)

CHAPTER III

EXPERIMENTAL PROCEDURES

3.1 Instruments and equipments

Thin Layer chromatography (TLC) was performed with aluminum sheets precoated silica gel (Merck Kieselgel 60 PF_{254}) and spots on the plate were observed under UV light or visualized by spraying with vanillin in ethanol followed by heating. The silica gel Merck Kieselgel 60 no. 7734 and 9385 was used for column chromatography. The melting points were obtained on a Fishers-Johns melting point apparatus and are uncorrected. The infrared (IR) spectra were recorded on a Nicolet Fourier Transform Infrared Spectrophotometer (FT-IR) model Impact 410 (KBr pellet). The Proton and Carbon-13 Nuclear Magnetic Resonance (^{1}H and ^{13}C-NMR) spectra were recorded at 400 and 100 MHz, respectively, on a Varian Mercury 400 NMR spectrometer. Chemical shifts are expressed in parts per million (ppm) using residual protonated solvents as reference. Solvents for NMR spectra were deuterated chloroform (chloroform–*d*). The mass spectra were obtained on Fisions Instrument model Trio 2000 operating at 70 eV ionization voltage.

3.2 Source of plant materials

The plant materials of *H. sabdariffa* Linn., *P. indica* (Linn.) Less., *C. alata* Linn., *E. scaber* Linn., *A. calamus* Linn., *A. comosus* (Linn.) Merr., *I. cylindrica* Beauv., *O. aristatus* Miq., *C. rotundus* Linn., and *S. sinense* Roxb. (Appendix I) used in this study were purchased from Vetchapong-osot, a medicinal plant vendor, Bangkok, Thailand.

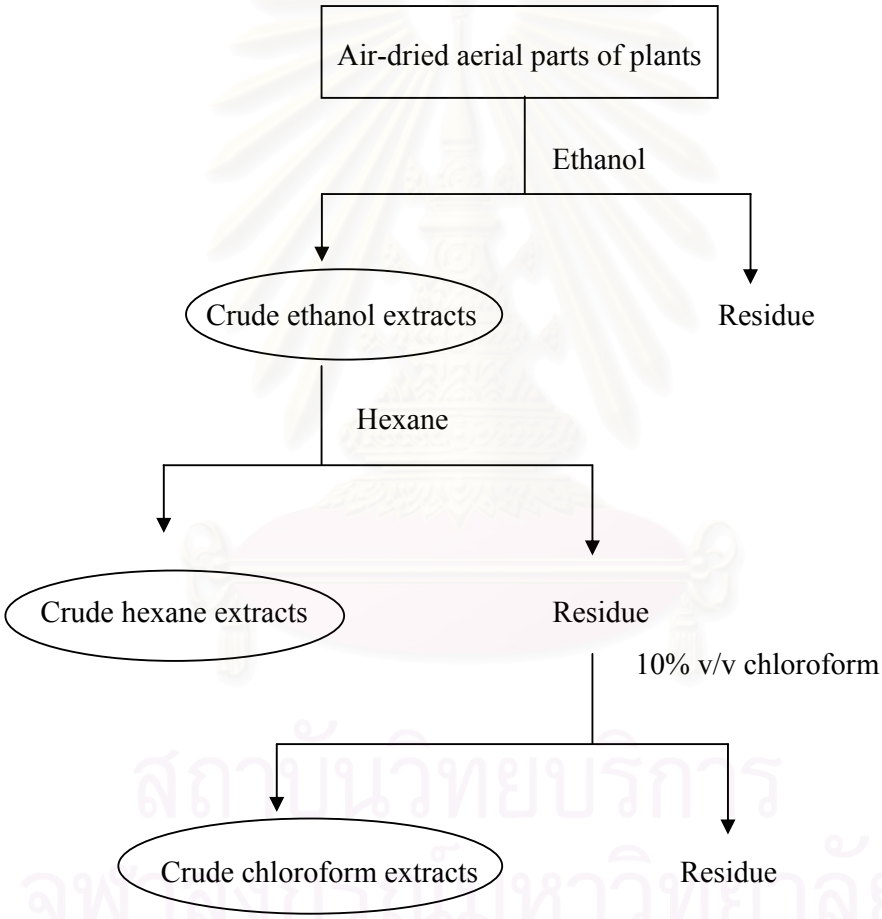
3.3 Solvents

All organic solvents used in this research such as hexane, chloroform, dichloromethane, ethyl acetate, methanol and ethanol were commercial grade and were re-distilled prior to use.

3.4 Extraction and Isolation

3.4.1 Extraction procedure

The air-dried plants were milled and then soaked in hexane for 7 days at room temperature. The solution was filtered and evaporated by a rotary vacuum evaporator to remove the solvent and obtain hexane extract. The residue was re-extracted with 95% ethanol for 7 days at room temperature. The filtered ethanol solution was evaporated to afford the ethanol extract. The extraction procedure for preliminary study of plant sample is shown in Scheme 2.2.



Scheme 3.1 The extraction and fractionation procedure of plant samples for preliminary screening

3.4.2 Extraction of the rhizomes of *C. rotundus*

The dried rhizomes of *C. rotundus* (5 kg) were milled and then soaked in hexane (7.5 liters) for 7 days at room temperature for one time. The solution was filtered and evaporated by a rotary vacuum evaporator to remove the solvent and obtain hexane crude extract (41.81 g, 0.84 % w/w) as a dark-brown oil. The residue was re-extracted with 95% ethanol (8 liters) for 7 days at room temperature for 1 time. The filtered ethanol solution was evaporated to afford 85.86 g of the ethanol extract (yield 1.72% w/w).

3.4.3 Extraction of the *E. scaber*

Whole plants of *E. scaber* 5 kg dry weight, were milled to fine powder which was then extracted by soaking in 95% ethanol for 7 days at room temperature. Evaporation of the solvent affording the crude ethanol extract as a greenish crude weighing 97.02 g (yield 1.94% w/w)

3.5 Preparation of brain microsomes from rat

All procedures of enzyme purification were carried out according to Urayama and Nakao, 1979. Rat were stunned by means of blow on the neck under an anesthetic with CO_2, decapitated, and exsanguinated. The brain (cerebrum) were quickly removed and the gray matter was minced with a pair of scissors after eliminating white matter and capillaries. The homogenizing medium (HM), consisting of 0.32 M sucrose, 5 mM Tris-HCl (pH 7.5), and 2 mM ethylenediamine tetraacetic acid (EDTA), was added at a ratio of 1 g of tissue to 9 ml of HM, and the tissue was homogenized with 10 strokes of a Potter-Elvehiem homogenizer, using a Teflon pestle. The homogenate was centrifuged at 7,700 x g for 10 min in a refrigerated centrifuge (HITACHI - HIMAC CS 100 Model) and the supernatant was re-centrifuged at 25,000 x g for 40 min. The pellet was suspended in 5 mM Tris-HCl (pH 7.5) containing 1 mM EDTA-Tris, washed twice with the same buffer.

3.5.1 Protein Assay

Protein concentration was determined by Bradford Assay with BSA as a standard (Appendices II).

3.6 In vitro assay of Na^+-K^+-ATPase activity

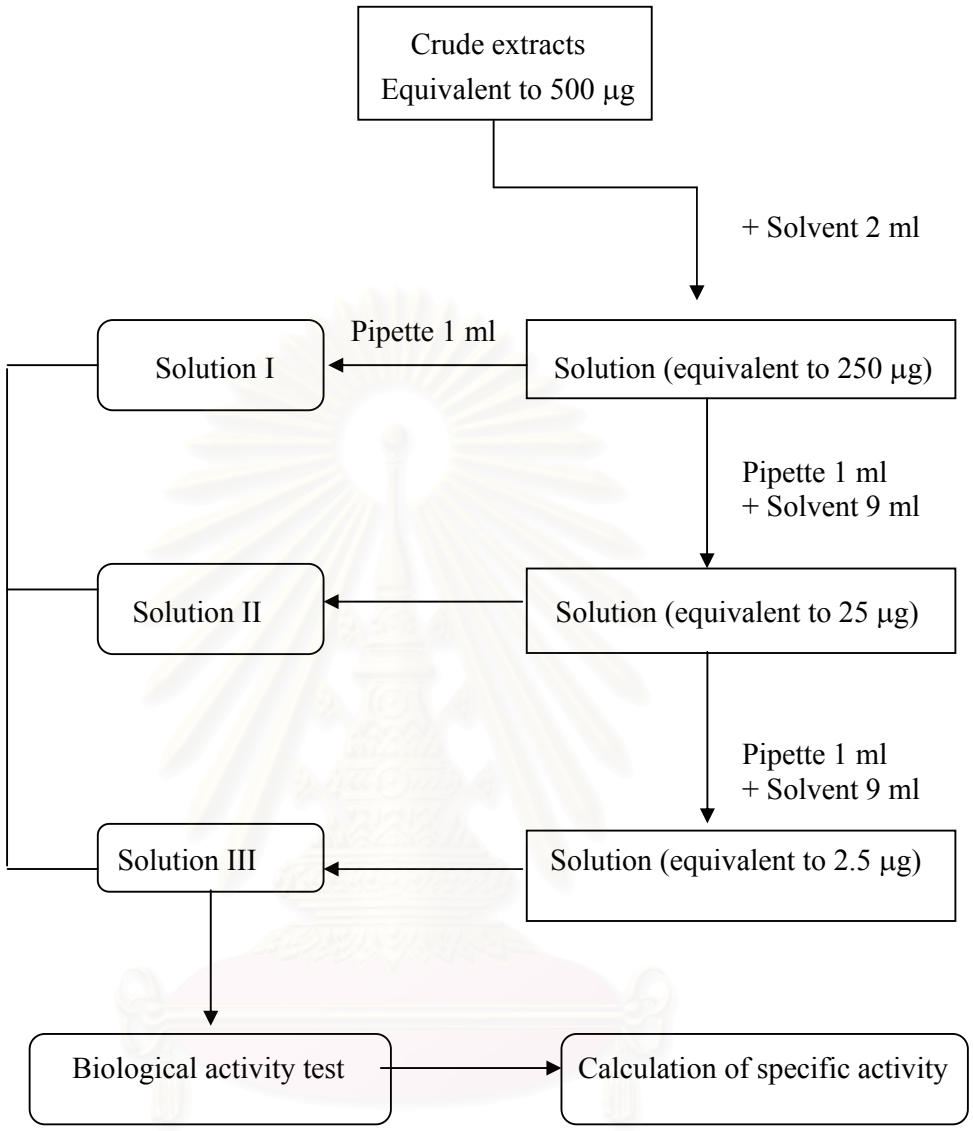
The standard assay mixture contained, in a final volume of 100 μl, 3 mM ATP-Tris, 5 mM $MgCl_2$, 0.5 mM EDTA, 140 mM NaCl, 14 mM KCl and 50 mM Imidazole (pH 7.2). The brain microsome was then diluted with washing buffer to a concentration of 5 μg /100 μl. The reaction was initiated by addition of ATP and incubation was carried out at 37 °C for 30 min. The reaction was stopped by using 50% trichloroacetic acid (TCA). The amount of inorganic phosphate liberated in the supernatant was measured colorimetrically according to the method of Fiske and Subbarow (Appendice III). One unit of specific activity is defined as the liberation of 1 μmol of inorganic phosphate per mg protein per min.

3.7 Experiments for bioassays

Na^+-K^+-ATPase inhibition test was used as a main bioassay to verify the bioactive compound presence. Selected plants for this investigation were traditional plants used as diuretic. Detailed bioassay experiments were as follows:

3.7.1 Experiment for crude extract

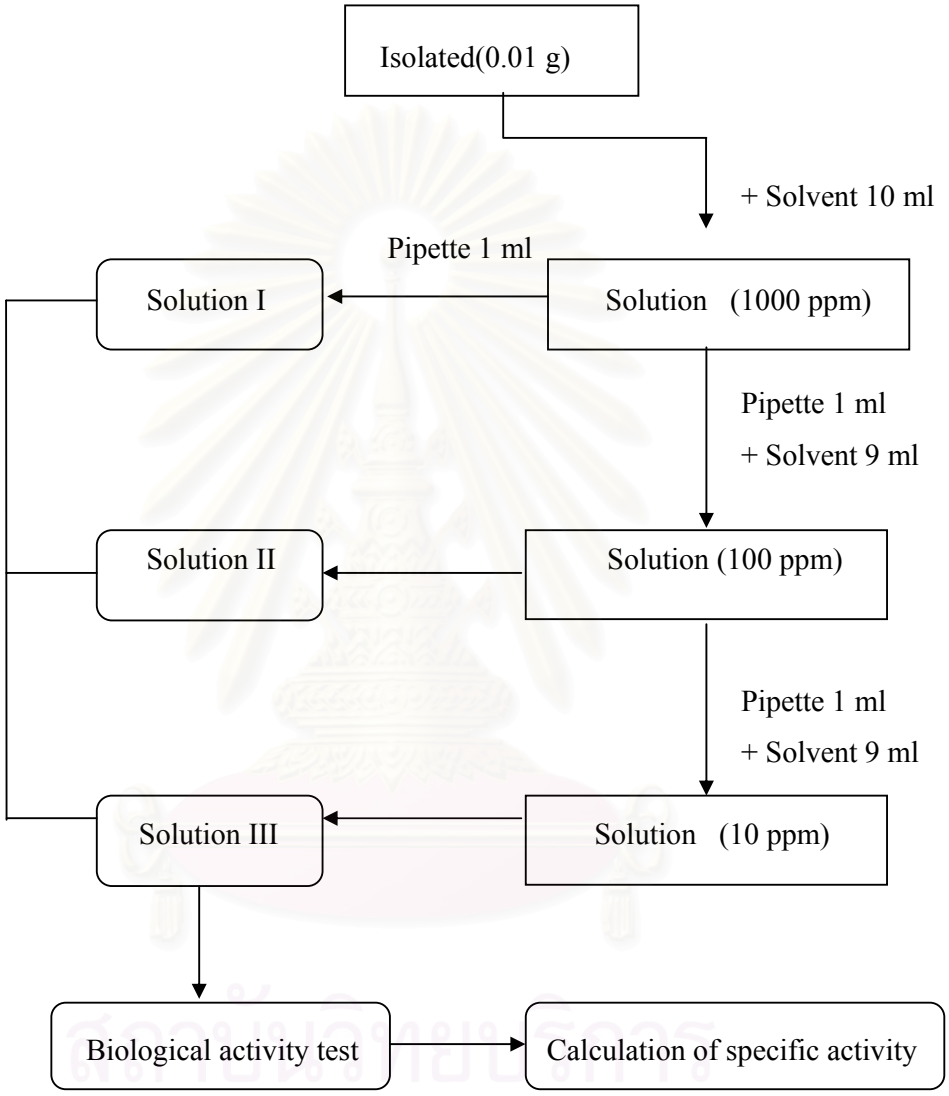
Crude extract of 2.5, 25 and 250 μg equivalent to dried materials was dissolved in 1 ml of dimethylsulfoxide (DMSO). These procedures could be summarized in Schemes 3.2. The mixture containing tested substance was added with and without ouabain. Other procedures were conducted in the same manner as in 3.6. Calculation of specific activity and inhibitory effect of substances were carried out using equation shown below. The absorbance of the enzyme mixture with DMSO is the same as the absorbance of blank which showed that DMSO had no effect on Na^+-K^+-ATPase activity. Each experiment was repeated in three replications.



Scheme 3.2 Bioassay test procedure for crude extracts

3.7.2 Experiment for isolated compounds

Isolated compounds were prepared in the concentrations of 10, 100 and 1000 ppm. The bioassay procedure was performed exactly as the same as that for crude extract. These procedures could be summarized in Schemes 3.3.



Scheme 3.3 Bioassay test procedure for pure compounds

Calculation:

$$\text{Specific Activity (SA)} = \frac{0.1x\ (A-B)}{C}$$

When "A" was the absorbance at 700 nm of mixture without ouabain

"B" was the absorbance at 700 nm of mixture with ouabain

"C" was the absorbance of standard phosphate (0.2 mM KH_2PO_4)

$$\%\ \text{Activity} = \frac{D \times 100}{E}$$

When "D" was SA of enzyme with testing sample

"E" was SA of enzyme with DMSO (vehicle)

CHAPTER IV

RESULTS AND DISCUSSION

Ten medicinal plants were extracted by hexane, chloroform and ethanol for preliminary screening tests for Na^+-K^+-ATPase activity. Crude extract with highest biological activity, was selected for further chemical constituents investigation.

4.1 Isolation of crude enzyme Na^+-K^+-ATPase from the rat brain

The crude enzyme was isolated in four baths from the brain of sprague-dawley rats weighting about 0.839-1.145 g. The results are summarized as shown in Table 3.1.

Table 4.1 The isolation of crude enzyme from the rat brain

Time of isolation	Total weight of the brains (g)	Total protein assay (mg protein)	Specific activity (μmol Pi /mg protein/hr)
1	1.98	11.63	23.2
2	3.65	19.39	15.2
3	7.69	27.01	28.0
4	7.81	30.48	12.8

The results revealed that specific activity of crude enzyme of the 1st and 3rd isolation were 23.2 and 28.0 μmol Pi/mg protein/hr, respectively, and were higher than other isolation. The crude enzyme of the 1st isolated was used for activity test with crude extracts of ten plants.

4.2 The optimal concentration of crude enzyme Na^+-K^+-ATPase for activity testing

ATPase activity of rat microsome Na^+-K^+-ATPase in relation to enzyme concentration at 5 and 10 μg/ 100 ml were $9.32x10^{-3}$ and $1.04x10^{-2}$ μmol Pi/min, respectively.

It was found that the activity of crude enzyme from rat brain between 5 and 10 μg/100 μl were not significantly difference. Thus, 5 μg/100ml of crude enzyme was chosen for activity tests.

4.3 Inhibition of Na^+-K^+-ATPase activity for preliminary screening tests of ten medicinal plants

Ten plants were minced to coarse powder and extracted according to the procedure described in Chapter III. Crude extracts were preliminary screened for various activities followed the procedures mentioned in Chapter III. All bioassay results are summarized in Tables 4.2 and Figure 4.1.

Table 4.2 Inhibition effect of each crude extract of ten medicinal plants on Na^+-K^+-ATPase activity

Crude Extracts	%Inhibition of each crude extracts at various concentrations (μg/ml)								
	Ethanol			Hexane			Chloroform		
	2.5	25	250	2.5	25	250	2.5	25	250
H. sabdariffa	12	22	22	27	17	36	10	5	48
P. indica	0	0	0	6	27	53	0	0	0
C. alata	27	35	70	0	0	0	0	0	35
E. scaber	8	24	88	0	0	0	0	0	0
A. calamus	4	14	21	18	32	32	11	7	63
A. comosus	0	0	84	0	31	87	0	73	80
I. cylindrica	0	8	0	0	0	0	0	0	36
O. aristatus	0	0	19	59	68	84	0	55	42
C. rotundus	38	20	88	83	85	100	10	13	23
S. officinarum	25	28	23	0	0	0	0	0	0

The results of inhibitory activity of Na^+-K^+-ATPase, it indicated that the hexane crude extracts of *C. rotundus* (100% inhibition) is higher than of those *A. comosus* (87%), *O. aristatus* (84%), *P. indica* (53%), *H. sabdariffa* (36%) and *A. calamus* (32%) at concentration of 250 μg/ml, respectively. For the chloroform crude extracts, *A. comosus* (80%) displayed the highest percent inhibition than those of *A. calamus* (63%), *P. indica* (48%), *O. aristatus* (42%), *I. cylindrica* (36%) and *C. alata* (35%) at concentration 250 μg/ml, respectively. For the ethanol crude extract of *C. rotundus* (88%) and *E. scaber* (88%) they exhibited higher activity than those of *A. comosus* (84%), *C. alata* (70%), *S. officinarum* (23%), *H. sabdariffa* (22%), *A. calamus* (21%) and *O. aristatus* (19%) at concentration 250 μg/ml, respectively. Therefore, the hexane and ethanol crude extract of *C. rotundus* and ethanol crude extract of *E. scaber* were selected for further study.

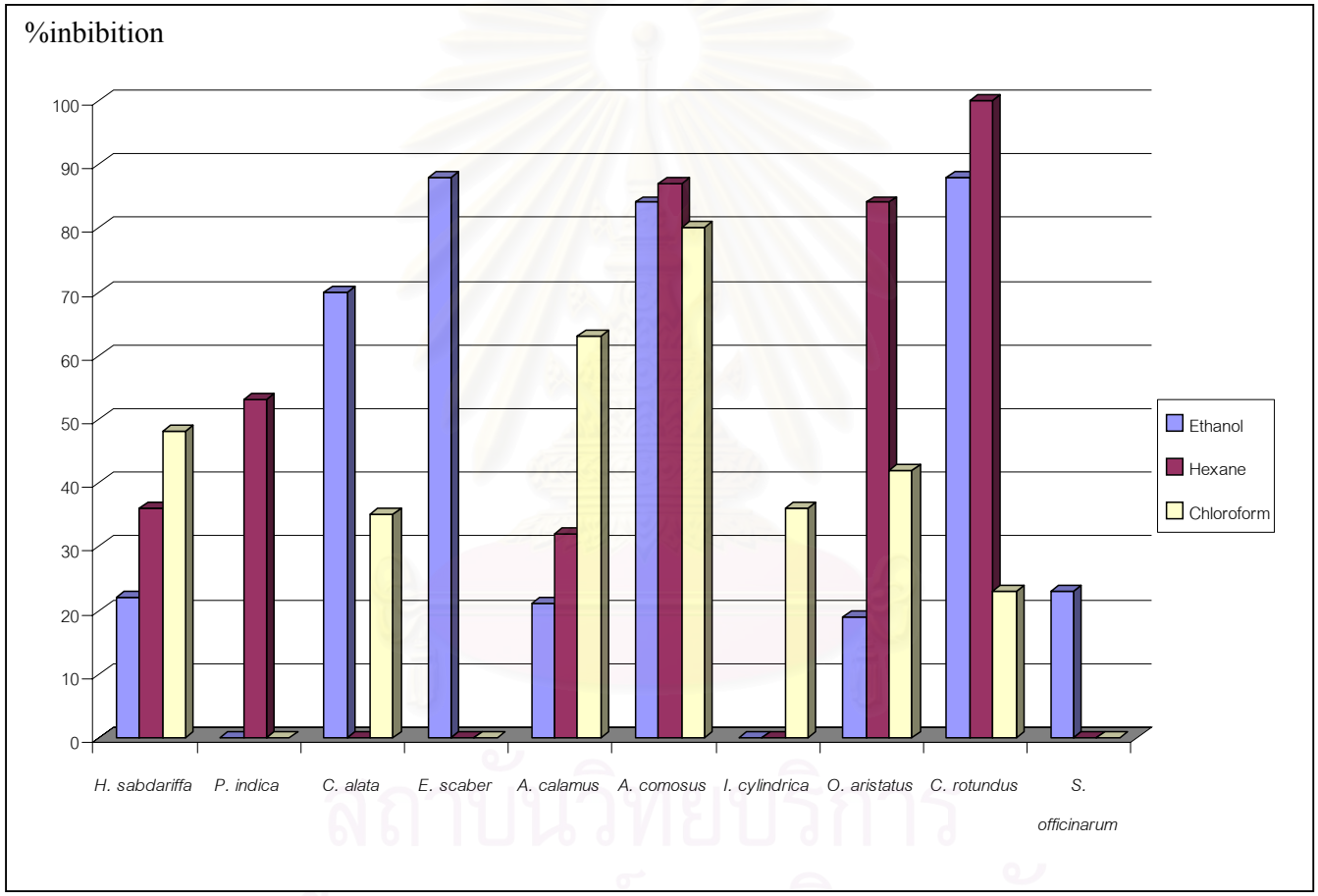


Figure 4.1 Effect of crude extracts of ten herbs on inhibition of Na^{+}-K^{+}-ATPase activity at 250 µg/ml concentration

4.4 Fractionation

According to preliminary study of Na^+-K^+-ATPase inhibition activities (see Table 4.2), hexane and ethanol crude extracts of *C. rotundus* and ethanol crude extract of *E. scaber* showed highest activity. The separation of crude extracts into small fractions was carried out and the biological activity test of each derived fraction was carefully monitored.

4.4.1 Fractionation of crude hexane extracts of *C. rotundus* (fraction I)

The hexane extract, fraction I, was concentrated togive 41.81 g of red-brown sticky liquid (0.836% w/w). It was separated by quick column chromatographic techniques using silica gel (no. 9358, 350.22 g) as absorbent. Crude extracts (26.36 g) were mixed with silica gel to dryness before being added on the top of a column and then the column was eluted with an increasing gradient of hexane, dichloromethane in hexane, dichloromethane, methanol in dichloromethane and finally methanol (approximately 300 ml per fraction). Every fraction was collected, concentrated to a small volume and then monitored by TLC. The fractions which contain the same compounds were combined, as shown in Table 4.4. Each compound was further purified by column chromatography and re-crystallization techniques.

Table 4.3 The separation of hexane extract by quick column chromatography

Solvent system (v/v)	**Fraction no.**
100% Hexane	1-5
10% CH_2Cl_2-Hexane	6-10
25% CH_2Cl_2-Hexane	11-15
40% CH_2Cl_2-Hexane	16-20
50% CH_2Cl_2-Hexane	21-27
60% CH_2Cl_2-Hexane	28-34
70% CH_2Cl_2-Hexane	35-40
80% CH_2Cl_2-Hexane	41-51
85% CH_2Cl_2-Hexane	52-55
90% CH_2Cl_2-Hexane	56-60
95% CH_2Cl_2-Hexane	61-65
100% CH_2Cl_2	66-71
5% MeOH- CH_2Cl_2	72-77
10% MeOH- CH_2Cl_2	78-83
20% MeOH- CH_2Cl_2	84-89
30% MeOH- CH_2Cl_2	90-95
40% MeOH- CH_2Cl_2	96-100

Table 4.4 Result of all fraction which contain the same R_f

Fraction No.	Fraction code	Remark	Weight (g)
1-3	I A	Yellow oil	2.93
4-6	I B	Yellow oil	1.84
7-15	I C	Yellow oil	2.57
16-24	I D	Yellow oil	2.49
25-31	I E	Yellow oil **(Mixture 1)**	1.38
32-40	I F	Yellow oil	0.96
41-49	I G	Yellow semi solid **(Mixture 2)**	1.07
50-65	I H	Yellow semi solid	1.12
66-73	I I	Yellow semi solid	0.98
74-78	I J	Dark brown oil **(compound 1)**	6.01
79-81	I K	Brown residue	3.18
82-84	I L	Brown residue	2.86
85-91	I M	Brown residue	2.32
92-98	I N	Brown residue	0.95

Each fraction was examined for its bioactivity on Na^+-K^+-ATPase inhibitory activity. The results are shown in Table 4.5 and Figure 4.2.

Table 4.5 Inhibitory activity of each fraction from hexane crude extract of *C. rotundus* on Na^+-K^+-ATPase activity

Fraction code	% Inhibition at various concentration	
	25 μg/ml	**250 μg/ml**
I A	6.66	9.17
I B	0	6.46
I C	12.47	22.09
I D	20.53	30.35
I E	7.67	21.15
I F	15.92	23.67
I G	10.12	23.89
I H	26.12	41.00
I I	7.95	46.57
I J	27.49	48.81
I K	33.05	52.63
I L	40.48	48.81
I M	39.73	58.86
I N	15.04	34.93

Fraction IM showed the highest inhibition (58.86%), followed by fractions IK, IL, IJ, II and IH with the inhibitory activity of 52.63, 48.81, 48.81, 46.57 and 41.00%, respectively at 250 μg/ml concentration. Accordingly, these fractions were separated to obtain active ingredients. Fractions IA and IB did not show good activity even at 250 μg/ml concentration.

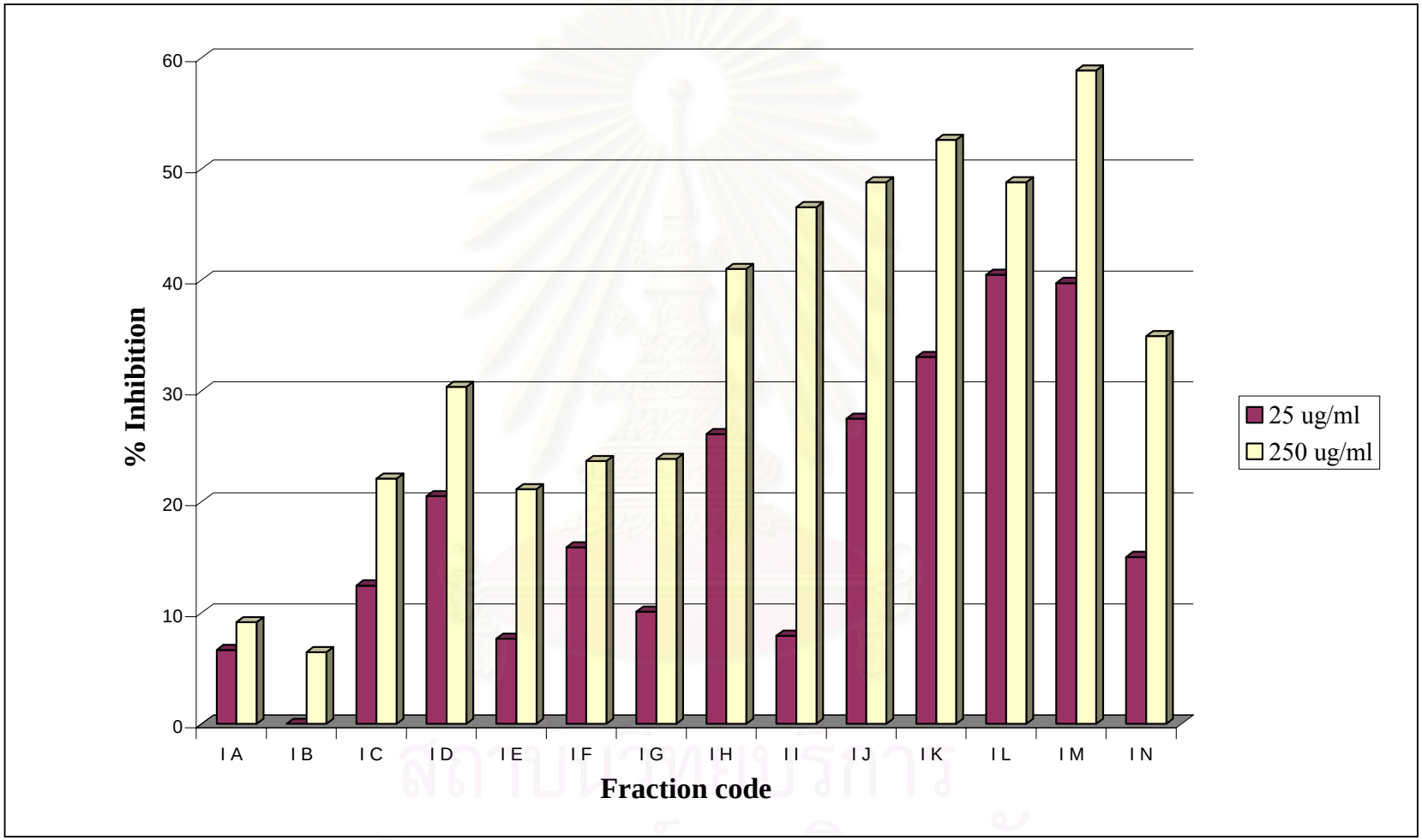


Figure 4.2 Inhibitory activity of hexane fraction of *C. rotundus* on Na^+-K^+-ATPase activity

4.4.2 Fractionation of crude ethanol extract of *C. rotundus* (fraction II)

Crude ethanol extract was separated by column chromatographic techniques. Silica gel no. 7734 (390.52 g) was used in column chromatography. Crude extracts (30.19 g) were mixed with silica gel to dryness before being added on the top of a column and then the column was eluted with an increasing gradient of hexane, ethyl acetate in hexane, ethyl acetate, methanol in ethyl acetate and finally methanol (approximately 50 ml per fraction). Each fraction was collected, concentrated to a small volume and then monitored by TLC. The fractions which contain the same compounds were combined, as shown in Table 3.4. Each compound was further purified by column chromatographic and re-crystallization techniques.

Table 4.6 The separation of crude ethanol extract by column chromatography

Solvent system (v/v)	Fraction no.
100% Hexane	1-20
20% EtOAc-Hexane	21-40
25% EtOAc-Hexane	41-49
30% EtOAc-Hexane	50-67
40% EtOAc-Hexane	68-83
50% EtOAc-Hexane	84-101
60% EtOAc-Hexane	102-117
70% EtOAc-Hexane	118-135
80% EtOAc-Hexane	136-154
90% EtOAc-Hexane	155-172
100% EtOAc	173-189
10% MeOH-EtOAc	190-205
20% MeOH-EtOAc	206-223
30% MeOH-EtOAc	224-241
50% MeOH-EtOAc	242-258
70% MeOH-EtOAc	259-266

Table 4.7 Result of all fractions which contain the same R_f

Fraction No.	Fraction code	Remark	Weight (g)
1-49	II A	Yellow oil	2.01
50-67	II B	yellow green oil	1.22
68-77	II C	Solid in yellow oil	1.95
78-86	II D	Yellow sticky oil	0.62
87-105	II E	Dark yellow oil	0.87
106-114	II F	Dark yellow oil	0.38
115-161	II G	Yellow sticky oil	1.51
162-177	II H	Brown oil	0.26
178-193	II I	Brown oil	0.38
194-210	II J	Pale brown oil	0.22
211-234	II K	Solid in brown oil	0.81
235-266	II L	Solid in brown oil	0.48

Each fraction derived from the separation of ethanol extract was carefully monitored with the biological activity test (Table 4.8).

Table 4.8 Inhibitory activity of crude ethanol extracts of *C. rotundus* on Na^+-K^+-ATPase activity

Fraction code	%Inhibition at various concentration	
	25 (µg/ml)	250 (µg/ml
II A	0	0
II B	0.95	6.67
II C	0	0
II D	3.49	25.51
II E	0	18.60
II F	6.98	23.26
II G	0	18.62
II H	33.05	52.83
II I	0	0
II J	28.57	33.33
II K	12.5	72.92
II L	29.17	70.42

The result of inhibitory activity on Na^+-K^+-ATPase from each fraction of ethanol extract of *C. rotundus* revealed that fraction IIH, IIK and IIL exhibited good inhibitory activity.

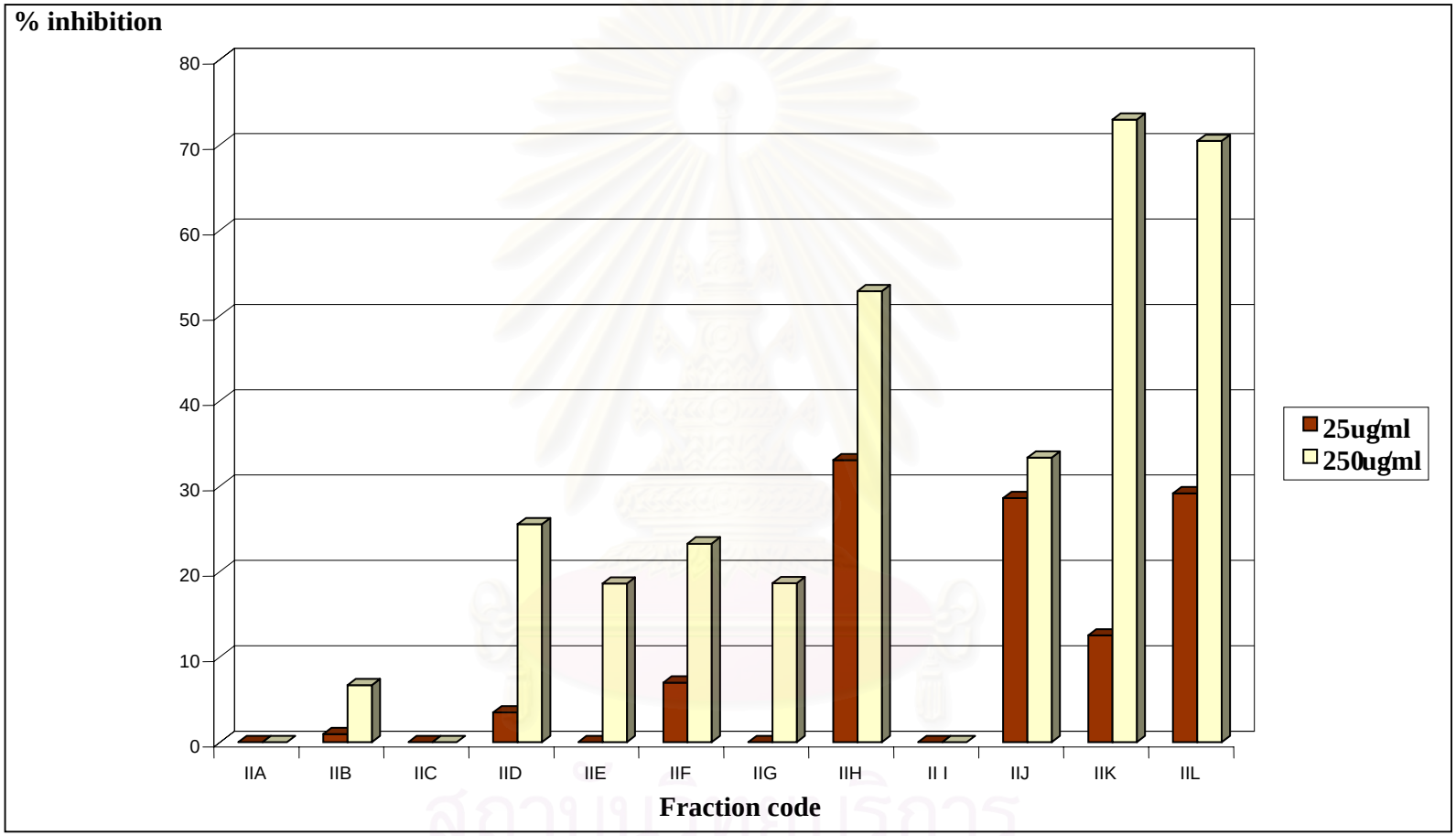


Figure 4.3 Inhibitory activity of crude ethanol extracts of *C. rotundus* on Na^{+}-K^{+}-ATPase activity

4.4.2.1 Isolation of fraction IIK and IIL from crude ethanol extract (fraction III)

The combined weight of fraction III is 1.292 g. It was further chromatographic using silica gel (No. 9358) as an adsorbent. The elution started from 50% ethyl acetate in hexane and collected approximately 10 ml per fraction. Eight fractions (EK1-EK8) were obtained, as shown in Table 4.10. The biological activity test of each fraction was carefully monitored (Table 4.11).

Table 4.9 The separation of fraction III by column chromatography

Solvent system (v/v)	Fraction no.
50% EtOAc-Hexane	1-108
60% EtOAc-Hexane	109-132
70% EtOAc-Hexane	133-157
80% EtOAc-Hexane	158-188
90% EtOAc-Hexane	189-220
100% EtOAc	221-243
10% MeOH-EtOAc	244-266
30% MeOH-EtOAc	267-287
50% MeOH-EtOAc	288-296
70% MeOH-EtOAc	297-304

Table 4.10 Combined fraction from fraction III separation

Fraction No.	Fraction code	Remark	Weight (mg)
1-40	III A	Yellow oil	61.5
41-135	III B	Yellow oil	30.7
136-204	III C	Brown oil	203.5
205-239	III D	Solid in dark brown oil	123.0
240-253	III E	Brown oil	34.7
254-270	III F	Brown oil	288.4
271-289	III G	Brown residue	136.2
290-304	III H	Dark brown residue	296.3

Table 4.11 Inhibitory activity of each fraction separated from fraction III on Na^{+}-K^{+}-ATPase activity

Fraction code	% Inhibition of various concentration	
	25 (µg/ml)	**250 (µg/ml)**
III A	0	10.21
III B	19.05	25.07
III C	5.69	23.58
III D	7.69	43.07
III E	2.04	0
III F	29.94	46.52
III G	79.10	76.76
III H	46.97	58.05

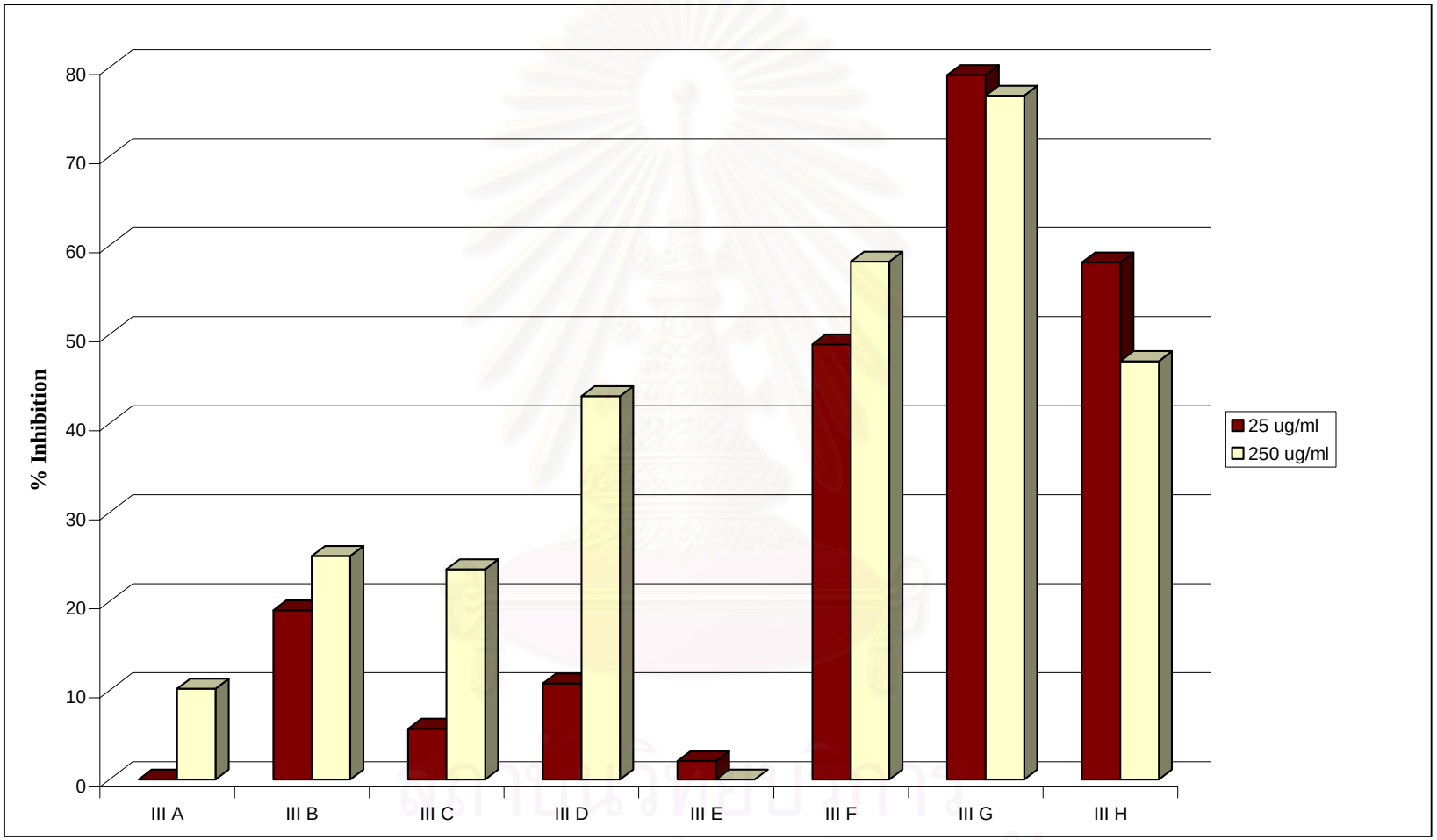


Figure 4.4 Inhibitory activity of fraction III A- III H of *C. rotundus* on Na^{+}-K^{+}-ATPase activity

4.4.3 The Separation of hexane crude extract of *E. scaber* (fraction IV)

The crude ethanol extract (20 g) was preadsorbed on silica gel no. 7734 and was fractionated by column chromatography. The column was eluted with hexane-ethylacetate gradient in a stepwise and finally methanol (approximately 50 ml per fraction). Each fraction was examined by TLC using 60% ethyl acetate in hexane as a developing solvent. Fractions with similar chromatographic patterns were combined, as shown in Table 4.12 and 4.13.

Table 4.12 The separation of hexane extract by quick column chromatography

Solvent system (v/v)	Fraction no.
100% Hexane	A,B, 1-11
5% EtOAc- Hexane	12-28
10% EtOAc- Hexane	29-34
20% EtOAc- Hexane	35-51
30% EtOAc- Hexane	52-66
40% EtOAc- Hexane	67-83
50% EtOAc- Hexane	84-97
60% EtOAc- Hexane	98-109
70% EtOAc- Hexane	110-125
80% EtOAc- Hexane	126-139
90% EtOAc- Hexane	140-153
100% EtOAc	154-166
5% MeOH-EtOAc	167-179
10% MeOH-EtOAc	180-193
20% MeOH-EtOAc	194-208
30% MeOH-EtOAc	209-217
50% MeOH-EtOAc	218-228
70% MeOH-EtAc	229-2362
100% MeOH	237-248

Table 4.13 The combined fractions having the same R_f

Fraction code	Fraction no.	Remark	Weight (g)
IV A	A,B, 1-37	Yellow oil	4.39
IV B	38-43	Yellow oil	3.85
IV C	44-48	Solid in Yellow oil	0.29
IV D	49-52	White solid **Compound 2**	1.02
IV E	53-60	Brown sticky oil	1.08
IV F	61-64	Dark Brown sticky oil	0.25
IV G	65-67	Dark Brown sticky oil	0.28
IV H	68-74	Black sticky oil	0.23
IV I	75-96	Green residue	0.71
IV J	97-103	Dark yellow residue	0.16
IV K	104-106	Dark green oil	0.23
IV L	107-119	Dark green oil	0.69
IV M	120-126	Green residue	0.16
IV N	127-135	Green residue	0.07
IV O	136-166	Brownish sticky oil	0.22
IV P	167-204	Dark yellow sticky oil	0.23
IV Q	205-209	Yellow residue	0.02
IV R	210-221	Dark green residue	1.33
IV S	222-248	Dark green residue	1.75

Table 4.14 Inhibitory activity of each fraction from crude ethanol extract of *E. scaber* on Na^+-K^+-ATPase activity

Fraction code	% Inhibition at various concentration	
	25 μg/ml	**250 μg/ml**
IV A	1.58	9.45
IV B	6.39	11.92
IV C	7.60	12.05
IV D	63.67	90.94
IV E	38.15	85.14
IV F	40.76	91.71
IV G	26.60	63.59
IV H	19.58	63.97
IV I	21.77	70.82
IV J	34.67	89.41
IV K	27.78	60.78
IV L	7.21	10.53
IV M	3.51	9.67
IV N	8.73	18.72
IV O	13.60	28.56
IV P	26.98	78.84
IV Q	0	0.29
IV R	45.38	85.77
IV S	37.79	86.03

From the above result, it was indicated that fraction IV F (91.71%), IV D (90.94%), IV J (89.41%), IV S (86.03%), IV R (85.77%), IV E (85.14%), IV P (78.84%), IV I (70.82%), IV H (63.97%), IV G (63.59%) and IV K (60.78%) had good inhibitory activity, respectively.

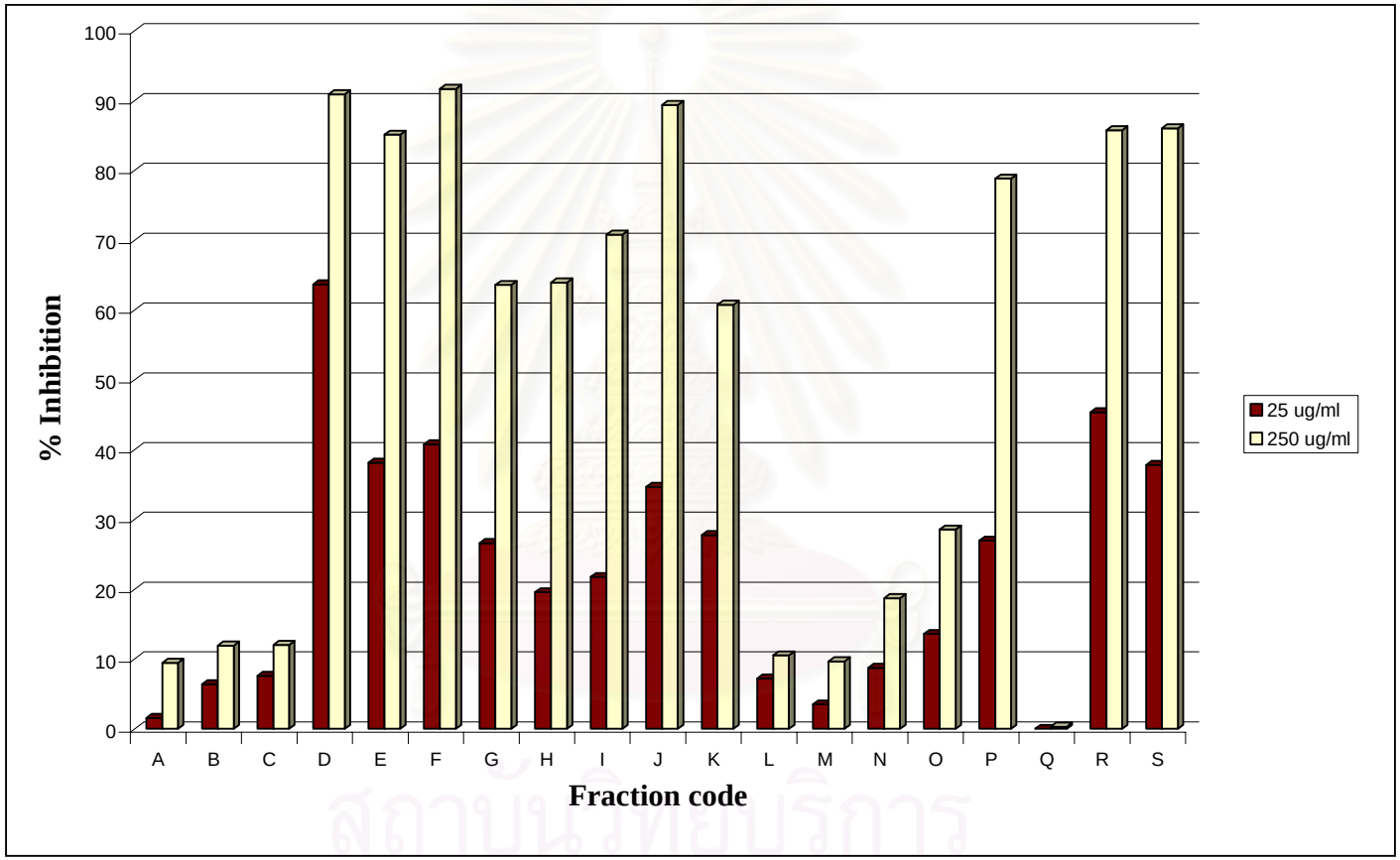


Figure 4.5 Inhibitory activity of fraction A- S of *E. scaber* on Na^{+}-K^{+}-ATPase activity

4.5 Structural elucidation of isolated compounds from *C. rotundus*

4.5.1 Structural elucidation of mixture 1

Mixture 1, white solid was obtained from fraction no. 25-31 of the hexane extract (fraction I) which was further fractionated by silica gel column chromatography eluting with 50% CH_2Cl_2-Hexane - 60% CH_2Cl_2-Hexane. This fraction was washed with hexane and filtered to yield mixture 1, 94.6 mg (0.23% w/w of fraction I), m.p. 142-144 ^{o}C. The IR spectrum (Figure 6.1) was summarized in Table 4.13.

Table 4.15 The IR absorption bands assignment of mixture 1

Wave number (cm^{-1})	Peak intensity	Tentative assignment
3424	Broad	O-H stretching vibration of alcohol
2937	Strong	C-H stretching vibration of $-CH_3$, $-CH_2$
1641	Medium	C=C stretching vibration
1470	Medium	C-H bending vibration of $-CH_3$
1057	Medium	C-O stretching vibration

The ^{1}H-NMR spectrum (Figure 6.2) showed that the signals of protons at chemical shift 0.66-2.26 ppm. Corresponding to those of methyl, methylene and methine groups ($-CH_3$, $-CH_2$, -CH, respectively) of the steroids. The proton adjacent to a hydroxyl group (-CH-OH) was shown as the multiple signal at 3.43-3.56 ppm. (2H, *J*=5.0 Hz) ppm. The signals at δ 5.00-5.40 ppm. were assigned to be the signals of olefinic protons (-CH=C-).

The ^{13}C-NMR spectrum (Figure 6.3) showed the olefinic carbon signals at 121.7, 129.3, 138.3 and 140.8 ppm. The carbon signal at 71.8 ppm. was the C-OH of the steroid.

Mixture 1 was identified as a mixture of β-sitosterol and stigmasterol by comparison of its ^{1}H and ^{13}C-NMR spectral data with those reported in the literature (Francisco *et al.*, 1994). The structures of these two steroids are shown in Figure 4.6.

Table 4.16 The ^{13}C-NMR chemical shift assignment of β-sitosterol, stigmasterol and mixture 1 (in $CDCl_3$)

Carbon	Chemical shift (ppm)		
	β-sitosterol	**stigmasterol**	**Mixture 1**
1	37.31	37.31	37.26
2	31.57	31.67	31.61
3	71.69	71.81	71.81
4	42.45	42.35	42.32, 42.26
5	140.76	140.80	140.74
6	121.59	121.69	121.74
7	31.92	31.94	31.90
8	31.92	31.94	31.90
9	51.17	50.20	50.11
10	36.51	36.56	36.52
11	21.11	21.11	21.25
12	39.81	39.74	39.77, 39.68
13	42.33	42.35	42.21
14	56.79	56.91	56.86, 56.76
15	24.32	24.39	23.38
16	28.26	28.96	28.27, 28.96
17	56.11	56.06	56.04
18	11.87	12.07	12.0, 11.87
19	19.40	19.42	19.42
20	36.17	40.54	36.17, 40.55
21	18.82	21.11	19.04, 21.09
22	33.95	138.37	33.93, 138.36
23	26.13	129.32	29.12, 129.26
24	45.85	51.29	50.14, 51.25
25	29.18	31.94	26.03, 29.74
26	19.84	21.26	18.79, 19.0
27	18.04	19.02	19.85, 21.1
28	23.09	25.44	23.05, 25.44
29	12.32	12.27	12.06, 12.29

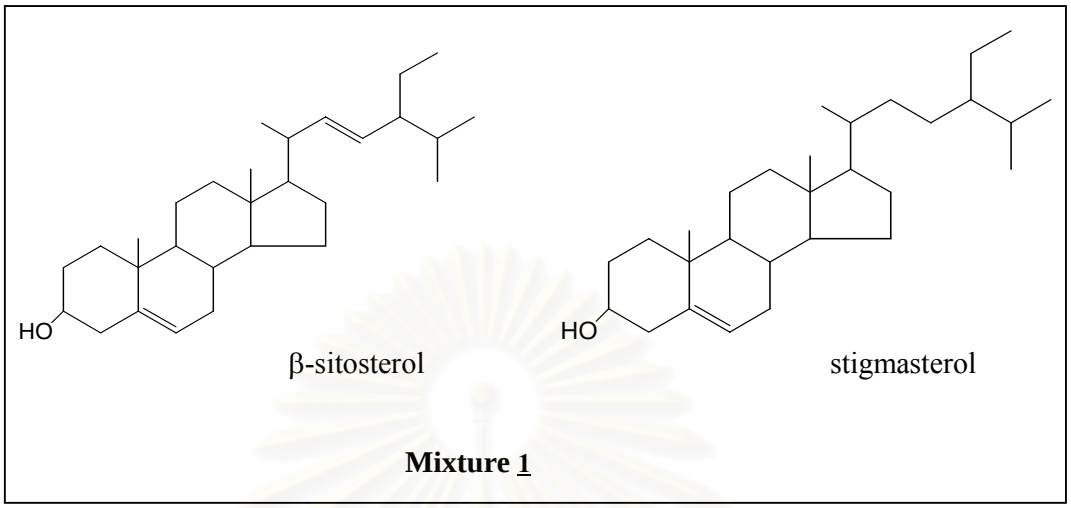


Figure 4.7 The structure of stigmasterol and β-sitosterol

4.5.2 Structural elucidation of mixture 2

Mixture 2 was obtained from fraction I of hexane extract (fraction no. 41-49) eluted with 80% CH_2Cl_2-Hexane. This mixture was a white solid (67 mg, 0.16% w/w of fraction I), m.p. 84.8-85.1 ^oC. The IR spectrum of mixture 2 is depicted as shown in Figure 6.4 and the tentative IR absorption band assignments of mixture 2 are presented as shown in Table 4.15.

Table 4.17 IR absorption band assignments of mixture 2

Wave number (cm^{-1})	Peak intensity	Tentative assignment
3438	Broad	O-H stretching vibration of carboxylic acid
2910, 2844	Strong	C-H stretching vibration of $–CH_3$,-CH_2-
1701	Strong	C=O stretching vibration of R-COOH
1466	Medium	C-H stretching vibration of $–CH_3$,-CH_2-
718	medium	C-H rocking mode of $–(CH_2)_n$-

The ^{1}H-NMR spectrum (Figure 6.5) of mixture 2 showed the proton signals of a methylene proton attach to a carboxyl group at δ 2.38 ppm and a methyl proton at δ 0.97 ppm.

The ^{13}C-NMR spectrum (Figure 6.6) of mixture 2 showed 12 signals. Ten signals of methylene carbons appeared at δ 34.0, 32.0, 29.7, 29.6, 29.5, 29.3, 29.1, 24.7 and 22.7 ppm and a signal of methyl carbon appeared at 14.2 ppm. The signal at δ 172.5 ppm should be the carboxyl group of carboxylic acid.

The EI mass spectrum (Figure 6.7) displayed the molecular ions at m/z 340, 368, 396, 424 and 452. They were separated by 28 mass units (-CH_2-CH_2-) and they showed the characteristic pattern of long chain hydrocarbons. Therefore, mixture 2 was assigned as a mixture of long chain carboxylic acids.

CH_3-$(CH_2)_n$-COOH

Saturated long chain carboxylic acid

4.5.3 Structural elucidation of compound 1

Compound 1 was obtained as white solid in yellow oil separated from fraction I, fraction no 74-78 eluted with 5% MeOH-CH_2Cl_2. This compound (71.53 mg, 0.17% w/w of fraction I), had m.p. 247.5 °C. The IR spectrum of this compound is shown in Figure 6.8 and the absorption peaks are assigned as summarized in Table 4.16. Unfortunately, this compound is insoluble in any common solvent, therefore, NMR spectral data could not be obtained. Thus, the identity of this compound is still unknown.

Table 4.18 The IR absorption band assignment of compound 1

Wave number (cm^{-1})	Peak intensity	Tentative assignment
3400	Broad	O-H stretching vibration of alcohol
2937, 2867	Strong	C-H stretching vibration of –CH_3, -CH_2
1731	Weak	C=O stretching vibration of R-COOH
1454, 1372	Medium	C-H bending vibration of –CH_3
1073, 1022	Medium	C-O stretching vibration

4.5.4 Structural elucidation of compound 2

Compound 2 was obtained as white solid from fraction IV. The IR spectrum of this compound is shown in Figure 6.9 and the absorption peaks were assigned as summarized in Table 4.19. The ^{13}C NMR spectrum of compound 2 are shown in Figure 6.10.

Compound 2 was identified as β-sitosterol by comparison of its ^{1}H and ^{13}C-NMR spectral data with those reported in the literature (Francisco *et al.*, 1994). The structures of these two steroids are shown in Figure 4.6.

Table 4.19 The IR absorption band assignment of compound 2

Wave number (cm^{-1})	Peak intensity	Tentative assignment
3396	Broad	O-H stretching vibration of alcohol
2945	Strong	C-H stretching vibration of $–CH_3$, $-CH_2$
1641	Weak	C=C stretching vibration
1450, 1376	Medium	C-H bending vibration of $–CH_3$
1038	Medium	C-O stretching vibration

4.6 Bioassay result

4.6.1 Bioassay result of isolated substances

From the fractionation and purification of *C. rotundus* crude hexane extract, 2 substances were isolated. The effect on Na^+-K^+-ATPase inhibitory activity is presented in Table 4.20 and Figure 4.8.

Table 4.20 The effect of isolated substances on Na^+-K^+-ATPase inhibitory activity

Substances	% Inhibition at various concentration (ppm)		
	10	100	1000
Mixture 1	7.25	29.61	38.13
Mixture 2	10.32	24.97	44.00

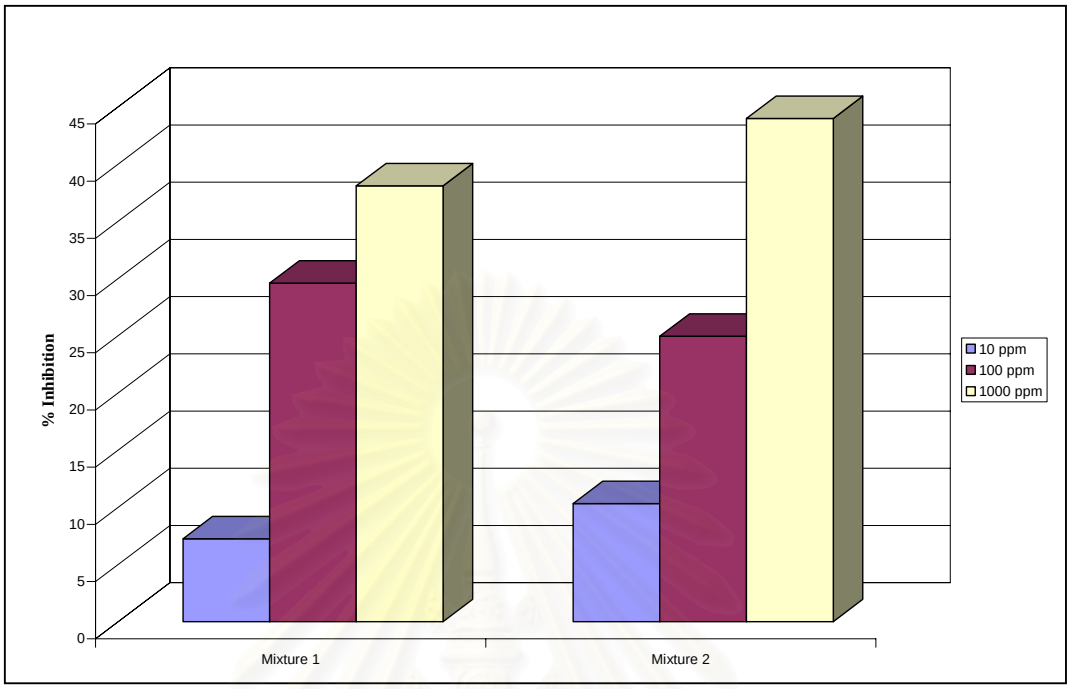


Figure 4.8 Effect of isolated substances on Na^{+}-K^{+}-ATPase inhibitory activity

CHAPTER V

CONCLUSION

The brains of two rats which total weight of 1.98 g were used to isolate crude Na^+-K^+-ATPase enzyme (11.63 mg protein, specific activity 23.2 μmol Pi/mg protein/hr). The optimum incubation time of crude enzymes is 30 min, 37 ^oC and concentration of crude enzyme is 5 μg/100 μl for biological activity. The preliminary results revealed that 50% inhibition of Na^+-K^+-ATPase activity was in the order of *C. rotundus* rhizomes (crude hexane and ethanol extracts) > *E. scaber* all parts (crude ethanol extracts) > *A. comosus* roots (all crude extracts) > *A. calamus* roots (crude chloroform extracts) > *O. aristatus* all parts (crude hexane extracts) > *P. indica* stems (crude hexane extracts) while 4 types --- *I. cylindrica, C. alata, S. sinense* and *H. sabdariffa* have activity lower than 50% at 250 μg/ml concentration. The activity was compared with ouabain which is the known Na^+-K^+-ATPase inhibitor. Then, compounds were isolated from crude hexane and ethanol extracts of *C. rotundus*, and crude ethanol extracts of *E. scaber* were separated using column chromatography. Four substances were isolated and purified and identified as a mixture of two steroids --- β-sitosterol and stigmasterol (mixture 1), a mixture of long chain carboxylic acids (Mixture 2) and two unidentified of compounds.

The result of biological activity of those compounds indicated that Mixture 1 and Mixture 2 gave 39 % and 44 % inhibition on Na^+-K^+-ATPase (27.07 mg protein, specific activity 28.0 μmol Pi/mg protein/hr) at dose level 1000 ppm, respectively. Leifert (2000) reported antiarrhythmic effect in adult rats with saturated fatty acid docosanoic acid. Alteration in the fatty acid composition of neural membrane and resulting changes in membrane fluidity may affect the binding of enzyme initiating activity (Slater *et al*, 2004). In 1999, Gerbi studied the inhibition of docosanoic acid (22:0) on Na^+-K^+-ATPase activity. They revalued that saturated acid from sunflower and soybean inhibited Na^+-K^+-ATPase activity in 32% and 68%, respectively.

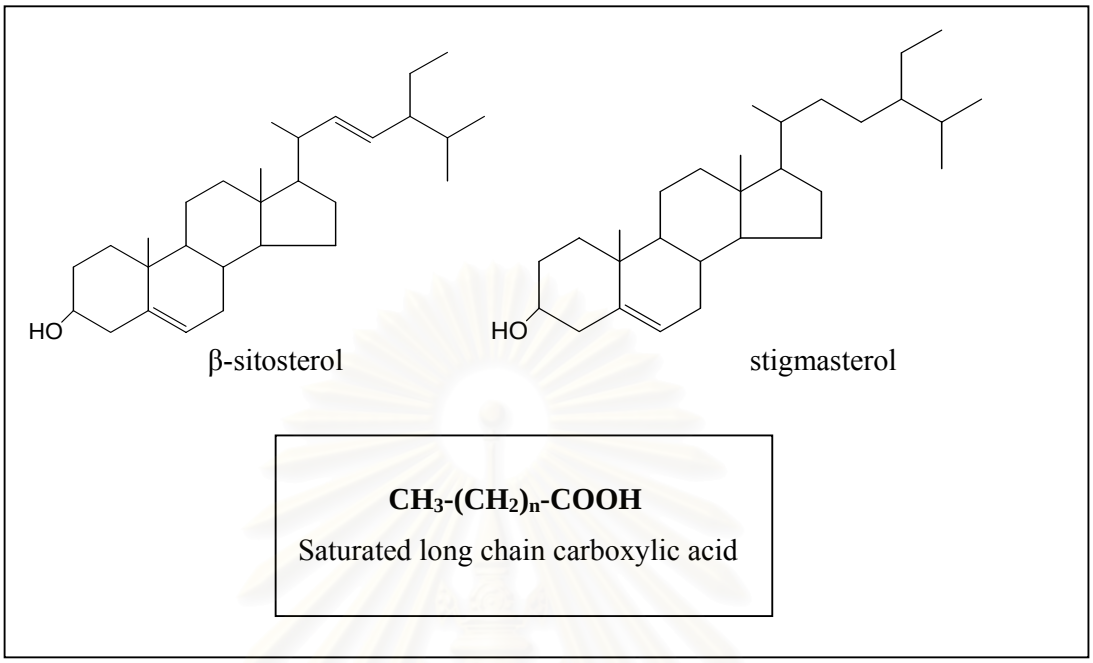


Figure 5.1 Isolates substance from hexane extracts of *C. rotundus*

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APPENDICES

APPENDICES I

PLANT ETHANOMEDICAL USES

***Acorus calamus* Linn.**

Family : Araceae

Common Name : Sweet flag, Waan-nam

Ethanomedical Uses : Rhizome is stated to act as a carminative, spasmolytic, diaphoretic, diuretic, increased sleeping and anti-arrhythmic. Traditionally it has been indicated for acute and chronic dyspepsia, gastritis and gastric ulcer, intestinal colic and anorexia (Newall, 1996).

***Ananas comosus* (Linn.) merr.**

Family : Bromeliaceae

Common Names : Pineapple, Sappparot (central)

Ethnomedical Uses : Roots use for the improvement of kidney function ; treatment of urinary stones, stiffness of joints, any disorder or diseases causing cachexia and urinary stones as a diuretic. Fruit use for the improvement of quality of menstrual blood , inhibition of tissue inflammation; as a mucolytic for viscous saliva, cough depressant and diuretic. (Farnsworth and Bunyapraphatsara, 1992).

***Cassia alata* Linn.**

Family : Leguminosae

Common Names : Candle bush, Acapulo, Ringworm bush, Calalabra bush, chumhet that (central)

Ethnomedical uses for constipation ; treatment of scabies and acne , skin disease, ringworm, Tinea versicolor, urinary stones and skin diseases ; as an anthelmintic, expectorant, diuretic and cardiotonic (Farnsworth and Bunyapraphatsara, 1992).

***Hibiscus sabdariffa* Linn**.

Family: Malvaceae

Common Name : Jamaican sorrel, Roselle of Rama, Krachiap, Krachiap prieo (central)

Ethnomedical uses for decreasing of body temperature, treatment of biliary diseases ; as a diuretic , anti-hypercholesterolemic, and element tonic (Farnsworth. and Bunyapraphatsara, 1992).

***Imperata cylindrica* (Linn.) P. Beauu**.

Family : Gramineae

Common Name : Yaa khaa

Ethnomedical uses for longevity ; treatment of any disorders of menstruation, infectious diaarhoea, abscesses, delirium due to high fever, infectious diseases, exanthematous fever, weakness associated with aphthous stomatitis and feeling hot inside the body and hematuria; as an antipyretic and diuretic (Farnsworth and Bunyapraphatsara, 1992).

***Orthosiphon aristatus* Miq**.

Family : Labiatae

Common Name : Kidney tea plant, Java tea, Yaa nuat maeo

Ethnomedical uses treatment of kidney diseases, any disorders which cause cachexia and wasting diseases, dysuria with urinary stones or discharges ; as a diuretic, antidiabetic (Farnsworth and Bunyapraphatsara, 1992).

***Pluchea indica* (Linn.) Less**.

Family : Compositae

Common Names : Indian marsh fleabane, Khlu Khlun

Ethnomedical uses treatment of dysuria with urinary stones or discharges and disorders of urination ; as a diuretic (Farnsworth and Bunyapraphatsara1992).

***Saccharum officinarum* Linn.**

Family : Graminae

Common Names : Noble sugar cane, Sugar Cane, Oi

Ethnomedicinal uses for eliminatio of vitiated lymph ; treatment of chronic gastrointestinal ailments of the children between the ages of 5 and 13 characterized by marked malnutrition, usually associated with intestinal parasitism, weakness associated with aphthous stomatitis and a hot feeling inside the body, hyperviscosity of sputum, internal injuries and sinusitis ; as water element tonic , cardiotonic, diuretic and antipyretic (Farnsworth and Bunyapraphatsara, 1992).

APPENDICES II

PROTEIN CONCENTRATION DETERMINATION

This assay has been modified from Bradford method (Bradford, 1976 and Walker, 1996).

Reagents ;

1) Dry stock

Coomassie Blue G (100 mg) is dissolved in 50 ml of 95% ethanol. The solution is mixed with 100 ml of 85% phosphoric acid and made up to 1 L with distilled water. The solution should be filtered through Whatman No.1 filter paper and then stored in chamber bottle at room temperature. It is stable for several weeks. However, during this time, dye may precipitate from solution, soothe stored reagent should be filtered before use.

2) Protein standard

Dissolved 1 mg of bovine serum albumin, BSA in 1 ml of distilled water.

Assay procedures ;

1) Pipette between 10 and 100 μg of protein in 100 μl total volume into a test tube. If the approximate sample concentration is unknown, assay a range of dilutions (1, 1/10, 1/100,1/1000). Prepare duplicates of each sample.

2) For the calibration curve, pipette duplicate volumes of 10, 20, 30, 40, 60, 80 and 100 μl of 1 mg/ml BSA into test tube, and make each up to 100 μl with distilled water. Pipette 100 μl of distilled water into an additional tube to provide the reagent blank.

3) Add 5 ml of dye stock to each tube, and mix well by vortex. Avoid foaming, which will lead to poor reproducibility.

4) Measure the absorbance at 595 nm of sample and standards against the reagent blank.

APPENDICES III

Na^+-K^+-ATPase ACTIVITY ANALYSIS

This assay has been modified from Fiske and Subbarow (1925). The analysis is 2 stage reactions which are (Podprasart, V. 1998):

1) Generation of Pi

ATP $\xrightarrow{\text{ATPase}}$ ADP + Pi

2) Color development of Pi using the method of Fiske-Subbarow reagent.

Na^+-K^+-ATPase requires presence of both Na^+ and K^+ as a catalist of the above reaction. The addition of ouabain affects the K^+-binding site of this specific ATPase, thus rendering this enzyme inactive.

Reagent ;

1) **Buffer solution**

Chemical	MW	g/0.5 liter	Concentration (mM)
Imidazole	68.08	2.835	50
EDTA-4H	292.24	0.116	0.5
$MgCl_2$	95.23	0.395	5
NaCl	58.44	6.820	140
KCl	74.56	0.870	14

Measure and adjust the pH of the solution with HCl to 7.2 and refrigerate until use.

2) **30 mM ATP** (disodium salt) from Sigma, MW = 551.1

3) **ANS solution**

Mixture of 1-amino-2-naphthol-4-sulfonic acid and sodium sulfite and sodium hydrogen sulfite ratio is 1:6:6.

4) **50% TCA (w/v), MW = 163.39**

Weighed 50 g of TCA and dissolved in a final volume of 100 ml in double deionized distilled water.

5) **20 mM Ouabain Octahydrate** from Sigma, MW = 728.8

6) **Sulfate-Molybdate reagent**, Prepared as the following:

Hexa-ammonium hepta-molybdate tetrahydrate (MW = 1235.86) 1.5 g was added with concentration of H_2SO_4 6.25 ml and adjust with double deionized distilled water to 450 ml. Store at 4 ^oC

7) **Phosphate standards**

Used KH_2PO_4 (MW = 136.09) of the highest quality available. The concentration of the phosphate standard was divided into 2 mM. Stored at 4 ^oC

Assay Procedures ;

For each sample, divided into 6 tubes; 3 tubes for determination using the assay mixture without ouabain, another 3 tubes for determination of ouabain sensitive Na^+-K^+-ATPase activity by adding ouabain.

Step 1: Generation of Pi

* 1) Pipetted 60 µl of buffer solution, crude enzyme 10 µl and ATP 10 µl into all 6 tubes, used 100 µl of double deionized distilled water for blank.

* 2) Added 2 µl of sample to 6 tube which 3 tubes added 10 µl of oubain and another 3 tubes without oubain. Adjust volume with double deionized distilled water to 100 µl then vortexed.

3) Incubated at 37 ^oC for 30 minutes.

4) Stopped the reaction by adding 20 µl of 50% TCA.

* Performed the experiment on the ice at 4 ^oC

Step 2 : Color development of Pi by Fiske & Subbarow Reducer.

5) Added 300 µl of Sulfate-Molydate reagent to all of the above tubes and mixed well.

6) Followed by adding 10 µl of ANS solution and vortexed.

7) Centrifuged approximately at 12,000 rpm for 1 minutes to precipitate denatured protein otherwise it would interfere with absorbance readings.

8) Read the optical density at 700 nm by micro trite plate reader (TECCAN-SUNPRISE).

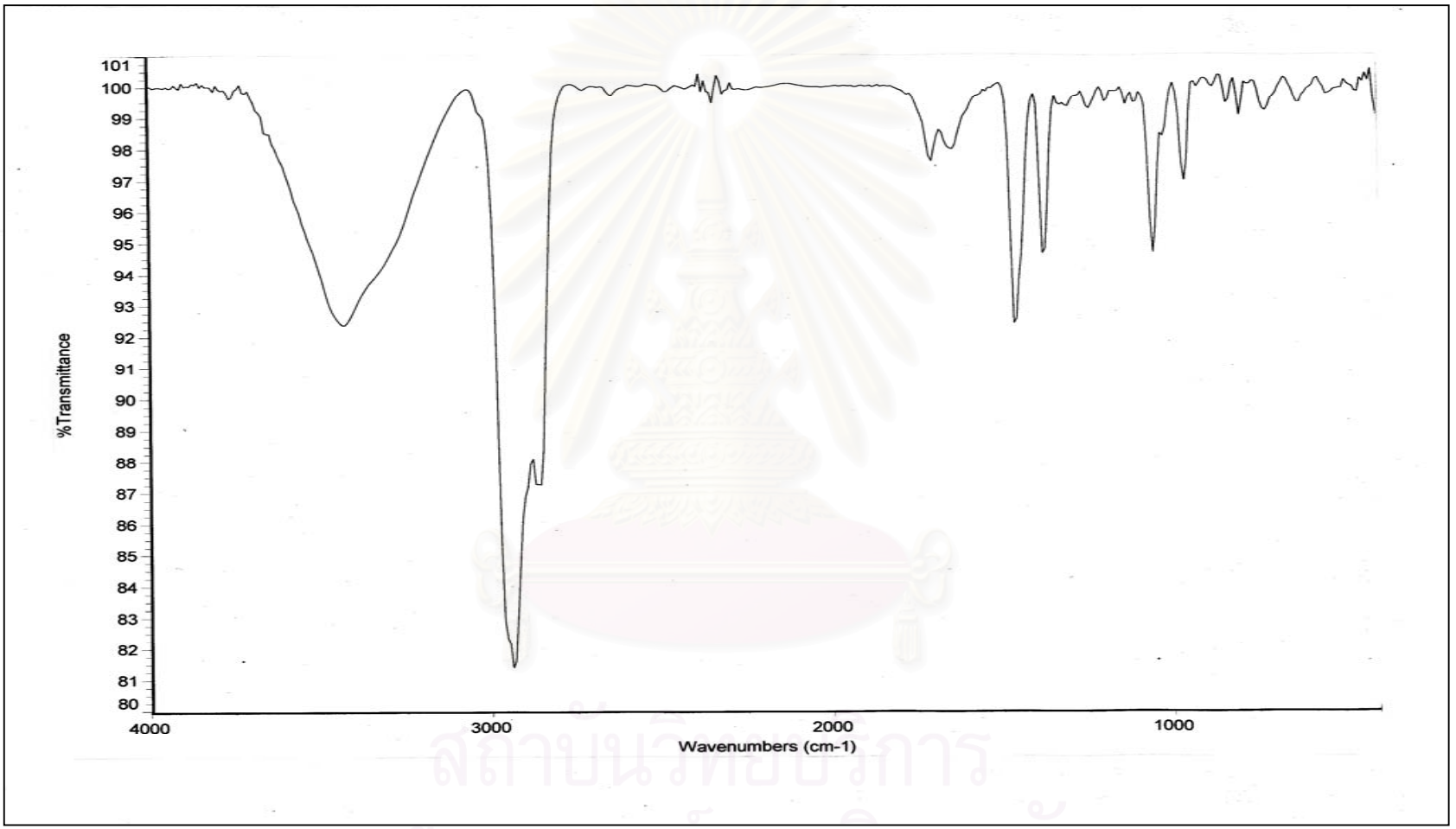

Figure 6.1 The IR spectrum of mixture 1

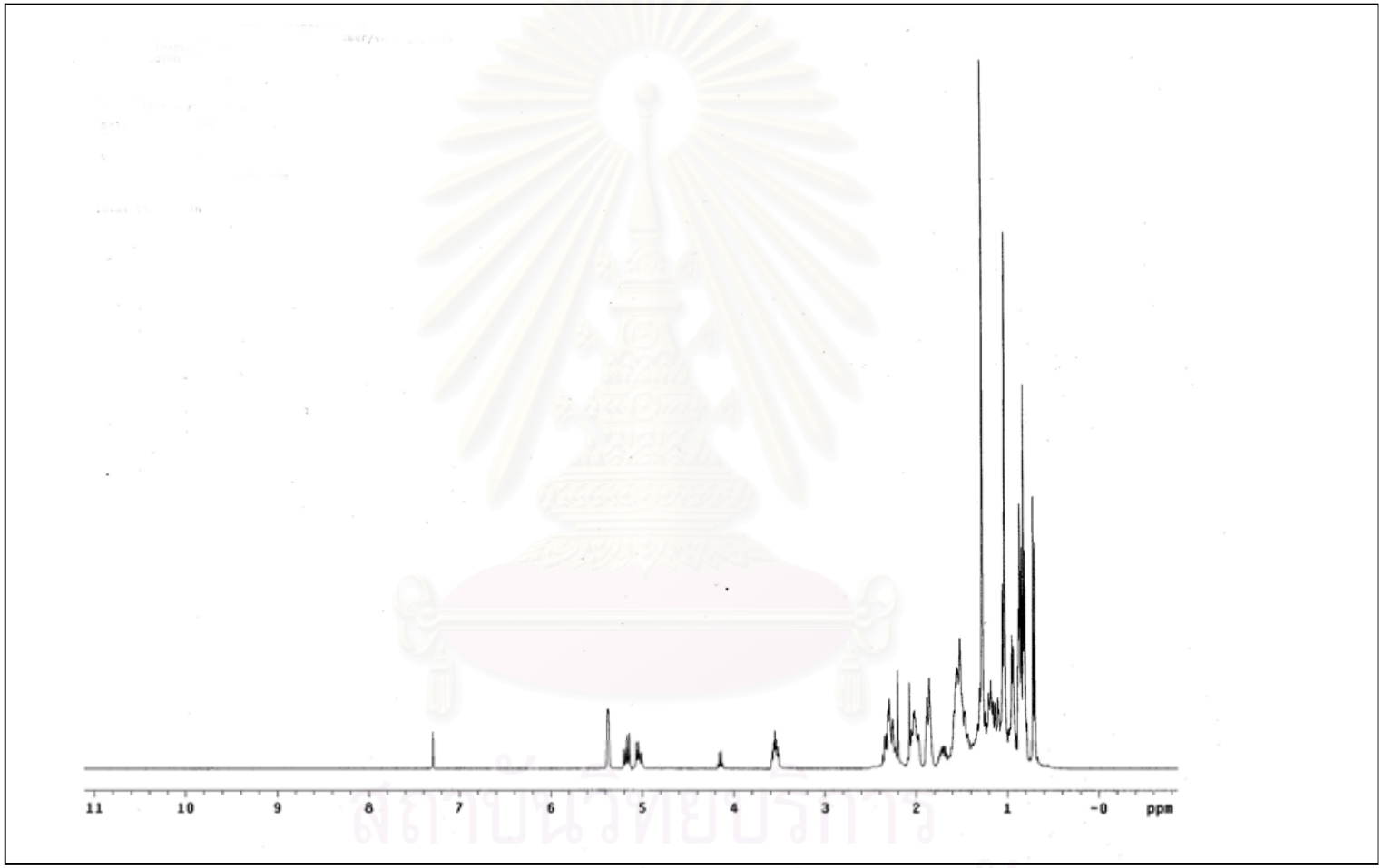

Figure 6.2 The ^{1}H-NMR spectrum of Mixture 1

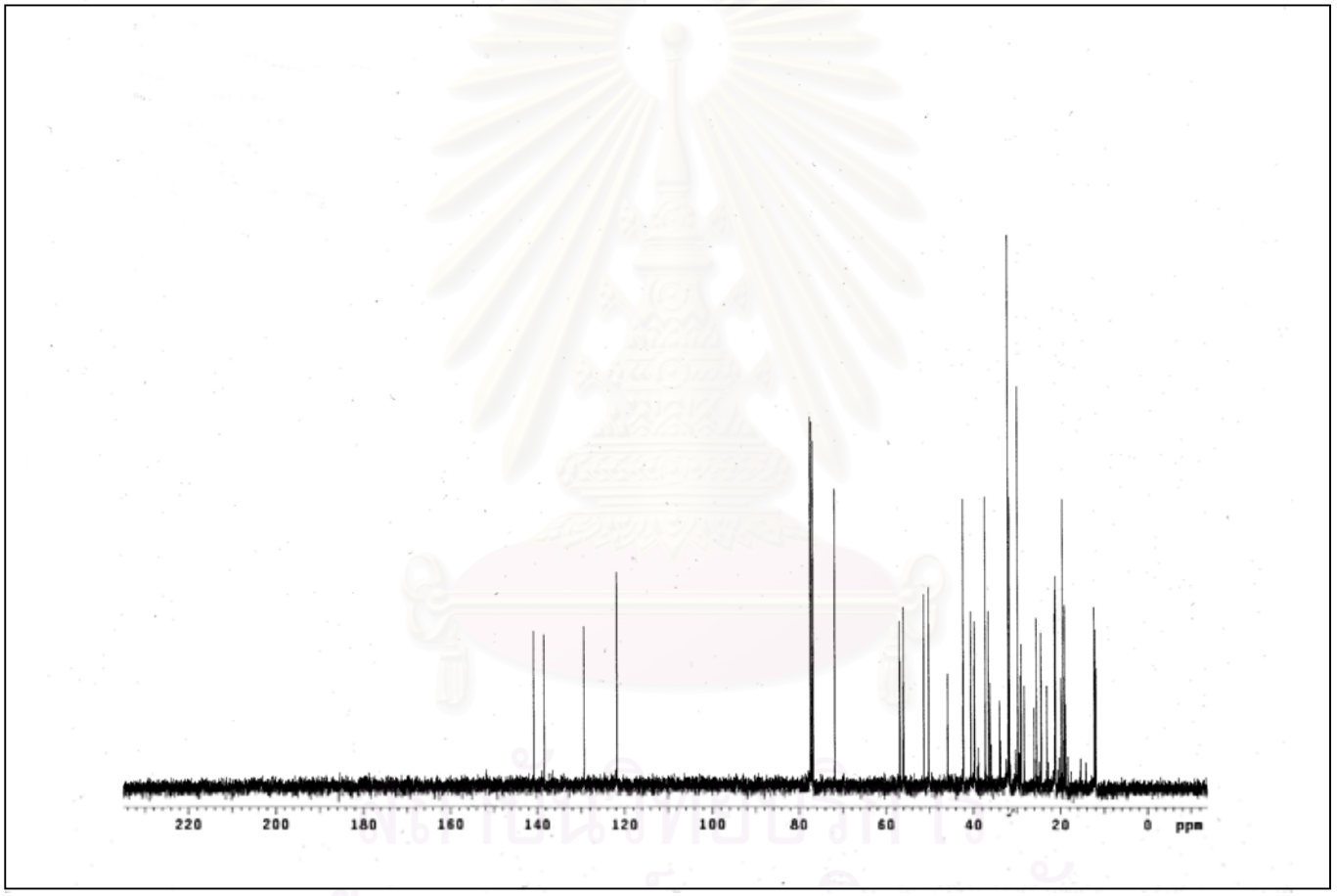

Figure 6.3 The ^{13}C-NMR spectrum of Mixture 1

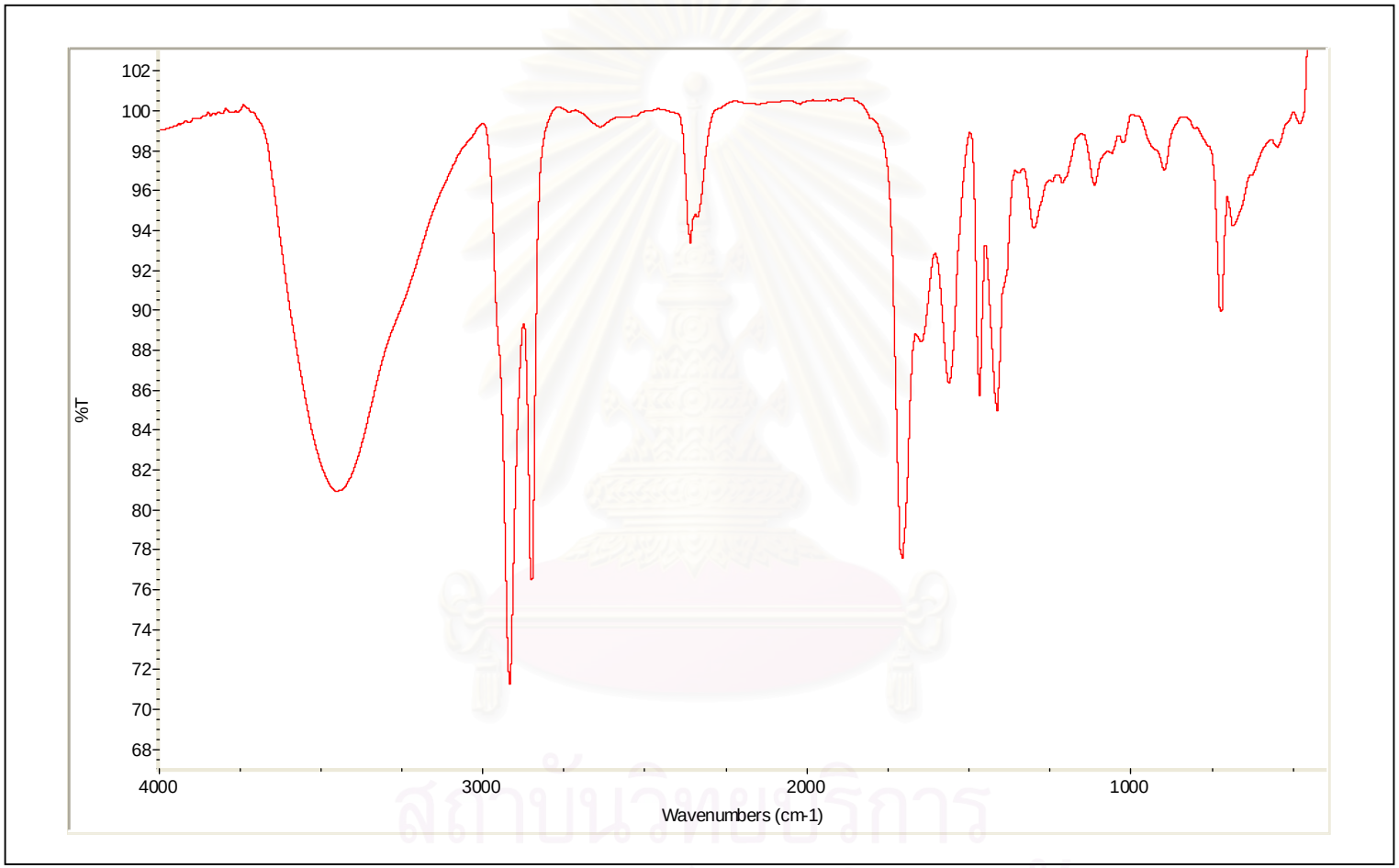

Figure 6.4 The IR spectrum of Mixture 2

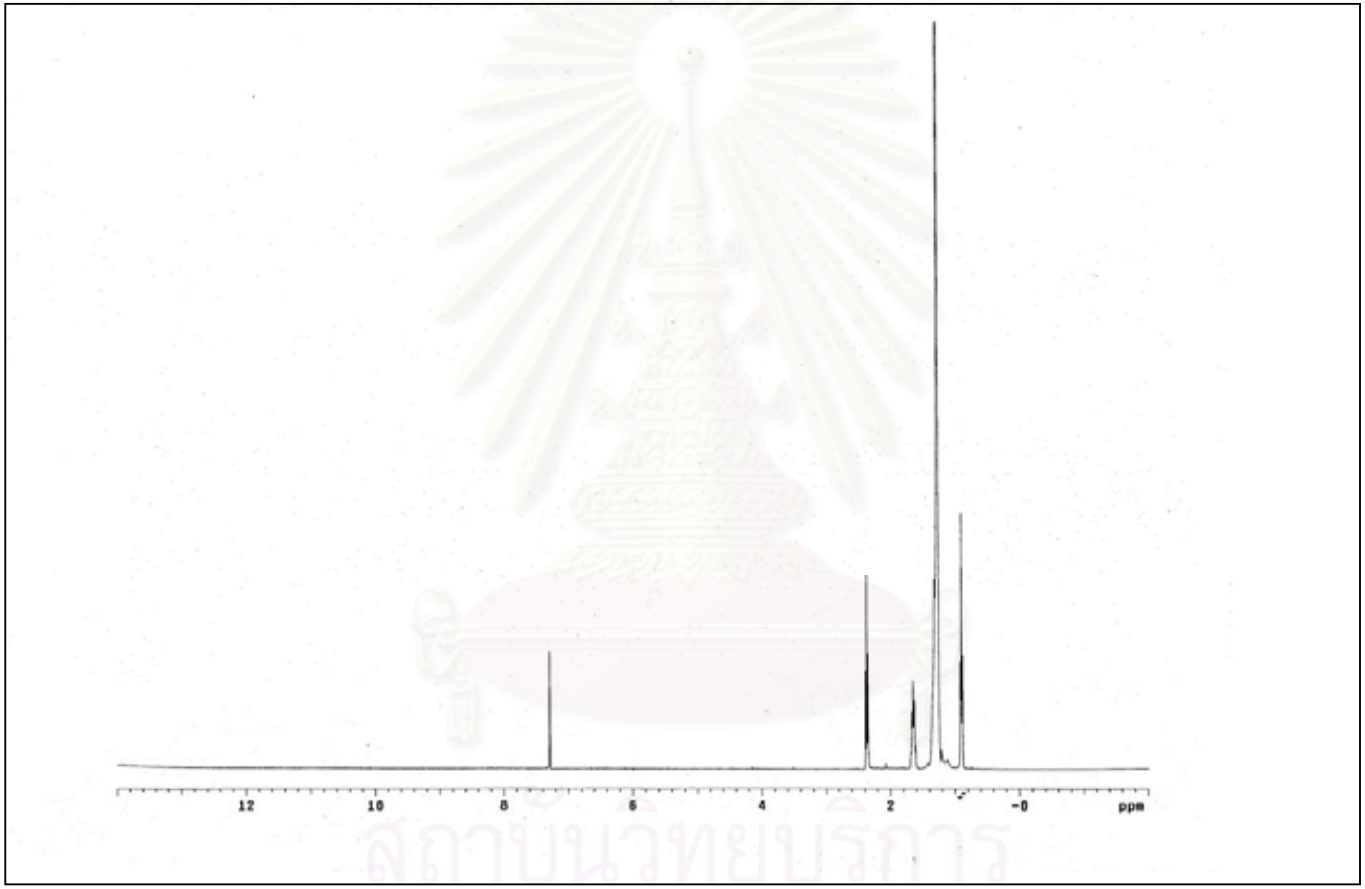

Figure 6.5 The ^{1}H-NMR spectrum of Mixture 2

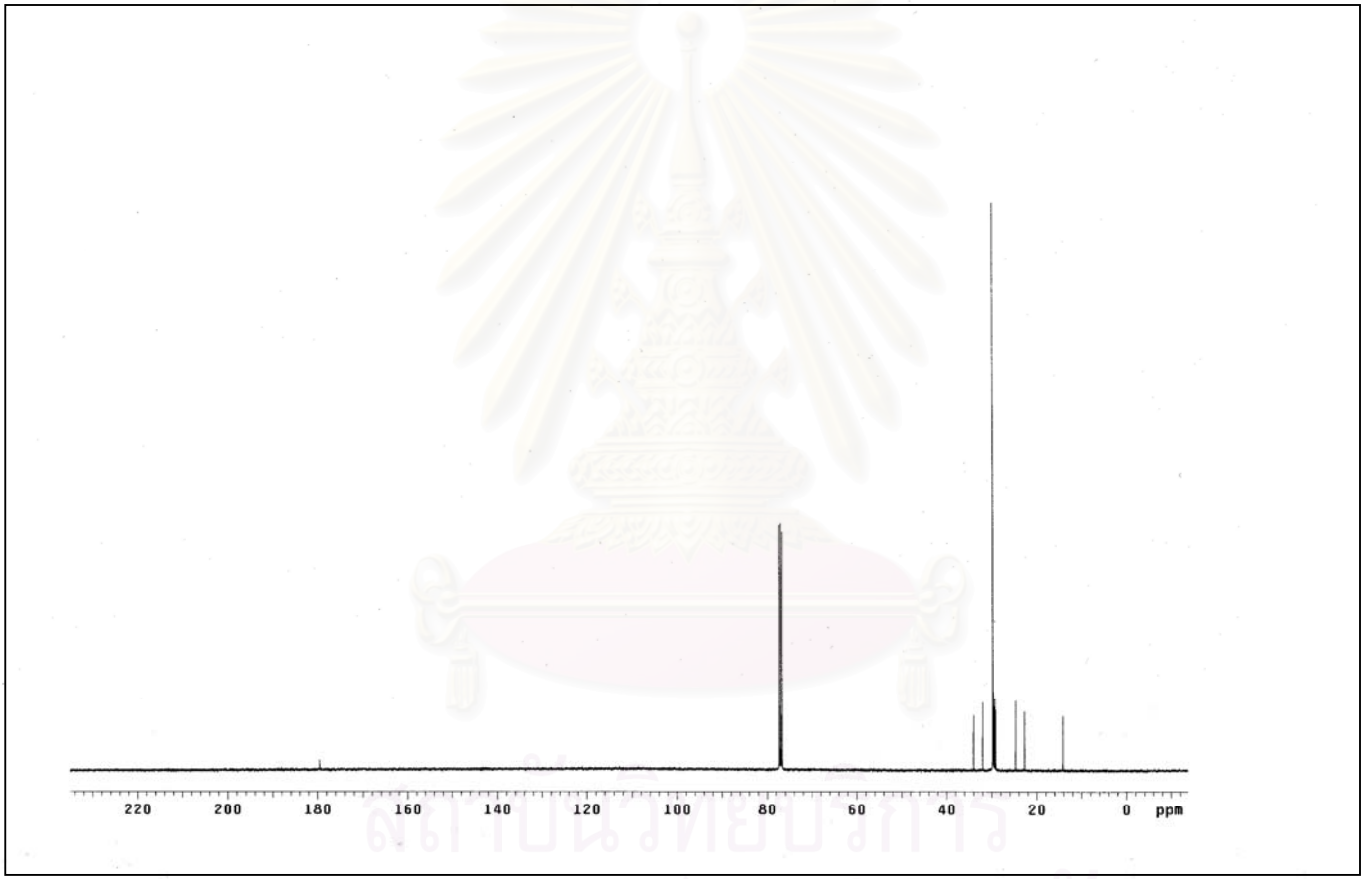

Figure 6.6 The ^{13}C-NMR spectrum of Mixture 2

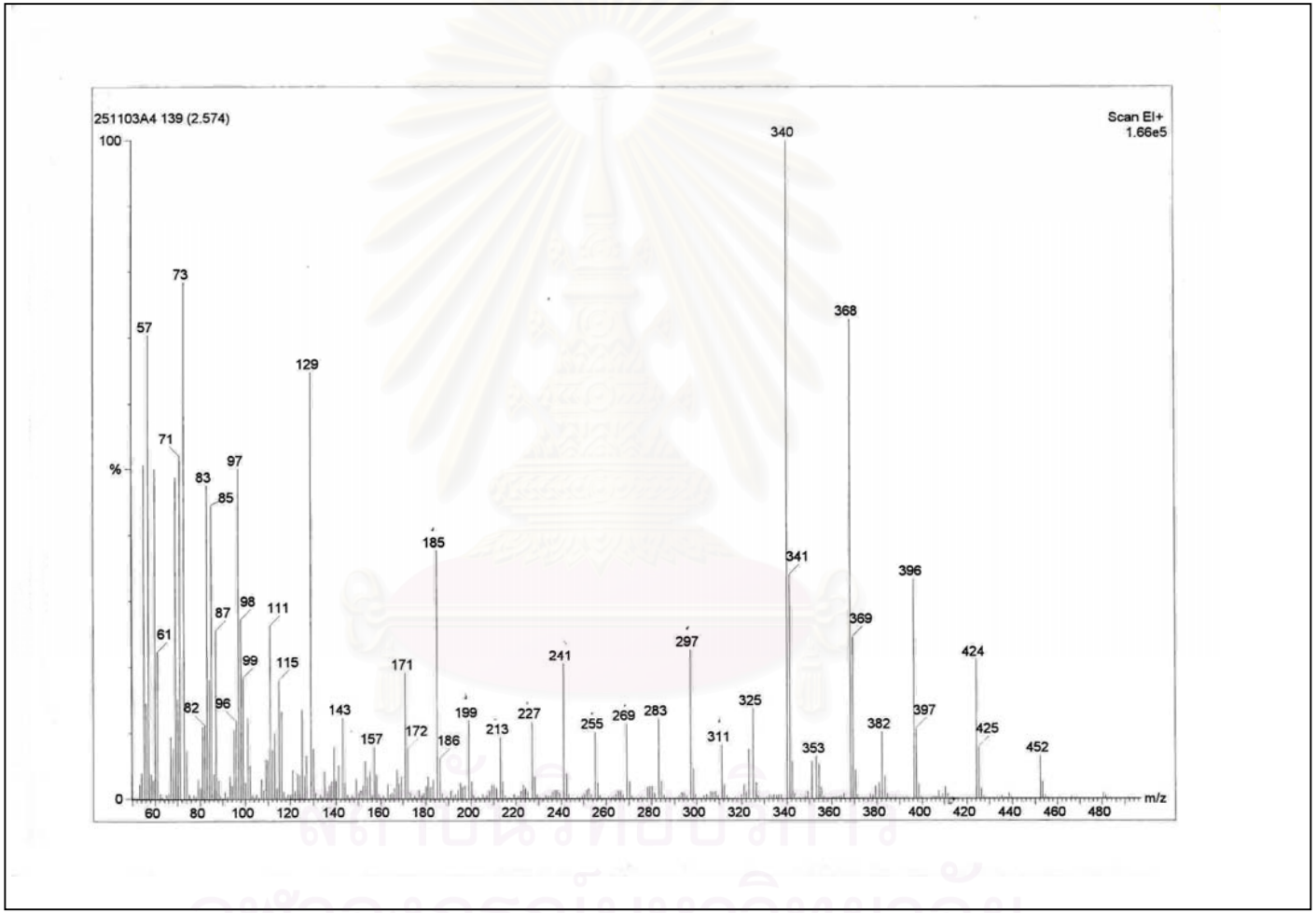

Figure 6.7 The mass spectrum of Mixture 2

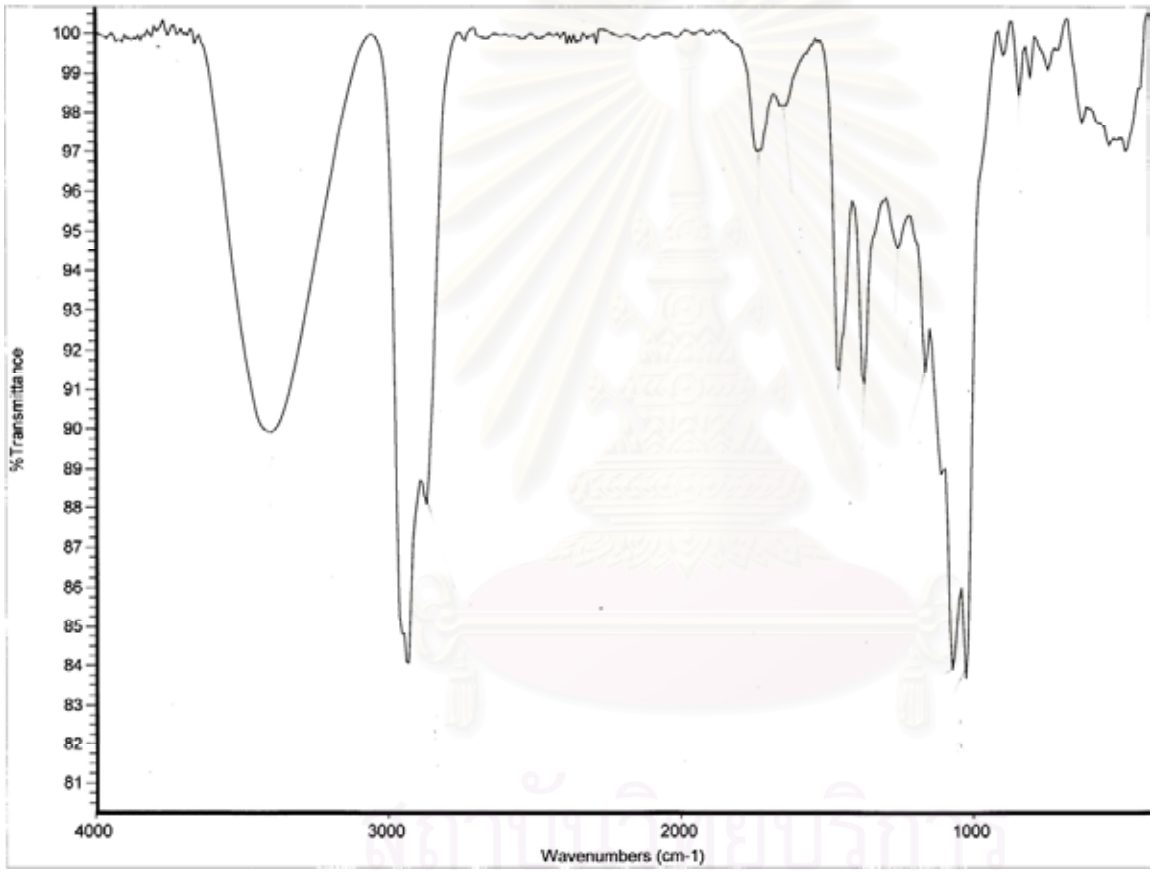

Figure 6.8 The IR spectrum of compound 1

Figure 6.9 The IR spectrum of compound 2

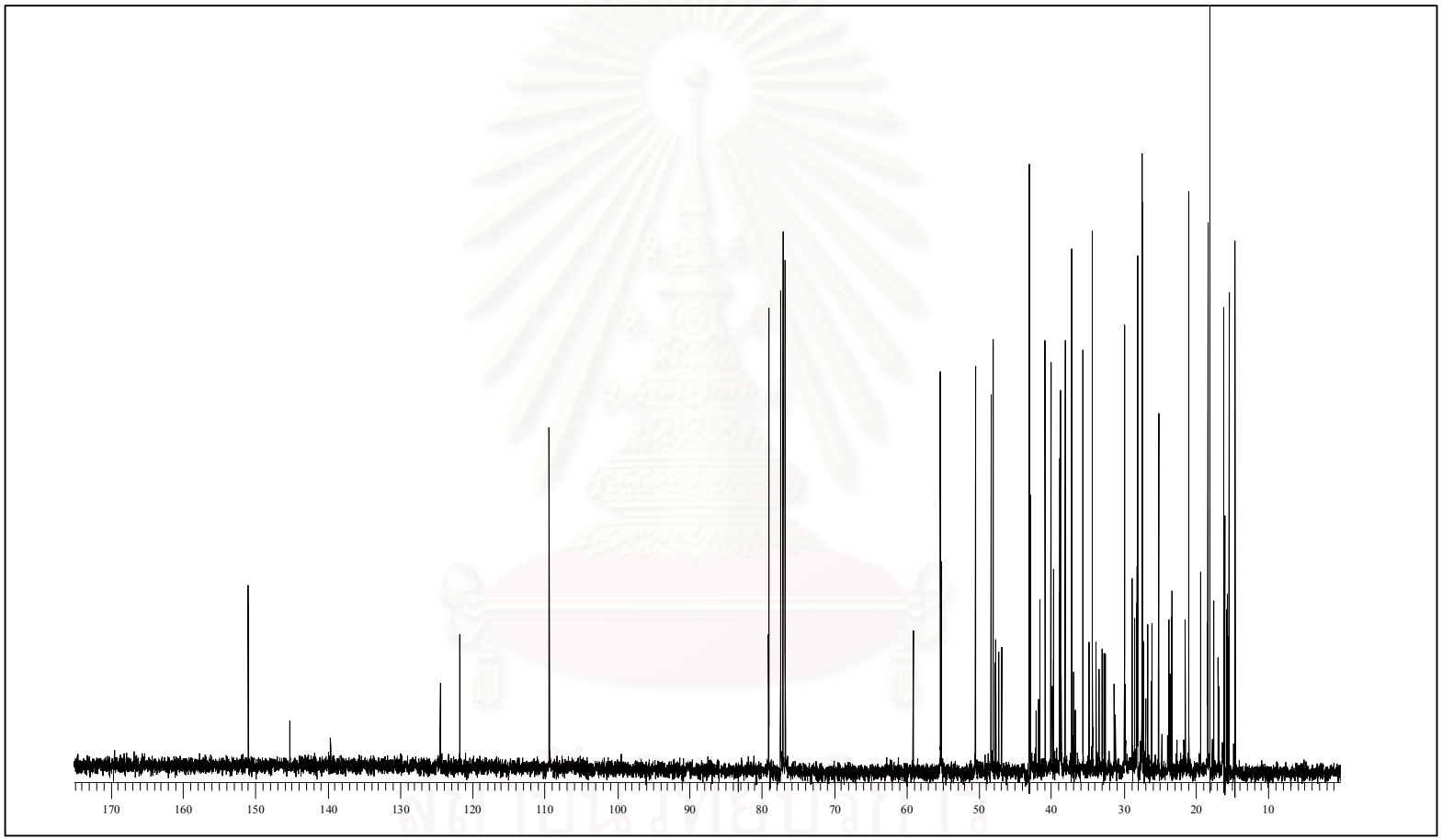

Figure 6.10 The ^{13}C-NMR spectrum of compound 2

VITA

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