การศึกษาสารที่เป็นองค์ประกอบทางเคมีจากกวาวเครือแดง

นางสาวสุดารัตน์ แทนพลกรัง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเคมี
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2554

STUDIES ON CHEMICAL CONSTITUENTS FROM *Butea superba* Roxb.

Sudarat Tanphonkrang

A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Master of Science in Chemistry

Suranaree University of Technology

Academic Year 2011

STUDIES ON CHEMICAL CONSTITUENTS FROM *Butea superba* Roxb.

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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สุดารัตน์ แทนพลกรัง : การศึกษาองค์ประกอบทางเคมีจากกวาวเครือแดง (STUDIES ON CHEMICAL CONSTITUENTS FROM *Butea superba* Roxb.) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.สันติ ศักดารัตน์, 83 หน้า.

กวาวเครือแดงเป็นพืชในวงศ์ Leguminosae จัดเป็นยาพื้นบ้านของไทยที่รู้จักและใช้กันมายาวนาน เมื่อนำส่วนสกัดด้วยอะซิโตนของหัวกวาวเครือแดงมาแยกด้วยเทคนิคทางโครมาโทกราฟี สามารถแยกสารบริสุทธิ์ได้ 3 ชนิดและพิสูจน์โครงสร้างของสารบริสุทธิ์ทั้งหมดที่แยกออกมาได้ โดยใช้ข้อมูลทางสเปกโทรสโกปีจาก UV-Vis IR MS ^{1}H-NMR และ ^{13}C-NMR ร่วมกับการเปรียบเทียบที่มีรายงานไว้แล้วพบว่าสารที่แยกออกมาได้ คือ 3,7-dihydroxy-4′-methoxyisoflavone (biochanin A), 3,7,4′-trihydroxyisoflavone (genistein) และ stigmasteryl-3-*O*-β-D-glucopyranoside

มหาวิทยาลัยเทคโนโลยีสุรนารี

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SUDARAT TANPHONKRANG : STUDIES ON CHEMICAL CONSTITUENTS FROM *BUTEA SUPERBA* ROXB. THESIS ADVISOR : ASSOC. PROF. SANTI SAKDARAT, Ph.D. 83 PP.

BUTEA SUPERBA ROXB, BIOCHANIN A, GENISTEIN, STIGMASTERYL-3-*O*-β-D-GLUCOPYRANOSIDE

Butea superba Roxb., a Thai traditional medicinal plant of family Leguminosae, is well-known plant used in Thailand for a long period of time. The acetone crude extract was purified by column chromatography and preparative thin layer chromatography to give three pure compounds. Structural elucidation of the isolated compounds was carried out on the basis of spectral analyses, including UV-Vis, IR, MS, ^{1}H-NMR and ^{13}C-NMR, as well as comparison with reported values. Two of these, were identified as isoflavonoids 3,7-dihydroxy-4′-methoxyisoflavone (biochanin A) and 3,7,4′-trihydroxyisoflavone (genistein). The other was stigmasteryl-3-*O*-β-D-glucopyranoside.

School of Chemistry Student's Signature_____

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Finally, I dedicate this work to my parents and the rest of my family. They provided me with every opportunity to succeed in life and the encouragement I needed to succeed.

Sudarat Tanphonkrang

CONTENTS

CONTENTS (Continued)

Page

CONTENTS (Continued)

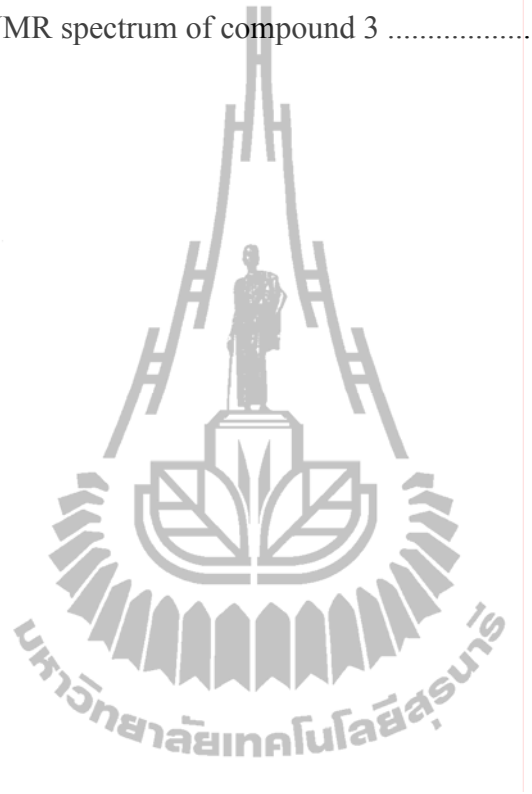
Page

มหาวิทยาลัยเทคโนโลยีสุรนารี

LIST OF TABLES

LIST OF FIGURES (Continued)

Figure **Page**



LIST OF SCHEMES

มหาวิทยาลัยเทคโนโลยีสุรนารี

LIST OF ABBREVIATIONS

AD	Anno Domini
br	broad
^{o}C	Degrees Celcius
$CDCl_3$	Chloroform-d_1
CD_3OD	Methanol-d_4
cm	centimeter
d	doublet
dd	doublet of doublets
DEPT	Distortionless Enhancement by Polarization Transfer
FT	Fourier Transform
g	gram
Hz	Hertz
IR	Infrared Spectroscopy
J	coupling constant in Hertz
kg	kilogram
LH	Luteinizing Hormone
m	multiplet
mL	milliliter
MS	Mass Spectroscopy
m/z	mass to charge ratio of an ion
nm	nanometer

มหาวิทยาลัยเทคโนโลยีสุรนารี

LIST OF ABBREVIATIONS (Continued)

NMR	Nuclear Magnetic Resonance Spectroscopy
ppm	part per million
prep-TLC	preparative Thin Layer Chromatography
s	singlet
t	triplet
TLC	Thin-Layer Chromatography
UV	Ultraviolet Radiation
UV-Vis	Ultraviolet-Visible Radiation
δ	chemical shift in ppm
λ_{max}	maximum absorption wavelength
ν_{max}	maximum absorption wavenumber

มหาวิทยาลัยเทคโนโลยีสุรนารี

CHAPTER I

INTRODUCTION

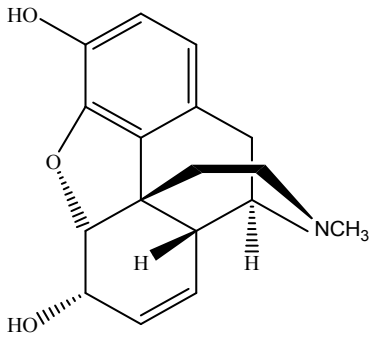
1.1 General Introduction

Natural products are organic compounds that are formed by living systems found in nature that usually have a pharmacological or biological activity for use in pharmaceutical drug discovery and drug design. It has been estimated that over 40% of medicines have their origins in these natural products, especially natural products from plants. Natural product research remains one of the main approaches of discovering bioactive compounds. Since little is known about the etiology of many human, animal, and plant diseases, it is difficult to design potentially active molecules for their treatment and therefore leads from natural sources will continue to be sought (Block, 1989).

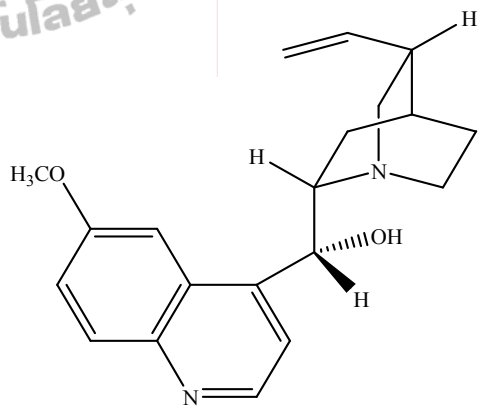
The traditional herbal medicines have been used for thousands of years in many oriental countries (China, Thailand, Japan, etc). Medicinal plants are widely used as therapeutic drugs or herbal medicines. Some of them are used as pharmaceuticals, fragrances, flavors, colors, stimulants, and cosmetics. The World Health Organization (WHO) estimated that 80% of the world's inhabitants mainly depend on traditional herbal medicines as sources for their health care (Farnsworth, Akerele, Bingel, Soejarto, and Guo, 1985). Over 100 chemical substances, derived from different plants, considered to be important drugs are either currently in use or have been widely used in one or more countries in the world. Approximately 75% of these substances

were discovered as a direct result of chemical studies focusing on the isolation of active substances from plants used in folk medicine (Gad, 2005).

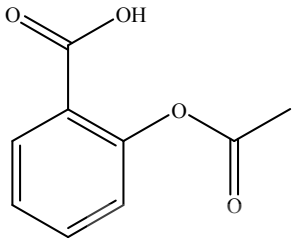
The first records, the roots of plants have been used for folk medicine (Cragg, Newman, and Snader, 1997). Currently some of these plants are still used as a medicine for the treatment of disease ranging from coughs and colds to parasitic infections and inflammation (Newman, Cragg, and Snader, 2000). Several "modern" drugs were first isolated from plants. For example, morphine (**1**) was first discovered from the opium poppy (*Papaver somniferum*) in 1816 (Cragg and Newman, 2001), quinine (**2**) was isolated in 1820 from *Cinchona officinalis* (Clark, 1996; Phillipson, 2001), aspirin (**3**) was isolated in 1829 from willow (*Salix* spp.) (Mann, 1992; Butler, 2004) and pilocarpine (**4**) was isolated in 1876 from jaborandi (*Pilocarpus mycrophyllus*) (Cragg, 2002).



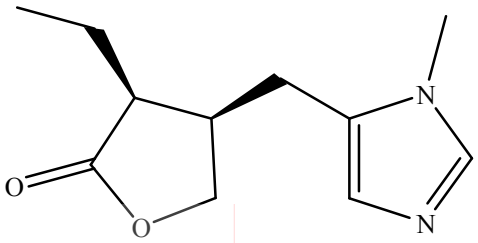
Morphine (**1**)



Quinine (**2**)

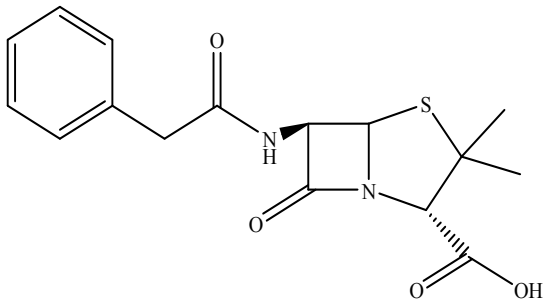


Aspirin (**3**)

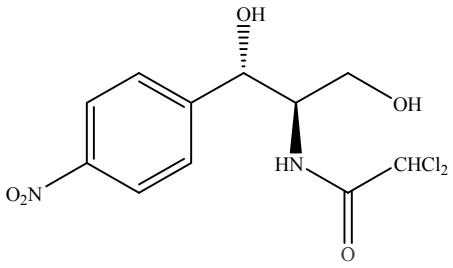


Pilocarpine (**4**)

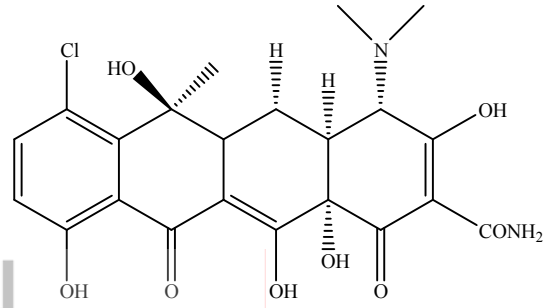
Moreover, in the research for natural products, a common tendency is to use screening techniques to monitor the bioactivity problems of current interest (Colegate, and Molyneux, 1993). In 1928 Fleming discovered penicillin (**5**) as an efficient antibacterial therapeutic from *Penicillium notatum* and opened a new method for researchers to the discovery of antibiotics. The output of this antibiotic research was prolific and included examples such as chloramphenicol (**6**), chlortetracycline (**7**) and erythromycin (**8**). All of these compounds are still in use as drugs today (Gad, 2005).



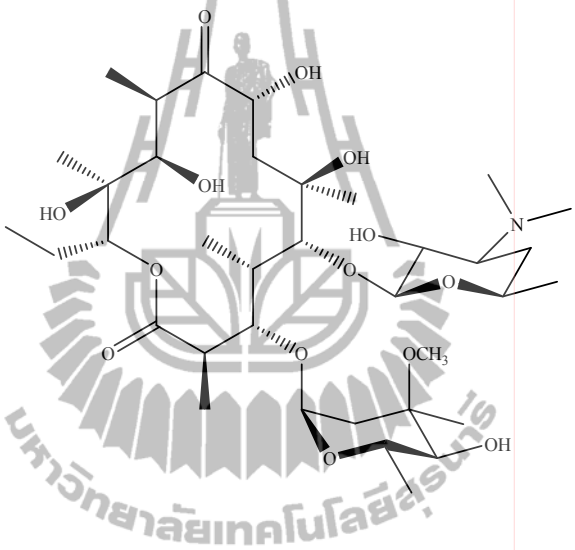
Penicillin (**5**)



Chloramphenicol (**6**)



Chlortetracycline (**7**)



Erythromycin (**8**)

1.2 History of Herbal Medicines in Thailand

Medicinal plants have been used in Thailand since at least the Sukhothai period (14th century AD) and the use of traditional drug formulae began during the Ayutthaya period. Traditional drugs have been popular in the Kingdom throughout the Ayutthaya and Rattanakosin periods. King Rama III (about 200 years ago) ordered the collection of traditional drug recipes, diagnosis of diseases, traditional massage, literature, and poetry, and their subsequent inscription on stone plates installed on the walls of two temples (Wat Phra Chetuphon Vimolmangklaram and Wat Raja

Orasaram). Again during the years 1895-1900 King Rama V ordered the collection of all knowledge of traditional medicines from the noble sand doctors, and the printing and distribution for the first time in the two volumes of the official pharmacopoeia called “Tamrapaettayasartsongkroh”.

Since the use of herbal medicines is quite often derived from empirical experience, standardization and quality control of these preparations are usually lacking, which hampers an increased utilization of such medicines. Thai pharmacists and scientists have therefore realized that this problem should be solved to upgrade the quality of herbal raw materials and their finished products (Dechatiwongse na Ayutthaya, 1997).

1.3 Concepts and Quality Control of Medicinal Plants

Despite the availability of a number of modern efficacious drugs, herbal medicines are still widely used and their importance is increasing because of the presence of certain incurable diseases such as AIDS and some chronic diseases. It is well known that any agent intended to be used as a drug should be uniform in quality regarding origin, the content of therapeutically active constituents and cleanliness. Some medicinal plant materials are sold under the same local name but derived from different origins; hence, their curative effects are different and some may even lead to toxicity. The natural variations of active constituents in herbal materials are also vital to the efficacy of herbal medicines. Additionally, these drugs are often contaminated with microorganisms, which may cause various infections to consumers. To overcome the risk of possible health hazards mentioned above, standard specifications and quality control procedures for these medicines are required for manufacturers as well

as by regulatory authorities to improve the herbal medicinal products (Dechatiwongse na Ayutthaya, 1997).

1.3.1 Important Factors of Quality Control

There are several important factors affecting the efficacy and safety of medicinal plant materials (Dechatiwongse na Ayutthaya, 1997 and Chavalittumrong, 2000).

Plant Name There are some difficulties with the plant names because some plants have the same local name but belong to different botanical origins or vice versa. If wrong medicinal plant materials are used, they may possess not only undesirable activities, but also unexpected toxicities.

Part Used The right part of a plant should be used. Different parts generally contain different substances.

Age The proper harvesting time for medicinal plants should be known to obtain high content of therapeutic principals.

Preparation and Storage Suitable processes of cleaning, cutting, drying, and preservation should be followed to prevent the contamination or deterioration of crude plant materials.

Quality Though we know the correct plant name, the right part used, the proper harvesting time, and suitable process of preparation and storage, we still cannot assume that raw materials of high quality can be obtained. Chemical constituents of plants are usually affected by external factors, *e.g*. climate, the physical features of the land, irrigation, and fertilizer.

Hence, it is essential to set up scientific criteria for standardization and quality control of herbal medicines to ensure that the consumers receive drugs of high quality.

The objectives of this research were as follows:

1. To extract and isolate the chemical constituents from the tuber root of *Butea superba* Roxb.

2. To identify the chemical structures of the isolated substances.

CHAPTER II

LITERATURE REVIEW AND OVERVIEW OF RESEARCH METHODOLOGY

2.1 *Butea superba* Roxb.

Butea superba Roxb. or "Kwao Krua Dang" is a Thai traditional medicinal plant that has been used in Thailand for a long period of time. *Butea superba* Roxb. helps to enhance human health. The medicinal use of tuber and stem of this plant is believed to give strength, power, and purpose of rejuvenation as well as maintain sexual performance or prevent erectile dysfunction (Suntara, 1931). According to its traditional use, the plant is believed to help elderly people to become as energetic as a teenager again, to stimulate male sexual desire and for physical treatment of degenerative conditions of male. People believe that it is one of the miracle herbs (Sutjit, 2003).

Because of its interesting biological activities and the fact that very little is known in the literature, it is desirable to phytochemically investigate this plant in detail.

2.2 Specification of *Butea superba* Roxb.

Scientific Name: *Butea superba* Roxb.

Family: *Leguminosae*

SubFamily: *Papilionaceae*

Common Names: Kwao kreu (Phayub), Jan-Kwao (Isaan), Ton-Jom-Thong (Chumporn), Thong-Kwao (Thai), Pho-Ta Ku (Karen-Kanchanaburi), Pho-Mue (Karen-Mae Hong Son)

Botanical Description: *Butea superba* Roxb. has a characteristic of being a crawler about 5-10 meters in length that wraps itself around large trees or scaffolds. The trunk has a diameter of about 4-10 centimeters, its wood is rather hard, and the bark has dark brown color. One branch has 3 leaves and the flowers are a yellowish orange color, about 4-5 centimeters in length that blossom when the plant sheds its leaves. The pod is flat and long with a length of about 10-15 centimeters, and contains only one large seed of a light brown color. This plant grows out in the open. The long tuber root of this plant is buried under ground like the tuber root of yam. The tuber roots of this plant have a length of 30-50 centimeters. If it is cut, the tuber roots will release red sap. Its morphological features are illustrated in Figure 2.1.

Ecology and Distribution: It is mostly found growing in forests in the northern, central, western, and northeastern regions of Thailand. It is found in the same habitats as *Pueraria mirifica* and also in mountain areas.

Propagation: It is normally propagated by seeds and roots.

Figure 2.1 Morphological illustration of *Butea superba* Roxb.

2.3 Pharmacological Study

Pharmacological studies of *Butea superba* Roxb. plant are exemplified as follows:

2.3.1 Effects of the tuber root powder of *Butea superba* Roxb. on blood testosterone and luteinizing hormone (LH)

Testosterone and LH were analyzed in *Butea superba* Roxb. treated male rats with 0, 10, 100, 150, and 200 mg/kg of plant powder to screen for potential androgen disruption. The results showed a dose-dependent decrease of only blood testosterone but no change in LH (Malaivijitnond, Ketsuwan, Watanabe, Taya, and Cherdshewasart, 2009). The testosterone disruption is significant, at least after 90 days of consumption of high doses of *Butea superba* Roxb. powder. The testosterone

disruption, clearly seen in this study, is an antagonist of the reported androgenic effect initiated by consumption of other plants (Cherdshewasart, Bhuntaku, Panriansaen, Dahlan, and Malaivijitnond, 2008).

2.3.2 Effects of *Butea superba* Roxb. on reproductive systems of rats

The male rats were fed daily with the powdered crude drug suspended in distilled water by a gastric tube at the dose of 2, 25, 250, and 1250 mg/kg body weight for 8 weeks. Rats fed with 1 mL of distilled water were used as a negative control. The weights of all vital organs in all treated groups were not different from the control. The percentage weight ratios of body weights of seminal vesicles and prostate glands were not different from the control, except that the testis of the group fed with 1250 mg/kg was significantly different from the control and the other treated groups. All treated groups gave higher sperm numbers than the control group. The sperm counts of the group fed with 1250 mg/kg were about 16% higher than those of the control group. Hematology as well as liver and kidney function of all treated groups showed no difference from the control. *Butea superba* Roxb. drug at 250 mg/kg which was 100 times more than the Thai FDA (Food and Drug Administration) recommended dose for humans appeared to be safe in rats (Boonyaratavej and Petsom, 1991). The crude drug has demonstrated a tendency to increase testis weight, and sperm counts in rats (Manosroi, Sanphet, Saowakon, Aritajat, and Manosroi 2006).

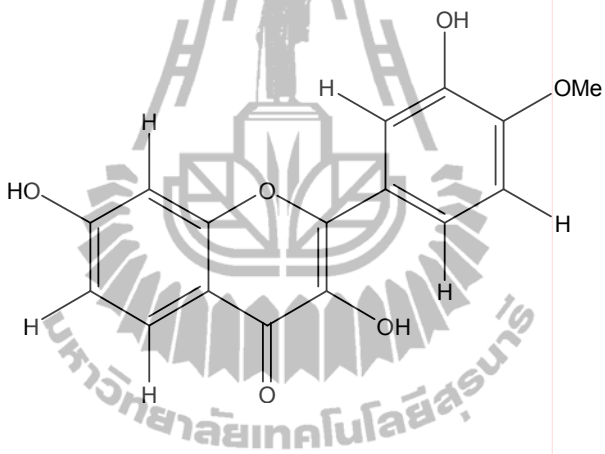
2.3.3 Effect of *Butea superba* Roxb. in inhibiting cAMP phosphodiesterase enzyme

The biological activity of 3,7,3'-Trihydroxy-4'-methoxyflavone (**9**) and 3,5'-dihydroxy-4'-methoxyflavone-7-O-β-D glucopyranoside (**10**) were isolated from *Butea superba* Roxb. It was found that both of these compounds were effective in inhibiting

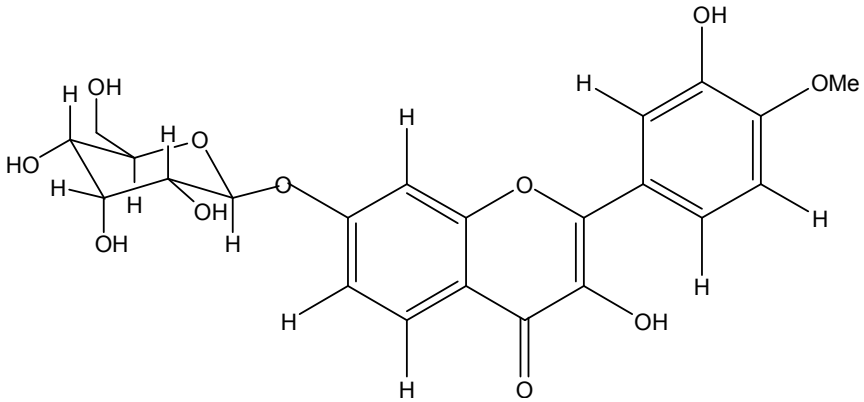
cAMP phosphodiesterase enzyme more than theophylline and caffeine, which have been shown to be important in controlling body function and involved in a large range of diseases (Roengsumran, et al. 2000).

2.4 Chemical Constituents

Roengsumran, et al. (2000) reported the isolation of 3,7,3'-Trihydroxy-4'-methoxyflavone and 3,5'-dihydroxy-4'-methoxyflavone-7-O-β-D glucopyranoside from the tuber roots of *Butea superba* Roxb.

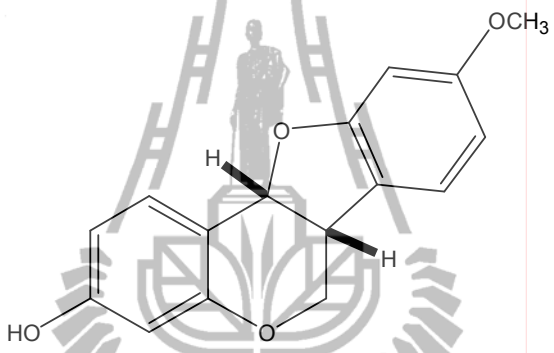


3,7,3'-Trihydroxy-4'-methoxyflavone (**9**)

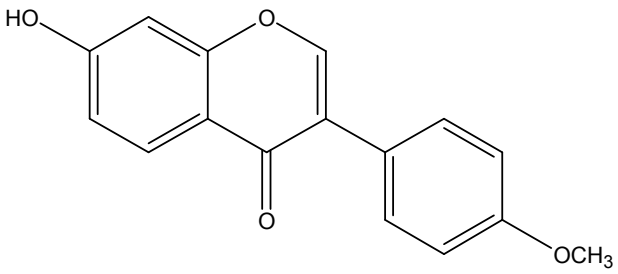


3,5'-Dihydroxy-4'-methoxyflavone-7-O-β-D glucopyranoside (**10**)

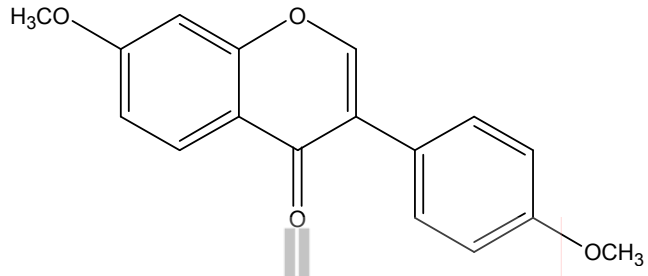
Ngamrojanavanich, et.al. (2007) reported the isolation of 3-hydroxy-9-methoxypterocarpan (medicarpin) (**11**) and four isoflavones, 7-hydroxy-4´-methoxy-isoflavone (formononetin) (**12**); 7,4′-dimethoxyisoflavone (**13**); 5,4′-dihydroxy-7-methoxy-isoflavone (prunetin) (**14**) and 7-hydroxy-6,4′-dimethoxyisoflavone (**15**) from the chloroform extract of the tuber roots of *Butea superba* Roxb.



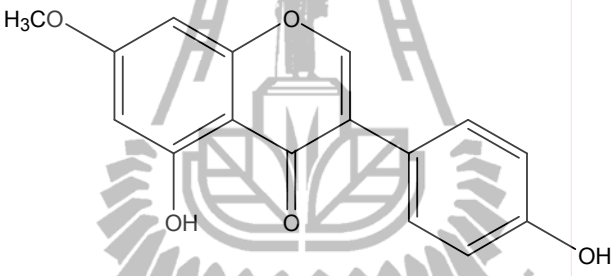
3-Hydroxy-9-methoxypterocarpan (medicarpin) (**11**)



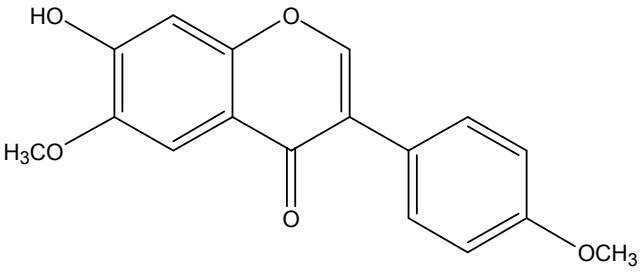
7-Hydroxy-4′-methoxy-isoflavone (formononetin) (**12**)



7,4′-Dimethoxyisoflavone (**13**)



5,4′-Dihydroxy-7-methoxyisoflavone (prunetin) (**14**)



7-Hydroxy-6,4′-dimethoxyisoflavone (**15**)

Since *Butea superba* Roxb. helps to enhance human health, it is very important to study the chemical constituents and their biological activity, including flavonoids. Flavonoids are compounds which possess the same C_{15} (C_6-C_3-C_6) flavone nucleus two benzene rings (A and B) linked through an oxygen-containing pyran or pyrone ring (C). This structure is common to 3-deoxyflavonoids (flavones, flavanones, isoflavones and neoflavones) and 3-hydroxyflavonoids (flavonols, anthocyanins, flavan-3,4-diols and flavan-3-ols) as shown in Table 2.1 (Khnau, 1976).

Table 2.1 The chemical structures of the flavonoid family.

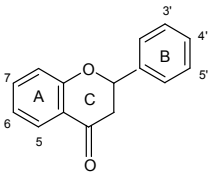
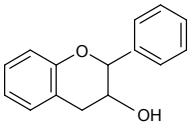
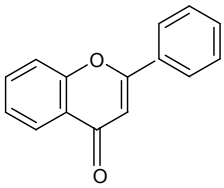
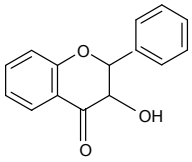
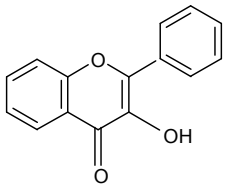
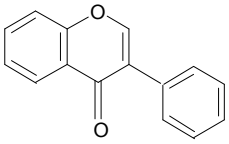
Structural formula	**Representative flavonoids**	**Substitutions**					
		5	**6**	**7**	**3′**	**4′**	**5′**
Flavanone							
	Eriodictyol	OH	H	OH	OH	OH	H
	Hesperitin	OH	H	OH	OH	OMe	H
	Naringenin	OH	H	OH	H	OH	H
Flavanol							
	Catechin	OH	H	OH	OH	OH	H
	Gallocatechin	OH	H	OH	OH	OH	OH
Flavone							
	Apigenin	OH	H	OH	H	OH	H
	Chrysin	OH	H	OH	H	H	H
	Luteolin	OH	H	OH	OH	OH	H

Table 2.1 (Continued).

Structural formula	Representative flavonoids	Substitutions 5	6	7	3′	4′	5′
Flavanonol							
	Taxifolin	OH	H	OH	OH	OH	H
Flavonol	Kampherol	OH	H	OH	H	OH	H
	Myricetin	OH	H	OH	OH	OH	OH
	Quercetin	OH	H	OH	OH	OH	H
	Galangin	OH	H	OH	H	H	H
Isoflavone							
	Daidzein	H	H	OH	H	OH	H
	Genistein	OH	H	OH	H	OH	H
	Glycitein	OH	OMe	OH	H	OH	H
	Formononetin	H	H	OH	H	OMe	H

Our research group has been interested in *Butea superba* Roxb. due to its wide use in traditional medicine, because of its interesting biological activities, and the fact that very little has been reported about it in the literature. Therefore, it is desirable to phytochemically investigate this plant in detail. A literature search for other plants in subfamily *Papilionaceae* give the results shown in Table 2.2.

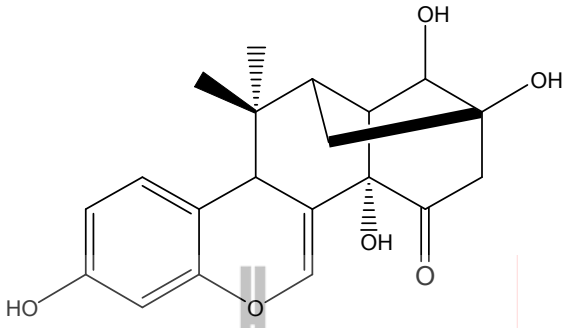
Table 2.2 Chemical constituents of plants in the subfamily *Papilionaceae.*

Scientific name	Plant part	Organic compounds	References
Pueraria mirifica	Root	miroestrol (**16**)	Hayodom, 1971.
		β-sitosterol (**17**)	
		stimasterol (**18**)	
Pueraria mirifica	Root	genistin (genistein-7-*O*-glucoside) (**19**)	Ingham, Tahara, and Dziedzic, 1989.
		puerain-6''-monoacetate (**20**)	
		mirificoumestan (**21**)	
		mirificoumestan hydrate (**22**)	Ingham, Tahara, and Dziedzic, 1988.
		mirificoumestan glycol (**23**)	Tahara, Ingham, and Dziedzic, 1987.
		kwakhurin (**24**)	Ingham, Tahara, and Dziedzic, 1986.
		daidzein (**25**)	
		daidzin (daidzein-7-*O*-glucoside) (**26**)	
		puerarin (**27**)	
		genistein (**28**)	
		coumestrol (**29**)	

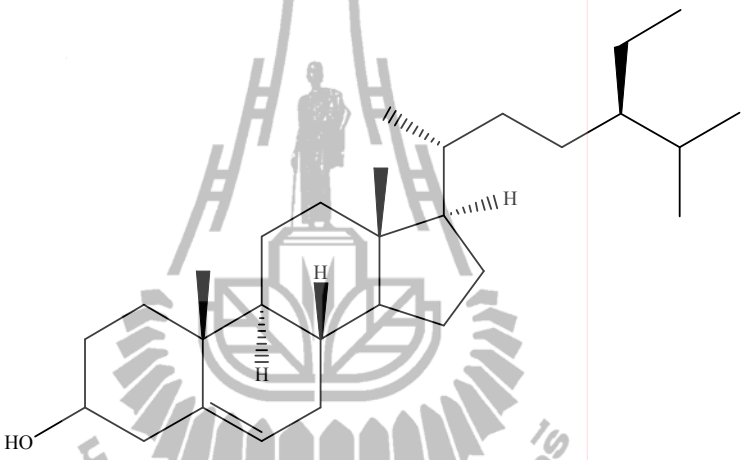
Table 2.2 (Continued).

Scientific name	Plant part	Organic compounds	References
Butea frondosa Roxb.	Flowers	butrin (**30**)	Hildebert, Bettina, Manfred, Yoshinobu, and Hiroshi, 1986.
		isobutrin (**31**)	
		coreopsin (**32**)	Gupta, Ravindranath, and Seahadri, 1970.
		isocoreopsin (**33**)	
		sulfurein (**34**)	
		monospermoside (**35**)	
	Seeds	butin (**36**)	Dixit, Agarwal, Bhargava, Gupta, and Jam, 1981.
		δ-lactone (**37**)	
		palasonin (**38**)	Biahnoi and Gupta, 1979.
			Chandra and Sabir, 1978.

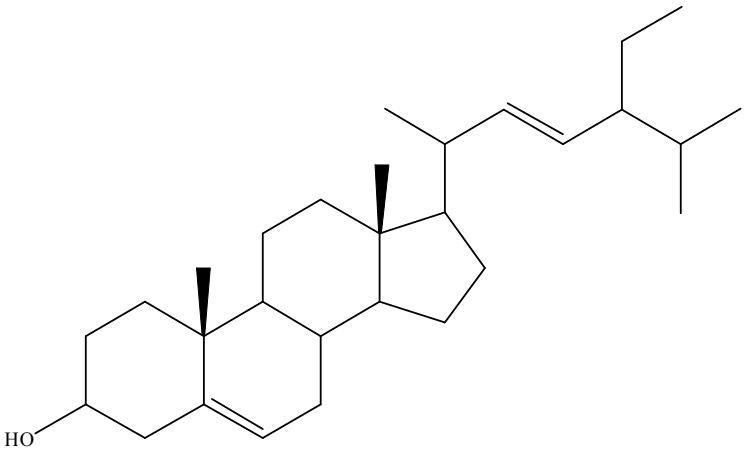
The following structures are reported of chemical constituents of plants in the subfamily *Papilionaceae*:



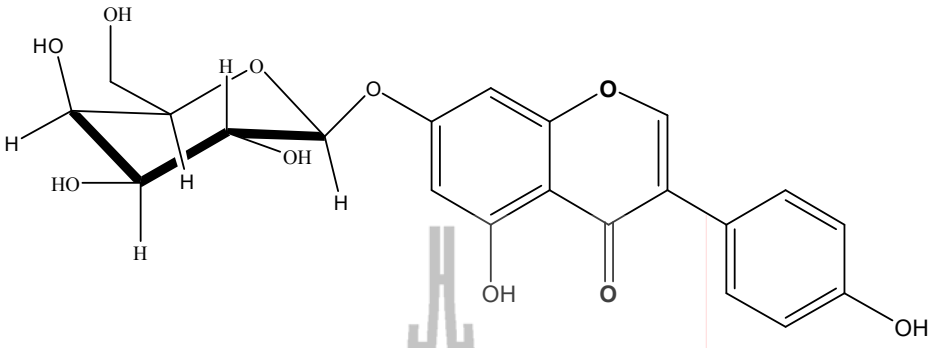
Miroestrol (**16**)



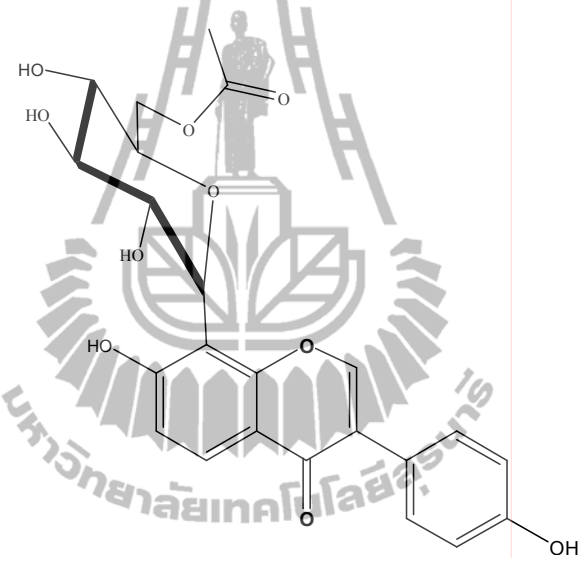
β-Sitosterol (**17**)



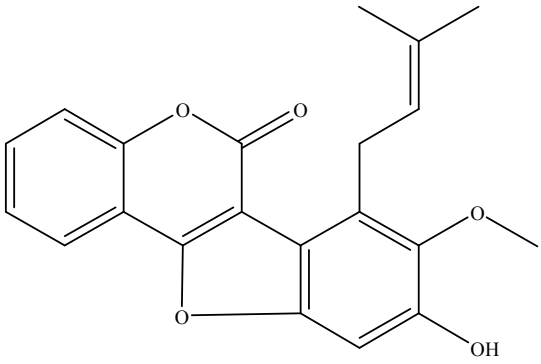
Stimasterol (**18**)



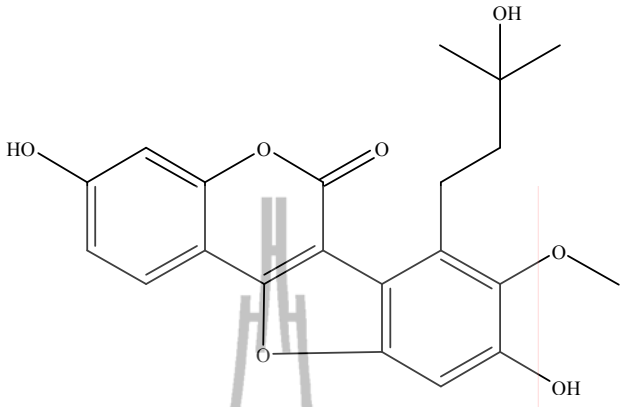
Genistin (genistein-7-*O*-glucoside) (**19**)



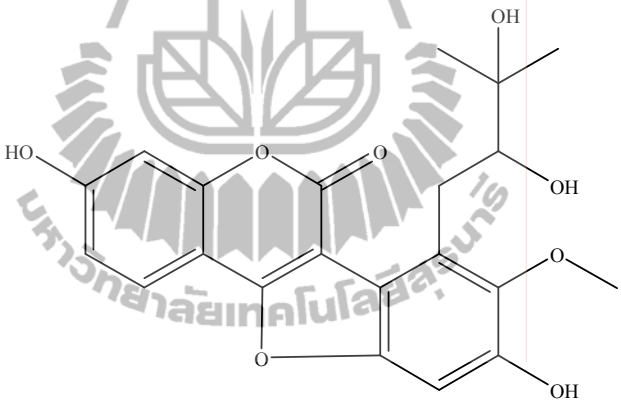
Puerain-6"- monoacetate (**20**)



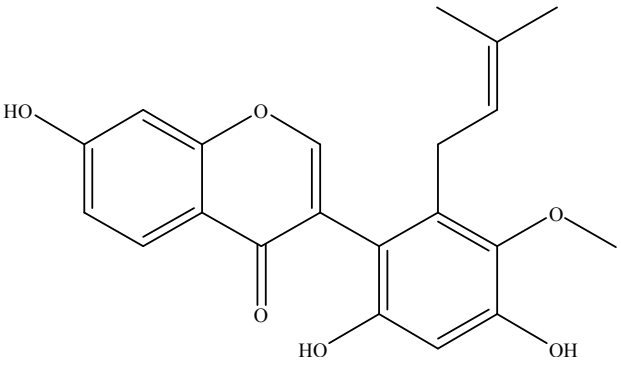
Mirificoumestan (**21**)



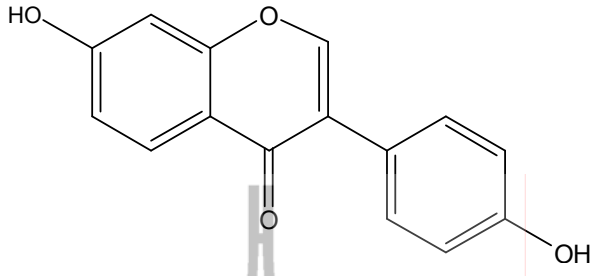
Mirificoumestan hydrate (**22**)



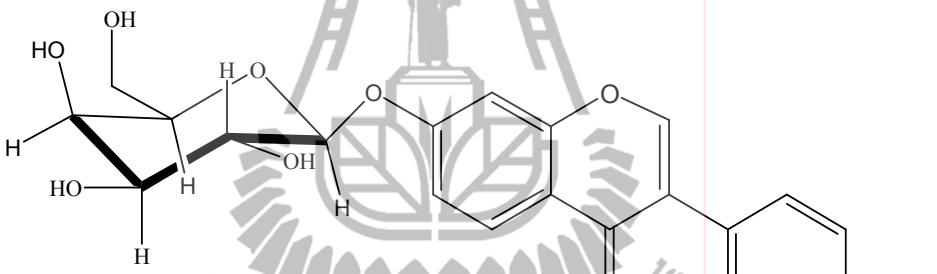
Mirificoumestan glycol (**23**)



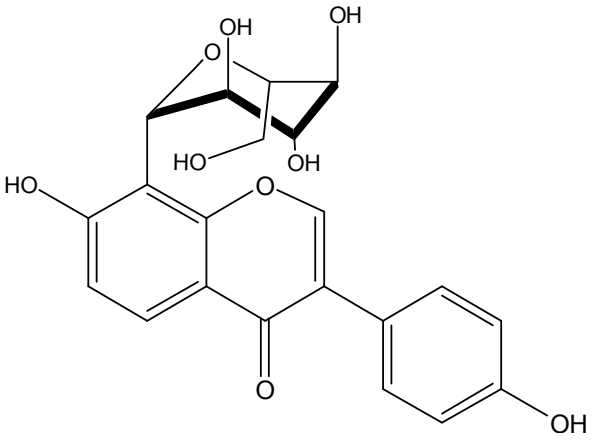
Kwakhurin (**24**)



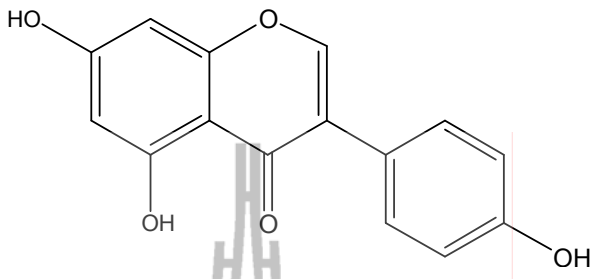
Daidzein (**25**)



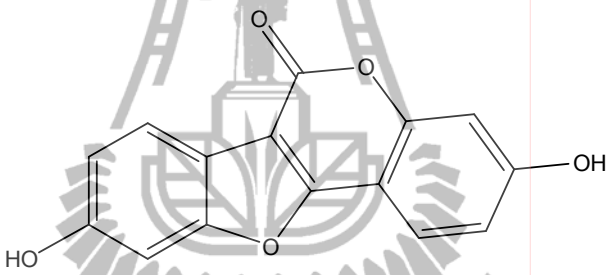
Daidzin (daidzein -7-*O*-glucoside) (**26**)



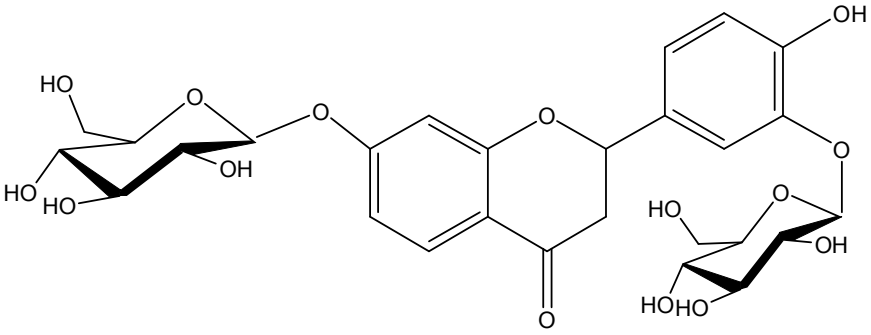
Puerarin (**27**)



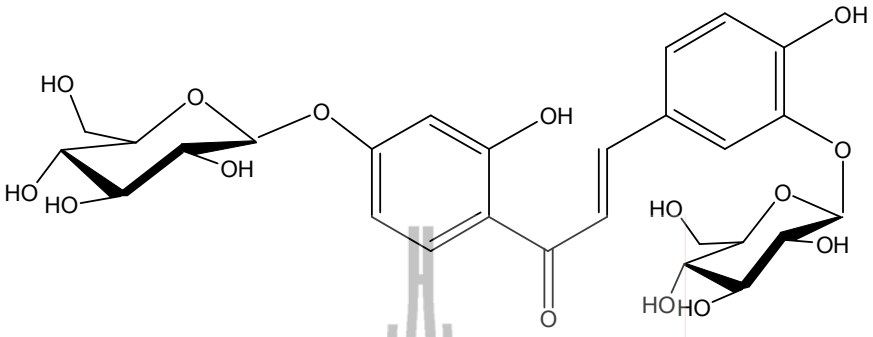
Genistein (**28**)



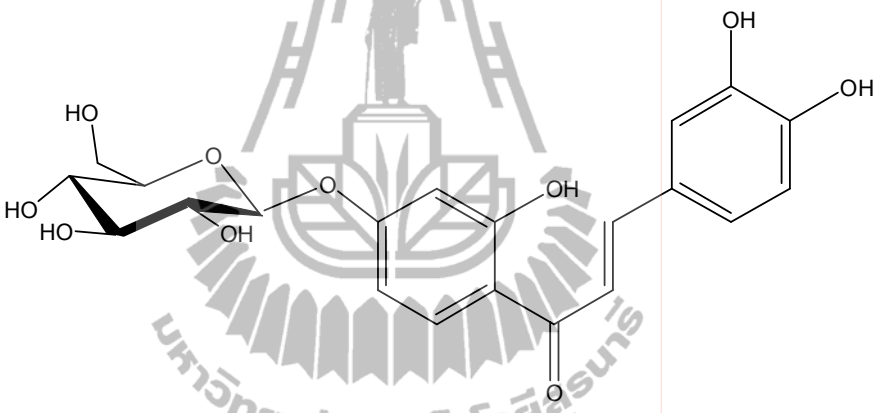
Coumestrol (**29**)



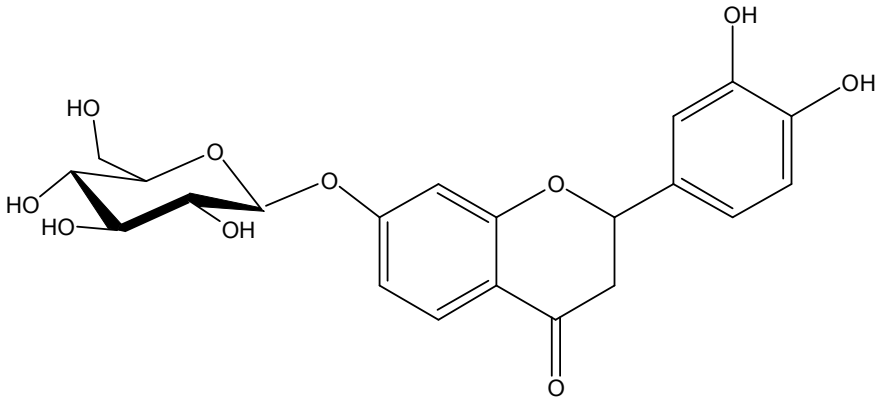
Butrin (**30**)



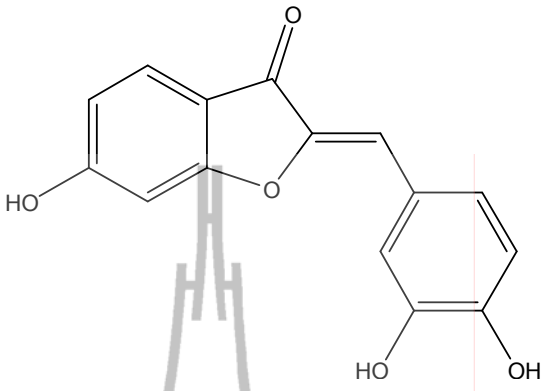
Isobutrin (**31**)



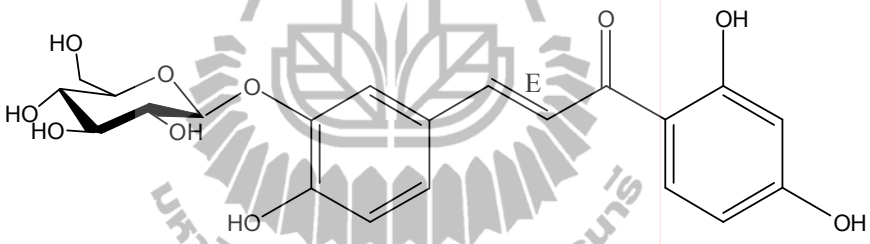
Coreopsin (**32**)



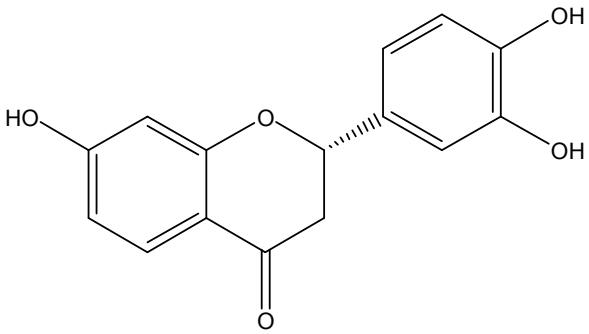
Isocoreopsin (**33**)



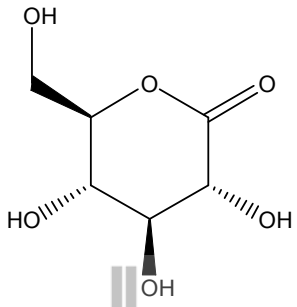
Sulfurein (**34**)



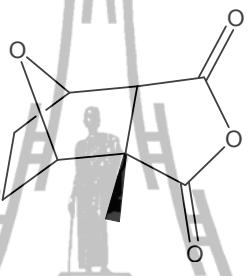
Monospermoside (**35**)



Butin (**36**)



δ-Lactone (**37**)



Palasonin (**38**)

2.5 An Overview of Research Methodology

2.5.1 Soxhlet Extraction

This method is rather convenient and widely used for extraction because it is a continuous process. The sample is put into a Soxhlet thimble which is held within the extraction apparatus. The solvent in the distillation flask is refluxed, and the condensed vapor collects within the thimble chamber, where it interacts with the sample. The solvent plus extract eventually siphons back into the distillation flask. This process continues until extraction is complete. A Soxhlet extractor is illustrated in Figure 2.1 (Wingrove, Thompson, Wafford, and Whiting, 1981).

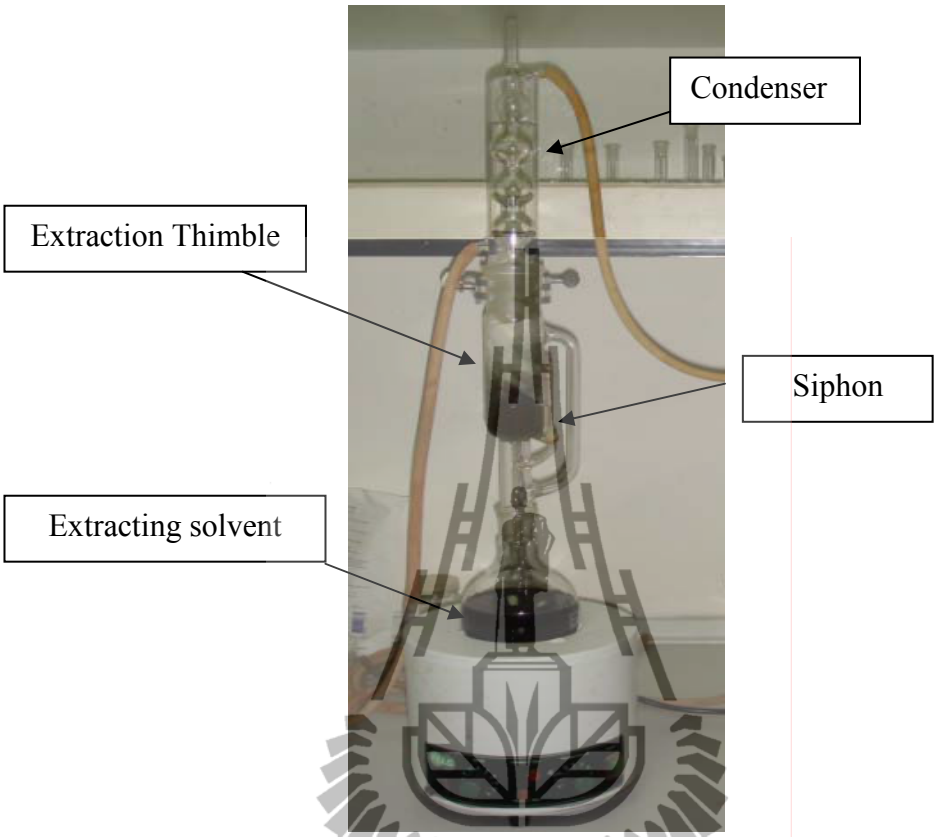


Figure 2.2 Soxhlet extraction apparatus.

2.5.2 Chromatography Techniques

The separation of the components of a mixture by chromatography involves a stationary phase (chromatographic support) through which a mobile phase (chromatographic solvent) is flowing. When a mixture is applied to the stationary phase and allowed to flow with the mobile phase, the various components of the mixture move at different relative rates. These differences in rates of movement are a function of their relative affinities for the stationary phase and the mobile phase. For instance, if substance A has a high affinity for the stationary phase and a low affinity for the mobile phase, it will move slowly, or not at all, as the mobile phase flows over

the stationary phase. Conversely, if substance B has high affinity for the mobile phase but a low affinity for the stationary phase, it will move rapidly (Wade, 1987).

Thin-Layer Chromatography (TLC)

TLC is an easy, quick, and inexpensive procedure that gives the scientist a quick answer as to how many components are in a mixture. TLC is also used to support the identity of a compound in a mixture. It involves the use of particulate solid adsorbent (usually silica or alumina) coated on glass, metal, or plastic as a stationary phase. A small amount of the mixture to be analyzed is spotted near the bottom of plate. The TLC plate is then placed in a shallow pool of a solvent in a developing chamber so that only the very bottom of the plate is in the liquid. This liquid, or the eluent, is the mobile phase, and it slowly rises up the TLC plate. For visualizing the spots, view the TLC plate under UV light (254 nm and 366 nm) and the compounds will appear as dark spots.

Preparative Thin Layer Chromatography

Prep-TLC has long been a popular method as a primary or final purification step in an isolation procedure. Separation can be effected rapidly and amount of material isolated is from 1 mg to 1 g. The adsorbent thickness of prep-TLC is 0.5-4 mm whereas that of analytical TLC is 0.1-0.2 mm. For commercially available prep-TLC plates, adsorbents (silica, alumina, C18, and cellulose) are usually 0.5, 1.0, and 2.0 mm in thickness.

Column Chromatography

In column chromatography, the stationary phase, a solid adsorbent, is placed in a vertical glass column and the mobile phase, a liquid, is added to the top and flows down through the column. Column chromatography is generally used as a purification

technique: it isolates desired compounds from a mixture. The mixture to be analyzed by column chromatrography is applied to the top of the column. The eluent is passed through the column by gravity or by the application of air pressure. Equilibrium is established between the solute adsorbed on the adsorbent and the eluting solvent flowing down through the column. Because the different components in the mixture have different interactions with the stationary and mobile phases, they will be out with the mobile phase to varying degrees and a separation will be achieved.

2.5.3 Structure Elucidation

Nuclear Magnetic Resonance (NMR) spectroscopy

NMR has become a very important spectroscopic method and the premier organic spectroscopy available to chemists to determine the detailed chemical structure of compounds isolated from natural product sources. NMR spectroscopy is routinely used by chemists to study chemical structure of simple molecules using simple one-dimensional techniques (1D-NMR). Two-dimensional techniques (2D-NMR) are used to determine the structure of more complicated molecules (Breitmaier, 2002).

Ultraviolet–Visible (UV-Vis) spectroscopy

UV-Vis spectroscopy is an analytical technique that provides useful information on the light absorption properties of molecules. Absorption occurs when the energy required for an electronic transition matches the energy of the incoming radiation. Compounds that can absorb UV or visible light must contain certain functional groups known as chromophores.

The maxima and minima of the absorption are recorded in nm and the intensity is recorded in terms of log ε with ε = A / c l (A: absorbance, c: molar concentration, l: length of sample cell).

It is evident that 95% ethanol is the common solvent used for UV. Other commonly used solvents are water, methanol, hexane, and petroleum ether.

Fourier transform-infrared (FT-IR) spectroscopy

The concept of IR spectroscopy is based on the vibrational transition energies of chemical bonds within the molecules. The infrared spectrum of a sample is measured by passing a beam of infrared light through the sample. The transmitted light shows how much energy was absorbed at each wavelength. From this process, a transmittance or absorbance spectrum can then be produced to show the positions of the absorbed wavelengths.

IR is used to probe bond vibrations in molecules. This is a simple method to reveal the types of functional groups present in a compound. The functional group region in the range from 4000-1600 cm^{-1} can be divided into three subregions:

- OH, NH, C-H
- C≡C, C≡N, X=C=Y (X, Y = C, O, N, S)
- C=O, C=C

The fingerprint region has very little information about functional groups and is used only to compare two compounds. The range is 1550-660 cm^{-1}. IR spectra can be measured either as solution or in the solid state mixed with KBr or as thin liquid film (Lambert, Leyns, Rooyen, Gosselé, Papon, and Swings, 1987).

Mass spectroscopy (MS)

Mass spectrometry is an analytical technique used to measure the mass-to-charge ratio of ions. This is a powerful, sensitive, and highly selective method to identify compounds. It provides both molecular weight and the fragmentation pattern of a compound. It relies on production of ions from a parent compound and the subsequent characterization of the patterns that are produced. Mass spectrometers can be divided into five fundamental parts, namely the sample inlet system, the ionization source, the analyzer, the detector, and the data analysis as shown in Figure 2.3.

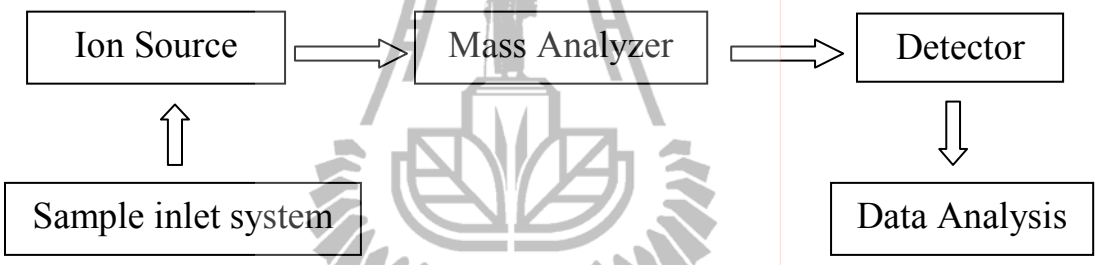


Figure 2.3 Diagram of the components of a Mass Spectrometer.

The sample has to be introduced into the ionization source of the instrument. Once inside the ionization source, the parent compound is bombarded by a electron stream and then converted to ions, because ions are easier to manipulate than neutral molecules. These ions are extracted into the analyzer region of the mass spectrometer where they are separated according to their mass-to-charge ratios (*m/z*) in a magnetic or electric field. The separated ions are detected by a detector and this signal is sent to a data system where the *m/z* ratios are stored together with their relative abundance for presentation in the format of an *m/z* spectrum (Lambert Leyns, Rooyen, Gosselé, Papon, and Swings, 1987; Hughes, Croley, Metcalfe, and March, 2001; Proestos, Sereli, and Komaitis, 2006).

CHAPTER III

EXPERIMENTAL

3.1 Source of Plant Materials

Fresh tuber roots of *Butea superba* Roxb. were collected from Chiang Rai province, Thailand. The specimen of this plant was authenticated with a voucher specimen at the Forest Herbarium, Bangkok, Thailand. The tuber roots were washed thoroughly and dried in an oven at 60 ^{o}C then the dried samples were ground to powder.

3.2 Chemicals

3.2.1 Solvents

The organic solvents used for extraction and as eluent for thin-layer and column chromatography were commercial grade and distilled before use. Other chemicals were analytical and HPLC grade as listed below:

Ethyl acetate	Carlo Erba
Hexane	Carlo Erba
Methanol	Carlo Erba
Dichloromethane	Carlo Erba
Chloroform	Carlo Erba
Acetone	Carlo Erba
Acetone-d_6	Aldrich

Chloroform-d_1	Aldrich
Methanol-d_4	Aldrich

3.2.2 Silica Gel

3.2.2.1 Merck silica gel 60 Art. 7734 (70-230 mesh ASTM) was used as adsorbent for normal column chromatography.

3.2.2.2 Merck silica gel 60 G Art. 7731 and 60 GF_{254} Art. 7730 were applied as adsorbent for preparative TLC.

3.2.2.3 Merck TLC aluminum sheet, silica gel 60 F_{254} precoated 20 cm x 20 cm in size with layer thickness of 0.2 mm was used to identify the identical fractions.

3.3 Instrumentation

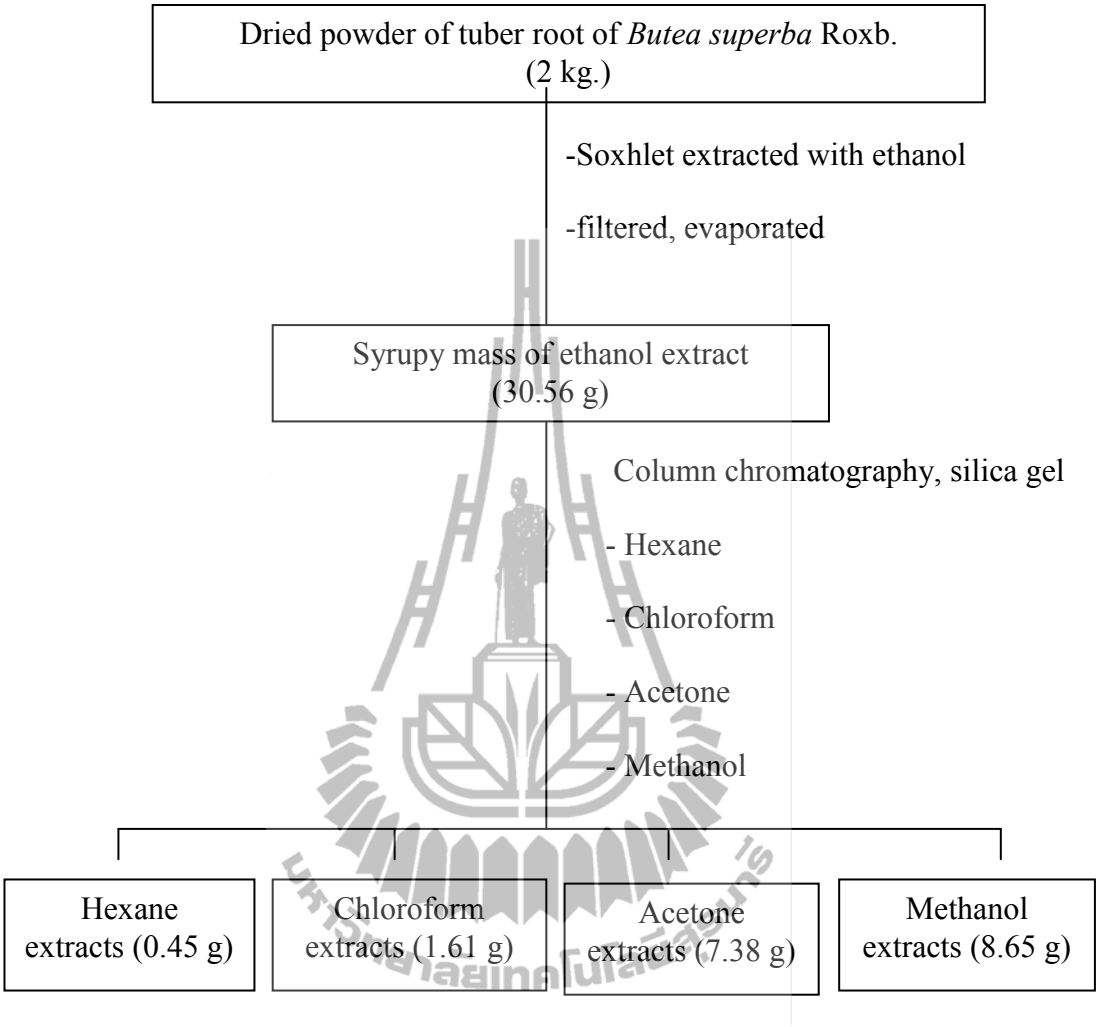
Rotary evaporator	Büchi
Heating bath: Büchi heating bath B-490	
Rotavapor: Büchi rotavapor R-200	
Controller: Büchi vacuum controller V-800	
UV/VIS Spectrophometer series CARY 1E	Varian
UV-Cabinet II	Camag
FT-IR Spectrophotometer Model	Nicolet
NMR Spectrometer INOVA 300	Varian

มหาวิทยาลัยเทคโนโลยีสุรนารี

3.4 Extraction

Dried powdered tuber roots of *Butea superba* Roxb. (2 kg) was extracted continuously with ethanol by Soxhlet extraction for 12 hours. The extracted solutions were then filtered through filter paper (Whatman No. 1). The filtrates were concentrated to remove solvent by evaporation under reduced pressure on a rotary evaporator leaving 30.56 g of syrupy dark brown gum. The ethanol extract (25 g) was separated by silica gel column chromatography. The column was eluted sequentially with hexane, chloroform, acetone, and methanol. All fractions were concentrated to give hexane crude extract (0.45 g), chloroform crude extract (1.61 g), acetone crude extract (7.38 g), and methanol crude extract (8.65 g). The extraction sequence is shown in Scheme 3.1.

มหาวิทยาลัยเทคโนโลยีสุรนารี

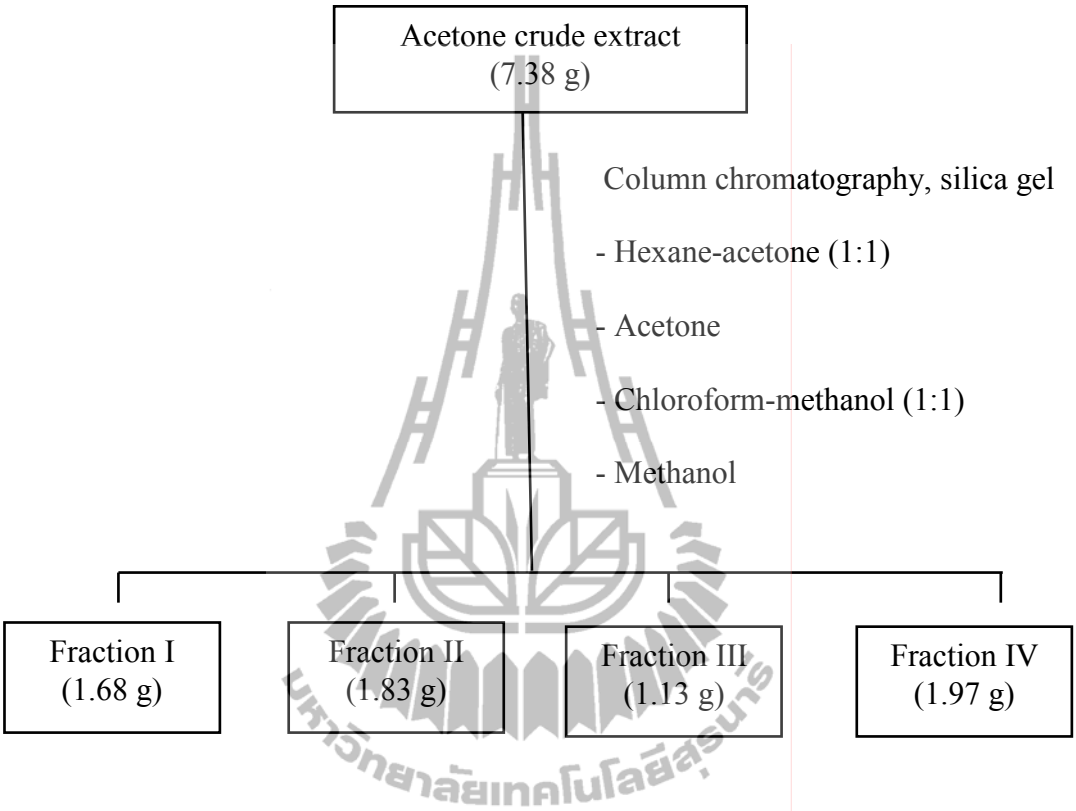


Scheme 3.1 The extraction of the tuber roots of *Butea superba* Roxb.

3.5 Isolation

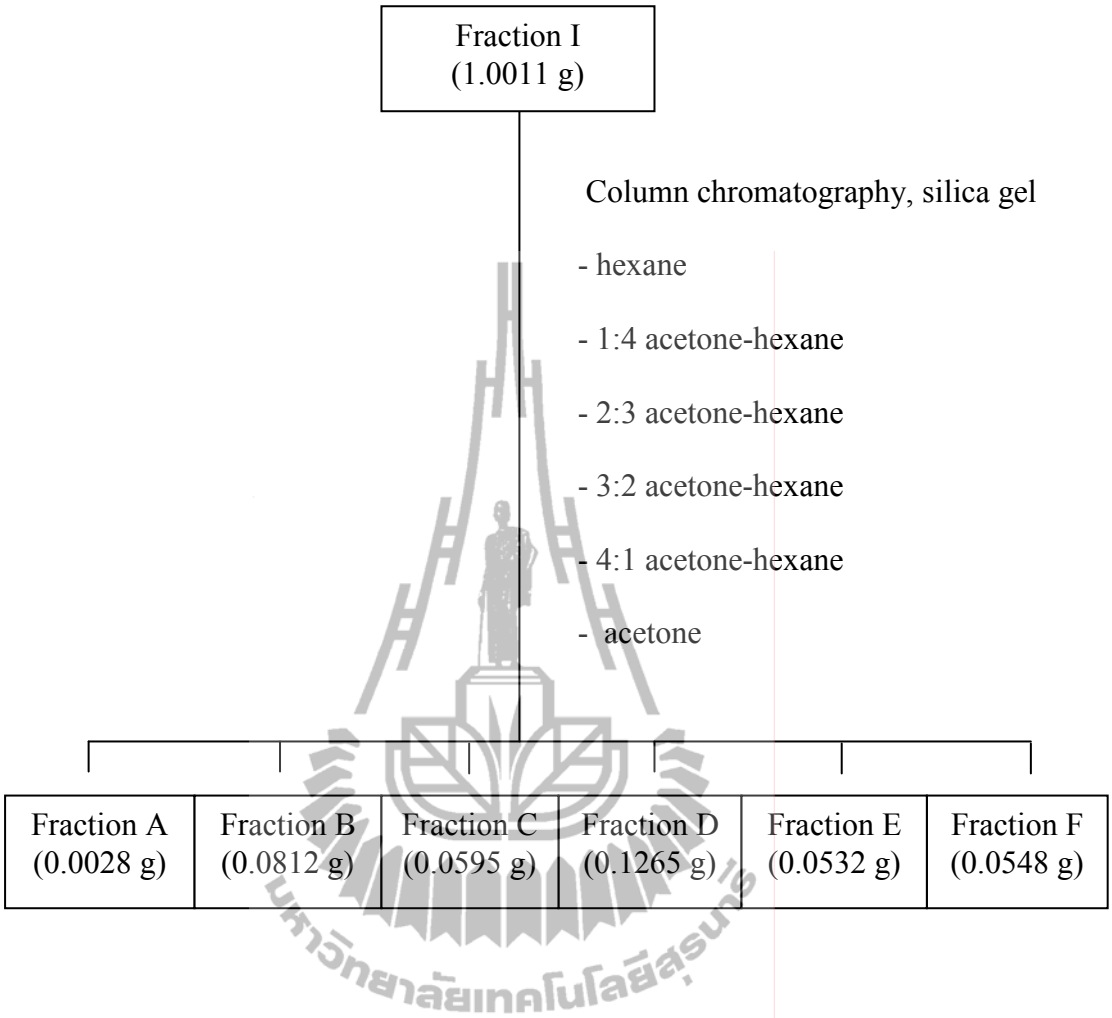
The acetone crude extract was subjected to silica gel column chromatography. The column was eluted successively with hexane-acetone (1:1), acetone, chloroform-methanol (1:1), and methanol. Every fraction of 1000 mL was collected and concentrated to a small volume and four major fractions (I 5.86 g, II 7.13 g, III 10.46 g and IV 6.97 g) were separated by monitoring with TLC (2 cm x 5 cm in size with

chloroform:methanol, 9:1 as developing solvent) in order to combine the fractions which had the same compounds. The separation sequence is shown in Scheme 3.2.



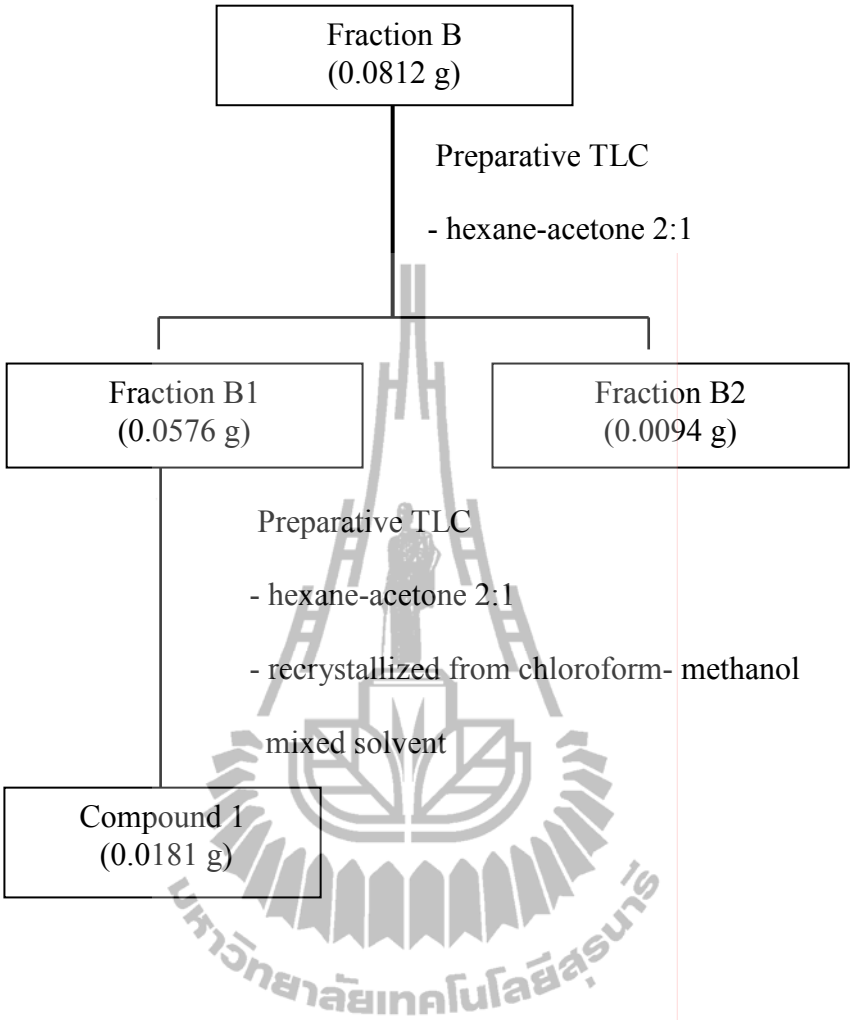
Scheme 3.2 The separation of acetone crude extract.

A portion of fraction I (1.0011 g) was chromatographed on silica gel 60 column. The column was eluted successively with hexane, 1:4 hexane-acetone, 2:3 hexane-acetone, 3:2 hexane-acetone, 4:1 hexane-acetone, and acetone respectively. Every fraction of 500 mL was collected and concentrated to a small volume and six fractions (see Scheme 3.3) were separated by monitoring with TLC (2 cm x 5 cm in size with hexane:acetone, 1:1 as developing solvent) in order to combine the fractions which had the same compounds. The separation sequence is shown in Scheme 3.3.



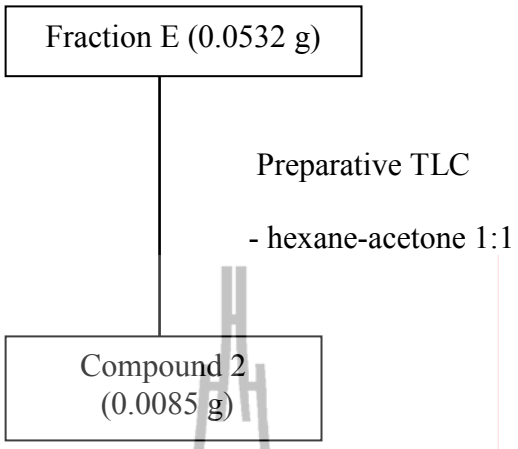
Scheme 3.3 The separation of fraction I from acetone crude extract.

Fraction B (0.0812 g) was further purified by preparative thin layer chromatography (hexane:acetone 2:1) to afford two fractions (B1 0.0576 g, B2 0.0094 g). Fraction B1 (0.0576 g) was further purified by preparative thin layer chromatography using the same developing solvent to give crude compound 1 (0.0211 g) which was recrystallized from chloroform-methanol mixed solvent to obtain pure compound 1 (0.0181 g) as light yellow powder. The separation sequence is shown in Scheme 3.4.



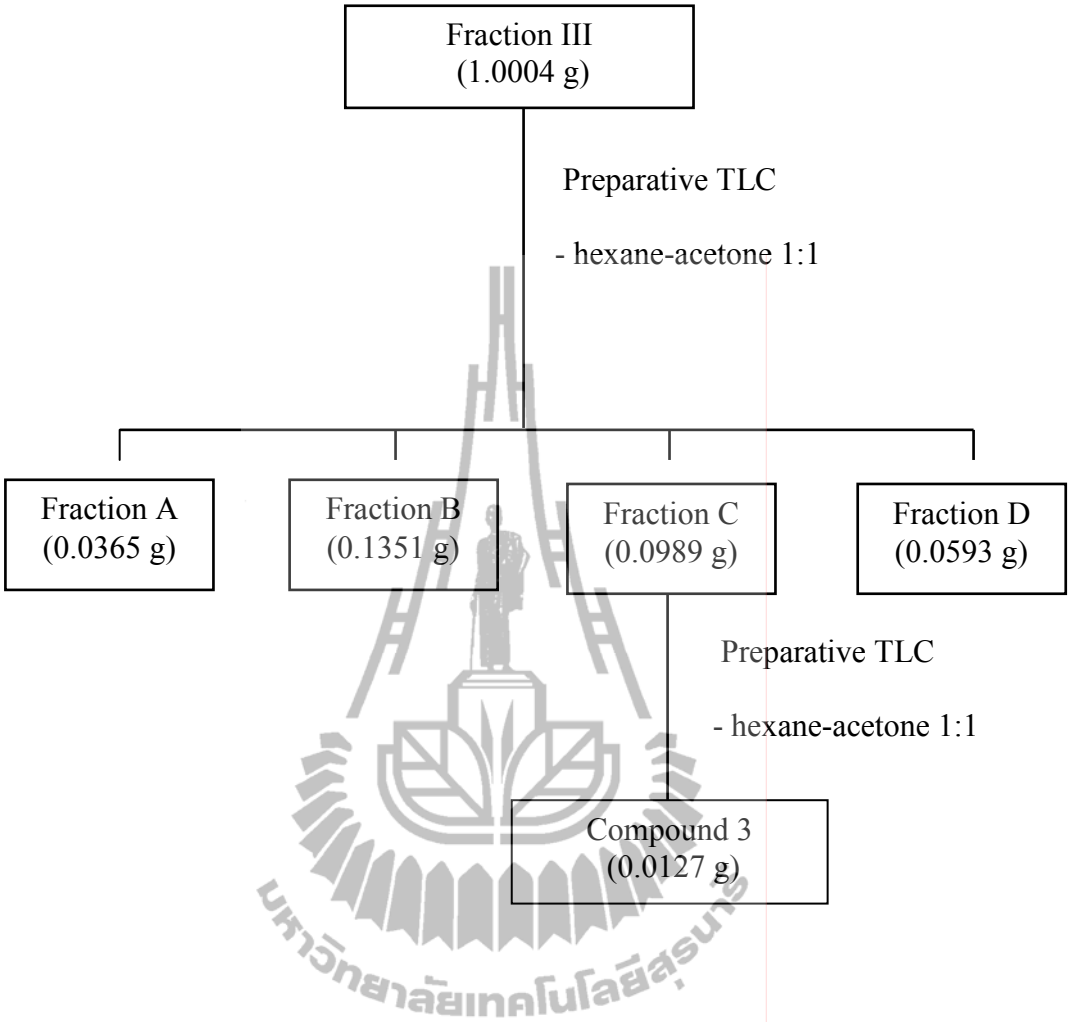
Scheme 3.4 The isolation of compound 1 from acetone crude extract.

Fraction E (0.0532 g) was further purified by preparative thin layer chromatography (hexane:acetone 1:1) to give crude compound 2 (0.0085 g) as a white powder. The separation sequence is shown in Scheme 3.5.



Scheme 3.5 The isolation of compound 2 from acetone crude extract.

A portion of fraction III (1.0004 g) was further purified by preparative thin layer chromatography (chloroform-methanol 9:1) to give four fractions (A 0.0365 g, B 0.1351 g, C 0.0989 g and D 0.0593 g). Fraction C (0.0989 g) was further purified by preparative thin layer chromatography (chloroform-methanol 4:1) to give crude compound 3 (0.0127 g) as a white powder. The separation sequence is shown in Scheme 3.6.



Scheme 3.6 The isolation of compound 3 from acetone crude extract.

3.6 Characterization of Isolated Compounds

3.6.1 Compound 1

UV-Vis (CH_3OH) λ_{max} values 260 and 325 nm (Figure A.1).

FT-IR spectrum (KBr) are 3100-3400, 1651, 1515, 1439, 1247, and 1183 cm^{-1} (Table 4.1 and Figure A.2).

^1H NMR δ (ppm) in acetone-d_6, are 12.99 (1H, *s*, 5-OH), 9.60 (1H, *s*, 7-OH), 8.15 (1H, *s*, 2-H), 7.53 (2H, *d*, *J* = 8.40 Hz, 2′,6′-H), 6.98 (2H, *d*, *J* = 8.40 Hz, 3′,5′-H), 6.40 (1H, *d*, *J* = 2.10 Hz, 8-H), 6.28 (1H, *d*, *J* = 2.10 Hz, 6-H), and 3.83 (3H,

S, 4′-OCH_3) (Table 4.2 and Figure A.3).

^{13}C NMR δ (ppm) in acetone-d_6, are 180.94 (4-C), 164.37 (7-C), 163.31 (5-C), 160.08 (4′-C), 158.42 (9-C), 153.82 (2-C), 130.46 (2′,6′-C), 123.58 (1′-C), 123.20 (3-C), 113.91 (3′,5′-C), 105.58 (10-C), 99.28 (6-C), 93.90 (8-C), and 54.97 (4′-OCH_3) (Table 4.3 and Figure A.4).

Mass spectrum EIMS, *m/z* (relative intensity) are 284 [M^+] (100), 283 [M-1]$^+$ (16), 269 (14), 152 (12), and 132 (36) (Figure A.5).

3.6.2 Compound 2

UV (CH_3OH) λ_{max} values 254 and 369 nm (Figure A.6).

FT-IR spectrum (KBr) are 3000-3500, 1659, 1576, 1500, 1369, and 1286 cm^{-1} (Table 4.4 and Figure A.7).

^{1}H NMR δ (ppm) in CD_3OD+$CDCl_3$ are 7.92 (1H, *s*, 2-H), 7.35 (2H, *d*, *J*= 8.70 Hz, 2′,6′-H), 6.89 (2H, *d*, *J*= 8.70 Hz, 3′,5′-H), 6.37 (1H, *d*, *J*= 2.10 Hz, 8-H), and 6.28 (1H, *d*, *J*= 2.10 Hz, 6-H) (Table 4.5 and Figure A.98.

^{13}C NMR δ (ppm) values, in CD_3OD+$CDCl_3$, are 181.24 (4-C), 164.57 (7-C), 162.49 (5-C), 158.56 (4′-C), 157.47 (9-C), 153.23 (2-C), 130.37 (2′,6′-C), 123.86 (1′-C), 123.18 (3-C), 115.57 (3′,5′-C), 105.54 (10-C), 99.46 (6-C), and 94.19 (8-C) (Table 4.6 and Figure A.9).

Mass spectrum EIMS, *m/z* (relative intensity) are 270 [M^+] (100), 269 [M-1]$^+$ (26), 153 (43), and 118 (14) (Figure A.10).

3.6.3 Compound 3

FT-IR spectrum ν_{max} (KBr) are 3500-3200, 2390, 1640, 1470, 1383, 1250, 1160, 1075, and 1019 cm^{-1} (Table 4.7 and Figure A.11).

^{1}H NMR δ (ppm) in pyridine-d_5, 0.95 (1H, *m*, 9-H), 2.00 (2H, *m*, 12-H), 5.07 (1H, *d*, *J* = 7.7 Hz, 1′-H), 4.08 (1H, *m*, 2′-H), 7.07 (1H, *d*, *J* = 4.0 Hz, 2′-OH), 6.40 (1H, *t*, *J* = 6.2 Hz, 6′-OH), 4.30 (1H, *m*, 3′-H), 1.60 (1H, *m*, 24-H), 4.00 (1H, *m*, 3-H), 0.95 (1H, *m*, 14-H), 0.77 (3H, *s*, 18-H), 0.95 (3H, *m*, 21-H), 1.45 (1H, *m*, 20-H), 1.40 (2H, *m*, 11-H), 1.40 (1H, *m*, 8-H), and 1.25 (2H, *m*, 15-H) (Table 4.8 and Figure A.12).

DEPT ^{13}C NMR δ (ppm) in pyridine-d_5, 141.4 (5-C), 122.4 (6-C), 139.3 (22-C), 130.0 (23-C), 122.4 (3-C), 37.4 (1-C), 57.3 (14-C), 30.0 (5-C), 40.5 (20-C), 40.5 (12-C), 56.8 (17-C), 13.0 (29-C), 103.1 (1′-C), 75.8 (2′-C), 79.1 (3′-C), 72.2 (4′-C), 78.9 (5′-C), and 63.4 (6′-C) (Table 4.9 and Figure A.13).

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CHAPTER IV

RESULTS AND DISCUSSION

4.1 Properties and Structure Elucidation of Compound 1

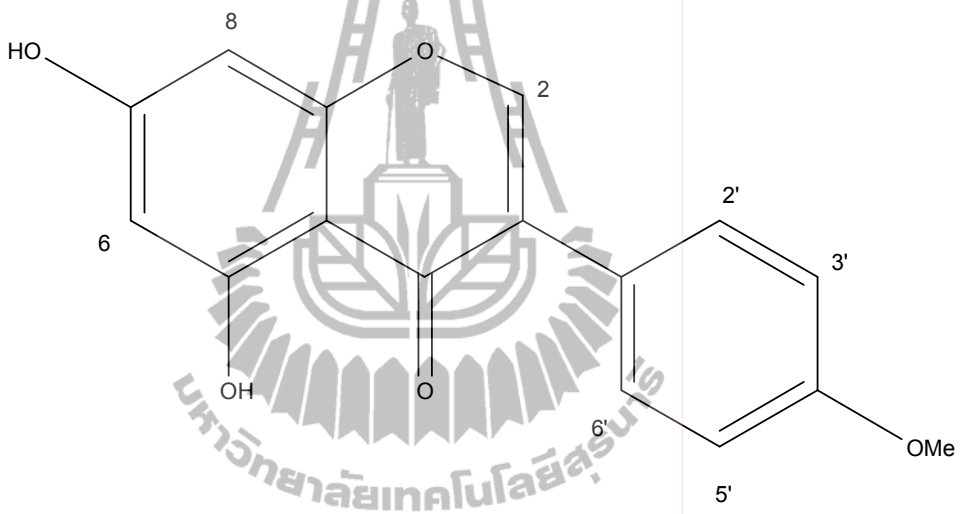

Figure 4.1 Structure of compound 1.

Compound 1 was obtained as a light yellow solid (0.0181 g, 0.0009% yields from dried powder). The UV-Vis spectrum (Figure A.1) showed absorption bands (λ_{max}) at 260 and 325 nm. The UV-Vis spectrum of compound 1 suggested the possibility of a flavonoid derivative compound (Maria, Anna, Lorenzo, Giovanni, and Franco, 2007).

The IR spectrum of compound 1 is shown in Figure A.2. It shows both a strong absorption band at 1651 cm^{-1} indicating the presence of an α,β-unsaturated carbonyl

carbon and a broad band of hydroxyl group at 3100-3400 cm^{-1}. A comparison of IR absorption bands of compound 1 with biochanin A (NIST, 2011) and the other absorption peaks were assigned as shown in Table 4.1.

Table 4.1 IR absorption band assignment of compound 1.

Wavenumber (cm^{-1})		Assignment
compound 1	biochanin A	
3100-3400	3000-3400	O-H Stretching
1651	1653	C=O Stretching
1622, 1609	1623, 1610	C=C Stretching
1247, 1183	1292, 1196	C-H Bending

The ^{1}H-NMR spectrum (Figure A.3) of compound 1 shows four signals of aromatic protons at δ 6.28 ppm (1H, *d*, *J* = 2.10 Hz), 6.40 ppm (1H, *d*, *J* = 2.10 Hz) for the H-6 and H-8 positions in the A ring, and a doublet signal at δ 7.53 ppm (2H, *d*, *J* = 8.40 Hz) for the H-2′ and H-6′ positions and a doublet signal at δ 6.98 ppm (2H, *d*, *J* = 8.40 Hz) for the H-3′ and H-5′ positions in the B ring. The B ring signals were easily assigned by consideration of symmetry. The aromatic region of the ^{1}H-NMR spectrum of compound 1 contains a characteristic resonance for H-2 of the isoflavone at δ 8.15 ppm (1H, *s*). This observation suggested that the characteristics of unsubstituted benzene ring and three singlet signals at δ 12.99 ppm (proton signal disclosed downfield shift) and 9.60 ppm suggesting hydroxyl groups at H-5 and H-7, position the presence of a methoxy group indicated by a singlet signal at δ 3.83 ppm.

The ^{13}C-NMR spectrum (Figure A.4) of compound 1 shows fourteen carbon signals for the fifteen skeletal carbon atoms (flavonoid characteristic). This compound

has one carbonyl group with the most downfield shift at δ 180.94 ppm, one methoxy carbon signal at δ 54.97 ppm, seven methine carbon on five signals at δ 153.83 ppm (C-2), 130.46 ppm (C-2′, C-6′), 113.91 ppm (C-3′, C-5′), 99.18 (C-6) and 93.90 (C-8), and seven quarternary carbon signals at δ 164.37 ppm (C-7), 163.31 ppm (C-5), 160.08 ppm (C-4′), 158.42 ppm (C-9), 123.58 ppm (C-1′), 123.20 ppm (C-3), and 105.58 ppm (C-10).

The mass (Figure A.5) of compound 1 showed the molecular ion peak at m/z 284 [M^+] and its molecular formula was determined as $C_{16}H_{12}O_5$ by EIMS.

Based on the above spectral data characteristics and comparison of the ^{1}H-NMR and ^{13}C-NMR chemical shifts of compound 1 with published data of biochanin A, the structure of compound 1 was identified as 5,7-dihydroxy-4′-methoxyisoflavone or biochanin A. A comparison of the ^{1}H-NMR and ^{13}C-NMR chemical shifts of compound 1 with biochanin A (Talukdar, Jain, De and Krishnamurty, 2000) are shown in Tables 4.2 and 4.3. These data indicate that compound 1 is biochanin A (Figure 4.1).

Table 4.2 The ^{1}H-NMR chemical shifts of compound 1$^{\#}$ and biochanin A*.

Proton position	Chemical shifts in ppm (coupling constant in Hz)	
	compound 1	biochanin A
2	8.15 (*s*)	8.20 (*s*)
5-OH	12.99 (*s*)	13.12 (*s*)
6	6.28 (*d*, *J* = 2.10)	6.32 (*d*, *J* = 2.5)
7-OH	9.60 (*s*)	9.80 (*s*)
8	6.40 (*d*, *J* =2.10)	6.44 (*d*, *J* = 2.5)
2′	7.53 (*d*, *J* =8.40)	7.40 (*d*, *J* = 8)
3′	6.98 (*d*, *J* = 8.40)	6.84 (*d*, *J* = 8)
4′-OCH_3	3.83 (*s*)	3.84 (*s*)
5′	6.98 (*d*, *J* = 8.40)	6.84 (*d*, *J* = 8)
6′	7.53 (*d*, *J* =8.40)	7.40 (*d*, *J* = 8)

$^{\#}$300 MHz ^{1}H, acetone d_6

*500 MHz ^{1}H, acetone d_6

Table 4.3 The ^{13}C-NMR chemical shifts of compound 1[#] and biochanin A [*].

Carbon position	Chemical shifts in ppm (coupling constant in Hz)	
	compound 1	biochanin A
2	153.82	154.2
3	123.20	121.9
4	180.94	180.1
5	163.31	162.0
6	99.28	99.0
7	164.37	164.4
8	93.90	93.7
9	158.42	157.6
10	105.58	104.4
1′	123.58	122.9
2′	130.46	130.1
3′	113.91	113.7
4′	160.08	159.1
5′	113.91	113.7
6′	130.46	130.1
4′-OCH_3	54.97	55.1

[#]75 MHz ^{1}H, acetone d_6

[*]125 MHz ^{1}H, acetone d_6

4.2 Properties and Structure Elucidation of Compound 2

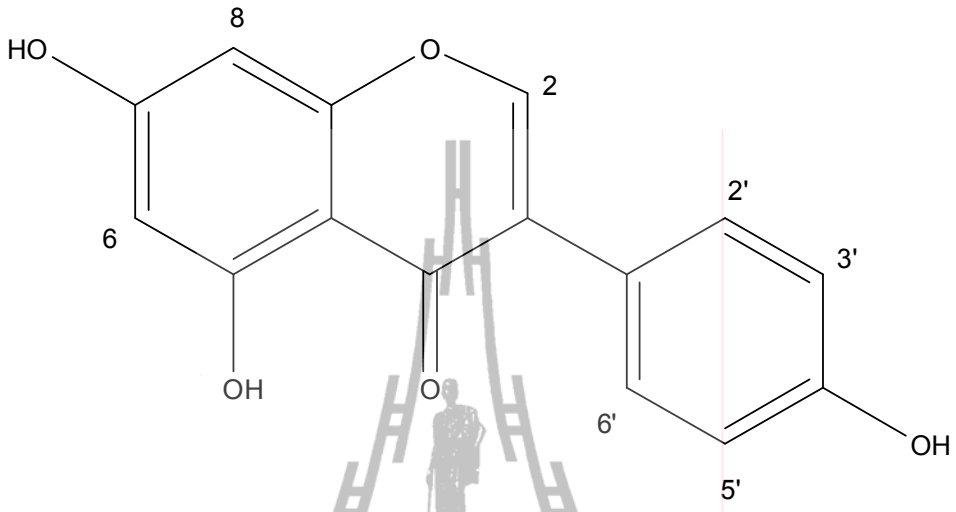

Figure 4.2 Structure of compound 2.

Compound 2 was obtained as a white solid (0.0085 g, 0.0004% yields from dried powder). The UV-Vis spectrum (Figure A.6) shows absorption bands (λ_{max}) at 254 and 369 nm. The UV-Vis spectrum of compound 2 suggests the possibility of a flavonoid derivative compound (Maria, Anna, Lorenzo, Giovanni, and Franco, 2007).

The IR spectrum (Figure A.7) of compound 2 showed a broad absorption band characteristic of a hydroxyl group at 3000-3500 cm^{-1} and strong absorption band at 1659 cm^{-1} shows the presence of α,β-unsaturated carbonyl carbon. A comparison of IR absorption bands of compound 2 with genistein (NIST, 2011) and the other absorption peaks were assigned as shown in Table 4.4.

Table 4.4 IR absorption band assignment of compound 2.

Wavenumber (cm^{-1})		Assignment
compound 2	genistein	
3000-3500	3000-3400	O-H Stretching
1659	1652	C=O Stretching
1622, 1576	1616, 1583	C=C Stretching
1286, 1255	1286, 1274	C-H Bending

The ^{1}H-NMR spectrum (Figure A.8) of compound 2 includes four signals of aromatic protons at δ 6.28 ppm (1H, *d*, *J* = 2.10 Hz) and 6.37 ppm (1H, *d*, *J* = 2.10 Hz) for the H-6 and H-8 positions in the A ring, and at δ 7.53 ppm (2H, *d*, *J* = 8.70 Hz) for the H-2′ and H-6′positions, and a doublet signal at δ 6.89 ppm (2H, *d*, *J* = 8.70 Hz) for the H-3′ and H-5′ positions in the B ring. The B ring signals were easily assigned by consideration of symmetry. The aromatic region of the ^{1}H-NMR spectrum of compound 2 contains a characteristic resonance for H-2 of the isoflavone at δ 7.92 ppm (1H, *s*).

The ^{13}C-NMR spectrum (Figure A.9) of compound 2 showed thirteen carbon signals for the fifteen skeletal carbon atoms (flavonoid characteristic). The most downfield carbon signal at δ 181.24 ppm indicated the presence of carbonyl carbon (C-4), seven methine carbon signals at δ 153.23 ppm (C-2), 130.37 ppm (C-2′, C-6′), 115.57 ppm (C-3′, C-5′), 99.46 (C-6) and 94.18 (C-8), and seven quarternary carbon signals at δ 164.57 ppm (C-7), 162.49 ppm (C-5), 158.56 ppm (C-4′), 157.47 ppm (C-9), 123.86 ppm (C-1′), 122.18 ppm (C-3), and 105.54 ppm (C-10).

The mass spectrum (Figure A.10) of compound 2 showed the molecular ion peak at m/z 270 [M^+] and its molecular formula was determined as $C_{15}H_{10}O_5$ by EIMS

On the basis of the spectral data, the structure of compound 2 was established as 5,7,4′-trihydroxyisoflavone or genistein. A comparison of the ^{1}H-NMR and ^{13}C-NMR chemical shifts of compound 2 with previous reports (Zhao, Zhang, Gao and Shao, 2009) are shown in Table 4.5 and 4.6. These data indicated that compound 2 is genistein (Figure 4.2).

Table 4.5 The ^{1}H-NMR chemical shifts of compound 2[#] and genistein[*].

Proton position	Chemical shifts in ppm (coupling constant in Hz)	
	compound 2	genistein
2	7.92 (*s*)	8.10 (*s*)
5-OH	-	12.90 (*s*)
6	6.28 (*d*, *J* = 2.10)	6.23 (*d*, *J* = 1.9)
7-OH	-	10.86 (*s*)
8	6.37 (*d*, *J* = 2.10)	6.36 (*d*, *J* = 1.9)
2′	7.35 (*d*, *J* = 8.70)	7.37 (*d*, *J* = 8.4)
3′	6.89 (*d*, *J* = 8.70)	6.82 (*d*, *J* = 8.4)
4′-OH	-	9.57 (*s*)
5′	6.89 (*d*, *J* = 8.70)	6.82 (*d*, *J* = 8.4)
6′	7.35 (*d*, *J* = 8.70)	7.37 (*d*, *J* = 8.4)

[#]300 MHz ^{1}H, CD_3OD+$CDCl_3$

[*]500 MHz ^{1}H, DMSO- d_6

Table 4.6 The ^{13}C-NMR chemical shifts of compound 2 [#] and genistein [*].

Carbon position	Chemical shifts in ppm (coupling constant in Hz)	
	compound 2	genistein
2	153.23	154.2
3	122.18	121.5
4	181.24	180.6
5	162.49	162.3
6	99.46	99.1
7	164.57	164.6
8	94.19	94.6
9	157.47	157.7
10	105.54	104.8
1′	123.86	122.6
2′	130.37	130.3
3′	115.57	113.4
4′	158.56	157.8
5′	115.57	115.4
6′	130.37	130.3

[#]75 MHz ^{1}H, CD_3OD+$CDCl_3$

[*]125 MHz ^{1}H, DMSO-d_6

4.3 Properties and Structure Elucidation of Compound 3

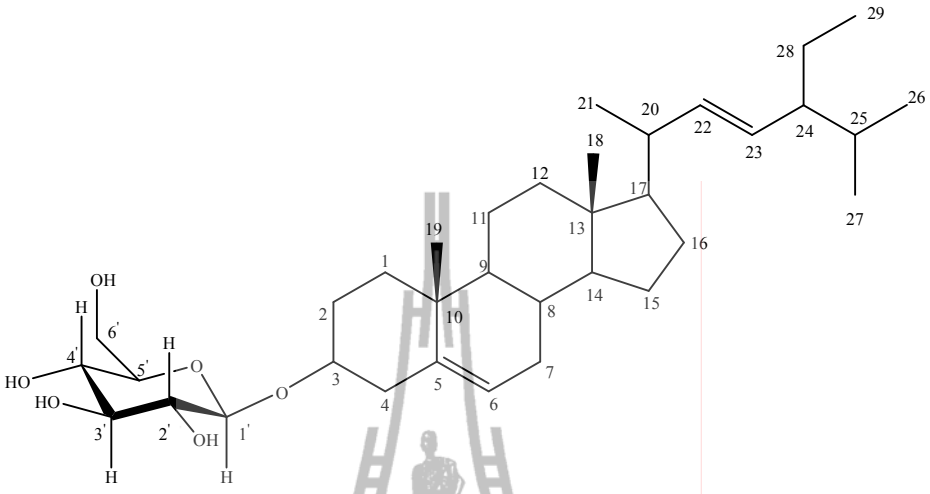

Figure 4.3 Structure of compound 3.

Compound 3 was obtained as a white solid (0.0127 g, 0.0006% yields from dried powder). The IR spectrum (Figure A.11) of this compound showed a broad absorption band of hydroxyl group at 3200-3500 cm^{-1}. A comparison of IR absorption bands of compound 3 with stigmasteryl-3-*O*-β-D-glucopyranoside (NIST, 2011) and other peaks were assigned as shown in Table 4.7

Table 4.7 IR absorption band assignment of compound 3.

Wavenumber (cm^{-1})		Assignment
compound 3	stigmasteryl-3-*O*-β-D-glucopyranoside	
3500-3200	3476-2944	O-H Stretching
1640	1646	C=C Stretching
1250	1214	C-O Stretching
1160	1168	C-O Stretching

The ^{1}H-NMR (Figure A.12) and DEPT ^{13}C-NMR (Figure A.13) spectra of compound 3 shows the presence of three olefinic proton signals at δ 5.37 ppm (m, H-6), 5.10 ppm (*dd*, *J* = 15.20, 8.80, H-22), and 5.08 ppm (*dd*, *J* = 15.20, 8.80, H-23). The six methyl groups occurred signals at δ 0.70 ppm (*s*, Me-18), δ 0.97 ppm (*m*, Me-19), δ 0.95 ppm (*m*, Me-21), 0.95 ppm (*m*, Me-26, Me-27), and 0.90 ppm (*m*, Me-29). These were found to be the signals of β-anomeric proton of glucopyranosyl a group of multiplet signals at δ 4.08 (*m*, H-2′), δ 4.30 (*m*, H-3′), δ 4.30 (*m*, H-4′), δ 4.00 (*m*, H-5′) and δ 4.43 (*m*, H-6′) ppm with corresponding carbons at δ 103.1 (C-1′), 75.8 (C-2′), 79.1 (C-3′), 72.2 (C-4′), 79.0 (C-5′) and 63.4 (C-6′) ppm.

A comparison of the ^{1}H-NMR and ^{13}C-NMR chemical shifts of compound 3 and stigmasteryl-3-*O*-β-D-glucopyranoside (Alam, Chopra, Ali, and Niwa, 1996) are shown in Tables 4.8 and 4.9.

Table 4.8 The ^{1}H-NMR chemical shifts of compound 3[#] and stigmasteryl-3-*O*-β-D-glucopyranoside[*].

Proton position	Chemical shifts in ppm (coupling constant in Hz)	
	compound 3	stimasteryl-3-*O*-β-D-glucopyranoside
1α	1.00 (*m*)	0.99
1β	1.75 (*m*)	1.81
1′	5.07 (*d*, *J* = 7.70)	4.21
2α	1.75 (*m*)	0.90
2β	2.15 (*m*)	1.27

Table 4.8 (Continued).

Proton position	Chemical shifts in ppm (coupling constant in Hz)	
	compound 3	stigmasteryl-3-*O*-β-D-glucopyranoside
2'	4.08 (*m*)	3.46
2'-OH	7.07 (*br*, *d*, *J* = 4.00)	
3	4.00 (*m*)	3.47
3'	4.30 (*m*)	3.40
3'-OH	7.13 (*br*)	
4α	2.50 (*m*)	1.14
4β	2.75 (*m*)	1.78
4'	4.30 (*m*)	3.11
4'-OH	7.13 (*br*)	
5'	4.00 (*m*)	3.62
6	5.37 (*br*, *m*)	5.32
6'α	4.43 (*m*)	4.21
6'β	4.58 (*m*)	4.41
6'-OH	6.40 (*br*, *t*, *J* = 6.20)	
7α	1.60 (*m*)	1.48
7β	1.90 (*m*)	
8	1.40 (*m*)	1.49
9	0.95 (*m*)	0.91
11	1.40 (*m*)	1.18

Table 4.8 (Continued).

Proton position	Chemical shifts in ppm (coupling constant in Hz)	
	compound 3	stigmasteryl-3-*O*-β-D-glucopyranoside
12β	2.00 (*m*)	1.96
12α	1.15 (*m*)	1.14
14	0.95 (*m*)	1.07
15	1.25 (*m*)	1.03
16α	1.30 (*m*)	1.64
16β	1.85 (*m*)	1.79
17	1.15 (*m*)	1.04
18	0.70 (*s*)	0.64
19	0.97 (*m*)	1.00
20	1.45(*m*)	1.31
21	0.95 (*m*)	0.98
22	5.10 (*dd*, *J* = 15.20, 8.80)	5.15
23	5.08 (*dd*, *J* = 15.30, 8.80)	5.01
24	1.60 (*m*)	0.92
25	1.75 (*m*)	1.44
26	0.95 (*m*)	0.89
27	0.95 (*m*)	0.81
28	1.30 (*m*)	1.05
29	0.90 (*m*)	0.82

[#]300 MHz ^{1}H, pyridine d_5;

[*]600 MHz ^{1}H, DMSO d_6 with TMS

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Table 4.9 The ^{13}C-NMR chemical shifts of compound 3 and stigmasteryl-3-*O*-β-D-glucopyranoside.

Carbon position	Chemical shifts in ppm (coupling constant in Hz)	
	compound 3	stigmasteryl-3-*O*-β-D-glucopyranoside
1	37.4	38.3
1’	103.1	100.8
2	30.8	33.3
2’	75.8	70.1
3	78.6	76.9
3’	79.1	76.7
4	39.9	46.8
4’	72.2	73.4
5	141.4	140.4
5’	78.9	76.7
6	122.4	121.1
6’	63.4	61.0
7	32.7	31.3
8	32.6	31.4
9	50.9	49.6
10	37.4	36.2
11	21.8	22.6
12	40.5	39.6

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Table 4.9 (Continued).

Carbon position	Chemical shifts in ppm (coupling constant in Hz)	
	compound 3	stigmasteryl-3-*O*-β-D-glucopyranoside
13	42.9	40.0
14	57.3	56.2
15	26.9	24.8
16	29.3	29.2
17	56.8	56.1
18	12.5	11.8
19	19.9	19.0
20	40.5	35.4
21	21.8	18.8
22	139.3	138.0
23	130.3	128.8
24	51.9	45.1
25	30.0	31.3
26	19.7	19.6
27	19.5	18.9
28	23.9	23.8
29	13.0	11.6

มหาวิทยาลัยเทคโนโลยีสุรนารี

[#]75 MHz ^{1}H, pyridine-d_5;

[*]150 MHz ^{13}C, DMSO-d_6 with TMS

The close agreement between our data and the literature values indicate that compound 3 is stigmasteryl-3-*O*-β-D-glucopyranoside (Figure 4.3).

CHAPTER V

CONCLUSION

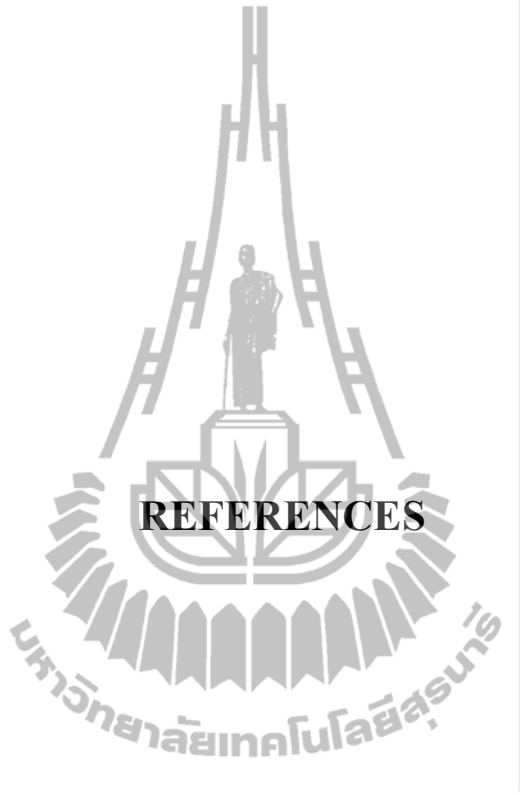
The dried powder of the tuber roots of *Butea superba* Roxb. was extracted with ethanol. The ethanol extract was separated by silica gel column chromatography by sequential elution with hexane, chloroform, acetone, and methanol. The acetone crude extract was separated by column chromatography and preparative thin-layer chromatography to give three pure compounds. The chemical structures of the isolated compounds were characterized by spectroscopic techniques including UV-Vis, FT-IR, MS, ^{1}H-NMR, and ^{13}C-NMR and compared with literature values. The structures of the three isolated compounds are summarized as follows.

Compound 1 was identified as 5,7-dihydroxy-4′-methoxyisoflavone or biochanin A. The spectroscopic evidence indicated the presence of the characteristic isoflavone group. The amount of compound 1 obtained was 0.0181 g (0.0009% yields from dried powder).

Compound 2 was identified as 5,7,4′-trihydroxyisoflavone or genistein. The spectroscopic evidence of compound 2 was similar to that of compound 1. The amount of compound 2 obtained was 0.0085 g (0.0004% yields from dried powder).

Compound 3 was identified as stigmasteryl-3-*O*-β-D-glucopyranoside. The spectroscopic evidence of compound 3 was in agreement with that obtained from the literature. The amount of compound 3 obtained was 0.0127 g (0.0006% yields from dried powder).

Genistein has been previously reported in *Pueraria mirifica*, also in the subfamily *Papilionaceae*, but this is the first report in *Butea superba* Roxb. This is the first report of biochanin A and stigmasteryl-3-*O*-β-D-glucopyranoside in both Butea superba Roxb. and in the subfamily *Papilionaceae*.



REFERENCES

REFERENCES

Alam, M. S., Chopra, N., Ali, M., and Niwa, M. (1996). Oleanen and stigmasterol derivatives from *Ambroma austa*. **Phytochemistry**. 41: 1197-1200.

Biahnoi, P. and Gupta, P. C. (1979). A new lactone from the seeds of *Butea frondosa*. **Planta Medica**. 53: 286-288.

Block, J. H. (1989). **Progress in the Design of Bioactive Molecules**. Washington D.C.: American Chemical Society.

Boonyaratavej, S. and Petsom, A. (1991). Chemical constituents of the roots of *Bridelia tomentosa* bl. **Journal of the Science Society of Thailand**. 17: 61-69.

Breitmaier, E. (2002). **Structure Elucidation by NMR in Organic Chemistry-A Practical**. New York: John Wiley and Sons. pp. 68-70.

Butler, M. S. (2004). The role of natural product chemistry in drug discovery. **Journal of Natural Products**. 67: 2141-2153.

Chandra, J. S. and Sabir, M. (1978). Modified method for isolation of Palasonin-The anthelmintic principle of *Butea frondosa* seeds. **Indian Journal of Pharmaceutical Sciences**. 40: 97-98.

Chavalittumrong, P. (2000). **Standard of Thai Herbal Medicine: *Andrographis paniculata* (Burm. f.) Nees**. Bangkok: E.T.O. Press.

Chavalittumrong, P. (2001). **Standard of Thai Herbal Medicine: *Curcuma longa* Linn**. Bangkok: E.T.O. Press.

Chavalittumrong, P. (2002). **Standard of Thai Herbal Medicine: *Cassia alata* Linn**. Bangkok: E.T.O. Press.

Cherdshewasart, W., Bhuntaku, P., Panriansaen, R., Dahlan, W., and Malaivijitnond, S. (2008). Androgen disruption and toxicity tests of *Butea superba* Roxb. a traditional herb used for treatment of erectile dysfunction, in male rats. **Maturatas**. 60: 131-137.

Clark, A. M. (1996). Natural products as a resource for new drugs. **Pharmaceutical Research**. 13: 1133-1141.

Colegate, S. M. and Molyneux, R. J. (1993). **Bioactive Natural Products**. Florida: CRC Press.

Cragg, G. M. (2002). Natural product drug discovery and development. **Puerto Rico Health Sciences Journal**. 21: 97-111.

Cragg, G. M. and Newman, D. J. (2001). Natural product drug discovery in the next millennium. **Pharmaceutical Biology**. 39: 8-17.

Cragg, G. M., Newman, D. J., and Snader, K. M. (1997). Natural products in drug discovery and development. **Journal of Natural Products**. 60: 52-60.

Dechatiwongse na Ayutthaya, T. (1997). Standardization and quality control of herbal medicines. In **International Symposium on the Standardization and Practice of Herbal Medicine**. (pp 1-16). Korea: Seoul National University.

Dixit, V. P., Agarwal, M., Bhargava S. K., Gupta R. S., and Jam G. C. (1981). Effect of *Butea Monosperma* seed extract fraction (Butin) on the testicular function of rats, dogs and presbytis monkey. **Iugosl Physiol Pharmacol Acta**. 17: 151-162.

Farnsworth, N. R., Akerele, O., Bingel, A. S., Soejarto, D. D., and Guo, Z. (1985). Medicinal plants in therapy. **Bulletin of the World Health Organization**. 63: 965-981.

Gad, S. C. (2005). **Drug Discovery Handbook**. New York: John Wiley and Sons.

Gupta, S. R., Ravindranath, B., and Seahadri, T. R. (1970). The glucosides of *Butea Monosperma-D*. **Phytochemistry**. 9: 2231-2235.

Hayadom, M. (1971). **Constituents of The Tuberous roots of *Pueranria mirifica***. M.S. thesis, Faculty of Science, Chulalongkorn University.

Hildebert, W., Bettina, G., Manfred, F., Yoshinobu, K., and Hiroshi, H. (1986). Isobutrin and butrin, the antihepatotoxic principles of *Butea monosperma* flowers. **Planta Medica**. 52: 77-79.

Hughes, R. J., Croley, T. R., Metcalfe, C. D., and March, R. E. (2001). A tandem mass spectrometric study of selected characteristic flavonoids. **International Journal of Mass Spectrometry**. 210(211): 371-385.

Ingham, J. L., Tahara, S., and Dziedzic, S. Z. (1986). Chemical investigation of *Pueraria mirifica* roots. **Journal of Biosciences**. 41: 403-408.

Ingham, J. L., Tahara, S., and Dziedzic, S. Z. (1988). Coumestans from the roots of *Pueraria mirifica*. **Journal of Biosciences**. 43: 5-10.

Ingham, J. L., Tahara, S., and Dziedzic, S. Z. (1989). Minor isoflavones from the roots of *Pueraria mirifica*. **Journal of Biosciences**. 44: 724-726.

Khnau, U. J. (1976). The flavonoids: a class of semi-essential food components their role in human. **World Review of Nutrition and Dietetics**. 24:117-191

Lambert, B., Leyns, F., Rooyen, L. V., Gosselé, F., Papon, Y., and Swings, J. (1987). Rhizobacteria of maize and. their antifungal activities. **Applied and**

Environmental Microbiology. 53(8): 1866-1871.

Lou, Z. C. (1993). Good manufacturing practices for pharmaceutical products, supplementary guidelines for the manufacture of herbal medicinal products. **WHO/Pharm/93.561/review1**. (pp 5-118). Geneva.

Malaivijitnond, S., Ketsuwan, A., Watanabe, G., Taya, K., and Cherdshewasart, W. (2009). Androgenic activity of the Thai traditional male potency herb, *Butea superba* Roxb. in female rat. **Journal of Ethnopharmacology**. 121: 123-129.

Malhotra, S. and Pal Singh, A. (2002). Hepatoprotective potential of phytomedicnes. **Gastroenterology**. 6: 76-77.

Mann, J. (1992). **Murder, Magic, and Medicine**. Oxford: Oxford University Press.

Manosroi, A., Sanphet, K., Saowakon, S., Aritajat, S., and Manosroi, J. (2006). Effects of *Butea superba* Roxb. on reproductive systems of rats. **Fitoterapia**. 77: 435-438.

Maria, C. B., Anna, R. B., Lorenzo, D. B., Giovanni, M., and Franco, F. V. (2007). Studies on the interactions between some flavonols and cyclodextrins. **Bioorganic and Medicinal Chemistry**. 17: 5744-5748.

Newman, D. J., Cragg, G. M., and Snader, K. M. (2000). The Influence of natural products upon drug discovery. **Natural Products Reports**. 17: 215-234.

Ngamrojanavanich, N., Loontaisong, A., Pengpreecha, S., Cherdshewasart, W., Pornpakakul, S., Pudhom, K., Roengsumran, S., and Petsom, A. (2007). Cytotoxic comstituents from *Butea superba* Roxb. **Journal of Ethnophamacology**. 109: 354-358.

NIST Standard Reference Database Number 69. (2011). **Chemistry** [On-line]. Available: http://webbook.nist.gov/chemistry/.

Phillipson, J. D. (2001). Phytochemistry and medicinal plants. **Phytochemistry**. 56: 237-243.

Pothisiri, P. (1996). **Medicinal use of botanicals an overview in Thailand food and drug administration**. Bangkok: E.T.O. Press.

Proestos, C., Sereli, D., and Komaitis, M. (2006). Determination of phenolic compounds in aromatic plants by RP-HPLC and GC-MS. **Food Chemistry**. 95: 44-52.

Roengsumran, S., Petsom, A., Ngamrojanavanich, N., Rugsilp, T., Sittiwicheanwong, P., Khorphueng, P., Cherdshewasart, W., and Chaichantipyuth, C. (2000). Flavonoid and flavonoid glycoside from *Butea superba* Roxb. **Journal of Scientific Research Chulalongkorn University**. 25: 169-176.

Suntara, A. (1931). **The Remedy Pamphlet of Kwao Krua Tuber of Luanganusarn-suntarakromkarnphiset**. Chiang Mai: Upatipongsa Press. pp. 1-11.

Sutjit, W. (2003). **The Antioxidant Test, Ames' Test and Micronuclei Test of Chemical Exteact from White Kwao Krua (*Pueraria mirifica*), Red Kwao Krua (*Butea superb* Roxb.), Black Kwao Krua (*Mucuna collettii*) and Kudza (*Pueraria labata*)**. M.S. thesis, Chulalongkorn University.

Sawasdimongkol, K. (1995). **Medicinal Plants and Thai Traditional Medicine**. Department of Medical Sciences: Ministry of Public Health, Thailand.

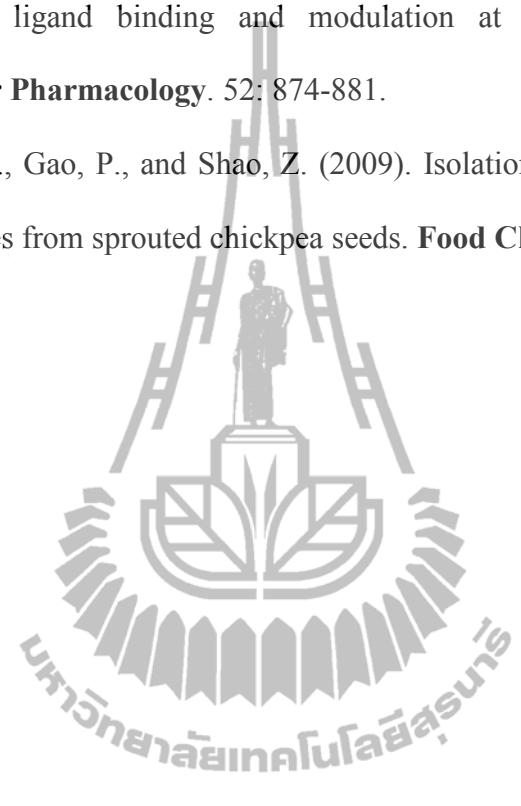
Tahara, S., Ingham J. L., and Dziedzic, S. Z. (1987). Structure elucidation of Kwakhurin, a new crenulated isoflavone from *Pueraria mirifica* roots. **Journal of Biosciences**. 42: 510-518.

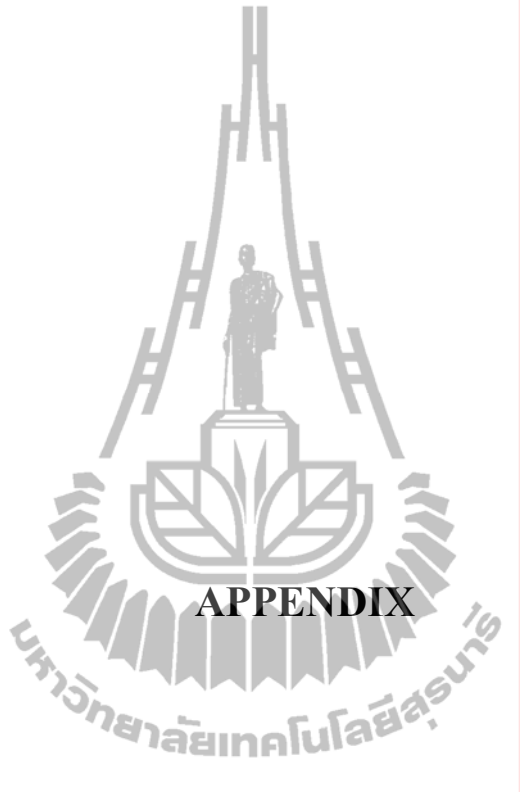
Talukda, A. C., Jain, N., De, S., and Krishnamurty, H. G. (2000). An isoflavone from *Myristica malabarica*. **Phytochemistry**. 53: 155-157.

Wade, L. G. (1987). **Organic Chemistry**. New Jersey: Prentice-Hall.

Wingrove, P. B., Thompson, S. A., Wafford, K. A., and Whiting, P. J. (1981). Key amino acids in the γ-subunit of the γ-aminobutyric acid receptor that determine ligand binding and modulation at the benzodiazepine site. **Molecular Pharmacology**. 52: 874-881.

Zhao, S., Zhang, L., Gao, P., and Shao, Z. (2009). Isolation and characterisisation of isoflavones from sprouted chickpea seeds. **Food Chemistry**. 114: 869-873.





APPENDIX

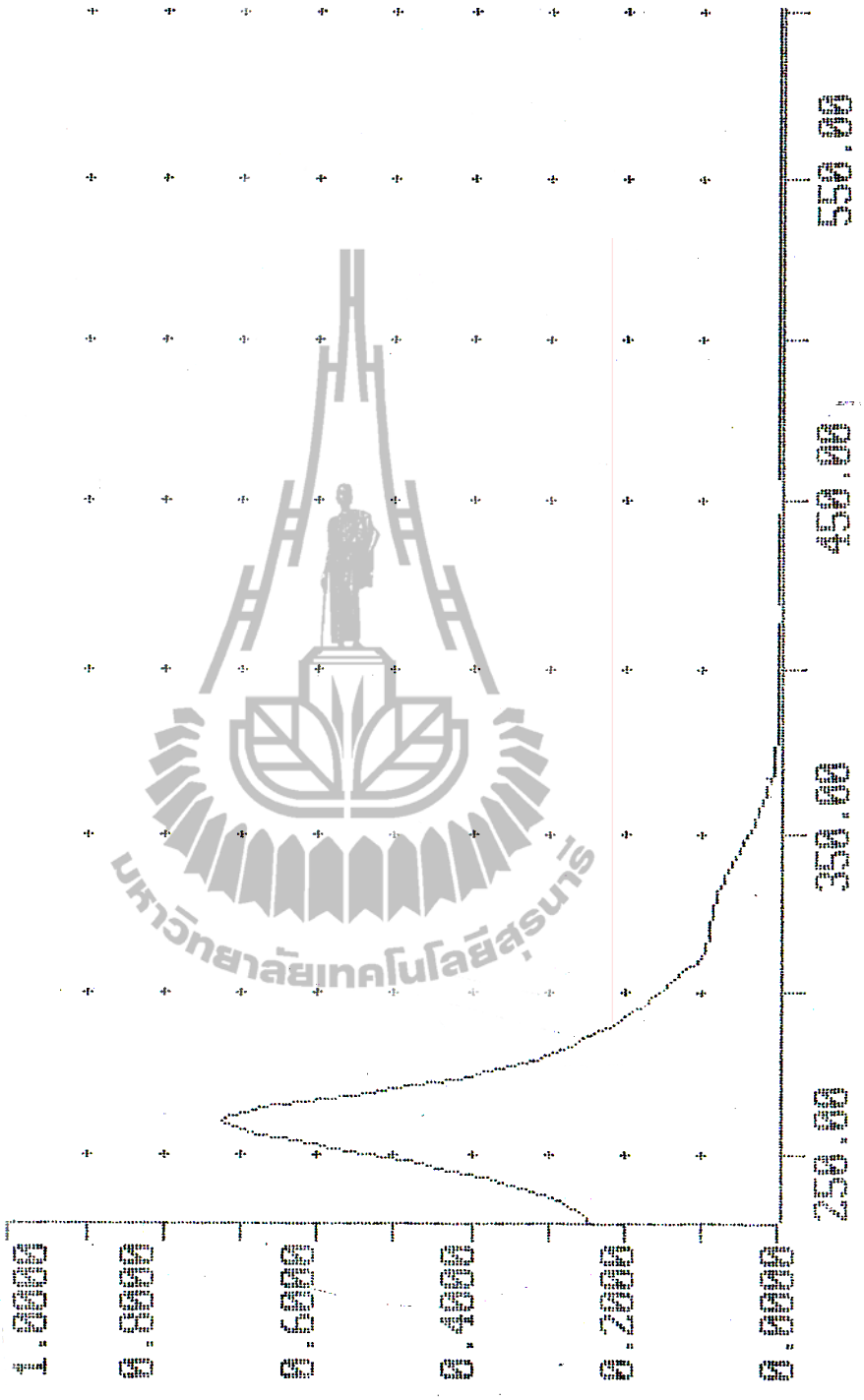


Figure A.1 UV-Vis spectrum of compound 1.

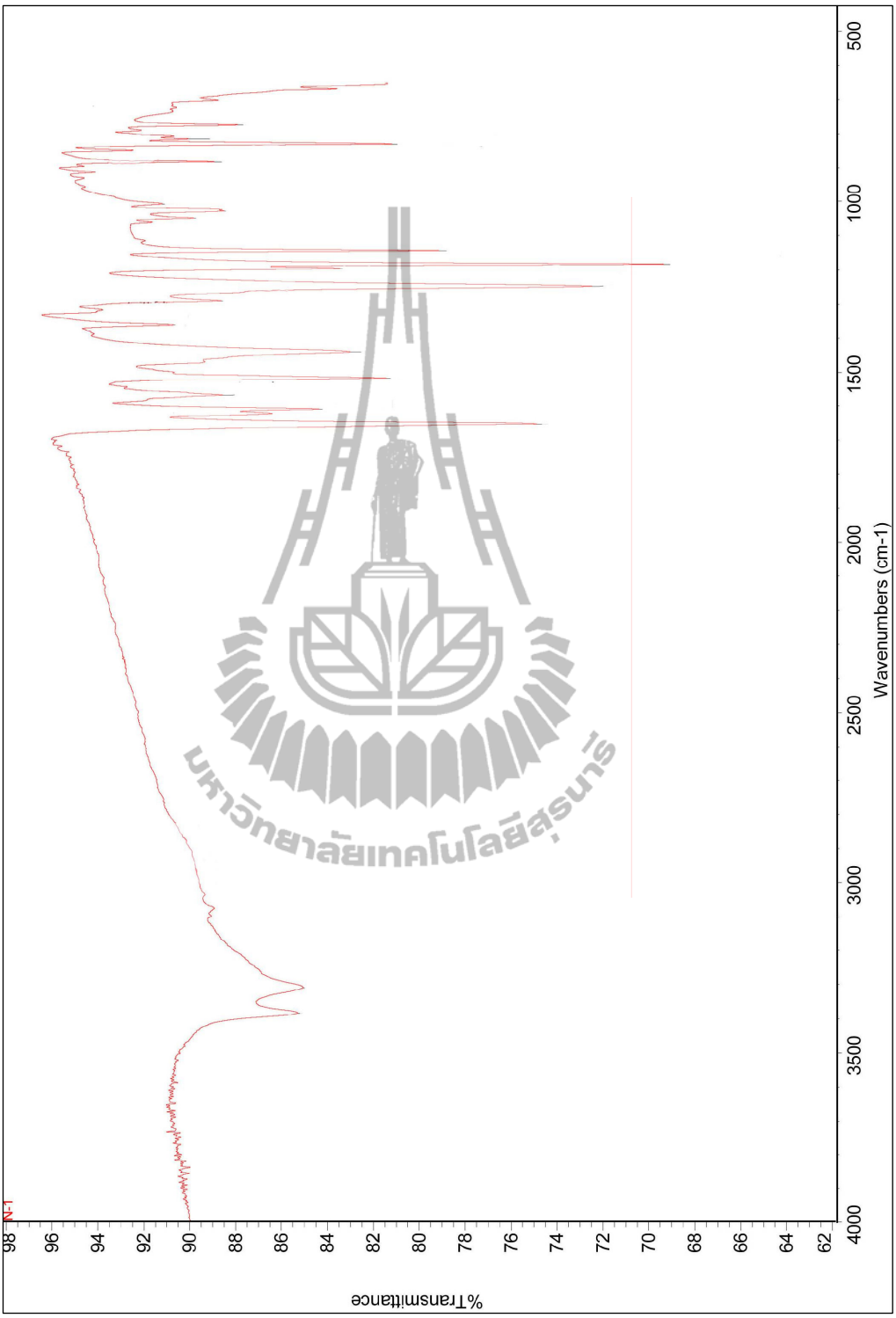


Figure A.2 IR spectrum of compound 1.

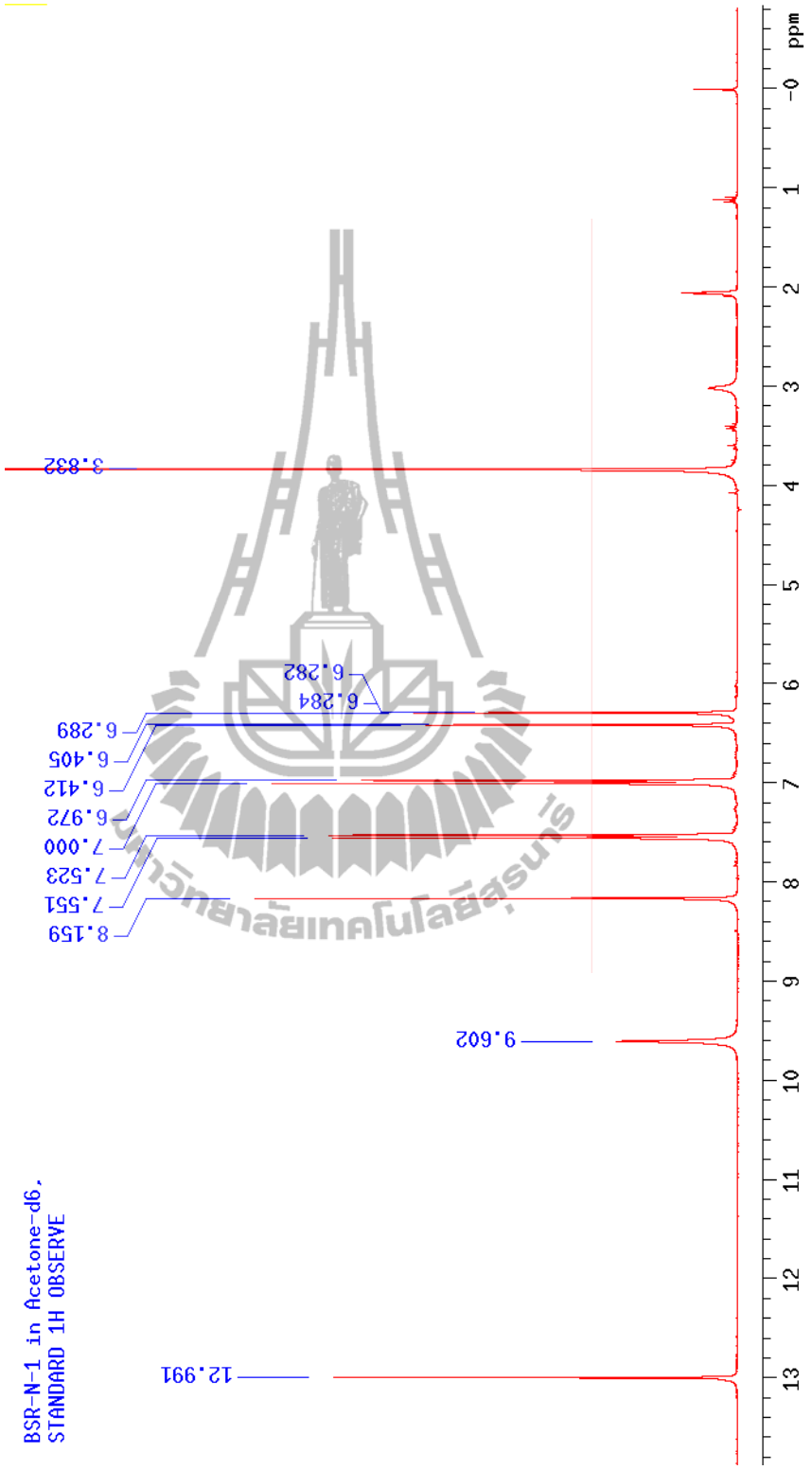


Figure A.3 ^{1}H-NMR spectrum of compound 1.

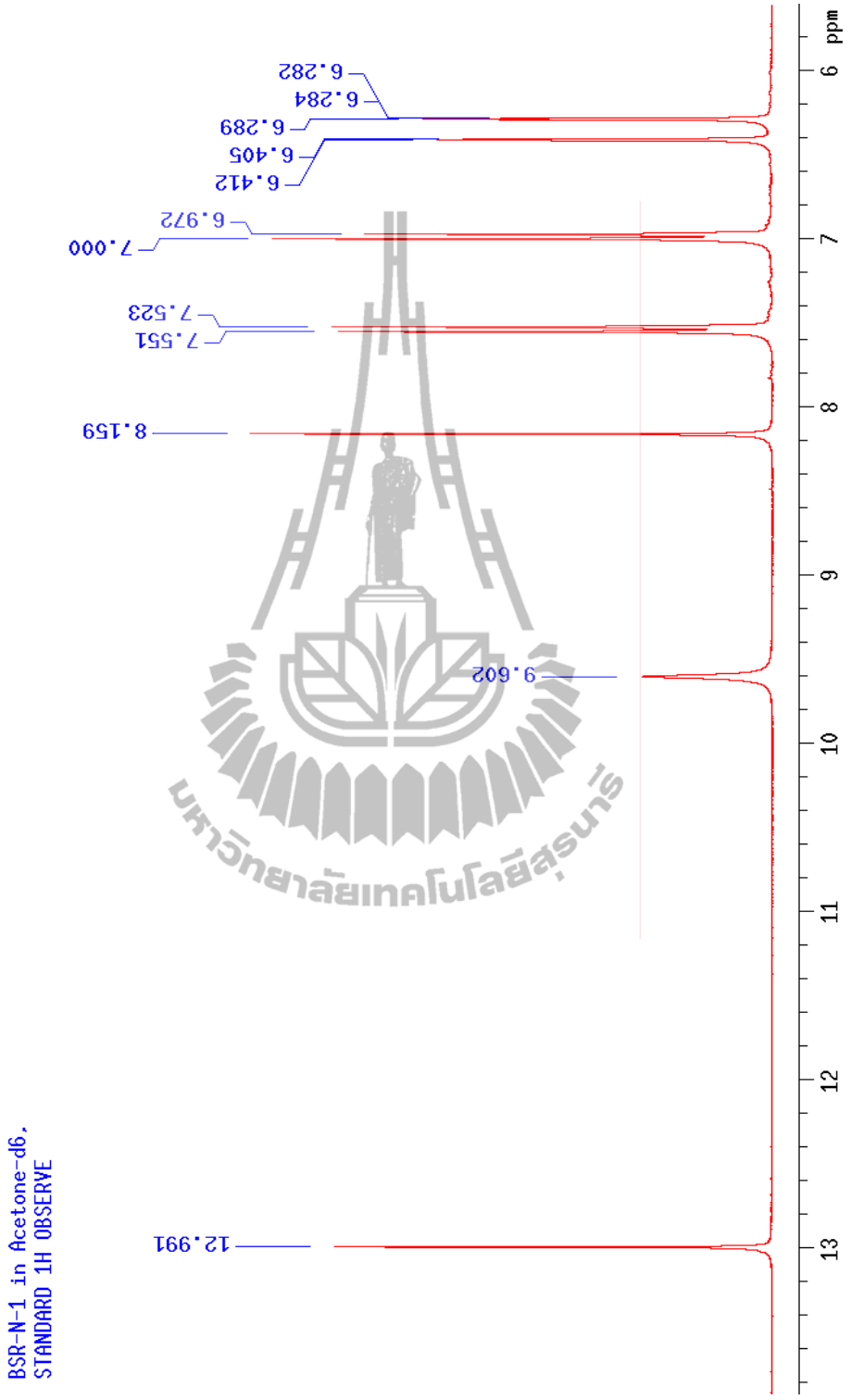


Figure A.3a expansion of figure A.3.

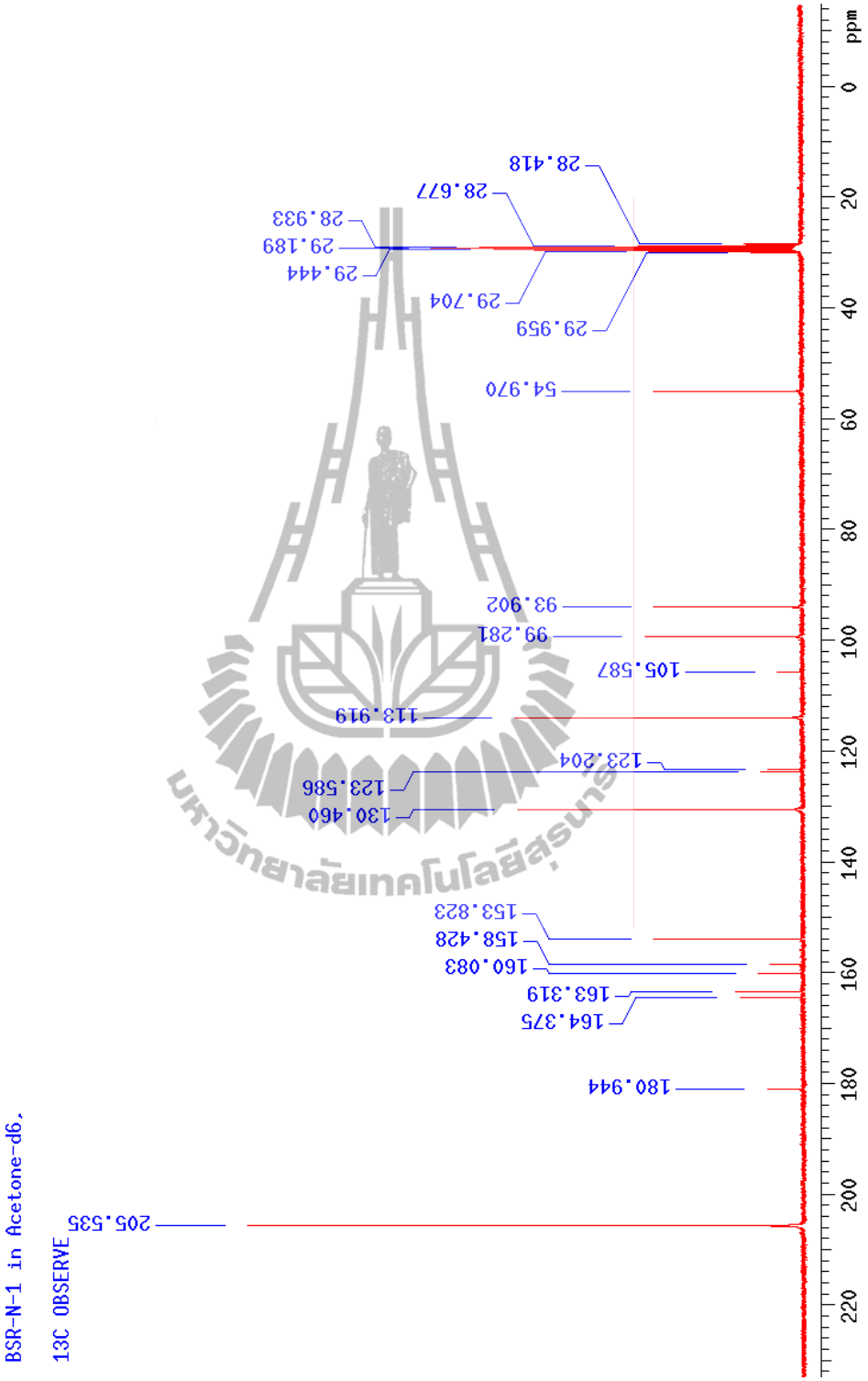


Figure A.4 ^{13}C-NMR spectrum of compound 1.

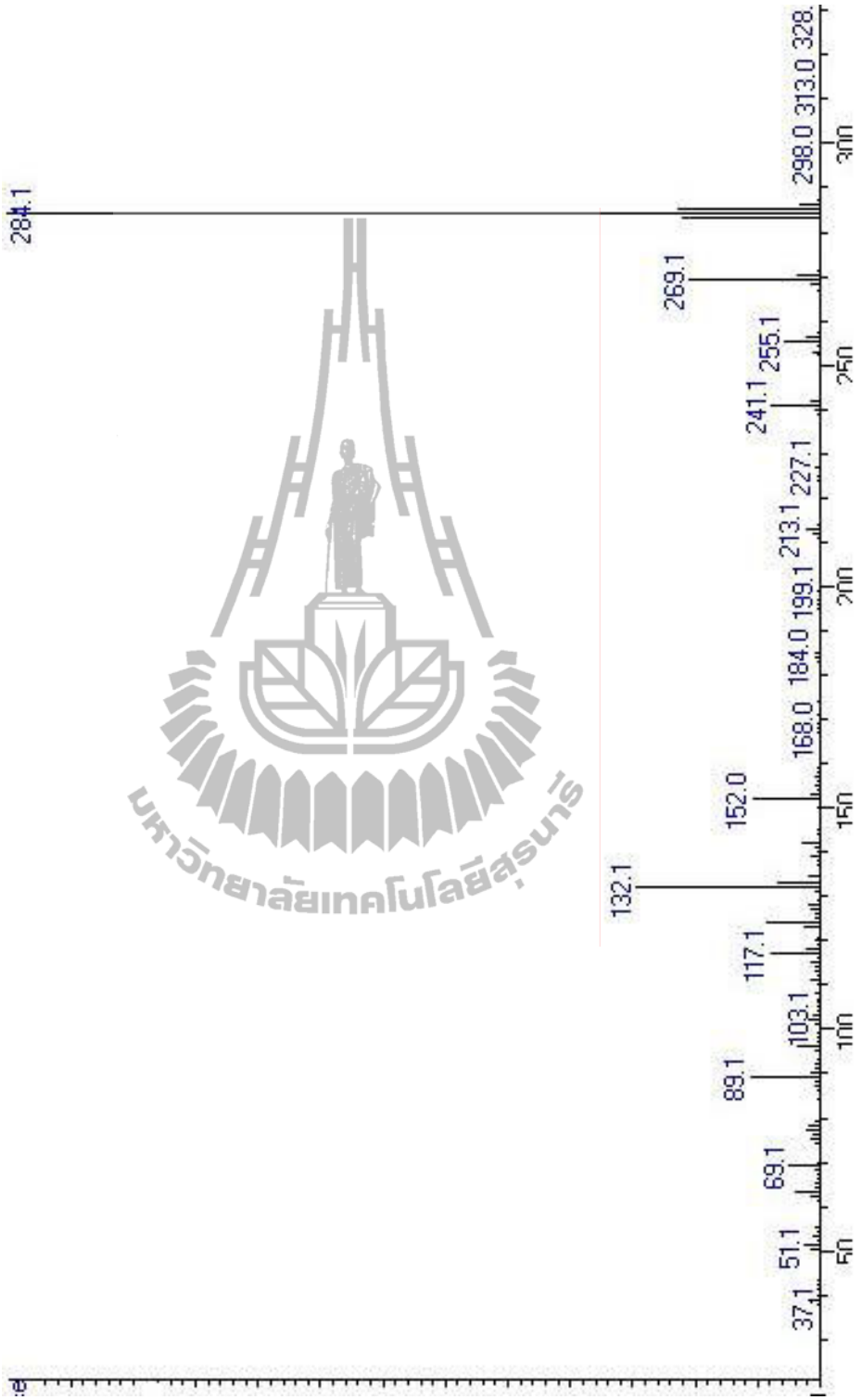


Figure A.5 mass spectrum of compound 1.

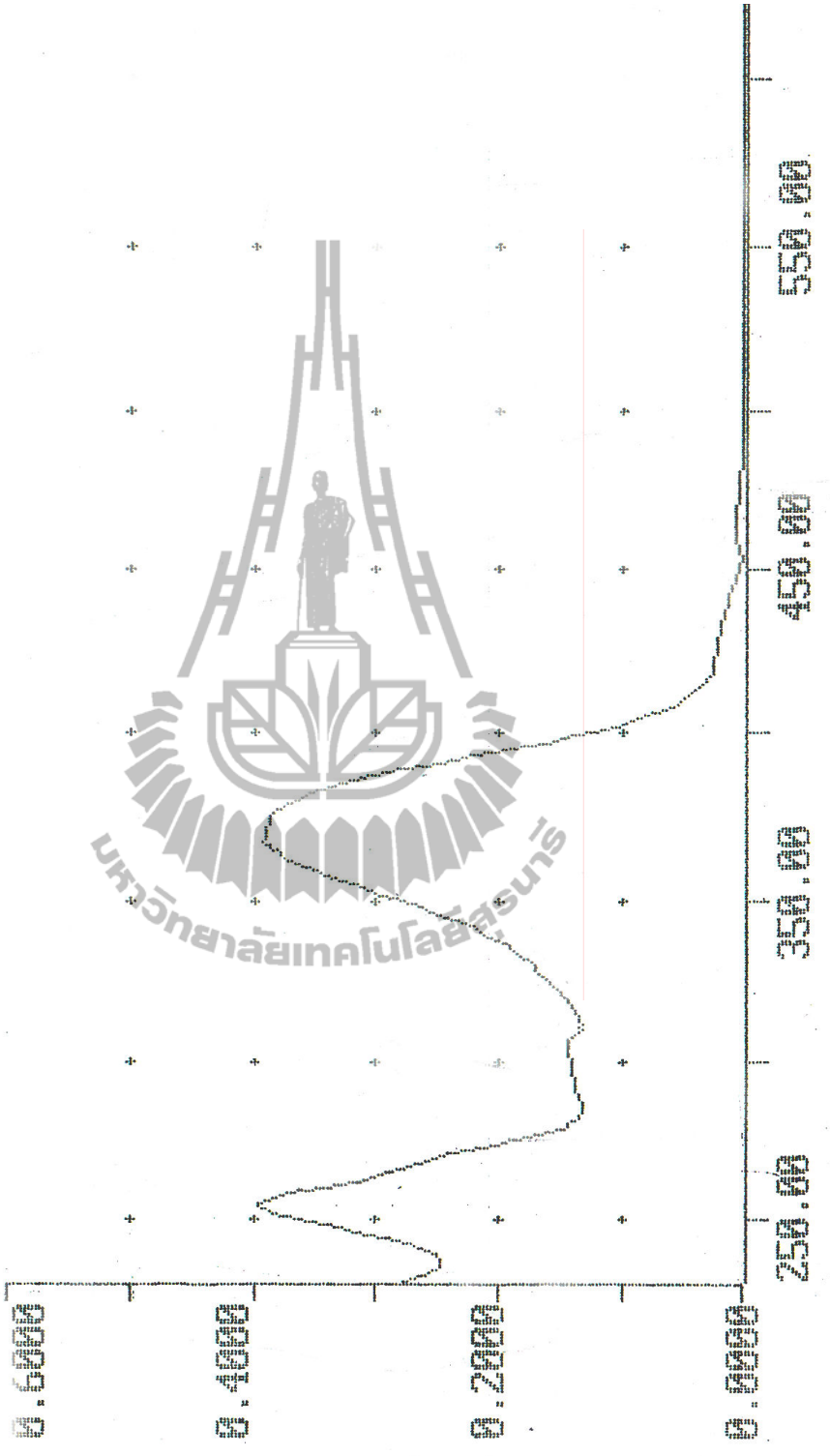


Figure A.6 UV-Vis spectrum of compound 2.

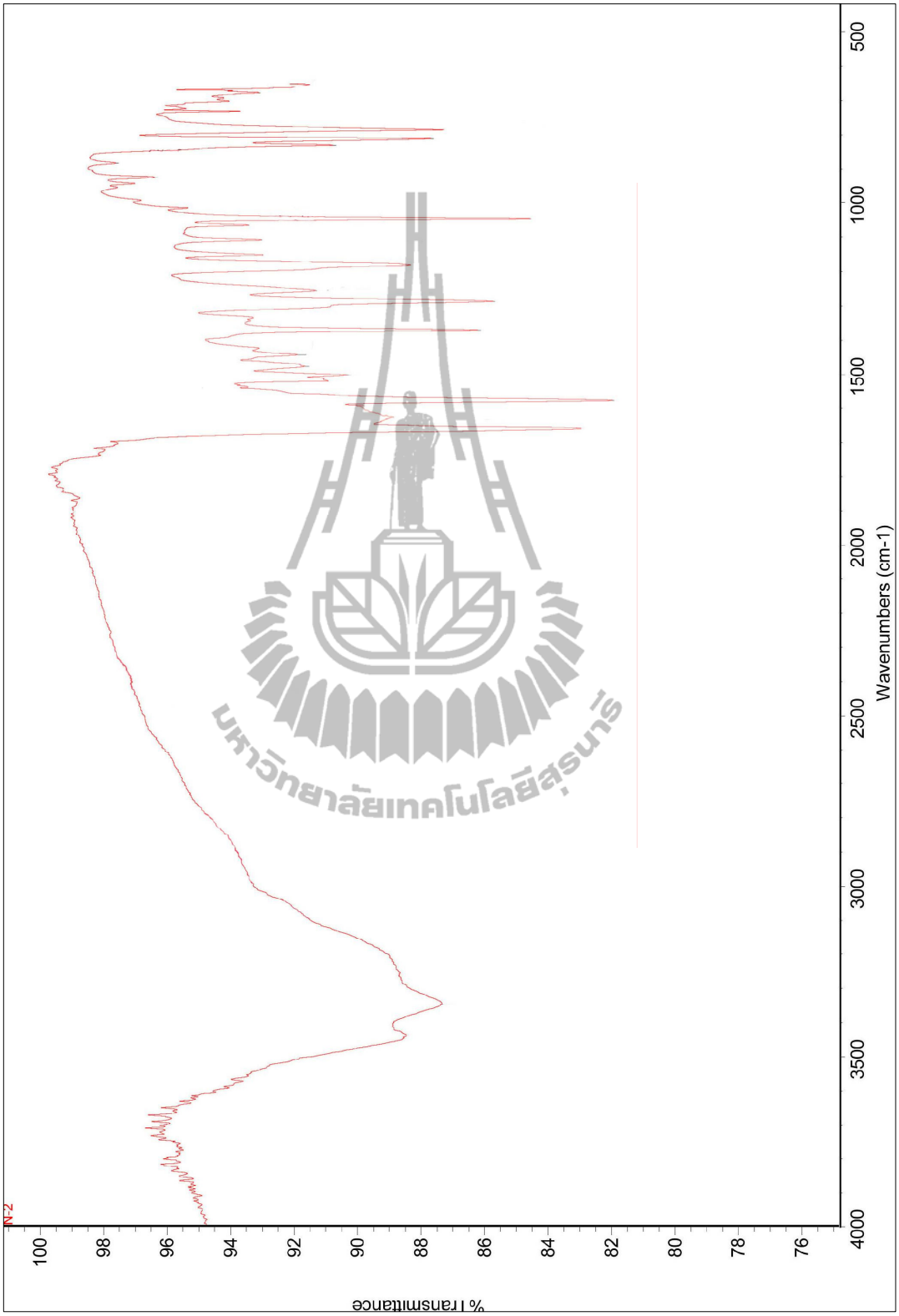


Figure A.7 IR spectrum of compound 2.

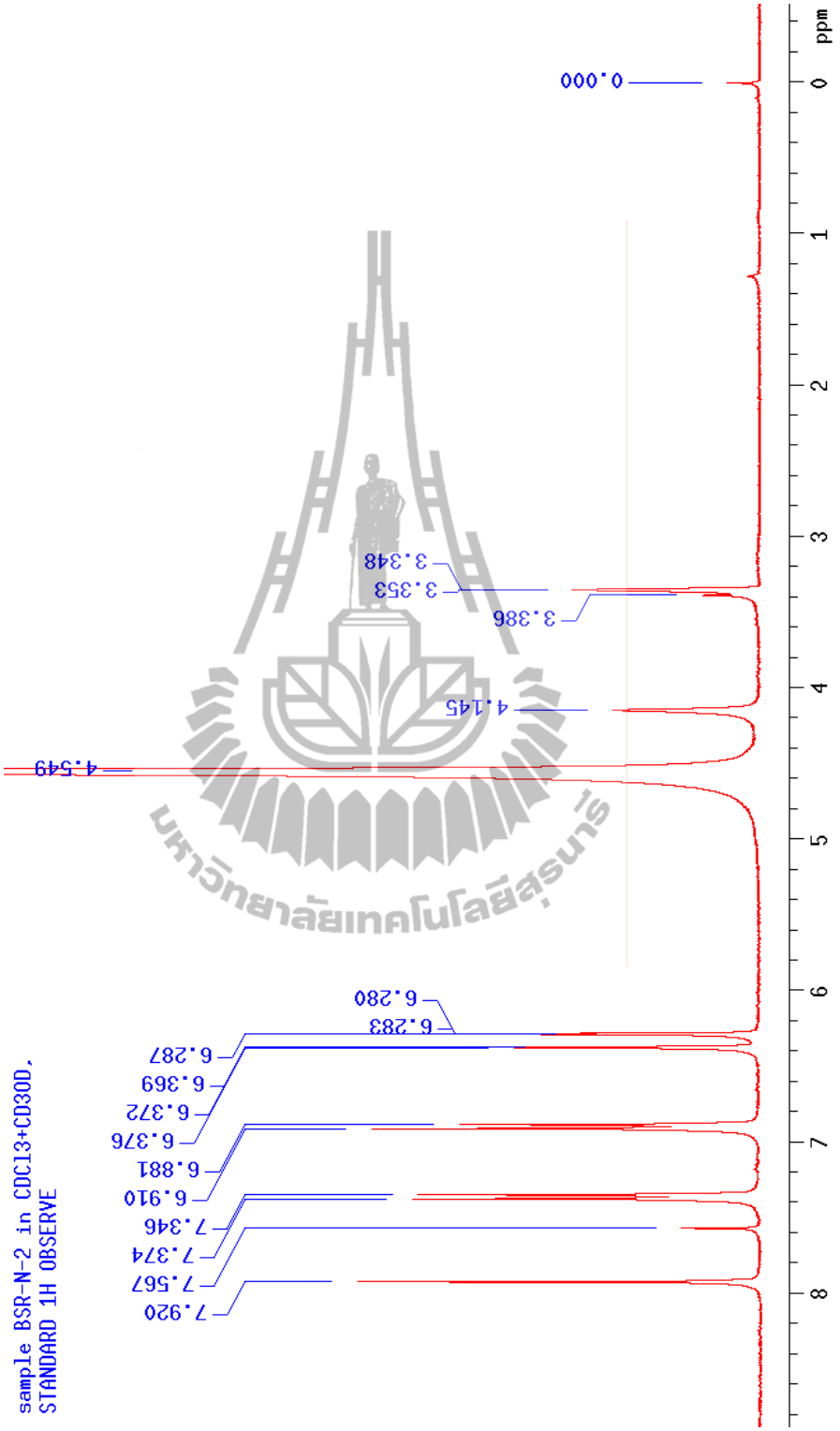


Figure A.8 ^{1}H-NMR spectrum of compound 2.

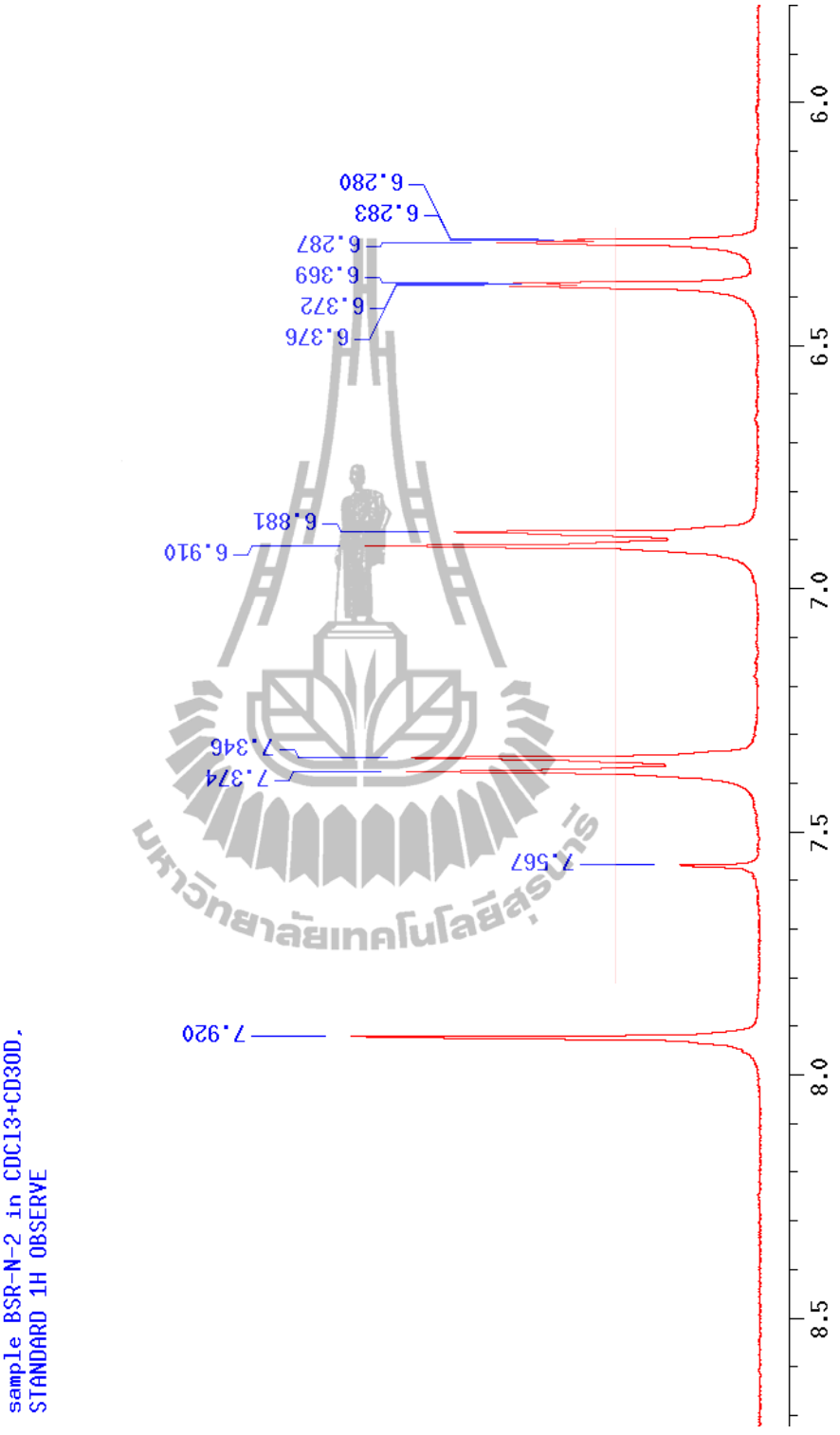


Figure A.8a expansion of figure A.8.

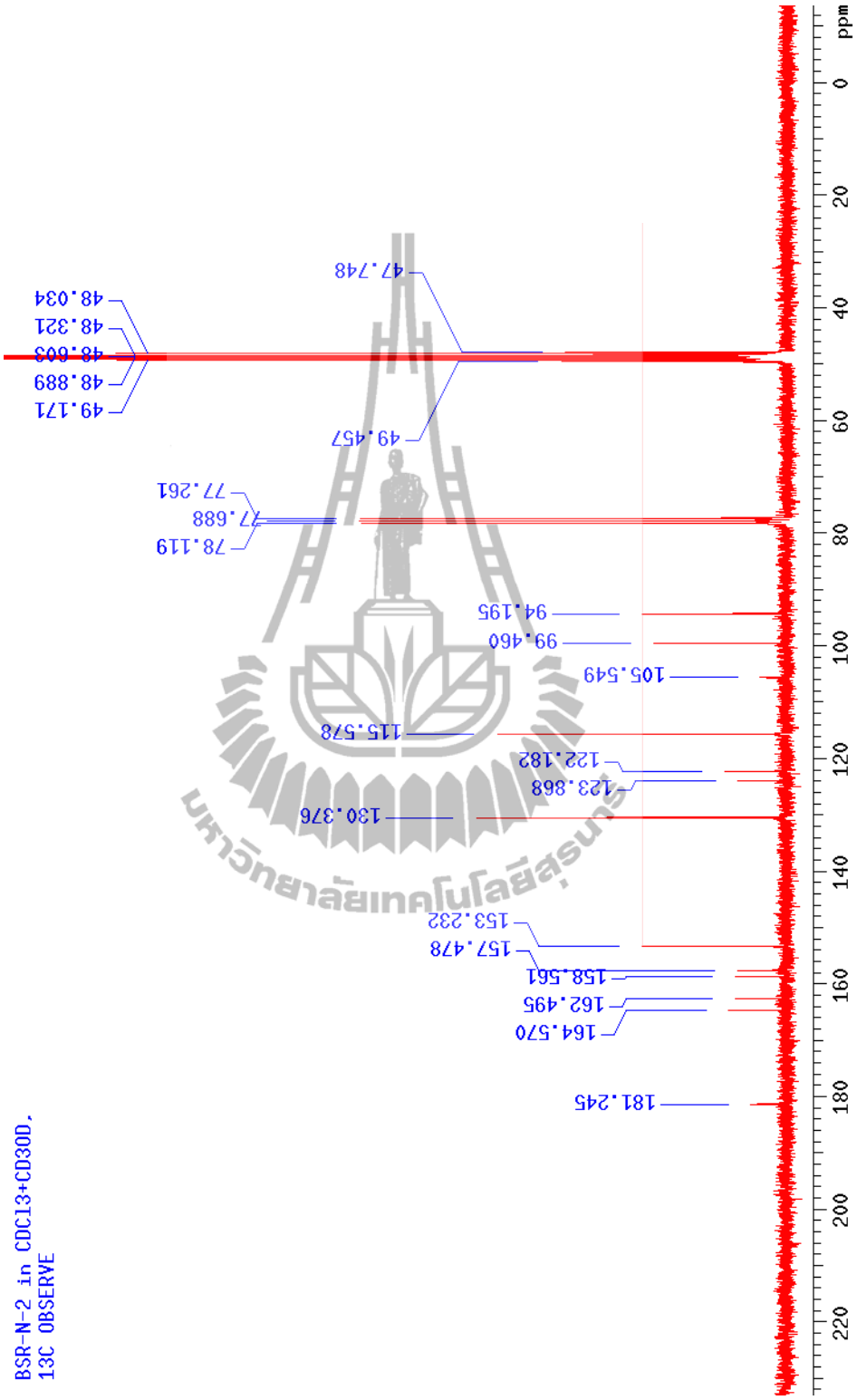


Figure A.9 ^{13}C-NMR Spectrum of compound 2.

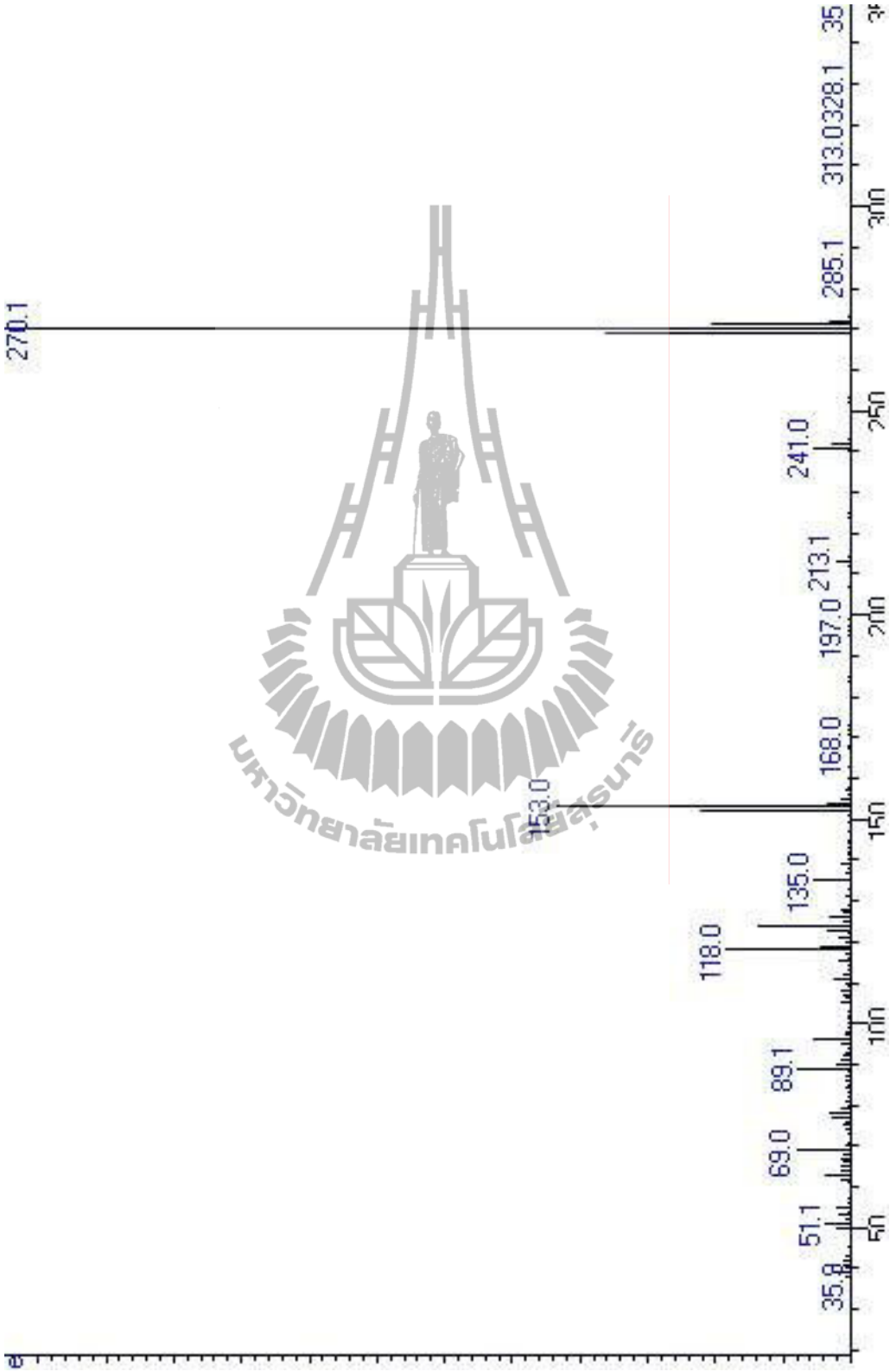


Figure A.10 mass spectrum of compound 2.

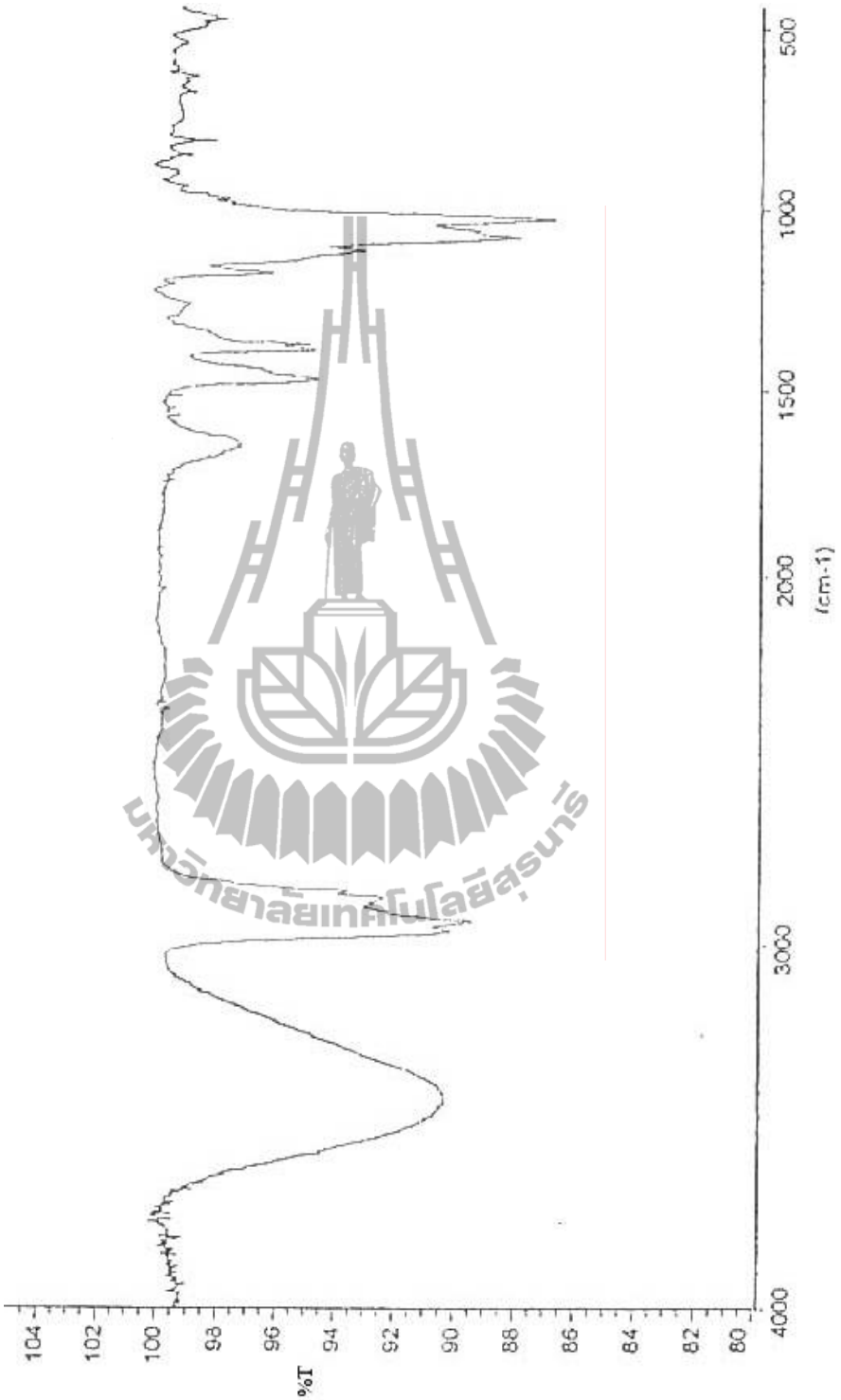


Figure A.11 IR spectrum of compound 3.

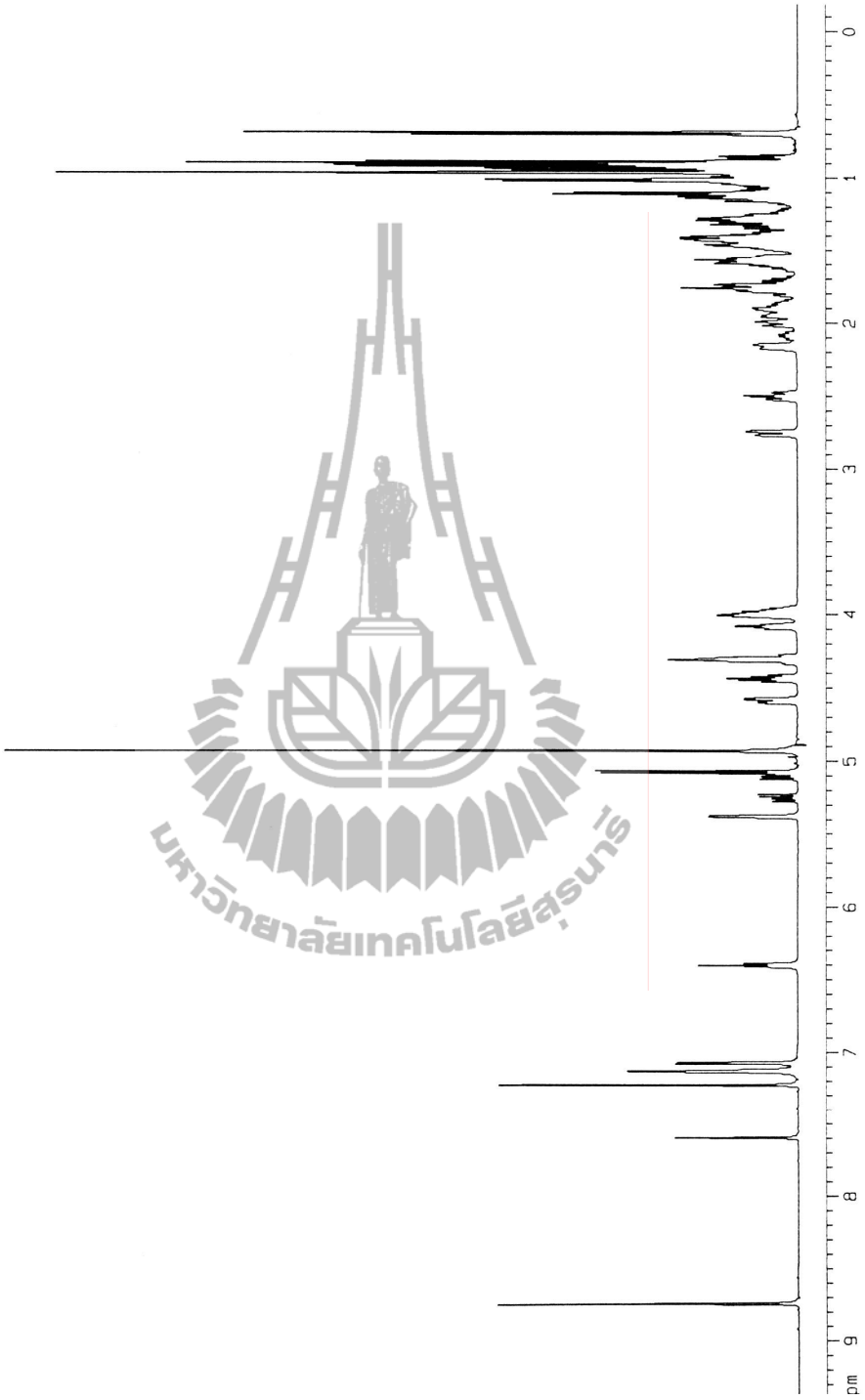


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Figure A.12 ^{1}H-NMR Spectrum of compound 3.

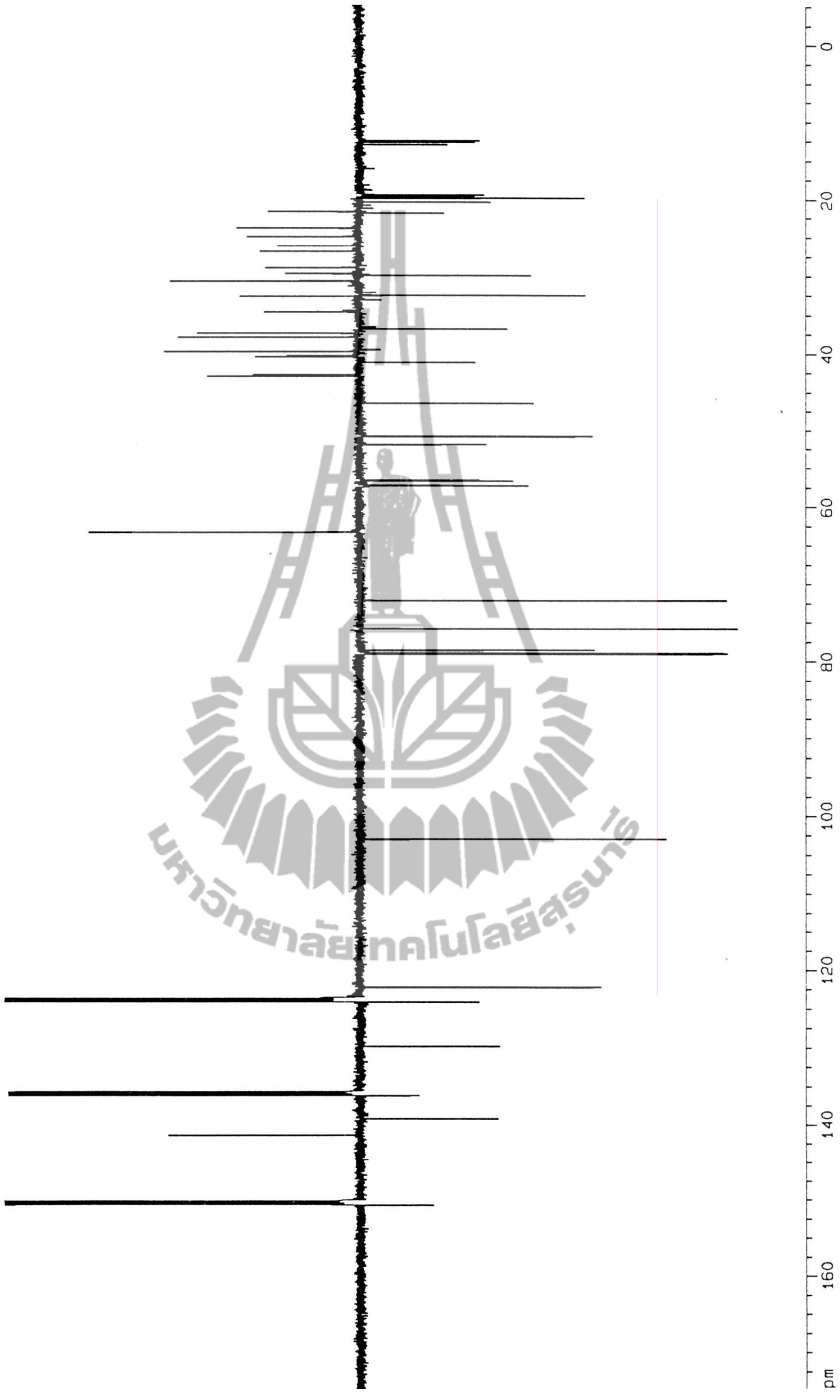

Figure A.13 DEPT ^{13}C-NMR spectrum of compound 3.

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