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CHEMICAL COMPOSITION INVESTIGATION OF THE *CLINACANTHUS NUTANS* (BURM. F.) LINDAU LEAVES

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CHEMICAL COMPOSITION INVESTIGATION OF THE *CLINACANTHUS NUTANS* (BURM. F.) LINDAU LEAVES

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ใบพญายอ ใช้เป็นยาพื้นบ้านที่นิยมใช้กันตั้งแต่อดีตจนถึงปัจจุบัน มีสรรพคุณในการรักษาการอักเสบอันเนื่องมาจากพิษสัตว์แมลงกัดต่อย โรคเริม โรคงูสวัด และอาการแพ้ต่างๆ เมื่อนำส่วนสกัดด้วยคลอโรฟอร์มของใบพญายอมาแยก ด้วยวิธีคอลัมน์โครมาโตกราฟี ได้สารบริสุทธิ์ 3 สาร การวิเคราะห์โครงสร้างของสารทั้งหมดที่แยกได้ใช้ข้อมูลทางสเปกโทรสโกปี (DEPT, COSY, NOESY, HSQC และ HMBC) ในการพิสูจน์โครงสร้างของสารทั้ง 3 สาร สารที่แยกได้ทั้ง 3 สารเป็นสารที่มีสูตรโครงสร้างคล้ายคลึงกับคลอโรฟิลล์ เอ และคลอโรฟิลล์ บี คือ 13^2–hydroxy–(13^2–*S*)–pheophytin b, purpurrin 18 phytyl ester และ pheophobide a ซึ่งสารเหล่านี้ยังไม่มีรายงานว่าพบในพืชชนิดนี้มาก่อน

สาขาวิชาเคมี ลายมือชื่อนักศึกษา_____

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CLINACANTHUS NUTANS/CHLOROPHYLL/PHEOPHYTIN/PHEOPHOBIDE

The leaves of *Clinacanthus nutans* have long been traditionally used in Thailand as an anti-inflammatory drug for the treatment of insect bites, herpes infection and allergic responses. The chloroform crude extract separated by column chromatography and further purified by preparative layer chromatography gave three pure compounds. Structures of isolated compounds were elucidated on the basis of spectral analysis, including DEPT, COSY, NOESY, HSQC and HMBC. Three isolated compounds were identified as chlorophyll a and chlorophyll b related, they are 13^2–hydroxy–(13^2–*S*)–pheophytin b, purpurin 18 phytyl ester, and pheophobide a, which are new compounds and not previously reported in this species.

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LIST OF ABBREVIATIONS

B_0	Static Magnetic Field
Br s	Broad singlet (for NMR spectra)
^oC	Degree Celcius
^{13}C NMR	Carbon-13 Nuclear Magnetic Resonance
COSY	Correlation Spectroscopy
d	Doublet (for NMR spectra)
dd	Doublet of Doublets (for NMR spectra)
DEPT	Distortionless Enhancement by Polarization Transfer
FID	Free Induction Decay
^1H-NMR	Proton Nuclear Magnetic Resonance
Hz	Hertz
HMBC	Multiple-Bond Heteronuclear Multiple-Quantum Coherence
HMQC	Heteronuclear Multiple Quantum Coherence
HSQC	Heteronuclear Single Quantum Coherence
IUPAC	International Union of Pure and Applied Chemistry
J	Scalar Coupling
mL	Millilitre
NOESY	NOE Spectroscopy
q	Quartet (for NMR spectra)
R_f	Retention Factor in Chromatography
s	Singlet (for NMR spectra)

LIST OF ABBREVIATIONS (Continued)

t	Triplet (for NMR spectra)
T_1	spin-lattice relaxation time
TLC	Thin Layer Chromatography
1-D	One-Dimensional
2-D	Two-Dimensional
3-D	Three-Dimensional
δ	Chemical Shift (ppm)

CHAPTER I

INTRODUCTION

1.1 Natural Products Isolation

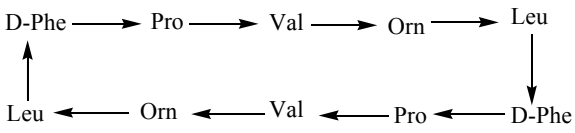
Marketed plants are very important items of trade in many parts of the world. The community uses these for a variety of purposes such as food, cosmetics, flavors, spices, and medicines. It seems that plants that are used for medicinal purposes form the most common category.

For at least five thousand years humankind has relied on natural products as the primary source for medicines. Herbs, bread mold, even leeches were employed to bring relief to the sick and infirm. There was little significant change over much of this time period; however, the last two centuries have brought an explosion of understanding of how these natural products are produced and how they interact with other organisms. The last two centuries have seen the isolation of the first commercial drug (morphine), the use of microbial products as medicines (penicillin), and even a use for the lowly leech (the anticoagulant, hirudin) (Newman *et al*., 2000). Who knows what the next two centuries will bring us. Now, at the start of a new millennium, it is estimated by the World Health Organization (Farnsworth *et al*., 1985). that 80% of the world's inhabitants must rely on traditional medicines for health care; these traditional medicines are primarily plant-based. Even in the remaining population, natural products are important in health care. It is estimated that 25% of all prescriptions dispensed in the USA contain a plant extract or active

ingredients derived from plants. It is also estimated that 74% of the 119 currently most important drugs contain active ingredients from plants used in traditional medicine. Another study of the most prescribed drugs in the USA indicated that a majority contained either a natural product, or a natural product was used in the synthesis or design of the drug. All of these estimations demonstrate the importance of natural products in drug discovery.

Until the 1970's, drug discovery was essentially based on serendipity. Rational drug discovery only began with the advent of molecular biology and computers. With the cost of drug development approaching $350 million (USA) per drug (Cragg *et al*., 1997), many discovery groups are debating "quantity" vs. "quality" strategies in an effort to reduce costs. The "quantity" approach is that of combinatorial chemistry, which can provide libraries of thousands of compounds in a short period of time. For example, this approach can provide thousands of analogs of the decapeptide gramicidin S1, Figure 1.1 (1), for testing, a task impossible for natural product isolation. However, these libraries tend to lack novelty and are usually based on natural product targets anyway. One "quality" approach is natural product screening and isolation, which provides fewer compounds over a longer period of time. Additionally, this strategy can lead to unforeseen novel molecular structures that combinatorial chemistry would be unlikely to reveal. The unique peroxy-bond of artemisinin (2), the ring system of paclitaxel (Taxol) (3), and the stereochemistry of erythromycin A (4) are synthetically challenging and are unlikely to have been discovered through a combinatorial approach. Many pharmaceutical companies are now pursuing both combinatorial and isolation strategies. Only time will tell which strategy will predominate, but to paraphrase a respected natural product chemist,

perhaps Nature is the world's best combinatorial chemist.



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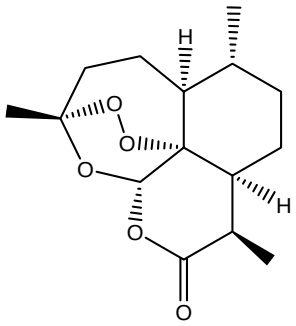

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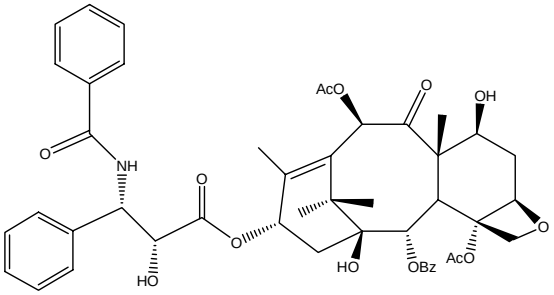

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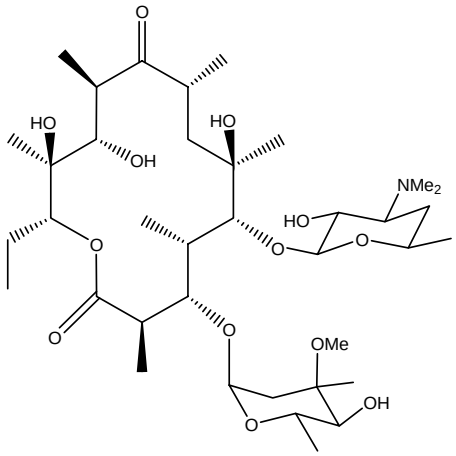

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Figure 1.1. Natural products.

Medicinal herbs are a significant source of synthetic and herbal drugs. In the commercial market, medicinal herbs are used as raw drugs, extracts, or tinctures. Isolated active constituents are used for applied research. For the last few decades, phytochemistry (study of plants) has been making rapid progress and herbal products are becoming popular. There has been dramatic rise in the sale of herbal products like *Allium sativum*, *Hypericum perforatum*, *Spirulina*, *Echinacea angustifolia*, *Ginkgo*

biloba, and *Silybum marianum*.

Owing to growing demand for herbals, the current need is to intensify research in the field of medicinal herbs and to get authentic information on the subject. Herbal products are often questioned for quality control and assurance. The majority of the herbal products fail in laboratory tests for the active constituents mentioned on the label. Extracts standardised to active constituents and marker compounds have definite advantage over the crude drugs (Pal Singh *et al*., 2002).

Clinacanthus nutans (Burm. f.) Lindau belongs to the family of *Acanthaceae.* This family includes many species, which are known to have medical properties. In Thailand, many Thai medicinal plants have been collected, including medicinal plants of the family *Acanthaceae* including: *Andrographis paniculata* (Burm. f.) Nees, *Acanthus ebrateatus* Vahl, *Barleria lupulina* Lidl, *Clinacanthus nutans* (Burm. f.) Lindau, *Justicia betonica* Linn, *Rhinacanthus nasutus* Linn, *Ruellia tuberosa* Linn, and *Thunbergia laurifolia* Linn (Hancharnlerd *et al*., 1994) (Table 1.1).

Table 1.1. Medicinal Plants of the Family *Acanthaceae* in Thailand and Their Traditional Use.

Plant Species	Characteristic	Seasonal Blooming	Traditional Use
1. *Acanthusebra teatus* Vahl	Shrub	Rainy season	All stem and seeds: healing of wounds, worm protection, fresh leaves for healthy hair

Table 1.1. (Continued).

Plant Species	Characteristic	Seasonal Blooming	Traditional Use
2. *Andrographis paniculata* (Burm. f.) Nees	Perennial shrub	All year	Leaves and stems: healing sore throat and diarrhea
3. *Barleria lupulina* Lindl.	Shrub	Rainy season	Blended leaves: healing inflammation after insect bites
4. *Clinacanthus nutans* (Burm. f.) Lindau	Shrub	In January	Fresh leaves: healing burns and scalds
5. *Justicia betonica* Linn	Shrub	All year	No information
6. *Rhinacanthus nasutus* Linn.	Small Shrub	In January	Leaves and roots: healing skin diseases Roots are antiseptic
7. *Ruellia tuberosa* Linn.	Perennial shrub (long life)	Rainy season	Roots: healing urinary tract inflammation and reducing toxicity
8. *Thunbergia laurifolia* Linn.	Shrub	Rainy season	Leaves: healing stomach ache

C. nutans has been traditionally used in Thailand for the treatment of herpes infections. Laboratory investigations in Thailand have indicated that the extract of this herb exhibits anti-viral properties against the herpes simplex virus (HSV) (Jayavasu *et al*., 1992, Thawaranantha *et al*., 1992) and varicella zoster virus (VZV) (Jayavasu *et al*., 1992). *C. nutans* cream was later clinically shown to be as efficacious as acyclovir in relieving pain, and healing herpes simplex and herpes zoster without causing a burning sensation, the side effect experienced by some patients using acyclovir (Jayavasu *et al*., 1992, Sangkitpporn *et al*., 1993, Sangkitpporn *et al*., 1993, Charuwichitratana *et al*., 1996, Jayavasu *et al*., 1998).

Typical *C. nutans* products are currently being used in many Thai hospitals to replace topical acyclovir in treating herpes simplex and herpes zoster. In addition, this herb exhibits an excellent and rapid acting, anti-inflammatory property (Chuakul, 1986, Tanasomwong, 1986, Satayavivad *et al*., 1996), making a *C. nutans* topical cream or lotion a good natural product for the relief of minor skin inflammation and insect bites (Satayavivad *et al*., 1996).

C. nutans has been considered as one of five medicinal plants in the National List of Essential Drugs A.D. 1999. It has been promoted for treatment of herpes simplex, herpes zoster, and skin pruritis in the Primary Health Care Programme (Minitry of Public Health, 1999).

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

1.2 Specification of *Clinacanthus nutans*

Scientific Name: *Clinacanthus nutans* (Burm. f.) Lindau.

Family: Acanthaceae.

Common Name: Slaed Pang Pon (Tua mia), Phayaa Yo, Phak Man Kai, Phak Lin Khiat (Chiang Mai), Phayaa Plong Thong, Phayaa Plong Kham (Lampang), Pho-so-chaang (Karen-mae Hong Sorn) (สมิตินันทน์, 2544).

Botanical Description: This plant is a shrub 1-3 m high with pubescent branches. Leaves are simple, opposite, narrowly elliptic oblong or lanceolate, 2.5-13 cm long, and 0.5-1.5 cm wide. The leaves have apex acute or acuminate; margin exsculptate-dentate or subentire; base cuneate, obtuse, rounded or truncate often oblique; pubescence on the nerves; petiole 3-15 mm long. Flowers are in dense cymes at the top of the branches and their branchlets; cymes 5-α flowered, often terminating drooping horizontal branches but themselves erect, subsecund, and combined into a large lax, leafy panicle. Each flower has calyx densely patently glandular-pubescent, about 1 cm long; corolla glandular-pubescent, about 3.5 cm, dull red with green base; lower lip (turned upwards) with yellow streaks, apically sordidly yellow or greenish yellow; stamens 2, inserted in the throat, more or less appressed against the upper lip. Ovary is compressed, 2-celled, 2 ovules in each cell; having style filiform, shortly bidentate. Capsule is oblong, basally contracted into a short, solid stalk 4-seeded (Backer *et al*., 1965, Perry, 1986). Its morphological features are illustrated in Figure 1.2 and Figure 1.3.

Ecology and Distribution: They are cultivated and found in deciduous forests.

Propagation: They are normally propagated by cuttings.

Loss on Drying: not more than 14%.

Total Ash: not more than 21%.

Acid-Insoluble Ash: not more than 4%.

Alcohol-Soluble Extract: not less than 27%.

Water-Soluble Extract: not less than 26%.

Ether-Soluble Extract: not less than 1%.

Ethnomedical Use: For the treatment of burn, herpes zoster and insect bite; antipyretic (Kharnngan, 1991).

Figure 1.2. Morphological features of twig, leave, and flower of *Clinacanthus nutans* (Burm. f.) Lindau.

Figure 1.3. Morphological illustration of *Clinacanthus nutans* (Burm. f.) Lindau.

Chemical Identification

1. Warm 0.5 g of the pulverized sample with 2 mL of acetic anhydride on a water bath for 2 minutes while shaking, then filter, and to the filtrate add carefully 1 mL of sulfuric acid to make two layers: a brownish red color develops at the zone of contact.

2. Boil 0.5 g of pulverized sample with 10 mL of water and filter. To 2 mL of the filtrate add 1 drop of ferric chloride TS: a brown precipitate is produced.

3. Boil 0.5 g of the pulverized sample with 10 mL of water for 2 minutes and filter. To 3 mL of the filtrate add 1 mL of Fehling's TS and warm the mixture a brick red precipitate is produced.

4. Boil 0.5 g of the pulverized sample with 10 mL of water for 2 minutes and filter. To 3 mL of the filtrate add a few drops of saturated lead acetate solution. Filter the solution when precipitation has completed. Add a few drops of lead subacetate TS until neutral or weakly alkaline: a yellow precipitate is produced.

1.3 Pharmacological Study

C. nutans has several pharmacological studies as exemplified below:

1.3.1 Activity Against Snake Venom

A water extract of leaves reduced mortality rate in snake venom treated mice from 100% to 63 ± 3.34%, while a 95% alcohol extract (2.0 g/kg) was ineffective when injected intravenously, intraperitoneally, or given orally (Thongharb *et al*., 1977). A water extract was also effective in counteracting snake venom when tested on rat phrenic nerve diaphram preparation (Cherdchu *et al*., 1977).

1.3.2 Antiinflammatory Activity

Leaf extracts, especially a butanol extract, reduced inflammation induced by carrageenin injection in rats. The active fraction was reported to be unstable. One of the active ingredients in *C. nutans* was identified as a flavonoid compound (Chuakul, 1986, Tanasomwong, 1986, Satayavivad *et al*., 1996).

1.3.3 Activity Against Herpes Simplex Virus (HSV)

In 1992 Jayavasu *et al*. and Thawaranantha *et al*. reported the ability of the ethanol extract of *C. nutans* leaves to inactivate HSV-2 compared with that of acyclovir. The activity was measured in vitro using a plaque reduction assay of HSV-2 in baby hamster kidney cell line. Based on 50 percent inhibited dose, the results showed the ethanol extract of *C. nutans* leaves inhibited plaque formation by HSV-2.

In 1992 Jayavasu *et al*. reported the clinical trial in the treatment of genital herpes patients with *C. nutans* extract, it showed that patients who used *C. nutans* as well as those who used acyclovir, could develop crust lesion within 3 days and healing within 7 days while the placebo group had crusting time in 4-7 days and healing time in 7-14 days or more. This meant that *C. nutans* and acyclovir have good efficacy in shortening the duration of infection and reducing severity. Besides, *C. nutans* has no sticky, burning, stinging pain and side effects. In conclusion, it is believed that *C. nutans* is suitable for the treatment of genital herpes patients.

In 1993 Sangkitporn *et al*. reported the results of treatment of recurrent Genital Herpes Simplex virus infection with *C. nutans* extract, the numbers of patients with lesion crusting within three days and with lesion crusting within seven days were significantly increased in the *C. nutans* extract-treated group and the acyclovir-treated

group compared with the placebo-treated group. There was no side effect of *C. nutans* during treatment. Mild, transient burning or pains associated with application of acyclovir were found in 21 male patients (24.7%) and in 18 female patients (23.1%).

1.3.4 Activity Against Varicella Zoster Virus (VZV)

In 1992 Jayavasu *et al*. reported the ability of the extract of *C. nutans* leaves to inactivate VZV. The activity was measured in vitro using a plaque reduction assay and DNA hybridization. Based on 50 percent inhibited dose, the results showed the extract of *C. nutans* leaves inhibited plaque formation by VZV. From the present findings, based on the result of inactivation assay, it was recognized that the in vitro antiviral activity of *C. nutans* might be a direct interaction of the extract with the virus.

In 1993 Sangkitporn *et al*. reported the results of treatment of herpes zoster with a topical formulation of 5 percent *C. nutans* extract. There was no difference of patient characteristics in the *C. nutans* extract-treated group and in the placebo group, but the numbers of patients with lesion crusting within three days and healing within seven days and ten days were significantly greater in the *C. nutans* extract-treated group than in the placebo group. Fourthermore, the pain score was reduced more rapidly in the *C. nutans* extract-treated group, and there were no side effects from the study medication. *C. nutans* cream is more effective than placebo in the treatment of herpes zoster and the patients healed faster than patients in the placebo group (Charuwichitratana *et al*., 1996).

1.3.5 Toxicological Study

Acute toxicity studies of ethanolic extract of *C. nutans* leaves given orally, subcutaneously, or intraperitoneally, did not produce any side effects of toxicity in the animal.

A subchronic toxicity study was performed by daily oral administration of the extract to rats for 90 days. Histopathalogical examination of internal organs did not show any abnormalities that could be due to the effect of the extract (Chavalittumrong *et al*., 1995). In addition ethanol extract of *C. nutans* was shown to inhibit growth of yellow head baculoving and also to induce phagocytosis activity in tiger prawn (Chavalittumrong *et al*., 1995). The results of these studies shows that *C. nutans* has strong potential to be developed as a novel source of medicine to treat herpes infections.

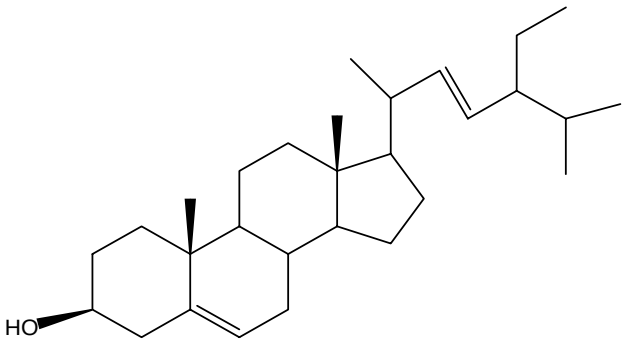
The Department of Medical Sciences, Ministry of Public Health transferred the technology of *C. nutans* antiviral cream, including extraction and the formulation of *C. nutans* cream, to the Government Pharmaceutical Organization to produce this cream for the market (Jayavasu *et al*., 1998). This case illustrates that traditional medicine can be very valuable and useful for modern scientific studies.

Consequently, a systematic procedure for the quality control of the crude drug and raw material has to be established. Currently, the control of *C. nutans* to produce antiviral cream uses a bioactivity test (plaque reduction assay) which requires considerable time and cost. As it is important to reduce the cost of such control, chemical study of active components should be conducted to obtain the quality control of crude extract of *C. nutans* by developing chemical reference standards for the active components isolated from *C. nutans*.

1.4 Chemical Constituents

C. nutans has been chemically investigated previously as below:

In 1976 and 1977 Dampawan *et al*. reported the isolation of stigmasterol (5), lupeol (6), and β-sitosterol (7) from a light petroleum extract of the leaves, stems, and roots of *C. nutans*.



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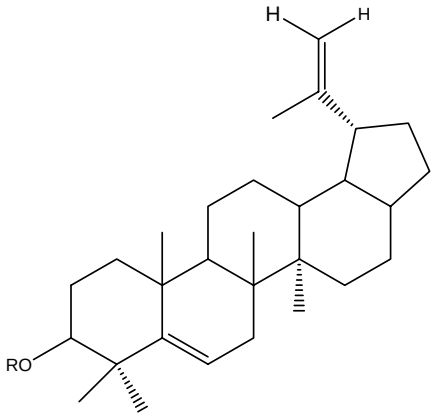

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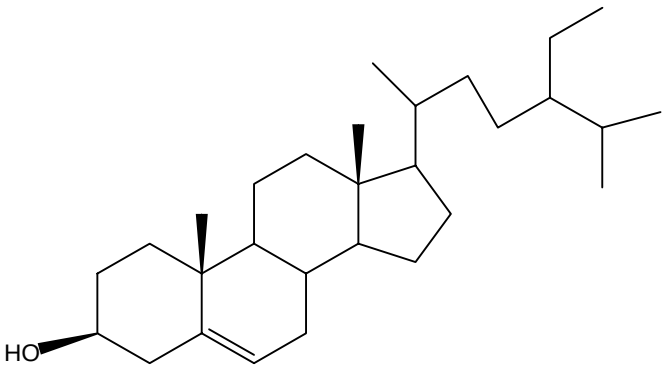
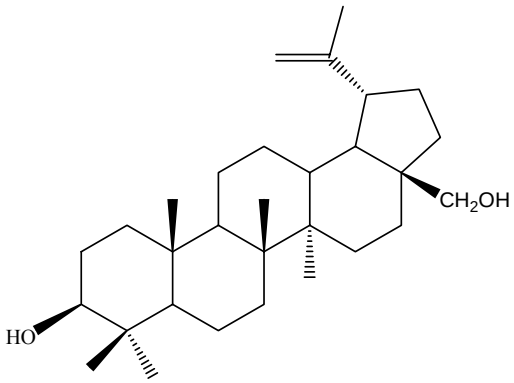


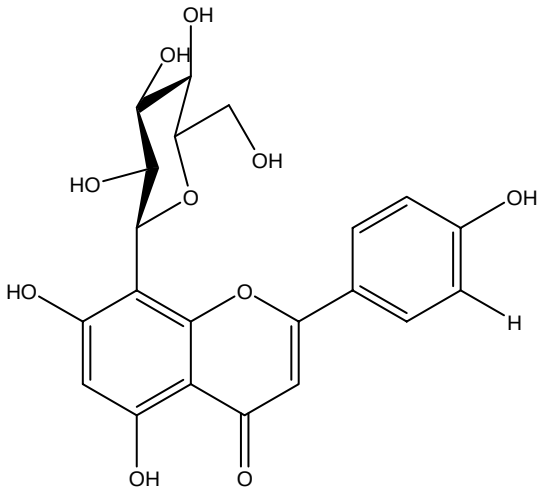
(7)

In 1983, Lin *et al*. reported the isolation of betulin (8), lupeol, and β-sitosterol from the roots of *C. nutans*.



(8)

Although chemical studies were started in 1976, very little advance was made at that time, because of unsystematic approaches in the isolation and lack of suitable techniques for the characterization of the components. Howerver, as a result of the development in instrumental and chemical techniques, in 1997 Teshima *et al*. isolated six known C-glucosyl flavones, (vitexin (9), isovitexin (10), shaftoside (11), isomollupentin-7-0-β-glucopyranoside (12), orientin (13), and isoorientin (14)) from the methanol extract of the stems and leaves of *C*. *nutans*.



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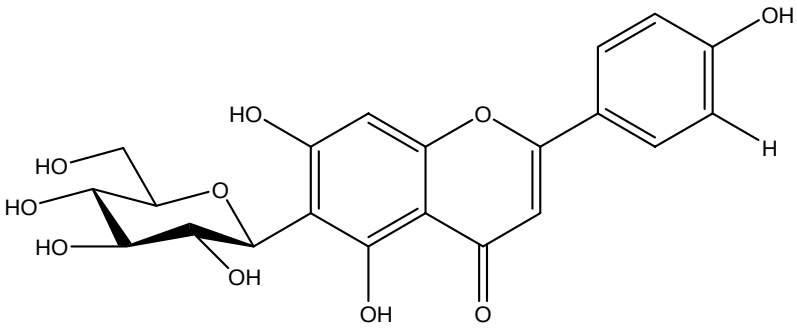
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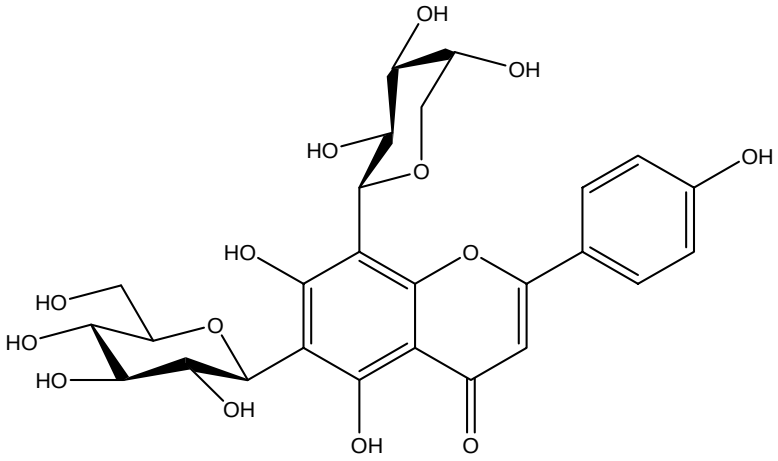
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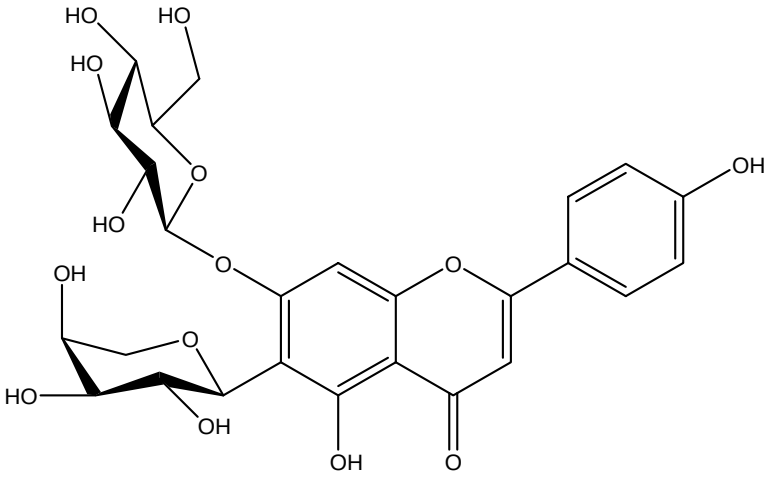
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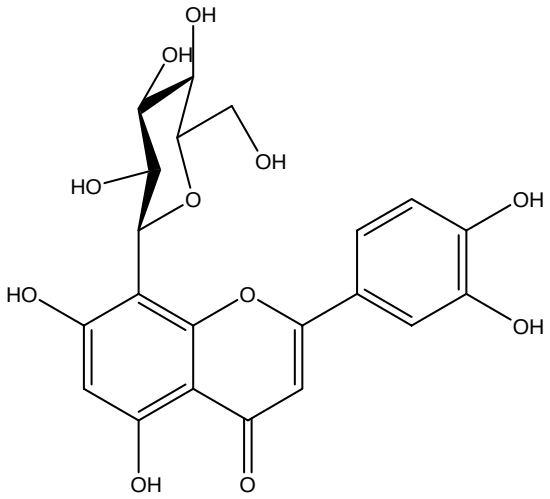

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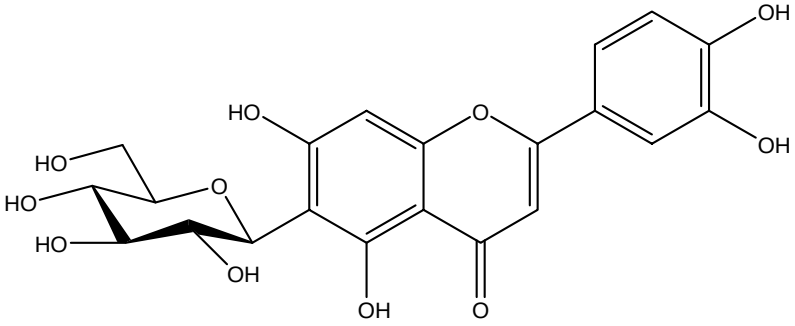
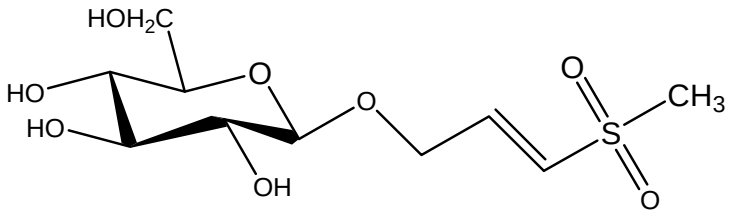


(14)

In 1998 Teshima *et al*. isolated five sulfur-containing glucosides, (clinacoside A (15), clinacoside B (16), clinacoside C (17), cycloclinacoside A1 (18), and cycloclinacoside A2 (19)) from the butanol and water soluble portions of the methanol extract of the stems and leaves of *C. nutans*.



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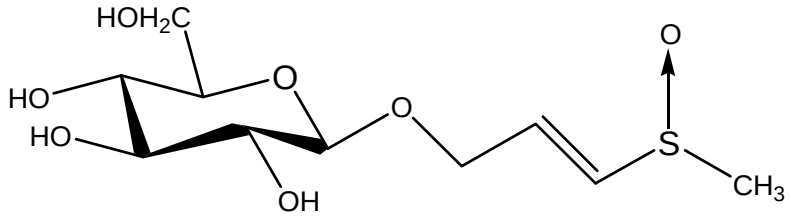

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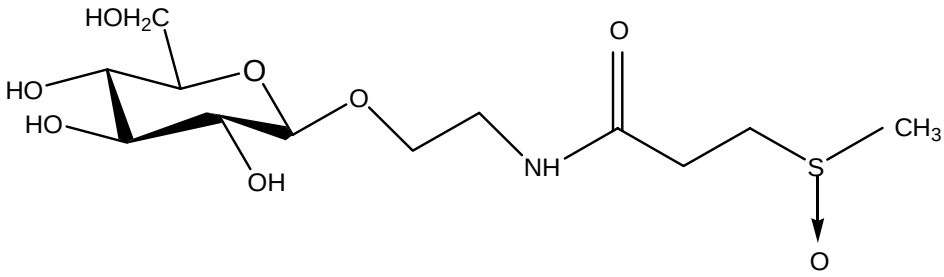

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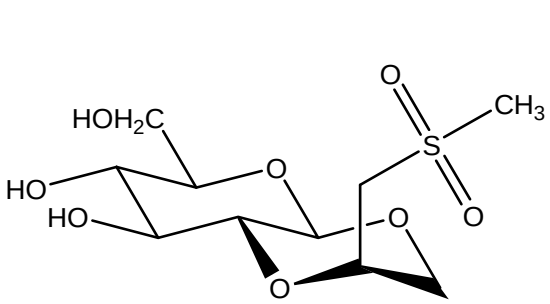

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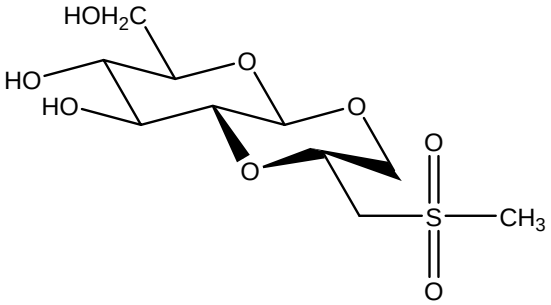
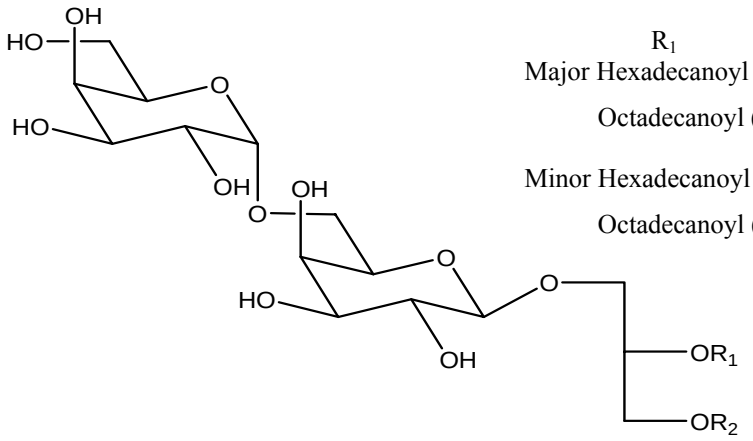


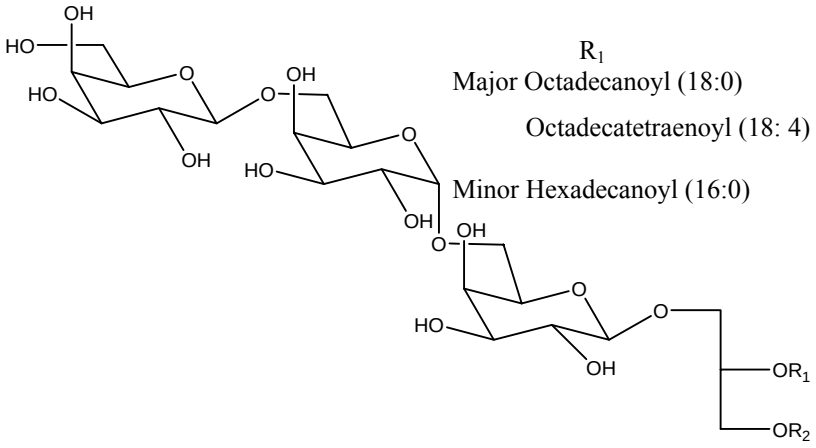
(19)

In 2001 Satakhum *et al*. reported the isolation of two glycoglycerolipids (1,2-O-dilinolenoyl-3-O-β-D-galactopyranosyl-glycerol (20) and 1-O-palmitoyl-2-O-linolenoyl-3-O-[α-D-galactopyranosyl-(1"→6')-O-β-D-galacctopyranosyl]-glycerol (21)) from the leaves of *C. nutans*. Further investigation by Suwanborirux *et al*. in 2003 found anti-HSV activity of both compounds (Suwanborirux *et al*., 2003).



	R_1	R_2
Major	Hexadecanoyl (16:0)	Hexadecatrienoyl (16:3)
	Octadecanoyl (18:0)	Hexadecatrienoyl (16:3)
Minor	Hexadecanoyl (16:0)	Octadecanoyl (18:0)
	Octadecanoyl (18:0)	Hexadecanoyl (16:0)

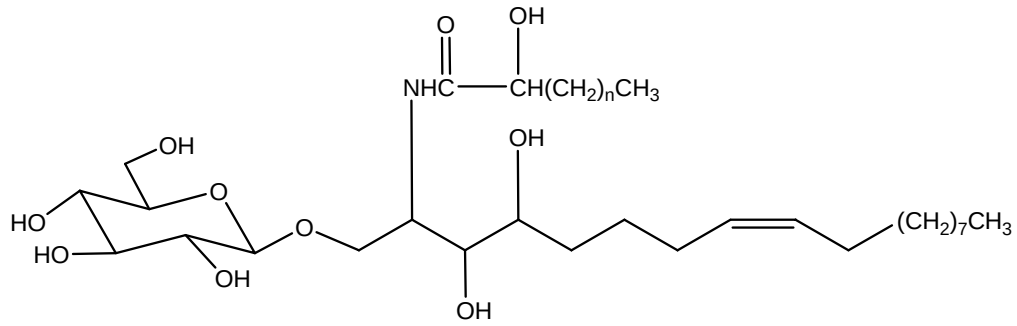
(20)



	R_1	R_2
Major	Octadecanoyl (18:0)	Octadecatetraenoyl (18:4)
	Octadecatetraenoyl (18: 4)	Octaadecanoyl (18: 0)
Minor	Hexadecanoyl (16:0)	Octadecatrienoyl (18:3)

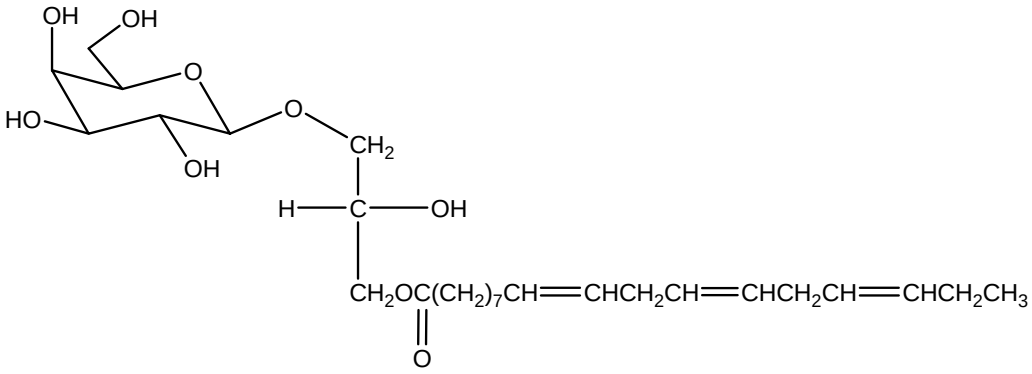
(21)

In 2004 Tuntiwachwuttikul *et al*. reported the isolation of a mixture of nine cerebrosides (22) and a monoacylmonogalactosylglycerol (23) from the leaves of *C. nutans*. The structures of the cerebrosides were characterized as 1-*O*-b-D-glucosides of phytosphingosines, which comprised a common long-chain base, (2*S*, 3*S*, 4*R*, 8*Z*)-2-amino-8(*Z*)-octadecene-1,3,4-triol with nine 2-hydroxy fatty acids of varying chain lengths (C16, C18, C20-26) linked to the amino group. The glycosylglyceride was characterized as (2*S*)-1-*O*-linolenoyl-3-*O*-b-D-galactopyranosylglycerol.



n= 13, 15, 17-23

(22)



(23)

Although the chemical constituents have been reported, the active components have not been identified. This project, attempts to isolate and characterize such chemical principles of *C. nutans*. Further studies on antiviral activity against HSV-2 of the isolated compounds should also be conducted.

Our research group has been interested in this plant due to wide use in traditional medicine. Because of its interesting biological activities, and the fact that very little is known about it in the literature, it was desirable to phytochemically investigate this plant in detail.

In this project, ten compounds were separated from the leaves of *C. nutans*. Three of the isolated compounds could be identified as chlorophyll a and chlorophyll b related compounds. Structures of isolated compounds were characterized on the basis of NMR spectra by comparison of their NMR spectral data with literature values.

Details on isolation and structure determination of chemical constituents will be discussed in the next section of this thesis.

Thus, the objectives of this research were as follows:

1. To extract and isolate chemical constituents from the leaves of *C. nutans*.

2. To identify the structural formula of the isolated substances.

CHAPTER II

CONCEPTS AND THEORETICAL BACKGROUND

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. Many herbal medicines being sold in the market are fake products. 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. Besides, these drugs are often contaminated with microorganisms, which may cause various infections to consumers. To overcome the risk of the possible health hazards mentioned above, standard specifications and quality control procedures for these medicines are required by manufacturers as well as by regulatory authorities to improve the image of herbal medicinal products (Dechatiwongse na Ayudhaya, 1997).

2.1 Importance of Standardization and Quality Control

There are some important factors affecting the efficacy and safety of medicinal plant materials, namely: (Dechatiwongse na Ayudhaya, 1997 and Chavalittumrong *et al*., 2000).

2.1.1 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 will possess not only undesirable activities, but also may have unexpected toxicities.

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

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

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

2.1.5 Quality Though we know the correct plant name, the right part used, the proper harvesting time, 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, fertilizer, *etc*.

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.

2.2 General Control Methods

The standard control of herbal remedies is performed by 2 aspects (Dechatiwongse na Ayudhaya, 1997, and Chavalittumrong *et al*., 2000, 2001, and 2002).

2.2.1 Establishment of Standard Specifications of Herbal Raw Materials

According to World Health Organization (WHO) documents and other useful information, the specifications for medicinal plant materials should include the following:

1. Name (Scientific Name, English Name, Local Name)
2. Synonyms
3. Definition
4. Constituents
5. Plant Description
6. Description of Plant Material
   - 6.1. General Appearance
   - 6.2. Organoleptic Properties
   - 6.3. Microscopic Characteristics
7. Packaging and Storage
8. Identification
   - 8.1. Chemical Reaction
   - 8.2. Thin-Layer Chromatographic Analysis
9. Purity Tests
   - 9.1. Microbiological
   - 9.2. Foreign Matter
   - 9.3. Total Ash
   - 9.4. Acid-Insoluble Ash

9.5. Water-Soluble Extract

9.6. Alcohol-Soluble Extract

9.7. Moisture

9.8. Pesticide Residues

9.9. Arsenic and Heavy Metals

9.10. Radioactive Residues

10. Determination of Major/Active Chemical Constituents

11. Medical Uses and Doses

2.2.2 Development of Good Manufacturing Practice (GMP) Guidelines for the Manufacture of Herbal Medicinal Products

The purpose of these guidelines is to outline steps which should be taken, as necessary and appropriate, by manufacturers of herbal medicinal products with the objective of ensuring that their products are of the nature and quality intended. Successful and concurrent implementation of these GMP guidelines will not only improve the image of the herbal medicinal products industry, but also promote international recognition and acceptance and consequently, escalate the export potential of these products. The Good Manufacturing Practices for Pharmaceutical Products, Supplementary guidelines for the manufacture of herbal medicinal products, WHO/Pharm/93.561 (Lou, 1993) should be followed to develop the above mentioned guidelines.

2.3 Quality Assurance and Regulatory Control of Herbal Medicines in Thailand

Medicinal plants have been used in Thailand for a long period of time. Documentarily, the first record appeared during the Sukothai period (14th century A.D.) and the use of traditional drug formulas began during the Ayudhya period. Traditional drugs have been popular in the Kingdom throughout the Ayudhya and Rattanokosin periods. King Rama III (about 200 years ago) ordered the collection of traditional drug formulas including the diagnosis of diseases, traditional massage, literature and poetry, and their subsequent inscription on stone plates installed on the walls of two temples (Wat Po and Wat Raja Orasa Ram). Again during the years 1895-1900 King Rama V ordered the collection of all knowledge of traditional medicine 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, the standardization and quality control of these preparations are usually lacking, which hamper 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 Ayudhaya 1997).

Generally, the term "herbal medicines" applies to medicinal products whose active ingredients consist exclusively of plant materials, or vegetable drug preparations. This notion of herbal remedies has broad international recognition since WHO has defined the term in the same way.

The chemistry of medicinal herbs is very complex. Not all the constituents present in the plant have therapeutic activity, some are poisonous *e.g*. pyrrolizidine and tropane

alkaloids. Phytochemistry deals with the study of the chemical composition of plant material. Plants are used in various forms varying from powders to extracts. Powder represents the drug in ground form and these types of preparations are considered to be crude. The Pharmacopoeia mentions standardized vegetable powders for therapeutic application (Sawasdimongkol, 1995).

Herbal systems of medicine have become increasingly popular in recent years. A recent study from America demonstrated that about 34% of the general population used one or the other system at least once a year. In India 76% of patients visiting the general medicine OPD of a tertiary care hospital use alternative therapies. In light of growing demand for herbal drugs, the quality control and assurance is primarily important. The standardized herbal extracts are considered to be more scientific than crude drugs. The commonly employed technique for removal of active substances from the crude drug is called extraction. Selection of the solvent is very critical in preparing extracts, because the active constituent of the plants have affinity for solvents.

Extracts are prepared by separating the soluble matter from vegetable tissues by application of a suitable solvent like alcohol, water, or ether. The resultant liquid is concentrated by evaporation to obtain liquid extract, or concentrated nearly to dryness to obtain solid extract. Depending on the solvent used, the extracts are classified as alcoholic, ethereal, or aqueous (the solid and liquid extract classification is based on method of preparation, the alcoholic, aqueous, and ethereal extract classification is based on type of solvent used) (Pothisiri, 1996).

The standardized herbal extract is a preparation, which contains a certain fixed proportion of the active constituent. For example, a standardized extract of *Papaver somniferum* contains not less than 9.5% of morphine. The concept of standardization has great impact on the quality of herbal products. Standardization helps in adjusting the

herbal drug formulation to a defined content of a constituent or constituents with therapeutic activity. The latest method of preparing herbal extracts is by successive macerating of the powdered drug in order of increasing polarity. This process is known as successive solvent extraction and is carried out in a special assembly known as a Soxhlet apparatus. The biological source of the drug has great impact on the finished product in herbal drug preparation. Proper identification of the drug is significant for phytochemical screening, which further exerts importance on therapeutic activity of the medicinal herb. Thus, presence of an identification standard is a must in the finished product of an herbal drug preparation. A constituent of a medicinal herb, which is used for quality control and assurance of a herbal product, is known as a marker compound. A marker compound may or may not have therapeutic activity. Commonly used herbal extracts are listed in Table 2.1 (Sawasdimongkol, 1995 and Malhotra *et al*., 2002).

Table 2.1. Report on Some Medicinal Standards.

Botanical Name	Standard	Percentage
1. *Achellia Millefolium*	Essential oil	0.04%
2. *Adhatoda Vasica*	Vasicine	0.5%
3. *Allium Sativum*	Allicin	0.6%
4. *Andrographis Paniculata*	Andrographolide	10%
5. *Asparagus Racemosus*	Saponin	30%
6. *Azadirachita Indica*	Azadiractin	2%
7. *Bacopa Monneri*	Bacoside	20%
8. *Boswellia Seratta Acid.*	Boswellic	40% &70%
9. *Camelia Sinensis*	Epigallocatechin Gallate	0.2%

Table 2.1. (Continued).

Botanical Name	Standard	Percentage
10. *Capsicum Frutescens*	Capsaicinoids	0.62%
11. *Centella Asiatica*	Asiaticoside	3%
12. *Cholorella Emersoni*	Chlorophyll	1%
13. *Commiphora Balsamdendron Mukul*	Guggulsterones	5%
14. *Cratageus Oxycanthus*	Vitexin	5%
15. *Curcuma Longa*	Curcumin	95%
16. *Cynara Scolymus*	Cynarin	1%
17. *Echinacea Angustifolia*	Echinacosides	4%
18. *Embelia Ribes*	Embellin	8%
16. *Cynara Scolymus*	Cynarin	1%
17. *Echinacea Angustifolia*	Echinacosides	4%
18. *Embelia Ribes*	Embellin	8%
19. *Ephedra Sinica*	Ephedrine	6%
20. *Garcinia Cambogia*	Hydroxy Cirtic Acid	50%
21. *Ginkgo Biloba*	Flavonoglycosides	24%
22. *Glycyrrihiza Glabra*	Glycyrrhizin	20%
23. *Gymnema Sylvestre*	Gymnemic Acid.	75%
24. *Hydrastis Canadensis*	Alkaloids	3%
25. *Hypericum Perforatum*	Hypericin	0.3%
26. *Huperzia Serrata*	Huperzine	5%
27. *Momordica Charantia*	Bitters	3%

Table 2.1. (Continued).

Botanical Name	Standard	Percentage
28. *Ocimum Sanactum*	Ursolic Acid	8%
29. *Passiflora Incarnata*	Vitexin	4%
30. *Phylanthus Niruri*	Bitters	2%
31. *Picrorrhiza Kurroa*	Kutkosides	10%
32. *Piper Methysticum*	Kavalactones	30%
33. *Pueraria Tuberosa*	Disogenin	7%
34. *Saraca Indica*	Tannins	8%
35. *Sereno Repens*	Fatty acids	20-25%
36. *Shilajit*	Fulvic acid	50%
37. *Silybum Marianum*	Silymarin	70%
38. *Spirulina Maxima*	Phycocyanin	2.5%
39. *Terminalia Arjuna*	Tannins	8%
40. *Terminalia Belerica*	Tannins	40%
41. *Terminelia* Chebula	Tannins	60%
42. *Tribulus Terrestris*	Saponin	20% & 40%
43. *Trigonella Foenum*	Graecum Saponin	10%
44. *Triphla*	Tannins	40%
45. *Uncaria Tomentosa*	Saponins	2%
46. *Valeriania Officinalis*	Valerenic acid.	0.8%
47. *Vitis Vinifera*	Proanthocyanidins	95%
48. *Withania Somnifera*	Withanolides	1.5%
49. *Zingiber Officinale*	Gingerols	5%

Nowadays, we talk about active constituents of phyto drugs. An active constituent is truly responsible for therapeutic activity of a medicinal plant. The extracts are further subjected to chemical tests for identification of the plant constituents. The isolated constituents are of further importance to the pharmaceutical industry for applied research.

2.4 Extraction and Isolation

2.4.1 Extraction Theory and Procedure

Extraction is a very common laboratory procedure for isolating or purifying a product. Organic chemists employ solid-liquid, liquid-liquid, and acid-base extractions. The following applies to solid-liquid extractions, which was be used for this thesis.

A solid-liquid extraction is often used to extract solid natural products from a natural source, such as a plant. A continuous solid-liquid extraction apparatus, called a Soxhlet extractor, is illustrated in Figure 2.1 (Wingrove *et al*. 1981).

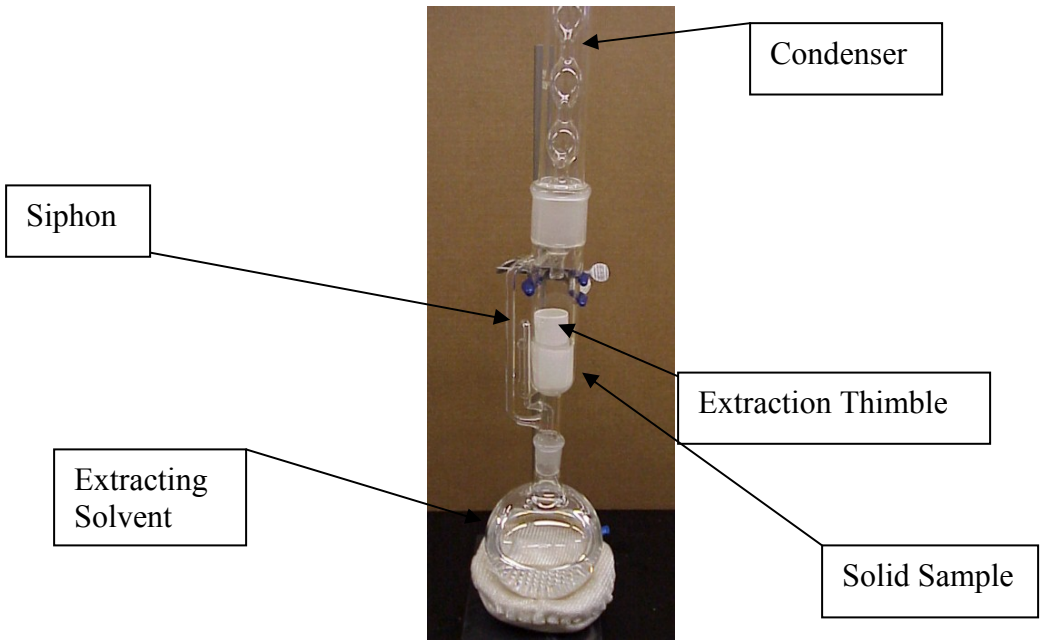


Figure 2.1. Soxhlet extraction apparatus.

As shown in Figure 2.1, 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 soxhlet thimble, where it interacts with the sample. The solvent plus extract eventually siphons back into the distillation flask. This process continues until extraction is complete.

Chemical composition of medicinal herbs is always a complex subject. Studying one substance among many is often difficult. Consequently, a number of techniques to separate complex mixtures into their individual components have been developed. Collectively, we call these methodologies chromatographic separation techniques;

Separation techniques allow individual substances to be isolated and studied. For example, once chlorophyll a and chlorophyll b were isolated using chromatography, the differences in their structures could be determined. A further use of separation techniques is for identification purposes. The principle behind the use of a separation technique for identification is that a substance will always behave the same way if it is treated identically, and one can know what it is by how it behaves.

2.4.2 Chromatography Theory

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).

2.4.2.1 Thin Layer Chromatography

Thin Layer Chromatography (TLC) is a solid-liquid technique in which the two phases are a solid (stationary phase) and a liquid (moving phase). Solids most commonly used in chromatography are silica gel (SiO_2.x H_2O) and alumina (Al_2O_3.x H_2O). Both of these adsorbents are polar, but alumina is more so. Silica is also acidic. Alumina is available in neutral, basic, or acidic forms. Thin Layer Chromatography (TLC) is a sensitive, fast, simple and inexpensive analytical technique. It is a micro technique; as little as one ng of material can be detected, although the sample size is from 1-100 µg. TLC involves spotting the sample to be analyzed near one end of a sheet of glass or plastic that is coated with a thin layer of an adsorbent. The sheet, which can be the size of a microscope slide, is placed on end in a covered jar containing a shallow layer of solvent. As the solvent rises by capillary action up through the adsorbent, differential partitioning occurs between the components of the mixture dissolved in the solvent the stationary adsorbent phase. The more strongly a given component of a mixture is adsorbed onto the stationary phase, the less time it will spend in the mobile phase and the more slowly it will migrate up the plate.

A spot or line of the mixture to be separated is applied at one end of a piece of the stationary phase. Then the chromatogram is developed by allowing the mobile phase to flow up by capillarity or down by gravity. After the mobile phase has reached the opposite end of the sheet of paper or thin layer medium, the chromatogram is removed from the mobile phase, the level to which the solvent has moved is marked

and the medium is dried. Each component of the mixture will have moved to a different level and some may not have moved at all. The ratio of the distance traveled by the solute (mixture component) to that of the solvent (mobile phase) is known as the R_f (retardation factor or flow rate) value.

$$R_f = \frac{\text{solute distance from origin}}{\text{solvent distance from origin}} \qquad (1)$$

If all conditions are kept the same (temperature, solvent system, nature of stationary phase, direction of flow, etc.), the Rf value is a characteristic of the solute and may be used to identify it. For identification of a mixture component, the unknown is chromatographed with a series of compounds of known identity and the Rf values compared. This is called co-chromatography (Pine, 1987).

2.4.2.2 Column Chromatography

In column chromatography, the stationary phase, a solid adsorbent, is placed in a vertical glass (usually) column and the mobile phase, a liquid, is added to the top and flows down through the column (by either gravity or external pressure). Column chromatography is generally used as a purification technique to isolate desired compounds from a mixture. The mixture to be analyzed by column chromatrography is applied to the top of the column. The liquid solvent (the eluent) is passed through the column by gravity or by the application of air pressure. An 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 carried along with the

mobile phase to varying degrees and a separation will be achieved. The individual components, or eluents, are collected as the solvent drips from the bottom of the column. The polarity of the solvent which is passed through the column affects the relative rates at which compounds move through the column. Polar solvents can more effectively compete with the polar molecules of a mixture for the polar sites on the adsorbent surface and will also better solvate the polar constituents. Consequently, a highly polar solvent will move even highly polar molecules rapidly through the column. If a solvent is too polar, movement becomes too rapid, and little or no separation of the components of a mixture will result. If a solvent is not polar enough, no compounds will elute from the column. Proper choice of an eluting solvent is thus crucial to the successful application of column chromatography as a separation technique. TLC is generally used to determine the system for a column chromatography separation (Pine, 1987).

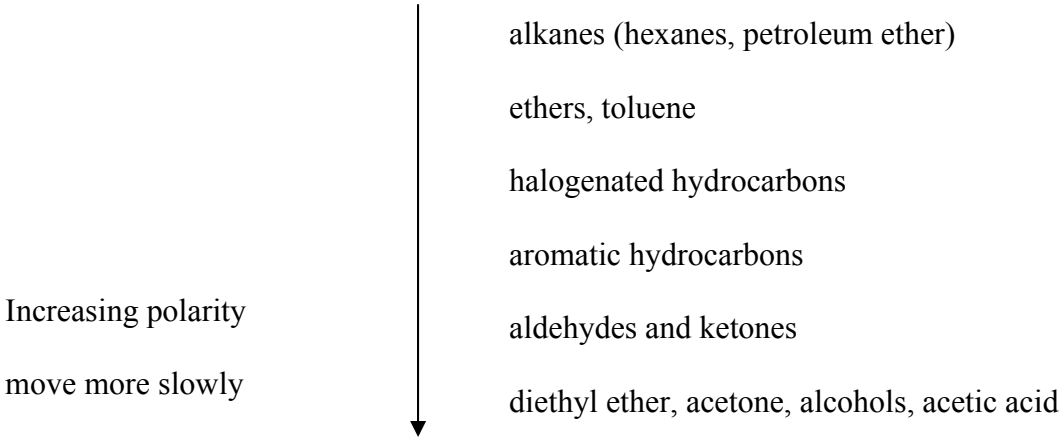
2.4.2.3 Determining Solvent Systems for Thin Layer Chromatography and Column Chromatography

For determing the best system to develop a TLC plate or chromatography column loaded with an unknown mixture, vary the polarity of the solvent in several trial runs. Carefully observe and record the results of the chromatography in each solvent system. When increasing the polarity of the solvent system, all the components of the mixture move faster. The ideal solvent system is simply the system that separates the components.

TLC elution patterns usually extrapolate to column chromatography elution patterns. Since TLC is a much faster procedure than column chromatography, TLC is often used to determine the best solvent system for column chromatography. For

instance, in determining the solvent system for a flash chromatography procedure, the ideal system is the one that moves the desired component of the mixture to a TLC R_f of 0.25-0.35 and will separate this component from its nearest neighbor by difference in TLC R_f values of at least 0.20. Therefore, a mixture is analyzed by TLC to determine the ideal solvent(s) for a flash chromatography procedure.

Because of toxicity, cost, and flammability concerns, the common solvents are hexanes (or petroleum ethers) and ethyl acetate (an ester). Diethyl ether can be used, but it is very flammable and volatile. Alcohols (methanol, ethanol) can be used. Acetic acid (a carboxylic acid) can be used, usually as a small percentage component of the system, since it is corrosive, nonvolatile, very polar, and has irritating vapors. Acetone (a ketone) can be used. Methylene chloride (halogenated hydrocarbon) is a good solvent, but it is toxic and should be avoided whenever possible. If two solvents are equal in performance and toxicity, the more volatile solvent is preferred in column chromatography because it will be easier to remove from the desired compound after isolation from a column chromatography procedure. The scheme below should help in solvent selection (Pine, 1987 and Wade, 1987).



Scheme 2.1. The expected elution order of organic classes.

2.5 Structure Elucidation

2.5.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

Nuclear magnetic resonance was first observed in 1946. Bloch and Purcell were jointly awarded the 1952 Nobel Prize for physics for their discovery. Also, the Nobel Prize for chemistry awarded in 1991 to Ernst of the ETH Zurich. The main application of NMR is in determining the structures of molecules. It is a very powerful tool for doing this and as such, has become, possibly, the most important of all spectroscopic methods.

2.5.1.1 1-D NMR Spectroscopy

1-D NMR was the only technique used until the 1970's, *i.e*. the spectrum has 2 axes, plotting frequency against intensity. Some of these techniques, often employing complex pulse sequences to obtain specific information, are still very useful in determining structures from spectra. Some of these are summarized below:

Table 2.2. Multiple Pulse 1-D NMR Techniques.

Experiment	Nuclei Observed	Types of Information, Applications
J-modulated spin-echo (attached proton test, APT)	^{13}C	CH and CH_3 carbon nuclei give positive signals, 4° and CH_2 carbons give negative signals. An aid to assignment.
INEPT	^{1}H / ^{13}C	The INEPT pulse sequence is used as a component of many 2-D experiments *e.g*. HSQC.

Table 2.2. (Continued).

Experiment	Nuclei Observed	Types of Information, Applications
DEPT	^{13}C	Tells how many hydrogen atoms are directly bonded to a carbon nucleus: CH, CH_2, CH_3. Disadvantage: no signals from 4° carbon atoms.
Selective TOCSY	^{1}H	Allows identification of all the protons belonging to a common coupled spin system.
1D-INADEQUATE	^{13}C	Exact ^{13}C-^{13}C coupling constants without the need to synthesise ^{13}C enriched compounds.

2.5.1.2 2-D NMR Spectroscopy

A 2-D spectrum is a spectrum in which both axes show chemical shifts, with the intensity of the peaks constituting a third dimension. This gives a contour plot. From a practical point of view, the most useful 2-D spectra are those that show either ^{1}H-^{1}H correlations or ^{1}H-^{13}C correlations.

2-D methods are based on the couplings between nuclear dipoles. These can be scalar (through bond) couplings, or of a dipolar type (through space, as in the nuclear overhauser effect).

In a normal pulsed NMR experiment, the excitation pulse is followed immediately by the data acquisition (detection) phase, in which the free induction decay, FID, is recorded and the data stored. In the specialised 1-D NMR techniques described

above, the spin system undergoes a preparation phase before data acquisition. In 2-D NMR experiments, these two phases are separated by a third phase, that of evolution and mixing.

Table 2.3. Two-Dimensional NMR Techniques.

Experiment	Nuclei Observed	Types of Information, Applications
Heteronuclear J-resolved ^{13}C NMR spectroscopy	^{13}C	^{13}C-^{1}H coupling constants, number of directly bonded protons (as in DEPT).
Homonuclear J-resolved ^{1}H NMR spectroscopy	^{1}H	Useful in determining chemical shift values in complicated spectra, identifying peaks of multiplets.
^{1}H-^{1}H COSY	^{1}H	Assigning signals in complicated spectra.
Long-range COSY	^{1}H	Assigning signals of protons separated by four or more bonds where the couplings are small.
^{1}H-^{13}C COSY (HMQC, Heteronuclear Multiple Quantum Coherence)	^{1}H, ^{13}C	Assigning signals in proton and carbon spectra, starting from known signals.
HMBC (Heteronuclear Multiple Bond Correlation)	^{1}H, ^{13}C	Assigning ^{1}H and ^{13}C signals on the basis of long range couplings.

Table 2.3. (Continue).

Experiment	Nuclei Observed	Types of Information, Applications
2-D TOCSY	^{1}H	Allows identification of all the protons belongng to a common coupled spin system.
NOESY	^{1}H	Gives evidence for spatial proximity of nuclei using nuclear Overhauser effect.
EXSY	^{1}H	Qualitative evidence of exchange processes.
2-D INADEQUATE	^{13}C	Assigning signals by detecting couplings between adjacent ^{13}C nuclei.

Assignment of peaks from correlated NMR spectroscopy (COSY, HMBC, HMQC, TOCSY, NOESY), results in many assignment problems, which can be solved in an elegant way with the help of 2-D correlated NMR spectroscopy. This is by far the most important form of 2-D NMR (Breitmaier, 1993).

2.5.1.3 Overview of the COSY Experiment

Pulse sequence; consists of a variable delay time, t_1, and acquisition time, t_2. The experiment is repeated many times with different values of t_1 and the data acquired during t_2 is stored in a computer. The value of t_1 is steadily increased throughout the experiment. During a typical experiment, something on the order of a thousand individual FID patterns, each incremented in t_1, must be recorded. This takes

approximately 30 minutes, longer for higher field instruments (500 MHz and above). The results of these are processed as summarized below.

1. After an initial relaxation delay, a radio frequency pulse prepares the system by rotating the bulk magnetization vectors of the nuclei by 90°.

2. This is the variable delay period, t_1. During this period, the spins evolve *i.e*. they relax under the influences of chemical shift and mutual spin-spin coupling.

3. At the end of this evolution period, a second 90° pulse is applied – this constitutes the mixing period. This has the effect of distributing the magnetization among the various spin states of the coupled nuclei. Magnetization that has been encoded by chemical shift during t_1 can then be detected at other chemical shifts during t_2, resulting in diagonal cross-peaks in the 2-D plot. This means that the cross-peaks show the correlation of pairs of nuclei by means of their spin-spin coupling. In the COSY spectrum of a complete molecule, the result is a complete description of the coupling partners in the molecule.

2.5.1.4 Overview of Heteronuclear Correlated (HETCOR) Spectroscopy Methods (HSQC, HMQC, HMBC)

As before, the magnetization vectors of the protons are allowed to relax at different rates according to their chemical shifts. This is done in the same way as for COSY, *i.e*. a 90° pulse followed by an evolution time t_1. After t_1, a 90° pulse is applied to both the protons and the carbons. This transfers magnetization from protons to carbons. Since the carbon magnetization was 'labeled' by the proton precession frequencies during t_1, the ^{13}C signals that are detected during t_2 are modulated by the chemical shifts of the coupled protons. The ^{13}C magnetization can then be detected in t_2

to identify a particular carbon carrying each type of proton modulation. What all this means is that in the techniques HSQC and HMQC we end up with a spectrum that shows the ^{1}H and ^{13}C spectra plotted against each other. Cross peaks occur where a proton chemical shift value corresponds to a proton which is attached to a ^{13}C nucleus which appears at its own carbon chemical shift value. Of the three techniques, HMQC is by far the most common, and useful. HMBC enables assignments of signals in cases where ^{13}C and ^{1}H nuclei are coupled through two or more bonds.

2.5.1.5 Overview of Nuclear Overhauser Effect Spectroscopy (NOESY)

NOE is a phenomenon involving polarization transfer between nuclei which do not have scalar (through bond) couplings, but are close together in space. (Ideally the nuclei being observed should be 3-5 Å apart.) This can be achieved by selective saturation of a particular signal, then watching for effects on other peaks in the 1-D spectrum. However, a much more satisfactory way of observing magnetization transfer to other nuclei is by 2-D NOESY. The pulse sequence for this experiment consists of three 90° pulses, the second and third of which are separated by a fixed delay τm in which magnetization is transferred between neighboring spins via the NOE. The result of this is a 2-D spectrum with cross peaks whose intensity depends on the efficiency of the transfer of magnetization between the two spins during τm. There are also diagonal peaks as in COSY, since the spins always have some magnetization left at the end of τm. The experiment works in exactly the same way in the event of a scalar coupling between two nuclei.

2.5.2 Structural Analysis on the Basis of NMR Parameters

The focus in the following three sections is on the analysis of the solution conformations of organic molecules utilizing NMR parameters, *i.e*. chemical shift, scalar coupling, and NOE.

2.5.2.1 Chemical Shift

There exists a great amount of tabular data in the NMR literature, according to which the chemical shift of a nucleus is indicative of a specific chemical structure. There are some computer programs available which can predict the chemical shift of a certain nucleus that is part of a defined structural subunit. However, chemical shifts provide information not only about the chemical structure of the molecule being studied, but also about its surroundings. Since the chemical shift of a nucleus depends on the local magnetic field around it, δ is affected by magnetic and electrostatic effects exerted by the surroundings of the nucleus. One example of this is the magnetic anisotropic effect of a neighbouring group, which can lead to a shielding or deshielding of the nucleus. In practice, δ-values of protons are the most sensitive for detecting anisotropy effects of molecular surroundings in organic compounds. Typical shielding or deshielding cones for groups of common anisotropy sources in organic molecules are depicted in Figure 2.2. The magnetic susceptibilities of the chemical bond, *e.g*. a carbonyl group and a carbon-carbon double bond, lead to the magnetic anisotropy effect in an external B_0-field. In aromatic compounds, such as benzene, the B_0-field induces a ring current that generates an additional magnetic field. The resulting anisotropy effect is stronger than those arising from double bonds. The strength of anisotropy is proportional to $1/r^3$, r being the distance from the anisotropy source. Thus, the δ_H-value of a proton-containing group can provide information about the spatial proximity of

another group having a known anisotropy effect. The ring-currents of larger π-systems produce such strong anisotropic effects in their proximity that they cover even more distance in space than NOE .

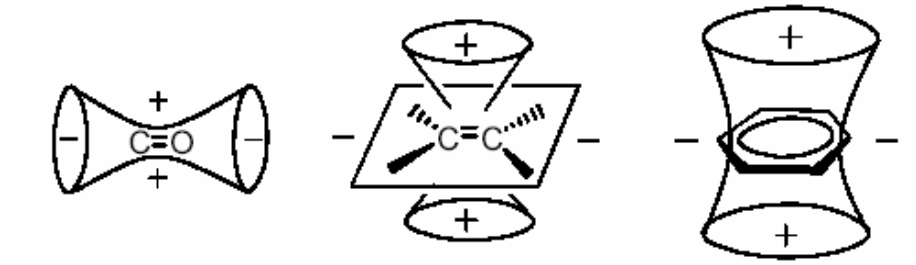


Figure 2.2. Schematic representation of the magnetic anisotropic effect. Carbonyl group, carbon double bond, and benzene ring. Shielding effect is denoted with (+)-sign and deshielding with (–)-sign.

Electric fields influence the electron densities of nuclei, and thus polarized charges (*e.g.* in amino, carbonyl, and nitro groups) affect their surroundings. The proton chemical shift can be strongly affected by hydrogen bonding. In a hydrogen bonded proton, the electron density is formally increased, but the electrostatic dipole field of the hydrogen bond produces a deshielding effect on the bonded hydrogen (Silverstein *et al*., 1963).

2.5.2.2 Scalar Coupling

In modern NMR spectroscopy, spectral assignment is largely based on the observed scalar couplings between NMR active nuclei in the molecules studied. Scalar spin couplings are mediated by bonding electrons, and thus the couplings are not only sensitive to the chemical structure, but also to bond conformations. The dependence of a vicinal coupling constant (^3JH-H) on the dihedral angle φ between H–C–C–H protons has been first theoretically formulated by Karplus with equation 2:

$$^{3}J\text{H-H} = A + B\cos\varphi + C\cos 2\varphi \qquad (2)$$

$$A = 4.22,\ B = -0.5 \text{ and } C = 4.5$$

Experimentally, the Karplus equation (Eq. 2) has been found to predict φ-angles relatively well when the molecular fragment studied resembles ethane. However, it has been shown that the vicinal coupling constant (^{3}JH-H) depends on electronegative substituents, solvent effects, bond-angles and bond-lengths. A number of variations for equation 2 exist in the literature in which the constants A, B, and C are readjusted, and/or trigonometric functions are added or altered to improve empirical correlation. The ^{2}JC-H or ^{3}JC-H values provide information similar to that given by the ^{3}JH-H values about the dihedral angle, but the former couplings have been more difficult to obtain until recent developments in the NMR techniques. Matsumori *et al.* have shown that the determination of the stereochemistry for acyclic natural products is possible utilizing the $^{2\text{ or }3}J$C-H values. Since the vicinal proton-carbon spin coupling constants (^{3}JC-H) obey a Karplus-type equation, the conformations of C–C–H fragments can be evaluated. Also geminal ^{2}JC-H values provide conformational information. Small ^{2}JC-H values have been measured for the β-alkoxy CH_2 group when the proton is in the *gauche* position with respect to the oxygen functionality of a neighbouring carbon atom, whereas for the *anti* conformation, large ^{2}JC-H values have been measured (Derome, 1987).

2.5.3 Fourier Transform Infrared (FT-IR) Spectroscopy

Infrared spectroscopy is a technique used to identify various functional groups in unknown substances through the identification of different covalent bonds that

are present in the compound. By identifying the different covalent bonds that are present in a compound, one can establish the types of functional groups present. By comparing the absorptions seen in an experimental spectrum to the literature absorptions of various functional groups, one can determine a list of possible identities for the bonds present. Sometimes the absorptions are given in a chart, called a correlation chart. A simplified correlation table is given in Table 2.4 (Lambert *et al*., 1987).

Table 2.4. A Simplified Correlation of Infrared Absorption with Organic Functional Groups.

Functional Group	Frequency (cm^{-1})	Intensity	Assignment
Alkanes Must have	3000-2800	s	−C-H Stretch
both peaks	1500-1450	s	−C-H Bend
Alkenes Must have	3100-3000	m	=C-H Stretch
both peaks	1675-1600	m	C=C Stretch
Alkynes Must have	3300-3200	w	≡C-H Stretch
both peaks	2200-2100	w	C≡C Triple Bond Stretch
Alcohols, Phenols	3600-3000	broad	O-H Stretch
	(1300-1000)		(C-O Stretch)
Aldehydes Must have	1750-1625	s	C=O Stretch
all three peaks	2850-2800	w	C-H Stretch off C=O
	2750-2700	w	C-H Stretch off C=O
Aromatic Rings (*i.e.*	3100-3000	w	=C-H Stretch
Benzene rings) Must	1600-1580	m	C=C Stretch
have all three peaks	1500-1450	m	C=C Stretch

Table 2.4. (Continued).

Functional Group	Frequency (cm^{-1})	Intensity	Assignment
Ketones	1750-1625	s	C=O Stretch
Carboxylic Acids	3400-2400	very broad	H-bonded O-H Stretch
Must have both peaks			across the entire range.
	1730-1660	s	C=O Stretch
	(1320-1210)	(s)	(C-O Stretch)
Ethers	(1300-1000)	(s)	(C-O Stretch)
Amines: Primary	3500-3200	m	N-H Stretch (two peaks in
Must have both peaks			this range)
	1640-1560	s	N-H Bend
Amines: Secondary	3500-3200	m	N-H Stretch (one peak in
Must have both peaks			this range)
	1550-1450	s	N-H Bend
Nitriles	2300-2200	m	C-N Triple Bond Stretch
Nitro Groups	1600-1500	s	N=O Stretch
Must have both peaks	1400-1300	s	N=O Stretch
	(2 peaks < 200		
	cm^{-1} apart)		
Amides	3500-3100	m	N-H Stretch
Must have all three	1670-1600	s	C=O Stretch
peaks	1640-1550	m	N-H Bend

Intensity Key: s= strong, m= medium, w= weak

CHAPTER III

EXPERIMENTAL

3.1 General Techniques

3.1.1 Chemical Solvents

The organic solvents were commercial grade and distilled prior to use for extraction and as eluents for thin layer and column chromatography. Reagent grade solvents were used for recrystallization.

3.1.2 Other Chemicals

3.1.2.1. Merck's silica gel 60 Art. 7734 (70-230 mesh ASTM) was used as adsorbent for normal column chromatography and 9385 (230-400 mesh ASTM) for flash chromatography.

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

3.1.2.3. Merck's TLC aluminum sheet, silica gel 60 F_{254} precoated 25 sheets, 20x20 cm^2, layer 0.2 mm was used to identify the identical fractions.

3.1.3 Chromatography

3.1.3.1 Thin Layer Chromatography (TLC)

Techniques: One way, ascending.

Adsorbent: Silica gel 60 F_{254} pre-coated on aluminum plate (Merck) size 2x5 cm^2.

Visualization: 1. Visualization under daylight.

2. Under ultraviolet light at 254 nm the compounds which contain unsaturated bonds especially conjugated systems are visible as quenching spots under UV light at 254 nm.

3. By staining TLC plates with anisaldehyde-sulfuric acid reagent (0.5% ethanolic solution of anisaldehyde with 5% sulfuric acid), the spot of organic compounds give specific color with this reagent after heating at 80-100^{o}C for a few minutes.

3.1.3.2 Column Chromatography

Adsorbent: Unless indicated otherwise, silica gel with particle size less than 0.63 mm (E. Merck) was used throughout the experiments.

Packing method: Slurry packing.

Sample loading: The sample was dissolved in a small amount of a suitable organic solvent, mixed with a small quantity of silica gel 60 with particle sizes 0.063-0.200 mm, air dried and added gently onto the top of column.

Elution: After loading of the sample, the column was eluted with a suitable solvent system using the gradient technique.

3.1.3.3 Preparative Chromatography

PLC plates were prepared as follows: A mixture of Merck's silica gel 60 GF_{254} for preparative layer chromatography (200 g) in water (480 mL) was shaken thoroughly until the suspension was homogeneous. It was allowed to stand in a stopper flask for 1-2 hr before being spread onto eight 20 x 20 cm^2 plates. The plates were left to dry in still air for 24 hr and then in an oven at 100^oC for 1 hr. Bands were detected by viewing under a UV lamp at 254 and 365 nm.

3.2 Spectroscopy

3.2.1 UV-Visible Spectrometer

UV-visible absorbance was measured on a Hewlett Packard 8452A diode array UV-VIS spectrophotometer.

3.2.2 FT-IR Spectrophotometer

IR spectra were recorded on a Perkin-Elmer FT-IR 2000 spectrophotometer. Spectra of solid samples were recorded as potassium bromide (KBr) pellets.

3.2.3 NMR Spectrometer

The ^{1}H-NMR and ^{13}C-NMR spectra were recorded at 500 MHz and 125 MHz, respectively on a Bruker DMX500 NMR spectrophotometer in deuterated

pyridine (C_6D_5N). The chemical shifts were recorded in parts per million with reference to the solvent signals.

The numbers of hydrogen atoms attached to each carbon atom were obtained from J-modulated spin-echo experiments. 2-D NMR spectroscopy was performed with standard COSY (^1H-^1H correlation), HSQC (^{13}C-^1H correlation) and HMBC (long range carbon-proton correlation) pulse sequences.

3.3 Source of Plant Materials

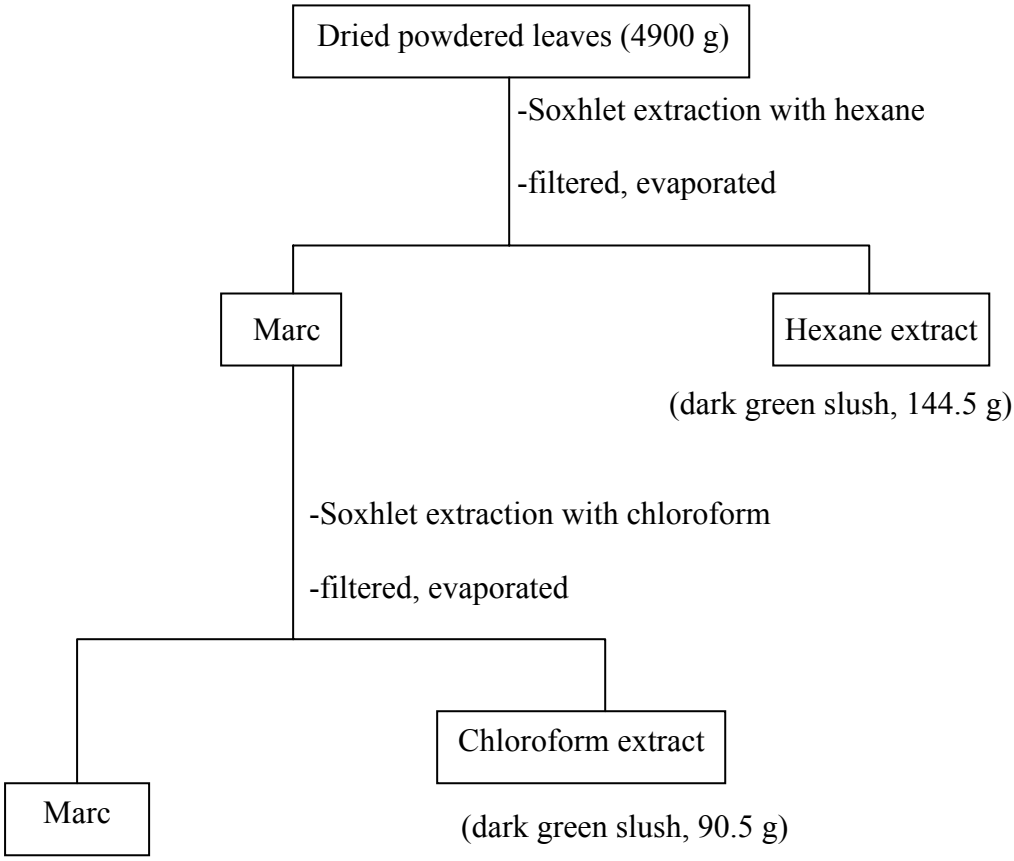
Fresh aerial parts of *C. nutans* were collected from Bangkok, Chanthaburi and Nakhon Pathom provinces, Thailand. The specimens were authenticated by the Botanical section, Medicinal Plant Research Institute, Department of Medical Sciences, Ministry of Public Health. The leaves were separated from the stems, washed thoroughly and dried in an oven at 50^oC. The dried samples were ground to powder.

3.4 Extraction

The dried powdered leaves (4900 g) were extracted (4 x 1225 g) consecutively with hexane and chloroform in a soxhlet extraction apparatus at reflux (3 x 4 liters for each solvent) The figure below shows the soxhlet extraction apparatus in the natural product chemistry section, Medicinal Plant Research Institute, Department of Medical Sciences, Ministry of Public Health.

Figure 3.1. Soxhlet extraction apparatus.

The extracted solutions were then filtered through filter paper, the filtrates were concentrated to remove solvent by evaporation under reduced pressure on a rotary evaporator giving 144.5 g (2.95% yield based on dry weight) of hexane crude extract as dark green slush, 90.5 g of chloroform crude extract (1.85% yield based on dry weight). The extraction sequence was as shown in Scheme 3.1.



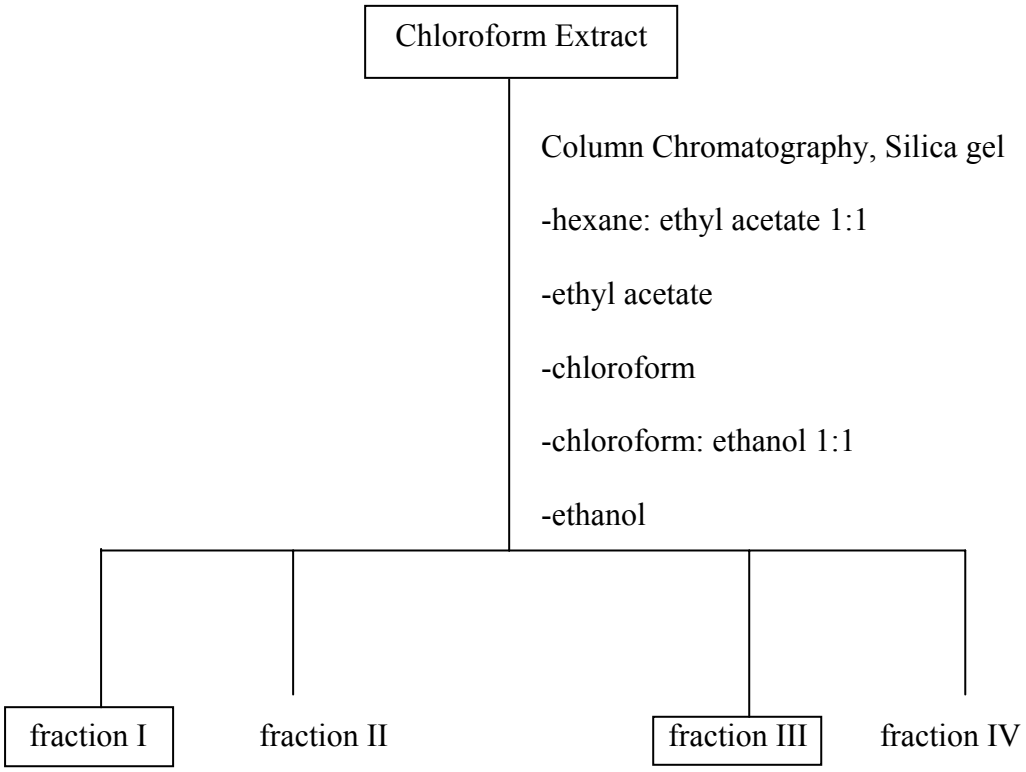
Scheme 3.1. Extraction of the leaves of *C. nutans*.

3.5 Isolation

Column Chromatographic Separation of Chloroform Crude Extract

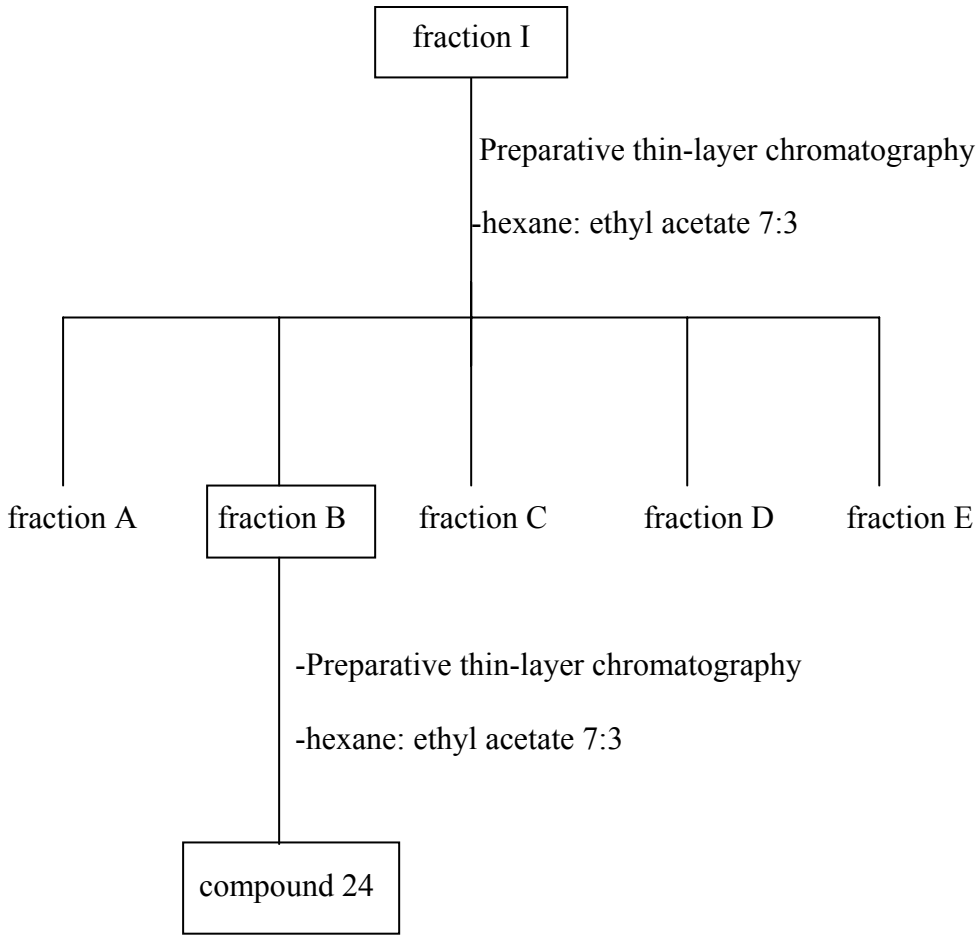
The chloroform crude extract was concentrated in vacuum to give a residue (90.5 g) which was chromatographed on a silica gel 60 column (4 x 855 g). The column was eluted successively with hexane–ethyl acetate (1:1), ethyl acetate, chloroform–ethanol (1:1), and ethanol. Every fraction was collected, concentrated to a small volume and four major fractions (I 32.92 g, II 6.50 g, III 30.51 g and IV 4.90 g) were

separated by monitoring with TLC in order to combine the fractions which had the same compounds. The isolation sequence was as shown in Scheme 3.2.



Scheme 3.2. Isolation of chloroform crude extract.

A portion of Fraction I (1.0017g) was further separated by preparative thin-layer chromatography (hexane–ethyl acetate 7:3) to afford five fractions (A 0.0450 g, B 0.0406 g, C 0.0791 g, D 0.0697 g and E 0.1469 g). Fraction C (0.0791 g) was further purified by preparative thin-layer chromatography using the same developing solvent to give crude compound 24 (0.0136 g), which was recrystallized from methanol (0.0058 g). The Isolation sequence of compound 24 was as shown in Scheme 3.3.

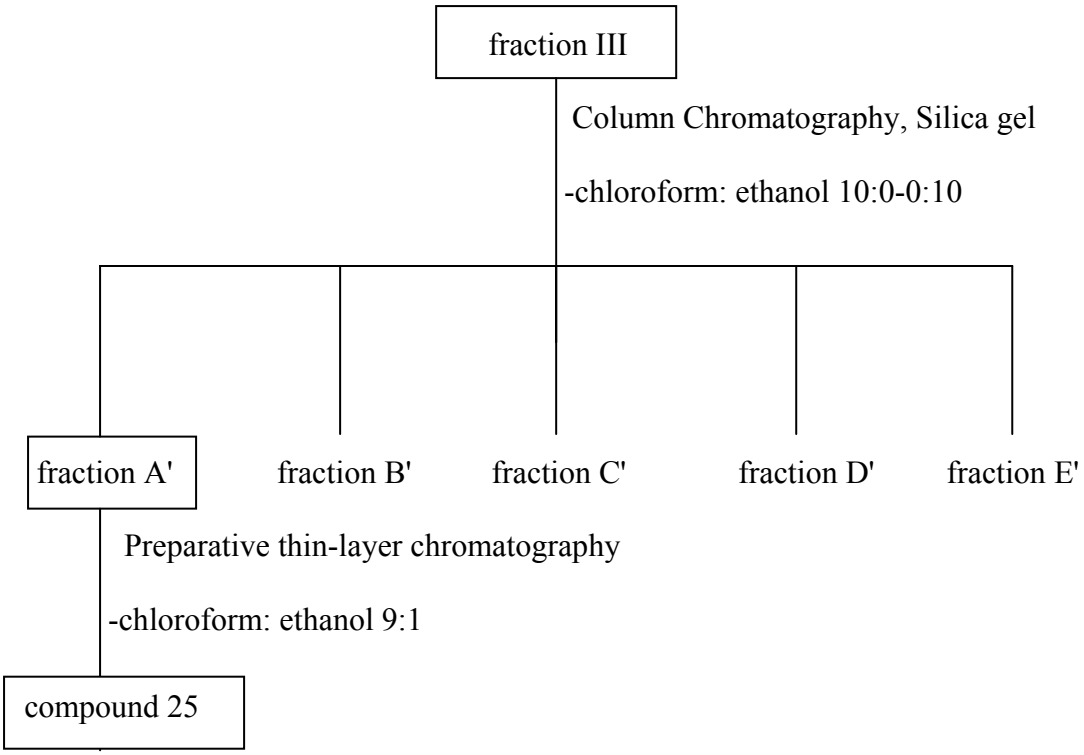


Scheme 3.3. Isolation of compound 24 from chloroform crude extract.

Faction III (30.51g) was chromatographed on a silica gel 60 column (855 g) and eluted successively with chloroform, chloroform containing increasing amounts of ethanol in gradient. Every fraction was collected, concentrated to a small volume and then monitored by TLC using the same solvent system as mentioned above in order to combine the fractions which had the same compounds. Five fractions (A' 1.39 g, B' 0.80 g, C' 0.49 g, D' 6.13 g and E' 2.55 g) were obtained.

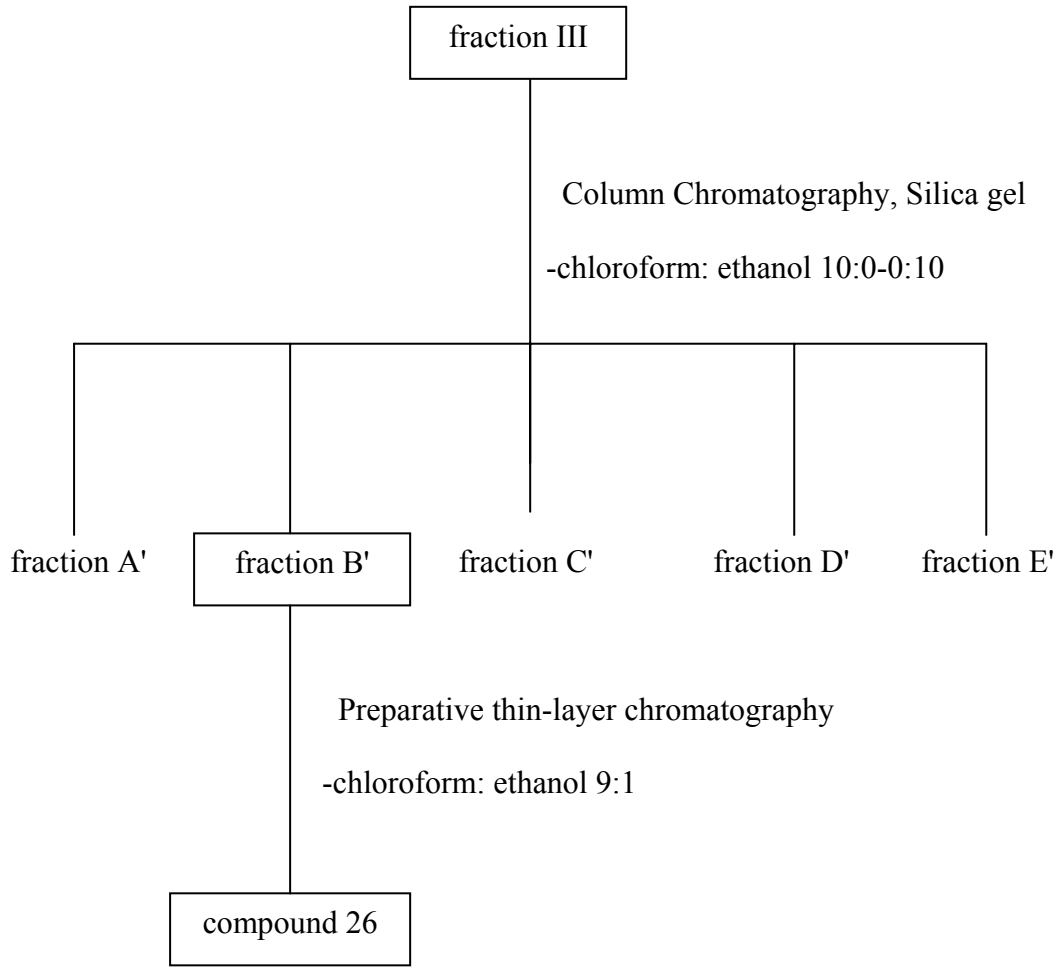
Fraction A' (1.39 g) was purified by PLC using chloroform–methanol (9:1) as the developing solvent to give crude compound 25 (0.3530 g). The crude compound was

further purified by preparative thin-layer chromatography using hexane–ethyl acetate (7:3) as the solvent system to provide pure compound 25 (0.0285 g). The isolation sequence of compound 25 was as shown in Scheme 3.4.



Scheme 3.4. Isolation of compound 25 from chloroform crude extract.

Purification of Fraction B' (0.80 g) was by preparative thin-layer chromatography, developing with chloroform–methanol (9:1), and recrystallizing from chloroform–ethanol to yield compound 26 (0.0136 g). The isolation sequence of compound 26 was as shown in Scheme 3.5.



Scheme 3.5. Isolation of compound 26 from chloroform crude extract.

3.6 Purification and Characterization of the Isolated Compounds

3.6.1 Purification and Characterization of Compound 24

Compound 24 was obtained from the first column chromatography, fraction I 1.0017 g of chloroform crude extract. Repetitive silica gel column chromatography as indicated in Scheme 3.3. The solvent was removed by rotary evaporator and the compound was recrystallized from methanol several times to

provide 0.0058 g (0.21% yield based on chloroform crude extract and 0.0039% yield from starting material). This compound is soluble in chloroform and dichloromethane.

UV ($CHCl_3$) λ_{max} ($CHCl_3$) 242, 315, 438, 520, 600, and 670 nm (Figure A.1).

FT-IR spectrum ν_{max} (KBr) 3429, 2925, 2852, 1721, 1637, 1300, 1277, and 1132 cm^{-1} (Table 4.2 p63 and Figure A.2).

^{1}H NMR δ ppm, in *pyridine-d_5*, 1.74 (3H, d, *J* = 6.3 Hz, 18^1-H),1.81 (3H, t, *J* = 7.55 Hz, 8^2-H), 3.45 (3H, s, 2^1-H), 3.72 (3H, s, 12^1-H), 3.85 (3H, s, 13^4-H), 4.19 (2H, q, *J* = 7.6 Hz, 8^1-H), 4.67 (1H, dq, *J* = 7.45Hz, 18-H), 5.23 (1H, m, 17-H), 6.21 (1H, dd, *J* = 12.6 Hz, 3^2-H), 6.54 (1H, dd, *J* = 17.85 Hz, 3^2-H), 8.92 (1H, s, 20-H), 10.16 (1H, s, 10-H), 11.01 (1H, s, 5-H), 11.48 (1H, s, 7^1-H) (Table 4.3 p65 and Figure A.3).

^{13}C NMR δ ppm, in *pyridine-d_5*, 194.9 (13^1-C), 188.0 (7^1-C), 175.6 (19-C), 91.3 (13^2-C), 174.0 (17^3-C), 144.0 (1-C), 137.5 (3-C), 138.1 (11-C), 133.5 (2-C), 129.5 (3^1-C), 123.0 (3^2-C), 108.2 (10-C), 102.6 (5-C), 95.1 (20-C), 53.6 (13^4-C), 51.5 (17-C), 51.1 (18-C), 32.7 (17^2-C), 32.4 (17^1-C), 23.3 (18^1-C), 19.9 (8^2-C), 19.6 (8^1-C), 12.5 (12^1-C), 12.4 (2^1-C) (Table 4.4 p66 and Figure A.4).

3.6.2 Purification and Characterization of Compound 25

Compound 25 was obtained from fraction III 30.5 g of first column chromatography of chloroform crude extract after repeated column chromatography using silica gel as an adsorbent, and appropriate solvent systems as indicated in Scheme 3.4. The final fraction (fraction A' 1.39 g) was chromatographed by preparative thin-layer chromatography to obtain 0.0285 g (0.031% yield based on chloroform crude extract and 0.00058% yield from starting material). This compound is soluble in chloroform and dichloromethane

UV ($CHCl_3$) λ_{max} 260, 360, 420, 545, 640, and 700 nm (Figure A.9).

FT-IR spectrum ν_{max} (KBr) 3430, 2925, 2856, 1734, 1705, and 1620 cm^{-1} (Table 4.9 p75 and Figure A.10).

^{1}H NMR δ ppm, in *pyridine-d_5*, 1.69 (3H, t, *J* = 7.7 Hz, 8^2-H), 1.83 (3H, d, *J* = 7.4 Hz, 18^1-H), 2.78, 3.02 (2H, m, 17^2-H), 2.33, 2.85 (2H, m, 17^1-H), 3.17 (3H, s, 7^1-H), 3.32 (3H, s, 2^1-H), 3.71 (2H, q, *J* = 7.7 Hz, 8^1-H), 3.84 (3H, s, 12^1-H), 4.67 (1H, q, *J* = 7.3 Hz, 18-H), 5.46 (1H, m, 17-H), 6.20 (1H, dd, *J* = 11.6, 1.1 Hz, 3^2-H), 6.41 (1H, dd, *J* = 17.8, 1.1 Hz, 3^2-H), 8.10 (1H, dd, *J* = 17.8 , 11.5 Hz, 3^1-H), 8.92 (1H, s, 20-H), 9.66 (1H, s, 5-H), 9.89 (1H, s, 10-H) (Table 4.10 p77 and Figure A.11).

^{13}C NMR δ ppm, in *pyridine-d_5*, 178.3 (16-C), 175.6 (19-C), 173.6 (17^3-C), 160.1 (13^1-C), 157.2 (6-C), 151.4 (9-C), 147.0 (8-C), 145.0 (1-C), 140.8 (12-C), 140.0 (14-C), 138.0 (4-C), 137.9 (3-C), 137.5 (7-C), 133.0 (2-C), 132.5 (11-C), 129.3 (3^1-C), 123.0 (3^2-C), 112.7 (13-C), 108.9 (10-C), 104.0 (5-C), 95.1 (20-C), 94.0 (15-C), 56.0 (17-C), 51.1 (18-C), 33.6 (17^2-C), 32.4 (17^1-C), 23.3 (18^1-C), 20.0 (8^1-C), 18.1 (8^2-C), 12.7 (12^1-C), 12.4 (2^1-C), 11.4 (7^1-C) (Table 4.11 p78 and Figure A.12).

3.6.3 Purification and Characterization of Compound 26

Compound 26 was obtained from fraction III of the first column chromatography of chloroform crude extract was further chromatographed over silica gel, using a combination of eluting solvents as indicated in Scheme 3.5. The final fraction (fraction B' 0.8 g) was chromatographed by preparative thin-layer chromatography obtain to 0.0136 g (0.015% yield based on chloroform crude extract and 0.00028% yield from starting material). This compound is soluble in chloroform and dichloromethane

UV ($CHCl_3$) λ_{max} 250, 280, 410, and 665 nm (Figure A15).

FT-IR spectrum ν_{max} (KBr) 3429, 2925, 2852, 1721, 1637, 1300, 1277, 1132, and 1095 cm^{-1} (Table 4.14 p84 and Figure A.16).

^{1}H NMR δ ppm, in *pyridine-d_5*, 1.71 (3H,t, *J* = 7.7 Hz, 8^2-H), 1.86 (3H, d, *J* = 7.2 Hz, 18^1-H), 2.70, 3.10 (2H, m, 17^1-H), 2.79, 2.99 (2H, m, 17^2-H), 3.26 (3H, s, 7^1-H), 3.38 (3H, s, 2^1-H), 3.69 (3H, s, 12^1-H), 3.75 (2H, q, *J* = 7.6 Hz, 8^1-H), 3.93 (3H, s, 13^4-H), 4.59 (1H, m, 17-H), 4.66 (1H, dq, *J* = 7.3, 2.0 Hz, 18-H), 6.21 (1H, dd, *J* = 11.6 Hz, 3^2-H), 6.40 (2H, dd, *J* = 17.8 Hz, 3^2-H), 6.90 (1H, s, 13^2-H), 8.22 (1H, dd, *J* = 17.8, 11.5 Hz, 3^1-H), 8.86 (1H, s, 20-H), 9.73 (1H, s, 5-H), 9.90 (1H, s, 10-H) (Table 4.15 p86 and Figure A.17).

^{13}C NMR δ ppm, in *pyridine-d_5*, 190.3 (13^1-C), 170.8 (13^3-C), 163.0 (16-C), 156.4 (6-C), 151.9 (9-C), 150.5 (14-C), 146.2 (8-C), 142.7 (1-C), 138.8 (11-C), 137.3 (7-C), 136.8 (3-C), 133.0 (2-C), 130.1 (13-C), 129.9 (3^1-C), 129.7 (12-C), 123.0 (3^2-C), 106.9 (15-C), 105.5 (10-C), 90.3 (5-C), 94.6 (20-C), 65.9 (13^2-C), 53.2 (13^4-C), 52.5 (17-C), 50.8 (18-C), 32.9 (17^2-C), 31.2 (17^1-C), 23.6 (18^1-C), 20.0 (8^1-C), 18.0 (8^2-C), 12.5 (2^1-C), 12.3 (12^1-C), 11.6 (7^1-C) (Table 4.16 p88 and Figure A.18).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Isolation and Separation of Chloroform Crude Extract of *Clinacanthus* nutans

Repeated chromatographic separations of chloroform crude extract led to isolation of several pure compounds. Structure determination of three isolated compounds were achieved by spectroscopic method.

Table 4.1. The Results of Separation of Chloroform Crude Extract by Column Chromatography.

Compounds	Physical appearance	Wt.	% Wt. by Wt
24	Green amorphous solid	0.0058 g	0.0039
25	Brown amorphous solid	0.0285 g	0.00058
26	Deep-green amorphous solid	0.0136 g	0.00028

4.2 Structure Elucidation of Isolated Compounds from the Leaves of *Clinacanthus* nutans

4.2.1 Structure Elucidation of Compound 24

The characteristic dark green color and the UV spectrum (Figure A.1) of

compound 24 suggested that compound 24 is a chlorophyll derivative compound (Watanabe *et al*.,1993).

The IR spectrum of compound 24 was shown in Figure A.2 and the absorption peaks were assigned as shown in Table 4.2.

Table 4.2. IR Absorption Band Assignment of Compound 24.

Wave number (cm^{-1})	Intensity	Tentative Assignment
3429	s	N-H Stretch
2925, 2852	m	C-H Stretch
1721, 1637	m	C=O Stretch
1300, 1277, 1132	m	C-H Bend

Information from ^{1}H-NMR and ^{13}C-NMR spectra showed the necessary diagnostic peaks to be identified as a pheophytin b derivative with a hydroxyl group on C-13^2. The 3H-2^1 and 3H-12^1 resonances occurred as sharp singlets at δ 3.45 and 3.72, respectively. H-5, H-10, and H-20 were found as sharp singlets at δ 11.01, 10.16, and 8.92, respectively. A vinyl group was present at C-3 as indicated by the H-3^1 doublet of doublets at δ 8.18 (J = 15.19, 11.39 Hz) and the H-3^2 (E) and the H-3^2 (Z) doublet of doublets at δ 6.54 (J = 15.19, 2.28 Hz) and 6.21 (J = 11.39, 2.28), respectively. An ethyl group was found to occur at C-8. The 2H-8^1 resonances appeared as a quartet at δ 4.19 (J = 7.65 Hz) and 3H-8^2 occurred as a triplet at δ 1.81 (J = 4.65 Hz). Similarly, as with pheophytin b, a five membered carbocyclic ring was presented at position 13. A keto group was found at C-13^1 as indicated by the fully substituted carbon resonance at δ 194.9. The C-13^2 resonances occurred at δ 91.3 as

with pheophytin b and a methyl ester was also presented at C-13^3. This was indicated by C-13^3 occurring as a fully substituted carbon resonance at δ 174.0 and the C-13^4 methyl carbon resonance being presented at δ 53.6. The H-13^4 resonance was found as a sharp singlet at δ 3.85. The characteristic signal of phytyl ester appeared at δ 5.41 (H-p2) and 4.72 (H-p1), the C-p1 and C-p2, resonances appeared at δ 61.8 and 121.3.

Compound 24 exhibited the ^{1}H-NMR and ^{13}C-NMR chemical shifts identical to 13^2-hydroxy-(13^2-*S*) pheophytin b (Nakatani *et al*., 1981). A comparison of the ^{1}H-NMR and ^{13}C-NMR chemical shifts of compound 24, 13^2-hydroxy-(13^2-*S*) pheophytin b and 13^2-hydroxy-(13^2-*S*) pheophytin a (Matsuo *et al*., 1996) are shown in Tables 4.3 and 4.4. These data indicated that compound 24 is 13^2-hydroxy-(13^2-*S*) pheophytin b (Figure 4.1).

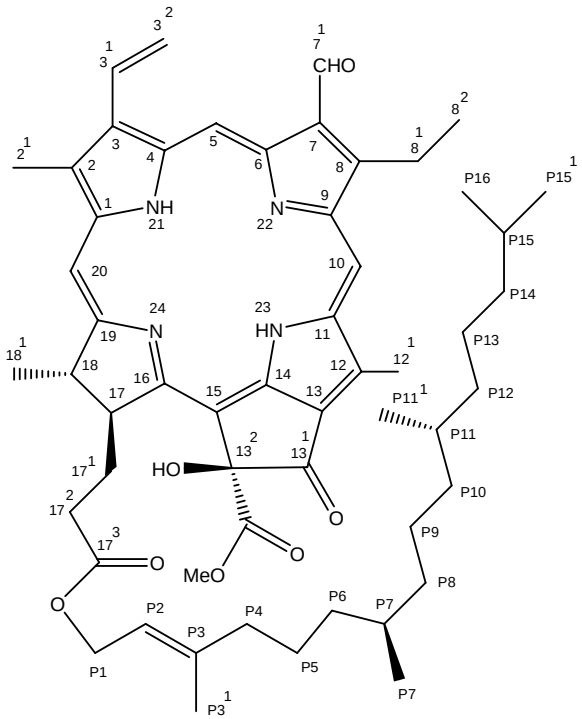


Figure 4.1. Structure of compound 24.

Table 4.3. The ^{1}H-NMR Chemical Shifts of Compound 24$^{\#}$, 13^2-Hydroxyl-(13^2-*S*) Pheophytin b$^{\&}$ and 13^2-Hydroxyl-(13^2-*S*) Pheophytin a* (Excluding Phytyl Moiety).

Proton Position	Chemical Shifts (ppm), Coupling Constant (Hz)		
	Compound 24	13^2-Hydroxyl-(13^2-S) Pheophytin b	13^2-Hydroxyl-(13^2-S) Pheophytin a
2^1	3.45 (s)	3.40	3.43 (s)
3^1	8.18 (dd, *J* = 11.65,10.9)	8.04	8.01 (dd)
3^2 (E)	6.54 (dd, *J* = 17.8, 2.5)	6.32	6.30 (dd)
3^2 (Z)	6.21 (dd, *J* = 12.6, 2.5)	6.32	6.20 (dd)
5	11.01 (s)	10.51	9.47 (s)
7^1	11.48 (s)	11.21	3.25 (s)
8^1	4.19 (q, *J* = 7.65)		3.70 (q)
8^2	1.81 (t, *J* = 7.55)		1.70 (t)
10	10.16 (s)	9.79	9.62 (s)
12^1	3.72 (s)	3.63	3.74 (s)
13^2-OH	6.52	5.59	5.53
13^4-OMe	3.85 (s)	3.75	3.62 (s)
17	5.23 (t, *J* = 6.4)	4.19	4.17 (ddd)
17^1	2.88		
17^1	2.67		
17^2	2.81		
17^2	2.46		

Table 4.3. (Continued).

Proton Position	Chemical Shifts (ppm), Coupling Constant (Hz)		
	Compound 24	13^2-Hydroxyl-(13^2-S) Pheophytin b	13^2-Hydroxyl-(13^2-S) Pheophytin a
18	4.67 (q, J = 7.3)	4.58	4.50 (dq)
18^1	1.74 (t, J = 6.95)		1.61 (d)
20	8.92 (s)	8.61	8.65 (s)
NH	0.66 (br, s), -1.33 (br, s)		

[#]500 MHz ^{1}H, pyridine d_5
[&]250 MHz ^{1}H, $CDCl_3$
* 400 MHz ^{1}H, $CDCl_3$

Table 4.4. ^{13}C-NMR Chemical Shifts of Compound 24[#] and 13^2-Hydroxyl-(13^2-*S*)-Pheophytin a* (Excluding Phytyl Moiety).

Carbon Position	Compound 24 (ppm)	13^2-Hydroxyl-(13^2-*S*)-Pheophytin a (ppm)
1	144.0	142.8
2	133.5	131.8
2^1	12.4	12.1
3	137.5	136.6
3^1	129.5	129.1
3^2	123.0	122.9
4	137.4	136.3

Table 4.4. (Continued).

Carbon Position	Compound 24 (ppm)	13^2-Hydroxyl-(13^2-*S*)-Pheophytin a (ppm)
5	102.6	98.0
6	160.2	155.4
7	138.9	136.2
7^1	188.0	11.3
8	148.3	145.8
8^1	19.6	19.5
8^2	19.9	17.4
9	151.8	151.1
10	108.2	104.3
11	138.1	137.8
12	138.8	129.4
12^1	12.5	12.3
13	128.9	127.0
13^1	194.9	192.0
13^2	91.3	89.0
13^3	174.0	172.8
13^4	53.6	53.4
14	151.1	149.8
15	91.3	107.7
16	166.8	162.5
17	51.5	51.8

Table 4.4. (Continued).

Carbon Position	Compound 24 (ppm)	13^2-Hydroxyl-(13^2-*S*)-Pheophytin a (ppm)
17^1	32.4	31.1
17^2	32.7	31.6
17^3	173.5	173.6
18	51.1	50.3
18^1	23.3	22.7
19	175.6	172.4
20	95.1	93.6

125 MHz ^{13}C, pyridine d_5
* 100 MHz ^{13}C, $CDCl_3$

Further studies of the structure of compound 24 were done by analysis of the COSY, HSQC, HMBC, and NOESY correlations (see also Tables 4.5-4.8).

The COSY spectrum (Figure A.5) indicated that one proton doublet of doublets at δ 8.18 (H-3^1) coupled to one proton doublet of doublets at δ 6.54 (H-3^2 E), and one proton doublet of doublets at 6.21 (H-3^2 Z). The signal at δ 5.41 (H-P2) correlated only with the signal at δ 4.72 (H-P1). The signal of one proton at 4.67 (H-18) showed a cross peak with a signal at δ 1.74 (H-18^1). Study of the COSY (H-H) spectrum of compound 24 led to the complete assignment of ^1H and ^{13}C shift signals as shown in Table 4.5.

Table 4.5. Observed Correlation in the COSY (H-H) Spectrum of Compound 24 (in Pyridine d_5).

δ ^{1}H-NMR (Position)	COSY (Coupled Hydrogen)
8.18 (H-3^1)	6.54 (H-3^2 E), 6.21 (H-3^2 Z)
4.19 (H-8^1)	1.81 (H-8^2)
5.23 (H-17)	2.88, 2.67 (H-17^1)
2.88, 2.67 (H-17^1)	2.81, 2.46 (H-17^2)
4.67 (H-18)	1.74 (H-18^1)
5.41 (H-P2)	4.72 (H-P1)

One Bond Correlation (HSQC) data revealed that the proton at δ 10.16 (H-10) was attached to the carbon at δ 108.2 (C-10), the proton at δ 11.01 (H-5) was attached to the carbon at δ 102.6 (C-5), the proton at δ 8.92 (H-20) was attached to the carbon at δ 95.1 (C-20), the proton at δ 8.18 (H-3^1) was attached to the carbon at δ 129.5 (C-3^1), the protons at δ 6.54 (H-3^2 E) and 6.21 (H-3^2 Z) were attached to the carbon at δ 123.0 (C-3^2), the proton at δ 5.23 (H-17) was joined with the carbon at δ 51.5 (C-17), the proton at δ 4.67 (H-18) was joined with the carbon at δ 51.1 (C-18), the proton at δ 3.72 (H-12^1) was joined with the carbon at δ 12.5 (C-12^1), the proton at δ 4.19 (H-8^1) was joined with the carbon at δ 19.6 (C-8^1), and the proton at δ 3.45 (H-2^1) was joined with the carbon at δ 12.4 (C-2^1). The protons attached to the carbons in compound 24 were assigned by the HSQC spectrum as shown in Figure A.6 and Table. 4.6.

Table 4.6. Observed Correlations in HSQC Vicinal (C-H) Spectrum of Compound 24 (in Pyridine d_5).

δ ^{1}H-NMR (Position)	HSQC (Attached Carbon)
3.45 (H-2^1)	12.4 (C-2^1)
8.18 (H-3^1)	129.5 (C-3^1)
6.54 (H-3^2 E)	123.0 (C-3^2)
6.21 (H-3^2 Z)	123.0 (C-3^2)
11.01 (H-5)	102.6 (C-5)
11.48 (H-7^1)	188.0 (C-7^1)
4.19 (H-8^1)	19.6 (C-8^1)
1.81 (H-8^2)	19.9 (C-8^2)
10.16 (H-10)	108.2 (C-10)
3.72 (H-12^1)	12.5 (C-12^1)
5.23 (H-17)	51.5 (C-17)
4.67 (H-18)	51.1 (C-18)
1.74 (H-18^1)	23.3 (C-18^1)
8.92 (H-20)	95.1 (C-20)

In the H-C long range coupling spectrum obtained by HMBC, the proton at δ 6.45 (H-3^2 E) showed cross peaks with the carbons at 129.5 (C-3^1) and 137.5 (C-3), the proton at δ 6.21 (H-3^2 Z) showed a cross peak with the carbon at 137.5 (C-3), the proton at δ 1.74 (H-18^1) showed cross peaks with the carbons at 175.6 (C-19), 51.1 (C-18), and 51.5 (C-17), the proton at δ 5.23 (H-17) showed a cross peak with the carbon at 23.3 (C-18^1), the proton at δ 3.45 (H-2^1) showed cross peaks with the

carbons at 137.5 (C-3) and 144.0 (C-1), and the proton at δ 8.92 (H-20) showed a cross peak with the carbon at 133.5 (C-2) (Figure A.7 and Table 4.7).

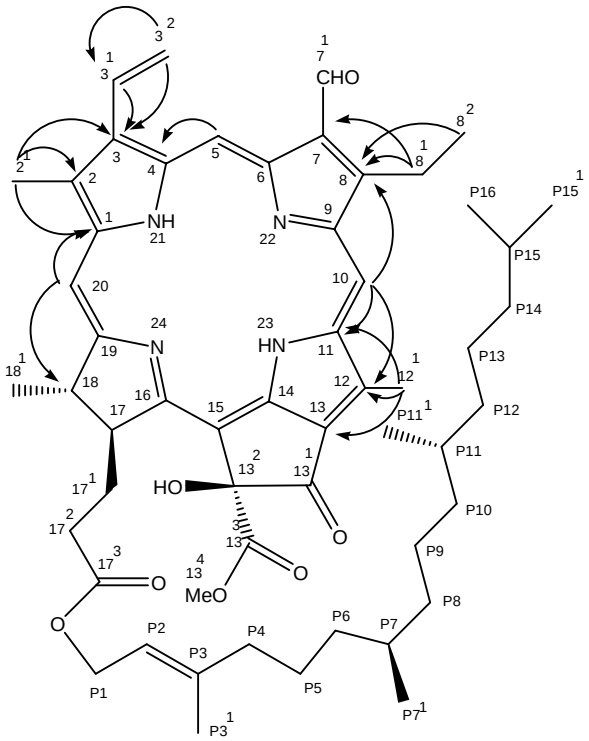


Figure 4.2. The HMBC correlations of compound 24.

Table 4.7. Observed Long Range C-H Correlations in the HMBC of Compound 24 (in Pyridine d_5).

δ ^{1}H-NMR (Position)	HMBC (Correlation Carbon)
3.45 (H-2^1)	144.0 (C-1), 133.5 (C-2), 137.5 (C-3), 138.1
6.45 (H-3^2 E)	129.5 (C-3^1), 137.5(C-3)
6.21 (H-3^2 Z)	137.5 (C-3)

Table 4.7. (Continued).

δ ^{1}H-NMR (Position)	HMBC (Correlation Carbon)
11.01 (H-5)	137.5 (C-3)
4.19 (H-8^{1})	19.9 (C-8^{2})
1.81 (H-8^{2})	19.63 (C-8^{1}), 138.1 (C-11)
10.16 (H-10)	138.1 (C-11)
3.72 (H-12^{1})	138.9 (C-12), 138.1 (C-11)
3.85 (H-13^{4})	174.0 (C-13^{3})
5.23 (H-17)	23.3 (C-18^{1})
4.67 (H-18)	23.3 (C-18^{1}), 32.4 (C-17^{1}), 32.7 (C-17^{2})
1.74 (H-18^{1})	175.6 (C-19), 51.0 (C-18), 51.4 (C-17), 53.6 (C-13^{4})
8.92 (H-20)	133.5 (C-2), 144.0 (C-1), 51.4 (C-17), 51.0 (C-18)

The NOESY correlation spectrum showed the appearance of coupling between the proton at δ 1.81 (H-8^{2}) ppm and the proton at 4.19 (H-8^{1}) ppm, coupling between the proton at δ 5.23 (H-17) ppm and the proton at δ 3.85 (H-13^{4}) ppm, coupling between the proton at δ 11.48 (H-7^{1}) ppm and the proton at δ 1.81 (H-8^{2}) ppm, 4.19 (H-8^{1}) ppm, coupling between the proton at δ 11.01 (H-5) ppm and the protons at δ 6.54 (H-3^{2} E) ppm, and 8.18 (H-3^{1}), coupling between the proton at δ 11.01 (H-5) ppm and the protons at δ 6.54 (H-3^{2} E) ppm, and 8.18 (H-3^{1}), The 3D structure of compound 24 could be drawn as shown in Figure 4.3 with the use of a molecular model. The NOE

effects obtained from the NOESY spectra could be seen as indicated in Figure A.8, Figure 4.3, and Table 4.8 which are in good agreement with the proposed structure.

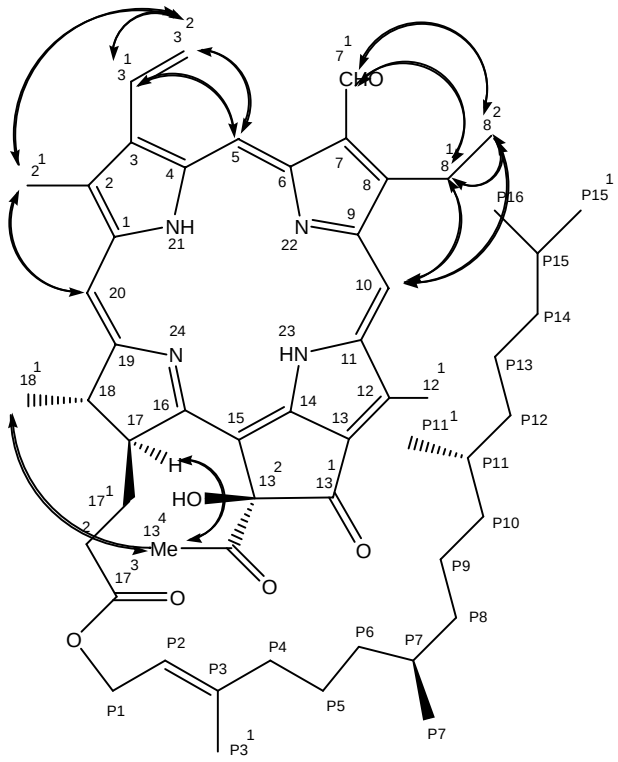


Figure 4.3. The NOESY correlations of compound 24.

Table 4.8. Observed NOE Correlation of Compound 24 (in Pyridine d_5).

δ ^{1}H-NMR (Position)	NOESY (Cross Peaks)
8.18 (H-3^1)	6.54 (H-3^2 E), 6.21 (H-3^2 Z)
6.54 (H-3^2 E)	3.45 (H-2^1), 11.48 (H-7^1)
11.01 (H-5)	6.54 (H-3^2 E), 8.18 (H-3^1)
11.48 (H-7^1)	1.81 (H-8^2), 4.19 (H-8^1)
1.81 (H-8^2)	4.19 (H-8^1)

Table 4.8. (Continued).

δ ^{1}H-NMR (Position)	NOESY (Cross Peaks)
10.16 (H-10)	1.81 (H-8^{2}), 4.19 (H-8^{1})
5.23 (H-17)	3.85 (H-13^{4})
1.74 (H-18^{1})	3.85 (H-13^{4})
8.92 (H-20)	3.45 (H-2^{1}), 3.85 (H-13^{4})

4.2.2 Structure Elucidation of Compound 25

Compound 25 was obtained as a grayish green solid. The UV spectrum (Figure A.9) showed absorptions of a chlorophyll derivative compound (Watanabe *et al*., 1993).

The IR spectrum of compound 25 is shown in Figure A.10 and the absorption peaks were assigned as shown in Table 4.9.

Table 4.9. IR Absorption Band Assignment of Compound 25.

Wave Number (cm^{-1})	Intensity	Tentative Assignment
3430	s	N-H Stretch
2925, 2856	m	C-H Stretch
1734, 1705, 1620	m	C=O Stretch

The ^{1}H-NMR spectrum (Figure A.11) of compound 25 showed the presence of the following signals: three olefinic methyl groups at δ 3.32 (s), 3.17 (s), and 3.84 (s); one vinyl group at δ 8.10 (dd, J = 11.5, 17.8 Hz); one ethyl group at δ 3.71 (q, J = 7.4 Hz); three olefinic protons at δ 9.66 (s), 9.89 (s), and 8.84 (s); two aliphatic protons at δ 5.46 (m), 4.67 (q, J =7.3 Hz), and one secondary methyl group at δ 1.74 (d, J = 7.4 Hz).; a $–CH_2CH_2C$ (=O)– group and a phytyl group. The ^{13}C-NMR spectrum of compound 25 displayed three carbonyl carbon signals at δ 160.1, 165.0, and 173.6. The ^{1}H-NMR and ^{13}C-NMR spectra of compound 25 closely matched that of purpurin 18 (Chan *et al*., 1999). The difference of the ^{1}H-NMR and ^{13}C-NMR spectra between compound 25 and purpurin 18, a methyl ester signal at δ_H 3.77 (s) and δ_C 51.63 (q) in purpurin 18 were replaced by the characteristic signals of phytyl in compound 25 at

δ_H: 5.46 (H-P2) and 4.76 (H-P1); δ_C: 117.8 (C-P2), 61.4 (C-P1) and 142.7 (C-P3). Thus, compound 25 was the phytyl ester of purpurin 18.

From the above results, compound 25 exhibited the ^{1}H-NMR and ^{13}C-NMR chemical shifts identical to purpurin 18 phytyl ester (Chan *et al*., 1999). A comparison of the ^{1}H-NMR and ^{13}C-NMR chemical shifts of compound 25, purpurin 18 phytyl ester and purpurin 18 (Watanabe *et al*., 1993). are shown in Tables 4.10 and 4.11. These data indicated that compound 25 is as purpurin 18 phytyl ester (Figure 4.4).

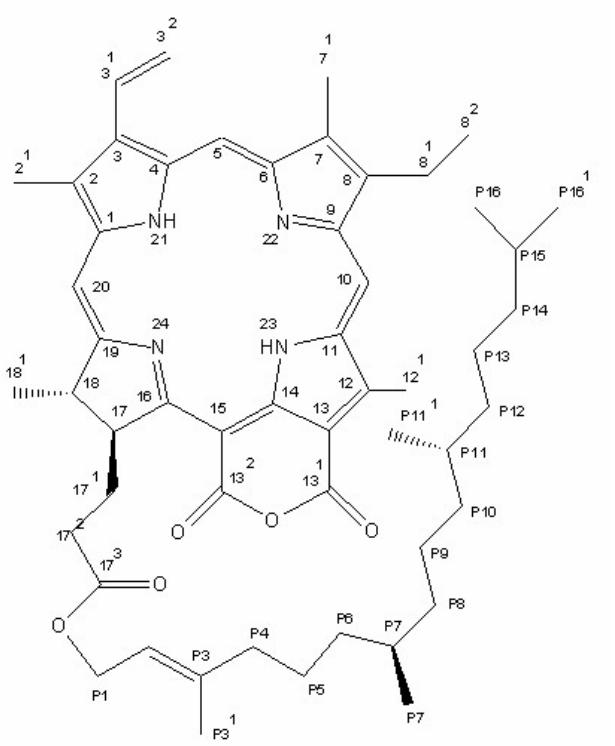


Figure 4.4. Structure of compound 25.

Table 4.10. The ^{1}H-NMR Chemical Shifts of Compound 25[#], purpurin 18 phylyl ester[*], and Purpurin 18* (Excluding Phytyl Moiety).

Proton Position	Chemical Shifts (ppm), Coupling Constant (Hz)		
	Compound 25	Purpurin 18 phylyl Ester	Purpurin 18
2^{1}	3.32 (s)	3.34 (s)	3.34 (s)
3^{1}	8.10 (dd, *J* = 17.8, 11.5)	7.87 (dd, *J* = 17.6, 11.6)	7.88 (dd)
3^{2} (E)	6.41 (dd, *J* = 17.8, 1.1)	6.28 (d, *J* = 17.6)	6.29 (dd)
3^{2} (Z)	6.20 (dd, *J* = 11.6, 1.1)	6.18 (d, *J* = 11.6)	6.19 (dd)
5	9.66 (s)	9.28 (s)	9.37 (s)
7^{1}	3.17 (s)	3.10 (s)	3.16 (s)
8^{1}	3.71 (q, *J* = 7.7)	3.55 (q, *J* = 7.4)	3.64 (q)
8^{2}	1.69 (t, *J* = 7.7)	1.61 (t, *J* =7.4)	1.66 (t)
10	9.89 (s)	9.43 (s)	9.60 (s)
12^{1}	3.84 (s)	3.72 (s)	3.78 (s)
17	5.46 (m)	5.20 (m)	5.19 (dd)
17^{1}	2.33 (m)	2.04 (m)	1.99 (m)
17^{1}	2.85 (m)	2.46 (m)	2.5 (m)
17^{2}	2.78 (m)	2.46 (m)	2.5 (m)
17^{2}	3.02 (m)	2.74 (m)	2.79 (m)
18	4.67 (q, *J* = 7.3)	4.40 (m)	4.39 (q)
18^{1}	1.74 (d, *J* = 7.4)	1.74 (d, *J* =7.2)	1.74 (d)
20	8.84 (s)	8.56 (s)	8.57 (s)

Table 4.10. (Continued).

Proton Position	Chemical Shifts (ppm), Coupling Constant (Hz)		
	Compound 25	Purpurin 18 phylyl Ester	Purpurin 18
NH	0.11 (br s)	0.11 (br. s)	0.23 (br. s)
NH	0.38 (br s)	-0.16 (br. s)	-0.07 (br. s)

[#] 500 MHz ^{1}H, pyridine d_5
*400 MHz ^{1}H, $CDCl_3$

Table 4.11. ^{13}C-NMR Chemical Shifts of Compound 25[#] and Known Purpurin 18* (Excluding Phytyl Moiety).

Carbon Position	Compound 25 (ppm)	Purpurin 18 (ppm)
1	145.0	144.1
2	133.0	131.8
2^1	12.4	11.9
3	137.9	137.7
3^1	129.3	129.9
3^2	123.0	128.3
4	138.0	136.6
5	104.0	103.0
6	157.2	156.1
7	137.5	136.6
7^1	11.4	10.9
8	147.0	145.9

Table 4.11. (Continued).

Carbon Position	Compound 25 (ppm)	Purpurin 18 (ppm)
8^1	20.0	19.3
8^2	18.1	17.3
9	151.4	149.9
10	108.9	107.5
11	132.5	139.1
12	140.8	131.5
12^1	12.7	12.2
13	112.7	111.4
13^1	160.1	177.2
13^2	165.0	176.4
14	140.0	164.1
15	94.0	92.5
16	178.3	159.3
17	56.0	55.0
17^1	32.4	31.0
17^2	33.6	32.2
17^3	173.6	177.5
18	49.8	49.2
18^1	24.4	23.8
19	177.7	176.6
20	96.2	95.0

[&] 125 MHz ^{13}C, pyridine d_5, [#] 100 MHz ^{13}C, $CDCl_3$

Further studies of the structure were done by analysis of HSQC and HMBC correlations (see also Tables 4.12 and 4.13).

One Bond Correlation (HSQC) data revealed that the proton at δ 9.89 (H-10) was attached to the carbon at δ 108.9 (C-10), the proton at δ 9.66 (H-5) was attached to the carbon at δ 104.0 (C-5), the proton at δ 8.84 (H-20) was attached to the carbon at δ 96.2 (C-20), the proton at δ 8.10 (H-3^1) was attached to the carbon at δ 129.3 (C-3^1), the protons at δ 6.41 (H-3^2 E) and 6.20 (H-3^2 E) were attached to the carbon at δ 123.0 (C-3^2), the proton at δ 5.46 (H-17) was joined with the carbon at δ 56.0 (C-17), the proton at δ 4.67 (H-18) was joined with the carbon at δ 49.8 (C-18), the proton at δ 3.84 (H-12^1) was joined with the carbon at δ 12.7 (C-12^1), the proton at δ 3.71 (H-8^1) was joined with the carbon at δ 20.0 (C-8^1), and the proton at δ 3.32 (H-2^1) was joined with the carbon at δ 12.4 (C-2^1). The protons attached to the carbons in compound 25 were assigned by HMQC spectrum as shown in Figure A.13 and Table 4.12.

Table 4.12. Observed Correlations in HSQC Vicinal (C-H) Spectrum of Compound 25 (in Pyridine d_5).

δ ^{1}H-NMR (position)	HSQC (attached carbon)
3.32 (H-2^1)	12.4 (C-2^1)
8.10 (H-3^1)	129.3 (C-3^1)
6.41 (H-3^2 E)	123.0 (C-3^2)
6.20 (H-3^2 Z)	123.0 (C-3^2)
9.66 (H-5)	104.0 (C-5)

Table 4.12. (Continued).

δ ^{1}H-NMR (position)	HSQC (attached carbon)
3.17 (H-7^{1})	11.4 (C-7^{1})
3.71 (H-8^{1})	20.0 (C-8^{1})
9.89 (H-10)	108.9 (C-10)
3.84 (H-12^{1})	12.7 (C-12^{1})
5.46 (H-17)	56.0 (C-17)
4.51 (H-18)	49.8 (C-18)
1.83 (H-18^{1})	24.4 (C-18^{1})
8.84 (H-20)	96.2 (C-20)

In the H-C long range coupling spectrum obtained by HMBC the proton at δ 9.89 (H-10) showed cross peaks with the carbons at 140.8 (C-12), and 174.0 (C-8), the proton at δ 1.86 (H-18^{1}) showed a cross peak with the carbon at 177.7 (C-19), the proton at δ 8.84 (H-20) showed a cross peaks with the carbons at 144.8 (C-1) and 133.0 (C-2), the proton at δ 6.41 (H-3^{2} E) showed a cross peaks with the carbons at 129.3 (C-3^{1}) and 138.0 (C-4), the proton at δ 5.46 (H-17) showed a cross peaks with the carbons at 178.3 (C-16), 32.4 (C-17^{1}), 23.3 (C-18^{1}), and 177.7 (C-19), and the proton at δ 4.67 (H-18) showed cross peaks with the carbons at 178.3 (C-16), 32.4 (C-17^{1}), and 177.7 (C-19) (Figure A.14 , Figure 4.5, and Table 4.13).

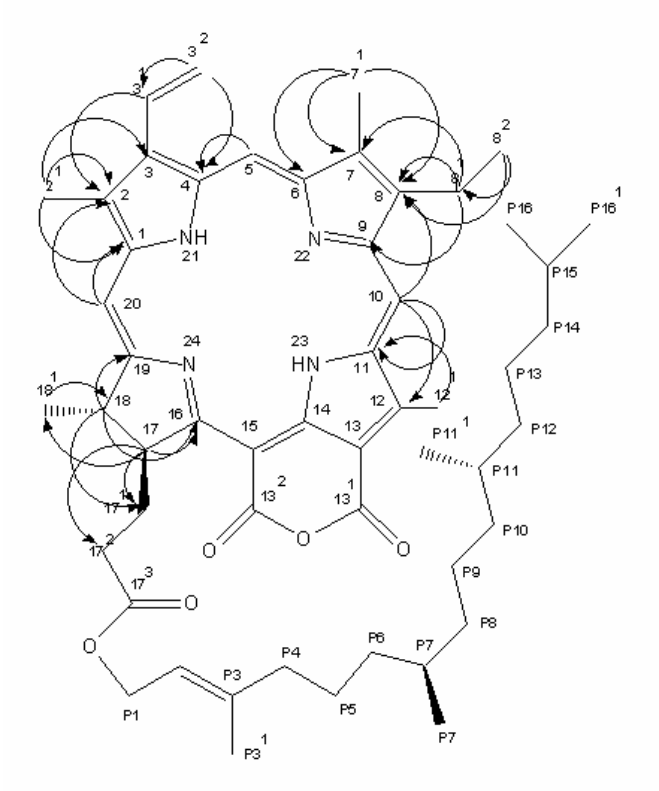


Figure 4.5. The HMBC correlations of compound 25.

Table 4.13. Observed Long Range C-H Correlation in the HMBC of Compound 25 (in Pyridine d_5).

δ ^{1}H-NMR (position)	HMBC correlation carbon
3.32 (H-2^1)	144.8 (C-1), 133.0 (C-2), 137.9 (C-3)
8.10 (H-3^1)	133.0 (C-2)
6.41 (H-3^2 E)	129.3 (C-3^1), 138.0 (C-4)
6.20 (H-3^2 Z)	138.0 (C-4)
9.66 (H-5)	138.0 (C-4)
3.17 (H-7^1)	137.5 (C-7), 147.0 (C-8), 157.2 (C-6)

Table 4.13. (Continued).

δ ^{1}H-NMR (position)	HMBC correlation carbon
3.71 (H-8^{1})	137.5 (C-7), 147.0 (C-8), 151.4 (C-9)
1.69 (H-8^{2})	147.0 (C-8), 20.0 (C-8^{1})
9.89 (H-10)	132.5 (C-11), 140.8 (C-12), 174.0 (C-8)
3.84 (H-12^{1})	95.1 (C-11), 140.8 (C-12)
5.46 (H-17)	178.3 (C-16), 56.0 (C-17), 32.4 (C-17^{1}), 33.6 (C-17^{2}), 23.3 (C-18^{1}), 175.6 (C-19)
4.51 (H-18)	178.3 (C-16), 32.4 (C-17^{1}), 175.6 (C-19)
1.83 (H-18^{1})	175.6 (C-19), 51.1 (C-18), 56.0 (C-17)
8.84 (H-20)	144.8 (C-1), 133.0 (C-2)

4.2.3 Structure Elucidation of Compound 26

The characteristic dark green color and its UV spectrum (Figure A.15) suggested that compound 26 is a chlorophyll derivative compound (Watanabe *et al*., 1993).

The IR spectrum of compound 26 is shown in Figure A.16 and the absorption peaks were assigned as shown in Table 4.14.

Table 4.14. IR Absorption Bands Assignment of Compound 26.

Wave Number (cm^{-1})	Intensity	Tentative Assignment
3429	s	N-H Stretch
2925, 2852	m	C-H Stretch
1721, 1637	m	C=O Stretch
1300, 1277, 1132, 1095	m	C-H Bend

Information from ^{1}H-NMR and ^{13}C-NMR spectra showed the necessary diagnostic peaks to be identified as a pheophorbide a. The 3H-7^1, 3H-2^1, and 3H-12^1 resonances occurred as sharp singlets at δ 3.26, 3.38, and 3.69, respectively. H-5, H-10, and H-20 were found as sharp singlets at δ 9.73, 9.90, and 8.86, respectively. A vinyl group was present at C-3 as indicated by the H-3^1 doublet of doublets at δ 8.22 (J = 11.5, 17.8 Hz) and the H-3^2(E) and the H-3^2 (Z) doublet of doublets at δ 6.40 (J = 17.8 Hz) and 6.21 (J = 11.6 Hz), respectively. An ethyl group was found to occur at C-8. The 2H-8^1 resonances appeared as quartet at δ 3.75 (J = 7.6 Hz) and 3H-8^2 occurred as a triplet at δ 1.71 (J = 7.7 Hz). Similarly, as with pheophytin b, a five membered carbocyclic ring was present at position 13. A keto group was found at C-

13^1, as indicated by the fully substituted carbon resonance at δ 190.3. The C-13^2 resonances occurred at δ 65.9 as with pheophorbide a and a methyl ester was also present at C-13^3. This was indicated by C-13^3 occurring as a fully substituted carbon resonance at δ 170.8 and the C-13^4 methyl carbon resonance being present at δ 53.2. The H-13^4 resonance was found as a sharp singlet at δ 3.93.

Compound 26 exhibited the ^{1}H-NMR and ^{13}C-NMR chemical shifts identical to pheophobide a (Ohshima *et al*., 1994). A comparison of the ^{1}H-NMR and ^{13}C-NMR chemical shifts of compound 26, pheophobide a and pheophorbide a methyl ester (Sakata *et al*., 1990) are shown in Tables 4.15 and 4.16. These data indicated that compound 26 is pheophobide a (Figure 4.6).

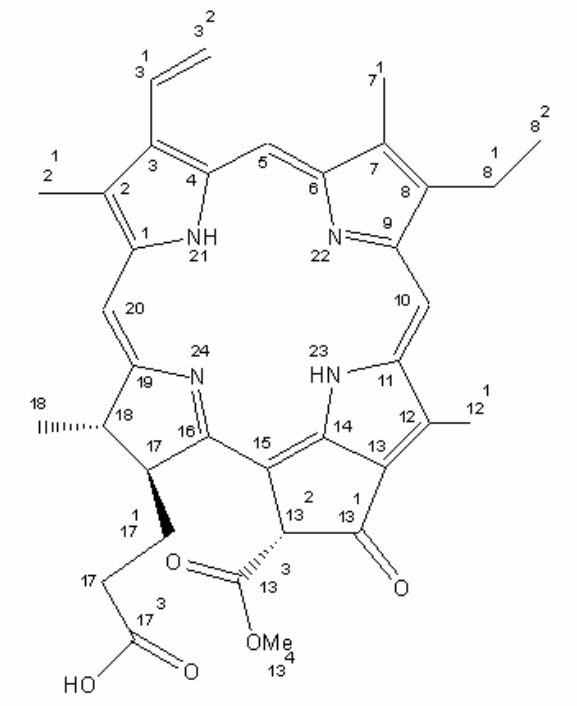


Figure 4.6. Structure of compound 26.

Table 4.15. The ^{1}H-NMR Chemical Shifts of Compound 26[&] and Pheophobide a[*] and Pheophobide a Methyl Ester*.

Proton Position	Chemical Shifts (ppm), Coupling Constant (Hz)		
	Compound 26	Pheophobide a	Pheophorbide a Methyl Ester
2^{1}	3.38 (s)	3.34 (s)	3.41 (s)
3^{1}	8.22 (dd, *J* = 17.8, 11.5)	7.89 (dd, *J* = 17.8, 11.5)	8.00 (dd, *J* = 18.0, 11.4)
3^{2} (E)	6.40 (dd, *J* = 17.8, 1.1)	6.11 (dd, *J* = 11.5, 1.5)	6.30 (dd, *J* = 18.0, 1.5)
3^{2} (Z)	6.21 (dd, *J* = 11.6, 1.1)	6.21 (dd, *J* = 17.8, 1.5)	6.19 (dd, *J* = 11.5, 1.5)
5	9.73 (s)	9.25 (s)	9.40 (s)
7^{1}	3.26 (s)	3.13 (s)	3.24 (s)
8^{1}	3.75 (q, *J* = 7.6)	3.57 (q, *J* = 7.3)	3.70 (q, *J* = 7.5)
8^{2}	1.71 (t, *J* = 7.7)	1.63 (t, *J* = 7.5)	1.70 (t, *J* = 7.5)
10	9.90 (s)	9.40	6.25 (s)
12^{1}	3.69 (s)	3.62 (s)	3.69 (s)
13^{2}	6.90 (s)	6.23 (s)	1.70 (t)
13^{4}	3.93 (s)	3.85 (s)	3.88 (s)
17	4.59 (m)	4.18 (m)	4.21, (dddt, *J* = 9.0, 3.2, 1.6, 0.2)

Table 4.15. (Continued).

Proton Position	Chemical Shifts (ppm), Coupling Constant (Hz)		
	Compound 26	Pheophobide a	Pheophobide a Methyl Ester
17[1]	3.10 (m)	2.28 (m)	2.64 (dddd, *J* = 14.0, 9.9, 6.7, 3.2)
17[1]	2.70 (m)		2.32 (dddd, *J* = 14.0, 9.3, 9.0, 5.2)
17[2]	2.99 (m)	2.59 (m)	2.52 (dddd, *J* = 15.7, 9.3, 6.7, 0.2)
17[2]	2.79 (m)		2.23 (dddd, *J* = 14.0, 9.3, 9.0, 5.2)
18	4.66 (q)	4.43 (dq, *J* =7.3, 2.0)	4.45 (dq, *J* = 7.3, 1.6)
18[1]	1.86 (d, *J* = 7.2)	1.80 (d, *J* =7.3)	1.81 (d, *J* = 7.3)
20	8.86 (s)	8.52 (s)	8.56 (s)
NH	0.89 (br. s), -1.30 (br. s)		0.56 (br, s), -1.61(br, s)

& 500 MHz ^{1}H, pyridine d_5
* 400 MHz ^{1}H, $CDCl_3$

Table 4.16. ^{13}C-NMR Chemical Shifts of Compound 26[&] and of Pheophobide a Methyl Ester*.

Carbon Position	compound 26 (ppm)	Pheophobide a Methyl Ester (ppm)
1	142.7	142.0
2	133.0	131.8
2^1	12.5	12.0
3	136.8	136.5
3^1	129.9	129.0
3^2	123.0	122.6
4	137.3	136.2
5	90.3	97.5
6	156.4	155.6
7	137.3	136.2
7^1	11.6	11.2
8	146.2	145.1
8^1	20.0	19.3
8^2	18.0	17.3
9	152.0	150.9
10	105.5	104.3
11	138.8	137.9
12	129.7	129.0
12^1	12.3	12.0

Table 4.16. (Continued).

Carbon Position	compound 26 (ppm)	Pheophobide a Methyl Ester (ppm)
13	130.1	129.0
13^1	190.3	189.6
13^2	65.9	64.8
13^3	170.8	69.6
13^4	53.2	51.6
14	150.5	149.7
15	106.9	105.3
16	163.0	161.2
17	52.5	51.2
17^1	31.2	31.1
17^2	32.9	29.9
17^3	177.8	173.3
18	50.8	50.2
18^1	23.6	23.1
19	173.8	172.2
20	94.6	93.1

& 125 MHz ^{13}C, pyridine d_5
* 100 MHz ^{13}C, $CDCl_3$

Further studies of the structure of compound 26 were done by analysis of COSY, HSQC, and HMBC correlations (see also Figures A.19-21 and Tables 4.17-4.19).

The COSY spectrum (Figure A.19) gave indication that one proton doublet of doublets at δ 8.22 (H-3^1) coupled to one proton doublet of doublets at δ 6.40 (H-3^2 E), and one proton doublet of doublets at δ 6.21 (H-3^2 Z). The signal at δ 3.75 (H-8^1) correlated only with the signal at δ 1.71 (H-8^2). The signal of one proton at δ 4.66 (H-18) showed a cross peak with the signal at δ 4.59 (H-17). Study of the COSY (H-H) spectrum led to the complete assignment of ^{1}H and ^{13}C shift signals as shown in Table 4.17.

Table 4.17. Observed Correlation in the COSY (H-H) Spectrum of Compound 26 (in Pyridine d_5).

δ ^{1}H-NMR (position)	COSY (coupled hydrogen)
8.82 (H-3^1)	6.40 (H-3^2 E), 6.21 (H-3^2 Z)
3.75 (H-8^1)	1.71 (H-8^2)
4.59 (H-17)	3.10, 2.70 (H-17^1)
4.66 (H-18)	4.59 (H-17), 1.86 (H-18^1)

One Bond Correlation (HSQC) data revealed that the proton at δ 9.90 (H-10) was attached to the carbon at δ 105.5 (C-10), the proton at δ 9.73 (H-5) was attached to the carbon at δ 90.3 (C-5), the proton at δ 8.86 (H-20) was attached to the carbon at δ 94.6 (C-20), the proton at δ 8.82 (H-3^1) was attached to the carbon at δ 129.9 (C-3^1), the protons at δ 6.40 (H-3^2 E) and 6.21 (H-3^2 Z) were attached to the carbon at δ 123.0 (C-3^2), the proton at δ 4.59 (H-17) was joined with the carbon at δ 52.5 (C-17), the proton at δ 4.66 (H-18) was joined with the carbon at δ 50.8 (C-18), the proton at δ 3.69 (H-12^1) was joined with the carbon at δ 12.3 (C-12^1), the proton at δ 3.75 (H-

8^1) was joined with the carbon at δ 20.0 (C-8^1), and the proton at δ 3.38 (H-2^1) was joined with the carbon at δ 12.5 (C-2^1).The protons attached to the carbons in compound 26 were assigned by HSQC spectrum as shown in Figure A.20 and Table 4.18.

Table 4.18. Observed Correlation in HSQC Vicinal (C-H) Spectrum of Compound 26 (in Pyridine d_5).

δ ^{1}H-NMR (position)	HSQC (attached carbon)
3.38 (H-2^1)	12.46 (C-2^1)
9.90 (H-10)	105.5 (C-10)
8.22 (H-3^1)	129.9 (C-3^1)
6.40 (H-3^2 E)	123.0 (C-3^2)
6.21 (H-3^2 Z)	123.0 (C-3^2)
9.73 (H-5)	90.3 (C-5)
3.26 (H-7^1)	11.6 (C-7^1)
3.75 (H-8^1)	20.0 (C-8^1)
3.69 (H-12^1)	12.3 (C-12^1)
4.59 (H-17)	52.5 (C-17)
4.66 (H-18)	50.8 (C-18)
1.86 (H-18^1)	23.6 (C-18^1)
8.86 (H-20)	94.6 (C-20)

In the H-C long range coupling spectrum obtained by HMBC, the proton at δ 6.90 (H-13^2) showed cross peaks with the carbons at 106.9 (C-15) and 150.5 (C-14), the

proton at δ 1.86 (H-18[1]) showed a cross peak with the carbon at 173.8 (C-19), the proton at δ 9.90 (H-10) showed a cross peaks with the carbons at δ 146.2 (C-8), 50.8 (C-18), and 52.5 (C-17), the proton at δ 9.73 (H-5) showed a cross peak with the carbon at 136.8 (C-3), the proton at δ 8.86 (H-20) showed a cross peaks with the carbons at 133.0 (C-2), 142.7 (C-1), and 50.8 (C-18), and the proton at δ 8.22 (H-3[1]) showed cross peaks with the carbons at 133.0 (C-2), 137.3 (C-7), and 137.3 (C-4) (see also Figure A.21, Figure 4.6, and Table 4.19).

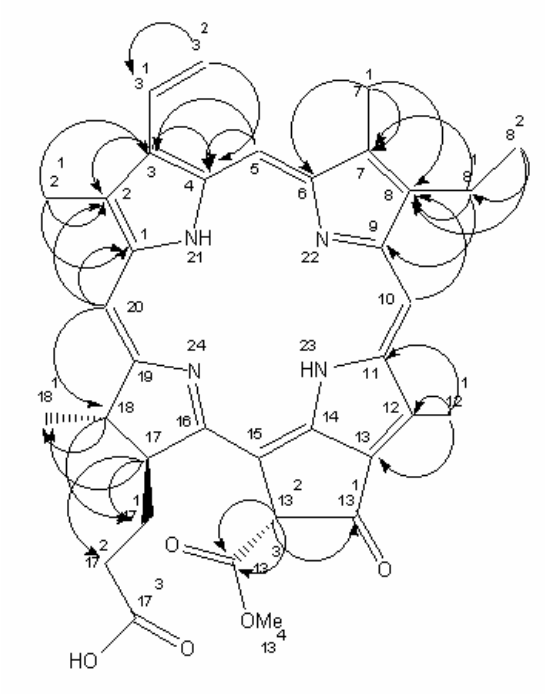


Figure 4.7. The HMBC correlations of compound 26.

Table 4.19. Observed Long range C-H Correlation in the HMBC of Compound 26 (in Pyridine d_5).

δ ^{1}H-NMR (Position)	HMBC (Correlation Carbon)
3.38 (H-2^1)	142.4 (C-1), 133.0 (C-2), 136.8 (C-3)
8.22 (H-3^1)	133.0 (C-2), 123.0 (C-3^2), 137.3 (C-7), 137.27 (C-4)
6.40 (H-3^2 E)	129.7 (C-3^1), 137.3 (C-4), 137.3 (C-7)
6.21 (H-3^2 Z)	137.3 (C-7), 137.3 (C-4)
9.73 (H-5)	136.76 (C-3), 137.27 (C-4)
3.26 (H-7^1)	137.3 (C-4), 156.4 (C-6), 137.3 (C-7), 146.2 (C-8)
3.75 (H-8^1)	146.2 (C-8), 151.7 (C-9), 137.3 (C-7), 137.27 (C-4)
1.71 (H-8^2)	146.2 (C-8), 20.0 (C-8^1)
9.90 (H-10)	146.2 (C-8), 50.8 (C-18), 52.5 (C-17)
3.69 (H-12^1)	138.8(C-11), 129.7(C-12), 130.0(C-13)
6.90 (H-13^2)	190.3 (C-13^1), 170.8 (C-13^3), 106.9 (C-15), 150.5 (C-14)
3.93 (H-13^4)	170.8 (C-13^3)
4.59 (H-17)	173.8 (C-19), 23.6 (C-18^1), 31.2 (C-7^1), 32.9 (C-17^2)
1.86 (H-18^1)	173.8 (C-19)
4.66 (H-18)	31.2 (C-17^1), 23.6 (C-18^1), 173.8 (C-19)
8.86 (H-20)	133.0 (C-2), 142.7 (C-1), 50.8 (C-18)

CHAPTER V

CONCLUSION

The dried powdered leaves of *C. nutans* were extracted consecutively with hexane and chloroform. The chloroform crude extract was separated by column chromatography and further purified by preparative thin layer chromatography giving three pure compounds. Structures of isolated compounds were elucidated on the basis of NMR spectral analysis, including DEPT, COSY, HMQC, HMBC, and NOESY with literature values. Three isolated compounds were identified as chlorophyll a and chlorophyll b related compounds, 13^2–hydroxyl-(13^2–*S*)-pheophytin b (24), purpurin 18 phytyl ester (25) and pheophobide a (26), which have not been previously reported in this species.

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APPENDIX

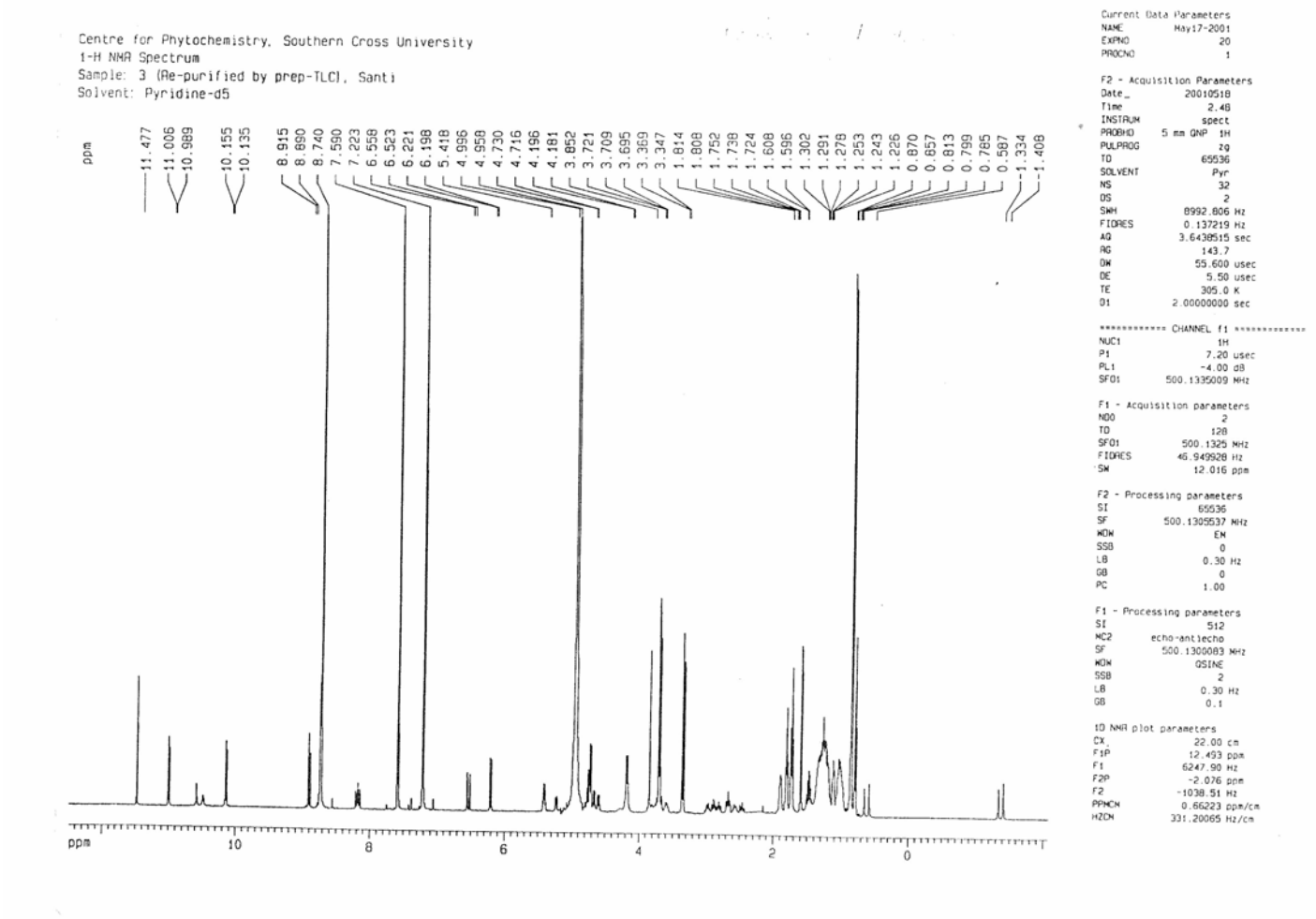

Figure A.3. 500 MHz ^{1}H-NMR spectrum of compound 24 in pyridine d_5.

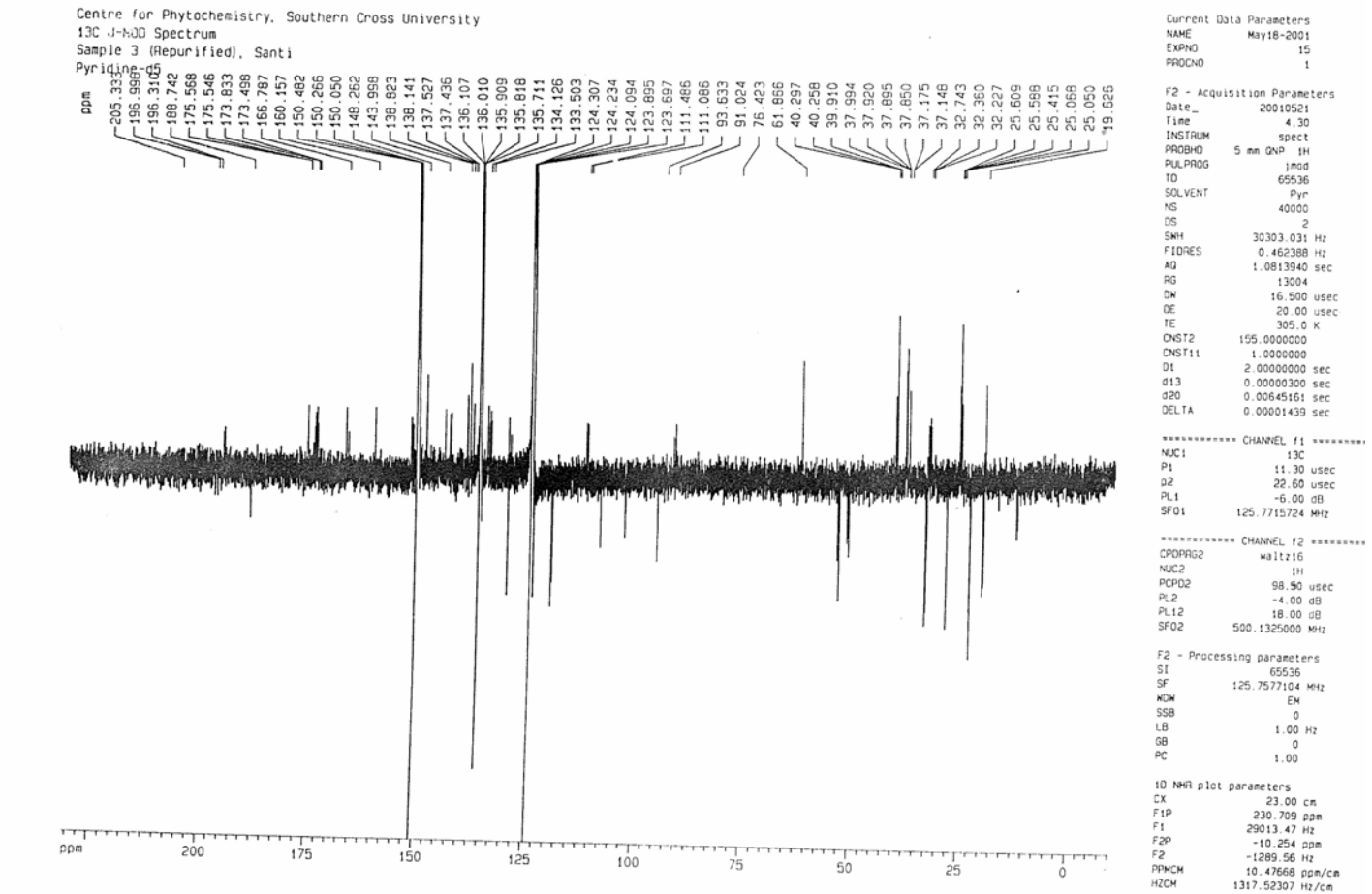

Figure A.4. 125 MHz ^{13}C- NMR spectrum of compound 24 in pyridine d_5.

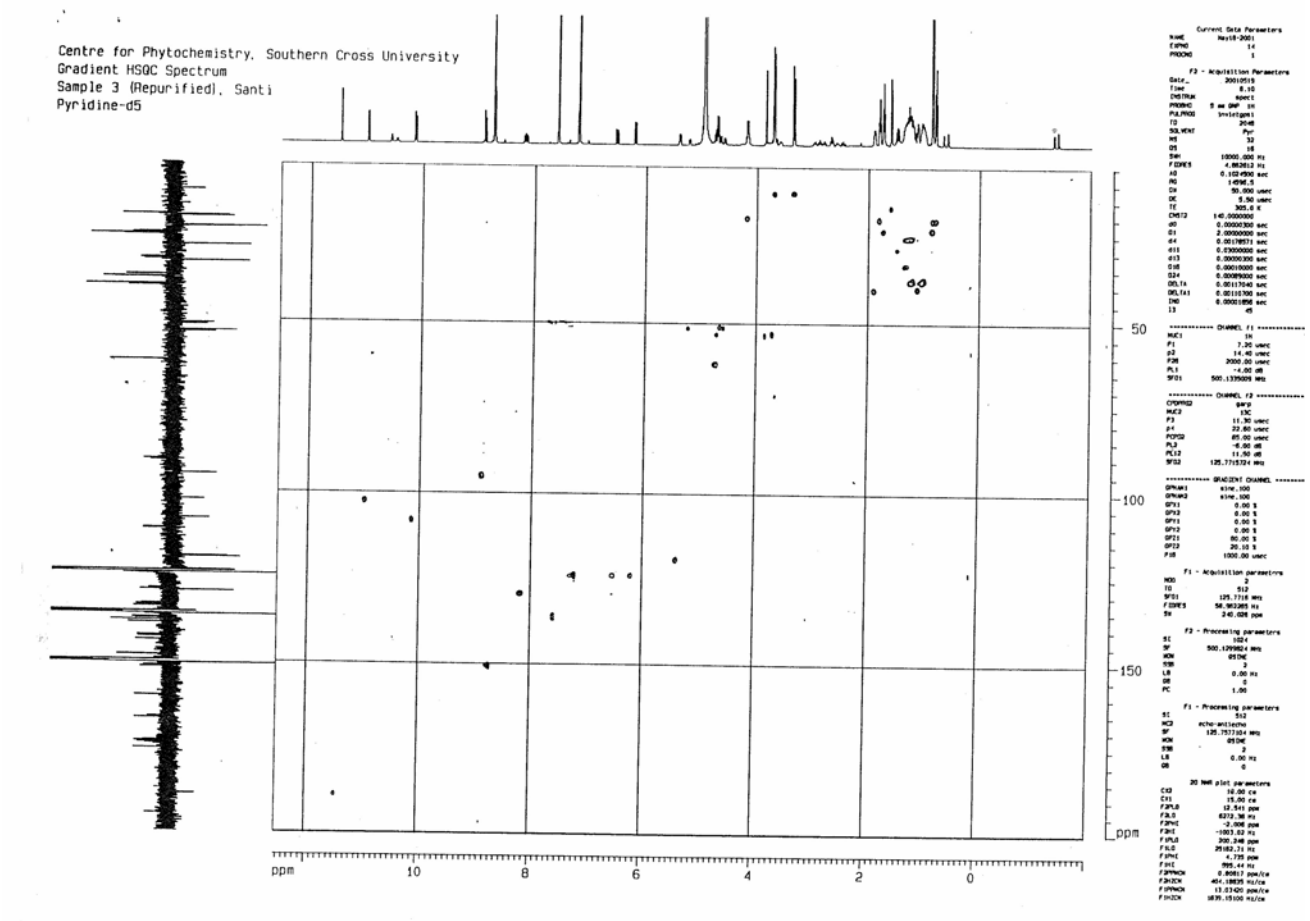

Figure A.6. Gradient HSQC spectrum of compound 24.

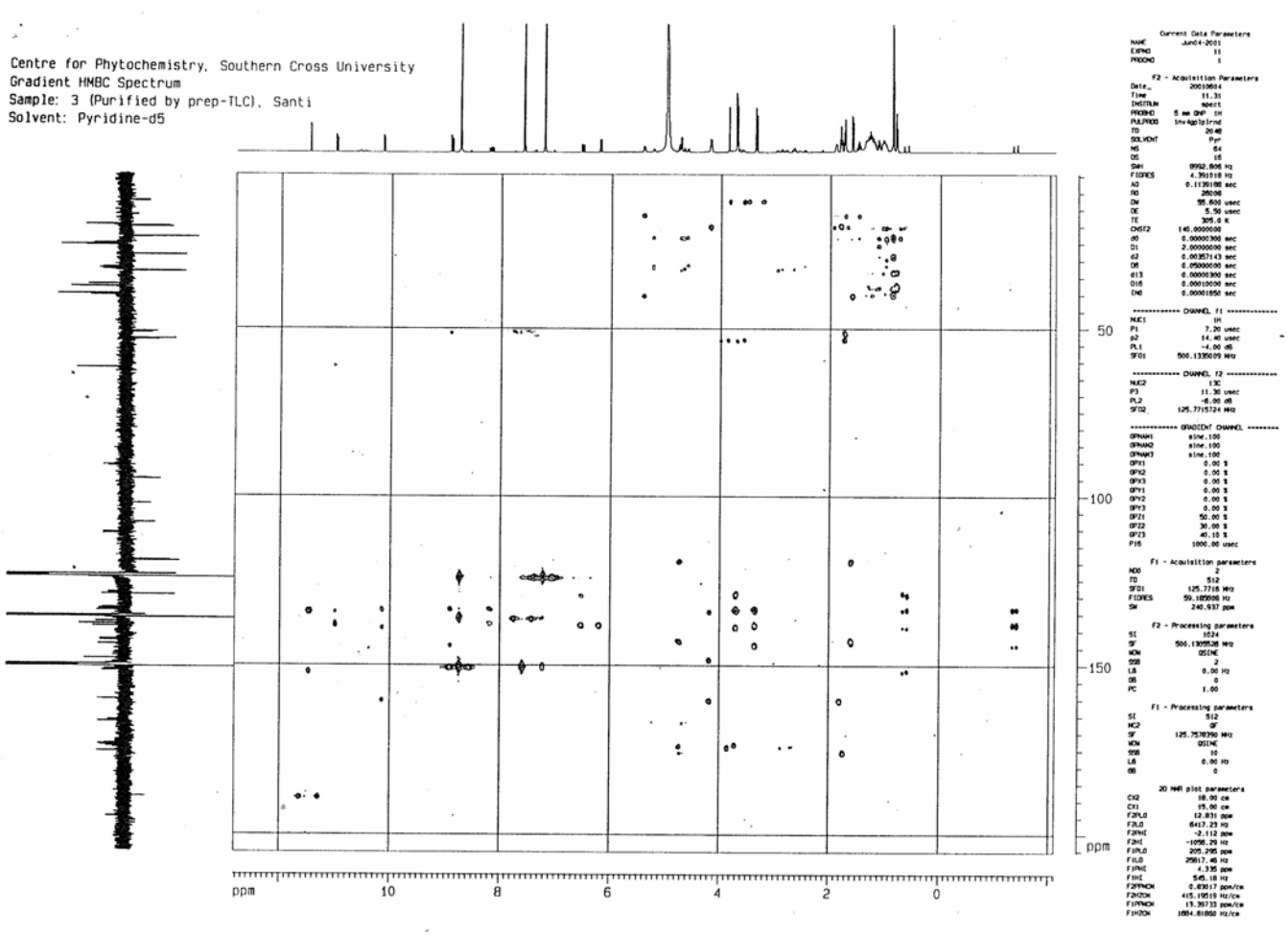

Figure A.7. Gradient HMBC spectrum of compound 24.

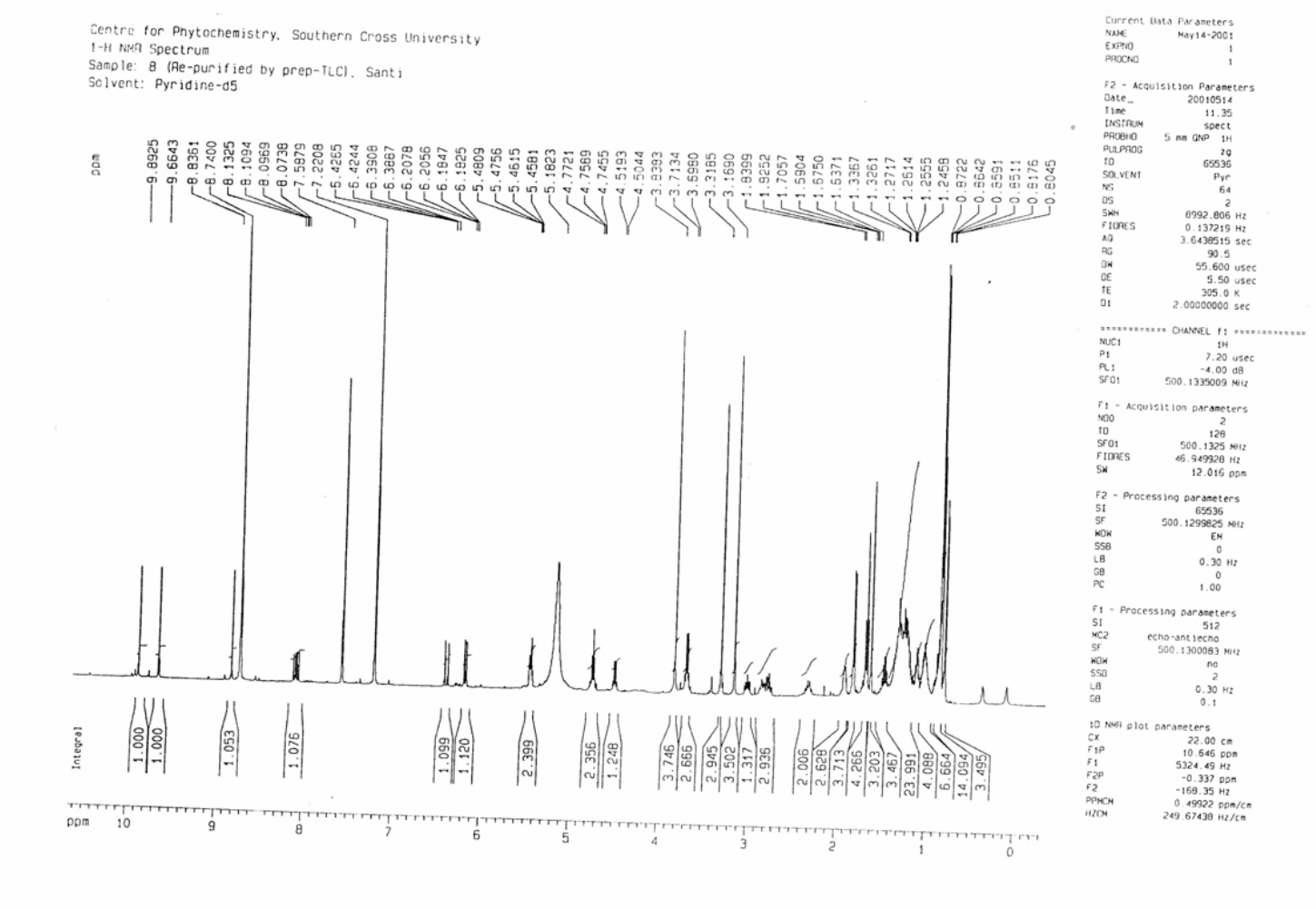

Figure A.11. 500 MHz ^{1}H-NMR spectrum of compound 25 in pyridine d_5.

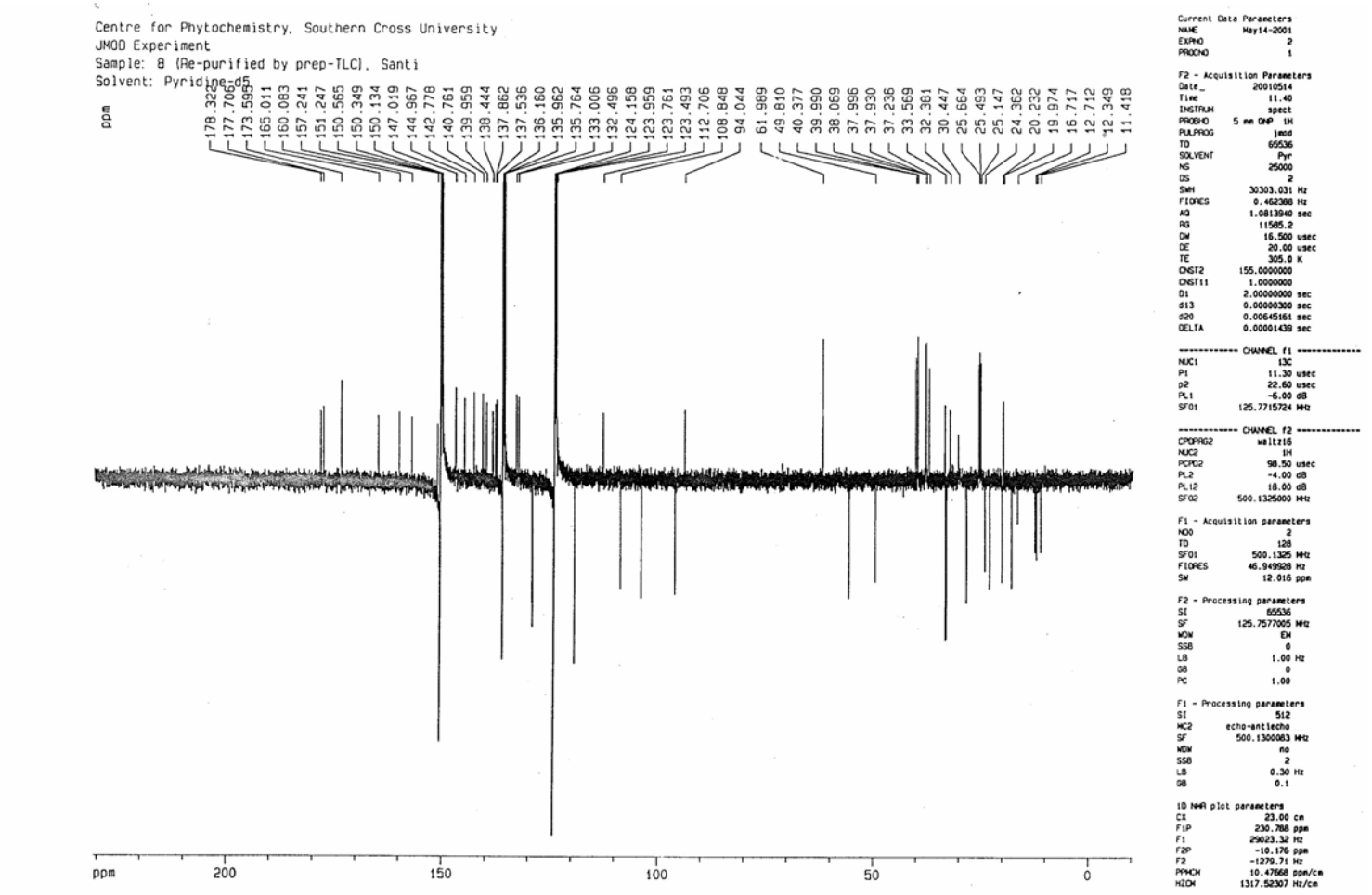

Figure A.12. 125 MHz ^{13}C- NMR spectrum of compound 25 in pyridine d_5.

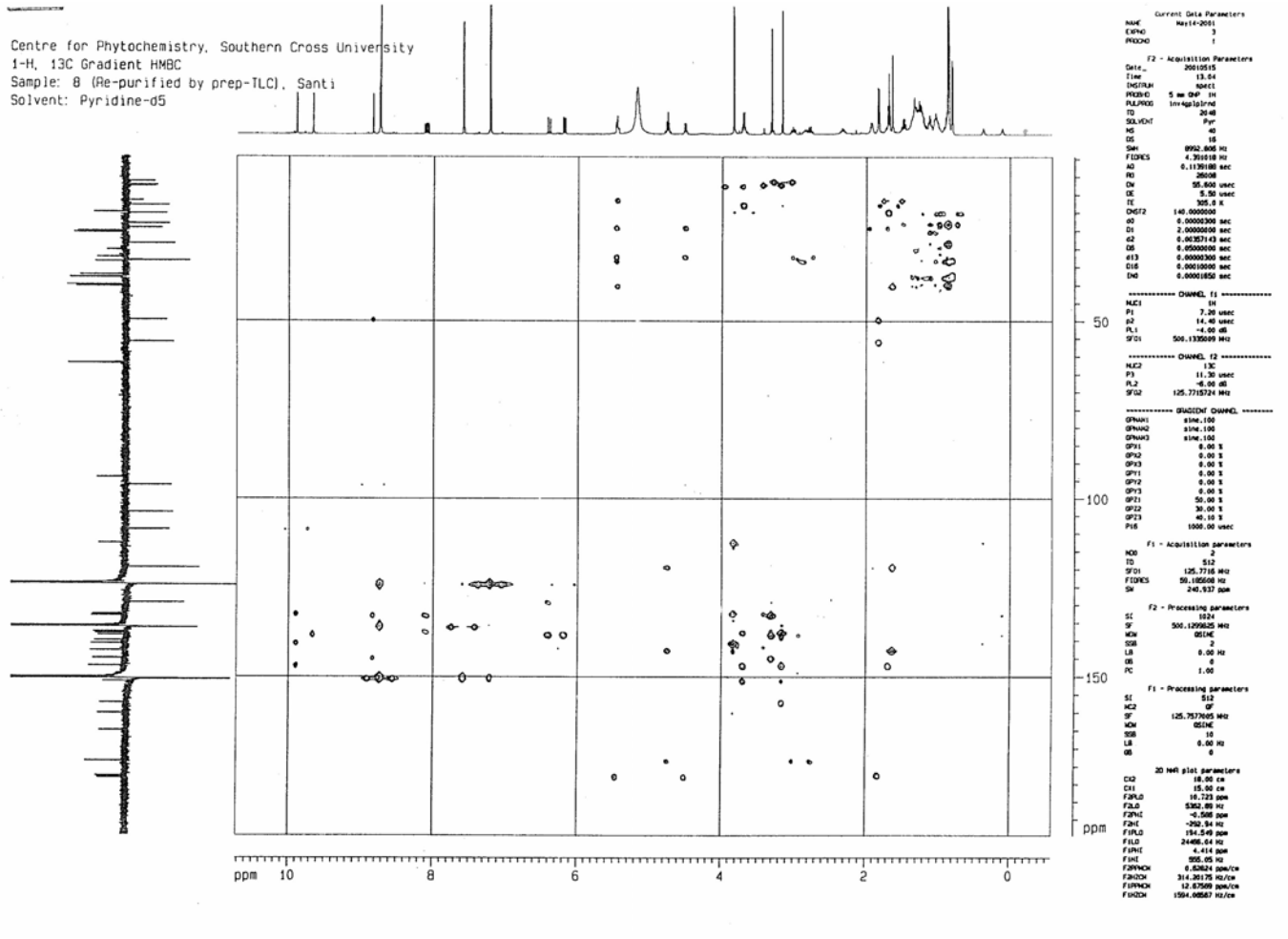

Figure A.14. Gradient HMBC spectrum of compound 25.

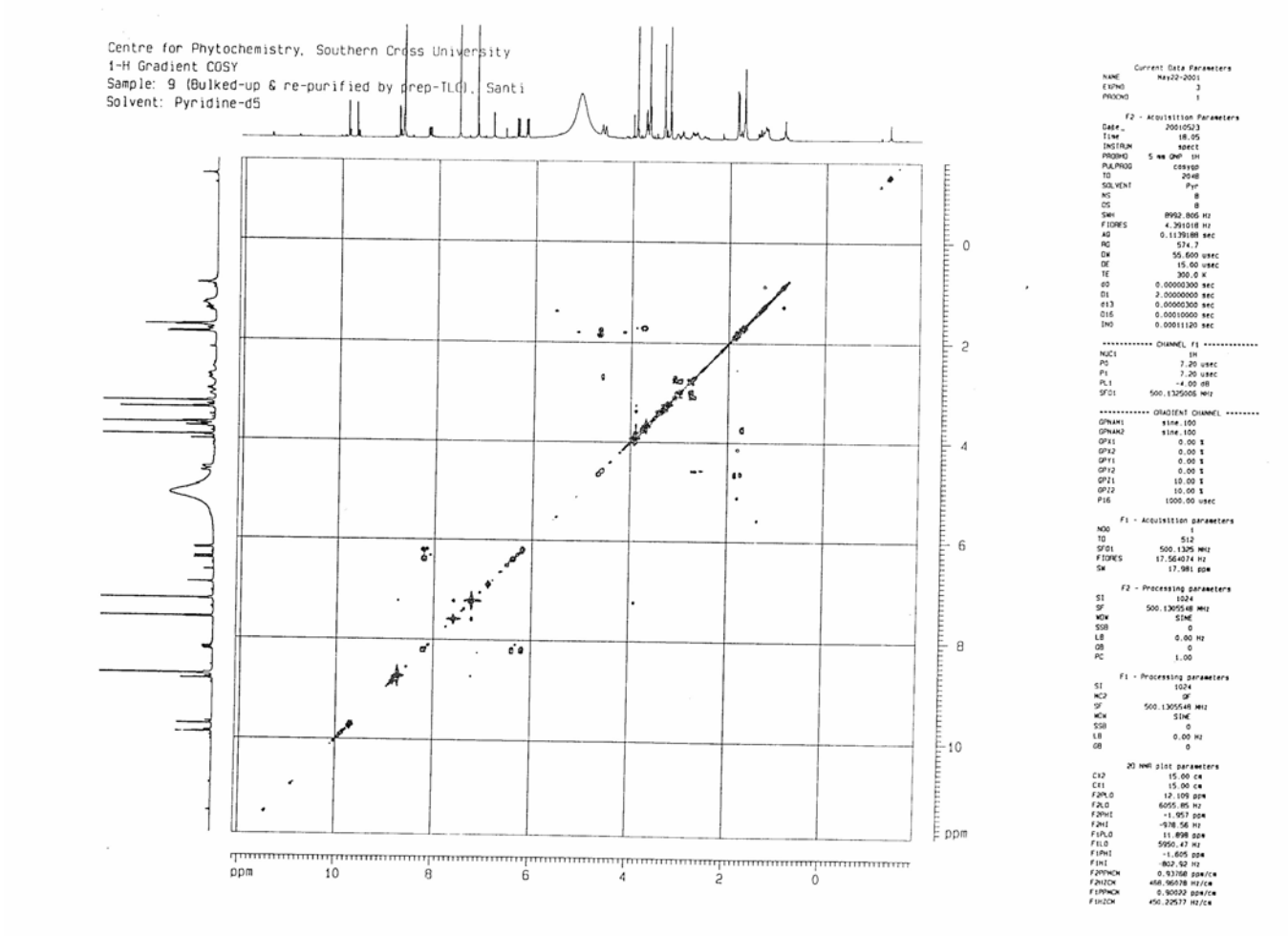

Figure A.19. Gradient COSY spectrum of compound 26.

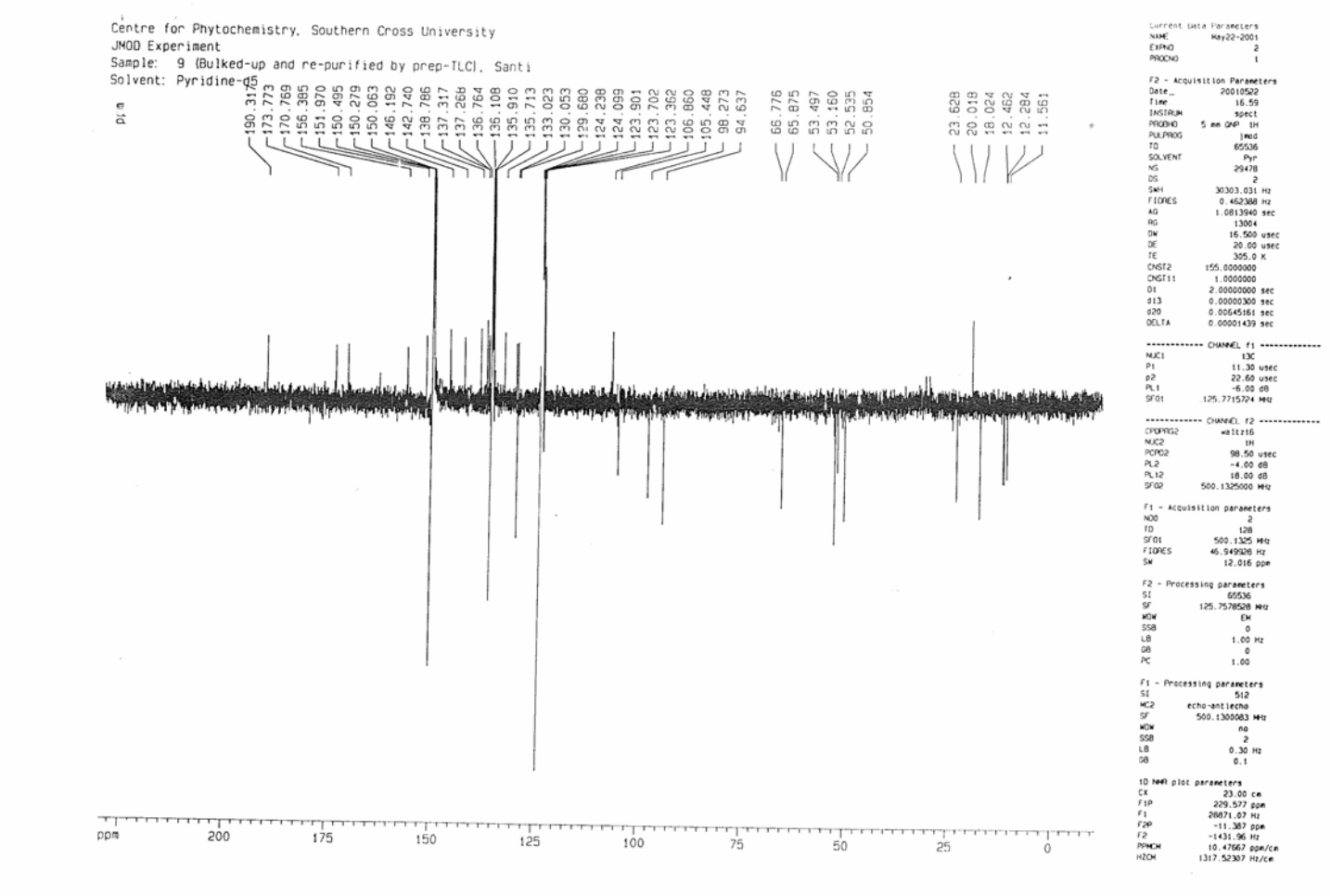

Figure A.18. 125 MHz ^{13}C- NMR spectrum of compound 26 in pyridine d_5.

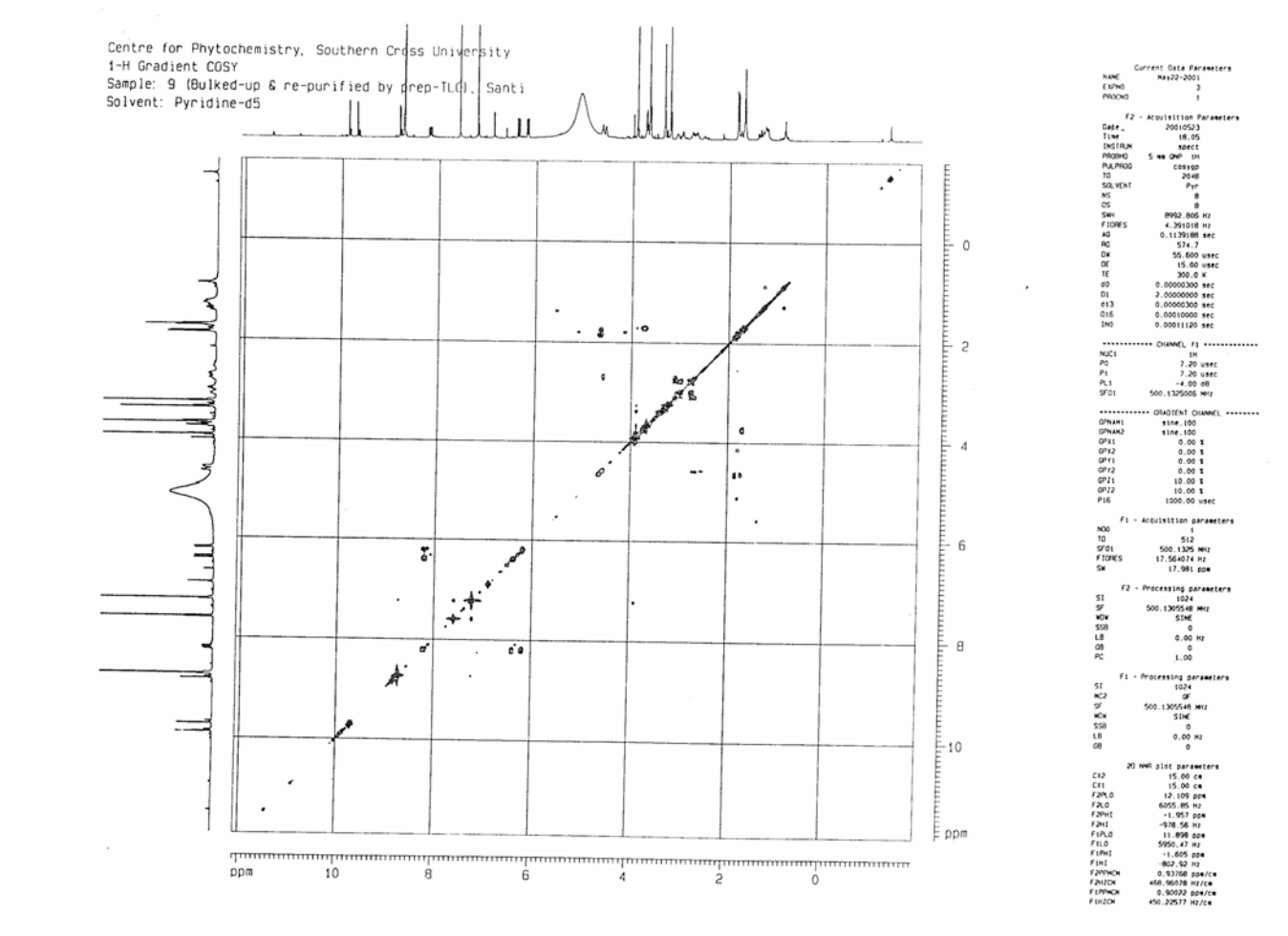

Figure A.19. Gradient COSY spectrum of compound 26.

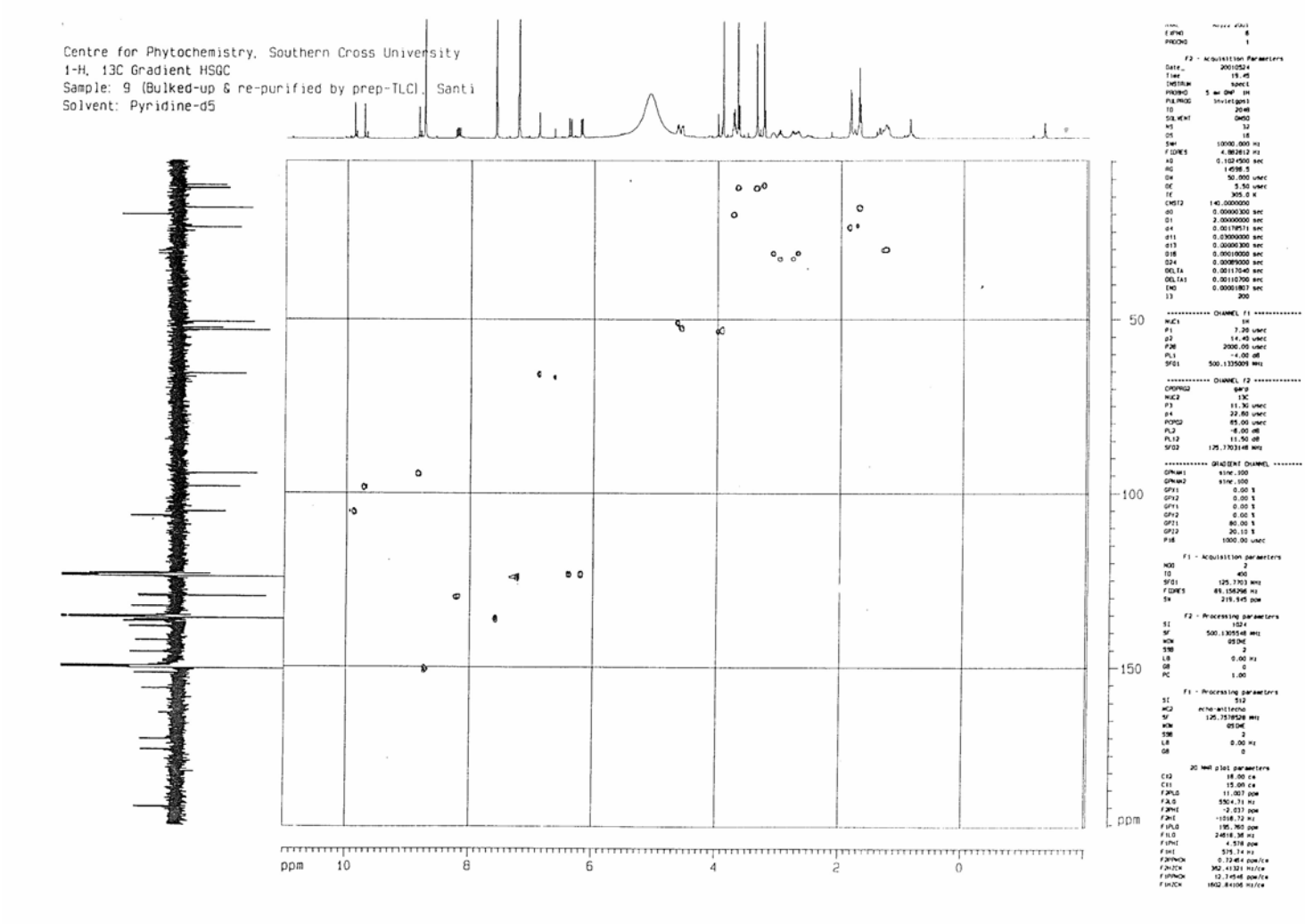

Figure A.20. Gradient HSQC spectrum of compound 26.

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