# PHYTOCHEMICALS AND BIOACTIVITIES OF *CRYPTOCARYA NIGRA* (LAURACEAE)

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**FACULTY OF SCIENCE**
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# PHYTOCHEMICALS AND BIOACTIVITIES OF *CRYPTOCARYA NIGRA* (LAURACEAE)

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**A DISSERTATION SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE**

**DEPARTMENT OF CHEMISTRY**
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# ABSTRACT

Phytochemical study and biological activity were conducted on *Cryptocarya nigra* (Lauraceae). *C. nigra* plant sample were collected from the Ulu Sat Reserved Forest, Machang, Kelantan. Isolation of the secondary metabolites involved extraction, fractionation, and purification, using various chromatographic techniques. The structural elucidations were carried out via spectroscopic methods; notably 1D and 2D NMR (COSY, HMQC, HMBC, NOE-DIFF), MS, IR, UV and comparison with published data.

In this study, a total of 8 known compounds were isolated from the samples of this plant species. Three phenanthrene alkaloids, namely atherosperminine **11**, 2-hydroxyatherosperminine **20**, noratherosperminine **47**, and a benzylisoquinoline alkaloid, (+)-*N*-methylisocolaurine **46** were isolated from the bark dichloromethane extract of *C. nigra.* The leaf alkaloid extract of *C. nigra* afforded two aporphines (isocorydine **48** and norisocorydine **49**), a phenanthrene alkaloid, argentinine **50** and a non-alkaloidal compound, benzamide **51**. This plant was being studied for the first time on their alkaloidal content.

A dichloromethane extract of the stem bark of *C. nigra* showed moderate *in vitro* inhibition of *Plasmodium falciparum* growth, with an $IC_{50}$ value of 2.82 µg/mL. (+)-*N*-Methylisococlaurine **46**, atherosperminine **11** and 2-hydroxy-atherosperminine **20** showed mild antiplasmodial activity ($IC_{50}$: 5.40, 5.80 and 0.75 µM, respectively). In addition, compound **46** and **11** showed mild antioxidant activity in DPPH assay ($IC_{50}$: 29.56 ug/mL and 54.53 ug/mL), FRAP assay (78.54 % and 70.66 %) and in the metal chelating assay ($IC_{50}$: 50.08 ug/mL and 42.87 ug/mL) respectively. No study has been reported on the antiplasmodial and antioxidant activities from *Cryptocrya nigra*.

# ABSTRAK

Kajian fitokimia dan aktiviti biologi telah dijalankan ke atas *Cryptocarya nigra* (Lauraceae). *C. nigra* telah diperolehi dari Hutan Simpanan Ulu Sat, Machang, Kelantan. Pengasingan metabolit sekunder melibatkan pengekstrakan, pengasingan, dan penulenan, melalui pelbagai kaedah kromatografi. Pengenalpastian struktur sebatian ditentukan melalui kaedah spektroskopi; iaitu 1D dan 2D NMR (COSY, HMQC, HMBC, NOE-DIFF), MS, IR, UV dan melalui perbandingan data literatur.

Dalam kajian ini, sebanyak 8 sebatian yang dikenali telah diasingkan daripada sampel ini spesies tumbuhan. Tiga alkaloid fenantrena, iaitu atherosperminina **11**, 2-hidroksiatherosperminina **20**, noratherosperminina **47**, dan benzilisokuinolina alkaloid, (+)-*N*-metilisokolaurina **46** telah diasingkan daripada ekstrak diklorometana batang *C. nigra*. Kajian ke atas ekstrak alkaloid daun *C. nigra* juga telah memberikan dua aporfina (isocoridina **48** dan norisocoridina **49**), satu alkaloid fenantrena, argentinina **50** dan sebatian bukan alkaloid, benzamida **51**. Pokok ini sedang dikaji untuk kali pertama bagi kandungan alkaloid mereka.

Satu ekstrak diklorometana batang *C. nigra* menunjukkan sederhana dalam perencatan *in vitro Plasmodium falciparum*, dengan nilai $IC_{50}$ 2.82 μg/mL. (+)-*N*-Methylisococlaurine **46**, atherosperminine **11** dan 2-hydroxy-atherosperminine **20** menunjukkan aktiviti antiplasmodial yang sederhana ($IC_{50}$: 5.40, 5.80 dan 0.75 μM, masing-masing). Di samping itu, sebatian **46** dan **11** menunjukkan aktiviti antioksidan yang sederhana dalam DPPH assay ($IC_{50}$: 29.56 μg/mL dan 54.53 μg/mL), FRAP assay (78,54% dan 70.66%) dan dalam kaedah chelating logam ($IC_{50}$: 50.08 μg/mL dan 42.87 μg/mL) masing-masing. Tiada kajian yang telah dilaporkan mengenai aktiviti antiplasmodial dan antioksidan daripada *Cryptocrya nigra*.

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# CONTENTS

# LIST OF SCHEMES

# LIST OF FIGURES

# LIST OF TABLES

# ABBREVIATIONS

| | |
|---|---|
| α | Alpha |
| β | Beta |
| λ | Maximum wave length |
| δ | Chemical shift |
| µM | Micromolar |
| µL | Microlitre |
| mM | Milimolar |
| µM | Micromolar |
| nM | Nanomolar |
| mg/ml | Miligram per mililitre |
| g | Gram |
| kg | Kilogram |
| ml | Mililitre |
| m | Meter |
| MHz | Mega Hertz |
| Hz | Hertz |
| UV | Ultraviolet |
| IR | Infrared |
| ppm | Part per million |
| MeOH | Methanol |
| $CHCl_3$ | Chloroform |
| $CH_2Cl_2$ | Dichloromethane |

| | |
|---|---|
| $OCH_2O$ | Methylenedioxy |
| $CH_3$ | Methyl group |
| $OCH_3$ | Methoxyl group |
| OH | Hydroxyl group |
| $NH_3$ | Ammonia |
| pH | Power of Hydrogen |
| HCl | Hydrogen Chloride |
| TLC | Thin layer chromatography |
| PTLC | Preparative thin layer chromatography |
| CC | Column Chromatography |
| NMR | Nuclear Magnetic Resonance |
| FT-NMR | Fourier Transform Nuclear Magnetic Resonance |
| $cm^{-1}$ | Per centimeter |
| *J* | Coupling constant |
| *d* | Doublet |
| *s* | Singlet |
| *dd* | Doublet of doublet |
| *t* | Triplet |
| *m* | Multiplet |
| 1D-NMR | One Dimension Nuclear Magnetic Resonance |
| 2D-NMR | Two Dimensional Nuclear Magnetic Resonance |
| $^1H$ | Proton NMR |
| $^{13}C$ | 13-Carbon NMR |
| COSY | $^1H$-$^1H$ Correlation Spectroscopy |

| | |
|---|---|
| DEPT | Distortioness Enhancement by Polarzation Transfer |
| HMQC | Heteronuclear Multiple Quantum Coherence |
| HMBC | Heteronuclear Multiple Bond Coherence |
| LC-MS | Liquid Chromatography-Mass Spectroscopy |
| MS | Mass Spectroscopy |
| EIMS | Electron Impact Mass Spectroscopy |
| FAB | Fast Atomic Bombardment |
| ESI | Electrospray Ionization |
| m/z | Mass per charge |
| $CDCl_3$ | Deuterated chloroform |
| MeOD | Deuterated methanol |

# CHAPTER 1

## INTRODUCTION

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# INTRODUCTION

## 1.1 General

By definition, the word *natural* is an adjective referring to something that is present in or produced by nature and not artificial or man-made. When the word *natural* is used in verbiage or written, many times it is assumed that the definition is something good or pure. However, many effective poisons are natural products. [1] The term *natural products* today is quite commonly understood refering to herbs, herbal concoctions, dietary supplements, traditional Chinese medicine, or alternative medicine. [2]

Naturally occurring compounds may be divided into three broad categories. Firstly, there are those compounds which occur in all cells and play a central role in the metabolism and reproduction of those cells. These compounds include the nucleic acids and the common amino acids and sugars. They are known as primary metabolites. Secondly, there are high-molecular-weight polymeric materials such as cellulose, lignins, and proteins which form the cellular structures. Finally, there are those compounds that are characteristic of a limited range of species. These are secondary metabolites. The secondary metabolites from the plants include terpenoids and steroids, alkaloids, flavonoids, coumarins, antraquinones and tannins. [3] Natural products have been the source of most of the active ingredients of medicines. This is widely accepted to be true when applied to drug discovery in ‘olden times’ before the advent of high-

throughput screening and the post-genomic era: more than 80% of drug substances were natural products or inspired by a natural compound. [4]

The development of high-throughput screenings, based on molecular targets, has led to a demand for the generation of large libraries of compounds. Malaysia, situated in the South East Asia, possesses one of the oldest and richest forests in the world. It is reported that there are ~8000 angiosperms residing in this great forest. Thus the Malaysian flora is an excellent candidate to provide natural chemical substances. The Malaysian tropical rain forest comprises of more than 15,000 plant species and many of them have been claimed to possess medicinal properties. [5] In recent years a rich harvest of novel natural products had been made, some of which possess cytotoxic or insecticidal activities. Some of the diverse classes of natural products, e.g. acetogenins, alkaloids, biflavonoids, coumarins, terpenoids and xanthonoids, are isolated from Malaysian plant families. [6] The variability of species that are commonly used as traditional medicines belongs to the families of Leguminocae, Rubiaceae, Lauraceae, Apocynaceae, Rutaceae, Moraceae, Meliaceae and Anonaceae. For examples, *Phyllanthus niruri* (dukung anak), *Andrographis paniculata* (hempudu bumi), *Eurycoma longifolia* (tongkat ali), *Labisia pumila* (kacip fatimah), *Pipper bettle* (sirih), *Centella asiatica* (pegaga), *Hibiscus rosasinensis* (bunga raya) and *Strobilanthes cripspa* (serus). [7-8]

In short, this thesis reports on the phytochemistry and bioactivity of *Cryptocarya nigra* from the family Lauraceae. This study mainly deals with isolation, structure elucidation of natural products, and their bioactivities. No study has been reported on the alkaloids, antiplasmodial and antioxidant activities from *Cryptocarya nigra*.

## 1.2 Objectives of Study

The objectives of this study are:

(1) To extract the crude alkaloids from the barks and leaves of *Cryptocarya nigra* (this is the first report on the alkaloids of *Cryptocarya nigra*) by using dicholoromethane ($CH_2Cl_2$) as a solvent and to purify the known and new alkaloids by using chromatographic techniques such as column chromatography (CC) and preparative thin layer chromatography (PTLC).

(2) To elucidate the structure of both novel and known compounds isolated from *Cryptocarya nigra* by using spectroscopic methods mainly 1D-NMR ($^{1}H$, $^{13}C$ and DEPT-135), 2D-NMR (COSY, HMBC, HMQC and NOESY), Ultraviolet (UV), Infrared (IR) and LCMS-IT-TOF.

(3) To evaluate the antiplasmodial and antioxidant activities of the isolated alkaloids and extracts of *Cryptocarya nigra*.

## 1.3 Lauraceae Family: General Appearance and Morphology

The family Lauraceae belongs to the Order Laurales that is classified in the Subclass Magnoliidae. [9-10] The family is pantropical, including about 50 genera with a probable number of 2500 to 3500 species world-wide, mostly from warm to tropical region, especially Southeast Asia and Brazil. [11-12]

Trees of the Laurel family predominate in the world's Laurel forests, which occur in a few humid subtropical and mild temperate regions of the northern and southern hemispheres, including the Macaronesian islands, southern Japan, Madagascar, and central Chile.

In Malaysia, there are 213 species of Lauraceae. In the lowland of Malaysia members of Lauraceae are typically small trees of the canopy except for a few species which may reach 30m tall. In the highland, the Lauraceae becoming more abundant reaching the top layer of the forest canopy and thus the term “oak laurel forest” is given to this vegetation which lies at altitude 1200-1600m. [13]

The timber of Lauraceae is light hardwood with trademark name *Medang* or *Tejur*. The timber is of no great commercial importance although trees of commercial size are felled. They are suitable for decorative work such as interior finishing, panelling, furniture and cabinetmaking. The timber is also suitable for plywood manufacture and the heavier species could be utilized for medium construction under cover [14]. The family of Lauraceae provided many useful economic products. Most of the economically important species other than sources of excellent wood are spices or flavoring agent. For example avocado (*Persea*) is one of important tropical fruit.

## 1.4 Classification of Tribe

There are many primitive and archaic features, which characterize the Lauraceae family. The determination is dependent on a combination of characters. List below illustrates the classification proposed by Hsuen Keng. There are over 30 genera, mainly tropical and sub-tropical Asia and America, about 16 are found in Malaysia. This classification is according to Malayan seed plants. [7] However, according to Judd *et al.* (2007), the classification proposed by Van Der Werff and Richter (1996) is presently used. They were classified based on both inflorescences structure and wood anatomy which divides the Lauraceae family into two sub-families; Lauroideae and Cassythoideae as shown in Scheme 1.1.

**Kingdom** : Plantae

**Divison** : Magnoliophyta

**Class** : Magnoliopsida

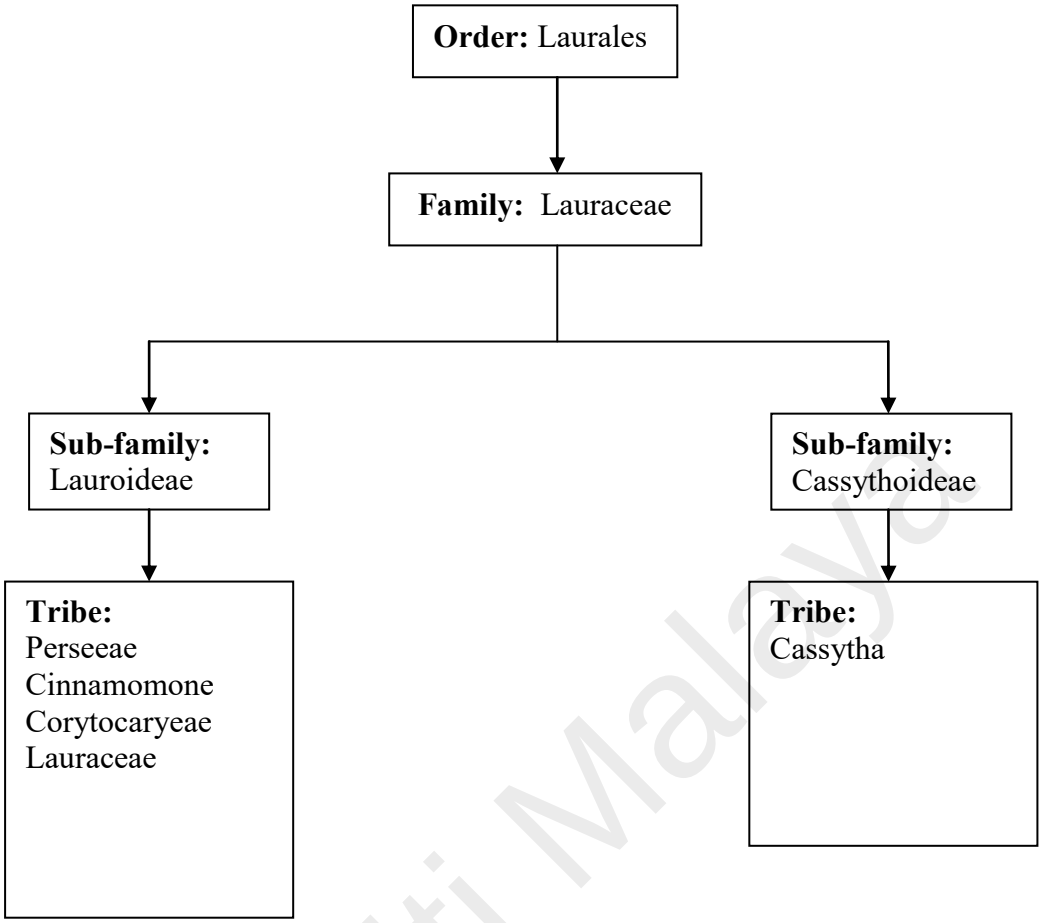
**Order** : Laurales

**Family** : Lauraceae

**Genera** :

| | | |
|---|---|---|
| *Actinodaphne* | *Adenodaphne* | *Aiouea* |
| *Alseodaphne* | *Anaueria* | *Aniba* |
| *Apollonias* | *Aspidostemon* | *Beilschmiedia* |
| *Brassiodendhron* | *Caryodaphnopsis* | *Cassytha* |
| *Chlorocardium* | *Cinnadenia* | *Cinnamomum* |

| | | |
|---|---|---|
| *Clinostemon* | *Cryptocarya* | *Dahlgrenodendrone* |
| *Dehaasia* | *Dicypellium* | *Dodecadenia* |
| *Endiandra* | *Endlicheria* | *Eusideroxylon* |
| *Gamanthera* | *Hexapora* | *Hypodaphnis* |
| *Iteadaphne* | *Kubitzkia* | *Laurus* |
| *Licaria* | *Lindera* | *Litsea* |
| *Machilus* | *Mezilaurus* | *Mochinnodaphne* |
| *Mutisiopersea* | *Nectandra* | *Neocinnamomum* |
| *Neolitsea* | *Notaphoebe* | *Ocotea* |
| *Paraia* | *Parasassafras* | *Persea* |
| *Phoebe* | *Phyllostemonodaphne* | *Pleurothyrium* |
| *Potameia* | *Potoxylon* | *Povedadaphne* |
| *Ravensara* | *Rhodostemonodaphne* | *Sassafras* |
| *Sextonia* | *Sinosassafras* | *Syndiclis* |
| *Triadodaphne* | *Umbellularia* | *Urbanodendron* |
| *Williamodendron* | *Yushunia* | |




Scheme 1.1: Classification of Family Lauraceae

## 1.5 The Genus *Cryptocarya*

The genus *Cryptocarya* belongs to the family Lauraceae, comprises more than 350 species [15] and 19 species are found in Malaysia. [16] This genus is distributed through the Neotropic, Afrotropic, Indomalaya and Australasia ecozones and also found in Southern Brazil. In Malaysia, they are called medang by local trade name while in Philpines, they were known as dugkatan and manayau. [17]

The species of the genus *Cryptocarya* found in Malaysia are *C. bracteolata, C. caesia, C. costata, C. crassinervia, C. densiflora, C. enervis, C. ferrea, C. griffithiana, C. impressa, C. infectoria, C. kurzii, C. laevigata, C. nigra, C. nitens, C. rugulosa, C. scortehinii, C. teysmanniana, C. tomantosa, C. wrayi and C. zollingeriana*.

*Cryptocarya* can be distinguished from other laurel genera by its bisexual and trimerous flowers, which are very typical in shape (usually slender, urceolate, apically narrowed tube and immersed ovary, six equal to subequal tepals, nine fertile stamens with disporangiate anthers, staminal glands only in the third androecial whorl), and by the characteristic fruit that is enclosed by the accrescent flower tube. [18] The leaves are alternate, pinnatinerved, rarely trinerved. Inflorescenses in axillary or sub terminal panicles. The flowers are bisexual (perianth tube turbinate or ovoid), lobes 6, stamens usually 9. The fruits entirely included in the enlarge perianth tube leaving only a minute opening at apex. [16] It is found in low elevation evergreen forests and littoral rainforest, usually on all type of soils. The seeds are readily dispersed by fruit-eating birds, and seedlings and saplings have been recorded from other habitats where they are unlikely to develop to maturity. [15]

Several species from this genus have been used widely in traditional medication, essential oil and also used as sources of timber for house construction and furniture due to the characteristic of the wood which are finely grained, more or less heavy and durable [19]. Many reported phytochemical and pharmacological studies on this genus shown the chemical constituents to consist typically pyrones and styrylpyrones which exhibit anticancer activities. Examples are *Cryptocarya massoia* which is used commercially to produce essential oil and *Cryptocarya alba* produces a very hard wood which is highly valued. *Cryptocarya woodii* leaves have been found in prehistoric settlements in Africa and are believed to have been used for insect control. [15]

## 1.6 *Cryptocarya nigra*

*Cryptocarya nigra* is a medium sized tree up to 10 m tall with about 15 cm diameters. The bark is white-grey brown and smooth. The inner bark is pinkish red and mottled white while the young twigs of this species are velvety hairy. *Cryptocarya nigra* leaves are spirally simple, thickly coriaceous from elliptic to elliptic lanceolate or elliptic ovate. They are apex shortly pointed, asymmetric, base obtuse with 8.0-14.5 cm x 5.0-6.5 cm. The upper surface is bright green with glaucous color below it. The young leaves secondary nerves 7-11 pairs which are raised below and faint above it. The tertiary nerves are in scalari form. The petioles are up to 1.5 cm long. *Cryptocarya nigra* flowers are yellow with inflorescence in axillaries and terminal panicles.

The species can be found in undisturbed mixed dipterocarp forests up to 300 m altitude in Sumatra, Borneo (Sarawak, Sabah, and East-Kalimantan), and usually on hillsides with sandy soils. In secondary forests this species usually present as a pre-disturbance remnant tree. [20]

Figure 1.1: Leaves of *Cryptocarya nigra* Kosterm

Figure 1.2: Bark of *Cryptocarya nigra* Kosterm

## 1.7 Medicinal Value

Sustainable management of traditional medicinal plant resources is important, not only because of their value as a potential source of new drugs, but due to reliance on traditional medicinal plants for health. [25] The World Health Organization (WHO) estimates that approximately 80 percent of the world's population relies primarily on traditional medicines as sources for their primary health care. [26] Natural products have been the single most productive source of leads for the development of drugs. Natural products have been the source of most of the active ingredients of medicines.

Plants have always been the single most productive source of lead compounds (e.g. morphine, cocaine, digitalis, quinine, tubocurarine, nicotine, and muscarine). Many of these lead compounds are useful drugs in themselves (e.g. morphine and quinine), and others have been the basis for synthetic drugs (e.g. local anaesthetics developed from cocaine). Clinically useful drugs which have been isolated from plants include the anticancer agent paclitaxel from the *yew* tree, and the antimalarial agent artemisinin from *Artemisia annua.* Plants provide a large bank of rich, complex and highly varied structures which are unlikely to be synthesized in laboratories. Furthermore, evolution has already carried out a screening process itself whereby plants are more likely to survive if they contain potent compounds which deter animals or insects from eating them. Even today, the number of plants that have been extensively studied is relatively very few and the vast majorities have not been studied at all. [27]

During the 19$^{th}$ century, organic chemists took up the study of many plant principles, the physiological effect of which had been recognized. A new impetus was given to the search for medicinal plant principles by the discovery of the clinical usefulness of alkaloids of *Rauwolfia* species and during the past dozen years this has

provided fresh stimulus for an enlarged and concentrated attack upon the still unexplored botanical resources of the world. [28]

*Cryptocarya massoy* is a native of New Guinea. In Peninsular Malaysia, the bark of this species is used by the women after childbirth, and also is added to tonics and cigarettes. In Indonesia, it is used against diarrhea and spasmodic bowel trouble, usually in combination with a *Cinnamon*. Furthermore, it is an ingredient of various native medicines and has a characteristic odor. In New Guinea, the aromatic bark of *Cryptocarya massoy* is employed to treat fever, and often it is used by the natives to treat bad cases of tuberculosis of the lungs. A small portion of the bark is placed so as to close a fresh wound. It is also chewed and the saliva rubbed over the limbs to ease muscular pains. In small quantities, mostly in combination with other vegetable drugs, it is ingested for violent headache, pain in the joints, puerperal infection, distention of the stomach, vomiting and chronic constipation; it is also mixed with cloves and sandalwood and used by the natives as rubefacient. The volatile oil of the bark contains pinene, limonene, dipentene, eugenol and safrol. [29]

Past studies of the species of *Cryptocarya chinensis* have found that it contains many pavine and proaphorpine alkaloids. [30-34] The pavine alkaloids have been noted to possess antiviral and immunological activity [35], behavioral and electrophysiological effects, and antiarrhythmic potential. [32] Several other compounds were isolated by Horn et al. from the leaves and bark of the South African plant. *Cryptocarya latifolia*, which has long been noted for its medicinal properties. [36] These range from the treatment of headaches and morning sickness to that of cancer, pulmonary diseases and various bacterial and fungal infections. [37] Phytochemical studies on stem bark and

leave of *Cryptocarya moschata* from Atlantic Rain Forest show the occurrence of styrylpyrones and flavonoid glycosides on this species. Some of the pyrones and styrylpyrones isolated from this genus have shown larvicidal and antifertility activities, as well as inhibition of breast cancer cell lines. [38]

In Brazilian Atlantic rain forest, the fruits of the species *Cryptocarya mandioccana* Meisner are distinct by their aroma and pungent flavor, being carminative and with stomachic properties. Its bark, bitter and scented is also considered to be stomachic and helpful in fighting colic and diarrhea. The tea from its seeds is used against stomachache, and it's crushed leaves against aches and colic. [39]

# CHAPTER 2

## GENERAL CHEMICAL ASPECTS

# CHAPTER 2

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## 2.1 Introduction

Natural products chemistry can be thought of as originating from mankind's curiosity about odor, taste, color, and cures for diseases. Folk interest in treatments for pain, for food-poisoning and other maladies, and in hallucinogens appears to go back to the dawn of humanity. For centuries China has led the world in the use of natural products for healing. Nowadays, due to the rising of interest concerning on research in the scope of medicinal importance, organic chemist had begun the investigation on alkaloids containing and its usage in either traditional or modern medicine.

The explorations of the Lauraceous plants have been intensified since many decades ago for alkaloids due to its medicinal value. The medicinal value of this plant is inestimable. For examples, herbalism and folk medicine, ancient and modern, have been the source of much useful therapy, pharmacology and taxonomy. These chemical compounds also important to economic and nutrition field. Besides alkaloids, they produced a wide range of non-alkaloidal compounds including carbohydrate, proteins, lipid, amino acids, terpenes, essential oils, acetogenins, polyphenols and aromatic compounds. [40]

The major source of alkaloids is the angiosperms, but alkaloids are also found in animals, insects, microorganisms, and lower plants. Human have utilized alkaloids as

medicines, poisons, and magical potions. The alkaloids are one of the most diverts groups of secondary metabolites found in living organisms and have an array of structure types, biosynthetic pathways, and pharmacological activities and are of limited distribution in the plant kingdom. It contains secondary, tertiary, or quaternary nitrogen atoms in their structures. Their amine character produce an alkaline solution in water and hence the origin of their name-alkaloid.

## 2.2 Alkaloid: Definitions

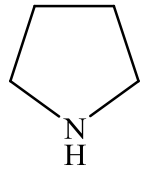
Human recognition of alkaloids is as old as civilization, since these substances have been used as drugs, in potions, medicines, teas, and poisons for 4000 years. The research on alkaloids is growing rapidly. Researchers are determined in attempting to decode the many secrets surrounding alkaloids. Thus, the level of scientific research activity in connection to alkaloids is high internationally.

The term alkaloid coined in 1819 by the pharmacist W. Meissner and meant simply, "*alkali-like*". [41] This term is applied to nitrogen containing molecules belonging to one of the largest and most diverse families of naturally occurring compounds which usually contain nitrogen as part of heterocyclic system. These compounds are grouped together by the presence of nitrogen atom in their structure. [42]

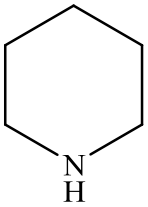
An "alkaloid" is a substance with nitrogen in the molecule, connected to at least two carbon atoms and must have at least one ring, but not necessarily heterocyclic was proposed by Nowacki and Nowacka (1965). Alkaloids are grouped in three main categories based on knowledge and speculation about their biogenesis. [16]

Alkaloids are a group of molecule with relatively large occurrence in nature around the Globe. They are very diverse chemicals and biomolecules, but they are all secondary compounds and derived from amino acids or from the transamination process. Similar alkaloids can have quite different biosynthetic pathways and different bioimpacts. Alkaloids are derived from l-lysine, l-ornithine, l-tyrosine, l-tryptophan, l-histidine, l-phenylalanine, nicotinic acid, anthranilic acid or acetate. The terpenoid, steroid and purine alkaloids are also important. Millions of people around the Globe use purine alkaloids every day whether starting the day with a cup of coffee or drinking a cup of tea in the afternoon. [43]

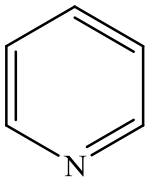
Alkaloid chemistry underlies the significance of the blocks, pathways and transamination reactions. The main criteria for alkaloid precursor determination are the skeleton nucleus of the alkaloid. The following is most important alkaloid nuclei exists; piperidine, indolizidine, quinolizidine, pyridon, pyrrolidine, imidazole, quinazoline, quinoline, pyridine, sesquiterpene, indole, and purine. Several examples of common alkaloid ring skeletons are illustrated in Scheme 2.1. They can be isolated, detected and modified. Modification of alkaloids by chemical and biological processes and bioengineering can produce new applications. Chemistry are not only investigates alkaloids, their structures and activities, but also develops method for their structural manipulation. [43]




Pyrolidine




piperidine

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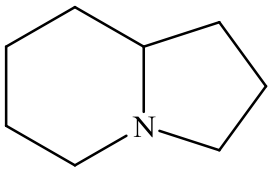


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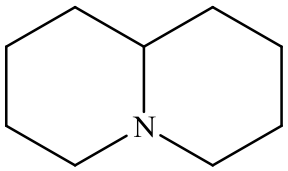
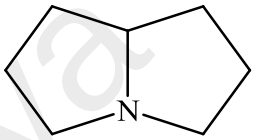
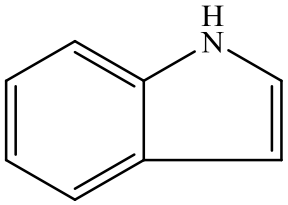


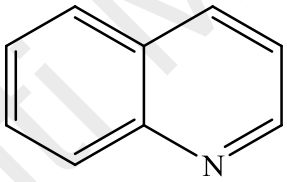

quinolizidine



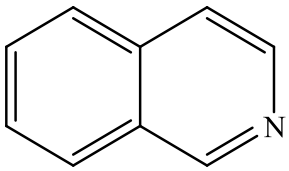

pyrrolizidine



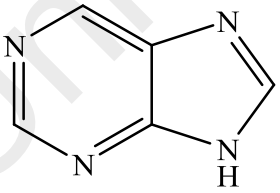

Indole



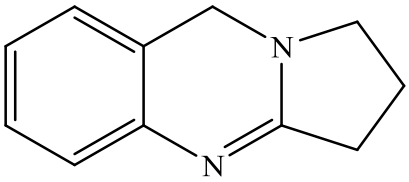

quinoline



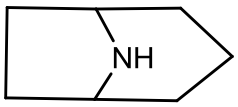

isoquinoline




Purine




quinazoline




tropane

Scheme 2.1: Examples of Alkaloid Ring Skeletons.

## 2.3 Alkaloids: Occurrence and Distribution [41,44]

The major source of alkaloids in the past has been the flowering plants, the Angiospermae, where about 20% contain this constituent, and it is estimated that they are present in only 10-15% of all vascular plants. The production of alkaloid has been found to be associated with living rather than dead cell tissue, and in some cases the amount of alkaloids present seems to vary with time and with degree of maturation of the plant. They are rarely found in cryptogamia (exception – ergot alkaloids), gymnosperms, or monocotyledons.

They occur abundantly in certain dicotyledons and particularly in the families of Apocynaceae (dogbane, quebracho, pereiro bark), Papaveraceae (poppies, chelidonium), Papilionaceae (lupins, butterfly-shaped flowers), Ranunculaceae (aconitum, delphinium), Rubiaceae (cinchona bark, ipecacuanha), Rutaceae (citrus, fagara) and Solanaceae (tobacco, deadly nightshade, tomato, potato, thorn apple). Well characterized alkaloids have been isolated from the roots, seeds, leaves or bark of some forty plant families and it is probable that the remaining families will provide only an occasional alkaloid-bearing plant.

It is generally true that a given genus or related genera yield the same or structurally related alkaloids; for example, seven different genera of Solanaceae contain hyoscyamine. In recent year an increasing numbers of examples of alkaloids have come from animals, insects, marine organisms, microorganisms, and lower plants.

## 2.4 Function of Alkaloids [45]

The reasons why plants produce alkaloids is mostly unknown. Although various theories have been proposed to explain their existence, but the function of alkaloids is still vague and not really understood by the chemist. Some of the suggested functions are listed as the following:

- As by-products of normal plant metabolism or waste products
- As storage reservoir of nitrogen for protein synthesis
- As protections against damage by ultraviolet light.
- As defense chemicals (agent as a toxic) for the plants against attack by predators (insects or herbivores). For this function, alkaloids must closely interact with specific targets in herbivores.
- As plants stimulants and regulators in such activities as growth, metabolism and reproduction.
- As substitutes of mineral in the plants such as potassium and calcium
- As a detoxification agent, which render harmless, by processes such as methylation, condensation, and ring closure, substances whose accumulation of which might otherwise cause damage to the plant.

In conclusion, one might say that while much has been learned about the biogenesis and metabolism of alkaloids, their functions in the plants, if any, are still largely unknown.

## 2.5 Classification of Alkaloids [46]

Chemical, pharmacological, botanical properties must all be considered in classifying a compound as an alkaloid. Alkaloids are grouped in three main categories (Scheme 2.2) based on both of knowledge and speculation about their biogenesis (Scheme 2.3).

- **True Alkaloid**

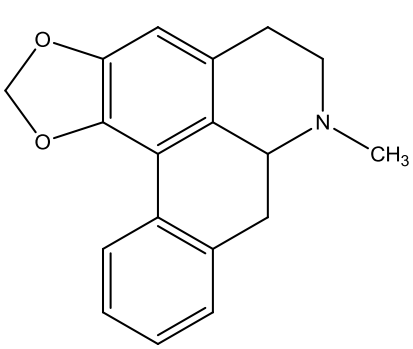
  The true alkaloids are compounds in which the nitrogen-containing heterocyclic system is derived from a biogenetic amine, formed by decarboxylation from an amino acid. They are usually found as salts in plant such as liriodenine **1** and morphine **2**.

- **Pseudo Alkaloid**

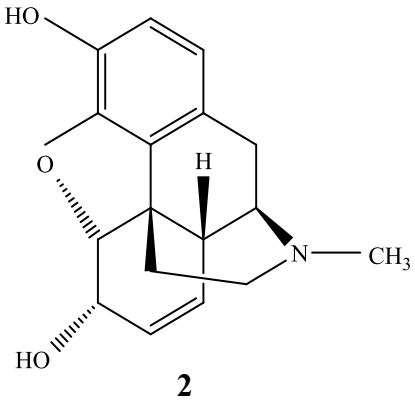
  Pseudo alkaloids are apparently unrelated to acid amino. They are nitrogen containing molecules but they have carbon skeletons derived from monoterpenes and other acetate derivatives and aliphatic polyketoacids such as coniine **3** and *β*-skytanthine **4**.

- **Proto Alkaloid**

  These compounds like true alkaloids, derived from an amino acid or biogenetic amine but they do not contain any heterocyclic system and represented in nature by biogenetic amine themselves and their methylated derivatives such as serotonins **5** and mescaline **6**.




**1**

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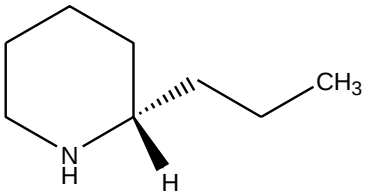


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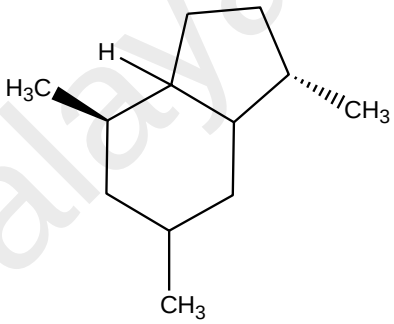
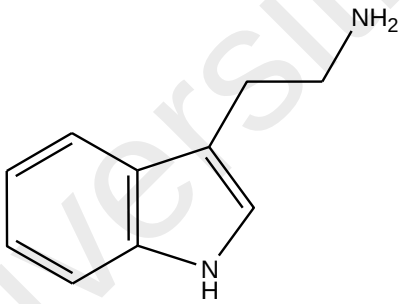
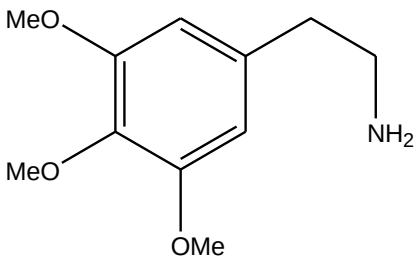



**4**



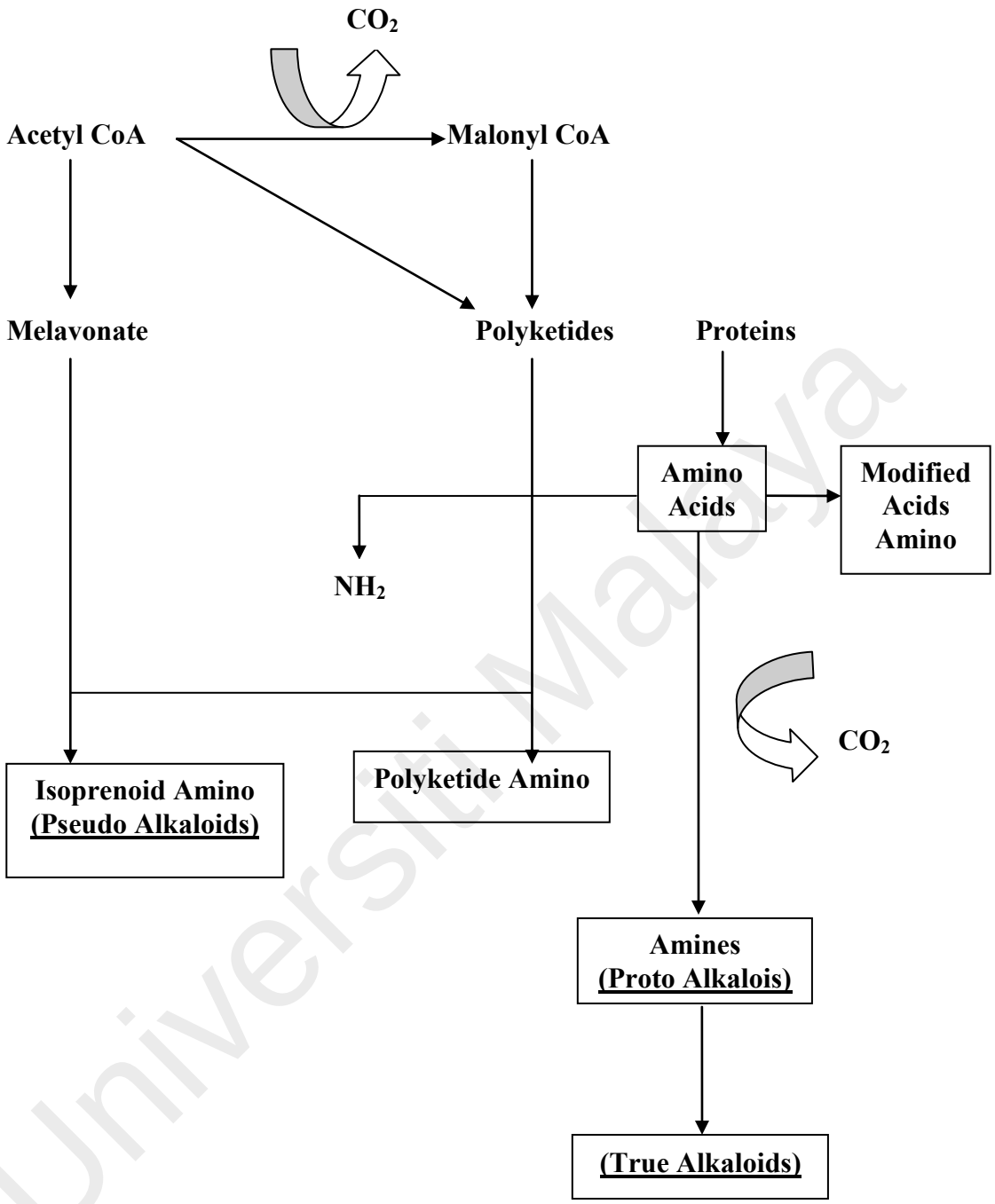

**5**




**6**



Scheme 2.2: Examples of Alkaloids Based on Three Main Categories

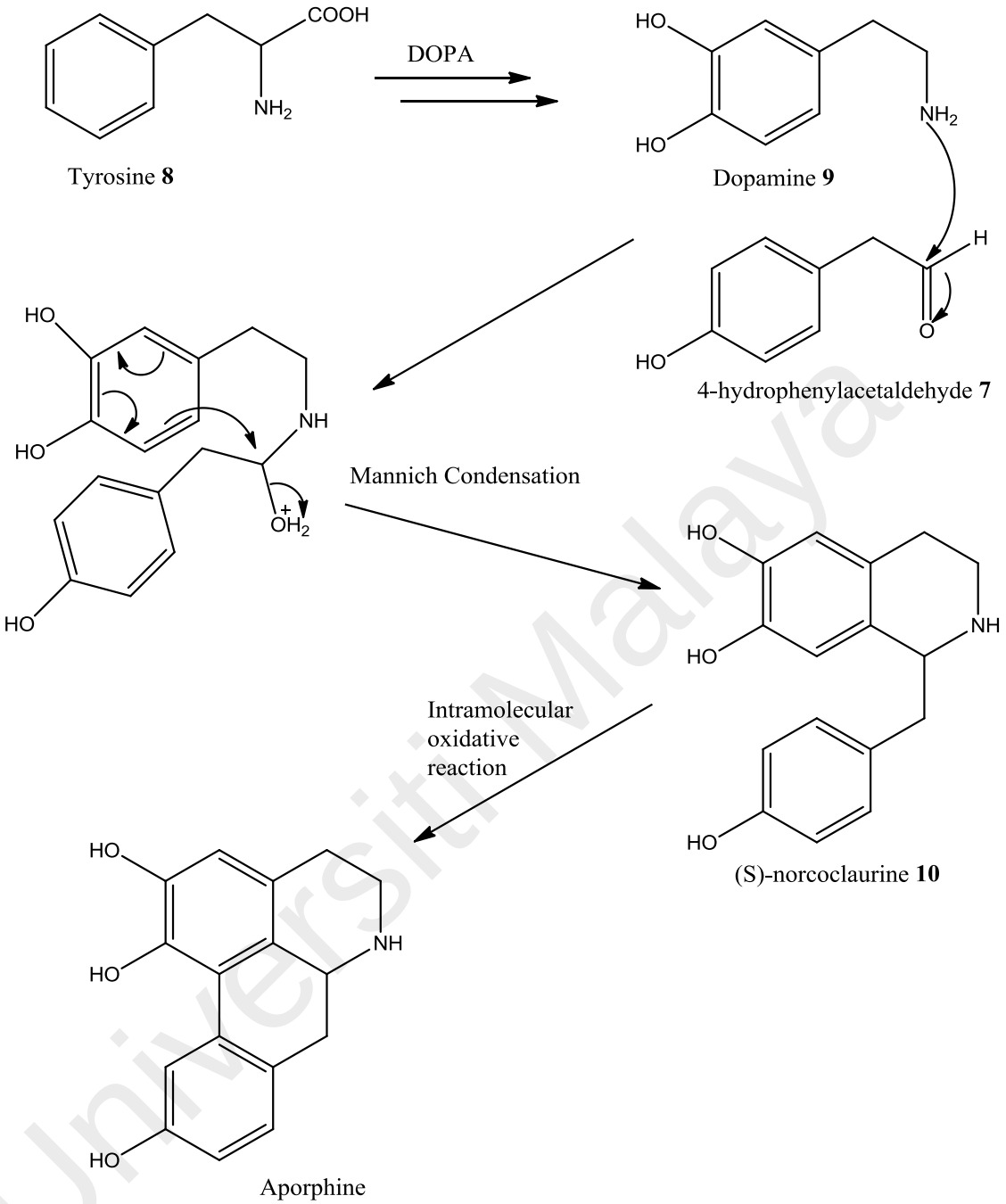



Scheme 2.3: Classification of Alkaloids with the Biogenesis Concept

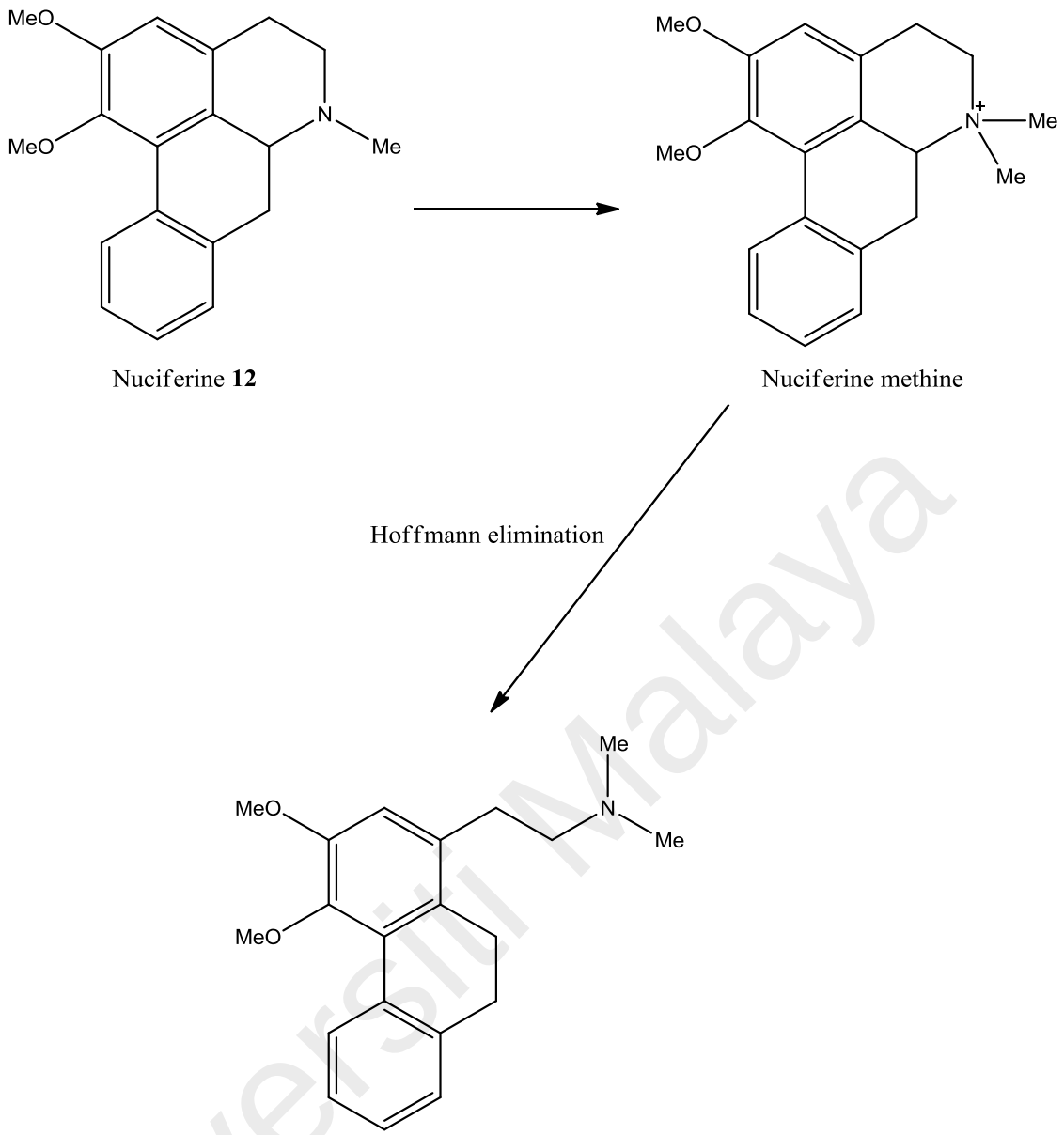
Classification of alkaloids can also be done based on their biogenesis criteria. Examples for biogenesis classification are aporphine and benzylisoquinolines (Scheme 2.4) and phenanthrene (Scheme 2.5) type of alkaloids. Biosynthesis is the experimental study of the formation of secondary metabolites. Thus, biogenesis is the hypothetical speculation on the precursor – product relationship in a biosynthetic pathway. Most of alkaloids of Lauraceae, are isoquinoline type derived from tetrahydrobenzylisoquinoline which was originated from tyrosine.

Experiment with labeled precursor and cell cultures showed that condensation between 4-hydrophenylacetaaldehyde **7** from amino acid tyrosine **8** and dopamine **9** yielded the first alkaloid intermediate (*S*)-norcoclaurine **10**, which mark the first and central intermediate of isoquinoline alkaloids. Intramolecular oxidative reaction from the benzylisoquinolines, (*S*)-norcoclaurine **10** gives aporphine structure [47-48] (Scheme 2.4).

Although no labeling experiments have yet done, there is complete agreement concerning the origin of phenanthrene alkaloids from aporphines. In fact, we may safely assume that phenanthrene alkaloids with a 2-dimethylamino side chain derive from quaternary aporphinium salts by *in vivo* Hoffmann elimination. Example, according to Batthacharya *et al.* mentioned that the phenanthrene alkaloid, atherosperminine **11** is formed from the Hoffman elimination product of aporphine alkaloid, (-) – Nuciferine **12** (Scheme 2.5) [49].




Scheme 2.4: Biosynthesis of Benzylisoquinoline and Aporphine Alkaloids from Tyrosine **8**




Nuciferine **12** Nuciferine methine

Atherosperminine **11**



Scheme 2.5: Biosynthesis of Phenanthrene Alkaloid **11** from Nuciferine **12**

## 2.6 Interest in Alkaloids

For many years, the nature of alkaloid in plant metabolism was a mystery. There are many explanations why plants, micro-organisms and animals produce alkaloids. Despite years of advanced research in the field, a final comprehensive biological explanation of the nature of alkaloid is still on the way. Furthermore, the discoveries of structure of alkaloids are unexpected and the biological activity is surprising. Based on the theoretical hypotheses on the nature of alkaloid compiled during the last 200 years, alkaloids are hypothesized as plant wastes and end products of metabolism. [50]

Almost all alkaloids have been found to possess some kinds of pharmacological activity, ranging from extreme toxicity to their use as valuable pharmaceuticals. Despite the increasing interest of public in phytomedicine, very few drugs from higher plants have attained any prominence in conventional medicinal practice in the last couple of decades. The most notable example is Taxal, a diterpenoid originally obtained from the bark of the Pacific yew tree (*Taxus brevifolia* Nutt.). Instead, major progress in the last decade has been in the field of phytomedicine, also referred to as botanicals or herbal medicine. In Germany and many European countries, these products are classified as drugs; and in USA they are sold as dietary supplements. [51]

Alkaloids applications can be found in different areas. Some alkaloids are still used in modern medicine today as natural or modified compounds. Their use is connected to the regulation of $Na^+$ ions and channels, mescaric, cholinergic receptor, acetylcholine-esterase, opiod and opiate receptors, glycine and other receptors, as well as the regulation of micro-tubules of the spindle apparatus. Moreover, alkaloids are

used in the regulation of microbial and schizonticide activity and as pharmaceuticals. Some alkaloids are used in food receptors as additional components or are consumed as part of the final product (caffeine, theophylline, piperine, and capsaicin). The use of alkaloids as a supplement in some products on the market is presently a matter of discussion, as they are considered a health risk (the case of ephedrine). Alkaloids can also be used as biological fertilizers and as control agents in plant protection. [52]

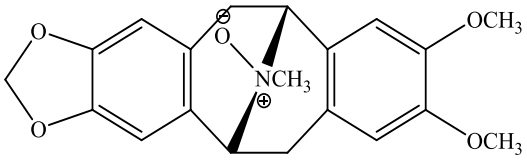
In summary, the main interests in the alkaloids at the present time are (a) the nature of, and the reason for their pharmacological effects in human; (b) the chemical and physical-chemistry properties associated with them, and (c) how and why they are synthesized in the plant (their biosynthesis).

## 2.7 Alkaloids Isolated from *Cryptocarya* species

Phytochemical studies on several species of *Cryptocarya* have been reported before as shown in Table 2.1. Several types of alkaloids were isolated and reported including some new compounds.

Table 2.1: Alkaloids Isolated from the Genus of *Cryptocarya*

| Plant | Alkaloids Isolated |
|---|---|
| ***Cryptocarya chinensis***[21] | (+)-eschscholtzidine-*N*-oxide **13**<br>(-)-12-hydroxycrychine **14**<br>(-)-12-hydroxy-*O*-methylcaryachine **15**<br>(-)-*N*-demethylcrychine **16**<br>isocryprochine **17**<br>isoamuronine **18**<br>(+)-8,9-dihydrostepharine **19** |
| ***Crptocarya crassinervia***[22] | 2-hydroxyatherosperminine **20**<br>*N*-demethyl-2-methoxyatherosperminine **21**<br>2-methoxyatherosperminine **22**<br>2-methoxyatherosperminine *N*-oxide **23** |
| ***Cryptocarya oubatchensis*** [23] | oubatchensine **24** |
| ***Cryptocarya konishii***[24] | oblongine **25**<br>methyloblongine **26** |
| ***Cryptocarya rugulosa***[53] | dicentrinone **27**<br>atheroline **28** |
| ***Cryptocarya phyllostemon***[54] | (-)-antotine **29**<br>(-)-phyllostemine **30**<br>(-)-phyllosteminine **31**<br>(+)-phyllocryptonine **32**<br>(+)-phyllocryptine **33** |

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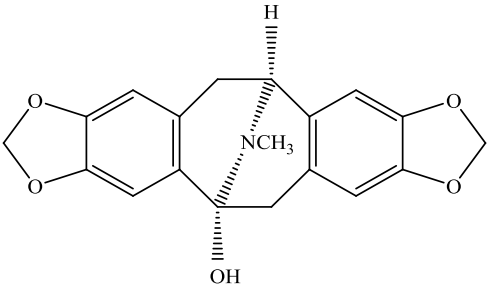


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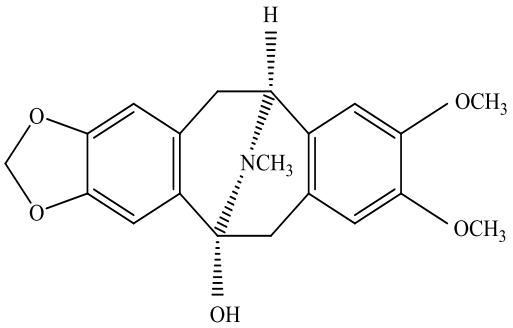


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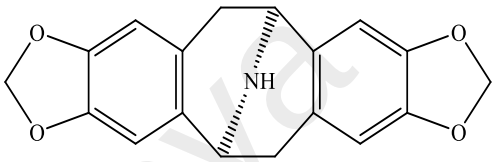


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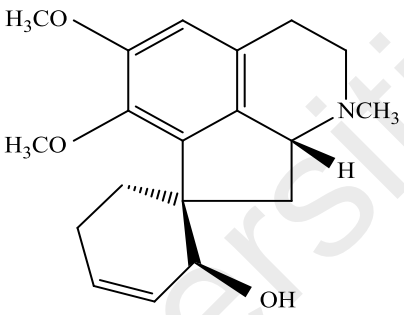


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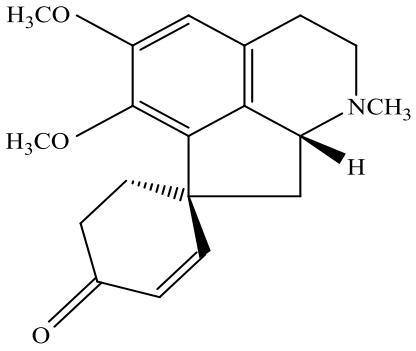


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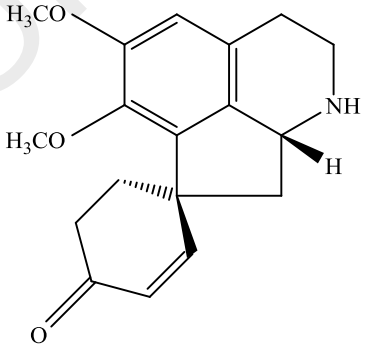


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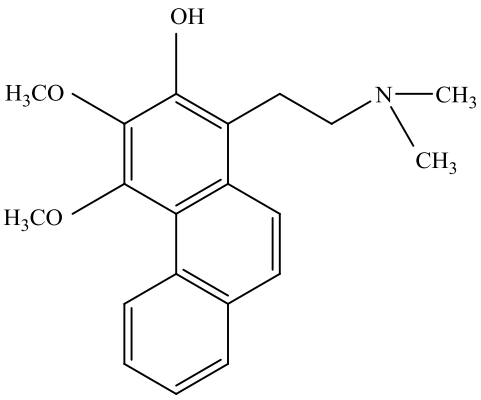
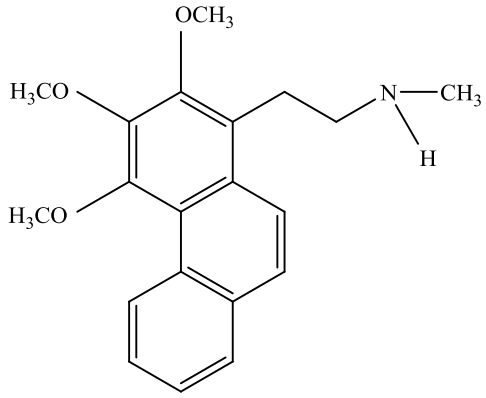



**20**

Figure 2.1: Structures of Alkaloids from the Cryptocarya

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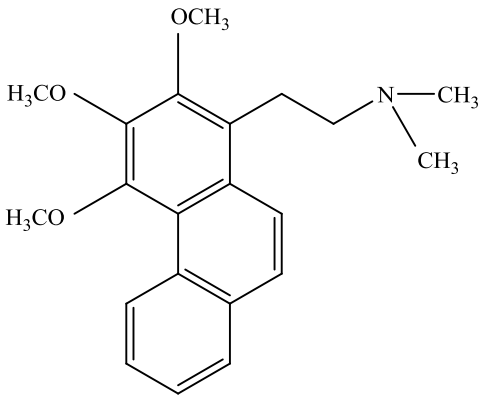


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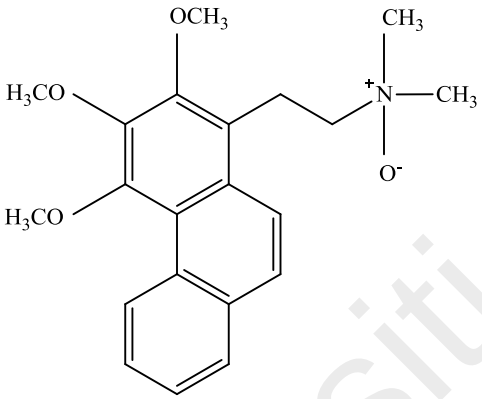


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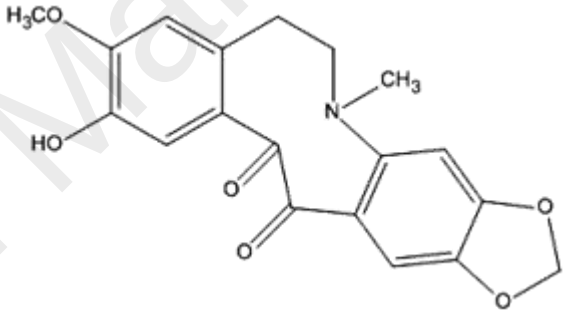


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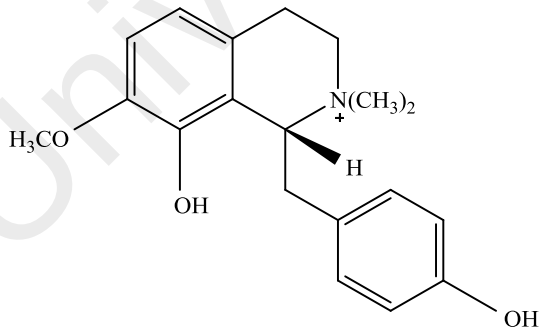


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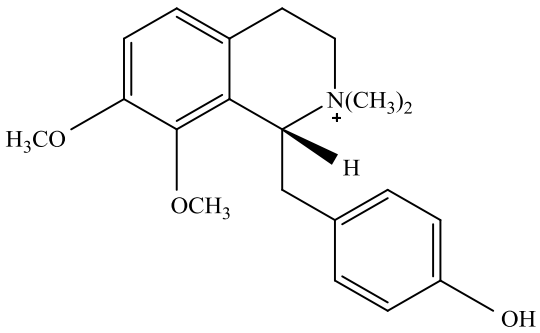


**26**

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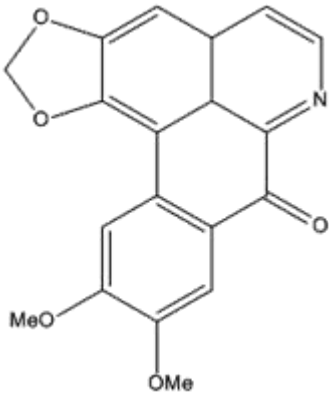


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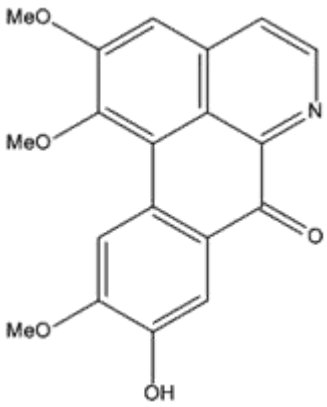


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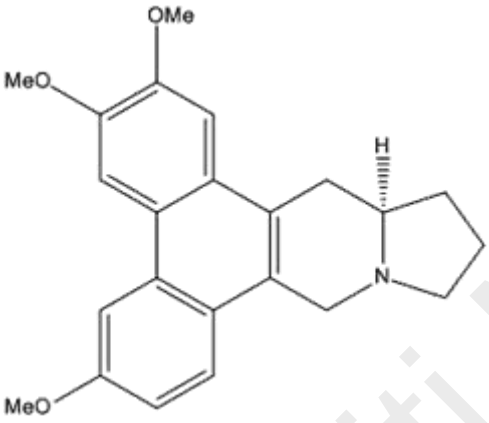


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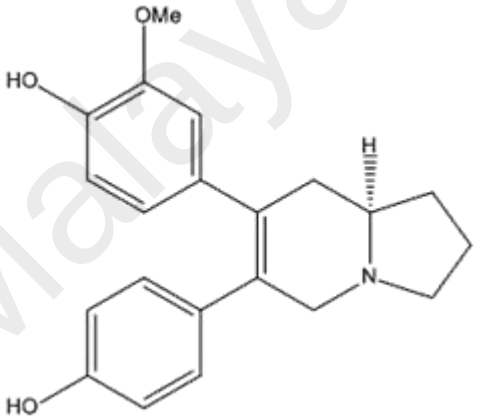


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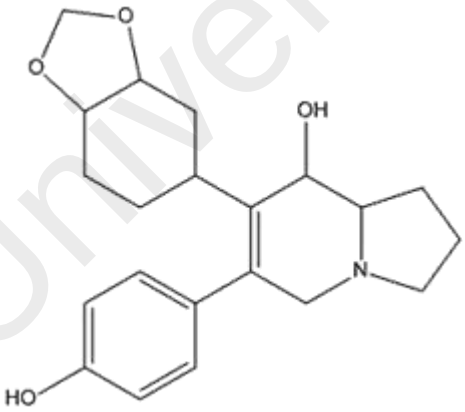


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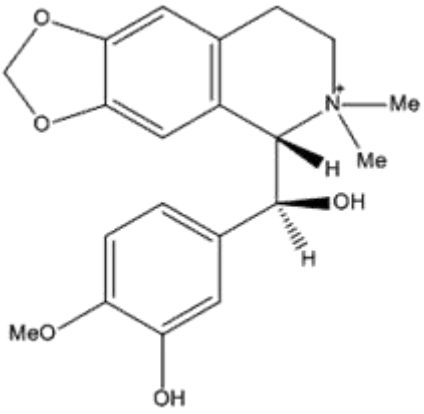
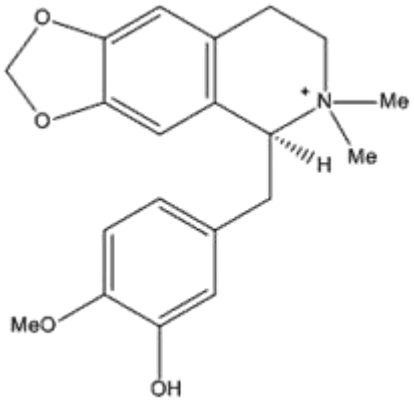



**32**

Figure 2.1: continued




**33**

Figure 2.1: continued

## 2.8 Isoquinoline Type of Alkaloids

Isoquinoline is chemically known as benzo [c] pyridine or 2-benzanine. The alkaloids that possess isoquinoline skeletons are known as isoquinoline alkaloids. Various types of isoquinoline alkaloids may be found in Lauraceae with aporphine being the major group of this type. [55]

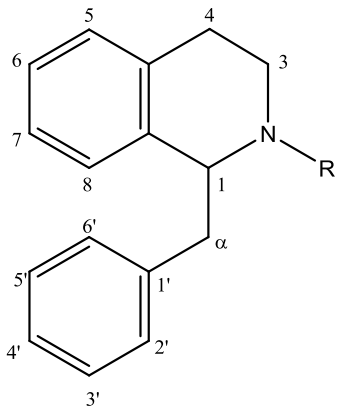
The isoquinoline backbone is biosynthesized from the aromatic amino acid tyrosine **8** (Scheme 2.4). Isoquinoline is a colourless hygroscopic liquid at room temperature and known as a weak base with *pKa* of 8.6 and has unpleasant odor. It is slightly soluble in water but well soluble in common organic solvents. Isoquinoline can be categorized into several classes based on the skeleton of the structure (Table 2.2). The benzylisoquinoline, aporphine and phenanthrene alkaloid will be discussed briefly in the next section since the author has isolated this type of alkaloids from the plant species studied.

Table 2.2: Categories of Isoquinoline Alkaloids [56]

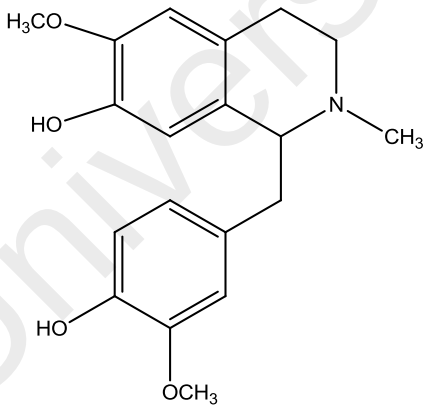
| | |
|---|---|
| Simple isoquinoline | Dibenzazonines |
| Benzylisoquinoline | Protoberberines and retroprotoberberines |
| Isoquinolones | Secoberberines |
| Pavines and isopavines | Benzophenantridhines |
| Bisbenzylisoquinolines | Arylisoquinolines |
| Baluchistanamines | Protopines |
| Cularines | Phthlideisoquinolines |
| Dibenzopyrrocolines | Rhoeadines |
| Proaporphines | Emetines |
| Aporphines | Phenethylisoquinolines |
| Proaporphine-benzylisoquinoline | Homoaporphines and homoproaporphines |
| Dimmers | 1-Phenylisoquinolines |
| Aporphine-pavine dimmers | *N*-Benzyltetrahydroisoquinolines |
| Oxoaporphines | 4-Arylisoquinolines |
| Aristolochic acid and aristolactams | Azafluoranthenes and tropolosoquinolines |
| Phenanthrene | 1, 6-Diazafluoanthenes |

The isoquinoline alkaloids are a large class of medicinally active alkaloids with a wide variety of properties. Their properties include being antispasmodic, antimicrobial, antitumour, antifungal, anti-inflammatory, cholagogue, hepatoprotective, antiviral, amoebicidal, anti-oxidant and can act as enzyme inhibitors.

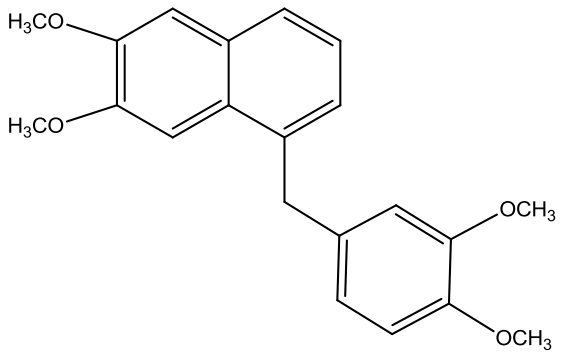
### 2.8.1 Benzylisoquinolines




The benzylisoquinoline types of alkaloids are derived from phenylalanine or tyrosine and are the parent skeleton of a wide variety of alkaloids possessing numerous different ring systems. There are two types of benzylisoquinoline, i.e. 1, 2, 3, 4-tetrahydro isoquinoline such as orientaline **34** and a fully aromatic type such as papaverine **35**.




**34**



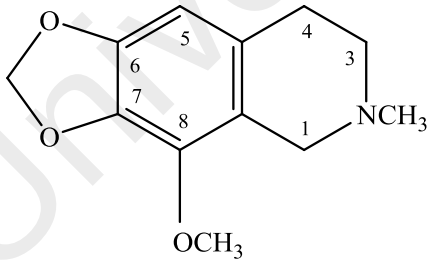

**35**

Benzylisoquinoline alkaloids are widely distributed in the family Anonaceae [57-59], Lauraceae [60-63], Menispermaceae [64-65], Papaveraceae [66-67], Fumariaceae [68-69], Ranuculaceae [70-71] and Berberidaceae [72-73].

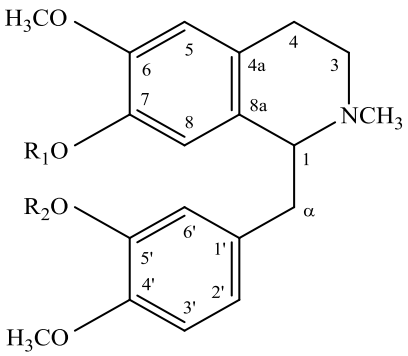
## $^1$H NMR

The $^1$H NMR data of benzylisoquinoline (Table 2.3) shows a number of interesting features due to the one asymmetric center at C-1. The H-1 showed a triplet or doublet-doublet ($J_1$ = 8-9, $J_2$ = 1.5-3 Hz) with chemical shift (in $CDCl_3$) between δ 3.6-3.7 while the aliphatic proton signals for H-3, H-4 and H-α, normally appeared as multiplet at δ 2.5-3.5.

The methylenedioxy protons resonate at δ 5.6-6.0. Base on the study of benzylisoquinoline compounds, this group can be attached to C-6,7 and C-3',4'. Methylenedioxy protons attached to C-6 and C-7, the signal showed as a doublet or singlet in the proton NMR spectrum. The methoxyl groups of the benzylisoquinoline generally resonate at δ 3.50-4.00. Normally, *N*-methyl groups resonated in the region of δ 2.4-2.6. Table 2.3 shows examples of chemical shift of $^1$H NMR for some benzylisoquinoline alkaloids, i.e. hydrocotarnine **36**, laudanosine **37**, and laudanine **38**.




**36**




**37** $R_1 = CH_3$, $R_2 = CH_3$

**38** $R_1 = CH_3$, $R_2 = H$

Table 2.3: $^{1}$H NMR (in $CDCl_3$, ppm) for hydrocotarnine **36**, laudanosine **37**, and laudanine **38**.

| Position of H | **36** | **37** | **38** |
|---|---|---|---|
| H-1 | 3.44 | 3.64 | 3.64 |
| H-3 | 2.60 | 2.73 and 3.12 | 2.74 and 3.09 |
| H-4 | 2.80 | 2.55 and 2.78 | 2.57 and 2.74 |
| H-5 | 6.31 | 6.50 | 6.45 |
| H-6 | - | - | - |
| H-7 | - | - | - |
| H-8 | - | 6.02 | 5.81 |
| H-α | - | 2.17 and 3.10 | 2.54 and 3.03 |
| H-2’ | - | 6.55 | 6.58 |
| H-3’ | - | - | - |
| H-4’ | - | - | - |
| H-5’ | - | 6.71 | 6.60 |
| H-6’ | - | 6.58 | 6.36 |
| 6-$OCH_3$ | - | 3.77 | 3.68 |
| 7-$OCH_3$ | - | 3.53 | 3.37 |
| 8-$OCH_3$ | 3.98 | - | - |
| 4’-$OCH_3$ | - | 3.78 | 3.68 |
| 5’-$OCH_3$ | - | 3.73 | - |
| *N*-$CH_3$ | 2.45 | 2.49 | 2.40 |
| $OCH_2O$ | 5.85 | - | - |

## $^{13}$C NMR

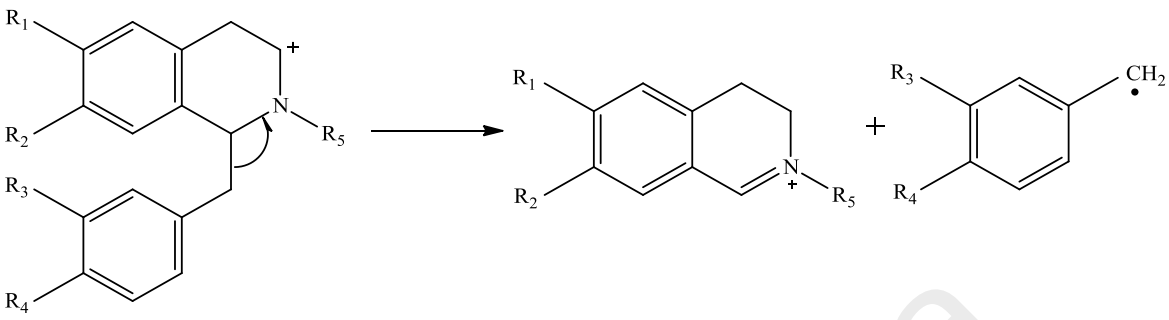
In the $^{13}$C NMR spectra, C-1 normally resonates at δ 52-58, but it resonates at higher field i.e. δ 60-67 in the presence of *N*-methyl group. Substituted carbons *N*-methyl, methoxyl and methylenedioxy appears at δ 40-45, δ 54-63 and δ 100-103, respectively. The quaternary carbon at the position 4a, 8a and 1' resonates at δ 115-132. The quaternary carbons with methoxyl and hydroxyl groups appear at δ 140-152. Unsubstituted $sp^2$ carbons usually appear at δ 100-130 and the $sp^3$ carbons at the position C-α and C-3 resonate at δ 38-40 and δ 45-46, respectively. The chemical shift of C-4 with *N*-methyl group in the structure appears at δ 23-24, but it will appear at δ 28-29 in the absence of the *N*-methyl group.

## Mass Spectrometry

In the mass spectra of benzylisoquinoline, the main cleavage occurs between C-1 and C-α to form an imine ion. The fragment ion corresponding to m/z 192 appears as a base peak indicating that C-6 and C-7 are substituted with methoxyl and hydroxyl groups respectively, and the structure bears a *N*-methyl group. If both C-6 and C-7 are substituted by methoxyl groups, the peak at m/z 206 corresponds to the base peak. The appearance of a base peak at m/z 176 indicates that C-6 and C-7 are attached to a methylenedioxy moiety without *N*-methyl in the structure.

Compounds having a methoxyl and hydroxyl groups in ring C displayed a peak at m/z 137. Two methoxyl groups attached to C-3' and C-4' showed the fragmentation peak at m/z 151 and a hydroxyl group in the ring C showed peak at m/z 107. [74] An

illustration of the mass fragmentation pattern of benzylisoquinoline is shown in Scheme 2.6.




m/z = 192 $R_1 = OCH_3$, $R_2 = OH$, $R_5 = CH_3$ ; m/z = 107 $R_3 = H$, $R_4 = OH$

m/z = 176 $R_1 = R_2 = OCH_2O$, $R_5 = H$ ; m/z = 121 $R_3 = OCH_3$, $R_4 = H$

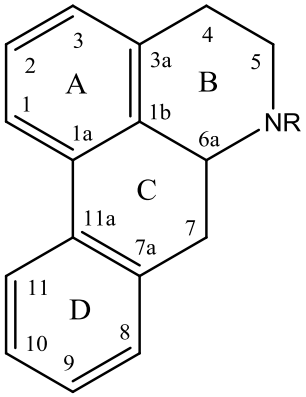
m/z = 206 $R_1 = R_2 = OCH_3$, $R_5 = CH_3$ ; m/z =137 $R_3 = O3\ H$, $R_4 = OCH_3$

Scheme 2.6: Illustration of the Mass Fragmentation Pattern of Benzylisoquinoline.

**Ultraviolet Spectrocopy**

The ultraviolet spectra of benzylisoquinoline show a maxima between 280 and 296 nm which has little effect by addition of aromatic substitution. Fully aromatic compounds having a methylenedioxy show more intensity absorption maximum.

### 2.8.2 Aporphines



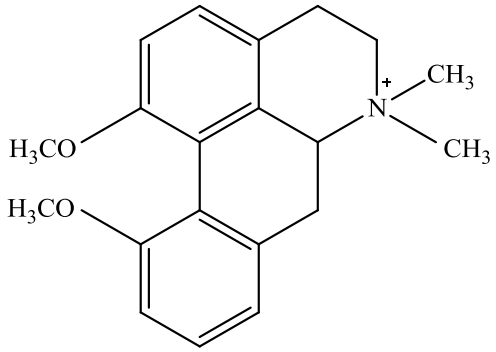

Aporphinoid alkaloids are known to possess medicinal properties. More than 600 aporphinoid alkaloids have been isolated from 19 plant families and many of those plants are used as folk medicines in different parts of the world for the treatment of several kinds of diseases. The family Lauraceae, Berberidacaeae, Magnoliaceae, Menispermaceae, Ranuculaceae, Monimiaceae, Pavaperaceae, and Annonaceae. [75-76]

Aporphine alkaloids are the largest group within the isoquinoline alkaloids. It contains a twisted biphenyl system. It consists of four rings, A, B, C, D. The whole aporphines alkaloids are based on the 4H-diben[de,g]quinoline structure or its *N*-methyl derivative commonly known as the aporphine nucleus. It can be divided into three groups: [77-78]

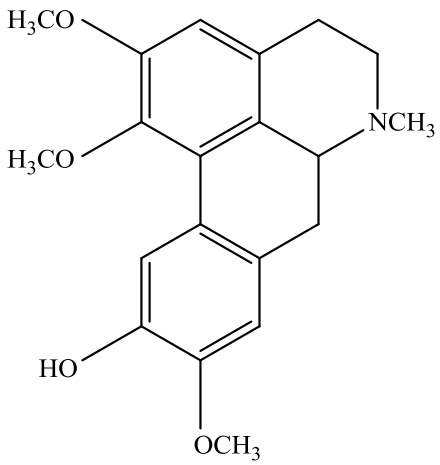
a) The quaternary aporphines salts, for example *N*, *N*-dimethylhernovine **39**

b) The aporphines which contain *N*-methyl moeity, lirioferine **40**, *N*-methyllauroteanine **41**

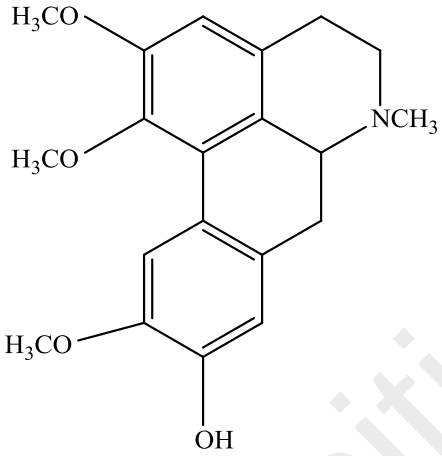
c) The noraporphine, which possess a secondary nitrogen atom, eg: norlirioferine **42**



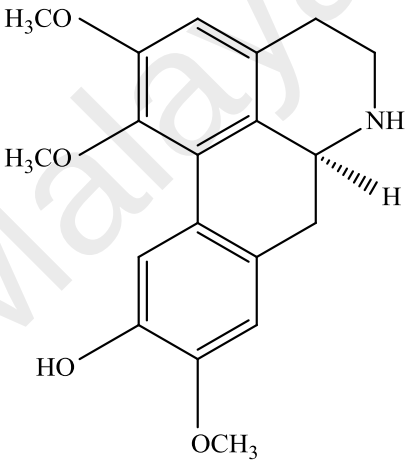

**39**




**40**




**41**




**42**

## $^{1}$H NMR [78]

The $^{1}$H NMR spectrum can provide important and valuable information leading to the structural elucidation of aporphines. The chemical shifts are very dependent on the position of the protons with respect to the aromatic rings. Several general features have been observed in the proton shifts of these alkaloids. The following chemical shifts have been observed.

**Methoxyl group** [79]

From the $^{1}H$ NMR spectrum, the aromatic protons appeared at δ 6.3-8.2 and the methoxyl protons at δ 3.3-3.9. The most deshielded methoxyl protons are those attached to C-2, C-9 and C-10 and the most shielded at C-1, except when C-1 is substituted by a methylenedioxy group. Protons for methoxyl group attached to C-1 usually appeared at high field due to the steric effect of ring D.

The most downfield aromatic proton (H-11) was observed between δ 7.5 and 8.2 depending on adjacent substituents at C-10. Substitution at C-10 will cause shielding of the H-11 proton. The most shielded aromatic proton is at C-3 and typically appeared as a singlet in the range of δ 6.5-7.5. The presence of a methoxyl group at C-11 significantly affects the chemical shift of H-8 and H-9. If C-11 bears a hydroxyl group, the chemical shift of H-8 and H-9 because overlapped and thus no coupling was observed. The position of C-2 is also substituted when position of C-1 and C-11 are substituted. This affects the methoxyl groups at C-1 and C-11 that would be sterically hindered. As a result, the methoxyl protons are pushed out of the aromatic plane, into a shielded area. In addition, ring A and ring D is positioned facing each other. Hence the protons of the methoxyl groups can arrange themselves on top of the adjacent ring, which happens to be a shielded zone giving a more upfield shift. [79]

**Methylenedioxy group** [77-78]

A methylenedioxy group usually shows resonances in the region of δ 5.87-6.02. The five possible locations for this group are C-1, 2; C-2, 3; C-9, 10; and C-10, 11. The presence of C-1, 2 methylenedioxy groups will cause an up field shift of the C-11 proton which will appear in the range δ 7.47-7.86; the resultant twisted biphenyl system can induce magnetic nonequivalence between the methylene protons, which then appeared as doublets at δ 5.9 and 6.1.

Protons for methylenedioxy group at C-9 and C-10 appear as a singlet whereas protons for those having methylenedioxy groups C-1 and C-2; C-2 and C-3; C-10 and C-11 appear as two doublets with coupling constant of about 1.5 Hz. This inequivalence arises from the torsion caused by the twisted biphenyl system of ring A and D. The appearance of the torsional effect on the methylenedioxy moiety depends on their positions and at position C-9, 10 the effect appears to be negligible.

**Aromatic protons** [78]

The hydrogens at C-3, C-8 and C-9 of aporphine are located upfield between δ 6.38-7.00 and cannot be easily differentiated from one another while hydrogen at C-11 is found relatively downfield between δ 7.57-8.05. Nevertheless, H-3 normally resonates at a higher field compared to the other aromatic protons (δ 6.50-6.70) when it is ortho to a hydroxyl or a methoxyl. This is due to induction effect. On the other hand, H-11 usually resonates at a lower field with respect to the other protons due to the deshielding effect imposed by the facing aromatic ring A and hydrogen bonding with the C-1 substituent in ring A.

**_N_-methyl group and aliphatic protons** [78-79]

The *N*-methyl protons were typically observed at δ 2.4-2.8. The aliphatic protons of C-4, C-5 and C-7 displayed a complex resonance pattern with absorption in the region of δ 2.40-4.40 whereas methyl protons resonated in the region of δ 2.50-2.60. A summary of the $^1$H NMR data of aporphine alkaloids are given in the Table 2.4.

Table 2.4: $^1$H NMR Data (δ/ppm) of Aporphine Alkaloids in $CDCl_3$

| Position of substitution | Methoxyl proton | Methylenedioxy proton | Aromatic proton | *N*-methyl proton | Aliphatic proton |
|---|---|---|---|---|---|
| C-1 | 3.70-3.55 | | | | |
| C-2 | 4.12-3.75 | | | | |
| C-3 | | | | | |
| C-8 | | | 7.00-6.38 | | |
| C-9 | 4.12-3.75 | | 7.00-6.38 | | |
| C-10 | 4.12-3.75 | | | | |
| C-11 | 3.75-3.65 | | 8.74-8.68 | | |
| C-1, 2 | | 5.87-6.02 | | | |
| C-2,3 | | 5.87-6.02 | | | |
| C-8, 9 | | 5.87-6.02 | | | |
| C-9, 10 | | 5.87-6.02 | | | |
| C-10, 11 | | 5.87-6.02 | | | |
| *N*-Me | | | | 2.50-4.44 | |
| C-4 | | | | | 2.40-4.00 |
| C-5 | | | | | 2.40-4.00 |

**$^{13}$C NMR [79]**

The general characteristic of the different type of carbons are mentioned below.

a) $sp^2$ carbon bearing hydrogen: δ 105-112.
b) $sp^2$ carbons at position 1a, 1b, 3a, 7a and 11a: δ 119-130.
c) $sp^3$ carbons at position 4(δ 28-30), 7(δ 35) and 5 and 6a (about δ 42 and 53 for noraporphine) and (about δ 53 and 62 for aporphine).
d) Carbon of the substituents: *N*-methyl (about δ 43), methoxyl (δ 56-62) and methylenedioxy (δ 100).

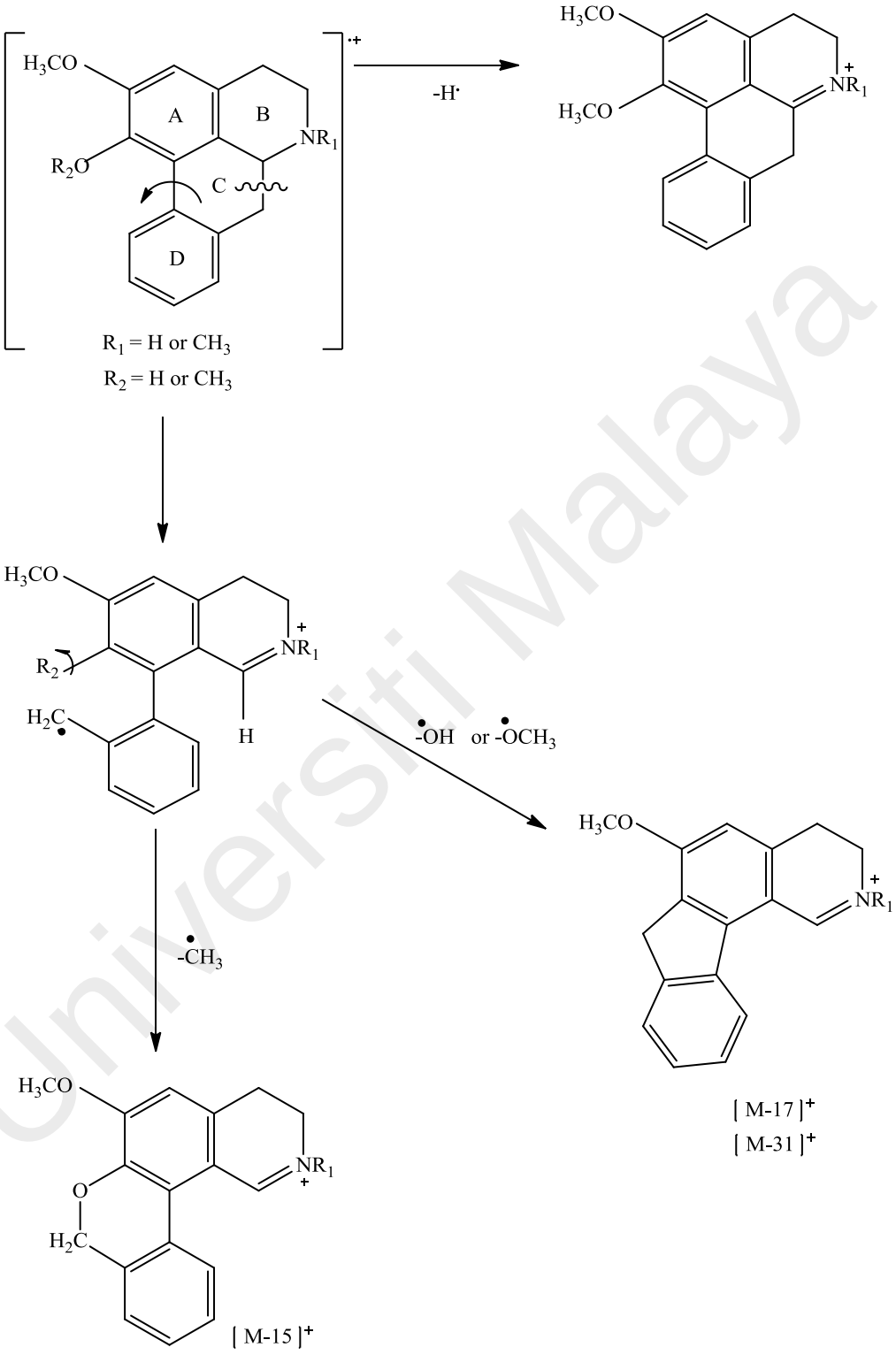
The quaternization of the *N*-atom causes deshielding of C-5 and C-6a and shielding of C-1b, C-3a, C-7 and C-7a.

**Mass Spectrometry [80]**

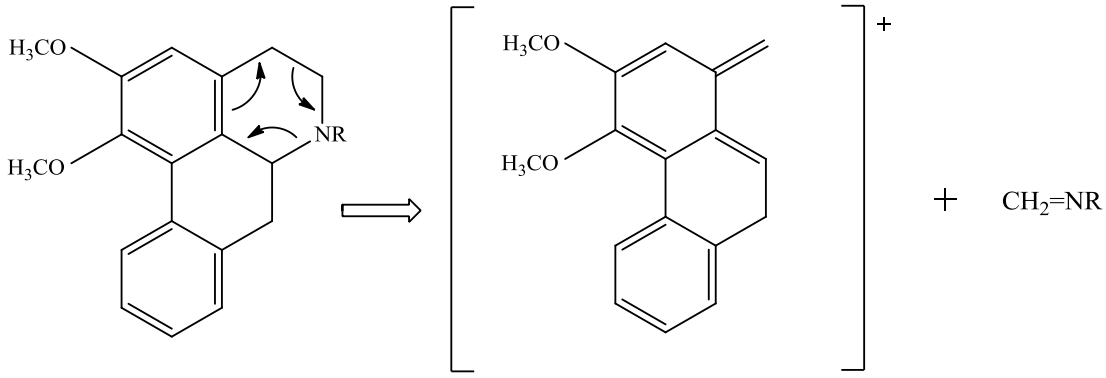
In the mass spectrum, the major fragmentation of the aporphine alkaloids is due to the loss of the hydrogen atom on C-6a. The $[M-1]^+$ peak always serves as the base peak of the molecule. If the molecule is substituted; $[M-15]^+$, $[M-17]^+$ and $[M-31]^+$ peak will also be observed due to the expulsion of a methyl, hydroxyl or methoxyl group respectively (Scheme 2.7). A hydroxyl group is substituted at ring D, a $[M-17]^+$ peak will be observed.

Aporphine compound having the *N*H or *N*-$CH_3$ groups will display peaks at $[M-29]^+$ and $[M-43]^+$ respectively which correspond to the lost of methylene imine group (-$CH_2$=NR) expelled via a retro Diels-Alder mechanism (Scheme 2.8) (or by the cyclic

process in ring B). The ion formed can further loose another methyl or methoxyl groupto produce peaks at $[M-74]^+$, $[M-58]^+$, $[M-60]^+$, and [M-44]. [80]




Scheme 2.7: The Principle Mass Fragmentation of




R = H or $CH_3$

If R = H, peak observed is $[M-29]^+$

If R = $CH_3$, peak observed is $[M-43]^+$

Scheme 2.8: The Mass Fragmentation of Aporphine with *N*-methyl or *N*-H Group.

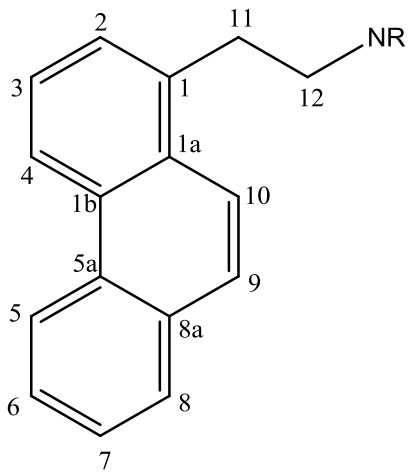
**Ultraviolet spectrum** [81]

The positions of the maximum absorptions in the ultraviolet spectra of aporphines depend mainly upon the location of the substituents. It is derived from the basic biphenyl system with the added influence of several auxochromes. The approximate absorption for various substitution patterns are listed as below.

| Position of substituents | Absorption maximums (nm) |
|---|---|
| 1, 2 | 234, 273, 312 |
| 1, 2, 9 | 233, 280, 305 |
| 1, 2, 10 | 226, 266, 275, 305 |
| 1, 2, 11 | 220, 265, 272, 300 |
| 1, 2, 9, 10 | 220, 282, 305 |
| 1, 2, 10, 11 | 220, 270, 305 |

The shape of the curve and the density of the latter two maxima depend on the substitution in ring D. Furthermore, the monophenolic aporphine position at C-3 and C-9 display a bathochromic shift at 315 nm and 350 nm in the alkaline environment. [81]

### 2.8.3 Phenanthrene Alkaloid




The phenanthrene alkaloids are derivatives of 1-(2-aminoethyl)phenanthrene. Even though they do not contain a nitrogen heterocycle, they are considered alkaloids because of their close relationship with aporphine alkaloids. Besides, they were found in the same plant families as aporphines, from which they are biogenetically derived, and they are usually included within the aporphinoids. Phenanthrene alkaloids with a 2-(methylamino)ethyl side chain are 6,6a-secoaporphines, while those with a 2-(dimethylamino)ethyl chain are often known as methines of the corresponding aporphine. [82]

Phenanthrene alkaloids are widely distributed and commonly co-occur with aporphine alkaloids. They are found in Menispermaceae, [83-84] Annonaceae, [85-87] Ranuculaceae, [88] Fumariaceae, [82] Hernandiaceae, [89] and Lauraceae. [90-91]

**$^1$H-NMR**

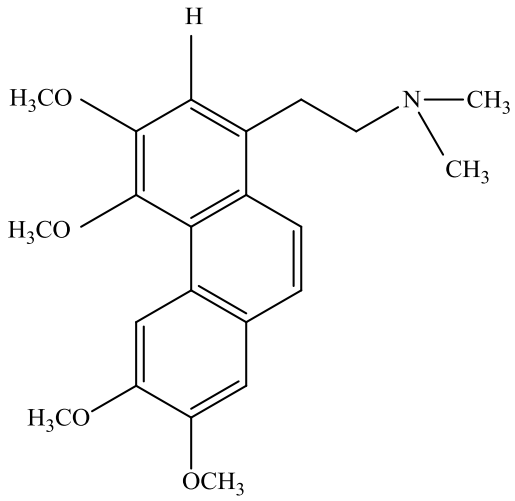
The $^1$H-NMR spectra displayed the average chemical shifts of methoxyl groups of phenanthrene alkaloids which resonated at δ 3.9-4.1. One sharp peak attributed to three or six hydrogen group attached to nitrogen, was usually observed at δ 2.4-2.8. Methylenedioxyl protons show a peak at δ 6.0-6.3.

As with aporphines, the C-5 proton appears quite upfield relative to the other aromatic protons which was observed at δ 9.3-9.6 in the $^1$H-NMR spectra. The presence of two doublets arising from the protons at C-9 and C-10 is a very characteristic feature of phenanthrene alkaloid skeleton. They appear close to each other at δ 7.3-7.9, with coupling constant of 9-10 Hz. The 2-aminoethyl side chain give rise to two batches of multiplets or doublet-doublet (J=15 Hz) at δ 3.2-3.6 and δ 2.6-3.0, each derived from two protons, which were typical of phenanthrene alkaloids for C-11 and C-12. [82]

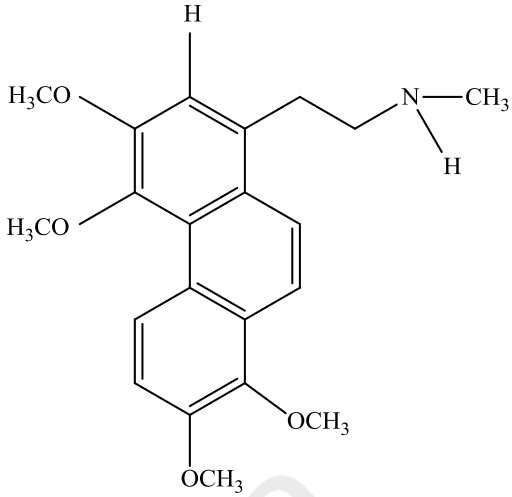
Table 2.5 showed the examples of chemical shift of the $^1$H NMR of some phenanthrene alkaloids, i.e. glaucine methine **43**, [86] and *N*-demethyl-2-methoxyatherosperminine **21**. [22]

Table 2.5: $^{1}H$ NMR (in $CDCl_3$, ppm) for Some Phenanthrene Alkaloids.

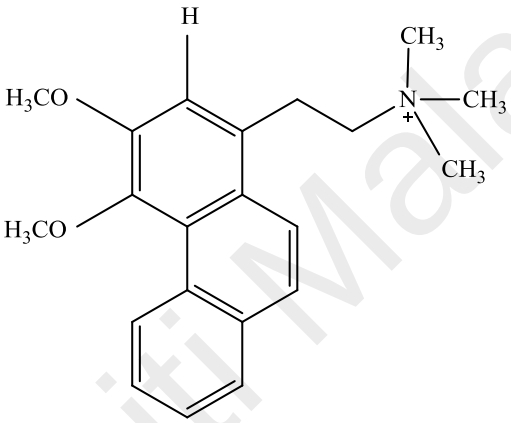
| Position of H | **43** | **21** |
|---|---|---|
| H-2 | 7.32 | - |
| H-5 | 9.30 | 9.53 |
| H-6 | - | 7.58 |
| H-7 | - | 7.53 |
| H-8 | 7.22 | 7.79 |
| H-9 | 7.80 | 7.67 |
| H-10 | 7.55 | 7.93 |
| H-11 | 3.30 | 3.43 |
| H-12 | 2.70 | 2.97 |
| 2-$OCH_3$ | - | 3.98 |
| 3-$OCH_3$ | 4.10 | 3.94 |
| 4-$OCH_3$ | 3.94 | 4.03 |
| 6-$OCH_3$ | 4.05 | - |
| 7-$OCH_3$ | 4.10 | - |
| *N*-$CH_3$ | 2.40 | 2.58 |



**43**



**44**



**45**

## $^{13}$C-NMR

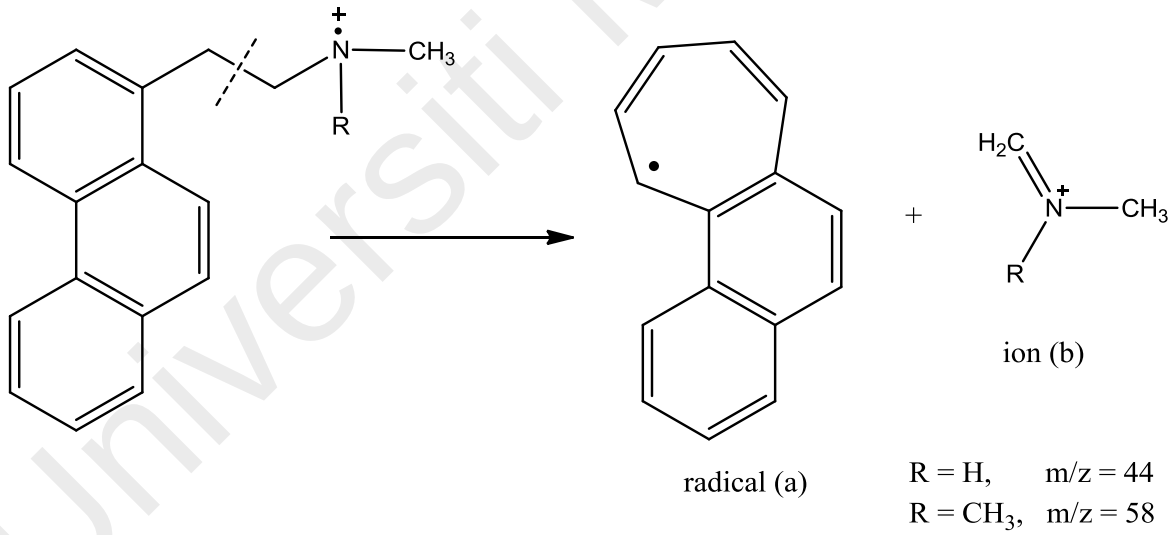
In the $^{13}$C-NMR spectra, the chemical shift of the methoxyl group appeared at δ 60.0-61.0, whereas the *N*-methyl resonated at δ 42.0-43.0 and the chemical shift of $sp^2$ carbon bearing the hydrogen aromatic appeared at δ 120.0-128.0. Examples of chemical shift of phenanthrene alkaloids, Noruvariopsamine **44** [93] and *N*-methylatherosperminium cation **45** [92] are shown in Table 2.6.

Table 2.6: $^{13}$C-NMR (in $CDCl_3$, ppm) for Some Phenanthrene Alkaloids.

| Position of H | **44** | **45** |
|---|---|---|
| C-1 | 130.7 | 129.2 |
| C-1a | 125.5 | 125.5 |
| C-1b | 124.1 | 124.3 |
| C-2 | 112.7 | 116.1 |
| C-3 | 151.0 | 150.8 |
| C-4 | 146.0 | 145.9 |
| C-5 | 113.0 | 122.1 |
| C-5a | 121.5 | 132.4 |
| C-6 | 110.4 | 126.5 |
| C-7 | 149.2 | 126.5 |
| C-8 | 153.7 | 128.3 |
| C-8a | 129.6 | 132.7 |
| C-9 | 121.4 | 127.4 |
| C-10 | 119.3 | 127.4 |
| C-11 | 31.0 | 26.2 |
| C-12 | 54.4 | 65.4 |
| 3-$OCH_3$ | 56.1 | 56.5 |
| 4-$OCH_3$ | 60.6 | 59.3 |
| 7-$OCH_3$ | 56.1 | - |
| 8-$OCH_3$ | 60.7 | - |
| *N*-$CH_3$ | 36.2 | 52.3 |

**Mass Spectrometry**

The mass of phenanthrene alkaloids are very informative regarding the nature of the side chains, which is the mode of cleavage of the amino group. The two fragmentations corresponding to loss of $CH_2NR_1R_2$ and the $CH_2{=}N + R_1R_2$ cation. This cation is usually the base peak and allows the identification of $R_1$ and $R_2$. Free bases and quaternary salts in this series generally undergo fragmentation *beta* to the nitrogen to generate a tropulium radiacal (a) and a mono-, di- or trimethylaminomethylene ion as the base peak (b). Thus, phenanthrenes with a 2-dimethylaminoethyl side chain present the base peak at 58 mass units, whereas those with a 2-monomenthylaminoethyl side chain show the base peak at 44 mass units. [82, 86] The mass fragmentation pattern is given in Scheme 2.9.




Scheme 2.9. The Mass Fragmentation of Phenanthrene Alkaloid

# CHAPTER 3

## RESULTS AND DISCUSSIONS

# 3.0 RESULTS AND DISCUSSIONS

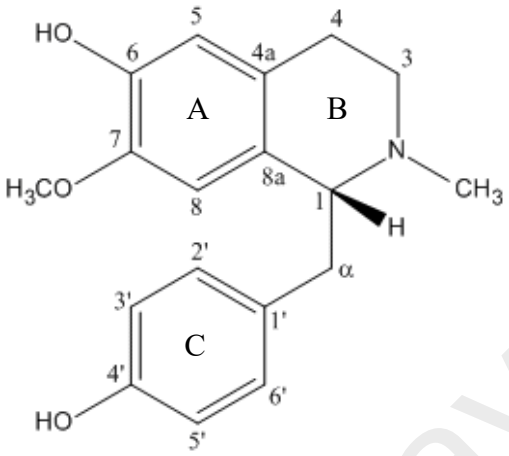
## 3.1 Chemical Constituents in *Crytocarya nigra* (Lauraceae)

Alkaloids from the bark and leaves of this species were extracted by the general procedure as described in the experimental section in Chapter 5.

The dichloromethane extract obtained were subjected through extensive column chromatography (CC) and by preparative thin layer chromatography (PTLC). Four alkaloids (*N*-methylisococlaurine **46**, atherosperminine **11**, 2-hydroxyatherosperminine **20**, and noratherosperminine **47**) were isolated from the bark and three alkaloids (isocorydine **48**, norisocorydine **49**, argentinine **50**) with one non-alkaloidal aromatic compound (benzamide **51**) were afforded from the leaves of *Crytocarya nigra*.

The following subchapter shall discuss briefly and concisely on the structural elucidation of each compounds isolated.

### 3.1.1 *N*-Methylisococlaurine 46




**46**

*N*-Methylisococlaurine **46** was isolated as a brownish amorphous solid with $[\alpha]_D^{27}$ = +29.4º (c 1.0, MeOH). The UV spectrum showed absorption bands at 301 nm. The IR spectrum (Figure 3.3) of this alkaloid showed strong absorption peak at 3266 and 2921 $cm^{-1}$ indicated the presence of hydroxyl group and stretching of C-H aromatic, respectively [94]. The LCMS spectrum (Figure 3.2) of compound **46** showed a pseudomolecular ion peak at m/z 300.16, $[M+H]^+$ corresponding to the molecular formula $C_{18}H_{21}NO_3$, with nine degrees of unsaturation (3 rings and 6 double bonds).

The $^1$H NMR spectrum (Figure 3.4) revealed six aromatic proton signals at the low field region. There were two singlets at δ 6.34 and δ 6.21 attributable to H-5 and H-8 respectively. Two signals centered at δ 6.78 (d, J=8.5, H-2' and H-6') and δ 6.50 (d, J=8.5, H-3' and H-5') appeared as doublets. The $^1$H NMR spectrum exhibited one methoxyl group and one *N*-methyl group at δ 3.82 and δ 2.42 respectively each appearing as a singlet. Furthermore, a total of seven proton signals were observed at a

higher region between δ 2.76-3.66 attributable to the aliphatic protons of H-$\alpha_a$, H- $\alpha_b$, H-1, H-$3_a$, H-$3_b$, H-$4_a$ and H-$4_b$. In fact, the correlation of vicinal protons H-$\alpha_a$/H-$\alpha_b$, H-2'/H-3', H-5'/H-6' and H-3/H-4 were observed in the COSY spectrum (Figure 3.7). The complete assignments for the proton and carbon signals are tabulated in Table 3.1 and $^{1}$H-$^{1}$H and $^{1}$H-$^{13}$C coupling pattern are shown in Figure 3.1.

The $^{13}$C NMR spectrum (Figure 3.5) is in agreement with the molecular formula deduced from the mass spectrum, accounting for all 18 carbons. While the DEPT 135 spectrum (Figure 3.6) showed there are one methoxyl, one *N*-methyl, three methylenes, seven methines, and six quaternary carbons in the molecule skeleton. The highest field signal in the $^{13}$C spectra was observed at δ 64.83 can be assigned to C-1, meanwhile two overlapping signals appeared at δ 130.4 and δ 115.5 corresponded to C-2'/C-6' and C-3'/C-5' respectively. The HMQC spectrum (Figure 3.8) displayed the cross-peaks of H-5/C-5 and H-8/C-8 at $\delta_H$ 6.34/ $\delta_C$ 110.8 and $\delta_H$ 6.21/ $\delta_C$ 113.9 ppm respectively.

The HMBC spectrum (Figure 3.9) revealed correlation H-5 to H-7 and H-8a, further supported the position of OMe group at C-7. In addition, the correlations of H-2' and H-6' to the carbon bearing hydroxyl group, C-4', were also observed. Another hydroxyl position at C-6 was deduced through correlation of H-8 to C-6, C-1 and C-4a. Long range coupling of H-5 to one of the C-4 protons is clearly seen in the HMBC spectrum.

Finally, assignment of all proton and carbon signals were established by DEPT, COSY, HMQC and through analysis of HMBC spectra and also by comparison with literature value [94,95] and thus confirmed the structure of *N*-Methylisococlaurine **46**.

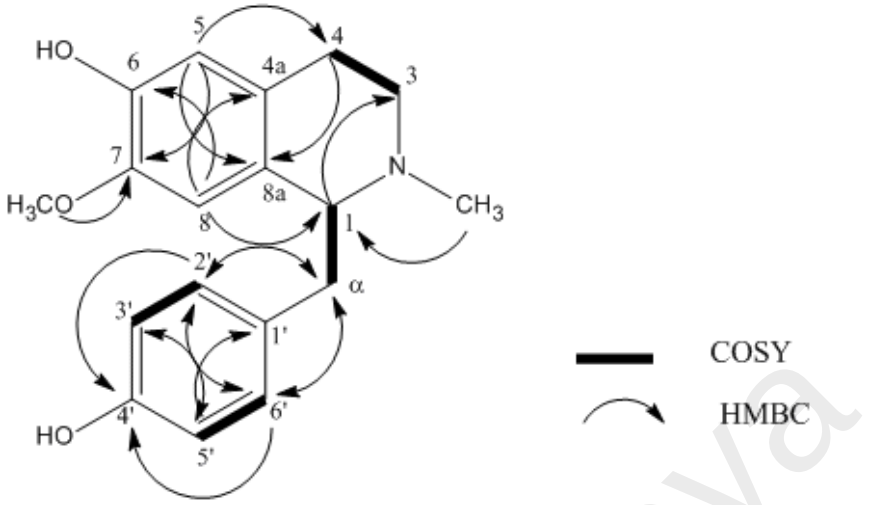



Figure 3.1: $^{1}H$-$^{1}H$ and $^{1}H$-$^{13}C$ Coupling Pattern Observed in COSY and HMBC Spectra of *N*-methylisococlaurine **46**

Table 3.1: $^{1}$H NMR (in $CDCl_3$, 400 MHz), $^{13}$C NMR (in $CDCl_3$, 400 MHz), HMBC and HMQC of **46** and comparison with $^{1}$H NMR literature values (Saidi *et al.* 2011)

| Position | $\delta_H$, ppm (J in Hz) (observed) | $\delta_C$ (ppm) (observed) | HMBC (H→C) | HMQC | $\delta_H$, ppm (J in Hz) (Saidi *et al.*, 2011) |
|---|---|---|---|---|---|
| 1 | 3.63-3.66, *t*, J=6.5 | 64.8 | α, 3, 8a | H-1 | 3.67-3.79, *t* |
| 3 | $H_a$: 2.96-3.01, *m*<br>$H_b$: 2.52-2.65, *m* | 44.6 | - | H-3 | $H_a$: 2.98-3.03, *dd*<br>$H_b$: 2.74-2.77, *m* |
| 4 | $H_a$: 2.52-2.65, *m*<br>$H_b$: 2.25-2.40, *m* | 24.7 | 3, 4a, 8a | H-4 | $H_a$: 3.17-3.24, *m*<br>$H_b$: 2.53-2.60, *m* |
| 4a | - | 125.2 | - | - | - |
| 5 | 6.34, *d,* J=2.2 | 110.8 | 4, 8a, 7 | H-5 | 6.51, *s* |
| 6 | - | 145.3 | - | - | - |
| 7 | - | 143.5 | - | - | - |
| 8 | 6.21, *s* | 113.9 | 1, 6, 4a | H-8 | 6.29, *s* |
| 8a | - | 130.1 | - | - | - |
| α | $\alpha_a$: 2.77-2.83, *dd*<br>$\alpha_b$: 2.52-2.65, *m*<br>J=14.4, 6.5 | 40.8 | 8a, 2', 6' | H-α | 2.80-2.86, *m* |
| 1' | - | 131.1 | - | | - |
| 2' | 6.78, *d*, J=8.5 | 130.4 | α, 4', 6' | H-2' | 6.86-6.88, *d*, J=8.3 |
| 3' | 6.50, *d*, J=8.5 | 115.5 | 1', 5' | | 6.54-6.56, *d*, J=8.3 |
| 4' | - | 154.6 | - | - | - |
| 5' | 6.50, *d*, J=8.5 | 115.5 | 1', 5' | H-5' | 6.54-6.56, *d*, J=8.3 |
| 6' | 6.78, *d*, J=8.5 | 130.4 | α, 6', 4' | H-6' | 6.86-6.88, *d*, J=8.3 |
| 7-OMe | 3.82, *s* | 55.9 | | - | 3.74, *s* |
| *N*-Me | 2.42, *s* | 42.3 | 1 | - | 2.37, *s* |

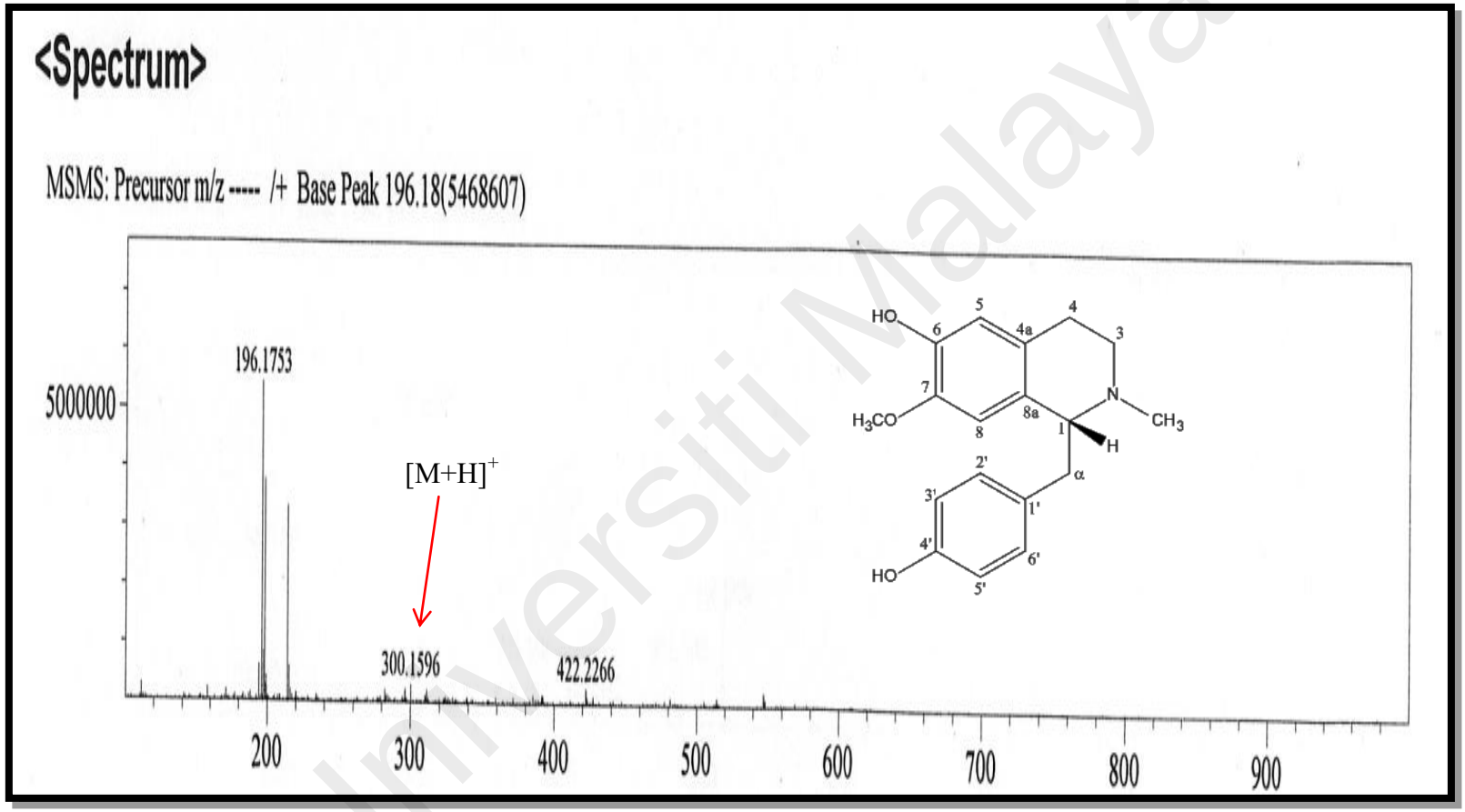



Figure 3.2: LCMS Spectrum of *N*-methylisococlaurine **46**

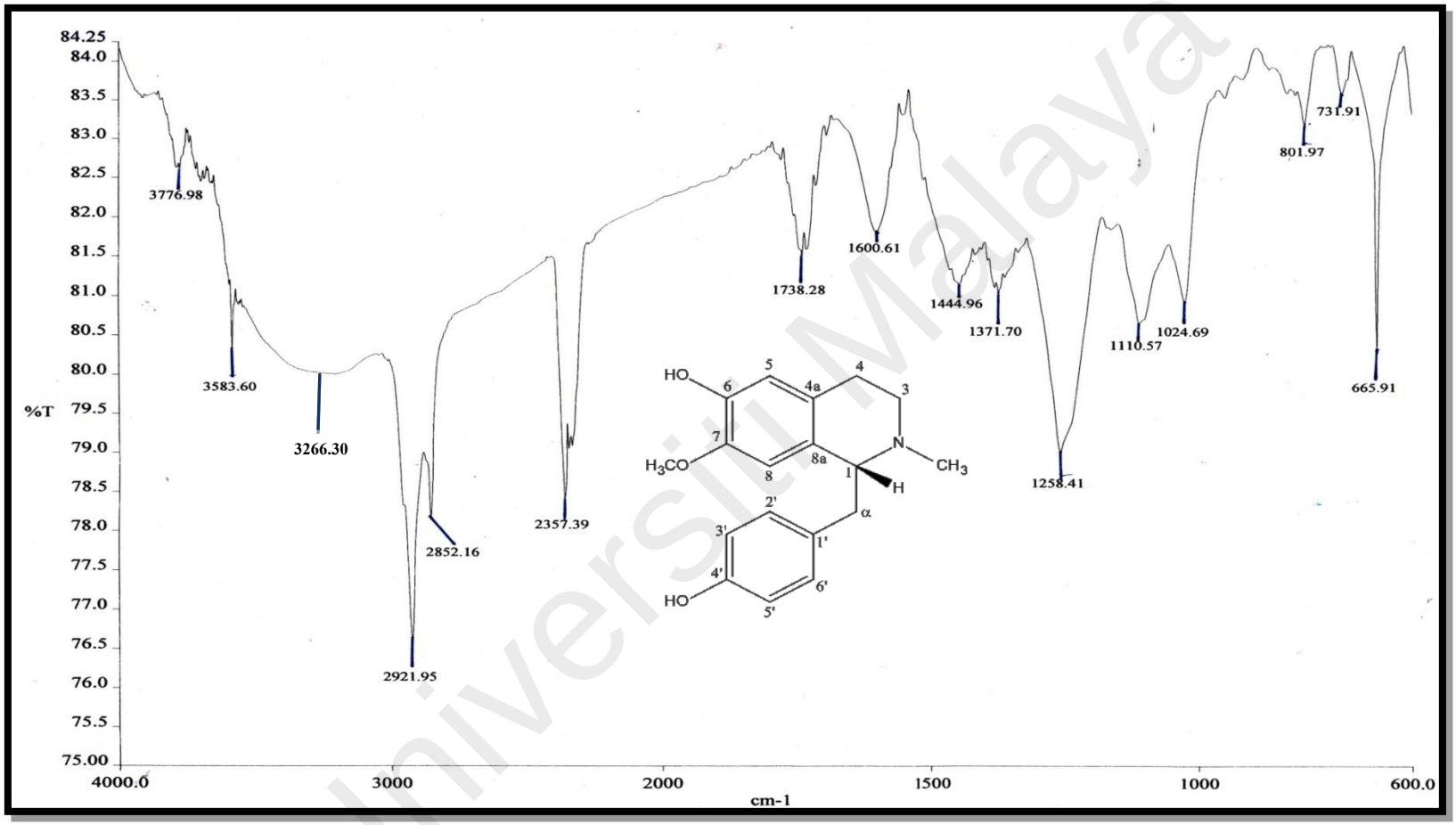



Figure 3.3: IR Spectrum of *N*-methylisococlaurine **46**

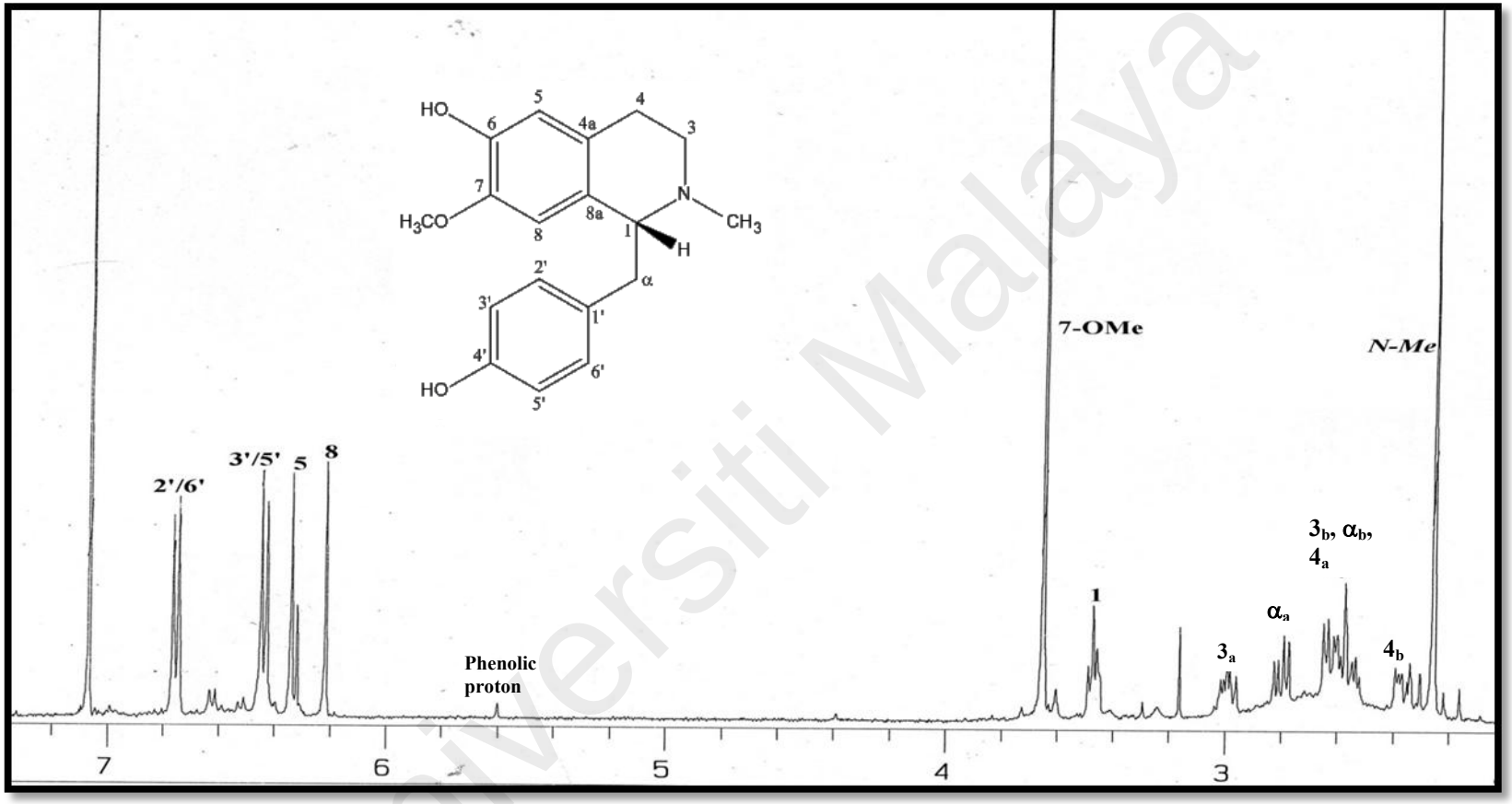



Figure 3.4: $^{1}$H NMR Spectrum of *N*-methylisococlaurine **46**

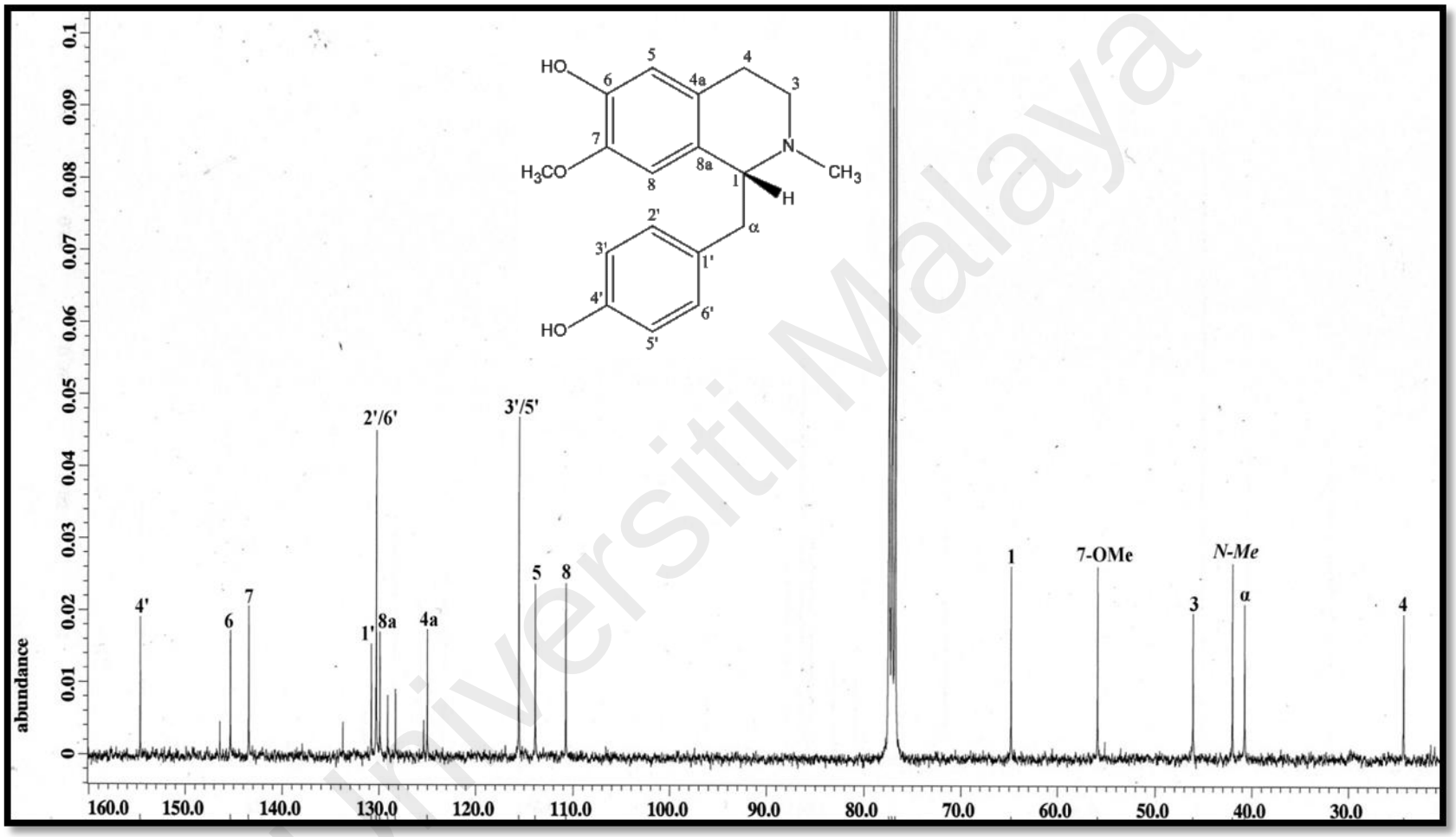



Figure 3.5: $^{13}$C NMR Spectrum of *N*-methylisococlaurine **46**

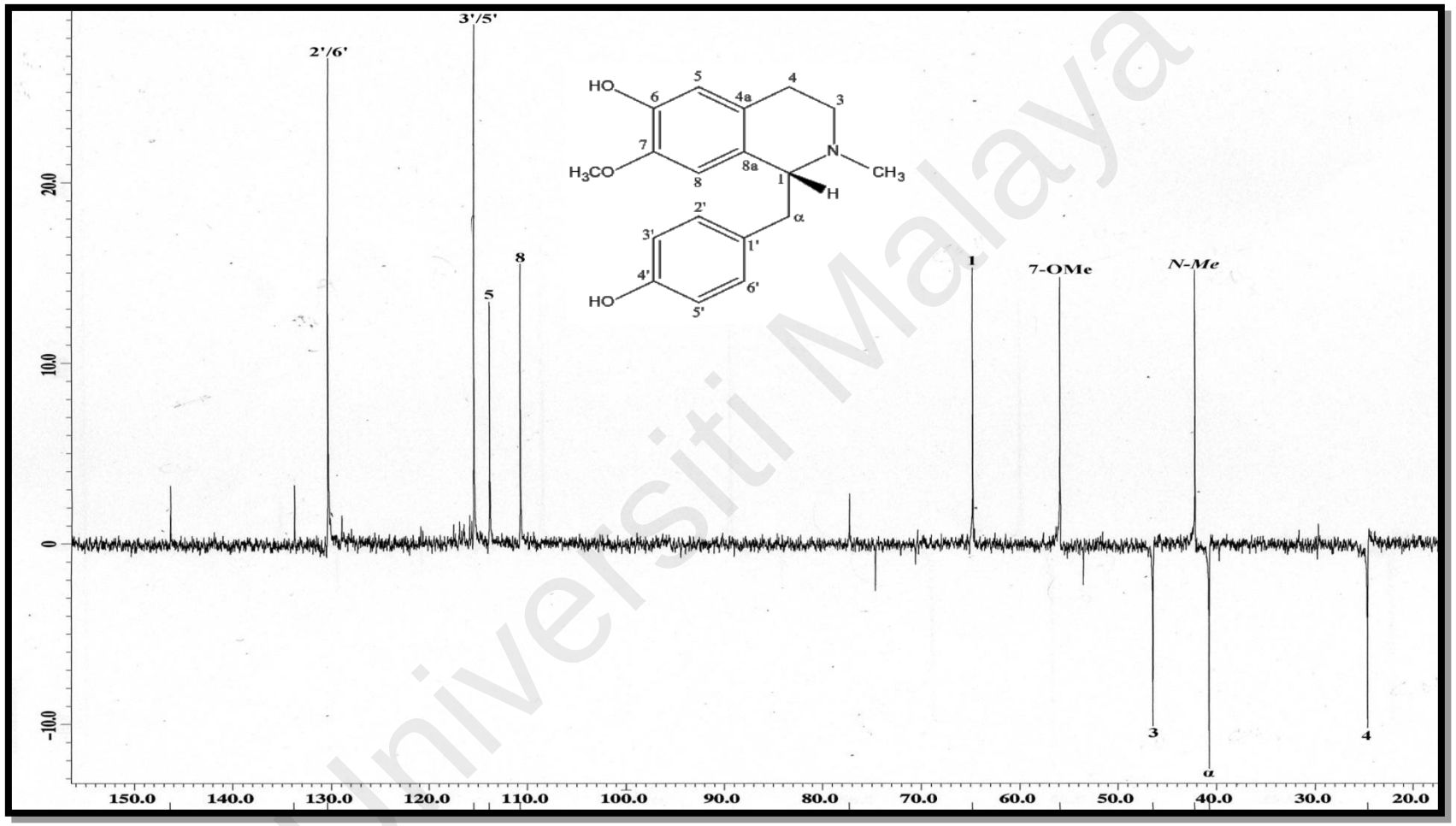



Figure 3.6: 135 DEPT NMR Spectrum of *N*-methylisococlaurine **46**

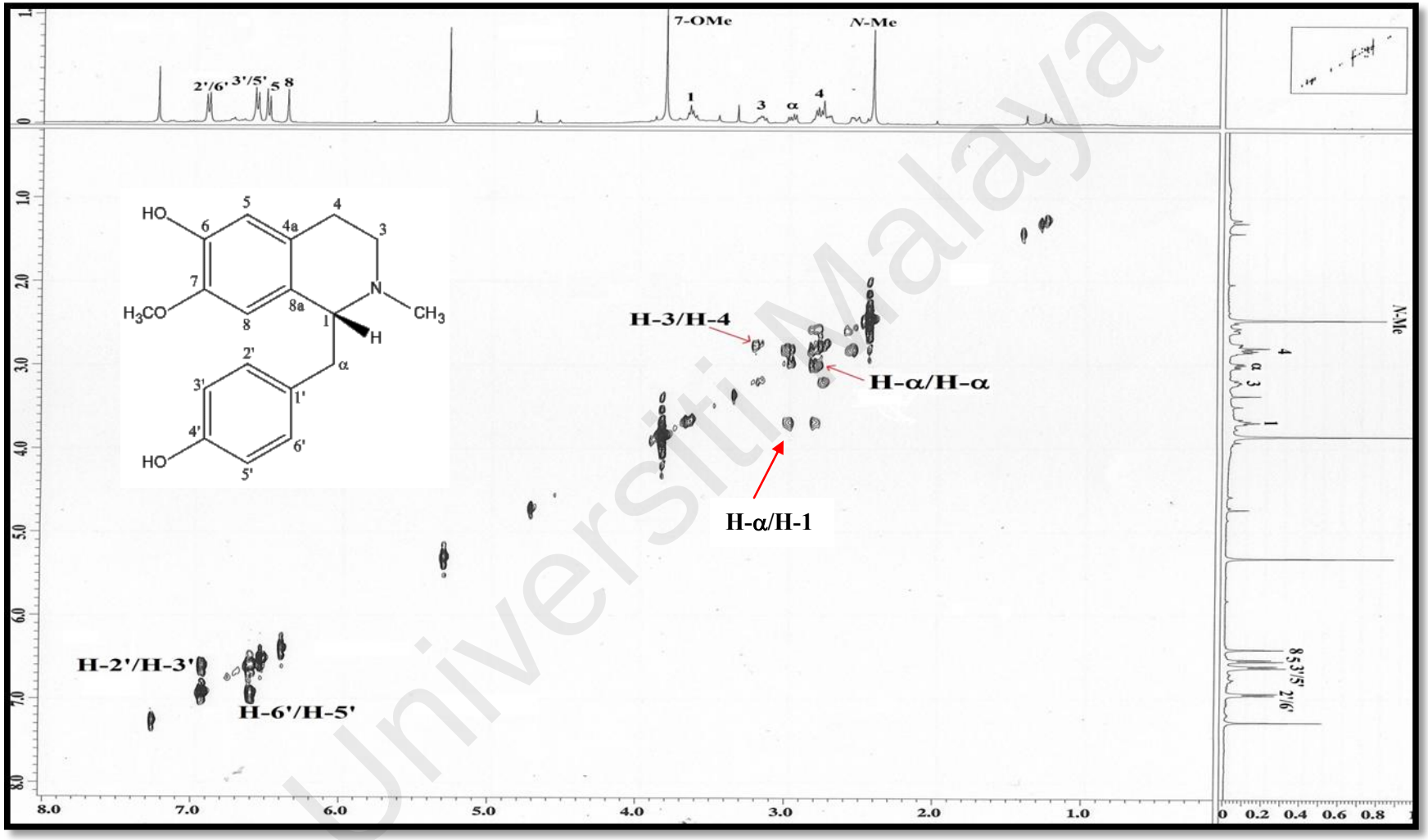



Figure 3.7: COSY Spectrum of *N*-methylisococlaurine **46**

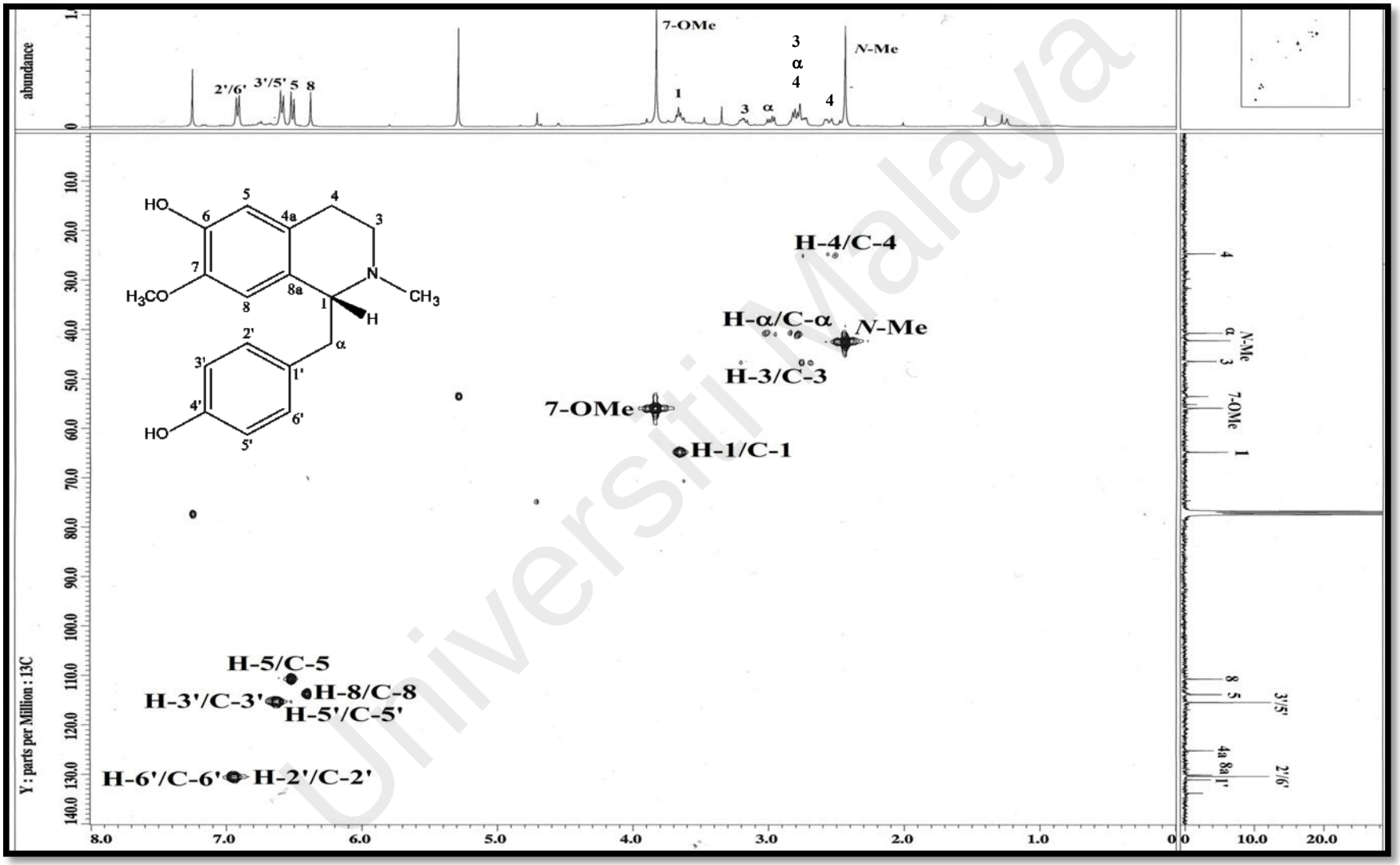



Figure 3.8: HMQC Spectrum of *N*-methylisococlaurine **46**

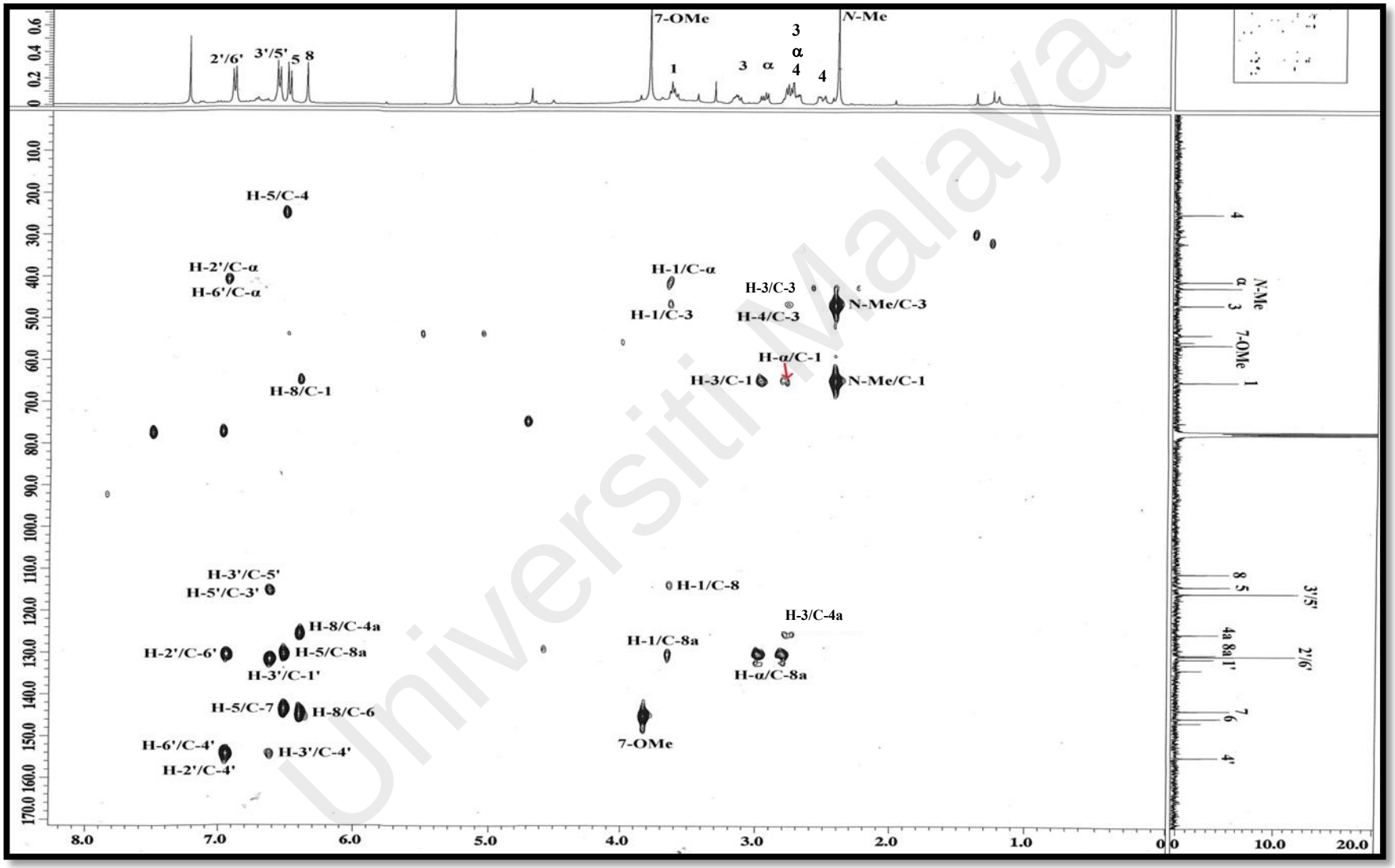
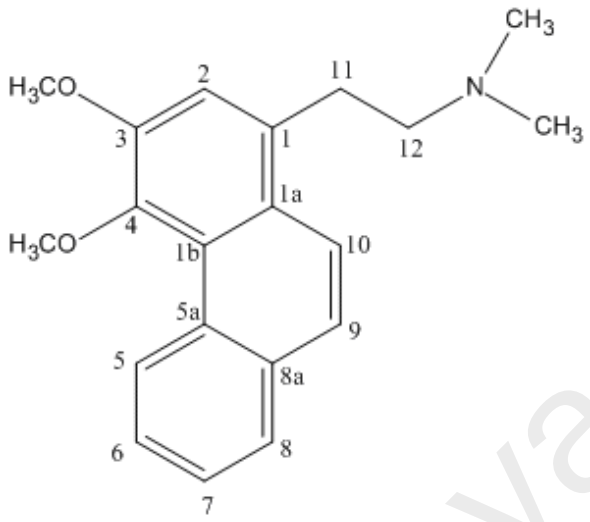



Figure 3.9: HMBC Spectrum of *N*-methylisococlaurine **46**

### 3.1.2 Atherosperminine 11




**11**

Atherosperminine **11** was isolated as brownish amorphous solid. Its UV spectrum exhibited absorption bands at 217, 227, 256, 306 and 345 nm.[96] The IR spectrum showed absorption at 1600 $cm^{-1}$ indicated the stretching of aromatic C=C. The LCMS spectrum (Figure 3.11) revealed a pseudomolecular ion peak, $[M+H]^+$ at m/z 310.18 suggesting a molecular formula of $C_{20}H_{23}NO_2$ with 10 degree of unsaturation.

The $^1$H-NMR spectrum (Figure 3.12) displayed 7 proton signals in the aromatic region including a doublet that belong to H-5 at δ 9.67 which represented typical characteristic of phenanthrene type. There were overlapping signals of doublet and doublet of doublets (*dd*) observed in the region δ 7.84-δ 7.87 which were assignable to H-10 and H-8. This spectrum also displayed a multiplet signal attributable to H-9, H-7 and H-6 which were present at δ 7.56- δ 7.64. In addition, a singlet peak at δ 7.25 belonged to proton H-2.

The $^1$H-NMR spectrum (Figure 3.12) also showed the presence of two methoxyl groups at δ 4.04 and δ 3.92 observed as a singlet. One sharp singlet attributable to six

protons attached to nitrogen was observed at δ 2.41. Furthermore, two sets of multiplets were present at δ 3.26-3.34 and δ 2.65-2.70, which were assigned to the methylene protons H-11 and H-12 respectively.

The $^{13}$C-NMR spectrum (Figure 3.13) revealed the presence of 20 carbon atoms which validated the molecular formula, $C_{20}H_{23}NO_2$. The spectrum showed the presence of two *N*-methyl signals, two methoxyls, two methylenes, seven methines, and seven quaternary carbons. The signals of *N*-methyl was observed at δ 45.6, while two methoxy carbon resonated at δ 56.7 and δ 59.9 which correspond to C-3 and C-4 respectively. Carbon in aromatic regions resonated at lower chemical shifts, δ 114.8, δ 122.5, δ 125.8, δ 126.6, δ 126.6 and δ 128.2 may belong to C-2, C-10, C-7, C-6, C-9 and C-5/C-8, whereas the signals of substituted aromatic carbon atoms were observed at higher chemical shift for C-3 and C-4 at δ 150.9 and δ 146.0 respectively. The higher chemical shift carbons were affected by the presence of electronegative substituents effect. The methylene carbon for C-11 and C-12 were observed at δ 32.6 and δ 61.1 typical for methylene position. 2D NMR experiments (COSY, HMQC, and HMBC) allowed the complete assignments of the protons and carbons chemical shift.

The COSY spectrum (Figure 3.14) showed the correlations of vicinal proton between H-5/H-6, H-7/H-8, H-9/H-10 and H-11/H-12. In the HMBC spectrum (Figure 3.16) showed long range heteronuclear interaction between *N*-CH3/C-12, H-12/C-11, H-11/C-2 and C-1a and H-5/C-1b and C-8a. Other COSY and HMBC correlations are summarized in Figure 3.10 and Table 3.2. The assignments of the protons and carbons were consistent with literature reviews [82,96] and thus confirmed the structure of atherosperminine **11**.

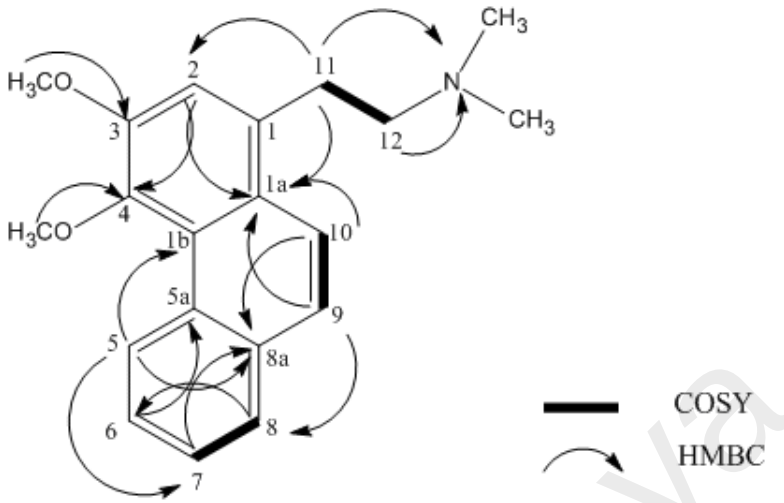



Figure 3.10: $^{1}$H-$^{1}$H and $^{1}$H-$^{13}$C Coupling Pattern Observed in COSY and HMBC Spectra of Atherosperminine **11**

Table 3.2: $^{1}$H NMR (in $CDCl_3$, 400 MHz), $^{13}$C NMR (in $CDCl_3$, 100 MHz), and HMBC of **11** and Comparison with $^{1}$H NMR Literature Values (Kini *et al.* 2004).

| Position | $\delta_H$, ppm (J in Hz) (observed) | $\delta_C$ (ppm) | HMBC (H→C) | $\delta_H$, ppm (J in Hz) (Kini *et al.*, 2004) |
|---|---|---|---|---|
| 1 | - | 130.2 | - | - |
| 1a | - | 126.2 | - | - |
| 1b | - | 125.3 | - | - |
| 2 | 7.25, *s* | 114.8 | 1, 1a, 3, 4, 11 | 7.20-7.50, *m* |
| 3 | - | 150.9 | - | - |
| 4 | - | 146.0 | - | - |
| 5 | 9.67, *d*, J=8.6 | 128.2 | 6, 8a | 9.67, *m* |
| 5a | - | 132.9 | - | - |
| 6 | 7.56-7.64, *m* | 126.6 | 5a, 7 | 7.20-7.50, *m* |
| 7 | 7.56-7.64, *m* | 125.8 | 5, 8, 8a | 7.20-7.50, *m* |
| 8 | 7.84-7.86, *dd*, $J_1$=7.3, $J_2$=2.0 | 128.2 | 6, 8a, 9 | 7.65, *m* |
| 8a | - | 133.3 | - | - |
| 9 | 7.56-7.64, *m* | 126.6 | 1b, 10 | 7.65, *m* |
| 10 | 7.86, *d*, J=9.3 | 122.5 | 8a, 1b | 7.65, *m* |
| 11 | 3.26-3.34, *m* | 32.6 | 1, 1a, 12 | 2.30-3.85, *m* |
| 12 | 2.65-2.70, *m* | 61.1 | 1, 11, *N*-$CH_3$ | 2.30-3.85, *m* |
| 3-OMe | 4.04, *s* | 56.7 | 3 | 3.90, *s* |
| 4-OMe | 3.92, *s* | 59.9 | 4 | 3.88, *s* |
| 2 *N*-Me | 2.41, *s* | 45.6 | 11, 12 | 2.25, *s* |

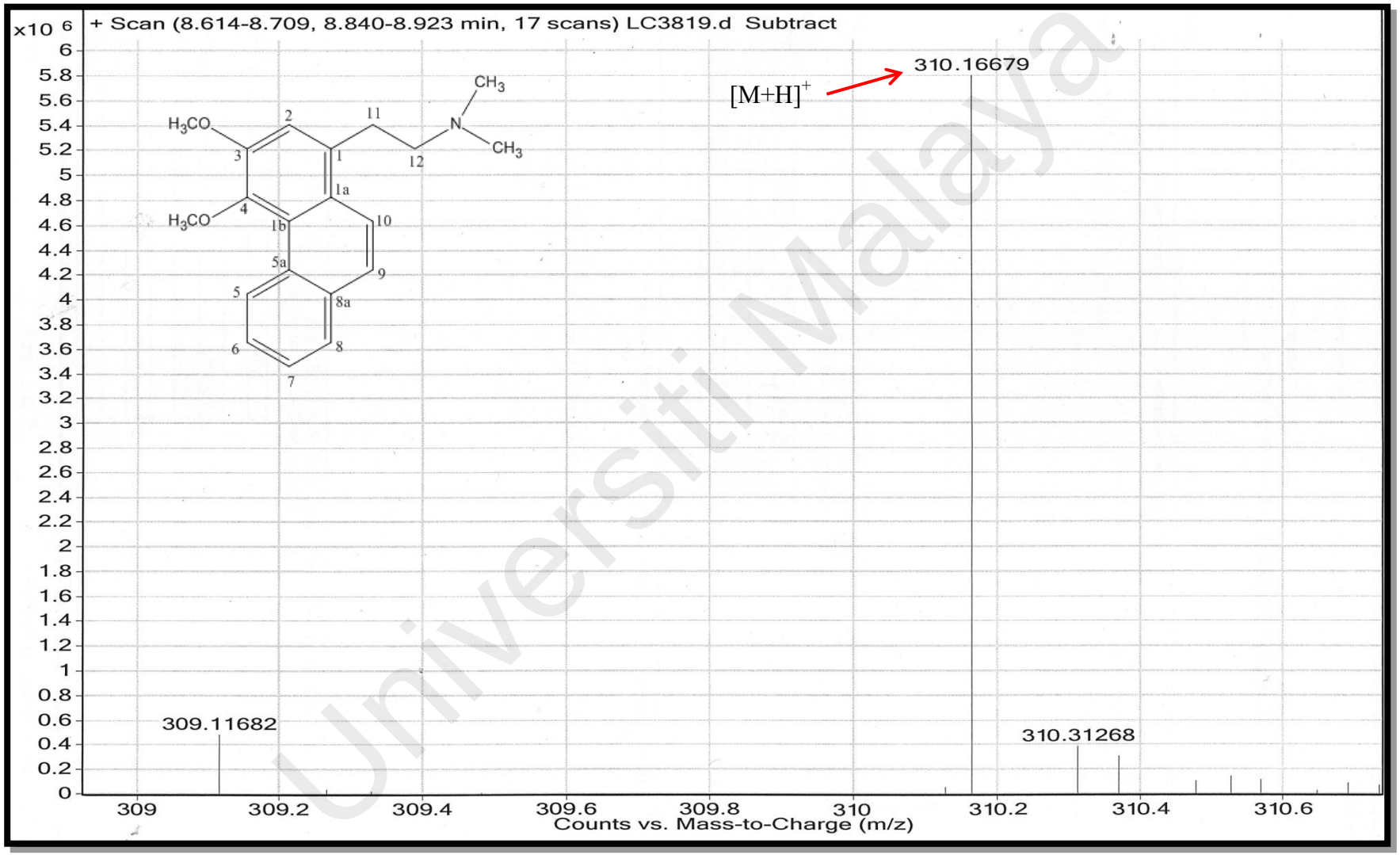



Figure 3.11: LCMS Spectrum of Atherosperminine **1**

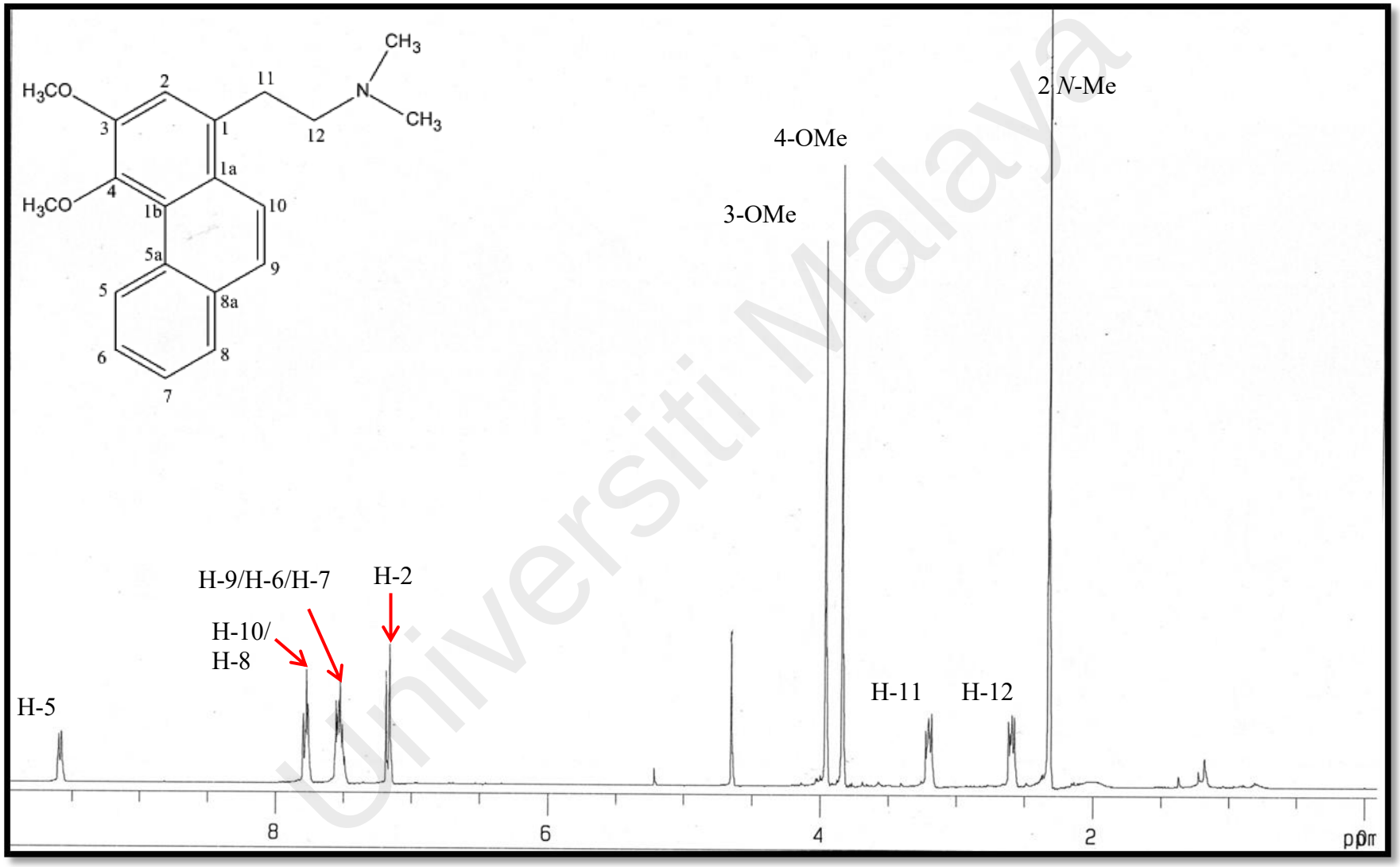



Figure 3.12: $^{1}$H NMR Spectrum of Atherosperminine **11**

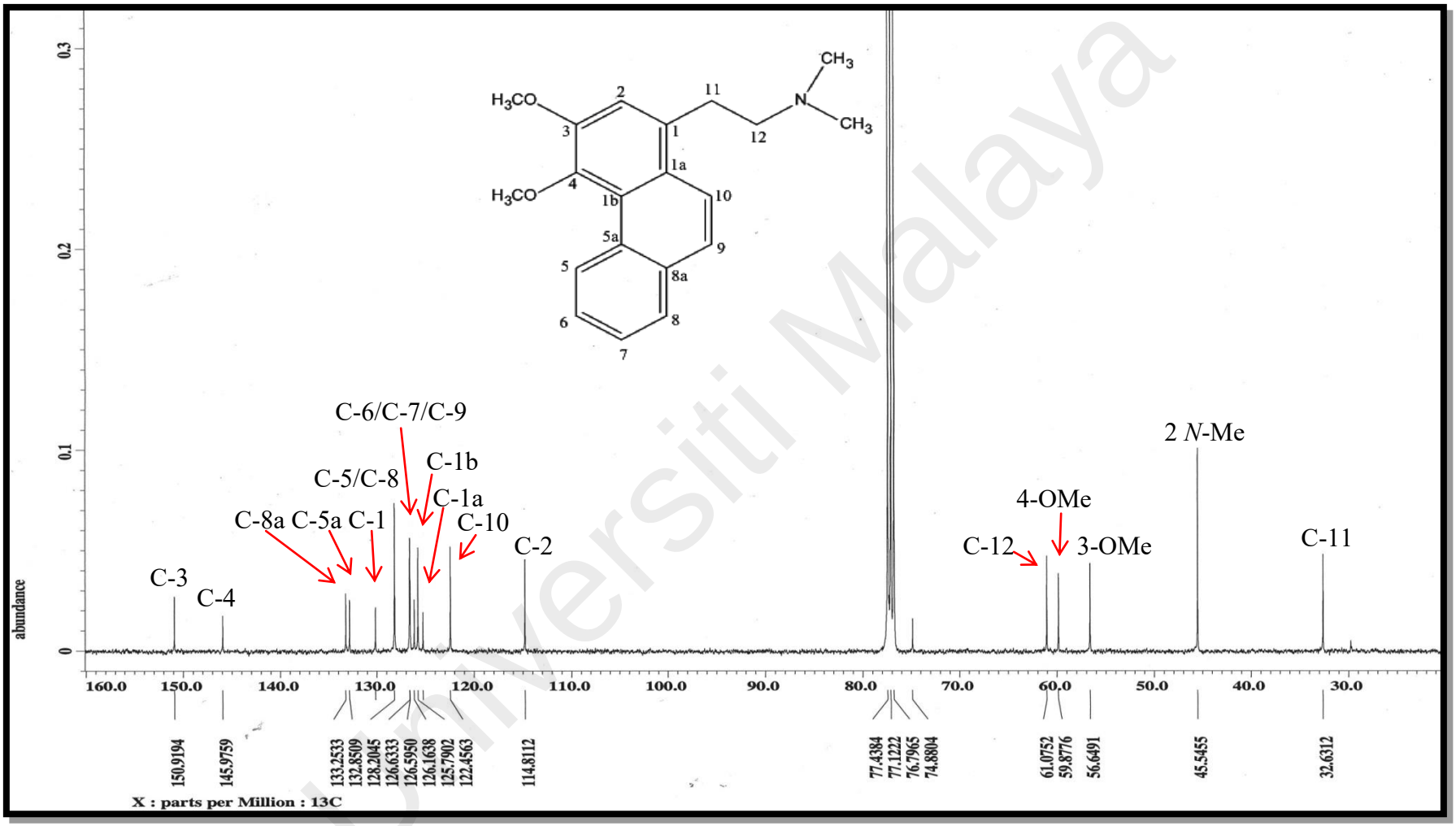



Figure 3.13: $^{13}$C NMR Spectrum of Atherosperminine **11**

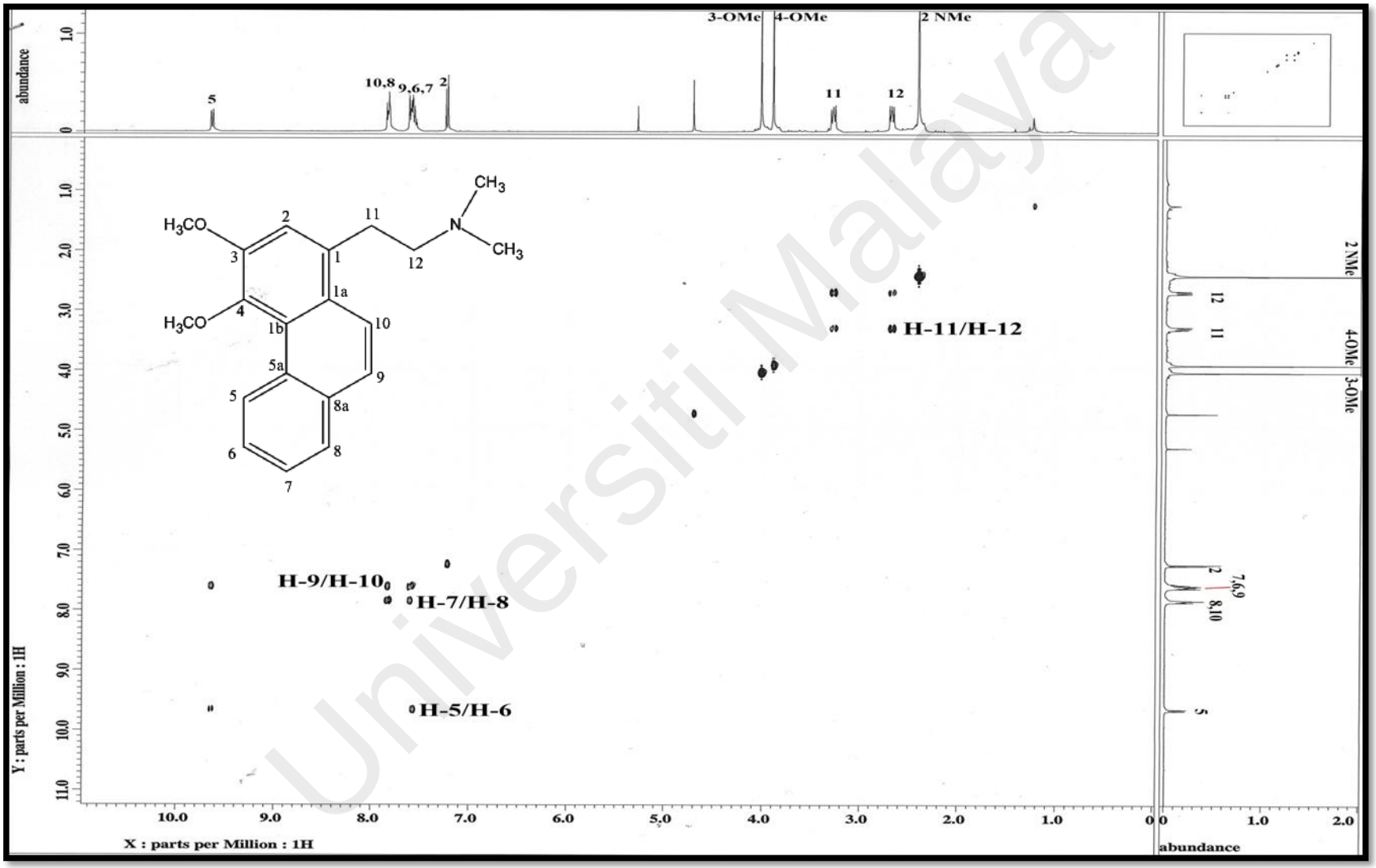



Figure 3.14: COSY Spectrum of Atherosperminine **11**

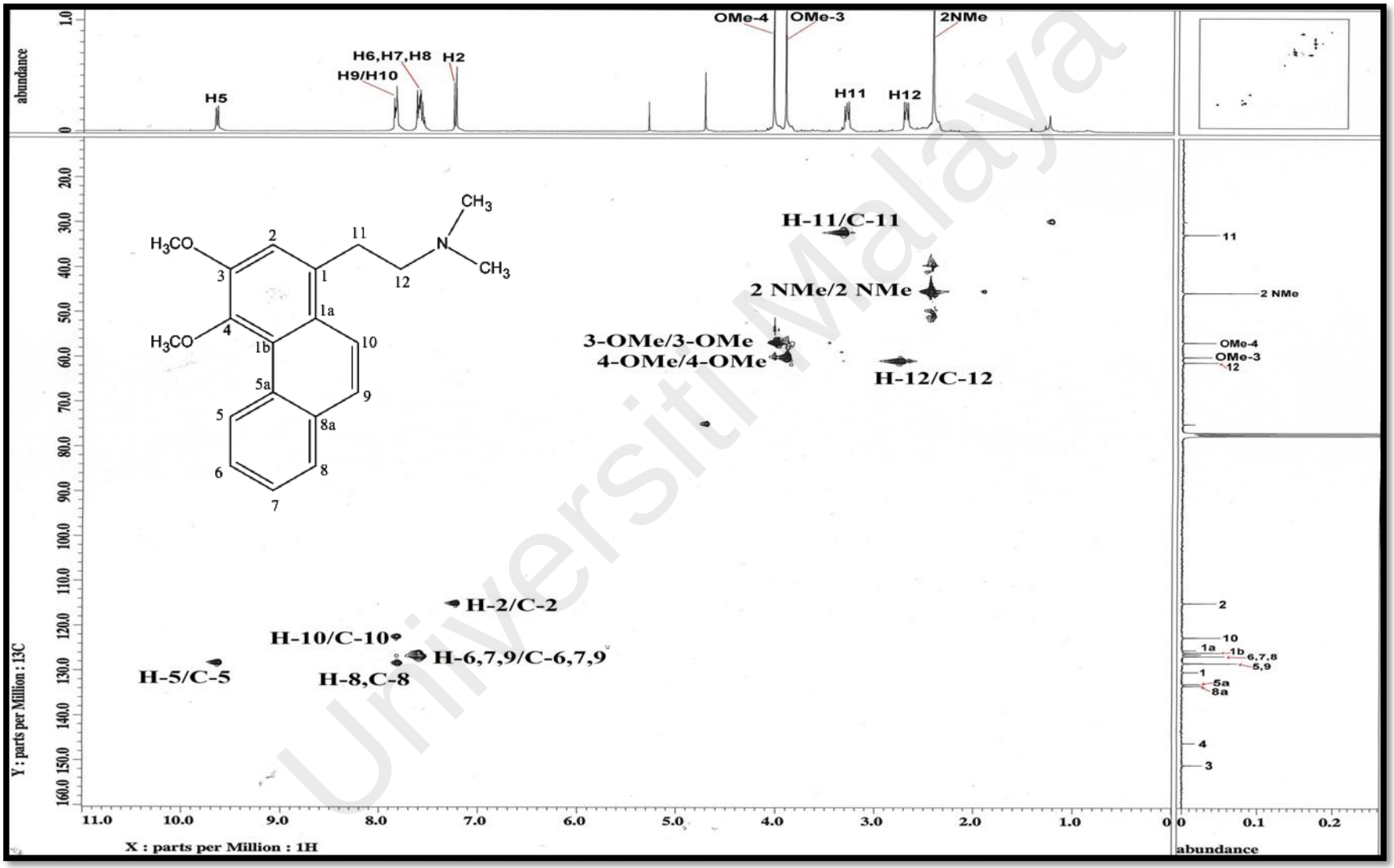



Figure 3.15: HMQC Spectrum of Atherosperminine **11**

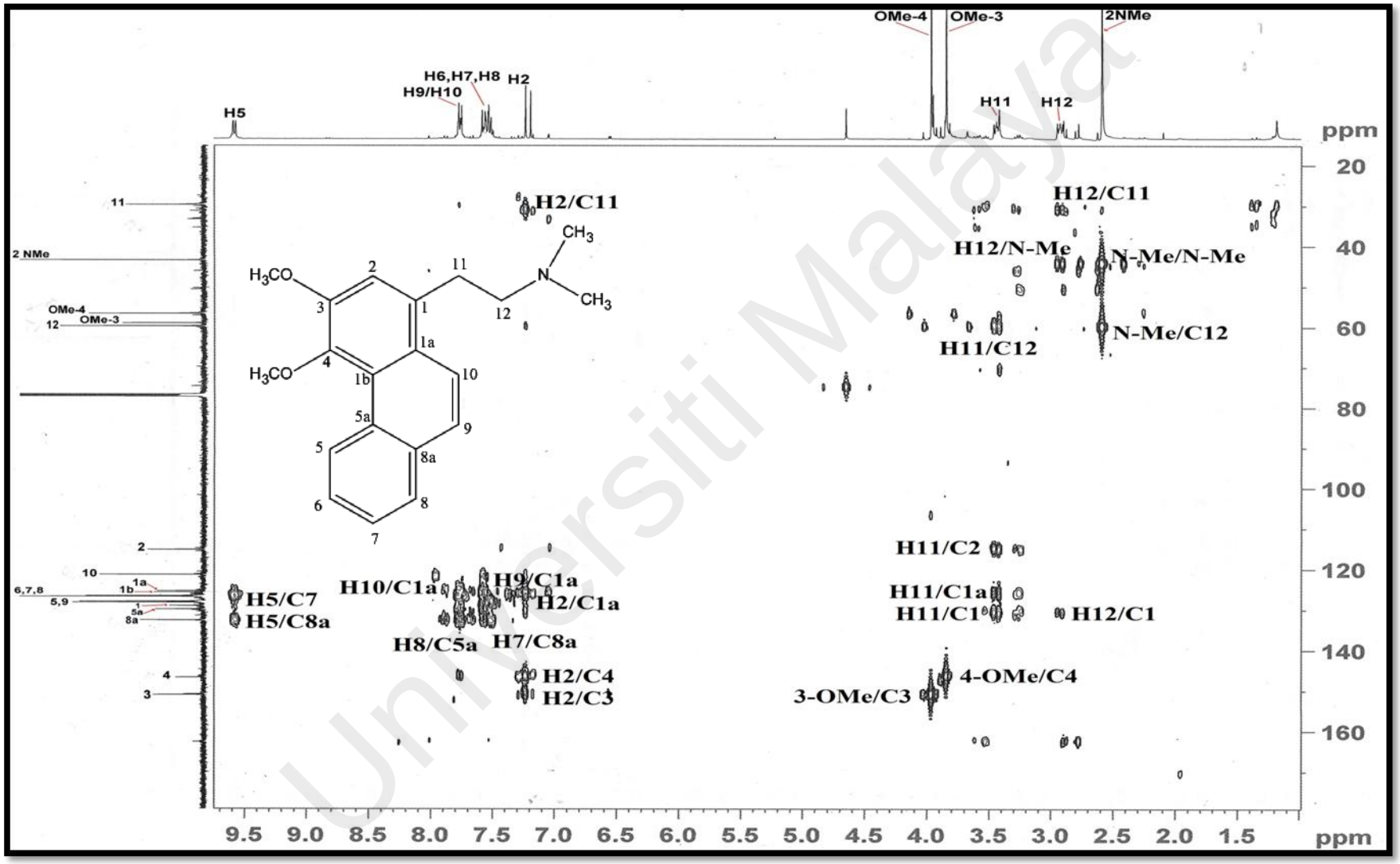
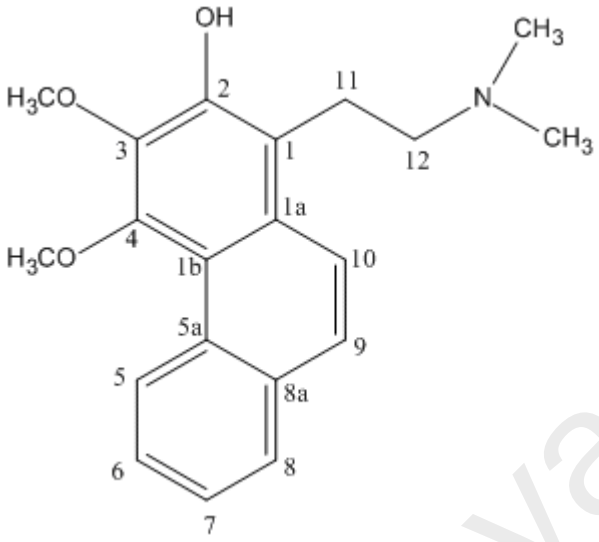



Figure 3.16: HMBC Spectrum of Atherosperminine **11**

### 3.1.3 2-hydroxyatherosperminine 20




**20**

2-Hydroxyatherosperminine **20** was obtained as a brownish amorphous solid. The IR spectrum (Figure 3.18) revealed an important peak at 3412 $cm^{-1}$ indicated to the presence of hydroxyl group. Its UV spectrum exhibited absorption bands at 296 and 286 nm. The LCMS spectrum (Figure 3.17) showed a pseudomolecular ion peak at m/z 326.18, $[M+H]^+$ corresponding to the molecular formula $C_{20}H_{23}NO_3$, with 10 degrees of unsaturation (3 rings and 7 double bonds). The peak at m/z 651.34973 may be considered as an artefact from LC-MS analysis; (325+325+1 = 651) which commonly appeared and many at times wrongly mistaken as a dimer. If it is a dimer, the molecular weight should be (M+M)-2, due to loss of two hydrogen from the formation of a bond rather than (M+M+1) as observed in this case.

Similar features were observed on $^1$H-NMR and $^{13}$C-NMR spectra of compounds **11** and **20**, which confirmed a close structural relationship between both compounds. However, there was obvious differences in the $^1$H-NMR spectrum of compound **20**, the H-2 proton signal at δ 7.25 which was apparent in compound **11** was absent in compound **20**, due to the presence of OH group at C-2 position.

In the $^{1}$H-NMR (Figure 3.19), there were two distinct methoxy signals appeared as a singlet at δ 3.90 and δ 4.02, most probably attached to C-4 and C-3 respectively. One sharp peak attributed to the six methyl hydrogen groups attached to the nitrogen was observed at δ 2.51. In addition, a very downfield chemical shift was observed at δ 9.64 (*d*, J=8.2 Hz), a characteristic of H-5 of phenantrene type. A doublet signal appeared at δ 7.83 (*d*, J=9.28) corresponded to two aromatic protons belong to H-8 and H-10. The remaining of the aromatic protons gave a series of multiplet corresponding to three protons at δ 7.55-7.63 for H-9, H-6 and H-7. This proved that the ring D was not substituted. Finally, four aliphatic protons were observed at δ 3.36-3.40 and δ 2.80-2.84 as multiplets which were typical of the phenantrene H-11 and H-12 methylene adjacent to *N* atom.

The $^{13}$C-NMR spectrum (Figure 3.20) showed the presence of two *N*-methyl signals, two methoxyl groups, two methylenes, six methines and eight quaternary carbons. The proton-proton correlations were determined using COSY experiment. COSY spectrum (Figure 3.21) displayed correlations of vicinal proton between H-11/H-12, H-10/H-9 and H-8/H-7. Due to the limitation of the sample amount, we were unable to get its 2D NMR spectrum including HMBC & HMQC spectrum.

The spectral data obtained were consistent with those found in the literature values [22], thus confirming the presence of 2-hydroxyatherosperminine **20**.

Table 3.3: $^{1}H$ NMR (in $CDCl_3$, 400 MHz) and $^{13}C$ NMR (in $CDCl_3$, 400 MHz) of **20** and Comparison with Literature Values (Awang *et al.* 2008)

| Position | $\delta_H$, ppm (J in Hz) (observed) | $\delta_C$ (ppm) (observed) | $\delta_H$, ppm (J in Hz) (Awang, 2008) | $\delta_C$ (ppm) (Awang, 2008) |
|---|---|---|---|---|
| 1 | - | 121.2 | - | 120.9 |
| 1a | - | 125.9 | - | 125.2 |
| 1b | - | 115.6 | - | 120.8 |
| 2 | - | 146.8 | - | 144.4 |
| 3 | - | 151.1 | - | 145.5 |
| 4 | - | 132.7 | - | 141.7 |
| 5 | 9.64, *d*, J=8.3 | 126.9 | 9.31-9.32, *d*, J=7.32 | 126.6 |
| 5a | - | 130.0 | - | 132.1 |
| 6 | 7.54-7.62, *m* | 126.8 | 7.47-7.52, *m* | 126.5 |
| 7 | 7.54-7.62, *m* | 127.9 | 7.47-7.52, *m* | 126.9 |
| 8 | 7.81-7.83, *m*, | 128.1 | 7.83-7.85, *dd*, $J_1$=7.80, $J_2$=1.72 | 128.4 |
| 8a | - | 128.3 | - | 128.8 |
| 9 | 7.62, *d*, J=9.2 | 127.0 | 7.68-7.71, *d*, J=9.04 | 126.8 |
| 10 | 7.83, *d*, J=9.2 | 125.5 | 7.95-7.98, *d*, J=9.04 | 121.9 |
| 11 | 3.36-3.40, *m* | 22.7 | 3.61, *m* | 21.5 |
| 12 | 2.80-2.84, *m* | 56.7 | 3.04, *m* | 57.7 |
| 3-OMe | 4.02, *s* | 59.9 | 4.04, *s* | 61.2 |
| 4-OMe | 3.90, *s* | 57.2 | 3.84, *s* | 60.3 |
| 2 *N*-Me | 2.51, *s* | 29.7 | 2.80, *s* | 43.0 |

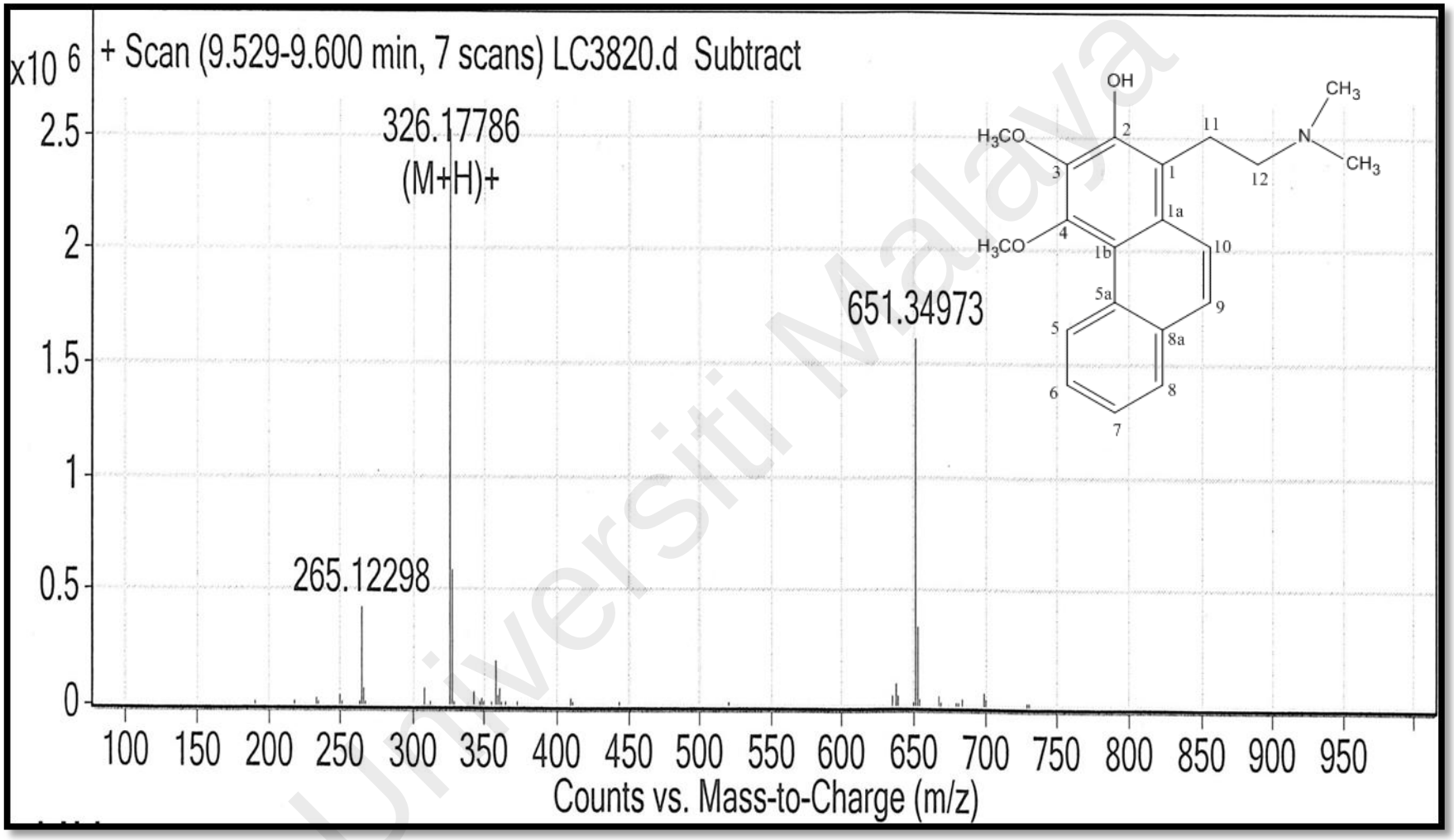



Figure 3.17: LCMS Spectrum of 2-hydroxyatherosperminine **20**

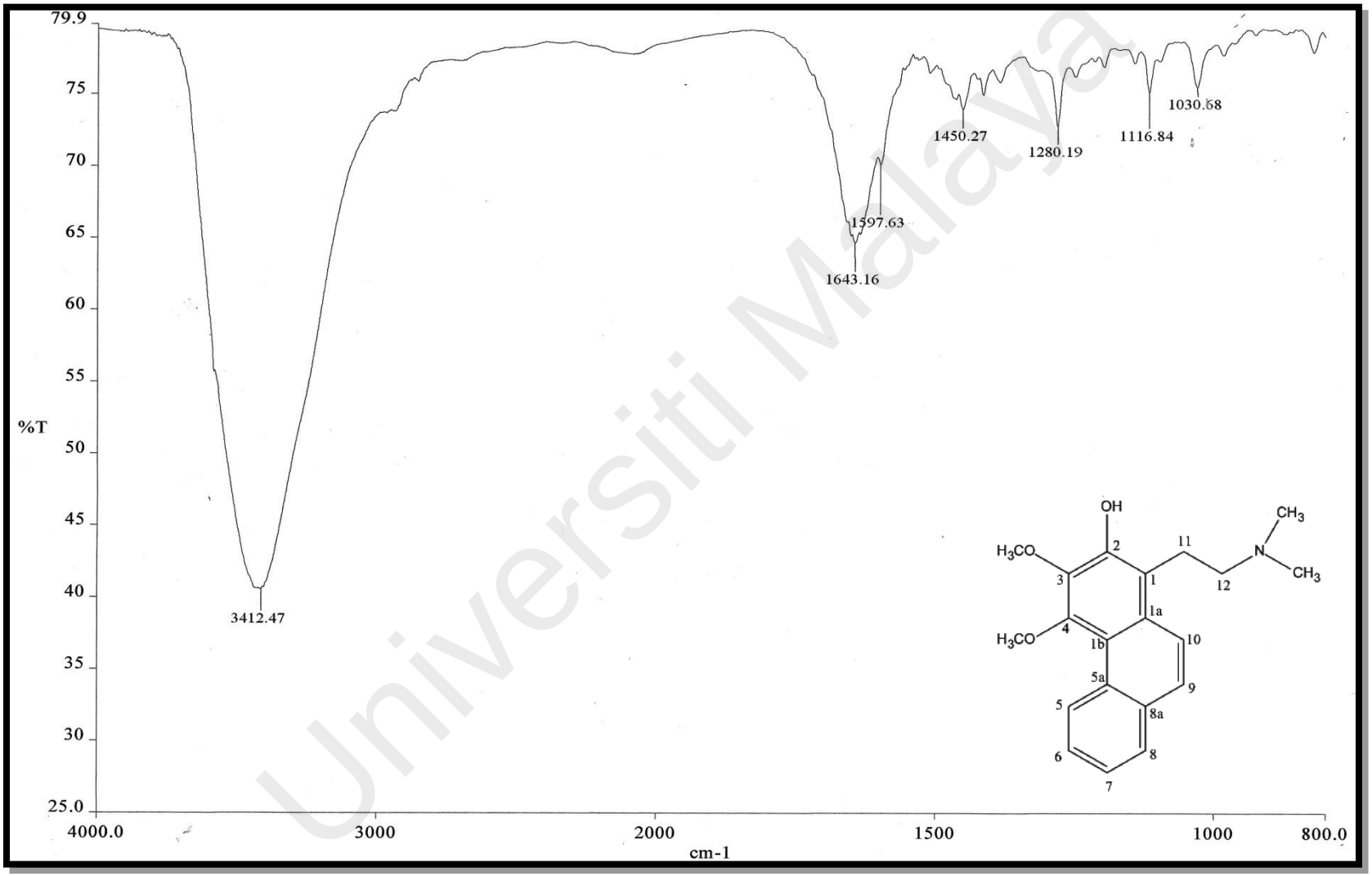



Figure 3.18: IR Spectrum of 2-hydroxyatherosperminine **20**

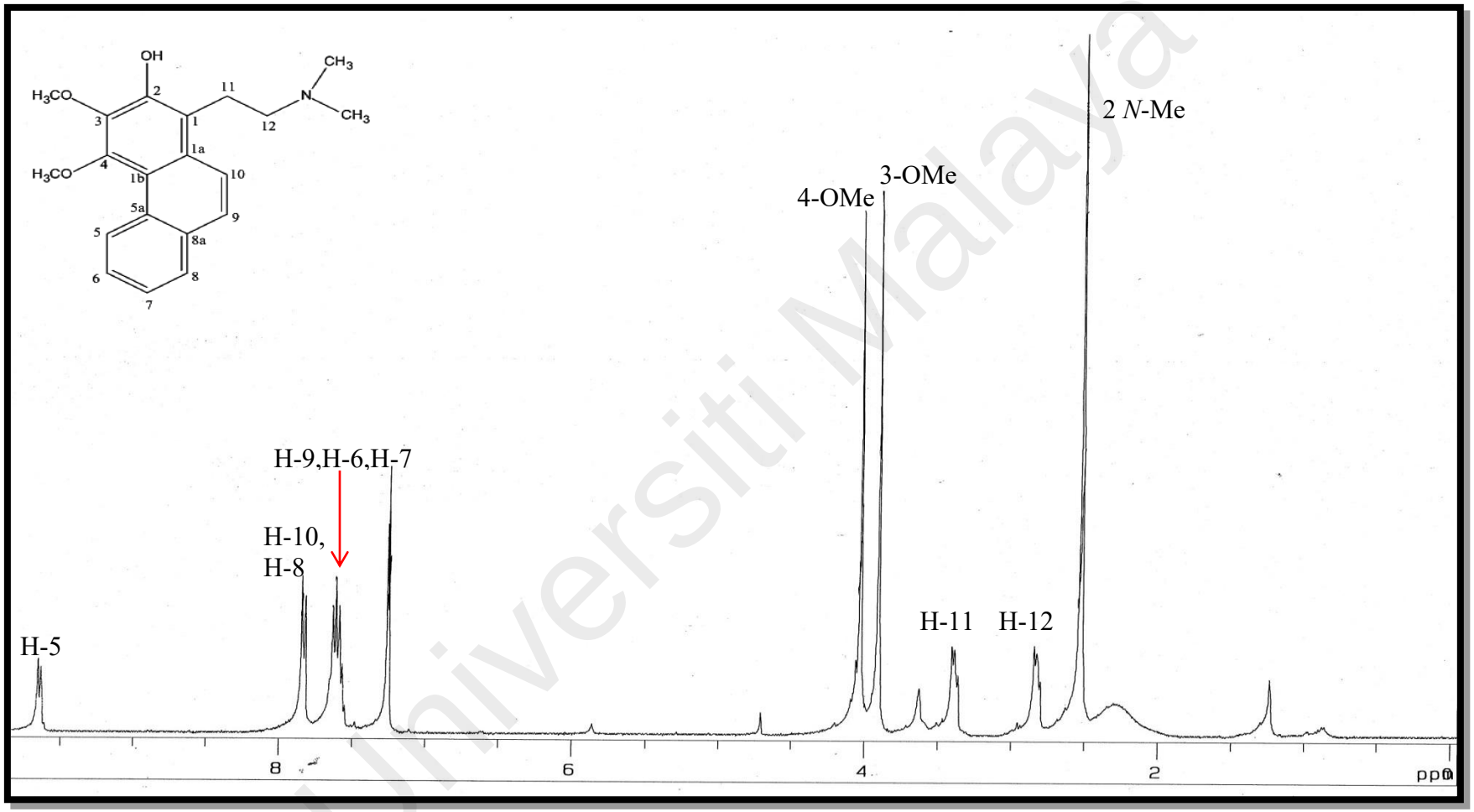



Figure 3.19: $^{1}$H NMR Spectrum of 2-hydroxyatherosperminine **20**

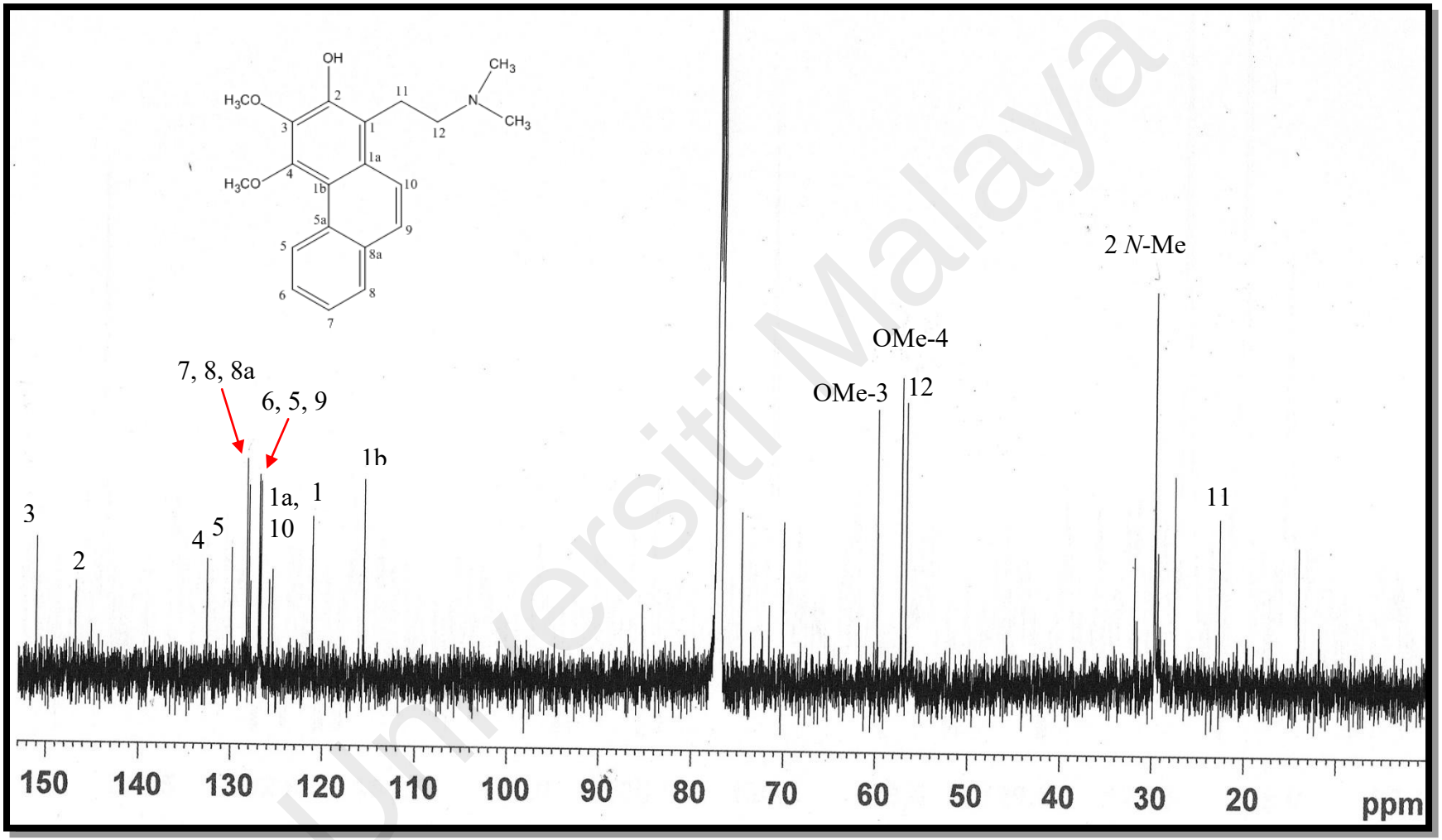



Figure 3.20: $^{13}$C Spectrum of 2-hydroxyatherosperminine **20**

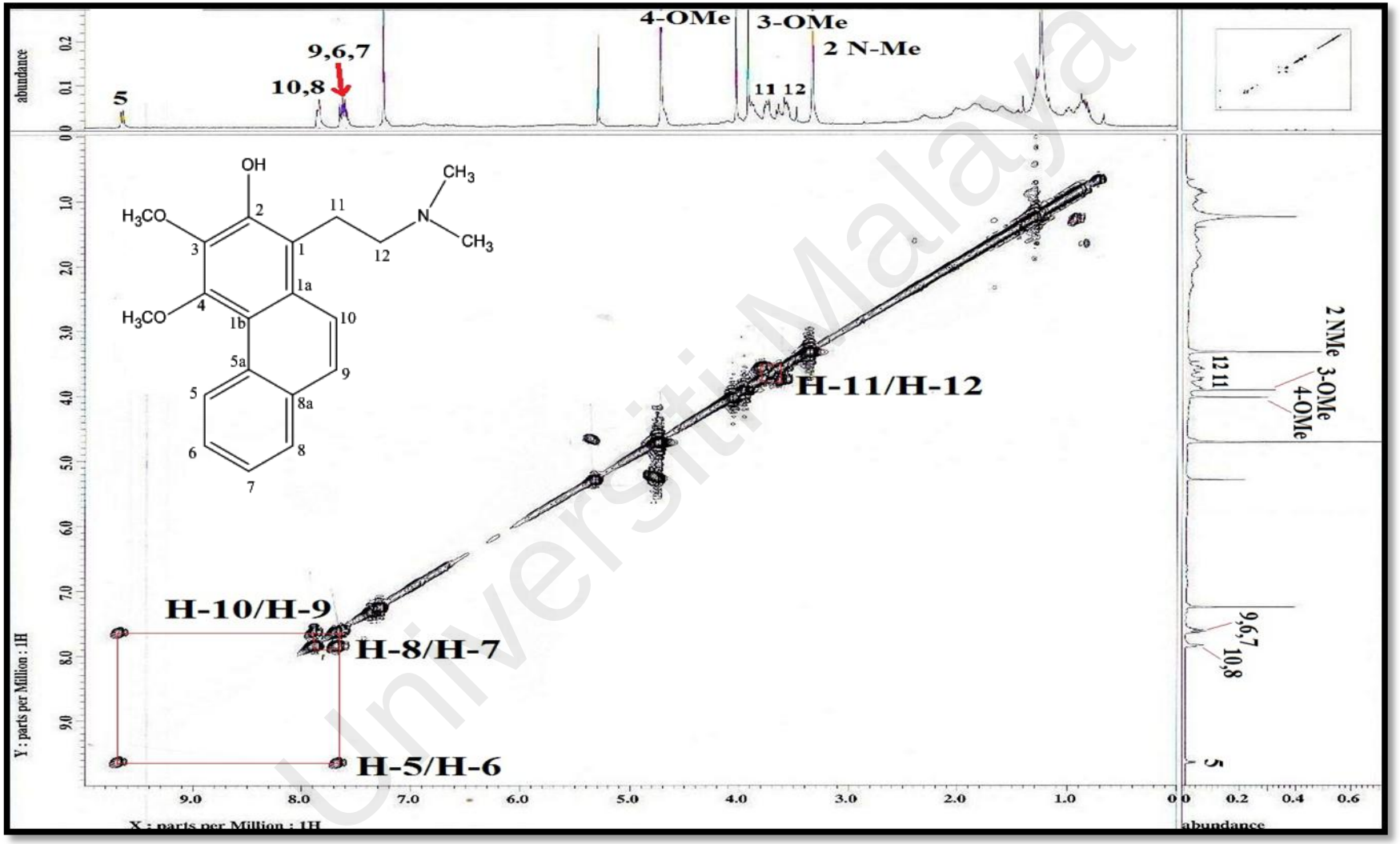
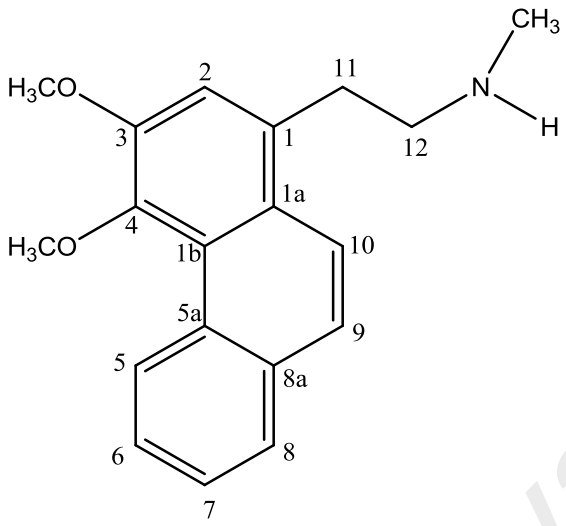



Figure 3.21: COSY Spectrum of 2-hydroxyatherosperminine **20**

### 3.1.4 Noratherosperminine 47




**47**

Noratherosperminine **47** was obtained as brownish amorphous solid. Its UV spectrum exhibited absorption bands at 258, 308 and 347 nm. The IR spectrum (Figure 3.23) gave absorption bands for aromatic ring (2917 and 2849 $cm^{-1}$) and another band at 3363 $cm^{-1}$ indicated the *N*-H stretching [97]. The LCMS spectrum (Figure 3.22) revealed a pseudomolecular ion peak, $[M+H]^+$ at m/z 296.16 suggesting a molecular formula of $C_{19}H_{21}NO_2$ with 10 degree of unsaturation (3 rings and 7 double bonds).

The structure of **47** was mainly deduced from the $^1$H-NMR spectrum (Figure 3.24). The $^1$H-NMR and $^{13}$C-NMR spectra features were also similar to those of atherosperminine **11** except the absence of one *N*-methyl group. The two methoxyl groups resonated at δ 4.01 and δ 3.90 as a singlet each which belongs to C-3 and C-4, respectively. One methyl proton attached to nitrogen atom was observed at δ 2.48. Furthermore, two sets of triplets were present at δ 3.32 and δ 2.99, which were assignable to the methylene protons H-11 and H-12 respectively.

There were 7 proton signals at the aromatic region were displayed in the $^{1}$H-NMR spectrum including a doublet belong to H-10 (δ 7.87), and a doublet of doublet belong to H-8 (δ 7.81-7.83). A downfield chemical shift was observed as a doublet at δ 9.64 with a coupling constant of 7.6 Hz typical of H-5. A multiplet signal was also present at δ 7.54-7.60 which assignable for H-9, H-6, H-7 and H-2.

The $^{13}$C-NMR spectrum (Figure 3.25) revealed the presence of 19 carbon atoms which validated the molecular formula, $C_{19}H_{21}NO_2$. The spectrum showed the presence of one *N*-methyl signal, two methoxyls, two methylenes, seven methines, and seven quaternary carbons. The signals of two methoxy carbons resonated at δ 51.5 and δ 56.7 which correspond to C-3 and C-4 respectively while *N*-methyl was observed at δ 34.4. The signals of substituted aromatic carbon atoms were observed at higher chemical shift for C-3 and C-4 at δ 151.0 and δ 146.4 respectively. The higher chemical shift carbons were affected by the presence of electronegative substituents effect. The methylene carbon for C-11 and C-12 were observed at δ 32.1 and δ 59.9 typical for methylene position.

The COSY spectrum (Figure 3.26) showed the correlations of vicinal proton between H-11/H-12, H-10/H-9 and H-5/H-6. Based on the available spectral data and investigation of the literature reviews [82,97], thus confirmed the structure of noratherosperminine **47**.

Table 3.4: $^{1}H$ NMR (in $CDCl_3$, 400 MHz) and $^{13}C$ NMR (in $CDCl_3$, 400 MHz) of **47** and Comparison with $^{1}H$ NMR Literature Values of (Leboeuf *et al.* 1980).

| Position | $\delta_H$, ppm (J in Hz) (observed) | $\delta_C$ (ppm) (observed) | $\delta_H$, ppm (J in Hz) (Leboeuf, 1980) |
|---|---|---|---|
| 1 | - | 130.2 | - |
| 1a | - | 126.8 | - |
| 1b | - | 128.1 | - |
| 2 | 7.54-7.60, *m* | 115.4 | 7.21, *s* |
| 3 | - | 151.0 | - |
| 4 | - | 146.4 | - |
| 5 | 9.64, *d*, J=7.6 | 128.3 | 9.63, *m* |
| 5a | - | 130.2 | - |
| 6 | 7.54-7.60, *m* | 128.3 | 7.45-7.93, *m* |
| 7 | 7.54-7.60, *m* | 128.3 | 7.45-7.93, *m* |
| 8 | 7.81-7.83, *dd*, $J_1$=7.3, $J_2$=1.9 | 128.3 | 7.45-7.93, *m* |
| 8a | - | 132.8 | - |
| 9 | 7.54-7.60, *m* | 128.2 | 7.45-7.93, *m* |
| 10 | 7.87, *d*, J=9.3 | 122.0 | 7.45-7.93, *m* |
| 11 | 3.32, *t*, J=7.1 | 32.1 | |
| 12 | 2.99, *t*, J=7.1 | 59.9 | |
| 3-OMe | 4.01, *s* | 51.5 | 3.99, *s* |
| 4-OMe | 3.90, *s* | 56.7 | 3.90, *s* |
| *N*-Me | 2.48, *s* | 34.4 | 2.51, *s* |

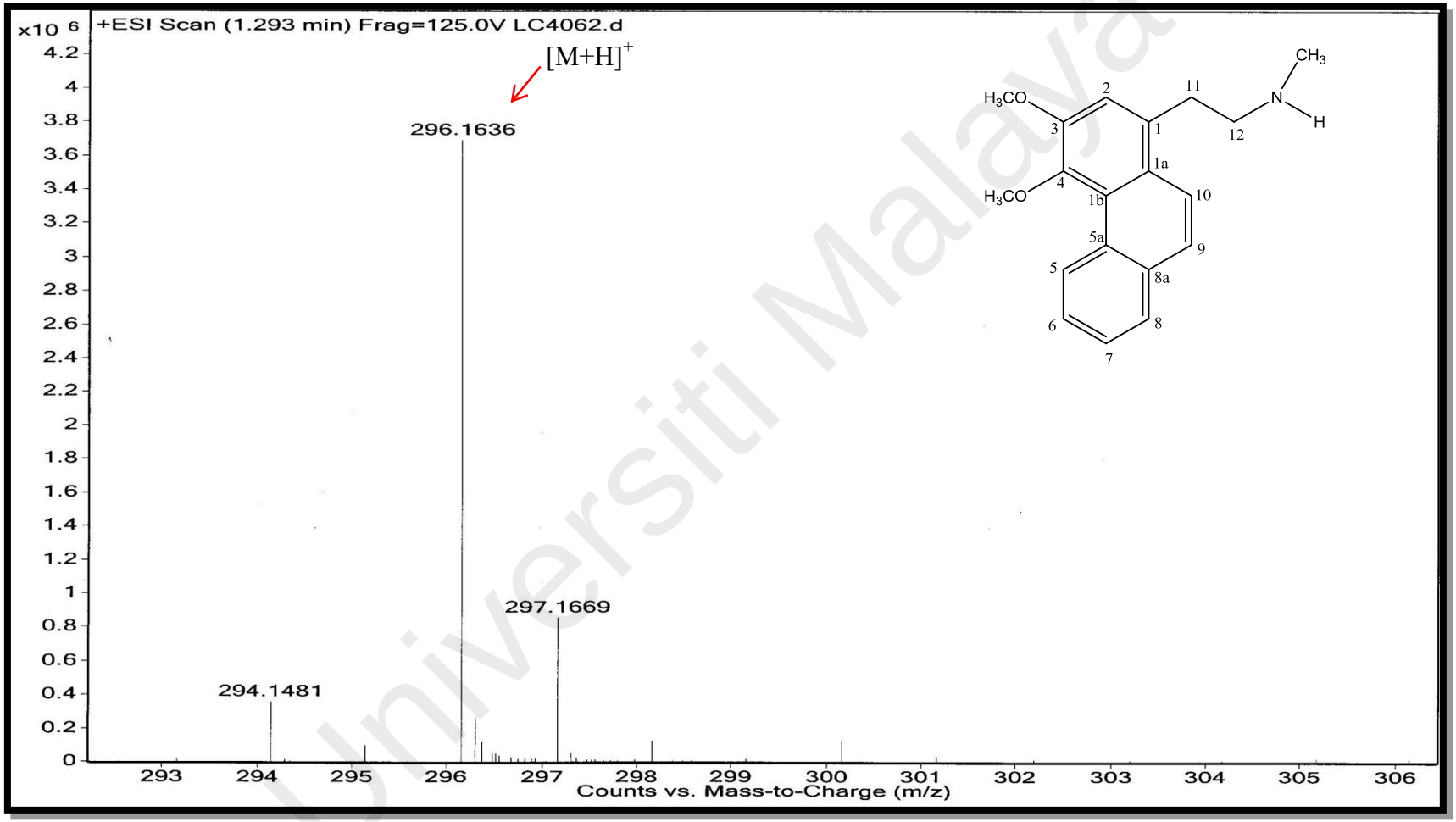



Figure 3.22: LCMS Spectrum of Noratherosperminine **47**

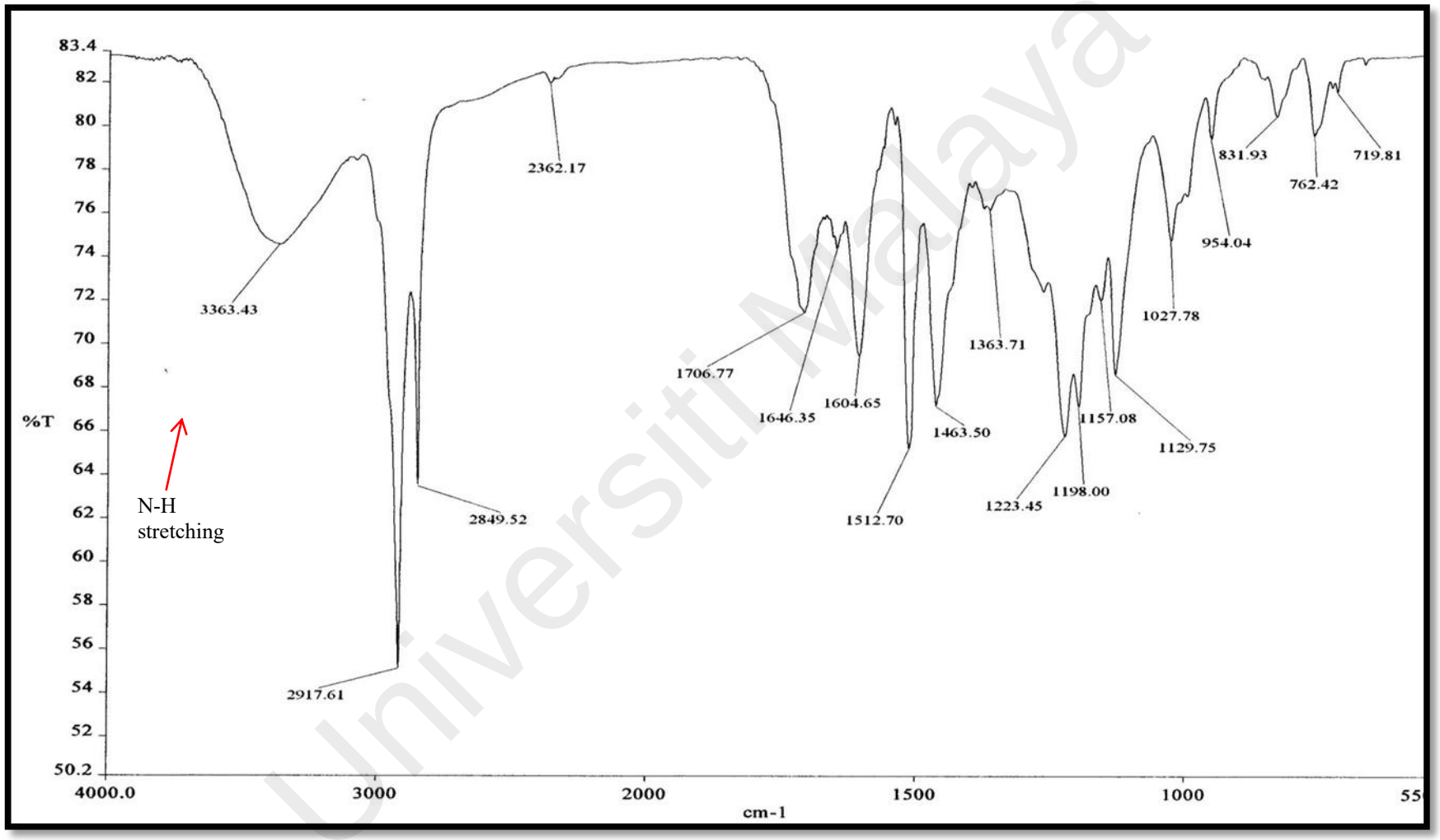



Figure 3.23: IR Spectrum of Noratherosperminine **47**

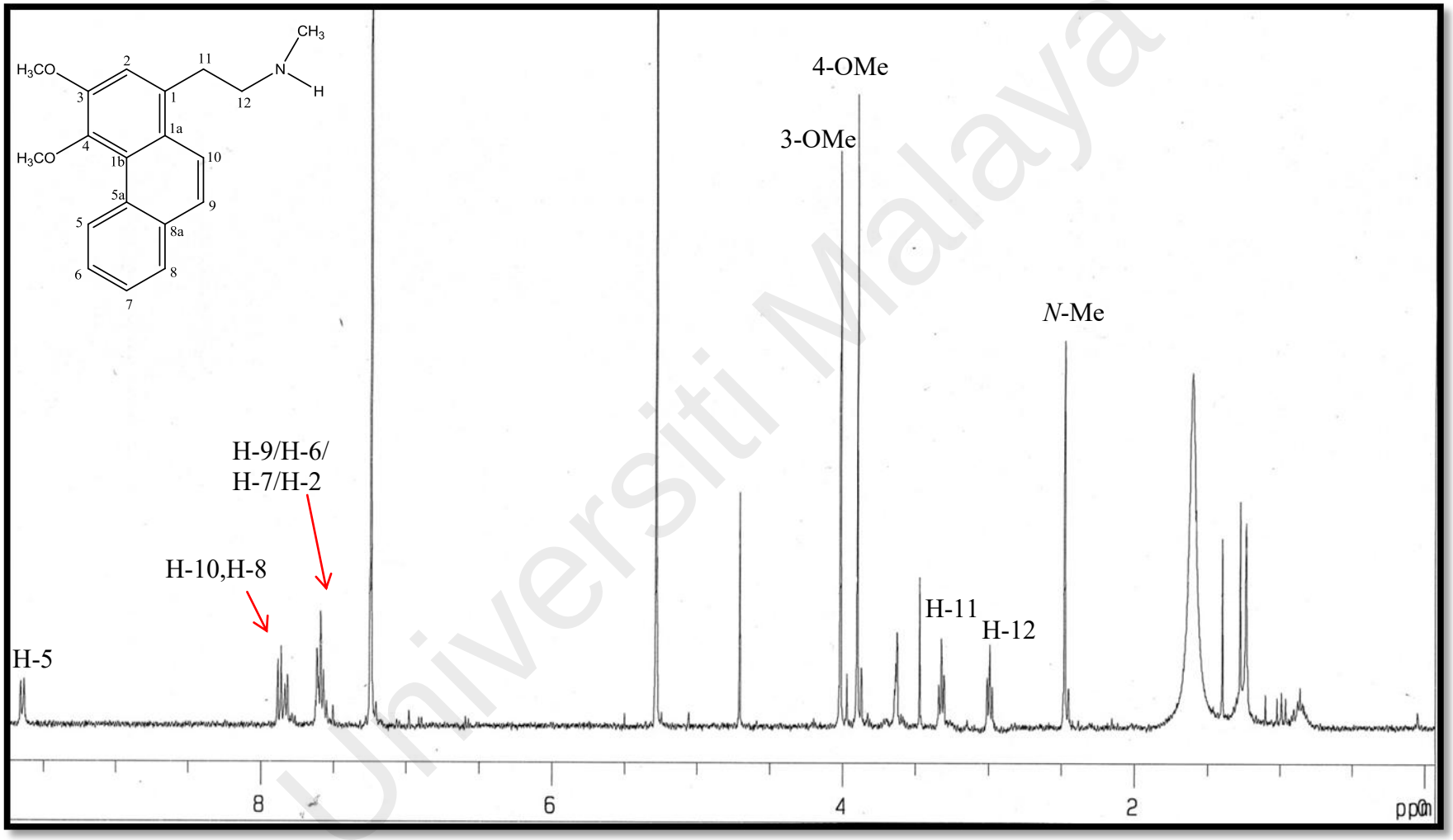



Figure 3.24: [1]H-NMR Spectrum of Noratherosperminine **47**

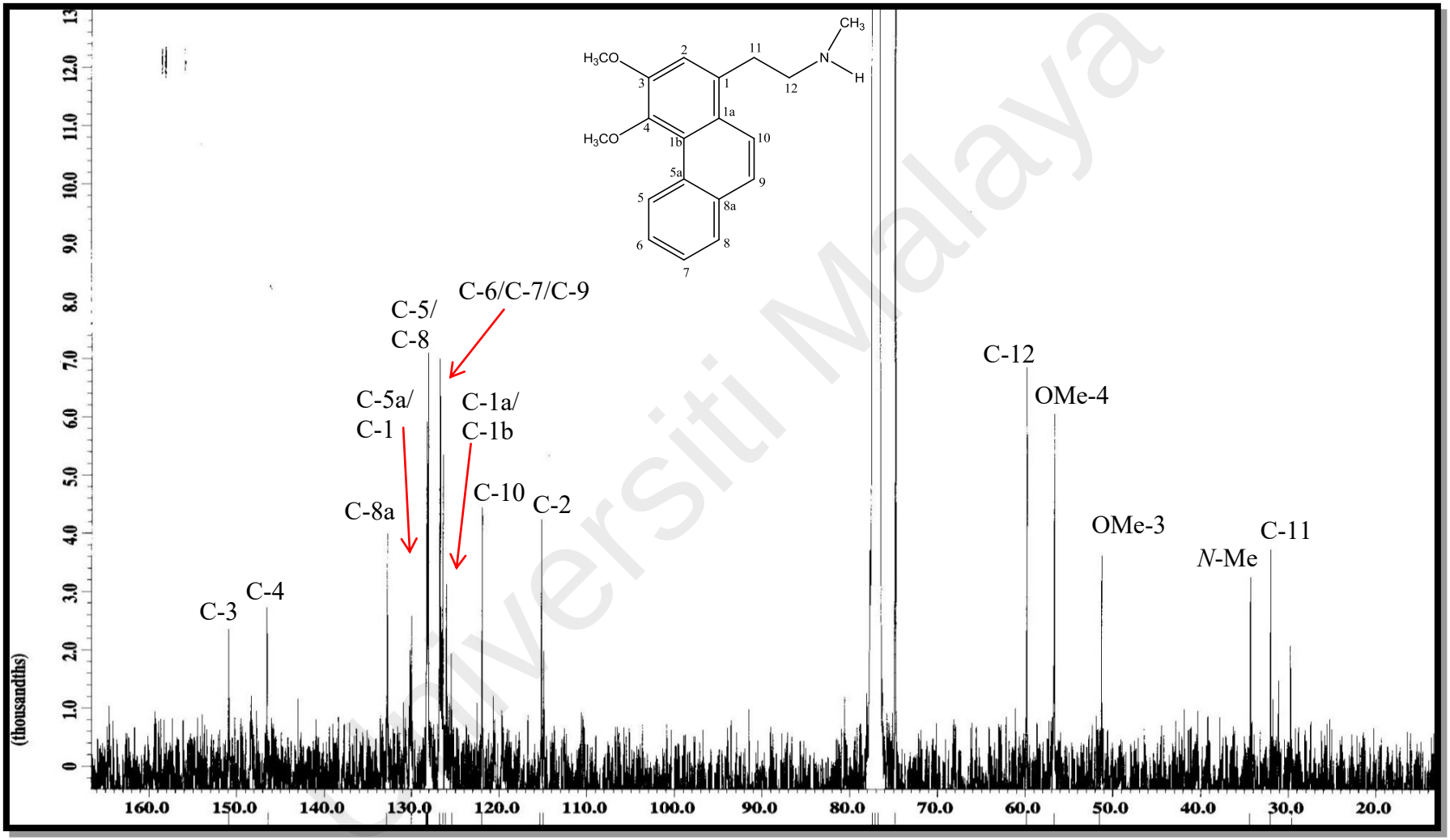



Figure 3.25: $^{13}$C-NMR Spectrum of Noratherosperminine **47**

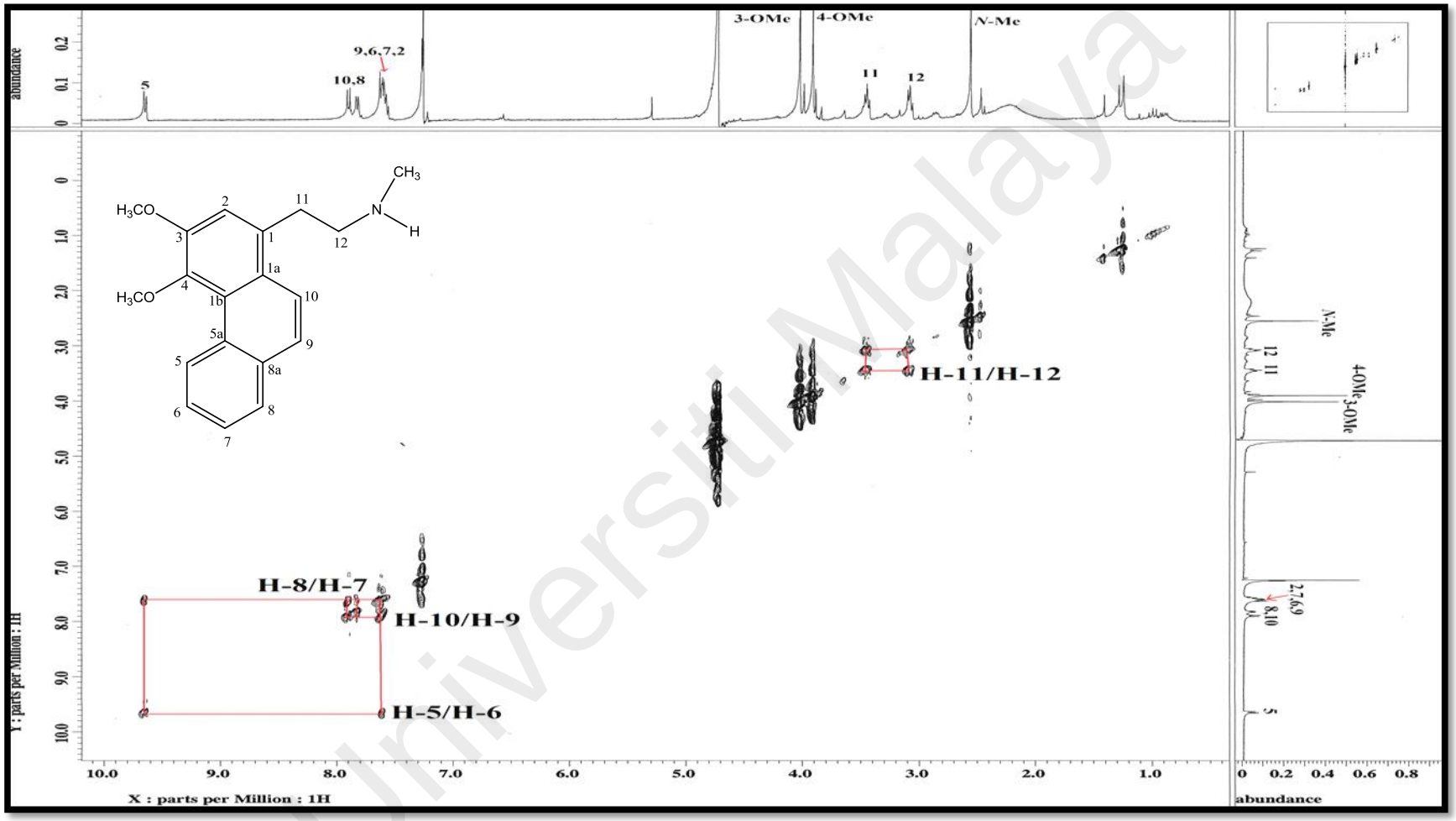
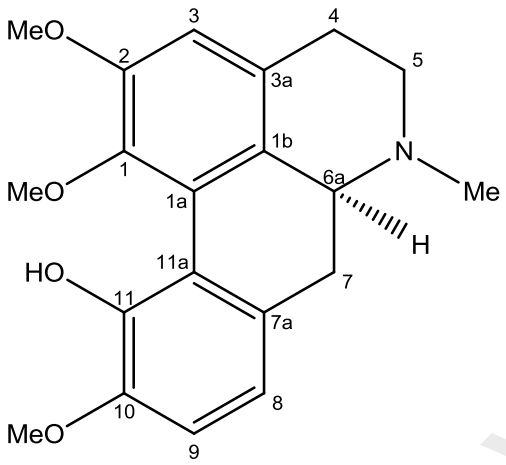



Figure 3.26: COSY Spectrum of Noratherosperminine **47**

### 3.1.5 Isocorydine 48




**48**

Isocorydine **48** was obtained as brownish amorphous powder with $[\alpha]_D^{25}$ +196º (c = 0.10, MeOH) indicated the absolute configuration at C-6a was *S* as in the known alkaloid [98]. Its UV spectrum exhibited absorption bands at 270 and 310 nm, which indicated the existence of the conjugated system. The IR spectrum revealed absorption band due to the highly conjugated hydroxyl group at 3202 $cm^{-1}$. The LCMS spectrum (Figure 3.27) revealed a pseudomolecular peak at m/z 342.2 suggesting a molecular formula of $C_{20}H_{24}NO_4$ with 10 degree of unsaturation (4 rings and 6 double bonds).

The $^1$H-NMR spectrum (Figure 3.28) established the presence of three methoxyl groups at δ 3.89, 3.88, and 3.68 which appeared as a singlet, corresponding to 10-OMe, 2-OMe, and 1-OMe respectively. H-3 was observed as a singlet at δ 6.68 indicating that C-1 and C-2 in ring A are substituted with methoxyl groups. Another peak presence as singlet at δ 2.50 corresponded to *N*-Me group in ring B. In addition, vicinal protons H-8 and H-9 signals appeared as a doublet at δ 6.81 and 6.84 with a coupling constant of 8.3 Hz. The aliphatic proton, H-5 appeared at deshielded area (δ 2.97-3.04) compared to H-

4 (δ 2.45-2.49) due to the withdrawing effect of the neighboring electronegative *N*-atom.

The $^{13}$C-NMR (Figure 3.29) and DEPT (Figure 3.30) spectra for Compound **48** showed 20 carbon signals consisting of four methyls, three methylenes, four methines and nine quaternary carbon signals. Three signals observed at δ 62.1, 56.1 and 55.9 corresponded to the OMe group attached to C-1, C-10 and C-2 respectively. The signals for quaternary carbons at δ 151.3, 149.5, 144.0, and 142.2 could be assigned to C-2, C-10, C-11 and C-1 respectively.

The COSY spectrum (Figure 3.31) showed cross peaks between $CH_2$-5/ $CH_2$-4, and $CH_2$-7/ CH-6a. An analysis of HMBC spectrum (Figure 3.33) revealed significant cross-peaks of H-9 to C-11 which bearing a hydroxyl group. The correlation of protons 10-OMe and H-8 to C-10 were also observed in the HMBC spectrum.

The spectral data obtained were consistent with those found in the literature reviews, thus confirming the structure for isocorydine **48** [98-100].

Table 3.5: $^{1}$H NMR (in $CDCl_3$, 400 MHz), $^{13}$C NMR (in $CDCl_3$, 400 MHz) and HMBC of **48** and Comparison with $^{1}$H NMR Literature Values (Ferreira *et al.* 2010).

| Position | $\delta_H$, ppm (J in Hz) (observed) | $\delta_C$ (ppm) | HMBC (H→C) | $\delta_H$, ppm (J in Hz) (Ferreira, 2010) |
|---|---|---|---|---|
| 1 | - | 142.2 | - | - |
| 1a | - | 125.9 | - | - |
| 1b | - | 129.2 | - | - |
| 2 | - | 151.3 | - | - |
| 3 | 6.68, *s* | 111.0 | 4,2,1,1b | 6.63, br *s* |
| 3a | - | 130.0 | - | - |
| 4 | 2.45-2.49, *m* | 29.3 | 1b,3a,3,5 | 4β: 2.64, *ddt*, J=16.5,3.5,1.3<br>4α: 3.11, *ddddd*, J=16.5,12.1,5.5,1.3,1.0 |
| 5 | 2.97-3.04, *m* | 52.7 | 4 | 5β: 2.41, *ddd*, J=12.1,11.3,3.5<br>5α: 2.94, *ddd*, J=11.3,5.5,1.3 |
| 6a | 3.11-3.20, *m* | 62.9 | 3a | 6aα: 2.80, *ddt*, J=13.2,3.5,1.3 |
| 7 | 2.66-2.71, *dd*, $J_1$=16.6, $J_2$= 3.2 | 35.9 | 1b,8 | 7β: 2.37, *ddd*, J=13.2,13.0,1.0<br>7α: 2.97, *dd*, J=13.0,3.5 |
| 7a | - | 130.2 | - | - |
| 8 | 6.81, *d*, J=8.3 | 119.0 | 7,11a,10 | 6.76, *dd*, J=8.0,1.0 |
| 9 | 6.84, *d*, J=8.3 | 111.1 | 7a,11 | 6.79, *d*, J=8.0 |
| 10 | - | 149.5 | - | - |
| 11 | - | 144.0 | - | - |
| 11a | - | 120.2 | - | - |
| 1-OMe | 3.68, *s* | 62.1 | 1,2-OMe | 3.64, *s* |
| 2-OMe | 3.88, *s* | 55.9 | 2,1-OMe | 3.84, *s* |
| 10-OMe | 3.89, *s* | 56.1 | 10 | 3.85, *s* |
| *N*-Me | 2.50, *s* | 43.9 | 5,6a | 2.46, br *s* |

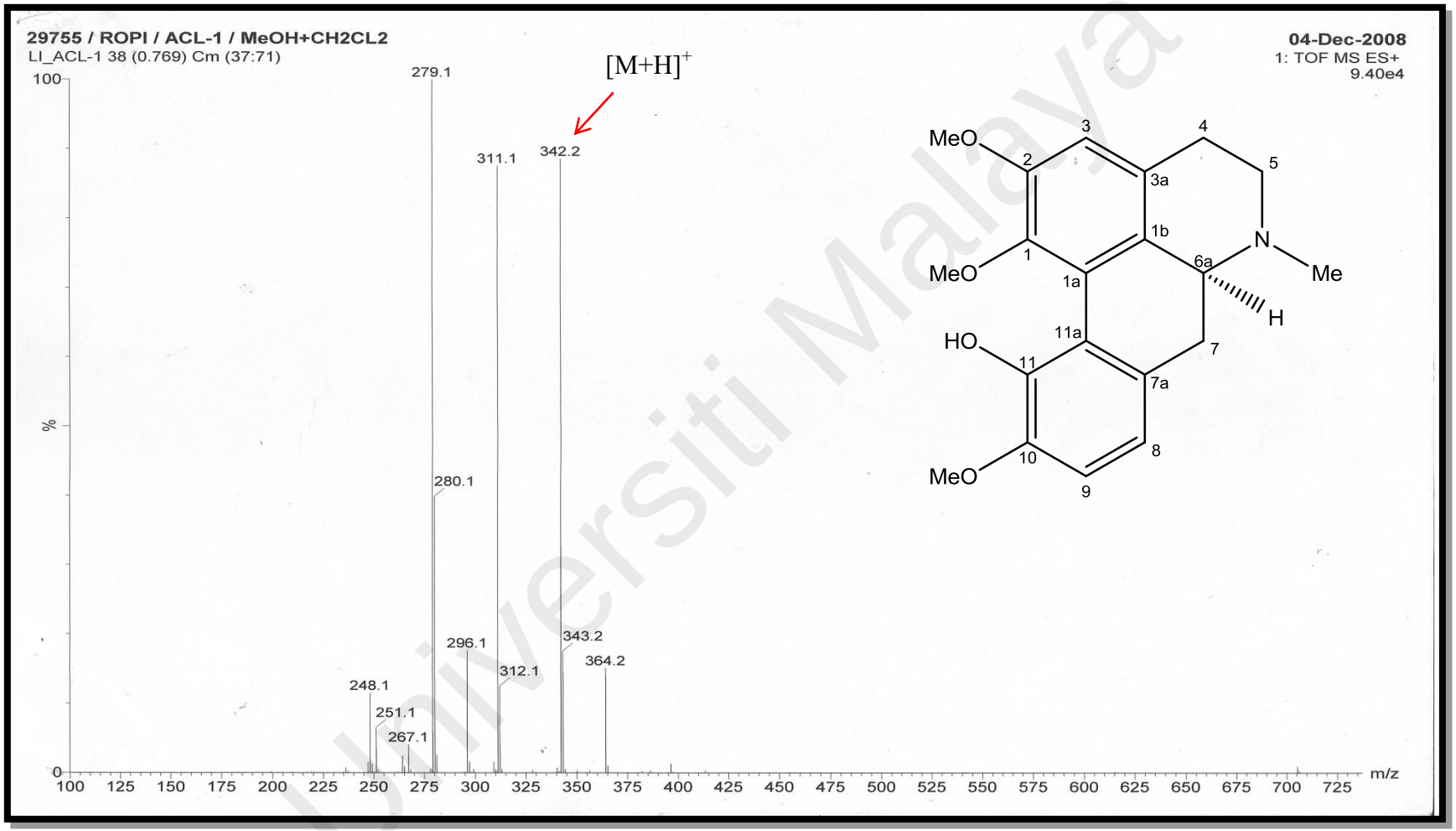



Figure 3.27: LCMS Spectrum of Isocorydine **48**

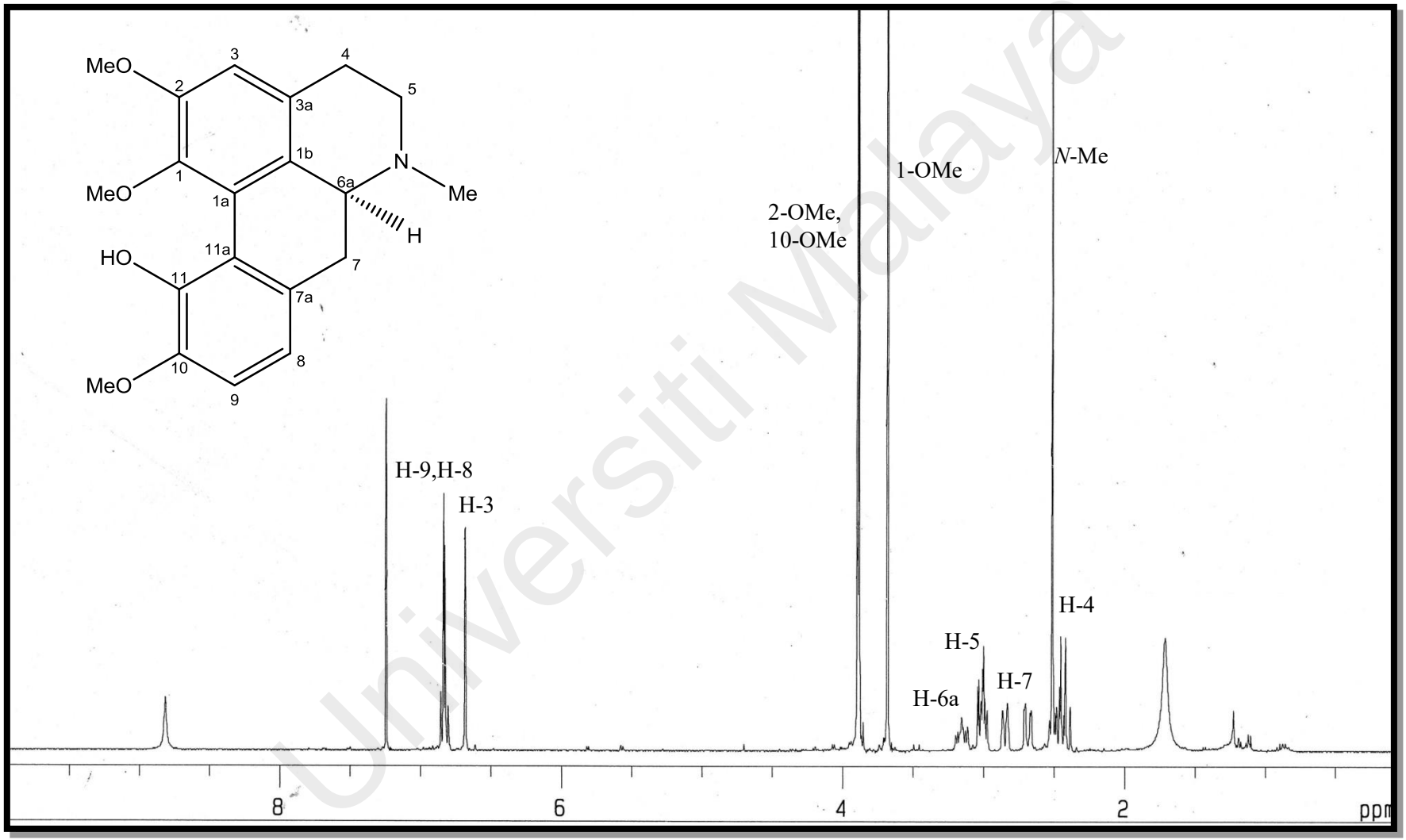



Figure 3.28: $^{1}$H NMR Spectrum of Isocorydine **48**

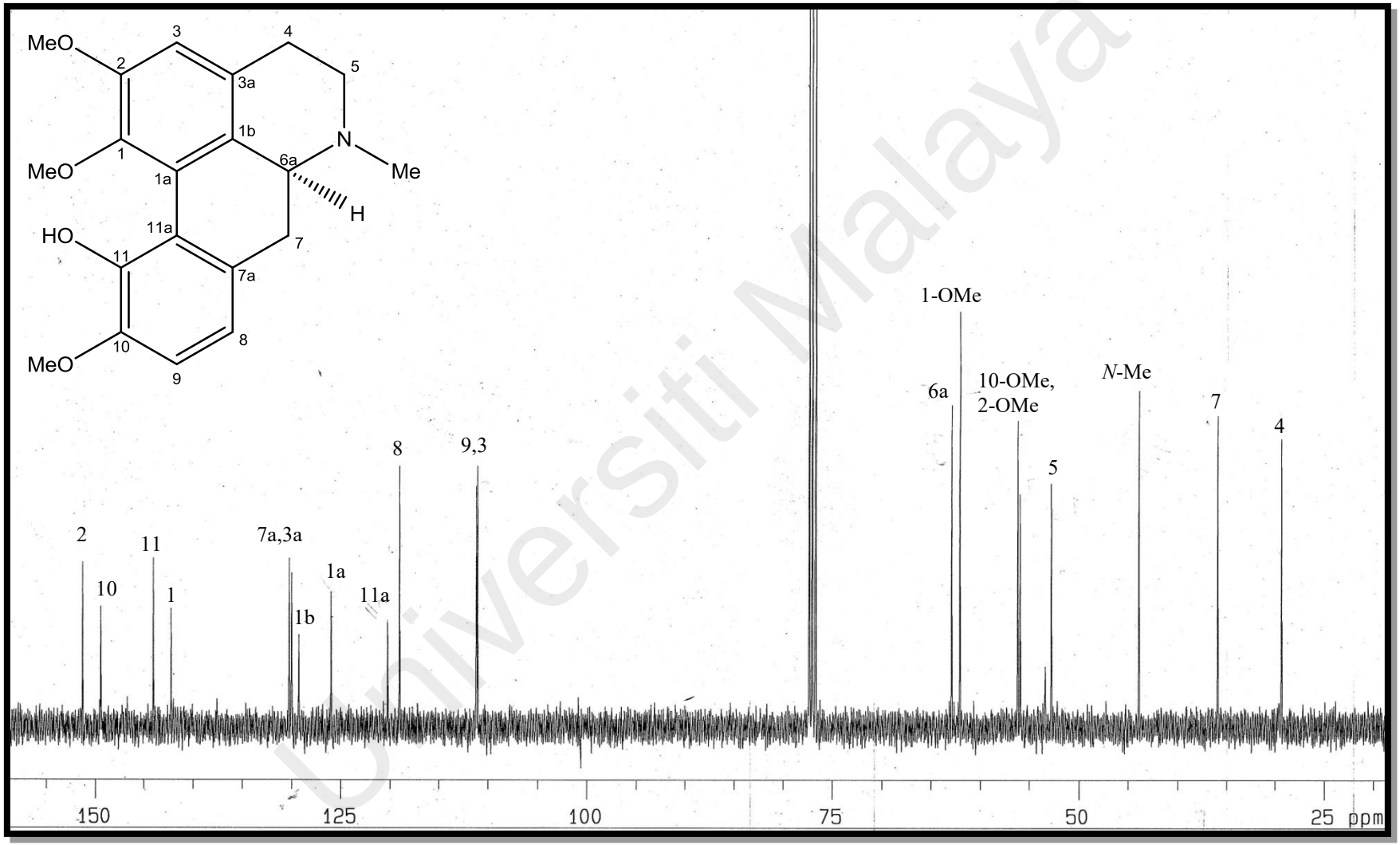



Figure 3.29: $^{13}$C NMR Spectrum of Isocorydine **48**

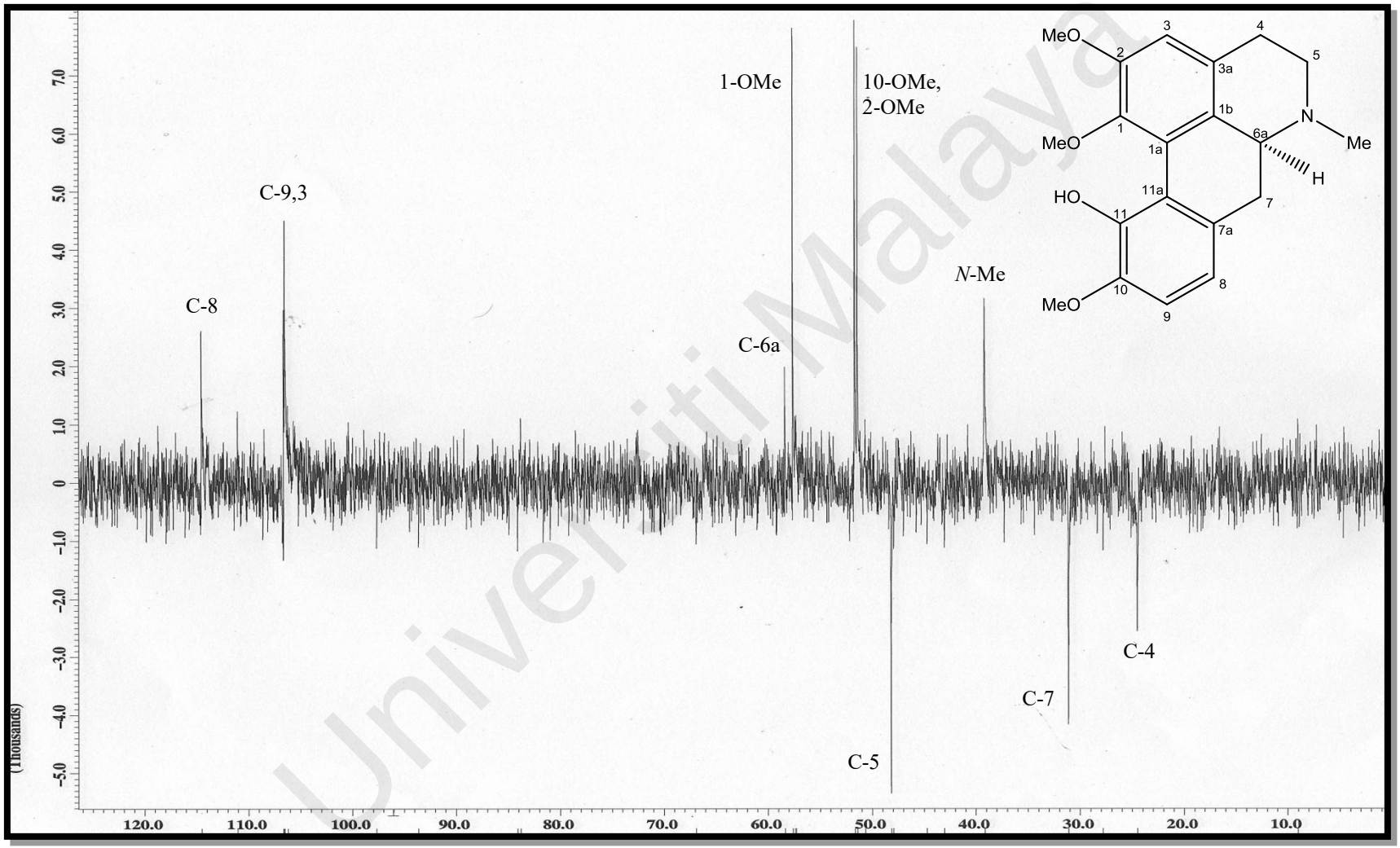



Figure 3.30: DEPT Spectrum of Isocorydine **48**

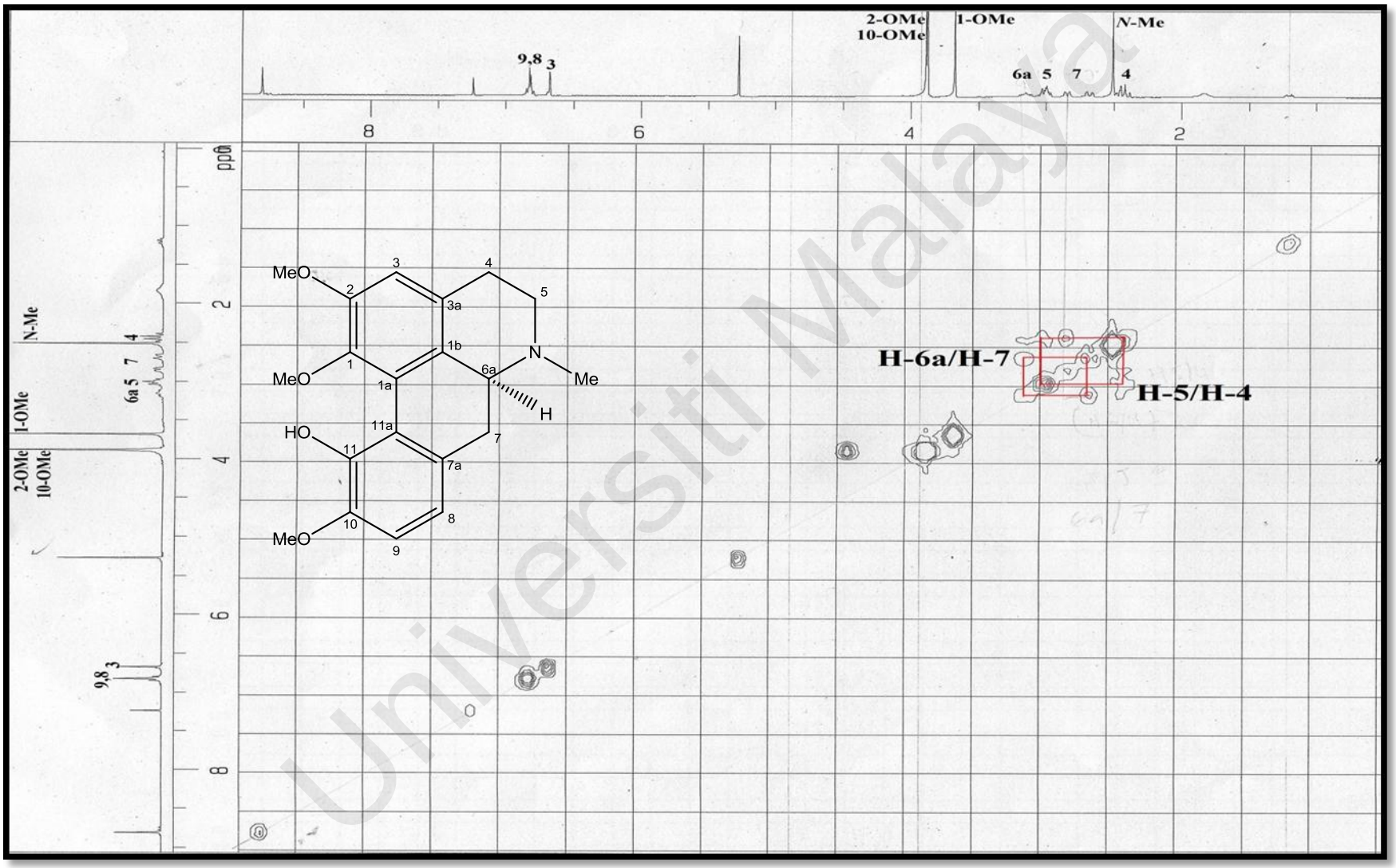



Figure 3.31: COSY Spectrum of Isocorydine **48**

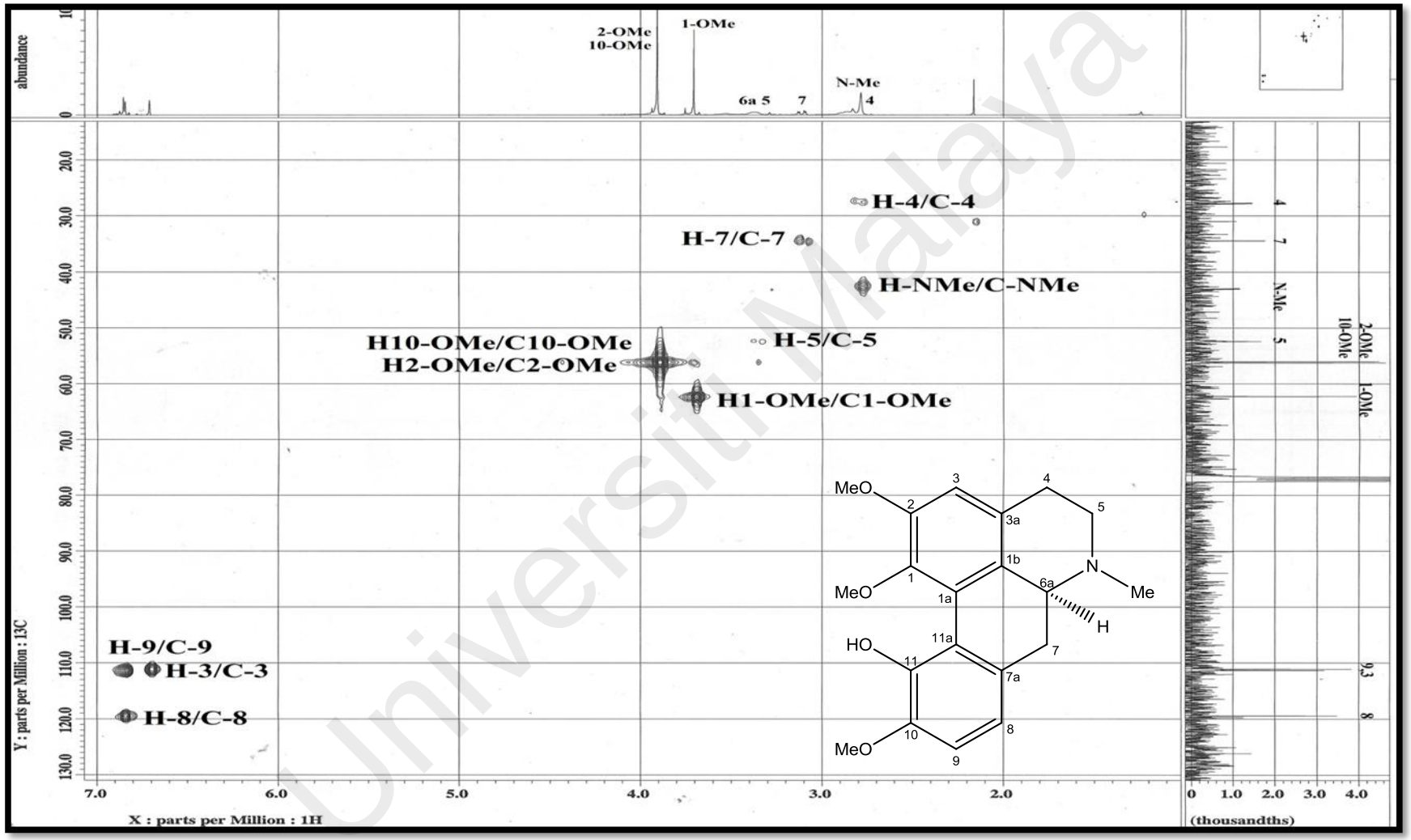



Figure 3.32: HMQC Spectrum of Isocorydine **48**

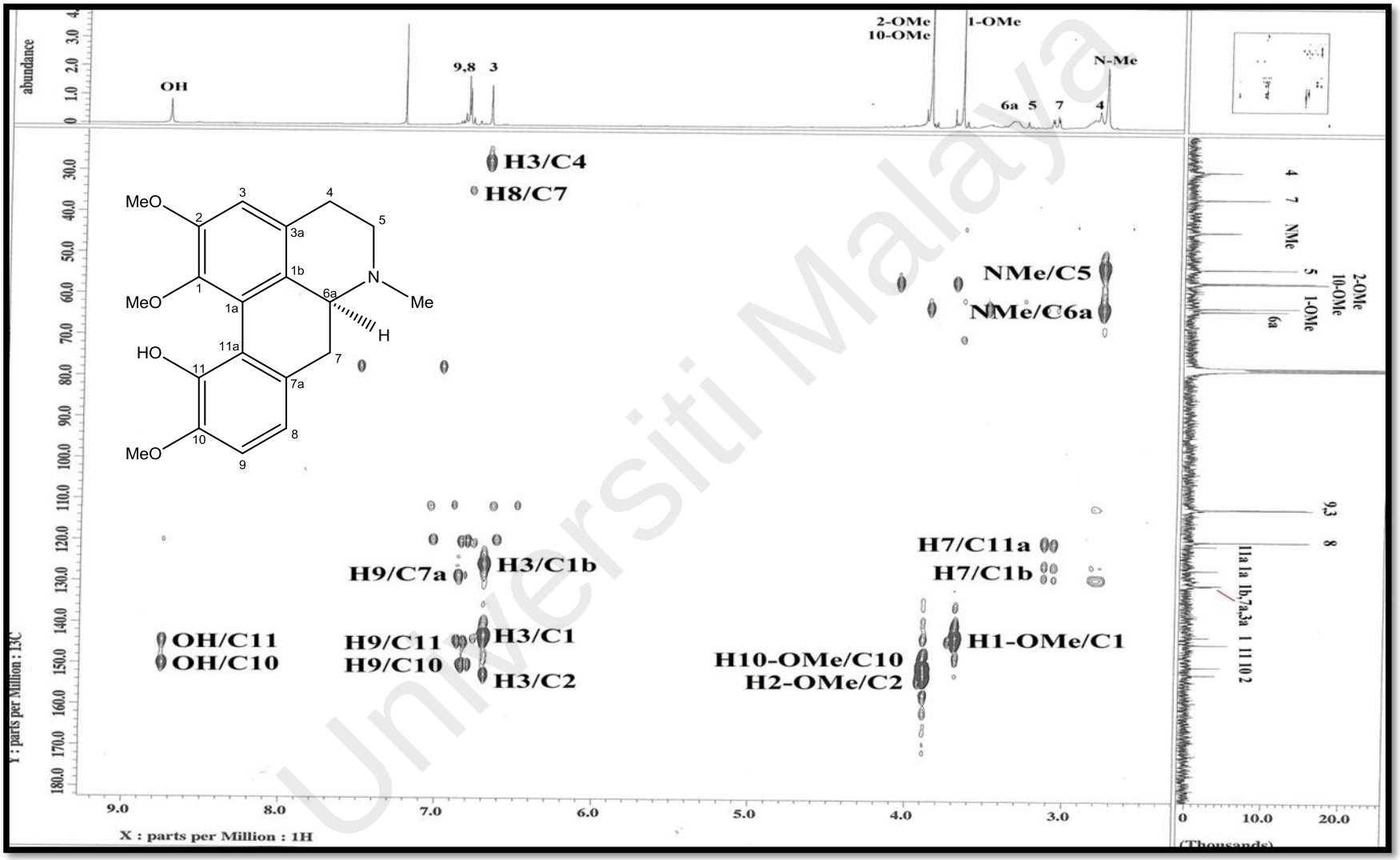
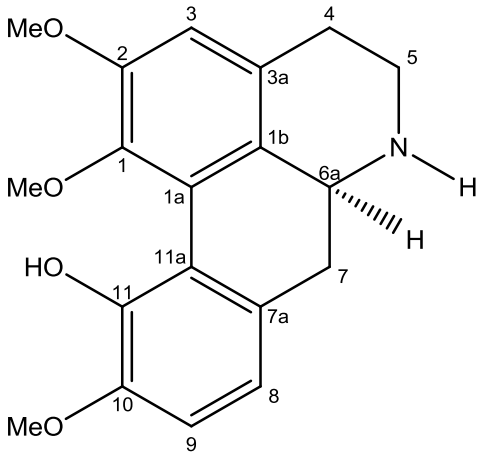



Figure 3.33: HMBC Spectrum of Isocorydine **48**

### 3.1.6 Norisocorydine 49




**49**

Norisocorydine **49** was isolated as brownish amorphous powder with $[\alpha]_D^{25}$ +158.5º (c = 0.10, EtOH) indicated the absolute configuration at C-6a was S as in the known alkaloid [99-101]. Its UV spectrum exhibited maxima at 270 and 310 nm, which supported a 1, 2, 10, 11-tetrasubstitued aporphine skeleton [102]. In addition, the IR spectrum gave a broad absorption bands between 3500 and 2936 $cm^{-1}$ due to the presence of OH and NH groups. The LCMS spectrum revealed a pseudomolecular peak at m/z 328.2 suggesting a molecular formula of $C_{19}H_{22}NO_4$ with 10 degree of unsaturation (4 rings and 6 double bonds). Other peak was observed at m/z 350.2 $[M+23]^+$, due to the addition of sodium, Na.

The $^1$H-NMR and $^{13}$C-NMR spectra of compound **49** were almost similar to the compound **48**, which confirmed a close structural relationship between both compounds. However, the only factor which differentiates compound **49** with compound **48** was the absence of an *N*-methyl signal at δ 2.50 in the $^1$H-NMR spectrum (refer Figure 3.35).

The $^{13}$C-NMR (Figure 3.36) and DEPT (Figure 3.37) spectra for compound **49** showed 19 carbon signals. The DEPT spectrum showed three methyls, three methylenes, four methines and nine quartenary carbon signals. Three signals of methoxyl group were observed at δ 62.3, 56.2 and 56.0 belong to OMe group at C-1, C-10 and C-2 respectively. The signals for quaternary carbons appeared at δ 151.5, 149.5, 144.2, and 142.1 which were assigned to C-2, C-10, C-11 and C-1 respectively.

In the COSY spectrum (Figure 3.38), the presence of methylene protons at position C-4 (δ 2.67-2.72, δ 2.95-3.04) and C-5 (δ 2.89-2.95, δ 3.29-3.33) were observed. An analysis of HMBC spectrum (Figure 3.40) revealed cross-peaks between the methylene protons and the methine protons with a quaternary carbon at δ 130.4 (C-3a) and δ 129.9 (C-1b). Thus, with this observation, the exact position of quaternary carbons could be determined. The other interaction of HMBC coupling pattern are summarized in Table 3.6.

The spectral data obtained were consistent with those found in the literature reviews, thus confirming the structure for norisocorydine **49** [99-101].

Table 3.6: $^{1}$H NMR (in $CDCl_3$, 400 MHz), $^{13}$C NMR (in $CDCl_3$, 400 MHz), and HMBC of **49** and Comparison with $^{1}$H NMR Literature Values (Chang *et al.* 2001).

| Position | $\delta_H$, ppm (J in Hz) (observed) | $\delta_C$ (ppm) | HMBC (H→C) | $\delta_H$, ppm (J in Hz) (Chang, 2001) |
|---|---|---|---|---|
| 1 | - | 142.1 | - | - |
| 1a | - | 125.8 | - | - |
| 1b | - | 129.9 | - | - |
| 2 | - | 151.5 | - | - |
| 3 | 6.69, *s* | 111.8 | 1,1b,2,4 | 6.90, *s* |
| 3a | - | 130.4 | - | - |
| 4 | α, 2.67-2.72, *m*<br>β, 2.95-3.04, *m* | 29.4 | 1b,3,5 | |
| 5 | α, 2.89-2.95, *m*<br>β, 3.29-3.33, *m* | 42.9 | 4,6a | |
| 6a | 3.57-3.65, *m* | 54.2 | 3a | 3.63, *d*, J=3.6 |
| 7 | α, 2.53, *t*, J=13.2<br>β, 2.72-2.77, *dd*, J=13.2,4.2 | 38.5 | 1b,6a,7a,8,11a | |
| 7a | - | 130.5 | - | - |
| 8 | 6.79, *d*, J=8.1 | 119.0 | 7,11a | 6.87, *d*, J=8.4 |
| 9 | 6.83, *d*, J=8.1 | 111.0 | 11 | 6.95, *d*, J=8.4 |
| 10 | - | 149.5 | - | - |
| 11 | - | 144.2 | - | - |
| 11a | - | 120.2 | - | - |
| 1-OMe | 3.69, *s* | 62.3 | 1,2-OMe | 3.67, *s* |
| 2-OMe | 3.89, *s* | 56.0 | 10 | 3.90, *s* |
| 10-OMe | 3.89, *s* | 56.2 | 2,1-OMe | 3.87, *s* |

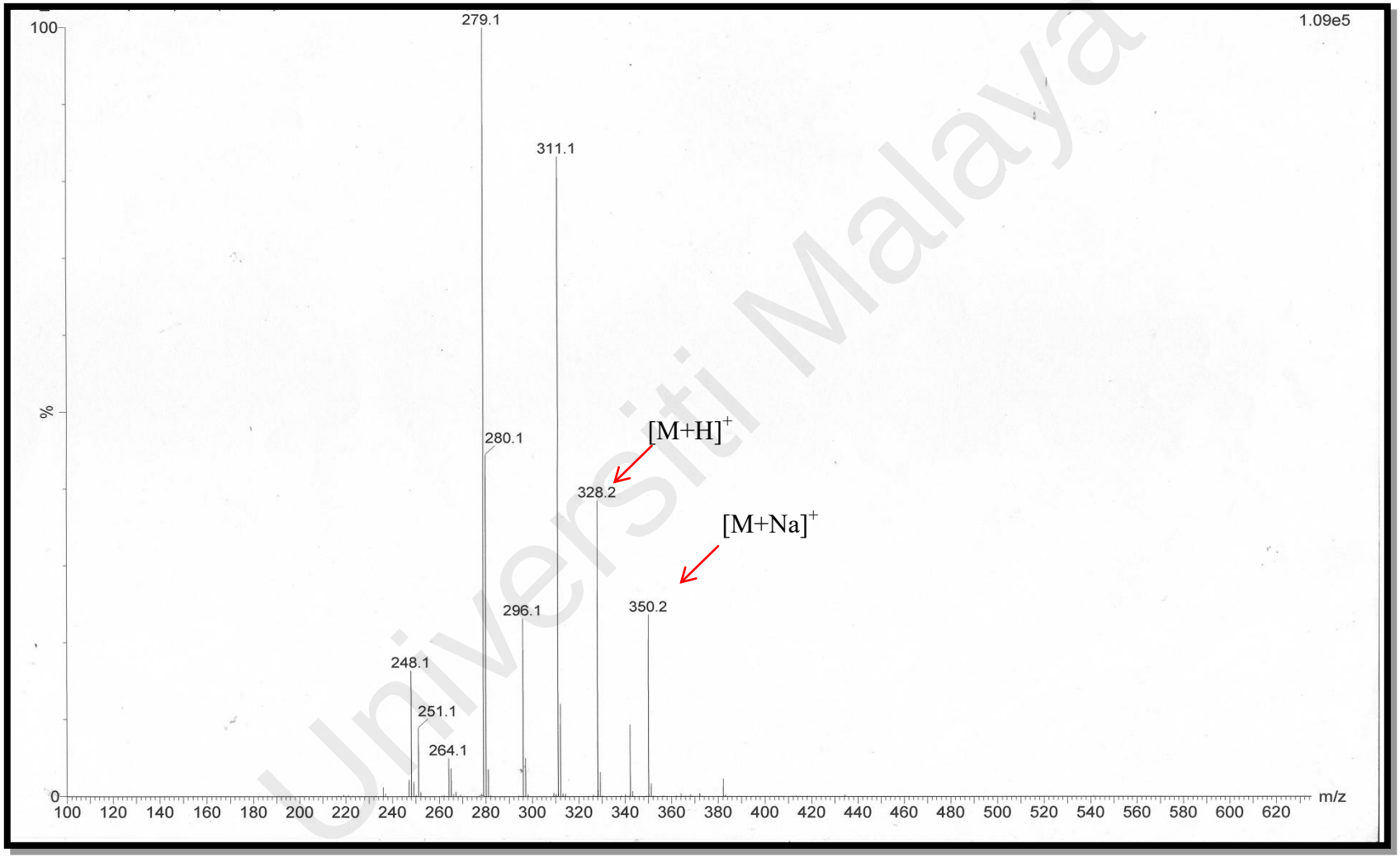



Figure 3.34: LCMS Spectrum of Norisocorydine **49**

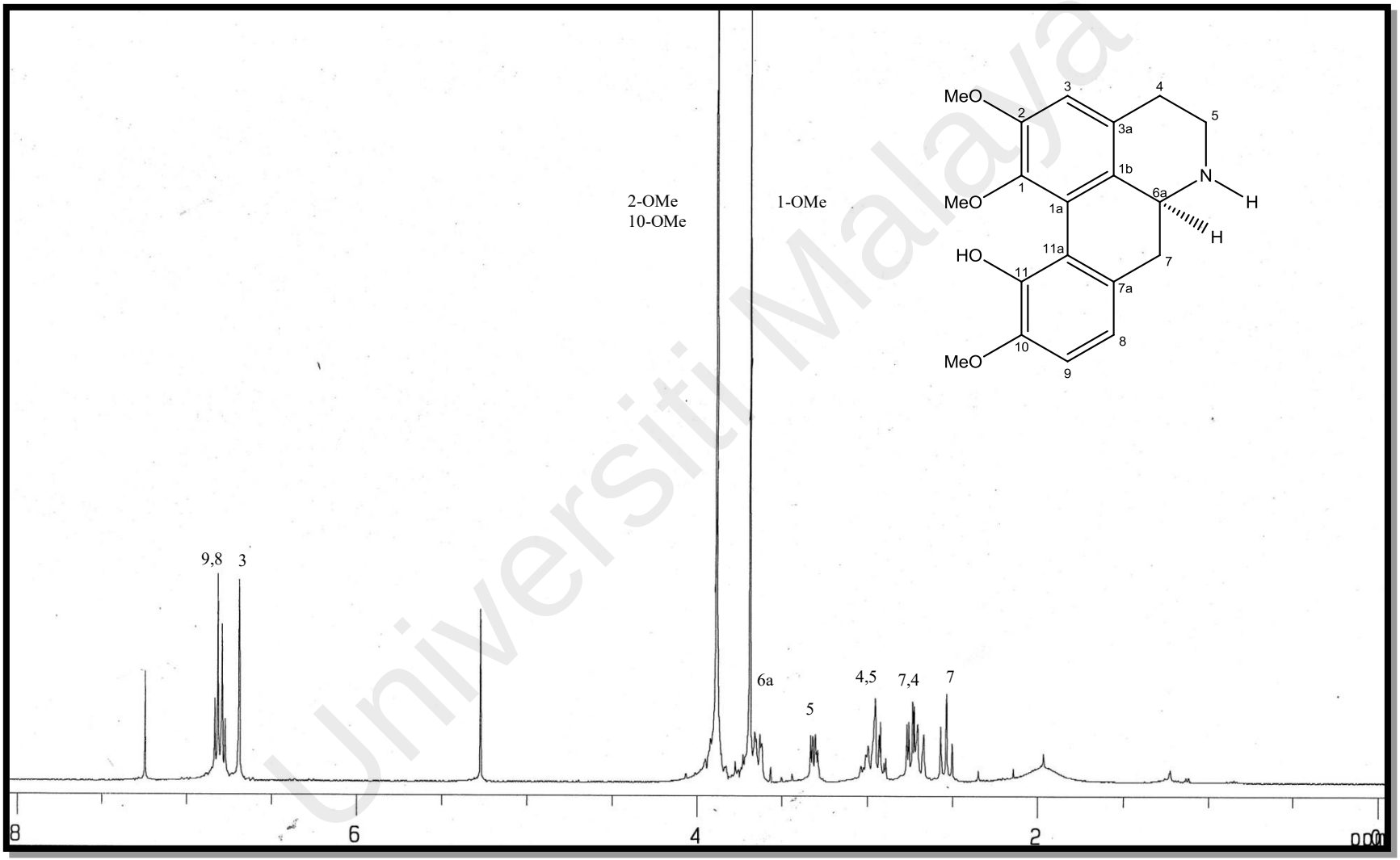



Figure 3.35: $^{1}$H NMR Spectrum of Norisocorydine **49**

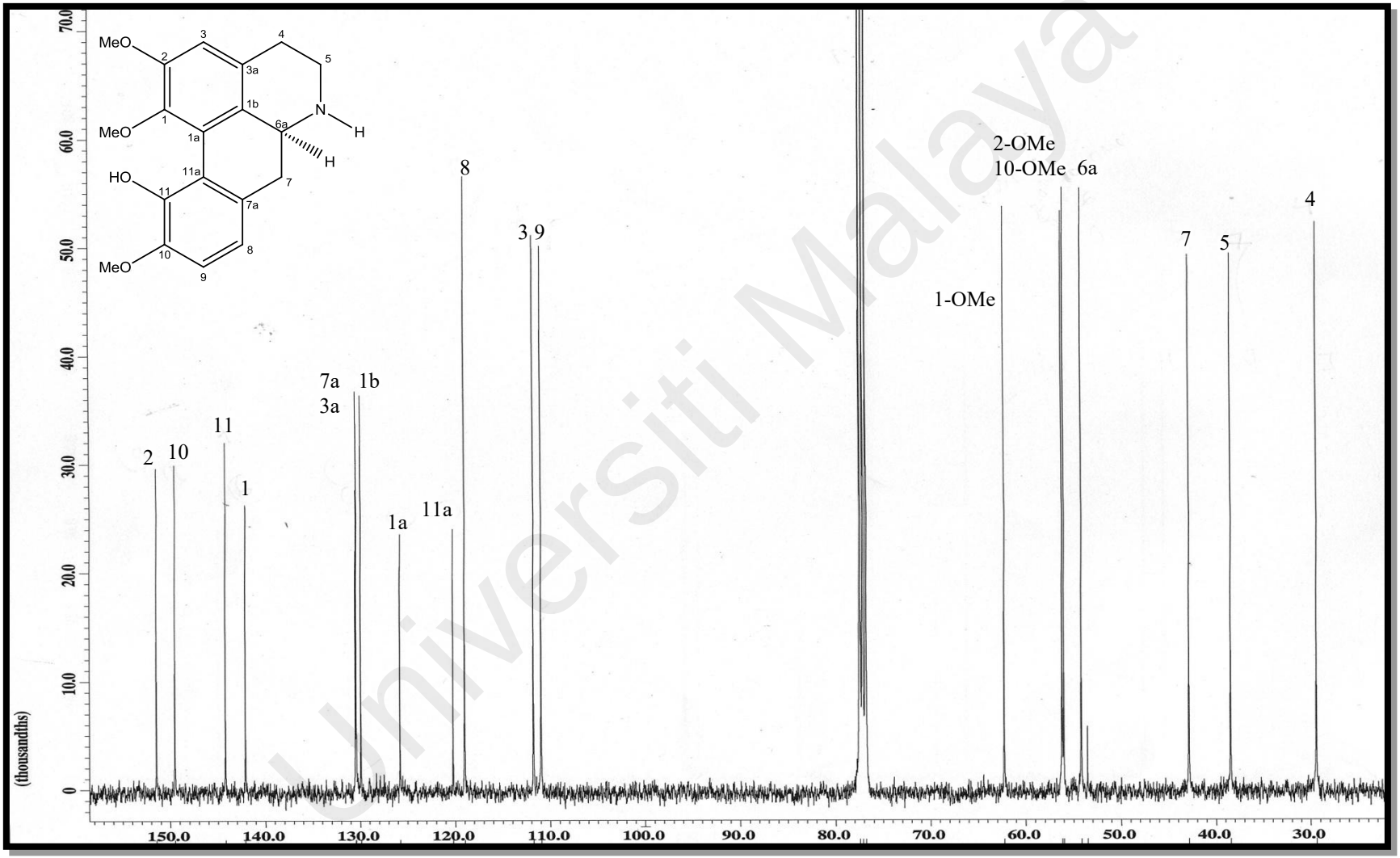



Figure 3.36: $^{13}$C NMR Spectrum of Norisocorydine **49**

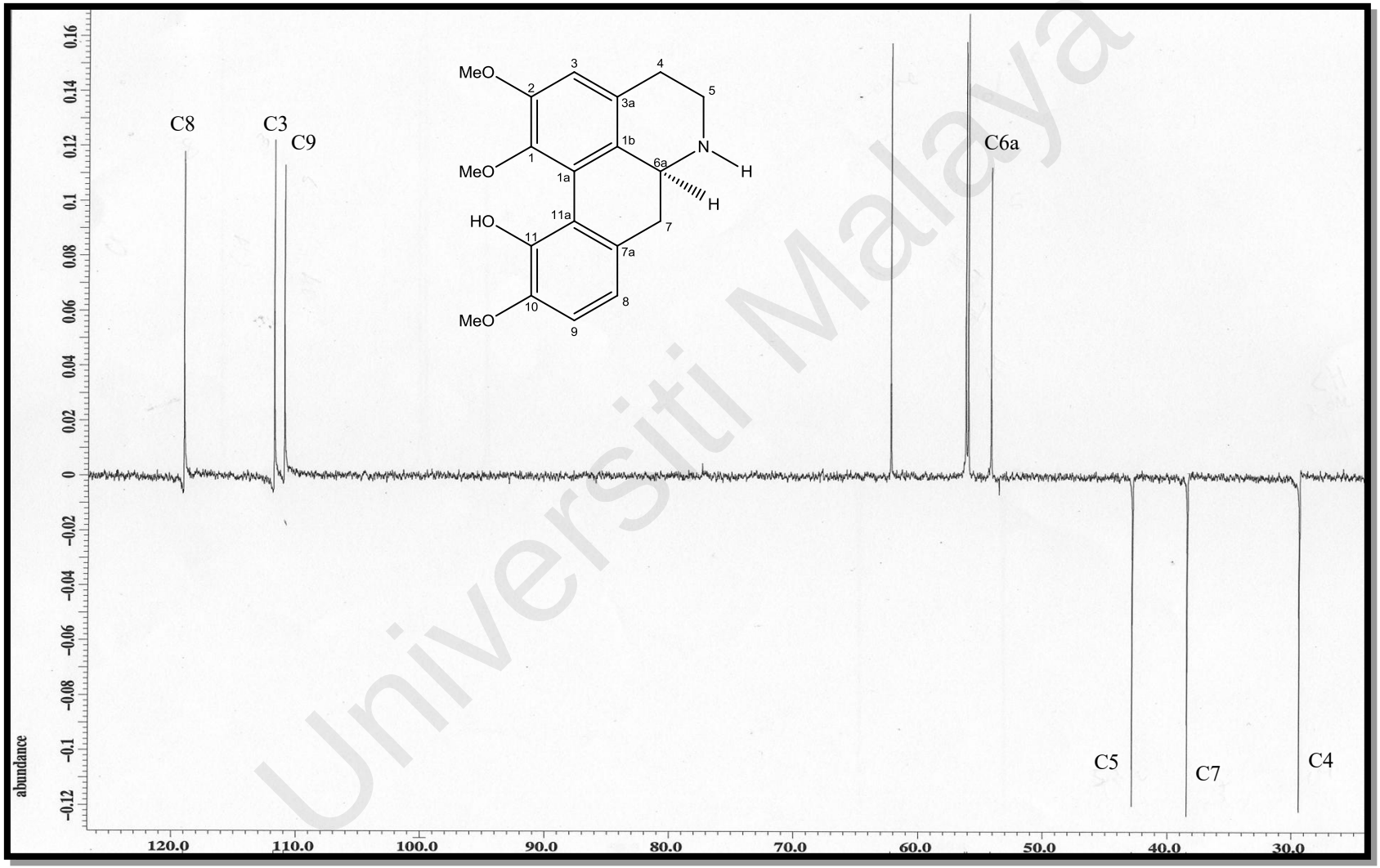



Figure 3.37: DEPT Spectrum of Norisocorydine **49**

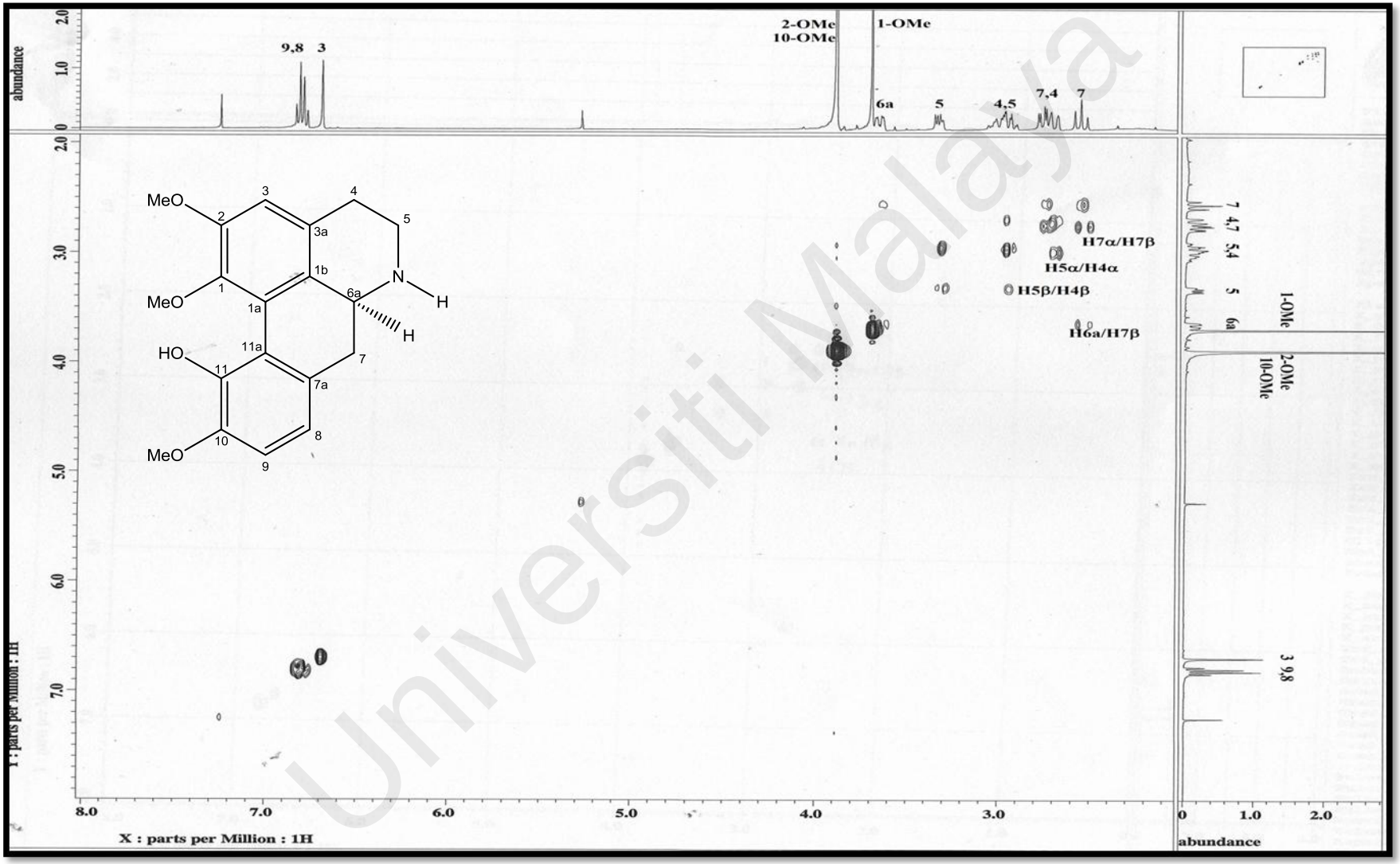



Figure 3.38: COSY Spectrum of Norisocorydine **49**

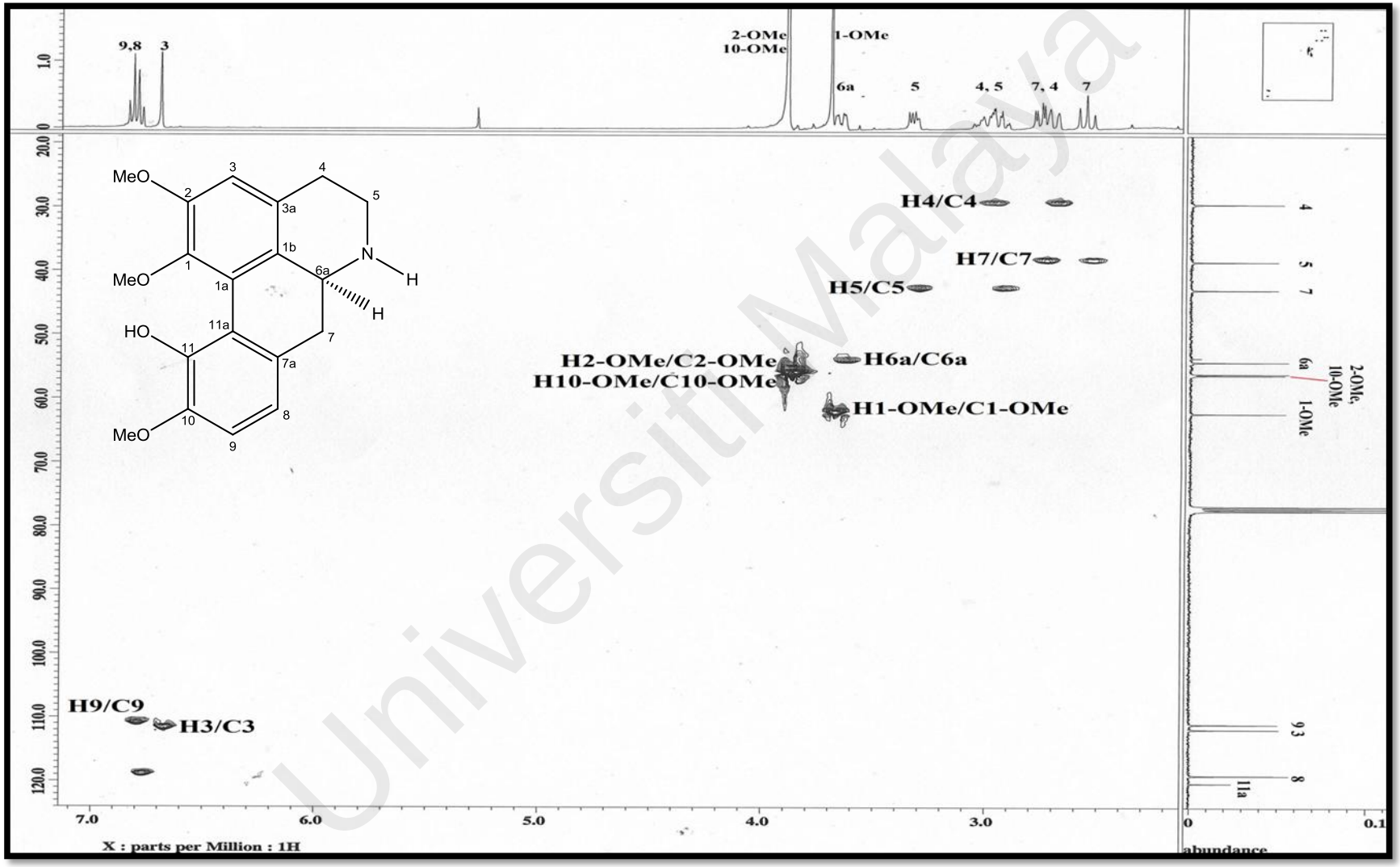



Figure 3.39: HMQC Spectrum of Norisocorydine **49**

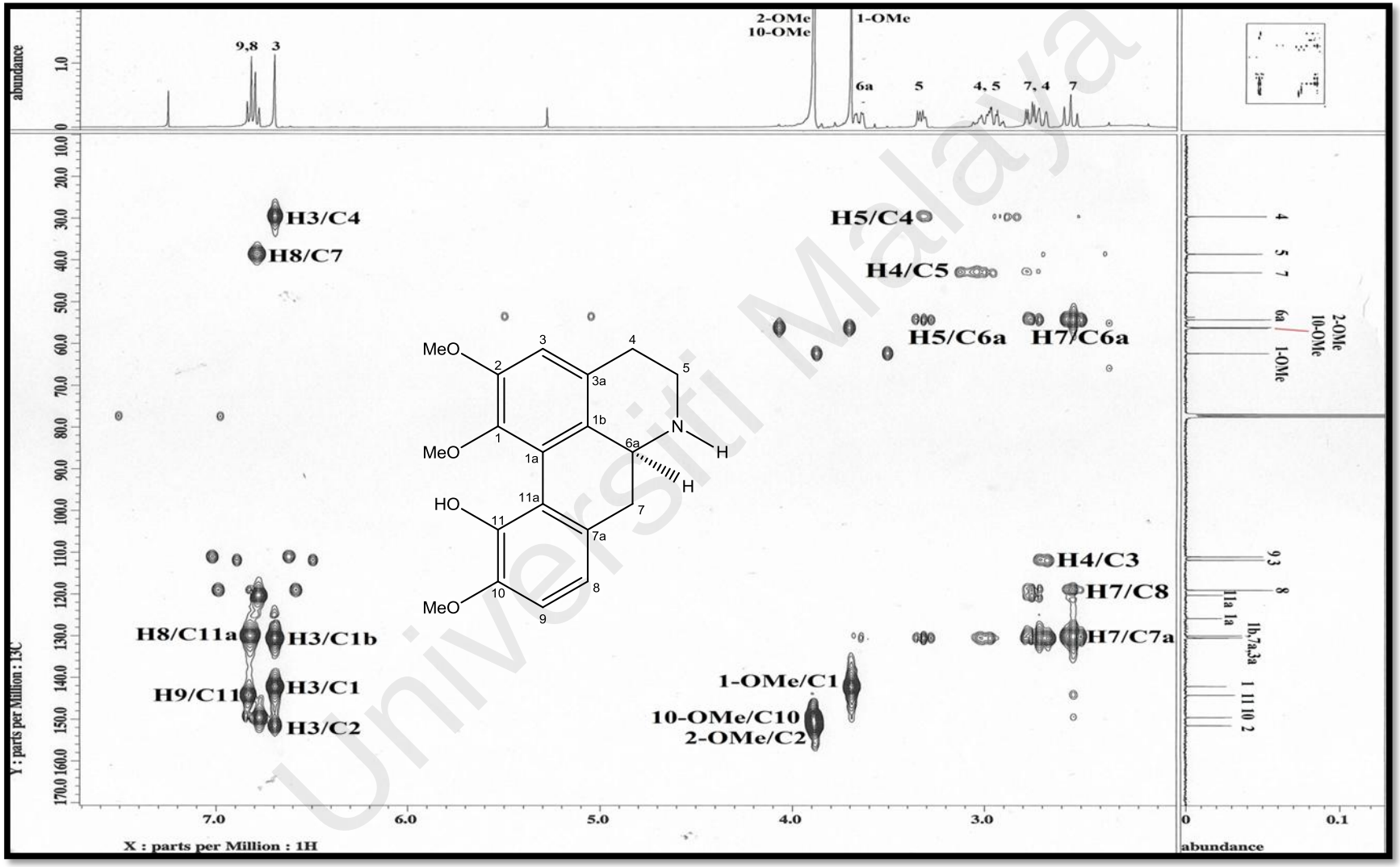
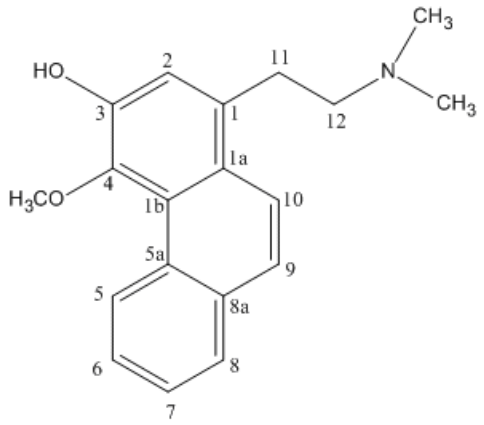



Figure 3.40: HMBC Spectrum of Norisocorydine **49**

### 3.1.7 Argentinine 50




**50**

Argentinine **50** was isolated as brownish amorphous solid. Its UV spectrum showed absorption bands at 245, 256, 291, and 345 nm. The IR spectrum exhibited strong and broad O-H stretching at 3367 $cm^{-1}$ and a strong C-H stretching for aromatic ring at 2922 and 2852 $cm^{-1}$. The LCMS spectrum revealed a pseudomolecular ion peak,$[M+H]^+$ at m/z 296 suggesting a molecular formula of $C_{19}H_{21}NO_2$ with 10 degree of unsaturation (3 rings and 7 double bonds).

The $^1$H-NMR spectrum (Figure 3.42) indicated the presence of singlets at δ 2.41 (6H) and δ 3.76 (3H), which corresponded to two *N*-methyl groups and methoxyl groups. In the aromatic region, two coupled protons at δ 7.56 (1H, *d*, J=9.4 Hz) and δ 7.82 (1H, *d*, J=9.4 Hz), characteristic of an AB system corresponding to H-9 and H-10 in the phenanthrene ring system, were detected. The aromatic proton at δ 9.34 (1H, *d*, J=7.6 Hz) corresponded to the H-5 in the phenanthrene alkaloids. This signal was consistent with the presence of a methylenedioxy group at C-3 and C-4 that resulted in an upfield shift of the H-5 proton signals to ≈ δ 9.0, since phenanthrene alkaloids with methoxy and/or hydroxyl substituents at C-3 and C-4 show H-5 signal downfield between δ 9.3 and δ 9.9. Finally,

two sets of multiplets were observed at δ 3.22-3.26 and δ 2.62-2.80, which were assignable to the methylene proton H-11 and H-12 respectively.

Comparison of the UV, IR, LCMS and $^{1}$H-NMR data of compound **50** with previous reported literature, thus confirming the structure for argentinine **50** [103].

Table 3.7: $^{1}$H NMR (in $CDCl_3$, 400 MHz) of **50** and Comparison with $^{1}$H NMR Literature Value (Javier *et al.* 2002).

| Position | $\delta_H$, ppm (J in Hz) (observed) | $\delta_H$, ppm (J in Hz) (Javier, 2002) |
|---|---|---|
| 1 | - | - |
| 1a | - | - |
| 1b | - | - |
| 2 | 7.17, *s* | 7.28, *s* |
| 3 | - | - |
| 4 | - | - |
| 5 | 9.34, *d*, J=7.6 | 9.42, *br d*, J=9.2 |
| 5a | - | - |
| 6 | 7.52-7.58, *m* | 7.86, *br d*, J=9.2 |
| 7 | 7.52-7.58, *m* | 7.61, *t d*, J=8.4, 1.6 |
| 8 | 7.78-7.82, *m* | 7.53, *br d*, J=8.8 |
| 8a | - | - |
| 9 | 7.56, *d*, J=9.4 | 7.68, *d*, J=9.2 |
| 10 | 7.82, *d*, J=9.4 | 7.91, *d*, J=9.2 |
| 11 | 3.22-3.26, *m* | 3.21, *ddd*, J=16.8, 11.6, 5.2 |
| 12 | 2.62-2.80, *m* | 3.59, *ddd*, J=16.8, 11.6, 5.2 |
| 4-OMe | 3.76, *s* | 3.84, *s* |
| 2 *N*-Me | 2.41, *s* | 2.84, *s* |

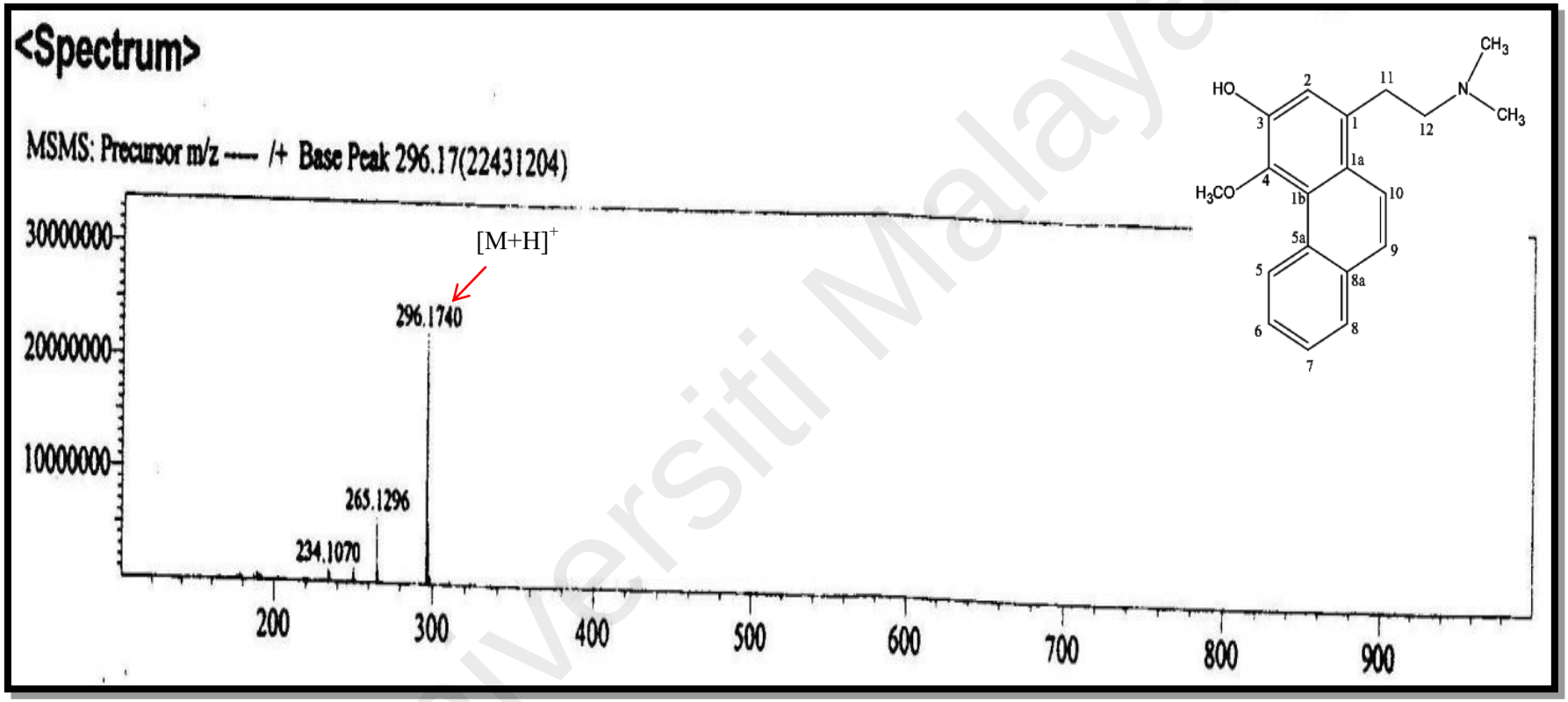



Figure 3.41: LCMS Spectrum of Argentinine **50**

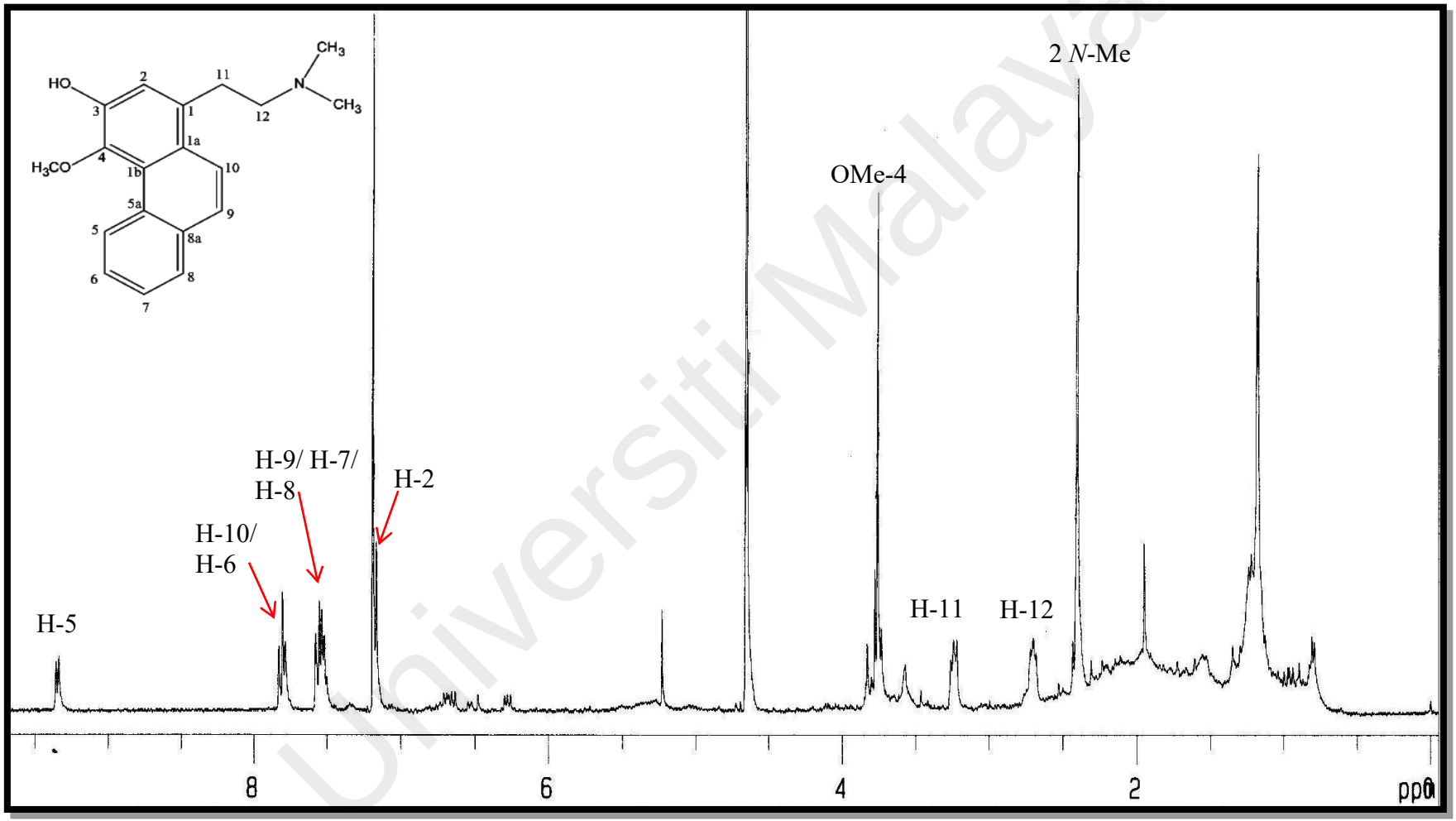
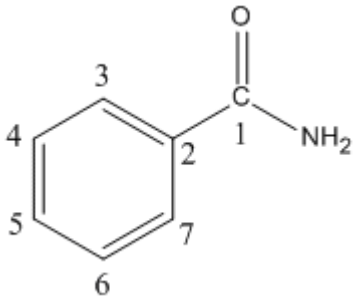



Figure 3.42: $^{1}$H-NMR Spectrum of Argentinine **50**

### 3.1.8 Benzamide 51




**51**

Benzamide **51** was the major compound isolated from the leaves of *Cryptocarya nigra* and obtained as crystalline needles with melting point 125-127ºC. Compound **51** showed UV absorption at 294 and 280 nm. In addition, the IR spectrum exhibited a strong absorption peak at 1646 $cm^{-1}$ attributed to a conjugated ketone function. The binding of $NH_2$ in the structure was indicated by the peak at 3368 $cm^{-1}$. The mass spectrum (Figure 3.43) showed a molecular ion at m/z 121 consistent with molecular formula $C_7H_7NO$. Other significant peaks were observed at m/z 105 $[M-16]^+$ (base peak) and 77 $[M-44]^+$, due to the loss of $NH_2$ and $CONH_2$, respectively.

The 400 MHz $^1H$ NMR spectrum (Figure 3.44) of compound **51** (Table 3.8) showed two sets of triplets and one doublet of aromatic proton signals at δ 7.43 (2H, *t*, J=7.4, H-4 and H-6), δ 7.51 (1H, *t*, J=7.5, H-5), and δ 7.80 (2H, *d*, J=3.3, H-3 and H-7) suggested the presence of mono substituted aromatic ring. In addition, it shows an extreme example of quadropole broadening, where the $NH_2$ absorption extends from δ 5.80-6.27 as a broad peak.

The $^{13}C$ NMR spectrum (Figure 3.45) was in agreement with the molecular formula deduced from the mass spectrum, accounting for all 7 carbons. In the $^{13}C$ NMR

spectrum, there are only 5 carbon peaks. There were 2 overlapping carbons appeared at the same chemical shift which has equivalent environment in the aromatic ring. These signals appeared at δ 127.3 (C-3 and C-7) and at δ 128.6 (C-4 and C-6). The DEPT experiment revealed five methines and two quaternary carbon signals. Finally, from the analysis of DEPT, HMQC and COSY and spectral data compared with literature values, it is confirmed that compound **51** was benzamide. [104]

Table 3.8. $^{1}H$ NMR (in $CDCl_3$, 400MHz) and $^{13}C$ NMR (in $CDCl_3$, 100MHz) of **51**

| Position | $\delta_H$, ppm (J in Hz) | $\delta_C$ (ppm) |
|---|---|---|
| 1 | - | 169.5 |
| 2 | - | 133.3 |
| 3 | 7.80, *d*, J = 3.3 | 127.3 |
| 4 | 7.43, *t*, J = 7.4 | 128.6 |
| 5 | 7.51, t, J = 7.5 | 132.0 |
| 6 | 7.43, *t*, J = 7.4 | 128.6 |
| 7 | 7.80, *d*, J = 3.3 | 127.3 |
| $NH_2$ | 5.80-6.27, br *s* | - |

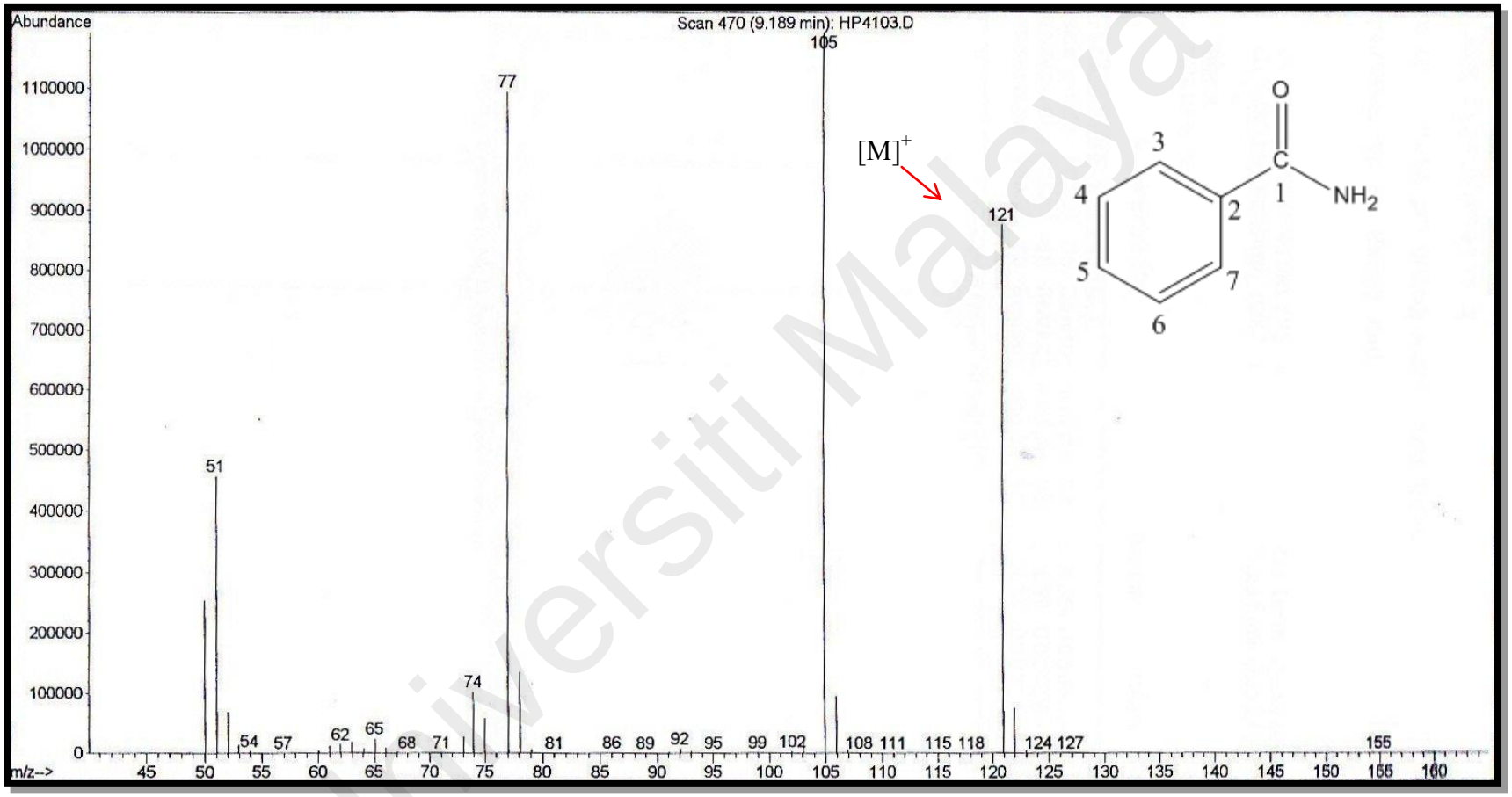



Figure 3.43: Mass Spectrum of Benzamide **51**

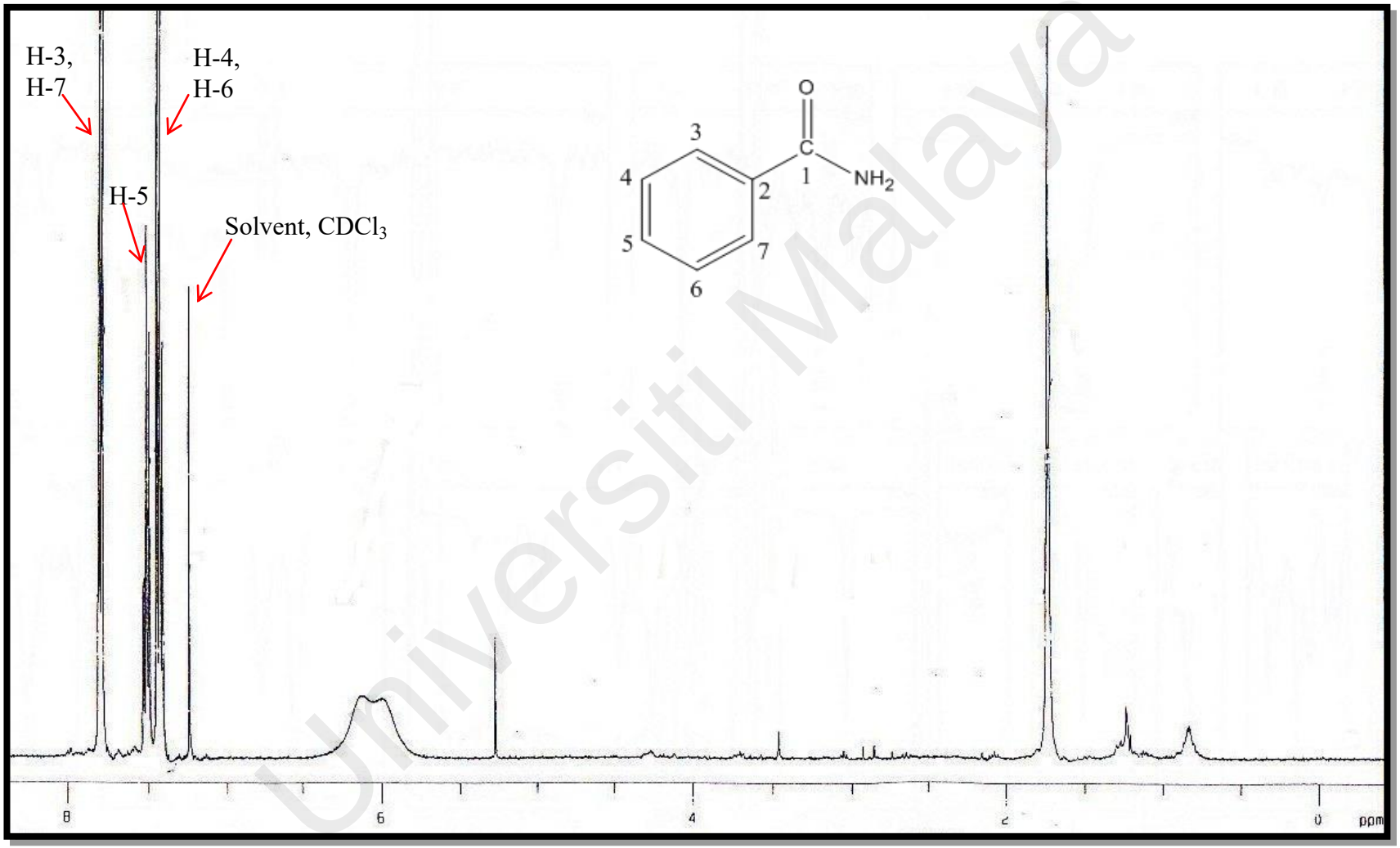



Figure 3.44: $^{1}$H NMR Spectrum of Benzamide **51**

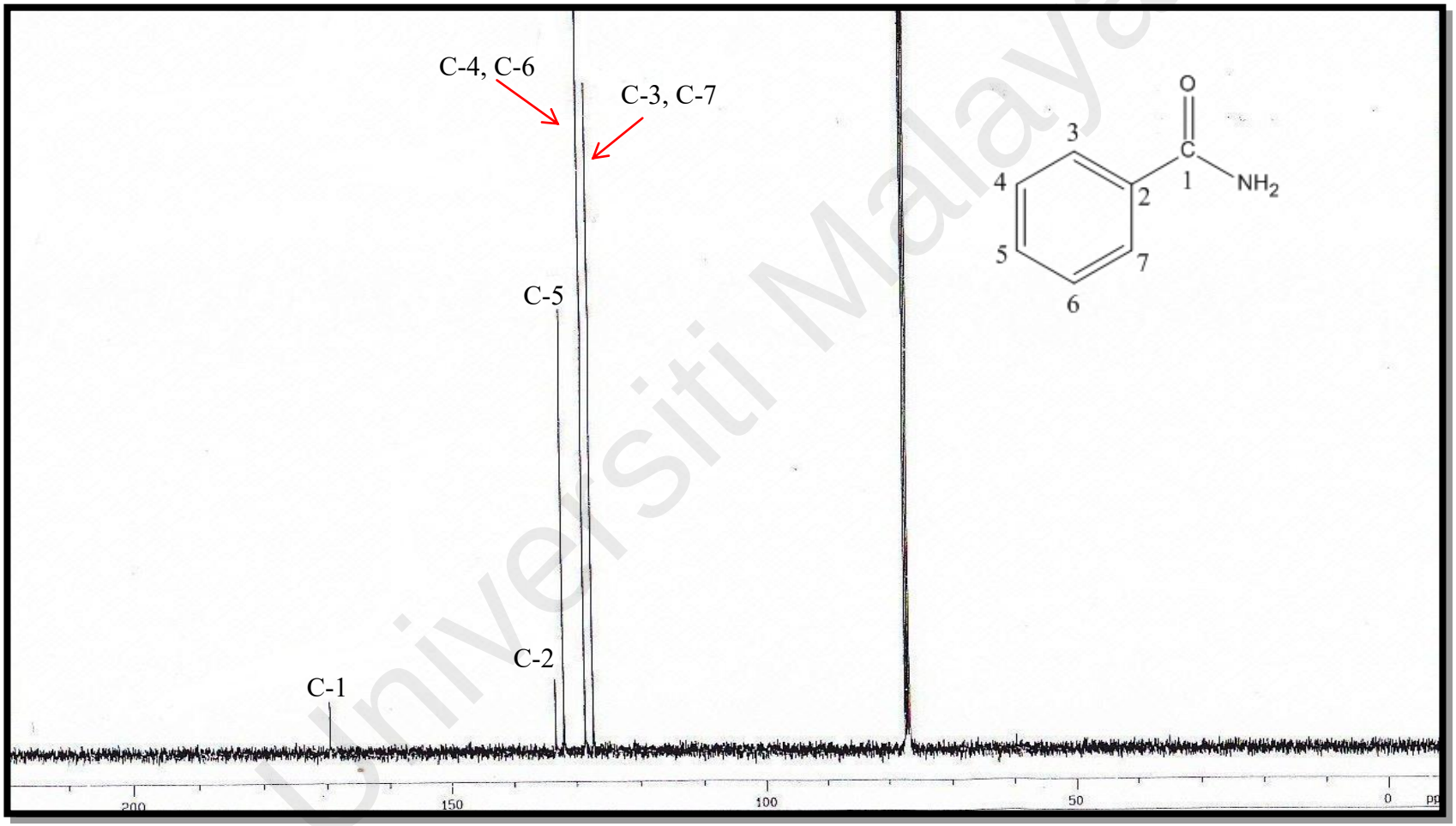



Figure 3.45: $^{13}$C NMR Spectrum of Benzamide **51**

## 3.2 Biological Activities of *Cyptocarya nigra* (Lauraceae)

### 3.2.1 Antiplasmodial Activities Against *Plasmodium falciparum* Strains

Malaria is caused by five species of parasites of the genus *Plasmodium* that affect humans (*P.falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*). Malaria due to *P. falciparum* is the most deadly form. According to the latest data by WHO, the estimation incidence of malaria has remained unacceptably high. There were about 219 million cases of malaria in 2010 and an estimated 660 000 deaths.[105] A major factor in the continuing burden of malaria is the spread of parasites resistant to front-line anti-malarials such as chloroquine and sulphadoxine/ pyrimethamine, and artemisinine.[106]

Table 3.9. *In vitro* Antiplasmodial Activities of Alkaloids from *Cryptocarya nigra*

| Sample | Antiplasmodial Activity ($IC_{50}$) | |
|---|---|---|
| | μg/mL | μM |
| Dichloromethane extract | 2.82 | - |
| *N*-Methylisococlaurine **46** | 1.62 | 5.40 |
| Atherosperminine **11** | 1.80 | 5.80 |
| 2-hydroxylatherosperminine **20** | 0.25 | 0.750 |
| Chloroquine | - | 0.090 ± 28.9 |
| Artemisinine | - | 0.002 ± 0.001 |

The compounds isolated from the bark of *Cryptocarya nigra* were tested for antiplasmodial activity based on the promising screening results of dichloromethane (DCM) crude extract ($IC_{50}$ = 2.82 μg/mL). The *in vitro* antiplasmodial activity of compounds isolated from *Cryptocarya nigra* against a chloroquine sensitive strain of *P. falciparum* (K1 strain) is summarized in Table 3.9. 2-Hydroxylatherosperminine **20** displayed the strongest inhibition activity, with an $IC_{50}$ value of 0.75 μM, followed by *N*-methylisococlaurine **46** and atherosperminine **11** with an $IC_{50}$ value of 5.40 μM and

5.80 μM respectively. These alkaloids have not been reported to have their antiplasmodial activity.

### 3.2.2 Antioxidant Activities

Recent development in medicinal field reports a number of disease associated with free radicals. The risk of diseases due to oxidative stress is compounded by unhealthy lifestyle, exposure of chemicals, pollution, cigarette smoking, drugs, illness, and stress etc. Antioxidants are the substances which can scavenge free radicals and help to decrease the incidence of oxidative stress induced damage. [119]

In the present study, the antioxidant activity of isolated compounds of *Cryptocarya nigra* was determined using DPPH radical scavenging assay, reducing power assay, and metal chelating assay. DPPH assay has been widely used to determine the free radical scavenging activity of various plants. FRAP assay was frequently used to study the antioxidant activity of plants. It measures the ferric to ferrous ion reduction in the presence of antioxidant. The reducing power of a sample may serve as important pointer of its potential antioxidant capacity. It showed the reducing capacity of a compound and is able to act as a significant indicator for antioxidant activity. Metal chelating activity is significant as it reduces the concentration of the catalyzing transition metal in lipid peroxidation through the Fenton reaction. [120]

*N*-Methylisococlaurine **46** showed the highest scavenging activity of DPPH free radicals with $IC_{50}$ = 29.56 μg/ml and reducing power at 78.54% in comparison to atherosperminine **11**. However atherosperminine **11** showed higher metal chelating activity than *N*-methylisococlaurine **46** at $IC_{50}$ = 42.87ug/ml and $IC_{50}$ 50.08ug/ml respectively. The high DPPH and reducing power antioxidant activity of *N*-methylisococlaurine **46** could be due to its hydroxyl group which donated the electron to form stable free radicals and possess the ability to chelate metal. Benzylisoquinoline

alkaloid of tetrandrine has been reported to have antioxidant activity [108]. The antioxidant activity of phenanthrene atherosperminine **11** is still not known. However there has been reported that the phenanthrene have shown an effect on glutathione reductase that increases antioxidant activity in fish of olive flounder, *Paralichthys olivaceus* [109].

Table 3.10. Antioxidant Activities of Alkaloids from *Cryptocarya nigra*

| Compound Name | $IC_{50}$ DPPH Activity (ug/ml) | % FRAP | $IC_{50}$ Metal Chelating Activity (ug/ml) |
|---|---|---|---|
| *N*-methylisococlaurine **46** | 29.56 | 78.54 | 50.08 |
| Atherosperminine **11** | 54.53 | 70.66 | 42.87 |
| Ascorbic acid (Standard) | 13.69 | | |
| EDTA (Standard) | | 83.74 | |
| BHA (Standard) | | | 19.60 |

In DPPH assay and FRAP assay, compound **11** and **46** showed moderate activity compared to standards reference used ascorbic acid and EDTA respectively, while in Metal Chelating Assay both compound shows weak activity compared to the standard reference, BHA. The results showed that the *N*-methylisococlaurine **46** and atherosperminine **11** exhibited both iron binding and antiplasmodial activity. These suggested that there are iron requirement in host-parasite interaction [110, 111] for oxygen transport, respiration and enzymes activities. Thus, metal chelating ability of compounds could be important criteria in antimalarial treatment. The mechanism of action of iron chelators are still not known and further investigation need to be carried out on *N*-methylisococlaurine **46** and atherosperminine **11** as antimalarial treatment through metal chelating mechanism. Since both compounds exhibited antiplasmodial effect, the antioxidant activities may be able to stimulate immune system [112, 113]. This will provide a good potential for antimalarial therapy.

Further investigations have been made to test the isolated compounds for their antiplasmodial and antioxidant activity. Only *N*-methylisococlaurine **46** and atherosperminine **11** were tested for both activities. According to Reis, et.al, the combination of chloroquine and two antioxidant agents, at the first signs of cerebral malaria prevented both inflammatory and vascular changes in the tissues of the brain, as well as the development of persistent cognitive damage. The addition of antioxidants did not diminish the efficacy of chloroquine in eliminating Plasmodia from the blood. The authors suggested that these antioxidant drugs should be studied as additive therapy for antimalarial drugs in clinical trials in order to investigate their potential to reduce or prevent cognitive damage after cerebral malaria [114]. Additionally, recent studies by Percario, et al. (2012), suggested in their studies on 'Oxidative Stress in Malaria' that the use of antioxidant supplements of synthetic or natural origin may constitute a far more effective adjuvant antimalarial strategy that causes less damage to the host [115].

# CHAPTER 4

## CONCLUSION

# CHAPTER 4

# CONCLUSION

*Cryptocarya nigra* of Lauraceae family has not been studied for their alkaloidal content. The chemical study on the dried barks and leaves of *Cryptocarya nigra* afforded eight known compounds namely (+)-*N*-methylisococlaurine **46**, atherosperminine **11**, 2-hydroxyatherosperminine **20,** noratherosperminine **47**, isocorydine **48**, norisocorydine **49**, argentinine **50** and benzamide **51**. The leaves of *Cryptocarya nigra* underwent the acid-base extraction, while the bark part was extracted without using acid-base. Both extracts were subjected to chromatographic analysis. The compounds obtained from both methods of study are listed in Table 4.1.

Table 4.1: Compounds Isolated from *Cryptocarya nigra*

| **Without acid base extraction method (Dried Bark)** | **Acid – base extraction method (Dried Leaves)** |
|---|---|
| *N*-Methylisococlaurine **46** | Isocorydine **48** |
| Atherosperminine **11** | Norisocorydine **49** |
| 2-hydroxyathersperminine **20** | Argentinine **50** |
| Noratherosperminine **47** | Benzamide **51** |

All the structures of the alkaloids were established by spectroscopic analysis, particularly NMR experiments.

The *Cryptocarya* genus is rich in various types of alkaloids. For example, pavine alkaloids was found in *Cryptocarya chinensis* [21], phenanthrene alkaloids occured in *Cryptocarya crassinerva* [22] and benzylisoquinoline alkaloids in *Cryptocarya rugulosa* [96]. Based from this studies, it can be concluded that the major type of alkaloids occurred in *Cryptocarya nigra* and are benzylisoquinolines, with (+)-*N*-

methylisococlaurine **46** as the major compound. On top of that, there is another type of alkaloid, namely aporphine or phenanthrene types of alkaloids were found in the *Cryptocarya nigra*. These types of alkaloids have never been isolated from this plant.

At present, there is no report on the antiplasmodial and antioxidant properties of *Cryptocarya nigra.* Out of 8 compounds isolated from this study, three are alkaloids, (+)-*N*-methylisococlaurine **46,** atherosperminine **11** and 2-hydroxyatherosperminine **20** which mildly inhibited *in vitro* growth of a chloroquine sensitive strain of *Plasmodium falciparum* (K1 strain), with the highest inhibition shown by compound **20**. In addition, both compounds **46** and **11** exhibited mild antioxidant activity. Therefore it can be summarized that the compounds tested showed some activity on antiplasmodial and antioxidant thus could be natural lead compounds for development of potential drugs for antimalaria since antioxidants are said to be an additive therapy for antimalarial, however further mechanistic studies must be performed first. The antiplasmodial and antioxidant data suggested that the barks extract of *Cryptocarya nigra* is a potential source of new antiplasmodial natural lead compounds having antioxidant potential.

# CHAPTER 5

## EXPERIMENTAL

# CHAPTER 5

# EXPERIMENTAL

## 5.1 General Experimental Procedures

### 5.1.1 Solvents

The solvents used in this work were dichloromethane, methanol and hexane. All solvents are from AR grade except those that are used for folk extractions (distilled). Other chemicals were hydrochloric acid, ammonia solution and sodium sulphate anhydrous.

### 5.1.2 Instrumentation

The ultraviolet spectra were obtained in methanol (MeOH) on Shimazu UV-250. Meanwhile, the infrared spectra were obtained with methanol as solvent on a Perkin Elmer 16000 spectrometer. Mass spectra were obtained using Agilent Technologies 6530 accurate-mass e-TOF LC/MS, with a ZORBAX Eclipse XDB-CI8 rapid resolution HT 4.6 mm x 50 mm x 1.8 mm column.

The $^{1}H$, $^{13}C$ and 2D NMR spectra was recorded in deuterated chloroform using JEOL LA 400 FT NMR and JOEL ECA 400 FT NMR spectrometer system; chemical shift are reported in part per million (ppm) scales and the coupling constant are given in hertz (Hz).

### 5.1.3 Chromatography

Alumina supported silica gel 60 $F_{254}$ plates were used for visualized isolated compounds based on the spot of TLC (thin layer chromatography). The plates were activated at 100˚C for one hour and stored in a desiccator until needed. TLC spots were

visualized under ultra-violet light (254 and 365nm) using the model UVGL-58 followed by spraying with the Dragendorff's reagent.

Silica gel 604 $F_{254}$ was used for preparative TLC. Mayer's reagent and Dragendorff's reagent were used for alkaloid screening and alkaloids spotting.

### 5.1.4. Reagents

#### 5.1.4.1. Mayer's Reagent (potassium mercuric iodide)

Mercuric iodide (1.4 g) in distilled water (60 ml) mixed with potassium iodide (5.0 g) in water (10 ml). The mixture was then made up to 100 ml solution. A positive test result was indicated by the formation of white precipitation when the aqueous layer (acidified) is treated with 2-3 drops of Mayer's reagent.

#### 5.1.4.2. Dragendorff's reagent (potassium bismuth iodide)

Solution A: Bismuth (III) nitrate (0.85 g) in a mixture of glacial acetic acid (10 ml) and distilled water (40 ml)

Solution B: potassium iodide (8.0 g) in distilled water (200 ml)

Stock solution: a mixture of equal volumes of both solution A and B

Spray reagent: the stock solution (20 ml) was diluted in a mixture of acetic acid (20 ml) and distilled water (60 ml)

A positive Dragendorff's test was indicated by the formation of orange spots.

## 5.2. Plant Material

The leaves of *Cryptocarya nigra* were collected at Hutan Simpan Ulu Sat, Machang, Kelantan by the phytochemical group of the Chemistry Department, Faculty of Science, University of Malaya. Identification was made by Mr Teo Leong Eng (University Malaya). Voucher specimen (KL 5272) was deposited at the herbarium of Department of Chemistry, University of Malaya, Kuala Lumpur, Malaysia.

## 5.3. Extraction of *Cryptocarya nigra*

Plant extractions were carried out by cold extraction or exhaustive extraction using the soxhlet extractor, following the general procedure describe below.

In order to aggregate the nitrogen-containing compound in the plants, dried leaves of *Cryptocarya nigra* (1.9 kg) were soaked with hexane for 3 days. After that, the hexane were extracted and filtered and dried the sample under room temperature. The dried sample were then basified and moistened with 10% ammonia solution and left overnight. The materials were then extracted with dichloromethane ($CH_2Cl_2$) in a big flask for 3 days. To get more benefit, the materials were extracted by using soxhlet extractor successively checking for a Mayer's negative test after each extraction.

The dichloromethane extract of the leaves will be concentrated by using rotavapor to a volume of about 500 ml and were examine for alkaloid contents using thin layer chromatography and spotting with Dragendroff's reagent. The dicholoromethane extracts were then repeatedly extracted with a solution of 5% hydrochloric acid until Mayer's test is negative. The acidic aqueous were then basified with 10% ammonia solution until pH 11 and then re-extracted with dicholoromethane. The crude alkaloid fraction was obtained as a dark gummy residue after washing the

combined dichloromethane extracts with distilled water, drying over anhydrous sodium sulphate and evaporation under pressure.

While the other part of *Cryptocarya nigra* was not subjected to the normal acid-base extraction. In order to aggregate the alkaloids in the plants, 2.0 kg of dried bark of *Cryptocarya nigra* were soaked with hexane for 3 days to remove wax, chlorophyll, terpenoids and flavonoids. Sample was filtered and dried in room temperature. After the dried sample was basified with concentrated ammonia solution (25%) for two hours, the material was then extracted with dichloromethane in the big flask (cold extraction) for three days. The dichloromethane extract was dried under reduce pressure to get crude alkaloids in dark solid form.

**5.4. Isolation and purification**

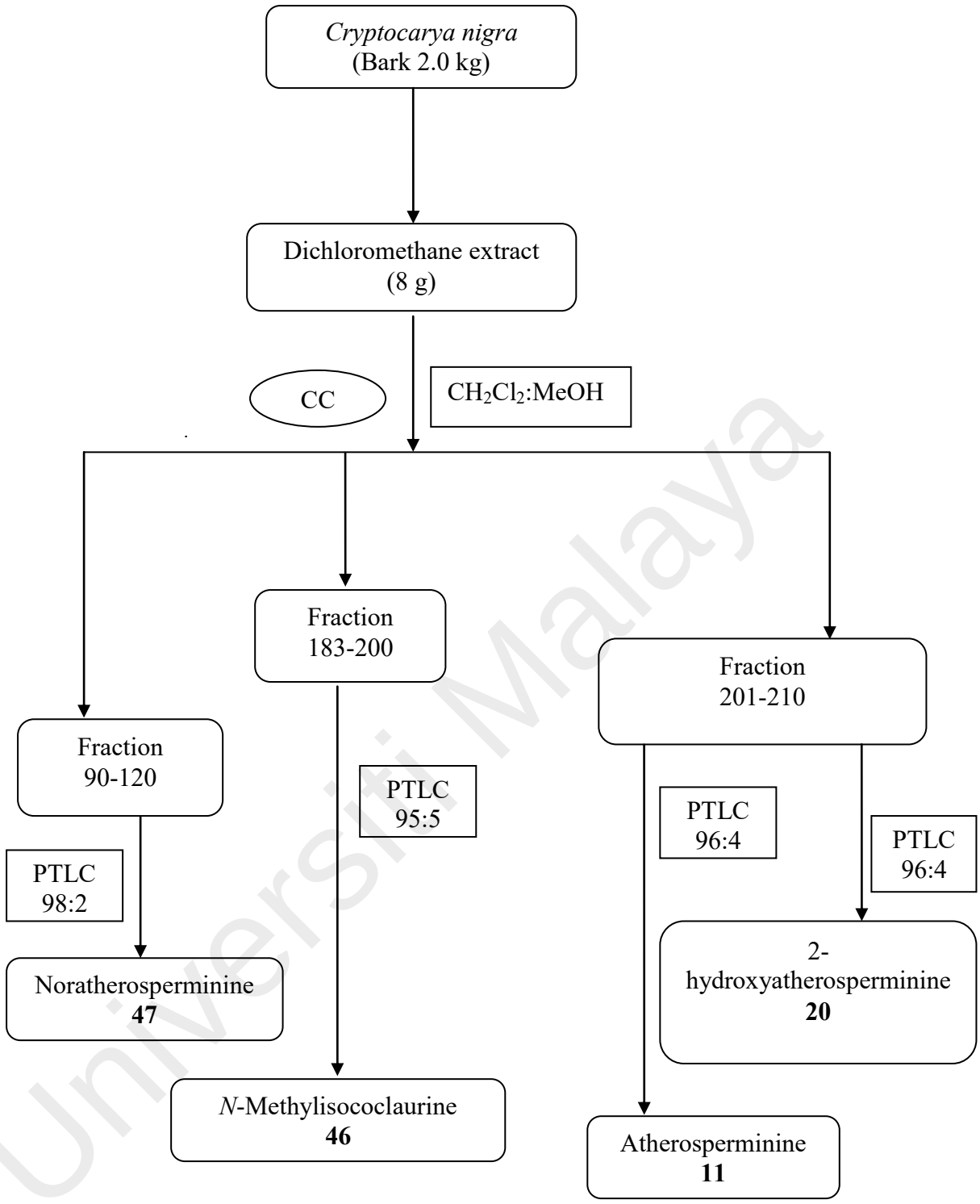
The crude alkaloid was subjected to column chromatography over silica gel 60 as stationary phase. The solvent system used for chromatography was dichloromethane with increasing portion of methanol (gradient elution system). The ratio of the solvent between $CH_2Cl_2$ and $CH_3OH$ were (100:0; 99:1; 98:2 96:4; 93:7; 90:10; 85:15; 80:10 and 50:50) and finally 100% MeOH. Fractions were collected every 100 ml and each fraction was tested with aluminium TLC plate for their alkaloids. The alkaloid spots were first detected by UV light (254 and 366 nm) and confirmed by spraying with Dragendroff's reagent. Fraction having spots with the same $R_f$ values and stains were combined and treated as a group. The combined groups were then treated separately to isolate and purify its alkaloid content either by extensive column chromatography or preparative TLC. The isolation and purification procedures were summarized in the Scheme 5.1 and 5.2

Table 5.1: Chromatography Results of the Chemical Constituents of *Cryptocarya nigra* (bark)

| Compounds | Solvent system ($CH_2Cl_2$: MeOH) | Weight/mg | % Yield |
|---|---|---|---|
| Noratherosperminine **47** | 98:2 | 1.5 mg | 1.8 % |
| Atherosperminine **11** | 96:4 | 15.0 mg | 18.8 % |
| 2-hydroxyathersperminine **20** | 96:4 | 5.0 mg | 6.3 % |
| *N*-Methylisococlaurine **46** | 95:5 | 25.0 mg | 31.3 % |

Table 5.2: Chromatography Results of the Chemical Constituents of *Cryptocarya nigra* (leave)

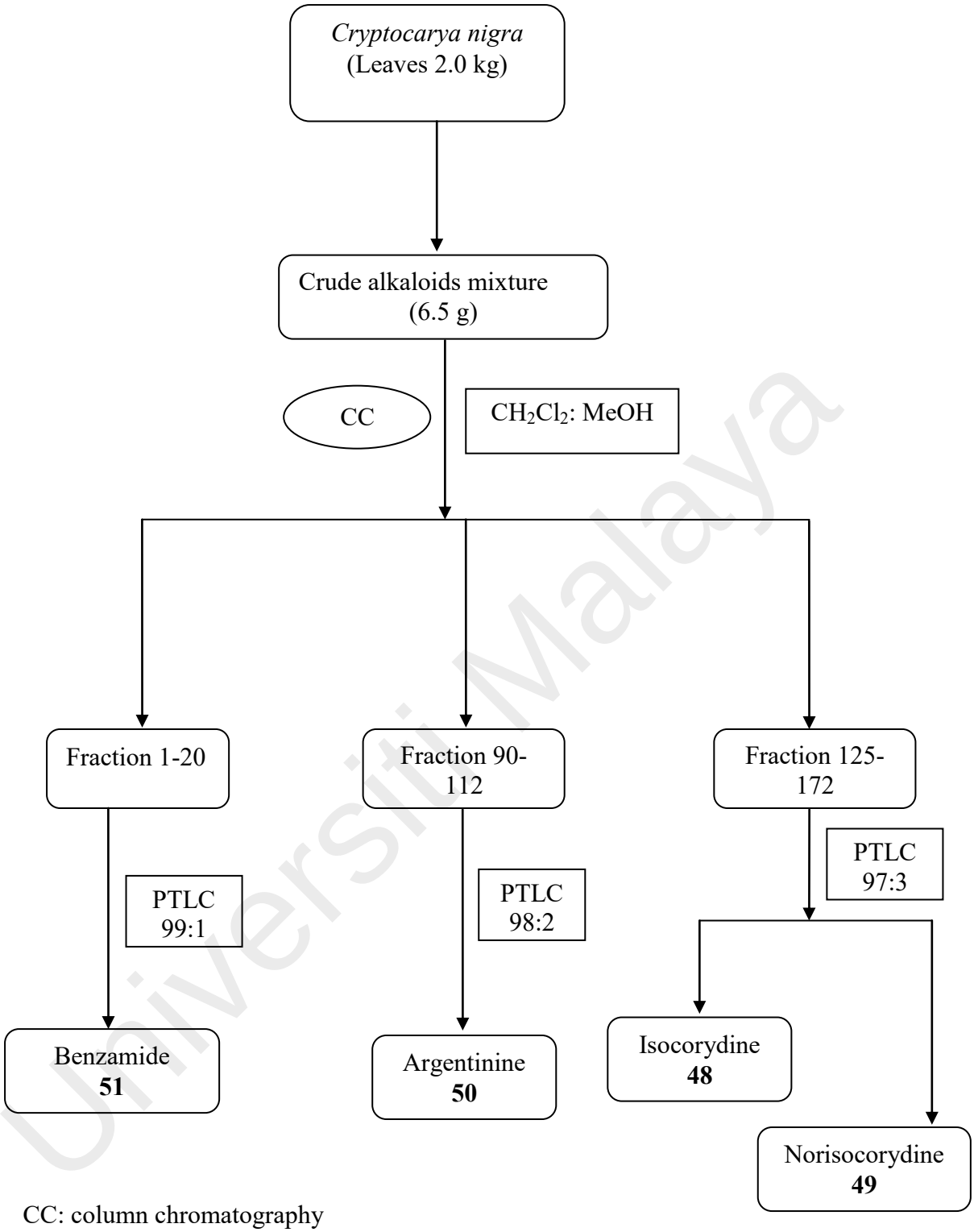
| Compounds | Solvent system ($CH_2Cl_2$: MeOH) | Weight/mg | % Yield |
|---|---|---|---|
| Benzamide **51** | 99:1 | 13.7 mg | 21.1 % |
| Argentinine **50** | 97:3 | 1.3 mg | 2.0 % |
| Isocorydine **48** | 94:6 | 5.3 mg | 8.2 % |
| Norisocorydine **49** | 94:6 | 8.8 mg | 13.5 % |




CC: column chromatography

PTLC: preparative thin layer chromatography

Scheme 5.1: Isolation and Purification of Alkaloids from the Bark of *Cryptocarya nigra.*




CC: column chromatography

PTLC: preparative thin layer chromatography

Scheme 5.2: Isolation and Purification of Alkaloids from the Leave of *Cryptocarya nigra*

## 5.5. Physical and Spectral Data of Isolated Compounds

### 5.5.1 *Cryptocaya nigra*

| | | |
|---|---|---|
| **N-Methylisococlaurine 46** | : | brownish amorphous solid |
| | : | $C_{18}H_{21}NO_3$ |
| $UV_{mx}$ (MeOH), nm | : | 301 nm |
| $IR\nu_{max}$ $cm^{-1}$ | : | 3266 and 2921 $cm^{-1}$ |
| Mass spectrum m/z | : | 300.16 |
| $[\alpha]_D^{27}$ | : | +29.4° |
| $^1H$ NMR ($CDCl_3$) δ, ppm | : | See Table 3.1 |
| $^{13}C$ NMR ($CDCl_3$) δ ppm | : | See Table 3.1 |

| | | |
|---|---|---|
| **Atherosperminine 11** | : | brownish amorphous solid |
| | : | $C_{20}H_{23}NO_2$ |
| $UV_{mx}$ (MeOH), nm | : | 217, 227, 256, 306 and 345 nm |
| $IR\nu_{max}$ $cm^{-1}$ | : | 1600 $cm^{-1}$ |
| Mass spectrum m/z | : | 310.18 |
| $^1H$ NMR ($CDCl_3$) δ, ppm | : | See Table 3.2 |
| $^{13}C$ NMR ($CDCl_3$) δ ppm | : | See Table 3.2 |

**2-hydroxyatherosperminine 20** : brownish amorphous solid

: $C_{20}H_{23}NO_3$

$UV_{mx}$ (MeOH), nm : 286 and 296 nm

$IR\nu_{max}$ $cm^{-1}$ : 3412 $cm^{-1}$

Mass spectrum m/z : 326.18

$^{1}H$ NMR ($CDCl_3$) δ, ppm : See Table 3.3

$^{13}C$ NMR ($CDCl_3$) δ ppm : See Table 3.3

**Noratherosperminine 47** : brownish amorphous solid

: $C_{19}H_{21}NO_2$

$UV_{mx}$ (MeOH), nm : 258, 308 and 347 nm

$IR\nu_{max}$ $cm^{-1}$ : 2917, 2849 and 3363 $cm^{-1}$

Mass spectrum m/z : 296.16

$^{1}H$ NMR ($CDCl_3$) δ, ppm : See Table 3.4

$^{13}C$ NMR ($CDCl_3$) δ ppm : See Table 3.4

**Isocorydine 48** : brownish amorphous powder

: $C_{20}H_{24}NO_4$

$UV_{mx}$ (MeOH), nm : 270 and 310 nm

$IR\nu_{max}$ $cm^{-1}$ : 3202 $cm^{-1}$

Mass spectrum m/z : 342.2

$[\alpha]_D^{25}$ : +196°

$^{1}H$ NMR ($CDCl_3$) δ, ppm : See Table 3.5

$^{13}C$ NMR ($CDCl_3$) δ ppm : See Table 3.5

**Norisocorydine 49** : brownish amorphous powder

: $C_{19}H_{22}NO_4$

$UV_{mx}$ (MeOH), nm : 270 and 310 nm

$IR\nu_{max}$ $cm^{-1}$ : 2936 and 3500 $cm^{-1}$

Mass spectrum m/z : 328.2

$[\alpha]_D^{25}$ : +158.5°

$^1H$ NMR ($CDCl_3$) δ, ppm : See Table 3.6

$^{13}C$ NMR ($CDCl_3$) δ ppm : See Table 3.6

**Argentinine 50** : brownish amorphous solid

: $C_{19}H_{21}NO_2$

$UV_{mx}$ (MeOH), nm : 245, 256, 291 and 345 nm

$IR\nu_{max}$ $cm^{-1}$ : 2922 and 3367 $cm^{-1}$

Mass spectrum m/z : 296

$^1H$ NMR ($CDCl_3$) δ, ppm : See Table 3.7

| | | |
|---|---|---|
| **Benzamide 51** | : | Crystalline needle |
| | : | $C_7H_7NO$ |
| Melting point | : | 125-127 ºC |
| $UV_{mx}$ (MeOH), nm | : | 294 and 280 nm |
| $IR\nu_{max}$ $cm^{-1}$ | : | 1646 and 3368 $cm^{-1}$ |
| Mass spectrum m/z | : | 121 |
| $^1H$ NMR ($CDCl_3$) δ, ppm | : | See Table 3.8 |
| $^{13}C$ NMR ($CDCl_3$) δ ppm | : | See Table 3.8 |

## 5.6. Experimental for Antioxidant Activities

### 5.6.1 DPPH Assay

The DPPH antioxidant assay was determined as described by Shimada *et al.* [116]. Briefly, 0.1 mM DPPH (1 mL) dissolved in ethanol was added to an ethanol solution (3 mL) of the tested compound at different concentrations (0, 50, 100, 150, 200 mg/mL). An equal volume of ethanol was added in the control test. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance at 517 nm was measured with a UV–VIS spectrophotometer. The percentage of scavenging of DPPH was calculated using the following equation:

DPPH scavenging effect (%) = (($A^o$ - A1) / $A^o$) x 100

Where $A_o$ is the absorbance of the control reaction and $A_1$ is the absorbance in the presence of the sample. Ascorbic acid (purity 99.0%) was purchased from Sigma used as the standard references.

### 5.6.2 Reducing Power Assay

The reducing power was determined using the method of Oyaizu, 1986 [117]. The tested compounds (0.5 mL) at different doses (0, 50, 100, 150, 200 mg/mL) were mixed with phosphate buffer (0.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (0.5 mL, 1%). The mixture was then incubated at 50°C for 20 min. A portion of trichloroacetic acid (0.5 mL, 10%) was added to the mixture, which was then centrifuged for 10 min at 3000 rpm (1000 g). The upper layer of solution (0.5 mL) was mixed with distilled water (0.5 mL) and $FeCl_3$ (0.1 mL, 0.1%) for 10 min, and then the absorbance was measured at 700 nm in a spectrophotometer. The EDTA (purity 99.0%) was purchased from Sigma and was used as the standard reference.

### 5.6.3 Metal Chelating Activity Assay

The chelation of ferrous ions was estimated according to the method of Dinis, et al. 1994 [118]. Briefly, 0.95 mL of tested compounds at different doses (0, 50, 100, 150, 200 mg/mL) were added to a solution of 50 µL $FeCl_2$ (2 mM). The reaction was initiated by the addition of 200 µL ferrozine (5 mM), and then the mixture was shaken vigorously and left standing at room temperature for 10 min. After equilibrium had been reached, absorbance of the solution was measured spectrophotometrically at 562 nm. Distilled water (200mL) instead of fferozine solution was used as a blank, which was used for error correction because of unequal colour of the sample solution. The percentage of inhibition of ferrozine-$Fe^{2+}$ complex (ferrous ion – chelating ability) of each sample was calculated according to the following formula:

$$\% \text{ inhibition} = [(Abs_{control} - Abs_{sample})/Abs_{standard}] \times 100$$

where, $Abs_{control}$ = absorbance reading of control

$Abs_{sample}$ = absorbance reading of sample

$Abs_{standard}$ = absorbance reading of standard reference

The IC50 value was determined from the graph of inhibition againts concerntration. BHA (purity 96.0%) was purchased from Acros organic and was used as the standard reference.

## 5.7. Experimental for Antiplasmodial Activities

The dichloromethane extract and the isolated compounds were assayed for *in vitro* antiplasmodial activity against the *Plasmodium falciparum*, K1 isolate (resistance strain). Briefly the methodology involves Malaria culture as discussed by Trager and Jensen, (1976) with some modification. The synchronization of the malaria culture to one stage is according to Lambros C and Vanderberg P., 1979. Chloroquine and artermisine were used as positive control**.** The antiplasmodial activity of each compound was expressed as an $IC_{50}$ value, defined as the concentration of the compound causing 50% inhibition of parasite growth relative to an untreated control.[107]

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# APPENDIX

## APPENDIX

Article in Proceedings/Presented at Conferences/Seminar and Publication

1. **Ayu Afiqah Nasrullah**, Azeana Zahari, Jamaludin Mohamad, Khalijah Awang. Antiplasmodial Alkaloids from the Bark of *Cryptocarya nigra* (Lauraceae). Molecules (2013), **18** (7), 8009-8017.

2. Alkaloids Isolated From *Cryptocarya nigra* (Lauraceae). **Ayu Afiqah Nasrullah**, Mat Ropi Mukhtar, Khalijah Awang, and A. Hamid A. Hadi. 7th Mathematics and Physical Science Graduate Congress (12-14 Dec 2011). Faculty of Science, National University of Singapore.

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*Article*

# Antiplasmodial Alkaloids from the Bark of *Cryptocarya nigra* (Lauraceae)

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**Abstract:** A dichloromethane extract of the stem bark of *Cryptocarya nigra* showed strong *in vitro* inhibition of *Plasmodium falciparum* growth, with an $IC_{50}$ value of 2.82 µg/mL. The phytochemical study of this extract has led to the isolation and characterization of four known alkaloids: (+)-*N*-methylisococlaurine (**1**), atherosperminine (**2**), 2-hydroxyathersperminine (**3**), and noratherosperminine (**4**). Structural elucidation of all alkaloids was accomplished by means of high field 1D- and 2D-NMR, IR, UV and LCMS spectral data. The isolated extract constituents (+)-*N*-methylisococlaurine (**1**), atherosperminine (**2**) and 2-hydroxy-atherosperminine (**3**) showed strong antiplasmodial activity, with $IC_{50}$ values of 5.40, 5.80 and 0.75 µM, respectively. In addition, (+)-*N*-methylisocolaurine (**1**) and atherosperminine (**2**) showed high antioxidant activity in a DPPH assay with $IC_{50}$ values of 29.56 ug/mL and 54.53 ug/mL respectively.

Compounds **1** and **2** also both showed high antioxidant activity in the FRAP assay, with percentages of 78.54 and 70.66 respectively and in the metal chelating assay, with $IC_{50}$ values of 50.08 ug/mL and 42.87 ug/mL, respectively.

**Keywords:** *Plasmodium falciparum*; *Cryptocarya nigra*; Lauraceae; antioxidant; antiplasmodial; activity

## 1. Introduction

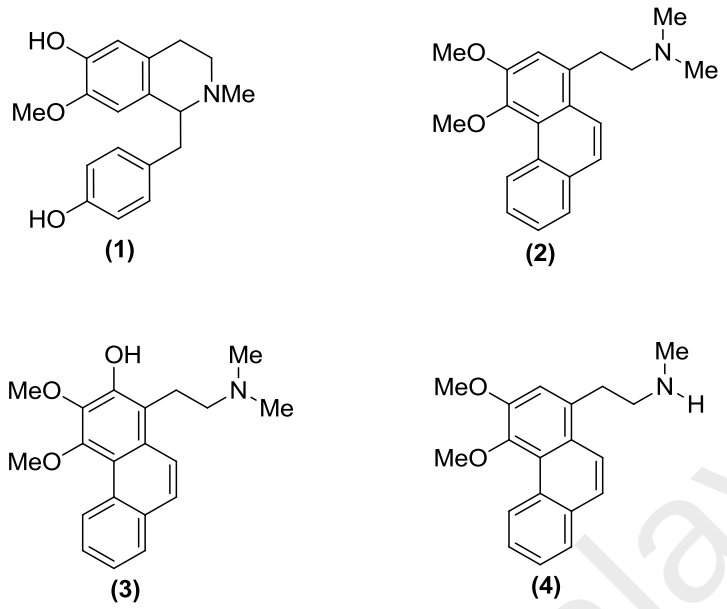
Malaria is caused by five species of parasites from the genus *Plasmodium*: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, *P. knowlesi* and *P. falciparum* is the most deadly form that affects humans [1]. According to the latest data from the WHO, the estimated incidence of malaria has remained unacceptably high. There were about 219 million cases of malaria in 2010 and an estimated 660,000 deaths [2]. A major factor in the continuing burden of malaria is the spread of parasites resistant to front-line anti-malarials such as chloroquine and sulphadoxine/ pyrimethamine, and artemisinin [3].

Malaysia, being host of one of the oldest and richest forest ecosystems in the World with a reported more than 15,000 plant species is an excellent source of natural bioactive compounds [4]. In our continuing efforts to search for new antiplasmodial agents [5–10], we report herein our phytochemical studies on *Cryptocarya nigra* (Lauraceae), whose dichloromethane extract showed potent antiplasmodial activity with an $IC_{50}$ value 2.82 µg/mL.

This plant is widely distributed in Peninsular Malaysia, Sumatra and Borneo, and it is locally known as '*Medang*' [11]. The genus *Cryptocarya* belonged to the family Lauraceae which comprises of more than 350 species of which 19 species are found in Malaysia [12,13]. This species is a medium sized tree that grows up to 10 m tall. Based on previously reported phytochemical and pharmacological studies, the genus *Cryptocarya* are known to be prolific producers of flavonoids, chalcones, lactones, α-pyrones and mainly alkaloids [14–24] with varied biological activities. A preliminary study on the leaves of the *Cryptocarya nigra* reported the presence of coumarins and flavonoids and their cytotoxic activity against murine leukemia P-388 cells [25]. However, no study has been reported on the alkaloidal content, antiplasmodial and antioxidant activities of *Cryptocarya nigra* extracts

The phytochemical study on the stem bark of *Cryptocarya nigra* has now led to the isolation of four known alkaloids; (+)-*N*-methylisococlaurine (**1**) [26], atherosperminine (**2**) [27], 2-hydroxyathersperminine
(**3**) [24], and noratherosperminine (**4**) [28] (Figure 1). Alkaloids **1**–**3** were evaluated for their antiplasmodial activity and alkaloids **1** and **2** were also subjected to antioxidant assays.

**Figure 1.** Structures of compounds **1**–**4**.




## 2. Results and Discussion

Extensive chromatographic analysis of the dicholoromethane extract (DCE) of the bark of *Cryptocarya nigra* afforded one benzylisoquinoline alkaloid, (+)-*N*-methylisococlaurine (**1**), and three phenanthrene alkaloids: atherosperminine (**2**), 2-hydroxyathersperminine (**3**), and noratherosperminine (**4**). The structure of each compound was characterized by analysis of its spectroscopic data and by comparison of its NMR, IR, UV and MS data with literature values [24,26–28].

Phenanthrene alkaloids are derivatives of phenanthrene with a 1-(2-aminoethyl) side chain. They are a very rare type also known as "seco-aporphine" alkaloids, probably formed biogenetically from an aporphine precursor through opening of ring B [29]. Phenanthrene alkaloids have been reported to exhibit antimicrobial activity, cytotoxicity, and dopamine receptor stimulation effects [27,30,31].

### *2.1. Antiplasmodial Activity*

The compounds isolated from the bark of *Cryptocarya nigra* were tested for antiplasmodial activity based on the promising screening results of the DCE ($IC_{50}$ = 2.82 µg/mL). The *in vitro* antiplasmodial activity of compounds isolated from *Cryptocarya nigra* against a chloroquine resistant strain of *P. falciparum* (K1 strain) is summarized in Table 1. Compound **3** displayed the strongest inhibition activity, with an $IC_{50}$ value of 0.75 µM, followed by compound **1** and compound **2** with $IC_{50}$ values of 5.40 µM and 5.80 µM, respectively. These alkaloids have no previous record of any antiplasmodial activity.

**Table 1.** *In vitro* Antiplasmodial Activities of Alkaloids from *Cryptocarya nigra*.

| Sample | Antiplasmodial Activity * The % of growth inhibition or $IC_{50}$ | |
|---|---|---|
| | μg/mL | μM |
| **1** | 1.62 | 5.40 |
| **2** | 1.80 | 5.80 |
| **3** | 0.25 | 0.75 |
| **4** | nt † | nt † |
| Chloroquine | 90.39 ± 28.85 ** | |
| Artemisinin | 2.42 ± 1.06 ** | |

* Results are recorded as the % of growth inhibition or $IC_{50}$. Notes: nt †—not tested; ** unit in nM.

*2.2. Antioxidant Activity*

Compound **1**, a benzylisoquinoline, showed high scavenging activity towards the free radical DPPH at $IC_{50}$ = 29.56 μg/mL and high reducing power at 78.54% as compared to compound **2** (Table 2). However compound **2** showed higher metal chelating activity than compound **1** at $IC_{50}$ = 42.87 ug/mL and $IC_{50}$ 50.08 ug/mL, respectively. The high DPPH and FRAP antioxidant activity of compound **1** may be due to its hydroxyl group that could donate an electron to free radicals and possesses the ability to chelate metals. In addition, Jee reported that the phenanthrenes have shown an effect on glutathione reductase that increases antioxidant activity in the fish *Paralichthys olivaceus* (olive flounder) [32]. Thus, compound **1** and compound **2** are good reductants with the ability to chelate metals and presented pro-oxidant activity.

**Table 2.** Antioxidant Activities of Alkaloids from *Cryptocarya nigra*.

| Compound Name | $IC_{50}$ DPPH Activity (ug/mL) | % FRAP | $IC_{50}$ Metal Chelating Activity (ug/mL) |
|---|---|---|---|
| *N*-Methylisococlaurine (**1**) | 29.56 | 78.54 | 50.08 |
| Atherosperminine (**2**) | 54.53 | 70.66 | 42.87 |
| Ascorbic acid (Standard) | 13.69 | | |
| EDTA (Standard) | | 83.74 | |
| BHA (Standard) | | | 19.60 |

The results showed that compounds **1** and **2** exhibited both iron binding and antiplasmodial activity. This suggests that there is iron requirement in host-parasite interactions [33,34] for oxygen transport, respiration and enzymes activities. These results indicate that there may be a correlation between antiplamodial activities and iron chelating in metal chelating [35]. Thus, metal chelating ability has potential in antimalarial treatment. The mechanism(s) of action of iron chelators are still not known and further investigation must be carried out on compounds **1** and **2** in order to elucidate their metal chelating mechanism and their potential as antimalarial agents. The antioxidant effect might play a major role in

inhibiting the actions of the parasites, but it may also stimulate the immune system of malarial victims [36], therefore being a good candidate for antimalarial therapy.

According to Reis *et al.*, the combination of chloroquine and two antioxidant agents, prevented both inflammatory and vascular changes in the tissues of the brain at the first signs of cerebral malaria, as well as the development of persistent cognitive damage. The addition of antioxidants did not diminish the efficacy of chloroquine in eliminating *Plasmodia* from the blood. The authors suggested that these antioxidant drugs should be studied as an additive therapy for antimalarial drugs in clinical trials in order to investigate their potential to reduce or prevent cognitive damage after cerebral malaria [37]. Additionally, recent studies by Percario *et al.* on oxidative stress in malaria [38] have suggested that the use of antioxidant supplements of synthetic or natural origin may constitute a far more effective adjuvant antimalarial strategy that causes less damage to the host.

## 3. Experimental

### *3.1. General*

Spectra were recorded on the following instruments: UV: Shimadzu UV-250 UV-Visible spectrophotometer; IR: Perkin Elmer 1600; NMR: JEOL ECA 400 MHz; LCMS-IT-TOF: Shimadzu. All solvents, except those used for bulk extraction were AR grade. Silica gel 60 F254 was used for column chromatography. Glass and aluminum-supported silica gel 60 F254 plates were used for preparative TLC. TLC spots were visualized under UV light (254 and 365 nm) followed by spraying with Dragendorff's reagent for alkaloid detection.

### *3.2. Plant Material*

The bark of *Cryptocarya nigra* was collected at Hutan Simpan Ulu Sat, Machang, Kelantan (Malaysia) by the phytochemical group of the Department of Chemistry, Faculty of Science, University of Malaya. A voucher specimen (KL5272) has been deposited at the Herbarium of the Department of Chemistry, University Malaya, Kuala Lumpur, Malaysia.

### *3.3. Extraction and Isolation*

The air-dried ground bark of the plant (2.0 kg) was first exhaustively extracted twice with hexane (14 L) for a 3-day period. The hexane extracts were combined and the solvent evaporated. The dried plant material were then made alkaline and moistened with 25% $NH_4OH$ (1 L) for 2 h. It was then macerated with $CH_2Cl_2$ (14 L) twice for a 3-day period. After filtration, the supernatant obtained was concentrated under reduced pressure using a rotary evaporator to a volume of 500 mL and tested for alkaloid content (using TLC and confirmed by spraying with Dragendorff's reagent). The extract was finally concentrated and dried to give 15.0 g of extract designated as the DCE. The crude alkaloid extract (8.0 g) was subjected to column chromatography over silica gel using $CH_2Cl_2$ and methanol mixtures (100:0, 99:1, 98:2, 97:3, 96:4, 95:5, 90:10, 80:20, and 70:30) and finally 100% methanol as

eluent to obtain twenty fractions. Further purification of fraction 16 by Preparative Thin Layer Chromatography (PTLC) yielded major alkaloid **1** (25.0 mg, MeOH-$CH_2Cl_2$; 95:5: saturated with $NH_4OH$). In addition, known compounds **2** (15.0 mg, MeOH-$CH_2Cl_2$; 96:4: saturated with $NH_4OH$), **3** (5.0 mg, MeOH-$CH_2Cl_2$; 96:4: saturated with $NH_4OH$), and **4** (1.5 mg, MeOH-$CH_2Cl_2$; 98:2: saturated with $NH_4OH$) were obtained after purification of fraction 17.

*3.4. Determination of Antiplasmodial Activity*

The samples were sent to the Institute for Medical Research, Kuala Lumpur (IMR) for antiplasmodial screening. DCE and the isolated compounds were assayed for *in vitro* antiplasmodial activity against the *Plasmodium falciparum*, K1 isolate (resistant strain). Briefly the methodology involves malaria culture as discussed by Trager and Jensen [39] with some modifications. The synchronization of the malaria culture to one stage is according to Lambros and Vandernerg [40]. Chloroquine and artemisinin were used as positive controls. The antiplasmodial activity of each compound was expressed as an $IC_{50}$ value, defined as the concentration of the compound causing 50% inhibition of parasite growth relative to an untreated control.

*3.5. Determination of Antioxidant Assay*

3.5.1. DPPH Assay

The DPPH antioxidant assay was determined as described by Shimada *et al.* [41]. Briefly, 0.1 mM DPPH (1 mL) dissolved in ethanol was added to an ethanol solution (3 mL) of the tested compound at different concentrations (0, 50, 100, 150, 200 µg/mL). An equal volume of ethanol was added in the control test. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance at 517 nm was measured with a UV–VIS spectrophotometer. The percentage of scavenging of DPPH was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = \frac{A^{\circ} - A1}{A^{\circ}} \times 100 \quad (1)$$

where $A^{\circ}$ is the absorbance of the control reaction and A1 is the absorbance in the presence of
the sample.

3.5.2. Ferric Reducing Power Assay (FRAP)

The reducing power was determined using the method of Oyaizu [42]. The tested compounds
(0.5 mL) dissolved in ethanol at different doses (0, 50, 100, 150, 200 µg/mL) were mixed with phosphate buffer (0.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [K3Fe(CN)6] (0.5 mL, 1%). The mixture was then incubated at 50 °C for 20 min. A portion of trichloroacetic

acid (0.5 mL, 10%) was added to the mixture, which was then centrifuged for 10 min at 3000 rpm (1000 g). The upper layer of solution (0.5 mL) was mixed with distilled water (0.5 mL) and $FeCl_3$ (0.1 mL, 0.1%) for 10 min, and then the absorbance was measured at 700 nm in a spectrophotometer. EDTA at different concentrations (2.5 µg/mL to 40 µg/mL) was used as standard reference. The reducing power activity was expressed as percentage of absorbance compared with EDTA.

3.5.3. Metal Chelating Activity Assay

The chelation of ferrous ions was estimated according to the method of Dinis *et al.* [43]. Briefly, tested compounds dissolved in ethanol (0.95 mL, at different doses of 0, 50, 100, 150, 200 µg/mL) were added to a solution of $FeCl_2$ (2 mM, 50 µL). The reaction was initiated by the addition of ferrozine (5 mM, 200 µL), and then the mixture was shaken vigorously and left standing at room temperature for 10 min. After equilibrium had been reached, absorbance of the solution was measured spectrophotometrically at 562 nm. The percentage of inhibition of ferrozine-$Fe^{2+}$ complex of each sample was calculated according to the following formula:

$$\% \text{ inhibition} = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100 \quad (2)$$

where, $Abs_{control}$ = absorbance reading of control and $Abs_{sample}$ = absorbance reading of sample. The $IC_{50}$ value was determined from the graph of percentage inhibition against concentration.

## 4. Conclusion

A phytochemical study on dried bark of *Cryptocarya nigra* afforded four known alkaloids **1**–**4**.

The alkaloids (+)-*N*-methylisococlaurine (**1**), atherosperminine (**2**) and 2-hydroxyatherosperminine (**3**) strongly inhibited *in vitro* growth of a chloroquine resistant strain of *Plasmodium falciparum* (K1 strain), with the strongest inhibition shown by compound **3**. In addition, both compounds **1** and **2** exhibited good antioxidant activity, therefore these compounds could be potential candidates as antimalarial agents since antioxidants are said to be an additive therapy for malaria. The antiplasmodial and antioxidant data suggested that the bark extract of *Cryptocarya nigra* is a potential source of new antiplasmodial agents having antioxidant potential.

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## Conflict of Interest

The authors declare no conflict of interest.

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