VOLATILE COMPOUNDS AND ALKALOIDS FROM THE AQUEOUS EXTRACT OF *MITRAGYNA SPECIOSA* AND THEIR *IN VITRO* AND *IN VIVO* ANTI-INFLAMMATORY STUDIES

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ABSTRACT

This work involved the aqueous extraction, supercritical fluid extraction (SFE) and hydro distillation extraction. Five compounds were isolated from the water extract and purified by using several chromatography techniques such as column chromatography (CC), thin layer chromatography (TLC) and preparative thin layer chromatography (PTLC). These compounds include mitragynine, speciociliatine, paynantheine, 3, 4, 5, 6-tetradehydromitragynine and 7-hydroxyspeciociliatine. The structures of these isolated compounds were elucidated with the aid of spectroscopic methods such as 1D NMR (^{1}H, ^{13}C, DEPT), 2D NMR (COSY, HMBC, HMQC/HSQC, NOESY), UV, IR, ESIMS/LCMS-IT-TOF and their data were compared with those in the literature. Fifteen compounds constituting 93.53% of the most active supercritical fluid (SCF) extract on nitric oxide (NO) inhibition have been identified. The prominent components were fatty acids, accounting for 39.01% of the total yield. The rest of the crude was made up of four hydrocarbons (36.67%), three phytosterols (9.32%), two esters (3.45%), two alcohols (2.30%) and a tocochromanol (2.78%). Palmitic acid (34.90%) was the most abundant compound followed by heptacosane (18.56%) and nonacosane (11.00%). Thirty-nine identifiable constituents of the essential oil that represented 85.29 ± 2.26% of the total peak area have been successfully characterized. Isophytol (23.51 ± 4.19%), (*E*)-phytol (9.17 ± 2.52%) and (2*E*, 6*E*)-farnesyl acetate (8.96 ± 2.54%) were among the major compounds. SFE extracts and the essential oil were analysed by means of gas chromatography and gas chromatography-mass spectrometry. The components of the extracts were identified by comparison using the NIST08s.LIB mass spectral database and their identifications were further confirmed through comparison with Kovats indices (*I*). The SFE and the aqueous leaves extracts were investigated in parallel with other solvent extracts for *in vitro* (NO inhibitory test) anti-inflammatory activity. M5S1 (the non-alkaloidal SFE crude) showed the highest NO inhibitory activity at 60.08 ± 10.02% without any cytotoxicity effect (cell viability, 91.98 ± 11.58%). Parameters for the extraction of M5S1 (extracted using pure SCF-CO_2 at 3000 psi of pressure and temperature at 60^{o}C) are determined as the optimal SFE condition to furnish the extract that possesses the highest anti-inflammatory activity without any toxicity. This finding provides the first evidence that the non-alkaloidal extract of the leaves of *M. speciosa* that possesses NO inhibitory potential. The aqueous extract of the leaves of *M. speciosa*, MSEA was further investigated for its *in vivo* (anti-ulcer test) anti-inflammatory activity. In comparison to ulcer control group, the animals pre-treated with MSAE showed significant reduction in gastric injuries grossly and histology. The gastric homogenate of the animals pre-treated with MSAE showed considerable decrease in the malondialdehyde (MDA) level, increased superoxide dismutase (SOD) activity and also protein concentration. Immunohistochemistry of these trial groups showed over-expression of heat-shock protein 70 (HSP70) and down-expression of pro-apoptotic BAX protein. In addition, the MSAE treated animals revealed significant increment in the pH of the stomach content. Acute toxicity study of the aqueous extract was safe up to 1000 mg/kg. This research provided first gastric protecting evidence of *M. speciosa* aqueous leaf extract as evaluated by biochemistry, histology and immunohistochemistry studies. Results of the present study indicate that both extracts, M5S1 and MSAE have potential property in preventing inflammatory diseases.

ABSTRAK

Dalam karya ini, kami memberi tumpuan kepada pengekstrakan akues, pengekstrakan cecair genting lampau (SFE) dan penyulingan hidro. Lima sebatian telah dipencilkan daripada ekstrak akues dan ditulenkan dengan menggunakan beberapa teknik kromatografi seperti kromatografi turus (CC), kromatografi lapisan nipis (TLC) dan kromatografi lapisan nipis persediaan (PTLC). Sebatian-sebatiannya termasuk mitraginina, spesiosiliatina, painanthina, 3, 4, 5, 6-tetradehidromitraginina dan 7-hidroksispeciociliatine. Struktur sebatian-sebatian terpencil telah ditentukan dengan bantuan kaedah spektroskopi seperti 1D NMR (^{1}H, ^{13}C, DEPT), 2D NMR (COSY, HMBC, HMQC/HSQC, NOESY), UV, IR, ESIMS/LCMS-IT TOF dan perbandingan dengan kajian-kajian terdahulu. Lima belas sebatian yang membentuk 93.53% daripada ekstrak SCF yang paling aktif dalam perencatan nitrik oksida (NO) telah dikenal pasti. Komponen utama ialah asid lemak, menyumbang 39.01% daripada jumlah hasil. Selebihnya terdiri daripada empat hidrokarbon (36.67%), tiga fitosterol (9.32%), dua ester (3.45%), dua alkohol (2.30%) dan satu tokokromanol (2.78%). Asid palmitik (34.90%) merupakan sebatian yang paling banyak diikuti oleh heptakosana (18.56%) dan nonakosana (11.00%). Tiga puluh sembilan juzuk yang boleh dikenal pasti daripada minyak pati yang mewakili 85.29 ± 2.26% daripada jumlah kawasan puncak telah berjaya dicirikan. Isofitol (23.51 ± 4.19%), (*E*)-fitol (9.17 ± 2.52%) dan (2*E*,6*E*)-farnesil asetat (8.96 ± 2.54%) adalah antara sebatian utama. Ekstrak SFE dan minyak pati yang diperolehi dianalisa dengan menggunakan kromatografi gas dan gas kromatografi-spektrometri jisim. Komponen-komponen ekstrak telah dikenal pasti melalui perbandingan menggunakan NIST08s. LIB pangkalan data spektra jisim dan pengesahan selanjutnya melalui perbandingan indeks Kovats (*I*). Ekstrak daun SFE dan akues disiasat secara selari dengan ekstrak-ekstrak pelarut lain untuk aktiviti anti-radang *in vitro* (ujian perencatan NO). M5S1 (ekstrak mentah SFE bukan alkaloidal) menunjukkan aktiviti perencatan NO tertinggi pada 60.08 ± 10.02% tanpa sebarang kesan sitotoksisiti (daya maju sel, 91.98 ± 11.58%). Parameter untuk pengekstrakan M5S1 (diekstrak menggunakan SCF-CO_2 tulen pada tekanan 3000 psi dan suhu pada 60 ^{o}C) ditentukan sebagai keadaan optimum SFE untuk memberikan ekstrak yang memiliki aktiviti anti-radang yang paling tinggi tanpa sebarang kesan toksik. Penemuan ini menyediakan bukti pertama bahawa ekstrak bukan alkaloidal daripada daun *M. speciosa* mempunyai potensi sebagai perencat NO. Ekstrak akues daun *M. speciosa*, MSEA seterusnya disiasat untuk aktiviti anti-radang *in vivo* (ujian anti-ulser). Berbanding dengan kumpulan kawalan ulser, haiwan pra-dirawat dengan MSAE menunjukkan pengurangan ketara dalam kecederaan perut terlalu dan histologi. Homogenat gastrik haiwan pra-dirawat dengan MSAE menunjukkan penurunan besar dalam tahap malondialdehid (MDA), peningkatan aktiviti superoxide dismutase (SOD) dan juga kepekatan protein. Immunohistokimia kumpulan percubaan ini menunjukkan lebih-ungkapan protein haba kejutan 70 (HSP70) dan peturun-ungkapan protein pro-apoptotik BAX. Di samping itu, haiwan yang dirawat dengan MSAE mendedahkan kenaikan ketara dalam pH kandungan gastrik. Kajian ketoksikan akut ekstrak akuas selamat sehingga 1000 mg/kg. Kajian ini menyediakan bukti pertama pelindungan gastrik oleh ekstrak akues daun *M. speciosa* seperti dinilai oleh kajian biokimia, histologi dan immunohistokimia. Keputusan kajian ini menunjukkan bahawa kedua-dua ekstrak, M5S1 dan MSAE mempunyai potensi dalam mencegah penyakit keradangan.

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Last but not least, this thesis is dedicated to the memory of my late father, Tohar bin Mohd. Apandi. This is for you, 'Abah'.

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List of Symbols and Abbreviations

α	Alpha
β	Beta
λ	Lambda
μ	Micro
δ	Chemical Shift
s	Singlet
d	Doublet
dd	Doublet of Doublet
m	Multiplet
t	Triplet
^{1}H	NMR Proton Nuclear Magnetic Resonance
^{13}C NMR	Carbon-13 Nuclear Magnetic Resonance
DCM	Dichloromethane
EtOAc	Ethyl Acetate
g	Gram
mg	Milligram
mL	Millilitre
m/z	Mass to Charge Ratio
nm	Nanometre
IC_{50}	Concentration Needed for Inhibition of 50% Activity
J	Homonuclear or Heteronuclear Coupling
Hz	Hertz
ppm	Parts Per Million
CH_2Cl_2	Chloroform
COSY	Correlation Spectroscopy
DEPT	Distortionless Enhancement by Polarization Transfer
HMBC	Heteronuclear Multiple Quantum Coherence
HPLC	High Performance Liquid Chromatography
HRESIMS	High-Resolution Electron Spray Ionization Mass Spectroscopy
HRFABMS	High-Resolution Fast Atom Bombardment Mass Spectroscopy
HSQC	Heteronuclear Single Quantum Coherence
IR	Infrared Spectroscopy
NMR	Nuclear Magnetic Resonance

NOESY	Nuclear Overhauser Effect Spectroscopy
PTLC	Preparative Thin Layer Chromatography
TLC	Thin Layer Chromatography
UV	Ultraviolet Spectroscopy
$\Delta^{x,y}$	Double Bond between C_x and C_y

CHAPTER 1: INTRODUCTION

Natural products, such as plants extract, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug discoveries because of the unmatched availability of chemical diversity. According to the World Health Organization (WHO), more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. The use of herbal medicines in Asia represents a long history of human interactions with the environment. Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases. Due to the development of adverse effects and microbial resistance to the chemically synthesized drugs, men turned to ethno pharmacognosy. They found literally thousands of phytochemicals from plants as safe and broadly effective alternatives with less adverse effect. Many beneficial biological activities such as anti-cancer, anti-microbial, anti-oxidant, anti-diarrheal, analgesic and wound healing activity were reported [1]. In many cases the people claim the good benefit of certain natural or herbal products. However, scientific studies are necessary to demonstrate the effectiveness of these products as to verify this traditional claim.

1.1 Medicinal Plants: Plant Extracts versus Isolated Active Constituents

The current scenario of drugs used in the treatment of diseases owes most of its part to medicinal plants, where ethno medicine and *in vitro* biological screening has always been a path to direct scientists for the search for new lead compounds. Many phytopharmaceuticals in the market are crude extracts and thus complex mixtures of compounds. Investigation of pharmacological activity on single isolated compounds versus the original plant extracts exerts polyvalent pharmacological effects. This might explain the pharmacological synergistic effects and the phenomenon that very often an

extract possesses a much better therapeutic effect than single isolated constituent. In consequence, the application of refined herbal extract rather than "isolated active principle(s)" may be favoured in order to reap benefit from the broad therapeutically and pharmacological action related to the special composition of the ingredients in the entire plant.

Clinical trials directed towards understanding of pharmacokinetics, bioavailability, efficacy, safety and drug interactions of these natural or herbal products and their formulations (extracts) require a careful evaluation. According to the World Health Organization (WHO), nearly 20,000 medicinal plants exist in 91 countries including 12 mega biodiversity countries [2].

1.2 From Traditional Preparation to Advance Technology

In traditional herbal medicine systems, herbal remedies are prepared in several rather standardized ways that usually vary based upon the plant utilized, and sometimes, what condition is being treated. Some of these methods include: infusions (hot teas), decoctions (boiled teas), tinctures (alcohol and water extracts) and macerations (cold-soaking). Others include preparing plants in hot baths (in which the patient is soaked in it or bathed with it), inhalation of powdered plants (like snuff), steam inhalation of various aromatic plants boiled in hot water and even aromatherapy. The well-trained herbalist will always thoroughly review the time-honoured method in which a plant has been traditionally prepared, it holds important information for preparing an effective herbal remedy.

The biological or therapeutic activity of a medicinal plant is closely related to the content of the plant chemicals. These chemicals can be classified into major groups of chemicals such as essential oils, alkaloids, acids, steroids, tannins, saponins and so

forth. Each one of these classes of chemicals may have preferred effective methods of extraction, which facilitates getting the chemicals out of the plant and into the herbal remedy that is being prepared. Most herbal remedies are in the form of water extract. Another extraction technique that is popular is the decoctions.

Supercritical Fluid Extraction (SFE) extracts chemical compounds by using CO_2 in its supercritical state in place of conventional organic solvents. The result is that the extract leaves no residual solvent, superior purity and yield, and lower operating costs as compared to traditional hydrocarbon-based solvent extraction systems. SFE provides a faster, safer and cleaner technology for analysts from the food, natural product, flavor, fragrance, pharmaceutical, nutraceutical, polymer and chemical industries. The supercritical fluid state occurs when a fluid is above its critical temperature and critical pressure, when it is between the typical gas and liquid phase. This supercritical phase allows CO_2 to take the properties of a gas (high diffusivity, low surface tension), as well as maintaining the solvating power of a liquid. Manipulating the temperature and pressure of the fluid influences its density, which can solubilize the material of interest that can be selectively extracted. The sample is placed in an extraction vessel and pressurized with CO_2 to dissolve the sample. Transferred to a fraction collector, the contents are depressurized and the CO_2 loses its solvating power causing the desired material to precipitate. The evaporated CO_2 can be recycled. Although the selectivity benefits and the cost-effectiveness of the technique have become widely recognized, one of the most tedious steps in SFE method is developing the optimum conditions (pressure, temperature, flow composition and co-solvent).

1.3 Inflammation: The Two Edged Sword

Inflammation is a wellness buzzword these days as it is the culprit behind all diseases.

Generally speaking, controlled inflammation is a beneficial response that can defend and protect the body from harmful factors but if the body's regulation of inflammation is dysfunctional, then inflammation will have an adverse effect on the body, such as the emergence of chronic inflammation and a series of chain reactions. Inflammation can be induced by many different stimulating factors, including physical damage, precursor chemicals, microbial invasion and immune responses [3]. A large number of inflammatory mediators lead to harmful effects on the body, including excessive degeneration, exudation, necrosis or the formation of abnormal granulation formation that result in different degrees of injury to the body. Examples of diseases and conditions with chronic inflammation include asthma, chronic peptic ulcer, tuberculosis, rheumatoid arthritis, chronic periodontitis, ulcerative colitis and Crohn's disease, chronic sinusitis, chronic active hepatitis (there are many more) [4]. Because inflammation involves many inflammatory mediators and pathways that lead to a wide range of changes in pathology, it is difficult to target the desired area when treating inflammation. Due to the resistance of such diseases to conventional treatments, as well as the side effects of presently available anti-inflammatory drugs, there is a pressing need for the development of novel anti-inflammatory drugs. Natural products are a valuable source of novel bioactive secondary metabolites. Recently, more attention has been focused on plants that have been established in folk medicine because of its time tested safety and efficacy. A huge number of natural product-derived compounds in various stages of clinical development highlighted the existing viability and significance of the use of natural products as sources of new drug candidates.

1.4 'Ketum': The Unique Plant with Stimulant and Sedative Properties

Rural communities in Malaysia commonly use herbs as food and traditional medicine. *Mitragyna speciosa*, locally known as 'ketum', is a unique plant that has been used

traditionally by some parts of the population as an alternative treatment for many diseases, especially to relieve drug addictions (Figure 1.1). The ethno pharmacological reputation of this medicinal plant is mainly for its medicinal uses as an analgesic, its ability to reduce withdrawal symptoms in opioid addicts, for treatment of diarrheal and diabetes, deworming, as a cough suppressant and for boosting immunity, to improve blood circulation and also as an energizing tonic [5-7]. To date, over 40 compounds have been successfully isolated and characterized from the leaves of *M. speciosa* grown in different regions [7, 8]. Scientific evidences for their efficacies have also been widely studied, however, almost all of the previous investigations were conducted on the alkaloidal fraction of the organic solvent extract, and not much information on the non-alkaloidal extract can be retrieved. In addition, systemic safety studies on this plant are also lacking. Therefore, in the present studies, evaluations were done on the non-organic solvent crudes that are of the carbon dioxide (SFE) and aqueous extracts. Study of the aqueous extract of the leaves is important as it mimics the consumption of the plant by locals and it is also essential to evaluate the toxicity of the aqueous extract in animals to ensure its safety.

Figure 1.1 The plant of *Mitragyna speciosa*

1.5 Research Objectives

M. speciosa is a misunderstood medicinal plant. The use of the leaves of this plant seems to have shifted from its folk remedy label to that of an opioid accomplice, branding it with an abuse potential. Despite the controversy, this study shall serve, as a decision making base for current potential medical application, as well as to identify future research needs.

This study has been conducted in hope to provide information that may bridge up the gaps in *Mitragyna speciosa* research. Thorough studies have been done on various solvent extracts of the plant parts by previous researchers but the aqueous extract has not been properly investigated, yet. The information regarding the aqueous extract is also still very limited. The SFE is a green technology that currently has received much attention in food, pharmaceutical and cosmetic industries. It represents a true alternative technique to organic solvents extraction of natural products when environmental and health safeties are in concern. Published reference on *M. speciosa* extracted by this method was not available when we first attempted this study and also to the best of our knowledge, this is the first report on the essential oils from the hydro distillation of *M. speciosa* leaves.

Objectives of the study are:

1. To evaluate the *in vitro* anti-inflammatory activity of *M. speciosa* leaves extracts.
2. To determine the chemical constituents of the most active *in vitro* anti-inflammatory extract.
3. To investigate the *in vivo* anti-ulcer effect and the acute toxicity of the aqueous extract of *M. speciosa* leaves (MSAE).
4. To isolate and identify chemical constituents of the aqueous extract (MSAE).

5. To characterize the volatile components from hydro distillation (HD) and supercritical fluid extraction (SFE) of *M. speciosa* leaves.

The wide availability of 'ketum' through the Internet reflects extensive demand for this product. It is now apparent that the international use of various 'ketum' chemo type preparations has spread beyond its traditional geographical boundaries. Though, it is clear that 'ketum' possesses wide range of medicinal values, due to its stimulant and euphoric effects, the plant is misused as an herbal drug of abuse, thus has been made illegal in Malaysia. This study is a crucial step to ensure the availability of a ready source of raw material as Malaysia moves up the chain to become a leader in the production of nutraceuticals and botanical drugs.

In this thesis, the studies will be reported in six chapters. Chapters 1 and 2 will deal with the introduction and a brief review on the biological and also chemistry aspects of the plant *M. speciosa*. Chapter 3 will elaborate on the biological activities performed (nitric oxide inhibition activity, gastro protective property and acute toxicity), Chapter 4 will discuss on the phytochemical and HPLC analytical studies, Chapter 5 will report on the volatile components and Chapter 6 will cover the conclusion.

CHAPTER 2: BIOLOGICAL AND CHEMICAL ASPECTS

Since time immemorial, plant-derived (phyto-) pharmaceuticals had been the concoction to treat and prevent a wide range of human maladies. Plants from genus of *Mitragyna* are credited with innumerable medicinal properties and are widely used by tribal people and other ayurvedic practitioners. In this chapter, the general biological and chemical aspects of the studied plant, *Mitragyna speciosa* Korth., in terms of classification and biogenesis were briefly discussed.

2.1 Taxonomic Classification

Taxonomically, this plant is classified as the following scheme:

Kingdom:	Plantae (Plants)
Subkingdom:	Tracheobionta (Vascular plants)
Superdivision:	Spermatophyta (Seed plants)
Division:	Magnoliophyta (Flowering plants)
Class:	Magnoliopsida (Dicotyledons)
Subclass:	Asteridae
Order:	Gentianales
Family:	Rubiaceae (Madder family)
Subfamily:	Cinchonoideae
Tribe:	Naucleeae
Genus:	*Mitragyna*
Species:	*speciosa*
Scientific name:	*Mitragyna speciosa* (Korth.) Havil.

Rubiaceae is a family of flowering plants, well known as the madder, coffee or bedstraw family. The Rubiaceae family is named after Rubia, a name used by Pliny the Elder (died in 79 AD) in his Naturalis Historia (one of the largest encyclopaedia to have survived from the Roman Empire to the modern day) for madder (*Rubia tinctorum*) [9]. The roots of this plant have been used since ancient times to extract alizarin and purpurin, two red dyes used for colouring clothes. The name rubia is therefore derived from the Latin word ruber, meaning red.

2.2 Rubiaceae: Distribution and Habitat

Rubiaceae consists of terrestrial trees, shrubs, lianas or herbs that are recognizable by simple, opposite leaves with interpetiolar stipules, inferior ovaries and sympetalous flowers [10]. It has a cosmopolitan distribution, found in nearly every region of the world, even on the Antarctic Continent with some species of *Coprosma, Galium* and *Sherardia* [11, 12]. A group of genera from Rubieae tribe is widely distributed in temperate regions [12]. However, the largest species diversity is distinctly concentrated in the humid tropical and subtropical floristic regions of the world. The highest number of species is found in Colombia, Venezuela and New Guinea. When adjusted for area, Venezuela is the most diverse, followed by Colombia and Cuba [13].

This family is predominantly by woody plants and the woody rubiaceous shrubs constitute an important part of the under storey of low- and mid-altitude rainforests [11]. Rubiaceae are tolerant of a broad array of environmental conditions (soil types, altitudes, community structures, etc.) and do not specialize in one specific habitat type (although genera within the family is often specialize).

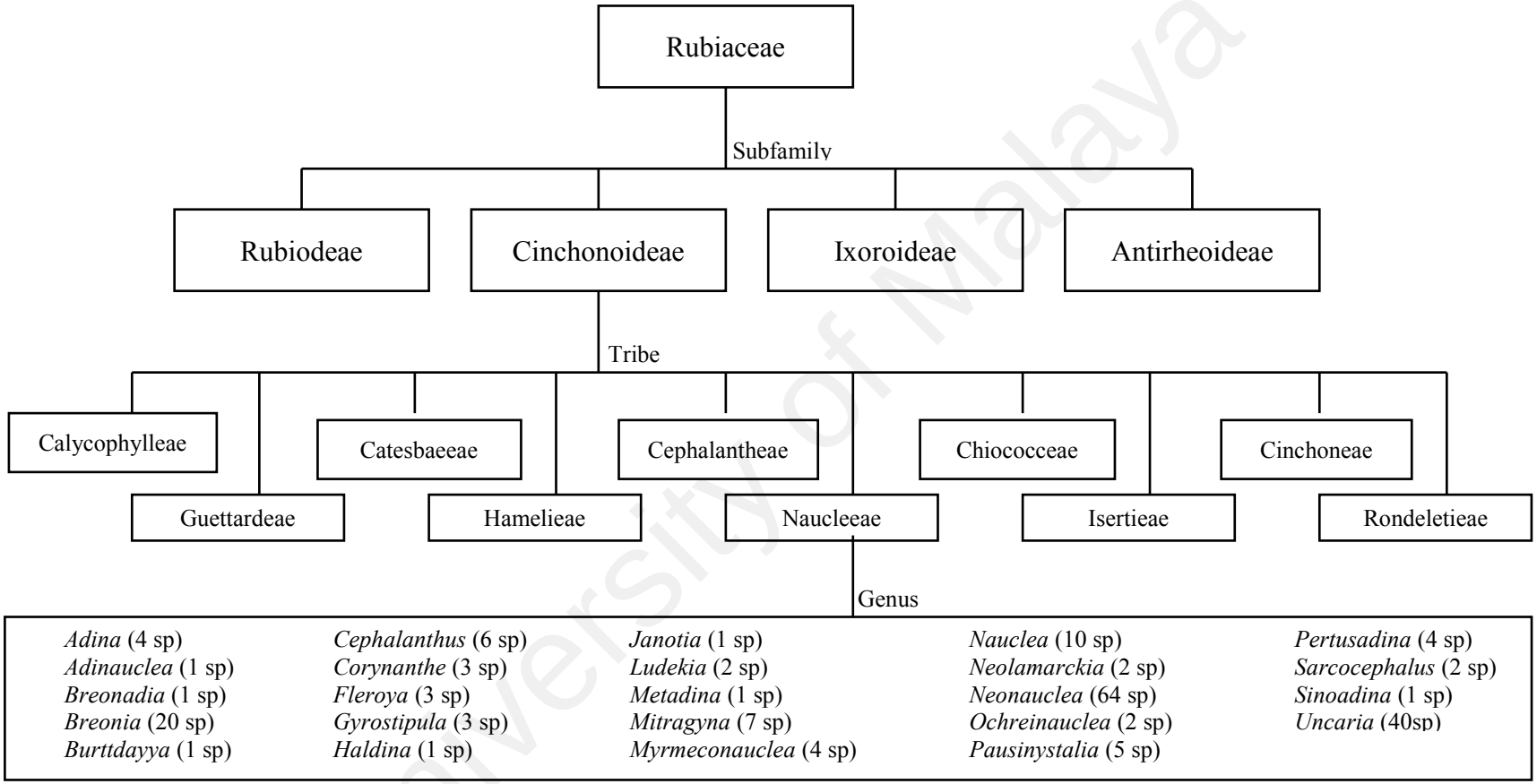
The most economically valuable genus is *Coffea*, the source of coffee, which is the world's most heavily traded primary commodity after crude oil [14]. Other

commercially important Rubiaceae are *Cinchona officinalis*, the source of quinine used to treat malaria, *Psychotria ipecacuanha* (ipecacuanha, an expectorant), *Pausinystalia johimba* (yohimbe, an aphrodisiac), *Genipa* spp. (genipapo, a beverage), *Uncaria gambier* (gambier, an important tannin source), *Uncaria* spp. (medicines), *Calycophyllum* spp. (lemonwood, a timber), *Neolamarckia* chinensis (timber trees), *Rubia tinctoria* (madder, a dye), and *Gardenia* spp. (perfume). The family also contains some of the most beautiful tropical ornamentals cultivars (Ixora, Gardenia, Mussaenda, *Pentas*, Portlandia, Serissa) [11].

The family comprises about 13,000 species in 611 genera, which makes it the fifth largest family of flowering plants by number of genera, and the fourth largest by number of species [11, 12]. Malaysia elaborates 80 genera with about 555 species. Forty five percent of the species in 49 genera are trees [15]. Four genera (*Aleisanthia, Klossia, Kochummenia* and *Perakanthus*) are endemic to Malaysia [15, 16].

2.3 Classification of Rubiaceae

Antoine Laurent de Jussieu described the family in 1789 [17]. During the twentieth century, the last subfamily classification solely based on morphological characters divided Rubiaceae into four subfamilies: Rubioideae, Cinchonoideae, Ixoroideae and Anti-rheoideae [18]. Currently, with the introduction of molecular phylogenetics, in most molecular study concerning Rubiaceae family, the classification with three subfamilies (Rubioideae, Cinchonoideae and Ixoroideae) is followed [19]. The genus *Mitragyna* is classified under the tribe Naucleeae, which is a member of the subfamily Cinchonoideae (Scheme 2.1) [20].



Scheme 2.1 Classification of Rubiaceae

2.4 Genus *Mitragyna*

The Dutch colonial botanist Pieter Korthals (1807-1892) was the first who formally described this genus in 1839 [21]. Korthals named the genus *Mitragyna* because the stigmas in the first species he examined resembled the shape of a bishop's mitre [22, 23]. However, as is the case with many plant genera and species, the nomenclature has frequently been confused and changed by various authors over the years. The genus has been variously named as *Nauclea, Sarcocephalus, Stephengyne* and *Uncaria* though consistently recognized as members of the tube Naucleeae in the sub-family Naucleoideae [22, 24]. In this genus, there are *Mitragyna diversifolia, Mitragyna hirsuta*, *Mitragyna inermis*, *Mitragyna parvifolia*, *Mitragyna parvifolia* var. *microphylla*, *Mitragyna rotundifolia*, *Mitragyna speciosa* and *Mitragyna tubulosa*, known as accepted species [20, 24, 25]. The genus grows exclusively in swampy humid territory in the tropical and subtropical regions of Asia and Africa [22].

The *M. parvifolia* (Roxb.) Korth., commonly known as ‘kaim’ is a most-investigated species in India [26]. *M. inermis* (Willd.) Kuntze (locally known as ‘uburu’, ‘abura’ or ‘gyayya’), the African species has gained much attention since 1930s from the French researchers and was previously documented under the name *M. africana* or *M. africanus* [6, 27]. The *M. speciosa and M. diversifolia* are commonly found in the Peninsular Malaysia. History of the former species, which is famous as ‘ketum’ among the natives has started way back in 1836 as described by Low and the popularity of this species keeps on blooming ever since, both in the scientific studies and also as an abused herbal drug [28, 29]. Table 2.1 contains a list of *Mitragyna* species, the accepted names (in bold), synonyms and their distributions.

Table 2.1 *Mitragyna* species and their distribution

Distribution	Names*	Status*
West Africa [11, 22, 30]	*Mitragyna africana* (Willd.) Korth. [Illegitimate]	Synonym of *Mitragyna inermis* (Willd.) Kuntze
	Mitragyna chevalieri K.Krause	Synonym of *Fleroya stipulosa* (DC.) Y.F.Deng
	Mitragyna ciliata Aubrév. & Pellegr.	Synonym of *Fleroya ledermannii* (K.Krause) Y.F.Deng
Mauritania east to Sudan [24]	***Mitragyna inermis* (Willd.) Kuntze**	**Accepted**
	Mitragyna ledermannii (K.Krause) Ridsdale	Synonym of *Fleroya ledermannii* (K.Krause) Y.F.Deng
	Mitragyna macrophylla Hiern	Synonym of *Fleroya stipulosa* (DC.) Y.F.Deng
	Mitragyna stipulosa (DC.) Kuntze	Synonym of *Fleroya stipulosa* (DC.) Y.F.Deng
East Africa [22]	*Mitragyna rubrostipulata* (K.Schum.) Havil.	Synonym of *Fleroya rubrostipulata* (K.Schum.) Y.F.Deng
Asia [22, 30, 31]	*Mitragyna brunonis* (Wall. ex G.Don) Craib	Synonym of *Mitragyna rotundifolia* (Roxb.) Kuntze
Combodia, China, Laos, Myanmar, Thailand, Malaysia, Indonesia, Vietnam, Philipines [22]	***Mitragyna diversifolia* (Wall. ex G.Don) Havil.**	**Accepted**
	Mitragyna diversifolia var. *microphylla* (Kurz) Craib	Synonym of *Mitragyna parvifolia* var. *microphylla* (Kurz) Ridsdale
Combodia, China, Laos, Myanmar, Thailand, Vietnam [24]	***Mitragyna hirsuta* Havil.**	**Accepted**
Java, Peninsular Malaysia [29]	*Mitragyna javanica* Koord. & Valeton	Synonym of *Mitragyna diversifolia* (Wall. ex G.Don) Havil.
	Mitragyna javanica var. *microphylla* (Kurz) Craib	Synonym of *Mitragyna parvifolia* var. *microphylla* (Kurz) Ridsdal

Table 2.1 Continued

India, Sri Lanka, Myanmar, Bangladesh [24]	***Mitragyna parvifolia* (Roxb.) Korth.**	**Accepted**
	Mitragyna parvifolia var. *parvifolia*	Synonym of *Mitragyna parvifolia* (Roxb.) Korth.
Bangladesh to Myanmar [11]	***Mitragyna parvifolia* var. *microphylla* (Kurz) Ridsdale**	**Accepted**
India, Bangladesh, Laos, Myanmar, Thailand, China [24]	***Mitragyna rotundifolia* (Roxb.) Kuntze**	**Accepted**
Malaysia, Indonesia, Thailand, Myanmar, Vietnam, Philippines, New Guinea, Papua New Guinea [24]	***Mitragyna speciosa* (Korth.) Havil.**	**Accepted**
Endemic to peninsular India [24]	***Mitragyna tubulosa* (Arn.) Kuntze**	**Accepted**

*In reference to World Checklist of Selected Plant Families (WCSP)-WCSP gives information on the accepted scientific names and synonyms of selected plant families. It includes more than 320,000 published names and allows the user to search for all the scientific names of a particular plant, or the areas of the world in which it grows (distribution). The data set counts upon the collaboration over 16 years of 132 specialists from 25 countries who have contributed data or acted as reviewers [20].

Natives of Africa, India, Malaysia, Thailand and other regions of Southeast Asia have used *Mitragyna* species in local folklore medicine for decades. The plants have been used to treat a wide variety of diseases such as fever, malaria, diarrhea, asthma, coughing, muscle pain, improving blood circulation and used for the expulsion of worms [32-34]. *M. speciosa* (Korth.) Havil, a species of particular medicinal importance, the leaves have been traditionally used by natives for their opium-like effect and coca-like stimulant ability to combat fatigue and enhance tolerance to hard work under the scorching sun. It has been used also as a substitute for opium and for weaning addicts off morphine [35]. However, the use of this plant has been banned in countries like Malaysia, Myanmar, Thailand (since 1946) and Australia (since 2005) because of its narcotic effect. The African species, *M. ciliate*, *M. inermis* and *M. stipulosa* are used to treat inflammation, hypertension, headache, rheumatism, gonorrhea and bronchopulmonary diseases. Meanwhile, decoction of the bark and

leaves of *M. Africana* (which is actually a synonym of *M. inermis* (Willd.) Kuntze) was reported to be used in Nigeria to treat mental illness and epilepsy [27, 36]. Apart from the medicinal usage, the plants also provide local indigenous populations with other importance such as body paints, dye, edible fruits and commercially in the timber, construction wood and paper industry [37]. Selected species of *Mitragyna* and their traditional usage were summarized in Table 2.2.

Table 2.2 Traditional medicine and other uses of the genus of *Mitragyna*

Name	Traditional uses	Ref
Mitragyna Africana (Willd.) Korth. Synonym of *Mitragyna inermis* (Willd.) Kuntze	• As a medicine for fever in Senegal.	[29]
	• Used in treatment for bacterial infections, dysentery, gonorrhoea, mental disorder and epilepsy.	[38]
	• In combination with other plant species, it is used to treat sleeping sickness (trypanosomiasis), sterility and mental illness.	[36, 38]
	• The bark provides yellow dye	[29]
Mitragyna ciliate Aubrév & Pellegr. Synonym of *Fleroya stipulosa* (DC.) Y.F.Deng	• The wood is used in construction, flooring, joinery, trim, furniture, sporting goods, toys, novelties, musical instruments, transmission poles, boxes, crates, canoes, oars and handles.	[24]
	• Preparations of the stem-bark have been used in treatment of amenorrhea, bronco-pulmonary diseases, colds, dysentery, fever, general weakness, gonorrhea, hypertension, inflammation, leprosy, malaria, rheumatism and to facilitate childbirth.	[24, 39, 40]
Mitragyna diversifolia (Wall. ex G.Don) Havil.	• The bark is used as a remedy for diarrheal in Bangladesh.	[41]
Mitragyna hirsuta Havil.	• Decoction or drink prepared from the stem-bark, wood, roots and mixed with *Polyalthia cerasoides* is used as galactogogue in Thailand.	[42, 43]

Table 2.2 Continued

Mitragyna inermis (Willd.) Kuntze	• The leaves and bark are febrifuge	[6, 27]
	• The plant (parts not specified) is diuretic. It is used in the treatment of constipation, stomach disorders, dysentery, rheumatism, malaria, gonorrhea, syphilis, leprosy, bilharzia, jaundice, and epilepsy.	
Mitragyna javanica Koord. & Valeton Synonym of *Mitragyna diversifolia* (Wall. ex G.Don) Havil.	• The leaves are frequently used to substitute *M. speciosa* as a stimulant but claimed to be less effective by the native population of Thailand.	[22]
Mitragyna macrophylla Hiern Synonym of *Fleroya stipulosa* (DC.) Y.F.Deng	• As a remedy for fever in Zambesi and Senegal	[29]
	• Its leaves, roots and bark are used for a variety of other complaints in west Africa	[29]
Mitragyna parvifolia (Roxb.) Korth.	• Bark and roots are used in treatment of fever, colic, cough, muscular pain, burning sensation, oedema, poisoning, gynaecological disorders and as aphrodisiac.	[33, 44-46]
	• Fruit juice augments the quantities of breast milk in lactating women and also as lactodepurant.	[46]
	• Leaves are put over wounds and ulcers to alleviate pain, swelling and for better healing.	[47]
	• The leaves are used as an opium substitute in Thailand	[22]
Mitragyna rotundifolia (Roxb.) Kuntze	• Decoction of the stems and roots is used to treat rheumatoid arthritis in Thailand.	[42, 43]
Mitragyna speciosa (Korth.) Havil.	• Used in a treatment of fever, coughs, diarrhea, diabetes and musculoskeletal pain.	[29, 34, 35, 48, 49]
	• Pounded leave are applied to wounds and heated leaves are dressed over enlarge spleens in Perak.	[29, 50]
	• A poultice of the leaves is used for expulsion of worms from children in lower Perak.	[29, 51]
	• The leaves and bark preparations are consumed as a stimulant, cure for opium and drug addiction or to substitute opium and drug in Thailand and Malaysia.	[22, 29, 51, 53]

Table 2.2 Continued

	• The wood was described to be suitable as veneer, coating plywood, non-structural laminated veneer lumber and profile moulding.	[24, 54]

As medicinal properties run through the genus, interest has been developed to perform scientific investigations. It has been communicated that *Mitragyna* plants possessed anti-pyretic, anti-diarrheal, euphoric, analgesic, anxiolytic and depressant effects. Members of this genus also acted to boost immunity, lower blood pressure and proved to possess anti-viral, anti-bacterial, anti-diabetic and appetite suppressing. African species, such as *M. inermis* was reported to have effect as anti-plasmodial that reflected the folklore used to treat malaria. *M. parvifolia*, which grows throughout India, was proved to possessed anti-anthelmintic activity which support the traditional used to deworm. The plant was also found to have analgesic and anti-inflammatory potential which is relevant when traditional healer use in the treatment of fever, colic muscular pain, burning sensation, cough, edema and as aphrodisiac [46]. Study has demonstrated the effect of *M. speciosa* in stimulating glucose transport in muscle cells, implicating the folkloric use of *M. speciosa* leaves for treating diabetes [55]. The advances in pharmacological activities of documented *Mitragyna* genus are presented in Table 2.3.

Table 2.3 Scientific studies on the genus of *Mitragyna*

Name	Biological activity	Ref
Mitragyna africana (Willd.) Korth. Synonym of *Mitragyna inermis* (Willd.) Kuntze	• Anti-stress - Methanol extract (50%) of the stem-bark showed the effect.	[27]

Table 2.3 Continued

Mitragyna ciliata Aubrév. & Pellegr. Synonym of *Fleroya ledermannii* (K.Krause) Y.F.Deng	• Vascular relaxant activity - Methanol extract of the stem bark induced a vasodilatation action on aorticvascular rings precontracted with noradrenaline or KCl.	[56]
	• Analgesic activity - Methanol extract exhibited potent analgesic property.	[57]
Mitragyna diversifolia (Wall. ex G.Don) Havil.	• Anti-diarrheal activity - Ethanol extract of the bark showed significant anti-diarrheal activity on gastrointestinal motility with barium sulphate milk model and castor oil induced diarrheal model in rats.	[58]
Mitragyna inermis (Willd.) Kuntze	• Cardiovascular activities - Aqueous extract possessed cardiacinotropic effect, induced an increase in coronary flow, induced the release of endothelial NO in porcine coronary arteries and produced smooth muscle relaxation in the rat-tail arteries.	[59]
	• Anti-plasmodial - Decoction in water of the powdered plant showed activity on *Plasmodium falciparum*	[60, 61]
Mitragyna parvifolia (Roxb.) Korth.	• Anti-inflammatory activity - Methanol extract of the stem-bark possessed significant and dose dependent anti-inflammatory effect. - Ethanol extract of the dried fruits and leaves exhibited the activity	[62] [46, 63]
	• Anti-nociceptive activity - Methanol extract of the stem-bark possessed significant and dose dependent anti-nociceptive effect.	[62]
	• Anthelmintic activity - Methanol extract of the dried stem-bark proved to be anthelmintic agent. - Ethanol and aqueous extracts of the leaves demonstrated anthelmintic activity	[47] [64]
	• Analgesic activity - Ethanol extract of the dried fruits and leaves were found to possessed analgesic effect.	[46, 65]

Table 2.3 Continued

	• Anti-microbial activity - Ethanol extract of the dried fruits has no anti-microbial activity. - Ethanol extract of the dried leaves has anti-microbial potential.	[46] [65]
	• Toxicity activity - Ethanol extract of the dried fruits and leaves were found to be non-toxic.	[46, 65]
	• Anti-oxidant activity - Ethanol extract of the dried leaves was anti-oxidant potential.	[63]
	• Anxiolytic activity - Methanolic, ethyl acetate extract and alkaloid rich fraction of the stem-bark was effective in dose dependent manner and proved statistically significant at higher doses. Alkaloid rich fraction was found to be more potent.	[66]
	• Anti-pyretic activity - The ethanol extract of the leaves showed reduction in normal body temperature and yeast provoked elevated temperature in a dose dependent manner. The effect became significant at 60 min at the highest dose of 100 mg/kg.	[67]
	• Anti-convulsant effect • The ethanol extract of the leaves suppressed tonic hind limb extensions (THLE) induced by maximal electroshock convulsive at the doses of 250 and 500 mg/kg and exhibited protector effect in pentylenetetrazole (PTZ)-induced seizures only at 500 mg/kg. The activity reported was dose dependent in both the models.	[68]
Mitragyna rotundifolia (Roxb.) Kuntze	• Anti-oxidant properties - Ethyl acetate and *n*-butanol extracts of the barks and leaves showed higher activities (DPPH and FRAP) than that of petroleum ether extract.	[69, 70]
	• Anti-diabetic activity - Ethyl acetate and *n*-butanol extracts of the barks and leaves had higher inhibition activity of *a*-glucosidase than that of petroleum ether extract.	[69]

Table 2.3 Continued

	• Hepatoprotective activity - Ethyl acetate and *n*-butanol extracts of the barks and leaves demonstrated a very good activity against carbon tetrachloride (CCl_4)-induced acute liver injury in mice and this maybe correlated with their anti-oxidant effects.	[71]
	• Analgesic activity - Methanol extract of the leaves exhibited potent analgesic effect.	[57]
	• Anti-inflammatory - Methanol extract of the stem-bark was significantly and dose dependently suppressed the development of carrageenan-induced rat paw edema	[39]
	• Anti-cancer activity - Aqueous extract showed strong anti-mutagenicity properties in TA98 and TA100 with the presence of metabolic activator S9 system.	[72]
	• Anti-diarrheal activity - Ethanol extract of the leaves at 50, 100, 200 and 400 mg/kg (p.o) respectively showed dose dependent protection against castor oil induced diarrheal in rats and inhibited intestinal transit.	[73]
Mitragyna speciosa (Korth.) Havil.	• Anti-oxidant properties - The DPPH IC_{50} values of aqueous, alkaloid and methanolic extracts of the leaves were 213.4, 104.81 and 37.08 μg/mL, respectively	[74]
	• Anti-microbial activity - Aqueous, alkaloid and methanolic extracts of the leaves showed anti-microbial property against *Salmonella typhi* and *Bacillus subtilis*.	[74]
	• Anti-diabetic - Aqueous, alkaloid and methanolic extracts of the leaves significantly increased the rate of glucose uptake.	[55]
	• Hepatoprotective activity - Alkaloid extract may contribute to an herb-drug interaction if consumed concomitantly with drugs that are substrates for human recombinant cytochrome P450 (CYP3A4, CYP2D6 and CYP1A2).	[75]

Even before the genus and species were described botanically, Low (1836) briefly stated *M. speciosa* (under the spelling 'beah') usage: "The Beah is the leaf of a moderately high tree so named, which opium-smokers substitute for that drug when it is not procurable." [29]. It has been said, but not proved, that the Malayan *M. speciosa* is the most potent species of a genus through the whole of which an alkaloid is distributed [29].

2.4.1 *Mitragyna speciosa*

M. speciosa has been used medicinally in Southeast Asia 'from time immemorial'. Traditionally it has been used to treat fever, malaria, diarrhea, muscle pain, inflammation and hypertension. Phytochemical research has shown that alkaloids, triterpenoids and flavonoids were main constituents in this plant. Pharmacology investigation demonstrated that the plant possess wide pharmacological effects, in anesthetic, anti-tussive, stimulant and opioid activities. In addition, recently *M. speciosa* has been used to produce energy drinks [5, 76]. Among those recognized species (Table 2.1), *M.speciosa* was famous for having dual properties as stimulant and sedative, which recently gained a lot of attention all over the world. This plant has been called by various names by the natives such as 'ketum' or 'biak-biak' in Malaysia, 'kratom', 'kraton', 'kakuam', 'ithang' or 'thom' in Thailand and 'kratom' or 'puri' in Indonesia.

2.4.1.1 Habitat

This species is found in tropical and subtropical Asian regions such as Thailand, Laos, Cambodia and Malaysia. It is also found in Borneo, Philippines, New Guinea, East and West Africa and India [22, 24]. Asian species are often found in rainforests, while African species are often in swamps. In general, it grows abundantly in wet, humid

areas rich with humus [22]. In Malaysia, this plant is widely found in northern half of Peninsular, Selangor and Negeri Sembilan [77].

2.4.1.2 Morphology and Physiology

Most are arborescent, large trunk tree and usually grow reaching the height of 50 feet with a branch spread of over 15 feet. The stem is erect and branching. A globular flowering head with 120 dark yellow florets attached to the leaf axils on long stalks characterizes this genus. It consists of fruit-like capsule with flat seeds. The ovate acuminate shaped leaves are dark glossy green colour, smooth and arranged opposite to one another [78]. Leaves can grow over 7 inches long and 4 inches wide. This plant is a type of tropical evergreen and non-seasonal, leaves constantly being shed and being replaced. However, it is very sensitive to extreme cold and drought. The leaves will fall abundantly during extremely hot dry season and grow plentiful during the rainy season [79]. There are two types of *M. speciosa* based on the colour of veins in the leaves. The Thai and Malay natives will distinguish between those with red and those with green (sometimes called white) midribs [22]. The taste of red vein is bitterer and its effect can last longer than the green vein. Therefore, most of the local people prefer red vein leaves than green one [80]. Somehow, the greenish-white veining variety has been claimed to possess stronger effect as a stimulant by most Thai users [35]. *M. speciosa* Korth., which has been traditionally used in tropical areas as a substitute for opium, is a rich source of Corynanthe-type indole alkaloids.

Figure 2.1 Bark of *Mitragyna speciosa* (KL5321)

Figure 2.2 Leaves of *Mitragyna speciosa* (KL5321)

Figure 2.3 Flower of *Mitragyna speciosa* (KL5321)

2.5 Phytochemistry of the Genus *Mitragyna*

The first compound isolated from the genus *Mitragyna* was actually an alkaloid extracted from the leaves of *M. speciosa* by Hooper (1907) and this was repeated in 1921 by Field who named the alkaloid mitragynine **1** [22, 29] . Following this, extensive phytochemical investigations have been carried out on *Mitragyna* species from Africa and South East Asia. *In vivo* and/or *in vitro* and clinical practices have demonstrated that *Mitragyna* and its active compounds possess wide-reaching pharmacological actions, including anti-tumour, analgesic, cardiovascular disease and anti-bacterial activity.

In India, the most studied species is *M. parvifolia* (Roxb.) Korth., which has been reported to possess pyroligneous acid, methyl acetate, ketones and aldehydes as the significant constituents. The plant has great potential for anti-inflammatory, analgesic and anti-microbial [46]. A related species, *M. javanica*, was documented to be used as a substitute to get around the Thai laws banning *M. speciosa*, but it is not considered as effective [22]. The dominant alkaloid in this *M. javanica* is mitrajavine **2**, which has not yet been pharmacologically tested. *M. speciosa* grows naturally throughout Southeast Asia and has been the most investigated species. Specimens from Papua New Guinea contained mitragynine **1**, speciogynine **3**, speciociliatine **4**, paynantheine **5**, specionoxeine **6** and isospecionoxeine **7** [22]. As for red and green/ white leaved plants of Thailand, the most common alkaloidal profile was mitragynine **1**, speciogynine **3**, speciociliatine **4**, paynantheine **5**, traces of ajmalicine **8**, traces of 9-methoxy-oxindoles, and traces of other indoles. Thai ‘kratom’ and Malay ‘ketum’ had the alkaloids mitragynine **1**, speciogynine **3**, speciociliatine **4**, paynantheine **5** and 7-hydroxymitragynine **9** in common. In both samples, mitragynine **1** was the most

dominant alkaloid, but in the Thai specimen it made up 66% of the total alkaloid while it made up only 12% of the alkaloids from the Malay sample [81].

The most abundant alkaloid in the leaves of Malaysian *M. speciosa* is mitragynine **1**, whereas that in the fruits is its diastereomer, speciociliatine **4** (around 1.91% of the total alkaloid extract) [81, 82]. Mitragynine **1** was assumed to be a physiologically active constituent having morphine-like properties. However, it should be noted that it might not be the most potent psychoactive component. In particular over more chronic utilization, credits should be given to 7-hydroxymitragynine **9** [83]. This minor constituent of the alkaloid from the leaves has demonstrated more potent opioid agonist effects, which structurally different from that of morphine [84-86]. In 2006, a new Corynanthe-type alkaloid, 7-hydroxyspeciociliatine **10** has been isolated from the methanol extract of the fruits of *M. speciosa* native to Malaysia. Even though the structure is similar to that of 7-hydroxymitragynine **9**, the compound was found to have a weak analgesic effect through stimulation of the μ–opioid receptor. This reminiscent the potency of specioliatine **4** in comparison with mitragynine **1** [82]. Table 2.4 summarized the known chemical compounds isolated from the genus *Mitragyna* and some of the pharmacological properties that have been documented.

Table 2.4 Chemical constituents and their pharmacological activities from genus of *Mitragyna*

Name	Chemical constituents	Pharmacological activities	Ref
	Alkaloid		
Mitragyna africana (Willd.) Korth. Synonym of *Mitragyna inermis* (Willd.) Kuntze	• Rhynchophylline **11** - oxindole	-	[29]

Table 2.4 Continued

Mitragyna ciliate Aubrév & Pellegr. Synonym of *Fleroya stipulosa* (DC.) Y.F.Deng	Non-alkaloid		
	• Arjunolic acid **12** - triterpenoid saponin	Anti-tumour promoter	[87]
Mitragyna inermis (Willd.) Kuntze	Alkaloid		
	• Mitraciliatine **13**	-	[22]
	• Mitranermine **14** - isolated by Raymond-Hamet, 1934	-	[22]
	• Speciogynine **3**	-	[22]
Mitragyna javanica Koord. & Valeton Synonym of *Mitragyna diversifolia* (Wall. ex G.Don) Havil.	Alkaloid		
	• Ajmalicine **8**	-	[22]
	• 3-Isoajmalicine **15**	-	[22]
	• Isomitraphylline **16**	-	[22]
	• Javaphylline **17**	-	[22]
	• Mitrajavine **2**	-	[22]
Mitragyna parvifolia (Roxb.) Korth.	Alkaloids		
	• Akuammigine **18**	-	[22]
	• Dihydrocorynantheine **19**	-	[22]
	• 16,17-dihydro-17*β*-hydroxy isomitraphylline **20**	-	[26, 62]
	• 16, 17-dihydro-17*β*-hydroxy mitraphylline **21**	-	[26, 62]
	• Hirsutine **22**	-	[22]
	• Isomitraphylline **16**	-	[26, 62]
	• Isopteropodine **23**	-	[22]
	• Isorhynchophylline **24**	-	[22]
	• Isorotundifoline **25** - Sherllard & Phillipson, 1964	-	[22]
	• Mitraphylline **26** - main alkaloid constituent of the leaves	-	[88]
	• Mitraversine **27** - Isolated by Field, 1921	-	[88]
	• Pteropodine **28**	-	[22]
	• Rhynchophylline **11**	-	[22]

Table 2.4 Continued

	• Speciophylline **29**	-	[22]
	• Tetrahydroalstonine **30**	-	[22]
	• Uncarine F **31**	-	[22]
	Flavonoids, glycosides and tannins	-	[22]
	Pyroligneous acid	-	[22]
Mitragyna rotundifolia (Roxb.) Kuntze	Alkaloid		
	• Rhynchophylline **11**	-	[22]
	Coumarin	-	
	• Scopoletin **32**	Anti-diabetic	[69]
Mitragyna rubrostipulata (K.Schum.) Havil. Synonym of *Fleroya rubrostipulata* (K.Schum.) Y.F.Deng	Alkaloid		
	• Mitraphylline **26** - oxindole - characterized by Michiels & Leroux, 1925	-	[22]
	• Isorhynchophylline **24** - oxindole - reported by Tondeur & Marison, 1930	-	[22]
	• Stipulatine **33**	-	[89]
Mitragyna speciosa (Korth.) Havil.	Alkaloid		
	• Ajmalicine **8** - raubasine - also found in *Rauwolfia serpentina*	Cerebrocirculant, anti-aggregant, anti-adrenergic (at alpha-1), sedative, anti-convulsant, smooth muscle relaxer.	[22, 90]
	• Akuammigine **18**	-	[88]
	• Ciliaphylline **34***	Anti-tussive, analgesic.	[91]
	• Corynantheidalinic acid **35**	-	[92]
	• Corynantheidaline **36**	-	[92]
	• Corynantheidine **37*** - also found in Yohimbe	μ -opioid antagonist.	[22, 86]
	• Corynoxeine **38***	Calcium channel blocker	[82]
	• Corynoxine A **39***	Calcium channel blocker, dopamine-mediating anti-locomotives.	[22, 93]
	• Corynoxine B **40***	Dopamine-mediating anti-locomotives.	[22, 93]
	• 3-Dehydromitragynine **41**	-	[77]
	• 9-Hydroxycorynantheidine **42**	Partial opioid agonist	[88]
	• 7-Hydroxymitragynine **9****	Analgesic, anti-tussive, anti-diarrheal, primary psychoactive.	[83-86, 94, 95]
	• 7-Hydroxyspecioliatine **10**	Opioid agonistic activity-weak stimulatory effect on μ–opioid receptors	[82]
	• 3-Isocorynantheidine **43**	-	[96, 97]

Table 2.4 Continued

	• Isocorynoxeine **44** - oxindole	-	[82]
	• Isomitraphylline **45*** - oxindole	Immunostimulant, anti-leukemic.	[22, 83, 98, 99]
	• Isomitrafoline **46*** - oxindole	-	[22, 93, 100]
	• 3-Isopaynantheine **47**	-	[88]
	• Isopteropodine **23**	Immunostimulant	[88]
	• Isorhynchophylline **24*** - oxindole	Immunostimulant.	[22, 96, 98, 101]
	• Isospeciofoline **48*** - oxindole	-	[22, 93, 100]
	• Isospecionoxeine **7** - oxindole	-	[22]
	• (-)-9-Methoxymitralactonine **49**	-	[88, 102]
	• Mitraciliatine **13***	-	[22, 103]
	• Mitrafoline **50*** - oxindole	-	[22, 93, 100]
	• Mitragynaline **51***	-	[92]
	• Mitragynalinic acid **52***	-	[92]
	• Mitragynine **1** - isolated by Hooper, before1921 and named by Field, 1921 • main alkaloid constituent of the leaves, roughly 66% of total alkaloid content found in leaves of Thai sample and 12% in Malaysian sample.	Protozoal poison, analgesic, anti-depressant properties, anti-tussive, anti-diarrheal, adrenergic, anti-malarial, anti-diabetic, possible psychedelic (5-HT2A) antagonist, suppressed PGE_2 production by inhibiting COX-2 expression in LPS-stimulated RAW264.7 macrophage cells.	[22, 29, 55, 104, 105]
	• Mitragynine oxindole B **53***.	-	[93]
	• (-)-Mitralactonine **54**	-	[88, 106]
	• Mitraphylline **26*** - Oxindole alkaloid	Vasodilator, anti-hypertensive, muscle relaxer, diuretic, anti-amnesic, anti-leukemic, possible immunostimulant.	[22, 83, 96, 98]
	• Mitraspecine **55** - isolated by Denis, 1930	-	[22]
	• Mitraversine **27** - isolated by Denis, 1930	-	[22]
	• Oxindole A **56***	-	[93]

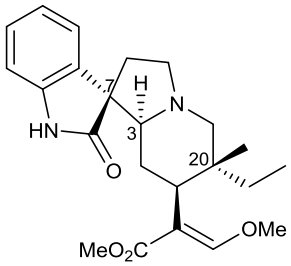
Table 2.4 Continued

	• Oxindole B **57***	-	[93]
	• Paynantheine **5*****	Smooth muscle relaxer.	[22, 83]
	• Rhynchophylline **11***	Vasodilator, anti-hypertensive, calcium channel blocker, anti-aggregant, anti-inflammatory, anti-pyretic, anti-arrhythmic, anti-thelmintic.	[22, 96, 98]
	• Speciociliatine **4***	Weak opioid agonist.	[22, 83, 103]
	• Speciofoline **58**	Analgesic, anti-tussive	[22, 100]
	• Speciogynine **3******	Smooth muscle relaxer.	[22, 83, 96, 103]
	• Speciophylline **29*** - oxindole	Anti-leukemic.	[22, 90, 99]
	• Stipulatine **33**	-	[89]
	• 3,4,5,6-Tetradehydromitragynine **59**	-	[81]
	• Tetrahydroalstonine **30**	Hypoglycemic, anti-adrenergic (at alpha-2)	[81]
	Non-alkaloid		
	• Epicatechin **60** - also found in dark chocolate	Anti-oxidant, anti-aggregant, anti-bacterial, anti-diabetic, anti-hepatitic, anti-inflammatory, anti-leukemic, anti-mutagenic, anti-peroxidant, anti-viral, potential cancer preventative, α-amylase inhibitor.	[81]
Mitragyna stipulosa (DC.) Kuntze Synonym of *Fleroya stipulosa* (DC.) Y.F.Deng	Alkaloid		
	• Rhynchophylline **11** - characterized by Larrieu, 1930	-	[22]
	• Rotundifoline **61** - characterized by Barger, Dyer, Sargent, 1930	-	[22, 91]
	• Isorotundifoline **25** - Isolated by Beckett *et. al.*, 1965	-	[22, 91, 107]

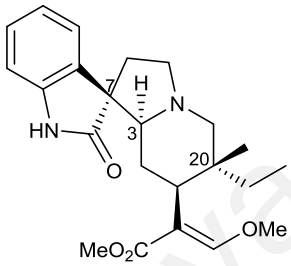
* < 1% of total alkaloid content found in the leaf.
** ≈ 2% of total alkaloid content found in the leaf.
*** 8.6% to 9% of total alkaloid content found in the leaf.
**** 6.6% to 7% of total alkaloid content found in the leaf

As depicted in Table 2.5, the most abundant type of compounds isolated were the alkaloids, specifically the indole alkaloids. Predominated by the Corynanthe type indole,

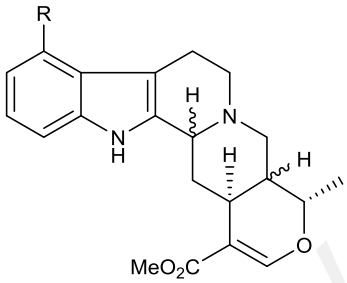
including oxindole derivatives that can occur in tetra- or penta-cyclic rings [22]. To date more than 40 alkaloids have been isolated and characterized from the genus.



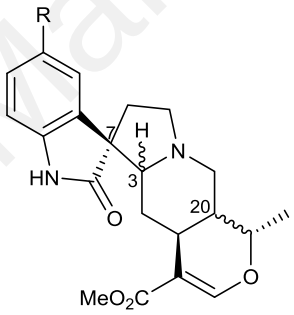
11



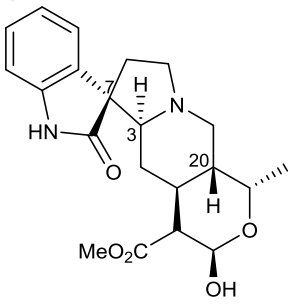
24



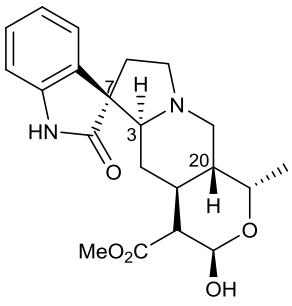
2: R=OMe, 3R, 20R
8: R=H, 3S, 20R
15: R=H, 3R, 20R
18: R=H, 3R, 20S
30: R=H, 3S, 20S



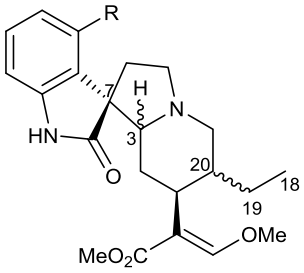
16: R=H, 3S, 20S
17: R=OMe, 3S, 20R
23: R=H, 3S, 20R
29: R=H, 3R, 20S



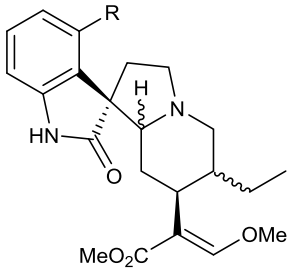
20



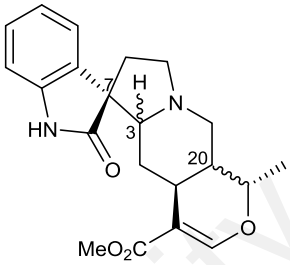
21



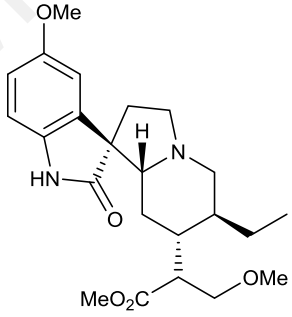
25: R=OH, 3S, 20R
38: R = H, 3S, 20R, $\Delta^{18,19}$
40: R = H, 3S, 20S
46: R = OH, 3S, 20S
53: R= OMe, 3S, 20S
58: R = OH, 3R, 20S



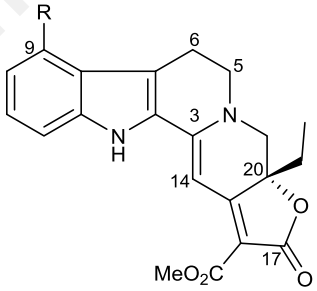
7: R = OMe, 3S, 20R, $\Delta^{18,19}$
39: R = H, 3S, 20S
44: R = H, 3S, 20R, $\Delta^{18,19}$
48: R = OH, 3R, 20S
50: R = OH, 3S, 20S
61: R =OH, 3S, 20R



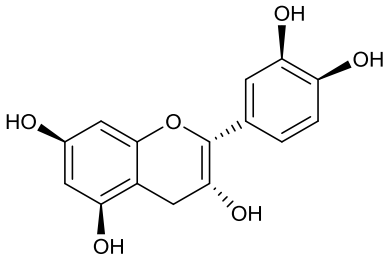
26: 3S, 20S
28: 3S, 20R
31: 3R, 20S



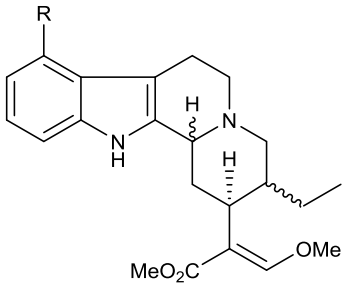
34



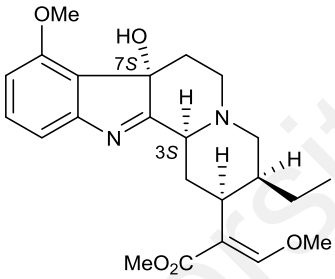
54: R=H
49: R=OMe



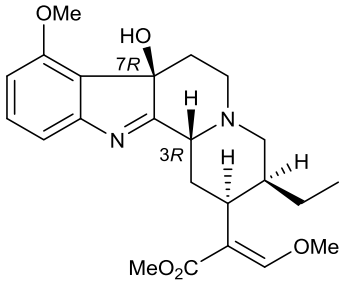
60



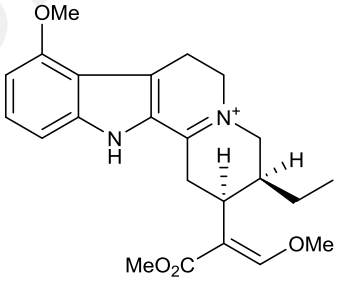
1: R = OMe, 3S, 20S
3: R = OMe, 3S, 20R
4: R = OMe, 3R, 20S
5: R = OMe, 3S, 20R, $\Delta^{18,19}$
13: R = OMe, 3R, 20R
19: R = H, 3S, 20R
22: R = H, 3R, 20R
37: R = H, 3S, 20S
42: R=OH, 3S, 20S
43: R = H, 3R, 20S
47: R = OMe, 3R, 20R, $\Delta^{18,19}$



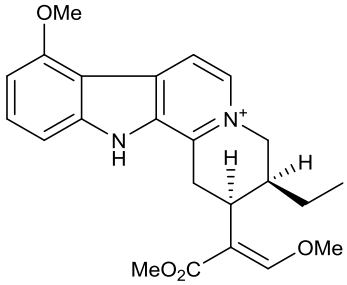
9



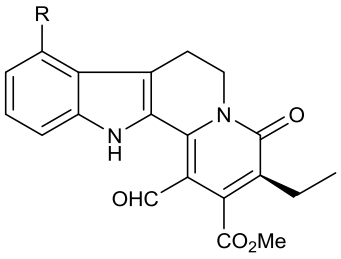
10



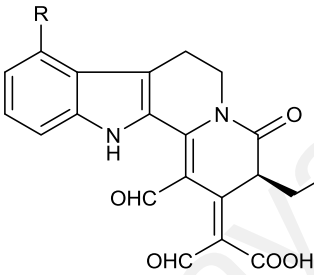
41



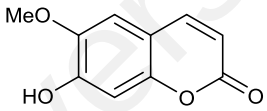
59



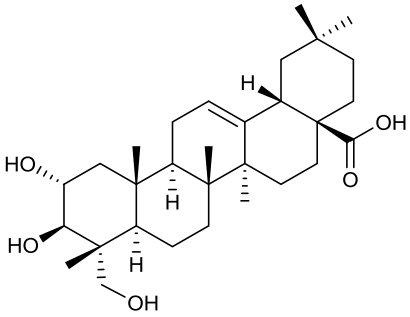
35: R=H
52: R=OMe



36: R=H
51: R=OMe



32



12

2.5.1 Alkaloid

The name ‘alkaloid’ was coined by the German pharmacist Carl Friedrich Wilhelm Meissner in 1819 referring to plant natural products (the only organic compounds known at that time) exhibiting basic behaviours similar to those of the inorganic alkalis [108]. The ending ‘-oid’ simply infers the alkali-like properties of the compounds.

An alkaloid is an organic compound contains of at least one nitrogen atom as the key atom in a heterocyclic ring. The naturally occurring compounds contain mostly basic nitrogen atoms. A side from that, alkaloids also include some related compounds with neutral and even weakly acidic properties. Some synthetic compounds of similar structure are also termed alkaloids. In addition to carbon, hydrogen and nitrogen, alkaloids may also contain oxygen, sulphur and, more rarely, other elements such as chlorine, bromine and phosphorus [108].

The boundary between alkaloids and other nitrogen-containing natural compounds is not clear-cut. Compounds like amino acid peptides, proteins, nucleotides, nucleic acid, amines and anti-biotics are usually not called alkaloids. Natural compounds containing nitrogen in the exocyclic position are usually classified as amines rather than as alkaloids.

Alkaloids are produced by a large variety of organisms including bacteria, fungi, plants and animals as a secondary metabolite. They can be purified from crude extracts of these organisms by acid-base extraction. Alkaloids have a wide range of pharmacological activities including anti-malarial (quinine), anti-asthma (ephedrine), anti-cancer (homoharringtonine), cholinomimetic (galantamine), vasodilatory (vincamine), anti-arrhythmic (quinidine), analgesic (emorphine), anti-bacterial (chelerythrine), and anti-hyperglycaemic activities (piperine). Many have found use in

traditional or modern medicine, or as starting points for drug discovery. Other alkaloids possess psychotropic (psilocin) and stimulant activities (cocaine, caffeine, nicotine, theobromine) and have been used in entheogenic rituals or as recreational drugs. Alkaloids can be toxic too (atropine, tubocurarine). Although alkaloids act on a diversity of metabolic systems in humans and other animals, they almost uniformly evoke a bitter taste [24].

2.5.2 Indole Alkaloids

Indole alkaloids are a class of alkaloids containing a structural moiety of indole; many indole alkaloids also include isoprene groups and are thus called terpene indole alkaloids. Many of them possess significant physiological activity and some of them are used in medicine. The amino acid tryptophan is the biochemical precursor of indole alkaloids [109]. These indole alkaloids can be divided into five major classes and each class consists of various skeletal types. An example of each skeletal is as listed in Table 2.5.

Table 2.5 Biogenetic classification of indole alkaloids [109]

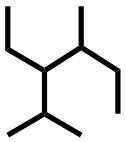
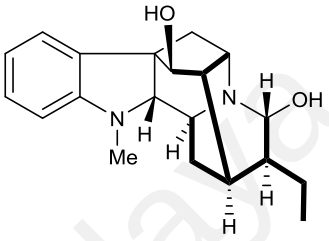
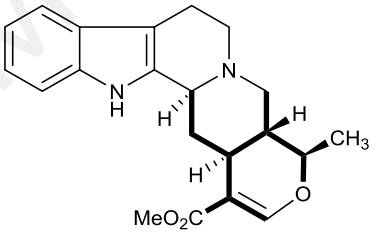
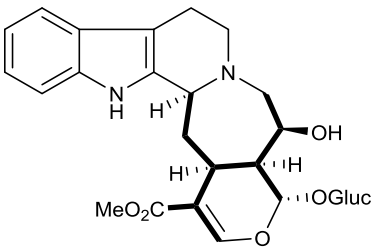
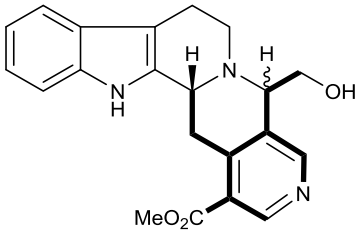
Class I Alkaloids 	
Group	**Example**
Ajmaline	 Ajmaline **61**
Ajmalicine	 Ajmalicine **8**
Cadambine	 3α-Dihydrocadambine **62**
Cadamine	 Cadamine **63**

Table 2.5 Continued

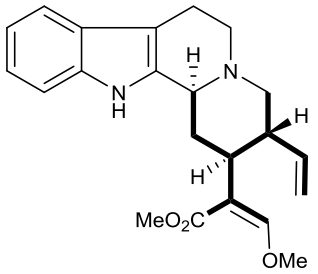
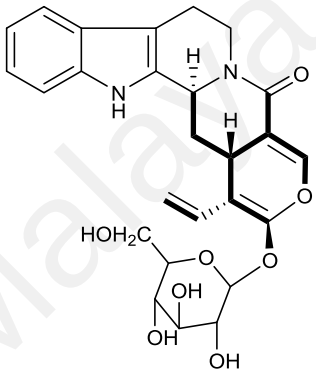
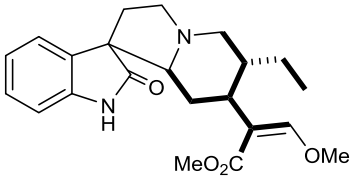
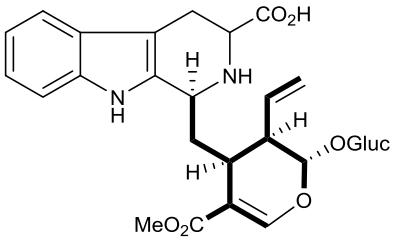
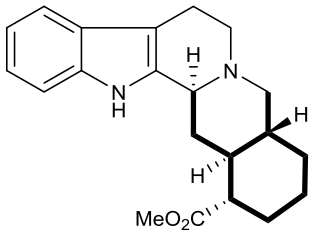
Corynantheine	 Corynantheine **64**
Strictosamide	 Strictosamide **65**
Rhyncophylline	 Rhyncophylline **11**
Vincoside	 5*α*-Carboxystrictosidine **66**
Yohimbine	 Yohimbine **67**

Table 2.5 Continued

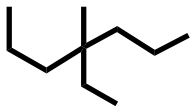
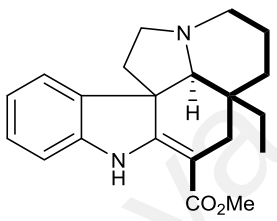
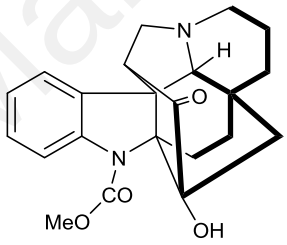
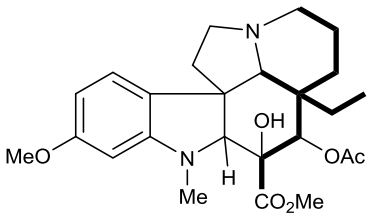
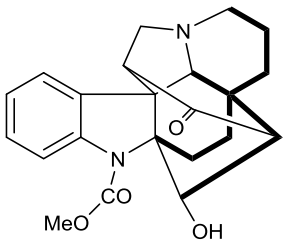
Class II Alkaloids 	
Group	**Example**
Aspidospermine	 Vincadifformine **68**
Kopsone	 Kopsine **69**
Vindoline	 Vindoline **70**
Fruticosine	 Fruticosine **71**

Table 2.5 Continued

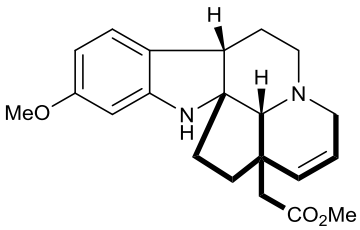
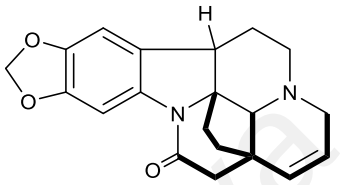
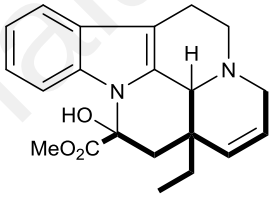
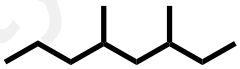
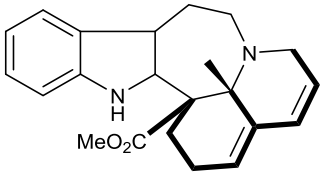
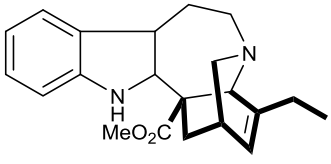
Schizophylline	 Schizophylline **72**
Schizozygine	 Schizozygine **73**
Vincamine	 Vincamine **74**
Class III Alkaloids 	
Group	**Example**
Andranginine	 Andranginine **75**
Catharanthine	 Catharanthine **76**

Table 2.5 Continued

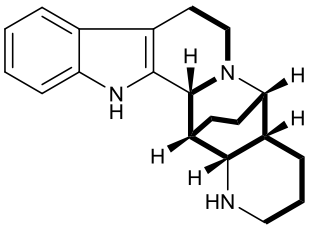
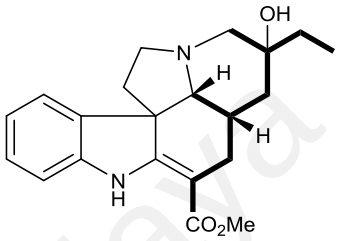
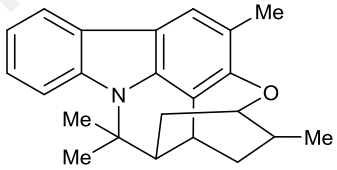
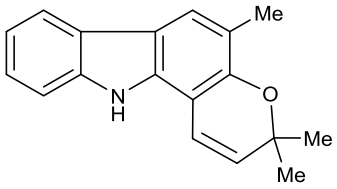
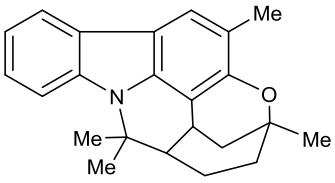
Nitramidine	 Nitrarine **78**
Pandoline	 Pandoline **79**
Class IV Alkaloids (not derived from secologanin)	
Non-tryptophan indole alkaloids (carbazole alkaloids)	
Group	**Example**
Currayangine	 Currayangine **80**
Girinimbine	 Girinimbine **81**
Mahanimbidine	 Mahanimbidine **82**

Table 2.5 Continued

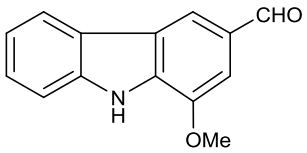
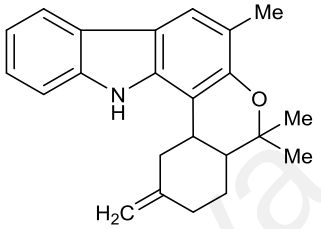
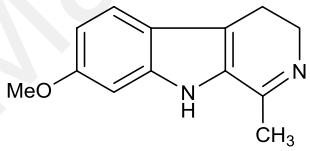
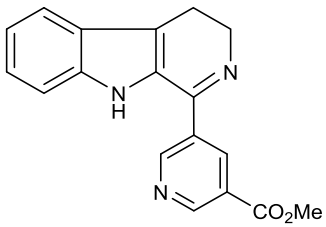
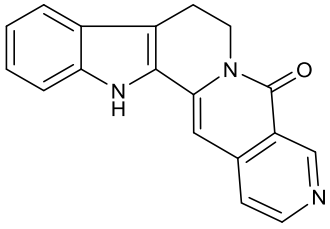
Murrayanine	 Murrayanine **83**
Murrayazolidine	 Murrayazolidine **84**
Non-isoprenoid tryptophan alkaloids	
Group	**Example**
Harmaline	 Harmaline **85**
Indolopyridine	 Indolopyridine 'A' **86**
Nauclefine	 Nauclefine **87**

Table 2.5 Continued

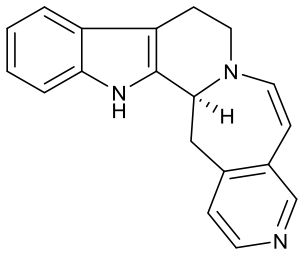
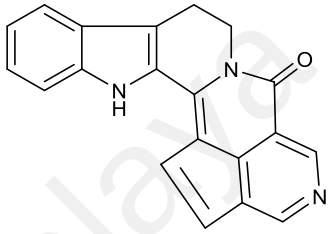
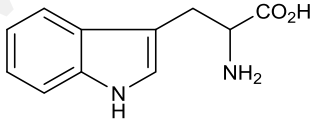
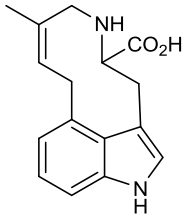
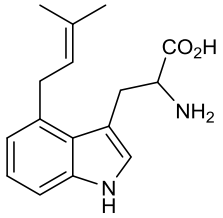
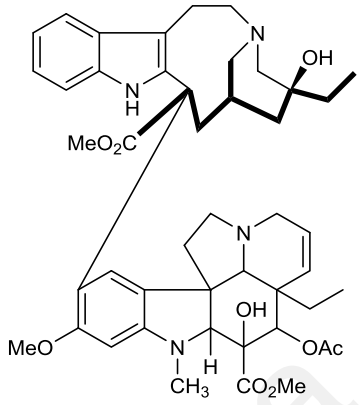
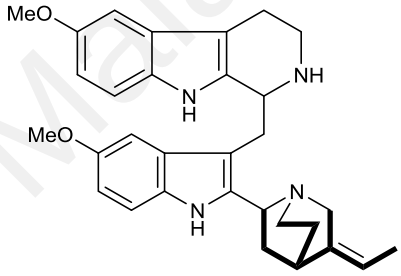
Naufoline	 Naufoline **88**
Nauclafine	 Nauclafine **89**
Tryptophan	 Tryptophan **90**
Isoprenoid tryptophan alkaloids (fungal indole alkaloids)	
Group	**Example**
Clavicipitic acid	 Clavicipitic acid **91**
Isopentenyl tryptophan	 4-isopentenyl tryptophan **92**

Table 2.5 Continued

Class V (binary indole alkaloids)	
Group	**Example**
II-III	 Vinblastine **93**
I-IV	 Cinochophyllamine **94**

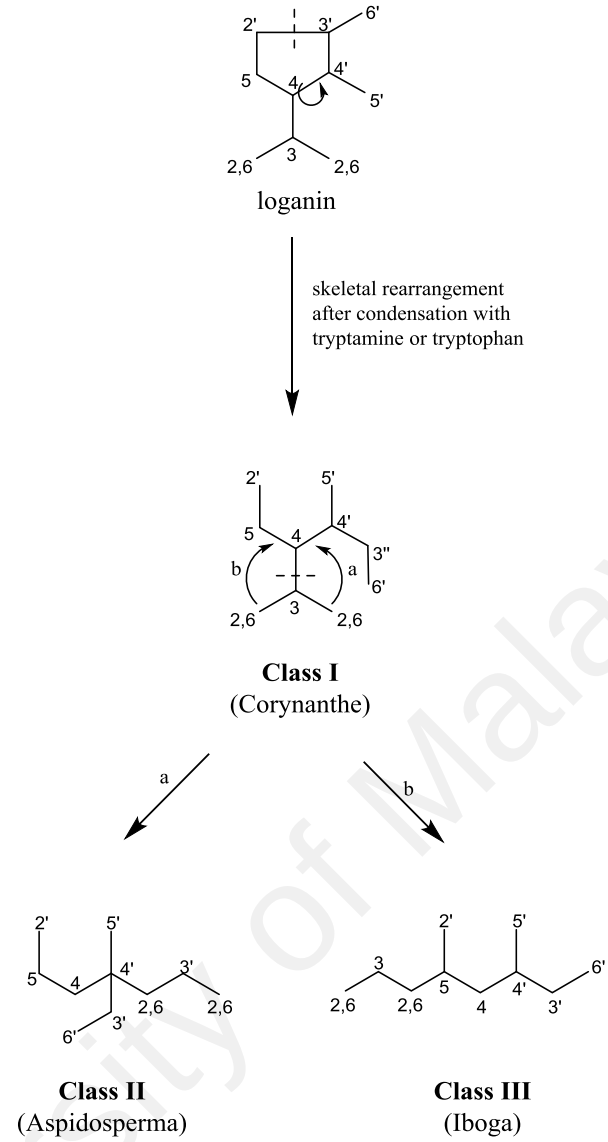
2.5.2.1 Indole Alkaloids of *Mitragyna speciosa*

Indole alkaloids from *M. speciosa* plant (Table 2.4) are all biogenetically derived from tryptophan **90** (tryptamine) and secologanine, which constitute the indole and terpenic portions, respectively (Scheme 2.2). Most alkaloids from this species belong to the Corynanthe type and its derivatives (Scheme 2.3). 25 alkaloids have been isolated and chemically characterized from *M. speciosa*, since 1960s [110]. The majority of the Corynanthe alkaloids from this species possess *S* or *α*- chirality at C15 [111].

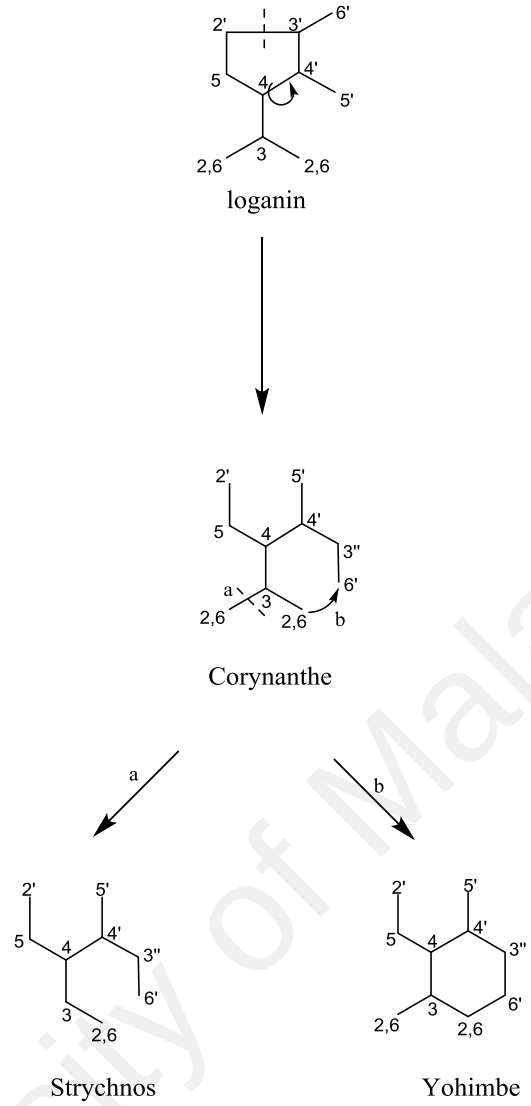
The Malaysian 'ketum' sample contained alkaloids such as mitragynine **1**, speciofoline **58**, ajmalicine **8**, speciogynine **3**, speciociliatine **4** and paynantheine **5**. The Malay

sample also had mitragynaline **51** and pinoresinol as minor components, as well as mitralactonal, mitrasulgynine and 3, 4, 5, 6-tetradehydromitragynine **59** [88]. Mitragynine **1** is the dominant alkaloid of the leaves and is exclusive to *M. speciosa*. Besides mitragynine **1**, speciociliatine **4** and paynantheine **5** are also unique to *M. speciosa* [22].

Working with the very young leaves of Malaysian 'ketum', 4 new types of indole alkaloids (corynantheidaline **36**, corynantheidalinic acid **35**, mitragynaline **51** and mitragynalinic acid **52**) were discovered by the Houghton and Said team. These new alkaloids were reported as having an unusual skeleton, featuring the presence of a carbon function at the C14 position (in comparison with previously known hitherto monoterpenoid indoles) [92]. However, another research team, led by Takayama, later revised the structure of the mitragynaline and corynantheidaline alkaloids, proving that there was actually no substitution on the 14 position [112].



Scheme 2.2 Three major skeletal of indole alkaloids from loganin

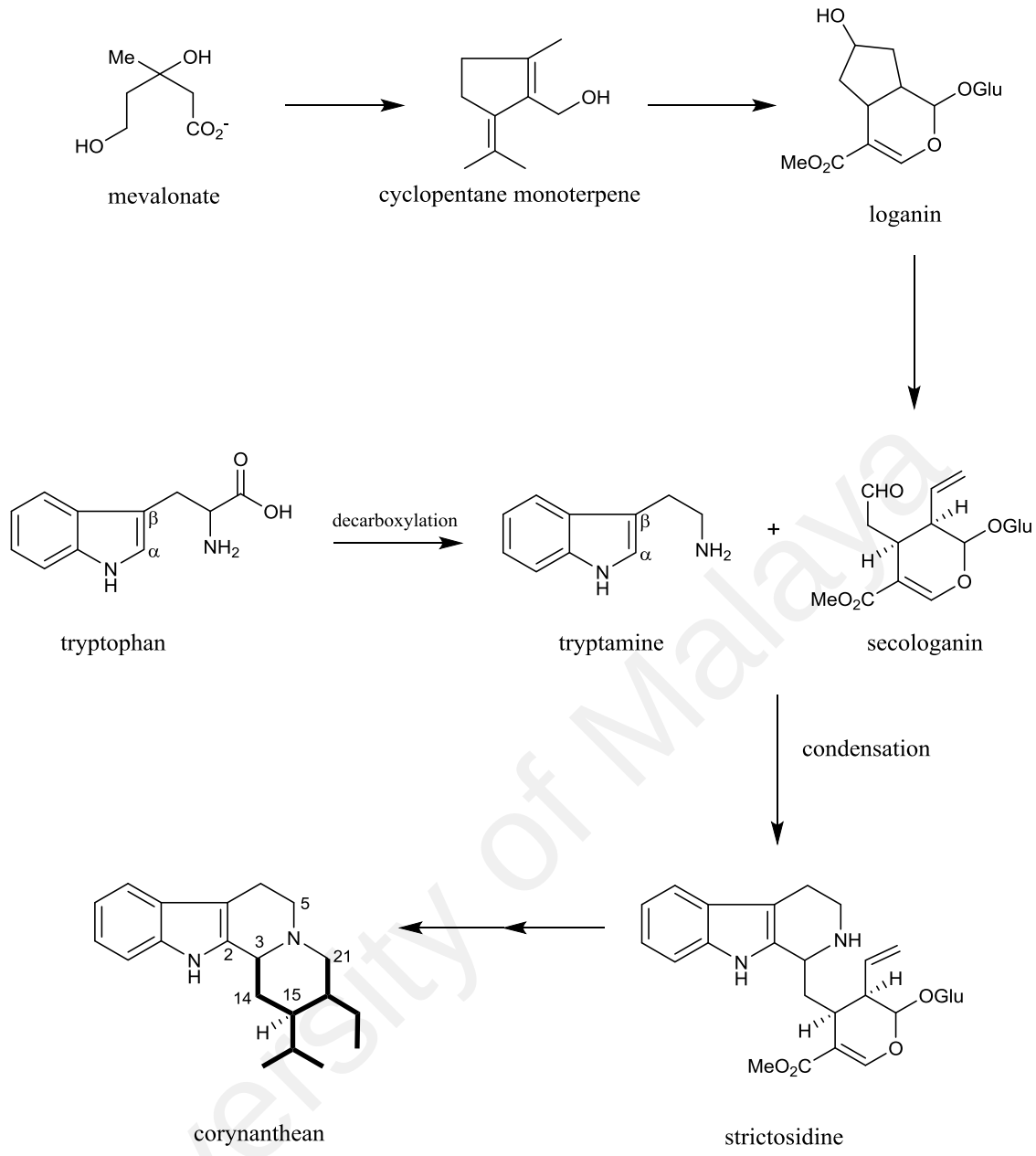


Scheme 2.3 Various skeletal types of indole alkaloids from rearrangement of Corynanthe type (Class I)

2.5.2.2 Biosynthesis of Corynanthean Indole Alkaloids

As mentioned earlier, most of the alkaloids from *M. speciosa* are of the Corynanthean type (Class I) in which an unrearranged secologanin unit can be recognized in combination with tryptamine/tryptophan (Scheme 2.4). Corynanthean alkaloids are made up of two major parts, indole and monoterpenoid. Both parts are linked at C3 and C21. The aromatic indole part of these alkaloids is derived from the decarboxylation of tryptophan to form tryptamine. This idea was proved by Battersby, Burnett and Parsons

(1969) where it was initially suggested by Perkin and Robinson (1919) [109]. The basic unit of indole alkaloids is derived from the Mannich condensation of tryptamine with an aliphatic aldehyde (bearing nine or ten carbons) at the α- or β–position of the indole nucleus to form a Schiff base. The monoterpenoid origin of the non-tryptophan portion (shown in heavy lines in the Corynanthean structure, Scheme 2.4) was formed from two mevalonate units to afford a cycpentane monoterpene. It has been firmly, established that all naturally occurring monoterpenoid indole alkaloids are derived from strictosidine (formerly known as isovincoside), which is the initial product of the condensation of secologanin and tryptamine [113].



Scheme 2.4 Origin of Corynanthean monoterpenoid indole alkaloid

CHAPTER 3: BIOLOGICAL STUDIES

The variety of pure constituents discovered in *Mitragyna speciosa* samples to this day still calls for further studies and experimentation, particularly, investigation on their specific activity, effects and potential applications. Through its traditional use, it is clear *M. speciosa* is much more than a simple opioid-like narcotic and mild stimulant. Many of the secondary chemicals found in this species are present in small yet appreciable quantities and their synergetic role and activity in the general pharmacology of *M. speciosa* is not yet fully understood, as thorough research has only just begun.

Prior to the late 1990's, nearly all of the chemical studies of *M. speciosa* activity focused on mitragynine with the assumption that this dominant constituent was the main active alkaloid. With 7-hydroxymitragynine now clearly identified out as the principal psychoactive alkaloid in *M. speciosa*, many elements of these studies need to be revised [83-86, 94, 95].

In our search for natural products with anti-inflammatory activity, we were inspired by the traditional usage of this unique plant as it is used in the treatment of fever, cough, muscle pain relief and also as a drinking tonic, which seems to indicate that there are anti-inflammatory potential [29, 34, 35, 48]. Previously, almost all of the studies have been conducted on the alkaloidal fractions of the organic solvents. Whereas in the current investigations, focus were given on the non-organic solvent crudes, which are the aqueous and SFE crude extracts that not only consist of the alkaloids but also the non-alkaloidal fractions.

In this chapter, a brief overview deals with inflammatory signalling, especially in relation to human neutrophils and eosinophils are discussed. Various types of extracts from the leaves of *M. speciosa* were subjected to *in vitro* nitric oxide (NO) inhibition

test via the Griess assay. Then, the anti-inflammatory study was further conducted on the aqueous extract (MSAE) through *in vivo* anti-ulcerogenic test using ethanol-induced gastric damaged in rats. This includes the anti-oxidant studies such as superoxide dismutase (SOD) activity and malondialdehyde (MDA) level. Immunohistochemistry was also studied by the effect of MSAE pre-treatment on the heat shock proteins (HSP70) and pro-apoptotic protein (BAX). Additionally, the acute toxicity of the MSAE was also determined.

3.1 Inflammation

The inflammatory process is usually tightly regulated, involving both signals that initiate and maintain inflammation and signals that shut the process down. An imbalance between the two signals leaves inflammation unchecked, resulting in cellular and tissue damage [114].

Due to resistance of such diseases to conventional treatments, as well as the side effects of presently available anti-inflammatory drugs, there is a pressing need for the development of novel anti-inflammatory drugs. Natural products are a valuable source of novel bioactive secondary metabolites. Various bioassays are available in which the anti-inflammatory activity of these natural products can be evaluated. Macrophages have been implicated in many of these assays, as they are the body's first line of defence and directly involved with the inflammatory response. The macrophage is a vital type of white blood cell that engulfs and digests debris (like dead cells) and foreign substances (antigens) that don’t belong in the body through the process of phagocytosis.

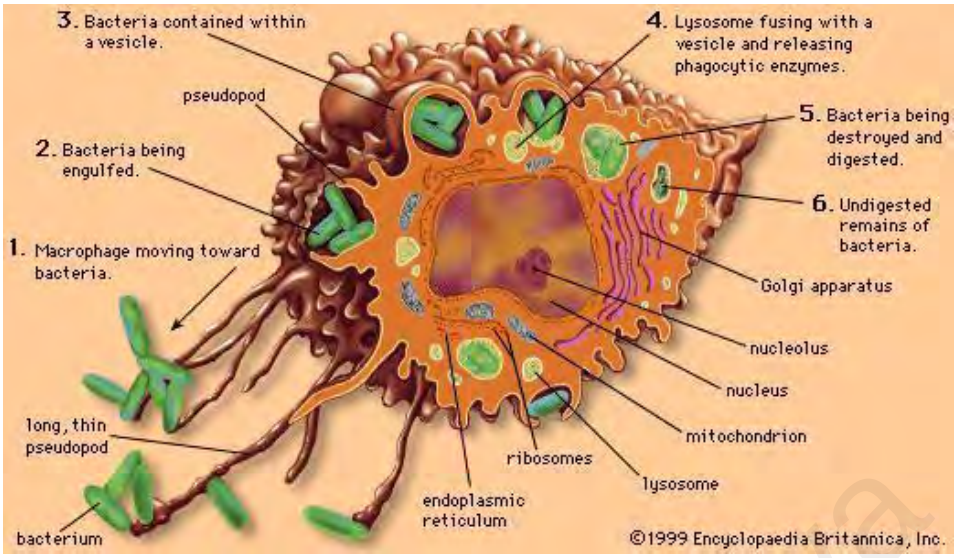


Figure 3.1 Macrophage cell

Macrophages are formed through differentiation of specific white blood cells called monocytes. Monocytes are originated from stem cells in the bone marrow and circulate throughout the blood stream. When there is tissue damage or infection, the monocytes migrate into the affected tissue or organ and undergo a series of changes to become macrophages. Once a monocyte leaves the blood, it matures into a *wandering macrophage* or a *fixed macrophage.* Wandering macrophages travel throughout both blood and lymph streams to perform their job; fixed macrophages strategically concentrate in specific areas that are more vulnerable to intruders like the lungs or the intestine. Macrophages can then be found in many areas in the body, like different tissues, lungs, skin, and also organs of the immune system like the spleen, lymph nodes, and bone marrow.

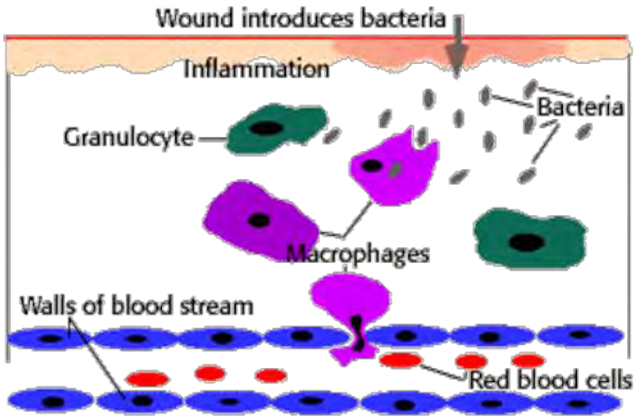


Figure 3.2 Migration of monocytes into the affected tissue or organ to become macrophages

In inflammation, macrophages have three major function; antigen presentation, phagocytosis, and immunomodulation through production of various cytokines and growth factors. Macrophages play a critical role in the initiation, maintenance, and resolution of inflammation. They are activated and deactivated in the inflammatory process. Activation signals include cytokines (interferon gamma, granulocyte-monocyte colony stimulating factor, and tumour necrosis factor alpha), bacterial lipopolysaccharide, extracellular matrix proteins, and other chemical mediators. Inhibition of inflammation by removal or deactivation of mediators and inflammatory effector cells permits the host to repair damages tissues. Anti-inflammatory cytokines (interleukin 10 and transforming growth factor beta) and cytokine antagonists that are mainly produced by macrophages can deactivate the activated macrophages. Macrophages participate in the auto regulatory loop in the inflammatory process. Because macrophages produce a wide range of biologically active molecules participated in both beneficial and detrimental outcomes in inflammation, therapeutic interventions targeted macrophages and their products may open new avenues for controlling inflammatory diseases.

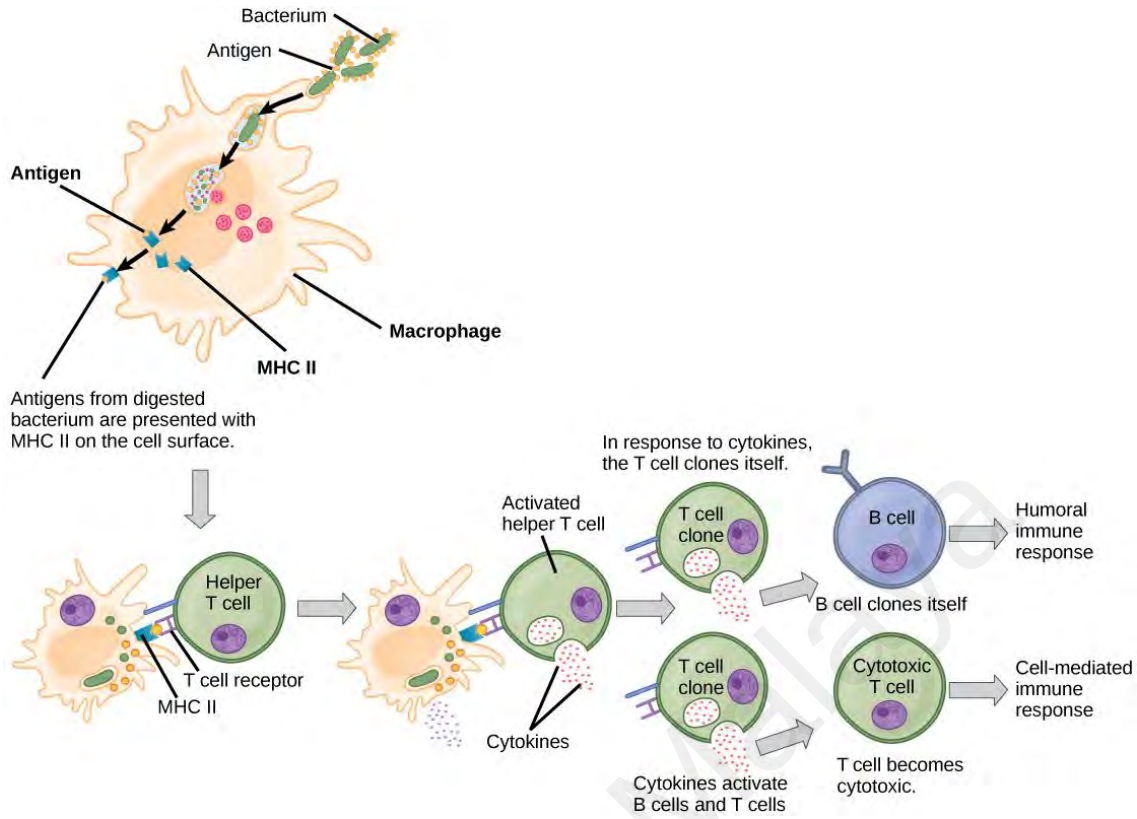


Figure 3.3 Macrophages major function as an antigen presentation, phagocytosis, and immunomodulation through production of various cytokines and growth factors

Nitric oxide (NO) is recognized as a mediator and regulator of inflammatory responses. It possesses cytotoxic properties that are aimed against pathogenic microbes, but it can also have damaging effects on host tissues. NO is produced by the oxidative deamination of the amino acid L-arginine by specific enzyme called nitric oxide synthases (NOS) [114]. There are three isoforms of this enzyme and an oxygenase and a reductase domain constitute them all. Neuronal NOS (nNOS or NOS1) is constitutively present in both the central and peripheral nervous systems, where NO acts as a neurotransmitter. Endothelial NOS (eNOS or NOS3) is constitutively expressed by endothelium and other cell types and is involved in cardiovascular homeostasis. In contrast, inducible NOS (iNOS or NOS2) is absent in resting cells, but the gene is rapidly expressed in response to stimuli such as proinflammatory cytokines. Once present, iNOS synthesizes 100-1000 times more NO than the constitutive enzymes and does so for prolong periods. This high concentration of NO may inhibit a large variety

of microbes, but may also potentially damage the host, thereby contributing to pathology [114, 115].

3.1.1 *In Vitro* Nitric Oxide Inhibitory Activity

NO reacts with soluble guanylate cyclase to form cyclic guanosine monophosphate (cGMP), which mediates many of the effects of NO. NO can also interact with molecular oxygen and superoxide anion to produce reactive nitrogen species that can modify various cellular functions. These indirect effects of NO have a significant role in inflammation, where NO is produced in high amounts by inducible nitric oxide synthase (iNOS) and reactive oxygen species are synthesized by activated inflammatory cells [115].

NO is a signalling molecule that plays a key role in the pathogenesis of inflammation. It gives an anti-inflammatory effect under normal physiological conditions. On the other hand, NO is considered as a pro-inflammatory mediator that induces inflammation due to over production in abnormal situations. NO is synthesized and released into the endothelial cells by the help of nitric oxide synthases (NOSs) that convert arginine into citrulline producing NO in the process. Oxygen and NADPH are necessary co-factors in such conversion. NO is believed to induce vasodilatation in cardiovascular system and furthermore, it involves in immune responses by cytokine-activated macrophages, which release NO in high concentrations. In addition, NO is a potent neurotransmitter at the neuron synapses and contributes to the regulation of apoptosis. It also involved in the pathogenesis of inflammatory disorders of the joint, gut and lungs [115].

3.1.2 *In Vivo* Gastroprotective Activity

A stomach ulcer or so-called gastric ulcer is an inflammation, irritation as well as

erosion, exfoliation or desquamation that occurs when there is imbalance between aggressive factors (endogenous: hydrochloric acid, pepsin; exogenous: *Helicobacter pylori* infection, ethanol, smoking) and protective factors (parietal cell, mucosal barrier, mucus and bicarbonate secretion, mucosal blood flow, cellular regeneration, prostaglandins and epidemic growth factors) [116, 117]. Such pathologic alteration will cause the defence mucus barrier to be disrupted and exposed the gastrointestinal wall to the corrosive acid and pepsin. In most cases, *Helicobacter pylori* infection and in rare cases, other infections such as tuberculosis, syphilis, viral infections, fungal infections, bacteria parasites and worms cause peptic ulcer diseases [118]. Ulcer can also be caused or worsened by some medications like ibuprofen, aspirin and diclofenac that are non-steroidal anti-inflammatory drugs (NSAIDs) and also potassium and iron supplements [119]. Inhibiting acid secretion is the general protocol for treating gastritis. This can be achieved by consuming acid-suppressing drugs [120]. However, the adverse effects and drug-drug interactions of the conventional therapies highlight the need of development for better treatment modalities for gastritis. Owing to the property of natural products as a source of novel bioactive secondary metabolites, they possess a valuable role as an alternative treatment [121]. In medical literatures and folk medicine, a huge number of traditional medicinal plant extracts were used for the remedy of various disorders including gastroprotection [122-128].

3.2 Plant Material and Extraction Procedures

The plant sample used (KL5321) was described under sub-heading 4.1 in Chapter 4. Hexane (MSH), ethyl acetate (MSEA), aqueous (MSAE), methanol (MSM) and carbon dioxide crudes (M1S1, M1S2, M2S2, M4S1, M4S2, M5S1, M5S2, M6S1, M6S2, M7S1, M7S2, M8S1, M8S2, M9S1, M9S2) were subjected to the *in vitro* NO inhibitory

test and the extraction methods of the crudes were as explained in 4.4.1, 4.4.2 and 4.4.3. The *in vivo* gastroprotective activity was performed on the MSAE.

3.3 Chemicals and Reagents

In vitro NO inhibition activity: Anti-biotic (5000 U/mL penicillin and 5000 μg/mL streptomycin) and Dulbecco's Modified Eagle's Media (DMEM) were obtained commercially from Flowlab^{TM}, Australia; fetal bovine serum (FBS) from Mycoplex^{TM} (PAA Lab. GmbH, Austria); recombinant mouse interferon gamma (IFN-γ) from BD Pharming, USA; N^{ω}-nitro-L-arginine methyl ester (L-NAME) hydrochloride (MW 269.7), lipopolysaccharide (LPS) from *Escherichia coli* (strain 0111:B4), sulphanilamide and naphtylethylenediamine from SIGMA Chemical Co., USA. Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific, USA and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MMT) from Fluka Chemie GmbH, Switzerland.

In vivo gastroprotective activity: All materials and reagents were acquired from Sigma (Sigma Aldrich, Germany), while superoxide dismutase (SOD) and malondialdehyde (MDA) assay kits were purchased from Cayman Chemical Company (Cayman, USA). Omeprazole as the anti-ulcer reference drug was obtained from the University of Malaya Medical Centre (UMMC). This drug was prepared by dissolving it in distilled water and administered orally at a dose of 20 mg/kg body weight (5mL/kg) according to Mahmood *et al*.

3.4 *In Vitro* Nitric Oxide Inhibition Activity

This *in vitro* test involved determination of various *M. speciosa* extracts effect on the inhibition of NO production by the macrophages cell using the Griess assay.

Consequently, the toxicity of each tested extract was revealed through the MTT cell viability assay.

3.4.1 Cell Culture and Treatment

Murine monocytic macrophages cell line (RAW 264.7 cells) was purchased from American Type Culture Collection (ATCC). The RAW 264.7 cells were established from an ascites of a tumour induced in a male mouse by intraperitoneal injection of Abselon Leukaemia Virus (A-MuLV). Cells will pinocytose neutral red and phagocytose zymosan. Those cells are also capable of anti-body dependent lysis of sheep erythrocytes and tumour targets and LPS can inhibit their growth.

In this study, RAW 264.7 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin under 5% CO_2 at 37 ^oC. Cells at 80-90% confluent were scrapped out and seeded into 96-well plate at 5×10^4 cells/50 μL per well and incubated for 2 h at 37 ^oC, 5% CO_2 to allow cell attachment. Attached cells were then induced with 100 U/mL of IFN-γ plus 5 μg/mL of LPS in the presence or absence of plant sample tested at a final concentration of 100 μg/mL. The final concentration of the sample vehicle (DMSO) in the reaction mixture was 0.1%. Untreated and drug controls were also stimulated with IFN-γ/LPS and received the same amount of DMSO in culture media. Cells were then incubated at 37 ^oC, 5% CO_2 for 17-20 h [129].

3.4.2 Griess Assay

The inhibitory activity of the plant extracts on NO production was determined by measuring the nitrite ion (NO_2^- - stable metabolite of NO) formation in the supernatants of spent cell culture media [130]. Briefly, 50 μL of Griess reagent (1% sulphanilamide

and 0.1% naphtylethylenediamine dihydrochloride in 2.5% H_3PO_4) was added to 50 μL of cell culture supernatant. After an incubation time of 10 min, the colour development was assessed at λ 550 nm on a micro plate reader (SpectraMax, Plus 384, Molecular Devices, Inc., USA) at room temperature. Fresh culture medium (DMEM only) was used as the blank in all the experiments. The amount of nitrite in the samples was calculated from a sodium nitrite standard curve (0-100 μM) freshly prepared in deionized water. The percentage of inhibition was calculated against control cells that were not treated with plant sample but induced with IFN-γ/LPS and contained 0.1% DMSO [129]. Percentage of inhibition was calculated as:

$$\text{NO inhibitory (\%)} = \frac{[NO_2^-]_{control} - [NO_2^-]_{sample}}{[NO_2^-]_{control}} \times 100\%$$

3.4.3 MTT Cell Viability Assay

Cell viability indicator (mitochondrial respiration) was assessed by the mitochondria-dependent reduction of MTT to formazan. After the removal of 50 μL culture media for the Griess assay, the cells remaining in the tissue culture plates were then assayed for cell viability. The wells in the micro titre plate were topped up with 100 μL of DMEM followed by the addition of 20 μL of MTT (5 mg/mL). The cells were incubated under 5% CO_2 at 37 ^oC for 4 h. Then the cell medium was removed and the formed formazan crystals were dissolved with 100 μL/well of 100% DMSO. The extent of reduction of MTT to formazan within the cells was quantified by measuring the optical density [129] and the absorbance was recorded at λ 570 nm on a micro plate reader (SpectraMax, Plus 384, Molecular Devices, Inc., USA). The OD_{570} of the samples were compared to that of control cells (untreated stimulated cells and contained 0.1 % DMSO) to obtain the

percentage viability using the formula stated below [129]:

$$\text{Cell Viability (\%)} = \frac{OD_{sample} \times 100\%}{OD_{control}}$$

3.4.4 Statistical Analysis

The results are expressed as mean ± standard deviation (SD). Data were statistically analysed using Graph pad prism version 5.0 for one-way analysis of variance (ANOVA) with Dunnett's test. Differences were considered significant at $p < 0.05$.

3.5 *In Vivo* Gastroprotective Activity

In vivo gastroprotective activity involved studies on the effect of MSAE in rats in terms of toxicity, inhibition of ulceration induced by ethanol, anti-oxidant activities (SOD activity and MDA level) and immunohistochemical activities (effect on HSP70 and BAX).

3.5.1 Acute Toxicity

The acute toxicity study was used to determine a safe dosage of MSAE following the 'fix dosage' technique of Organization for Economic Cooperation and Development (OECD) Guideline No. 420. Thirty-six healthy Sprague Dawley rats were obtained from the Experimental Animal House, Faculty of Medicine, University of Malaya. The rats (5-7 weeks old) weighed between 150-160 g were assigned equally into 3 groups (six female and six male in each group): vehicle (distilled water), 500 mg/kg and 1000 mg/kg of MSAE, respectively. The animals were fasted overnight (accessible to tap water) prior to the dosing. Food was further withheld for 3 to 4 h after dosing. The

animals were observed for every 30 min following the administration for general behavioural, signs and symptoms of toxicity indications and finally for mortality after 24 h over a period of 2 weeks. The animals were sacrificed on the 15th day. Serum biochemical, haematological and histological (liver and kidney) parameters were evaluated. The study was approved by the Ethics Committee for Animal Experimentation, Faculty of Medicine, University of Malaya, Malaysia with the Ethic no. PM/08/06/2012/MMA (R). Throughout the experiments, all animals received human care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the United States National Academy of Sciences and published by the National Institutes of Health [131].

3.5.2 Animals of Gastric Ulcer Evaluation

Healthy mature Sprague Dawley rats (two months old, 200–220 g weight) were obtained from The Experimental Animal Unit, University of Malaya. The rats were housed individually in cages of broad line net under standard laboratory conditions (25 ± 2 ^{o}C; 12 h light/dark cycle). Rats were maintained on standard pellet diet and tap water, which then were deprived of food one night prior to the experiment. Water was removed only sixty minutes before the test was conducted. The animal experimental design of this study was constructed according to the rule of Animal Care and Employ Committee from Laboratory Animal Science Centre, Faculty of Medicine, University of Malaya.

3.5.3 Dose Selection for Anti-ulcer Assessment

In reference to the results of acute toxicity study, two dose levels of MSAE were chosen in such a way that the low dose (100 mg/kg) was one-tenth of the highest dosage tested

in acute toxicity study (1000 mg/kg) while the high dose (200 mg/kg) was two times of the low dosage.

3.5.4 Ethanol-induced Gastric Ulcer

The following pre-treatment was constructed according to the recommendations of Abdulla *et al*. [132]. The 24 hours fasted rats were divided randomly into 5 groups of 6 rats each. Groups 1 and 2 orally administered with distilled water and act as the normal control and ulcer control groups, respectively. Group 3, the reference group received an oral dose of 20 mg/kg Omeprazole in distilled water (5 mL/kg). Groups 4 and 5, the 'investigational groups' were given MSAE*,* at dose level of 100 and 200 mg/kg, respectively. At 1 h after pre-treatment, Group 1 rats were orally administered with distilled water (5 mL/kg), while those of Groups 2–5 received absolute ethanol (5 mL/kg). An hour later, the animals were euthanized under high dose of xylazine and ketamine anaesthesia followed by cervical dislocation technique to assure their euthanasia. Then their stomachs were immediately removed and reserved in normal saline [132].

3.5.5 Gastric Juice Acidity Assessment

The excised stomachs were rapidly dissected along the greater curvature. The gastric juice was collected and centrifuged at 3000 rpm for 10 min and then was subjected to titration against 0.01 N NaOH to pH 7 [133].

3.5.6 Gastric Wall Mucus (GWM) Assessment

The customized method of Corne *et al*. was used to analyse the gastric wall mucus [134]. In brief, the glandular segments of the rat stomachs were each transferred to 1%

Alcian blue solution (in sucrose solution, buffered with sodium acetate, pH 5) immediately after removing and weighing them. The segments were then washed with sucrose solution to remove the excess dye. The dye that was in complex with the gastric wall mucus was then extracted with $MgCl_2$ solution. An amount of 4 mL of the blue extract was then shaken with an equal volume of diethyl ether. The resulting emulsion was centrifuged and absorbance of the supernatant was then measured at 580 nm. GMW was calculated by the amount of Alcian blue extracted /g glandular tissue. The stomach samples were then washed with iced phosphate buffered saline (PBS) and prepared for ulcer area measurement.

3.5.7 Macroscopic Gastric Lesions Estimation

The lesions due to gastric mucosal injury appeared in the form of elongated haemorrhagic bands parallel to the long axis of the stomach. The gastric mucosa of all experimental animals was examined for estimate damage. A planimeter (10 x 10 mm^2 = ulcer area) was used to measure length and width of the lesion bands under a dissecting microscope (1.8x). Each ulcer lesion's region was calculated by totalling the small squares (2 x 2 mm^2) covering the ulcer band. Consequently, the sum of the lesion areas was incorporated in the calculation for the ulcer area (UA) of each gastric according to recommendation of Abdulla *et al*., [132] as follows:

$$UA\ (mm^2) = \text{the sum of small squares} \times 4 \times 1.8$$

To calculate the inhibition percentage (I%), the following formula was applied:

$$(I\%) = \frac{(UAcontrol - UAtreated)}{UAcontrol} \times 100\ \%$$

3.5.8 Preparation of Gastric Homogenate

The samples of stomach glands were rinsed thoroughly with ice-cold normal saline. The glandular gastric homogenates (10% (w/v)) were prepared using cold PBS (50 mM, pH 7.4) contained cocktail of mammalian protease inhibitor. The homogenates were centrifuged at 4,500 rpm for 15 min at 4 ^{o}C. The supernatants were then divided into aliquots and kept at -80 ^{o}C for further experiments.

3.5.8.1 Measurement of Anti-oxidant Activities of Stomach Homogenate

SOD activity and MDA level in gastric were assessed according to the SOD and MDA commercial assay kits company's manuals (Cayman Chemical Co., Mich, USA). The supernatants of the homogenates were centrifuged for the second time at 12,000 rpm for 20 min at 4 ºC before assayed for SOD activity. Then, 30 μL of the sample was mixed with 1 mL of reactant (50 mM phosphate buffer, 100 nM EDTA and 13 mM L-methionine, pH 7.8), 150 μL of 75 μM NBT and 300 μL of 2 μM riboflavin in a dark chamber. The mixture was then exposed to fluorescent light (15 W) for 15 min prior to measurement of spectrophotometer absorbance at λ 560 nm. While MDA production due to lipid peroxidation was determined by mixing well 0.2 mL of the stomach homogenate with 8.1% sodium dodecyl sulphate, 20% acetate buffer (pH 3.5) and 0.8% thiobarbituric acid (TBA) for 3 min and then incubated at 95 ^{o}C for 60 min. After chilling, MDA (TBA-reactive substance) was extracted with 1 mL of H_2O_2 and 2.5 mL of *n*-butanol:pyridine mixture (15:1, v/v). The upper layer, which was the organic layer that contained MDA, was measured at λ 532 nm.

3.5.8.2 Measurement of Protein Concentration

Protein concentration in all the stomach tissue homogenates was measured by Biuret

reaction according to method described by Lowry [135]. Briefly, the method combines the reactions of copper ions with the peptide bonds (involves reduction of the Folin–Ciocalteu reagent) with the oxidation of aromatic protein residues (mainly tryptophan also tyrosine). The Lowry method is best used with protein concentrations of 0.01–1.0 mg/mL. The concentration of the reduced Folin reagent is measured by absorbance at 750 nm. Thus, the total concentration of protein in the sample can be deduced from the concentration of tryptophan and tyrosine residues that reduce the Folin–Ciocalteu reagent.

3.5.9 Histopathology of Gastric Wall

3.5.9.1 Preparation of Tissue Sections

Specimens of the gastric walls were fixed in 10% phosphate buffered formalin for 24 h at room temperature. These were then processed using automated paraffin tissue-processing machine (Leica, Germany). The tissues were embedded in paraffin wax and then cut into sections adopted at a thickness of 5 μm.

3.5.9.2 Haematoxylin and Eosin

Stomach sections were stained with haematoxylin and eosin for histological evaluation [136].

3.5.9.3 Study of Mucosal Glycoproteins

Another part of sections was stained with periodic acid Schiff (PAS) for gastric mucosal glycoprotein evaluation [137].

3.5.10 Immunohistochemical Staining

The slides containing stomach tissue sections were heated at 60 ^{o}C for 25 min in a microwave oven. Then the tissue sections were deparaffinised by dipping the slides in xylene and then rehydrated with alcohol. Antigen retrieval process was performed via microwave boiling of samples in 10 mM sodium citrate buffer. Immunohistochemistry staining procedure was conducted according to Dako manufacturer's protocol (Dako-Cytomation, USA). In brief, 0.03% H_2O_2 containing sodium azide (peroxidase block) was used to block the endogenous peroxidase enzyme for 5 min. Carefully washed with wash buffer, tissue sections were then incubated with BAX (1:200) and HSP70 (1:500) biotinylated primary anti-bodies for 15-20 min. Following incubation and gentle washing with wash buffer, the sections were placed in a buffer bath, and then re-incubated with streptavidin-HRP (streptavidin conjugated to horseradish peroxidase in PBS containing an anti-microbial agent) in a humidified chamber for 15 min. Later, the sections were washed again with wash buffer and placed in the buffer bath prior to the Diaminobenzidine substrate-chromogen incubation. After 5 seconds of incubation, tissue slides were washed following counterstained with haematoxylin for another 5 seconds. Then the sections of stomachs were dipped in weak ammonia (0.037 mol/L) for 10 times. Followed by rinsing with distilled water prior to the mounting of the cover slips. The target antigens should be seen as brown stains when examined under light microscope.

3.5.11 Statistical Analysis

Experimental data values were reported as mean ± standard error mean (S.E.M). The statistical significance of differences between groups was analysed using one-way ANOVA, followed via post hoc analysis. A probability value of $p < 0.05$ was

considered as statistical significant.

3.6 Results and Discussion

In vitro nitric oxide inhibitory test was performed on the M5S1 extract that was obtained by means of SFE while the *in vivo* gastroprotective activity was conducted on the aqueous extract of the leaves of *M. speciosa*.

3.6.1 *In Vitro* Nitric Oxide Inhibitory Activity

In murine macrophage RAW 264.7 cells, LPS stimulation alone has been demonstrated to induce iNOS transcription and its protein synthesis, with a corresponding increase in NO production. Furthermore, LPS stimulation has also been shown to induce IκB proteolysis and NF-κB nuclear translocation [138]. Therefore, this cell system is an excellent model for drug screening and the subsequent evaluation of potential inhibitors against iNOS and NO production. In our attempt to investigate the anti-inflammatory activity of *M. speciosa*, a total of 19 different extracts from the leaves of the plant were tested for their inhibitory effect of NO release in RAW 264.7 cells. The overall efficacy of all plant extracts on nitrite production and cell viability in IFN-γ/LPS-activated macrophage are presented in Table 3.1.

The plant extracts were considered as having either a strong, moderate or weak activity, if the inhibition of NO release was more than 90%, between 50% and 89%, or less than 50%, respectively, as compared to the control. Meanwhile, the viability of RAW 264.7 cells assessed by MTT method must be above 85% to prove that the decrease in NO production was not a result of cell death [139]. NOS inhibitor, L-NAME was used as the positive control in this assay strongly (86.65%) inhibited the production of NO without any cytotoxic effect at the concentration of 250 μM (0.674 μg/mL). Of the 19

kinds of extracts, one extract exhibited more than 100%, 6 extracts demonstrated 51 to 64% and 12 extracts with 16 to 48% inhibitory activity towards NO production when tested at a final concentration of 100 μg/mL. Although MSH showed strong inhibition (101.29 ± 10.05 %), its MTT assay indicated cytotoxic effect (cell viability, -0.29 ± 1.00%). Meanwhile, among those samples that moderately reduced NO production, M5S1 showed the highest NO inhibitory activity at 60.08 ± 10.02% without any cytotoxicity effect (cell viability, 91.98 ± 5.58%).

Table 3.1 The effect of *M. speciosa* extracts on NO production and cell viability of RAW 264.7 macrophage cells tested at final concentration of 100 μg/mL

Sample	Type of Extract / SFE Condition	Result	
		NO Inhibition (%)	Cell Viability (%)
MSH	Hexane Extract	101.29 ± 10.05	-0.29 ± 1.00
MSEA	Ethyl Acetate Extract	48.13 ± 8.02	61.37 ± 5.50
MSM	Methanol Extract	22.07 ± 4.05	61.58 ± 5.05
MSAE	Aqueous Extract	37.17 ± 4.05	107.16 ± 9.55
M1S1	1300 psi, 40 °C, 100% CO_2	21.13 ± 4.10	65.41 ± 16.06
M1S2	1300 psi, 40 °C, 20% EtOH	56.86 ± 8.83	68.51 ± 15.55
M2S2	1300 psi, 60 °C, 40% EtOH	63.83 ± 4.94	69.40 ± 16.58
M4S1	3000 psi, 40 °C, 100% CO_2	54.37 ± 7.19	79.39 ± 6.26
M4S2	3000 psi, 40 °C, 40% EtOH	42.22 ± 4.54	88.46 ± 9.47
M5S1	3000 psi, 60 °C, 100% CO_2	60.08 ± 10.02	91.98 ± 5.58
M5S2	3000 psi, 60 °C, 60% EtOH	24.56 ± 9.78	88.10 ± 15.22
M6S1	3000 psi, 80 °C, 100% CO_2	44.79 ± 1.62	94.45 ± 11.35
M6S2	3000 psi, 80 °C, 20% EtOH	40.35 ± 11.57	75.61 ± 6.38
M7S1	5000 psi, 40 °C, 100% CO_2	51.44 ± 15.02	95.28 ± 8.66
M7S2	5000 psi, 40 °C, 60% EtOH	19.88 ± 6.02	92.92 ± 14.08
M8S1	5000 psi, 60 °C, 100% CO_2	48.24 ± 0.88	88.33 ± 13.65
M8S2	5000 psi, 60 °C, 20% EtOH	46.65 ± 2.31	93.95 ± 14.70
M9S1	5000 psi, 80 °C, 100% CO_2	54.91 ± 2.84	91.32 ± 11.00
M9S2	5000 psi, 80 °C, 40% EtOH	16.68 ± 1.28	72.24 ± 6.32
L-NAME[a]		86.65 ± 6.12	95.52 ± 3.50

Values are expressed as means ± SD of three independent experiments.
[a] Positive control used for Griess assay

Furthermore, in review of CO_2 extracts, it revealed that the extracts obtained at 3000 and 5000 psi, using pure CO_2 showed stronger inhibitory activity (lower secretion of NO) than those extracted at the same temperature but in the presence of modifier (Figure 3.4). Hence suggesting that extraction with 100% CO_2 alone is sufficient enough to enrich extract with compound/s of potent anti-inflammatory property. Thus, the extraction conducted using pure CO_2 at 3000 psi of pressure and temperature at 60 ^oC was the optimal conditions determined from this test to furnish an extract (M5S1) that possesses the strongest anti-inflammatory activity with no cytotoxicity effect. The chemical constituents of M5S1 were identified via GC and GC-MS analysis as depicted in Table 4.8 and Figure 4.29 in Chapter 4.

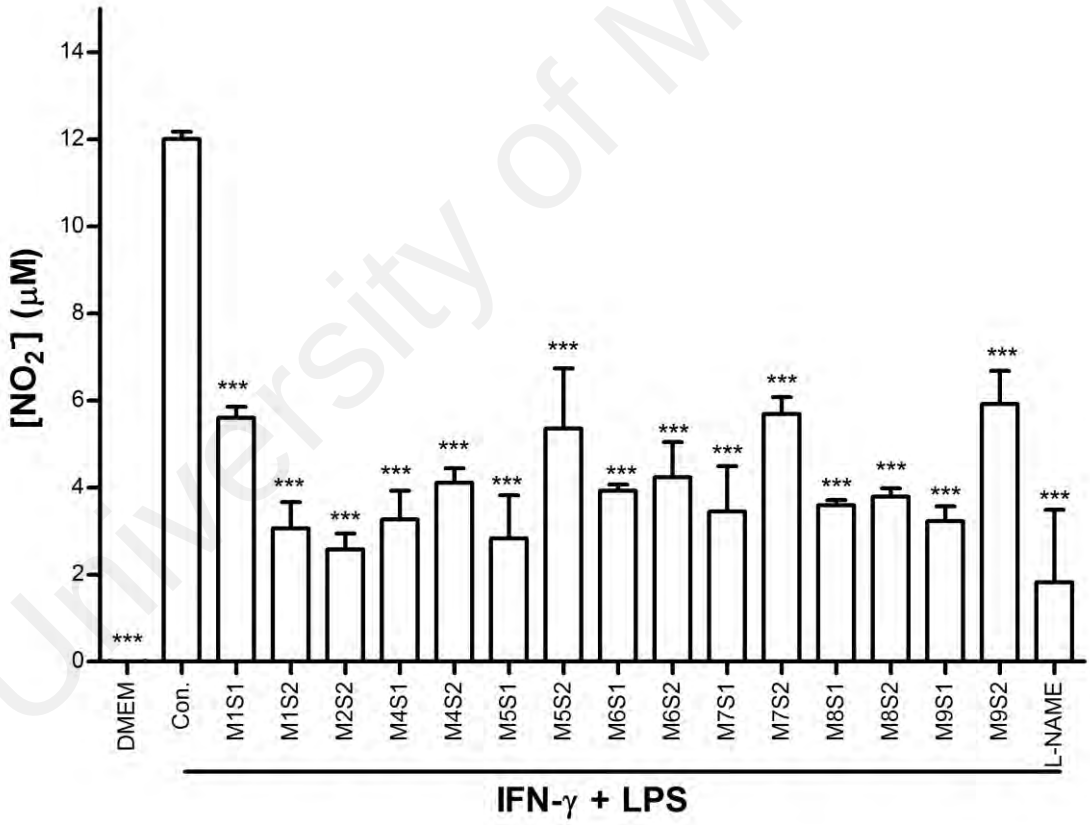


Figure 3.4 Effect of *M.speciosa* SFE extracts on NO production in RAW 264.7 cells
Cells were stimulated for 17-20 h with 100 U/mL IFN-γ and 5 g/mL LPS. Concentrations of NO_2^- in the media were determined by the Griess assay. All values are the means ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significantly different from the IFN-γ/LPS untreated control group (Con.). M2S1, M3S1 and M3S2 were not tested due to the scarcity of the extracts.

Table 4.8 in Chapter 4, listed the identifiable constituents of M5S1, the relative peak areas of these constituents and their experimental retention indices on the HP-5 column. Fifth- teen compounds, constituting 93.53% of the extract were identified as depicted in Figure 4.29 (pg. 154). The characterization of M5S1 by GC and GC-MS did not show any present of alkaloid compound, which is in agreement with the TLC profile sprayed with Dragendorff's reagent. This is the first report showing non-alkaloidal extract of *M. speciosa* exhibits NO inhibition (anti-inflammatory) activity.

The fatty acid constituents (palmitic acid, methyl ester oleic acid and stearic acid) clearly dominated M5S1 profile, contributing to 39.01% of the total yield, though this figure was largely due to palmitic acid (34.90%) alone. Fatty acids are involved in the formation of healthy cell membranes, proper development and functioning of the brain and nervous system, regulating blood pressure and viscosity, immune and inflammatory responses [140, 141]. Palmitic acid as the most abundant component in M5S1 has been communicated for its anti-inflammatory activity. Ester bond hydrolysis of membrane phospholipids by phospholipase A_2 and consequent release of fatty acids are the initiating steps of inflammation. Thus, inhibition of phospholipase A_2 is one of the ways to control inflammation. The structural and kinetics studies have proved that palmitic acid (*n*-hexadecanoic acid), is an inhibitor of phospholipase A_2, hence, is an anti-inflammatory compound [142]. As M5S1 is rich in palmitic acid, therefore it merits a further study to establish palmitic acid as a potential to inhibit the production of NO by iNOS. This may explain the anti-inflammatory property exhibited by *M. speciosa*.

In addition, the presence of phytosterols may also enhance the anti-inflammatory property exhibited by M5S1 as they have been claimed to possess anti-inflammatory [143], anti-oxidant 144] and angiogenic activity [145]. Sitosterol, stigmasterol and campesterol were present at an appreciable amount, 6.67%, 0.99% and 1.66%

respectively (Table 4.8 and Figure 4.29 in Chapter 4). Along with these phytosterols, the appearance of tocopherol which constituted 2.78% of the extract, although at low concentration might also help account to the anti-inflammatory activity as it has been associated with the anti-oxidant properties, especially against lipid peroxidation in biological membranes [146, 147].

Interestingly, the aqueous extract, even though showed relatively low inhibition activity at 37.17% but it was the least toxic with cell viability of 107.16%. The aqueous decoction of the 'ketum' leaves was the main preparation used by the traditional healer in the folk medicine as treatment for many diseases, especially to treat muscle pain, intestinal infections, to reduce coughing, fever and diarrhea [6, 29, 35, 50, 148-151]. With respect to the fact that the aqueous extract of *M. speciosa* leaves closely mimic its traditional herbal drug preparation and not toxic, the aqueous extract was further investigated for *in vivo* anti-inflammatory activity through the anti-ulcer test.

3.6.2 *In Vivo* Gastroprotective Activity

3.6.2.1 Acute Toxicity Assessment

Animals pre-treated with 500 mg/kg and 1000 mg/kg of MSAE were observed for a period of 14 days. No mortalities were recorded; in fact all animals were healthy and did not manifest any symptoms of toxicity-related macroscopic or behavioural abnormalities at these dosages. Furthermore, blood biochemistry parameters were within the normal ranges and no major structural destruction was noticed in the histology of liver and kidney as compared to the control group.

The animals were fasted overnight (food but not water) prior to MSAE and vehicle administration in order to eliminate food from inside the gastrointestinal tract that might

complicate absorption of the test substance. An extract is considered to be safe if no animal death is registered within 14 days or no clinical toxicity signs were observed [152]. Based on this acute toxicity finding, it is therefore evident that oral administration of MSAE up to 1000 mg/kg is save. This is in accordance with acute toxicity study of the standardized aqueous extract conducted by Kamal *et al.* [153].

3.6.2.2 Gastric pH

The effect of MSAE on the gastric pH was depicted in Figure 3.5. From the results, the pH of the stomachs in the MSAE-treated animals improved to 4.08 ± 0.49 in case of MSAE 100 mg/kg and 5.93 ± 0.31 (p < 0.001) in case of MSAE 200 mg/kg as compared to ulcer group (2.95 ± 0.36). In addition, no significance was recorded between the highest dose MSAE-treated and Omeprazole group (6.6 ± 0.22) indicating that the gastroprotective effect of MSAE is dose-dependent.

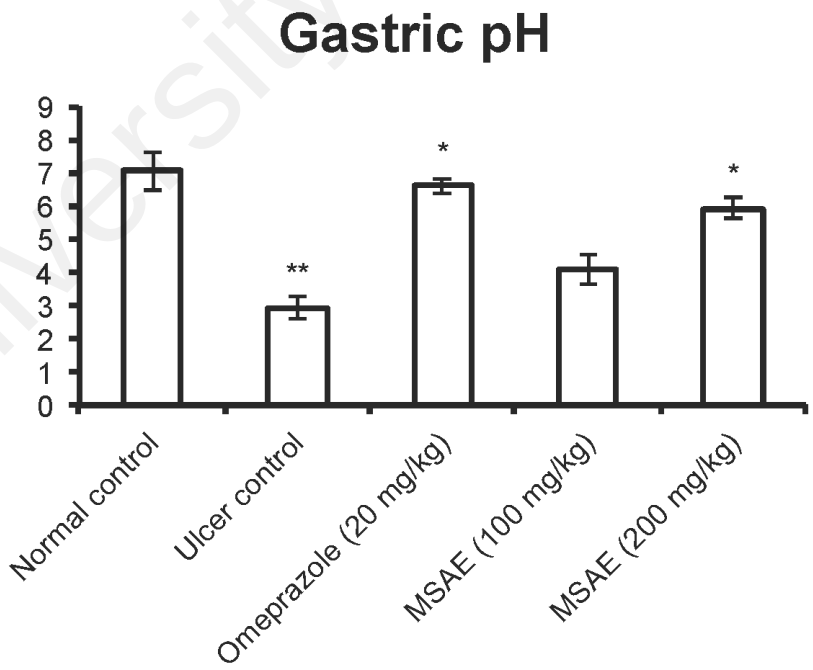


Figure 3.5 Effects of MSAE on pH of the stomach mucus
All data shown are calculated as mean ± SEM (n = 6). ***p* <0.001 compared to normal group. **p*<0.001 compared to ulcer group.

3.6.2.3 Effect of MSAE on Gastric Wall Mucus (GWM)

Absolute ethanol significantly ($p < 0.001$) decreased the Alcian-blue binding capacity of gastric mucus to (75.17 ± 2.89 mg Alcian blue/g tissue) in ulcer control group compared to normal group rats (125.33 ± 4.76 mg Alcian blue/g tissue). In the investigational groups, pre-treatment with MSAE 100 and 200 mg/kg appreciably ($p < 0.001$) enhanced the mucosal binding capacity reaching 201.50 ± 7.04 and 285.17 ± 7.34 mg Alcian blue/g tissue respectively (Figure 3.6).

Mucus secretion is a crucial defensive factor in protecting the lining of the gastrointestinal tract. The gel mixture of water and glycogens that make up the mucus is reported to provide a kind of protective barrier against acidic aggressive attacks on the lining of gastrointestinal tract [154]. As expected, orogastric intubation of ethanol significantly ($p < 0.001$) reduced the mucosal lining resulting in high acidic condition as indicated by the ulcer control group. On the other hand, pre-treatment with MSAE or Omeprazole in the other groups managed to elevate the mucus contents above the level observed in the normal control group. In fact, the elevation of the mucus was in accordance with the restoration of the pH level in a concentration dependent fashion (Figure 3.5). Pre-treatment with 100 mg/kg MSAE raised the average mucus weight to 201.50 ± 7.04 mg, while the average pH level was raised to 4.08 ± 0.49. Increasing the MSAE dosage to 200 mg/kg resulted in an increment of mucus contents (285.17 ± 7.34 mg) and gastric contents pH (5.93 ± 0.31), a level that is similar to that of Omeprazole group. The enhancement in mucus production in the pre-treatment groups could be one of the factors that protected the newly formed gastric lining cells from further ethanol-induced gastric injury. Similar reports on the raise of mucosal secretion resulting in gastroprotection against ethanol-induced gastric ulcer by plant extract have been recorded [155, 156].

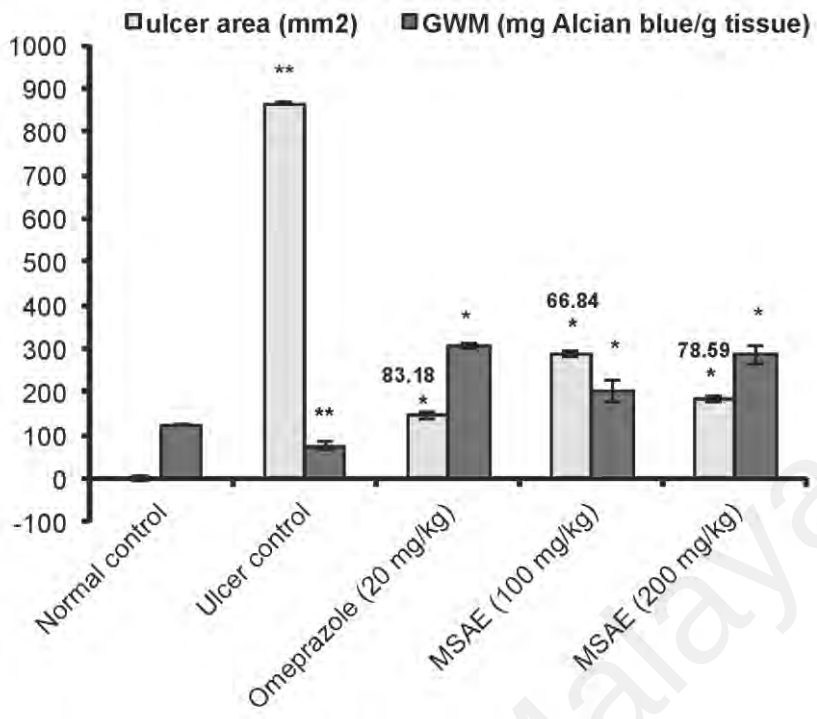


Figure 3.6 Effects of MSAE on experimental ulcer area, % inhibition and gastric wall mucus
All data shown are calculated as mean ± SEM (n = 6). ***p* < 0.001 compared to normal group. **p* < 0.001 compared to ulcer group. % Inhibition is indicated above the columns.

3.6.2.4 Macroscopic Estimation and Histology of Gastric Lesions

Macroscopical analysis of gross lesions induced by ethanol was shown in Figure 3.6, which recorded 865.00 ± 9.95 mm^2 ulcer area in the ulcer control group compared to normal group ($p < 0.001$). Pre-treatment of the animals with MSAE (100 and 200 mg/kg) have considerably ($p < 0.001$) abridged the ulcer area to 286.83 ± 25.61 and 185.17 ± 23.35 mm^2, respectively. This translated to an observed percentage in ulcer inhibition of 78.59% in the animals pre-treated with 200 mg/kg, reaching a value close to the reference drug group pre-treated with Omeprazole (83.18%). The results depicted the relationship between gastric wall mucus and the ulcerogenic area. As shown in (Figure 3.6), reduction in mucus contents resulting in wider ulcer area formed. The observation was substantiated by the observed decreasing of necrotic lesions in the histopathological

micrographs (Figure 3.7) upon treatment with either Omeprazole or MSAE. In fact, the gross appearance of the necrotic lesions was detected to reduce significantly upon MSAE pre-treatment in a dosage reliant way compared to the ulcer control group. Pre-treatment with 100 mg/kg MSAE incurred a marked reduction of haemorrhagic lesion when compared to the ulcer control group (Figure 3.7E). However, 200 mg/kg MSAE dosage resulted in a more prominent reduction of the lesion with remarkably flattened mucosal walls (Figure 3.7D) to a level similar to that of the positive control Omeprazole-treated group (Figure 3.7C).

Researchers commonly applied orogastric administration of absolute ethanol to induce intense gastric mucosal damage in experimental animals [157]. Grossly, the damage appeared as haemorrhagic bands lesions and restricted to the glandular parts. The injuries begin through vascular epithelium disturbance resulting in increased vascular permeability, edema and necrotic lesions plummeting the discharge of bicarbonates and production of mucus [123, 158]. Results of our study revealed that MSAE pre-treatment was capable of suppressing the gastric lesions caused by ethanol. This effect could be due to the inhibition of leucocyte infiltration of the gastric wall in rats pre-treated with MSAE. It has been documented that up-regulation of COX-2 via COX-1 inhibition is the key factor in inflammatory reactions associated with ulcers [159].

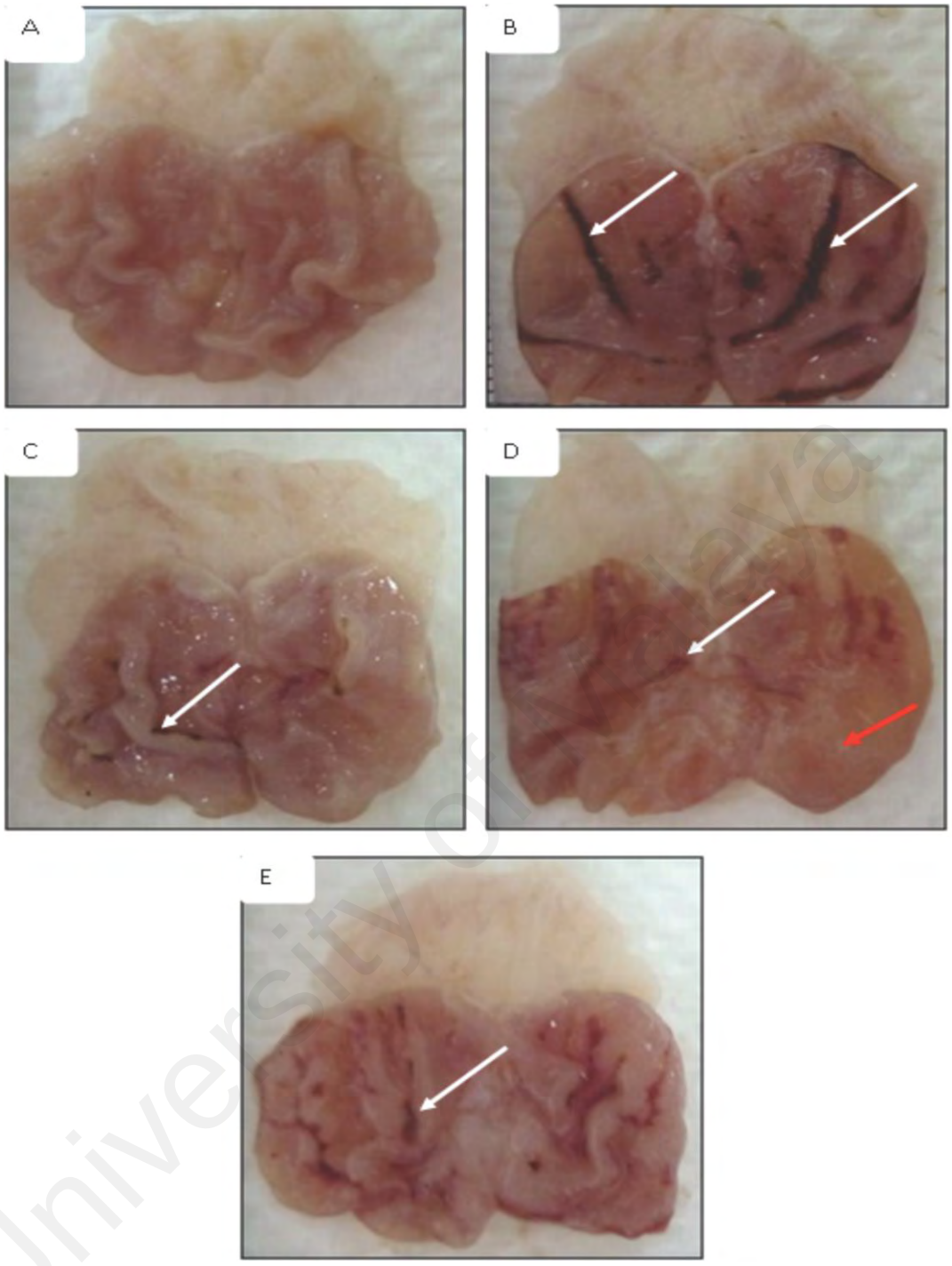


Figure 3.7 Effects of MSAE on macroscopical manifestation of the stomach walls in rats (n = 6)

(A) Normal stomach mucosal walls in the normal group. (B) Severe mucosa harm due to absolute ethanol administration producing haemorrhagic lesions and necrosis. (C) Omeprazole group 20 mg/kg (reference control) shows significant mucosal protection compared to ulcer control group. (D) Animals pre-fed with 200 mg/kg show minor damage with flattening of gastric mucosal walls (red arrow) indicating gastoprotection. (E) Rats received 100 mg/kg of MSAE show moderate gastric lesions. The white arrow indicates the haemorrhagic lesions.

3.6.2.5 Anti-oxidant Activity

Effect of MSAE on SOD Activity:

Ethanol significantly ($p < 0.001$) reduced the SOD activity of the ulcer control group (11.09 ± 0.57 U/mg protein) compared to normal control group (29.28 ± 0.93 U/mg protein) as revealed in Figure 3.8. However**,** treatment with MSAE one hour before ethanol administration, significantly ($p < 0.001$) altered the results, elevating the SOD activity to 23.71 ± 1.00 and 28.09 ± 1.49 U/mg protein in both MSAE doses 100 mg/kg and 200 mg/kg correspondingly. Furthermore, the low dose group recorded considerable ($p < 0.001$) value compared to Omeprazole-treated group (30.77 ± 0.71 U/mg protein). Results indicated that the enhancement gastric protection effect of MSAE to the endogenous enzymatic activity of the gastric mucosa was dose dependent.

Superoxide dismutase (SOD) is an enzyme found in all living cells that alternately catalyse the conversion (dismutation or partitioning) of the harmful superoxide (O_2^-) radical into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). O_2^- is produced as a by-product of oxygen metabolism and, if not regulated will causes many types of cell damage. Hydrogen peroxide is also damaging, but less so, and is degraded to water by other enzymes such as catalase. Reduction of the SOD action in the ulcer control group in this study is suggested to be due to the high utilization of SOD in the decomposition of O_2^- generated by lipid peroxidation [160, 161]. Thus, SOD is an important anti-oxidant defence in nearly all-living cells exposed to oxygen.

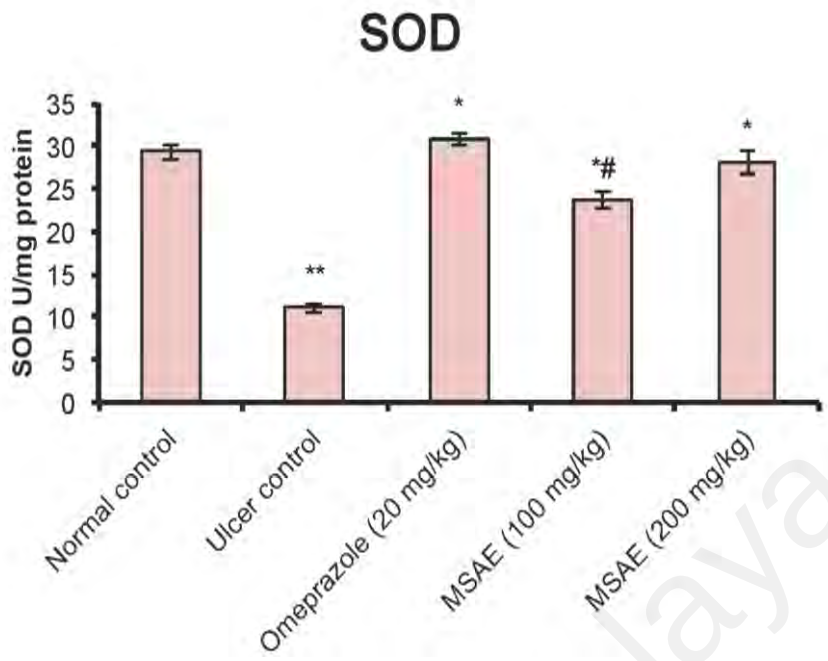


Figure 3.8 Effect of *M. speciosa* aqueous extract (MSAE) pre-treatment on the superoxide dismutase (SOD) activity of all experimental rats
Data were measured as mean ± SEM (n = 6). $^{**}p < 0.001$ compared to normal group. $^{*}p < 0.001$ compared to ulcer group. $\#p < 0.001$ compared to Omeprazole-treated group.

Effect of MSAE on the Gastric MDA Level:

Ethanol administration to the rats significantly ($p < 0.001$) increased the MDA level to 178.00 ± 5.98 μmol/g proteins in the ulcer control group as compared to the normal group (87.27 ± 1.83 μmol/g proteins). Whereas, in comparison to the ulcer rats, MDA level of rats fed with 100 mg/kg MSAE decreased to 115.17 ± 6.02 μmol/g proteins ($p < 0.001$). On increasing the MSAE concentration to 200 mg/kg the MDA level was found to be further reduced to 102.07 ± 3.37 μmol/g protein ($p < 0.001$), approaching the MDA level of Omeprazole-treated group (99.50 ± 2.63 μmol/g protein) (Figure 3.9).

Malondialdehyde (MDA) is one of the most prevalent byproducts of lipid peroxidation during oxidative stress. Cellular lipids membranes that contain unsaturated fatty acids with more than one double bond are said to be more susceptible to the oxidizing action of free radicals. Ethanol induced gastric ulcer model in associated with the disruption in

the mucus bicarbonate barrier and the damage in the blood vessels of the stomach walls are probably due to lipid peroxidation and reactive oxygen species accumulation [115]. This leads to an increased level of MDA, which reacts, with the bases of DNA inducing mutagenic lesions and eventually cell necrosis and death of the cells [117]. The MDA analysis revealed the inhibition activity of MSAE on lipid peroxidation.

In the present study, the ulcer control group showed an increased level of MDA (Figure 3.9) and decreased level of SOD (Figure 3.8), which is an indication of oxidative stress. Treating animals with MSAE prior to ethanol administration has significantly protected the gastric mucosa from the necrotic effect of ethanol by decreasing the MDA level and increasing the activity of endogenous anti-oxidant SOD in the MSAE-treated groups in the same manner as Omeprazole-treated group. The flavonoids and phenol content of this plant might have attributed towards the anti-oxidant effect of the MSAE observed in this study [74].

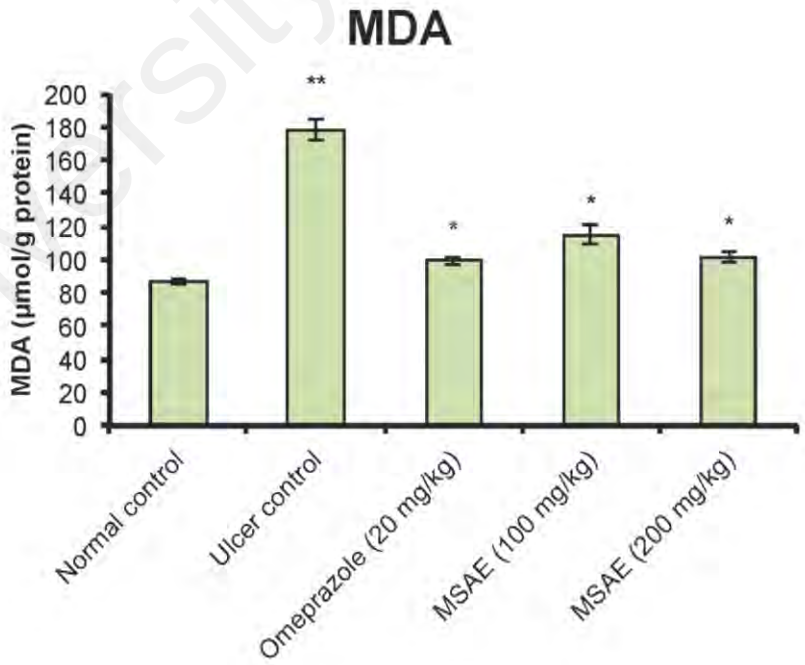


Figure 3.9 Effect of *M. speciosa* aqueous extract (MSAE) pre-treatment on the level of malondialdehyde (MDA) of all experimental rats
Data were measured as mean ± SEM (n = 6). $^{**}p < 0.001$ compared to normal group. $^{*}p < 0.001$ compared to ulcer group. No significance was recorded between MSAE-treated and Omeprazole-treated groups.

3.6.2.6 Effect of MSAE on the Gastric Protein Concentration

The results of MSAE pre-treatment on the gastric protein concentration are shown on Figure 3.10. Results revealed significant ($p < 0.001$) declined in the gastric protein concentration of the ulcer control group (7.50 ± 0.75 mg protein/mL tissue) compared to normal animals (13.25 ± 0.8 mg protein/mL tissue) due to the toxic effect of ethanol administration. Pre-treatment of the rats with MSAE has significantly increased the level of gastric protein to reach 10.04 ± 0.41 mg protein/mL tissue for the low dose (100 mg/kg) and 11.48 ± 0.60 mg protein/mL tissue for the high dose (200 mg/kg) indicating significant improvement in the protein content of the gastric tissue compared to ulcer rat's group.

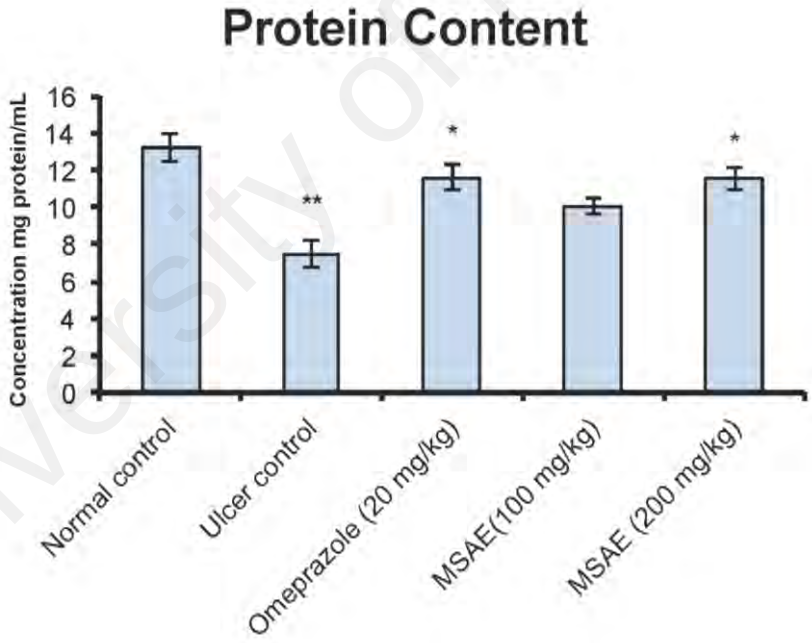


Figure 3.10 Effect of *M. speciosa* aqueous extract (MSAE) pre-treatment on the gastric protein content of all experimental rats
Data were measured as mean ± SEM (n = 6). $^{**}p < 0.001$ compared to normal group. $^{*}p < 0.01$ compared to ulcer group. No significance was recorded between MSAE-treated and Omeprazole-treated groups.

3.6.2.7 Histological Lesions

In ulcer control group, absolute ethanol induced remarkably severe gastric mucosal injury penetrated deeply into walls accompanied by edema together with infiltration of leukocytes compared to rats fed with plant extract MSAE as illustrated in Figure 3.11.

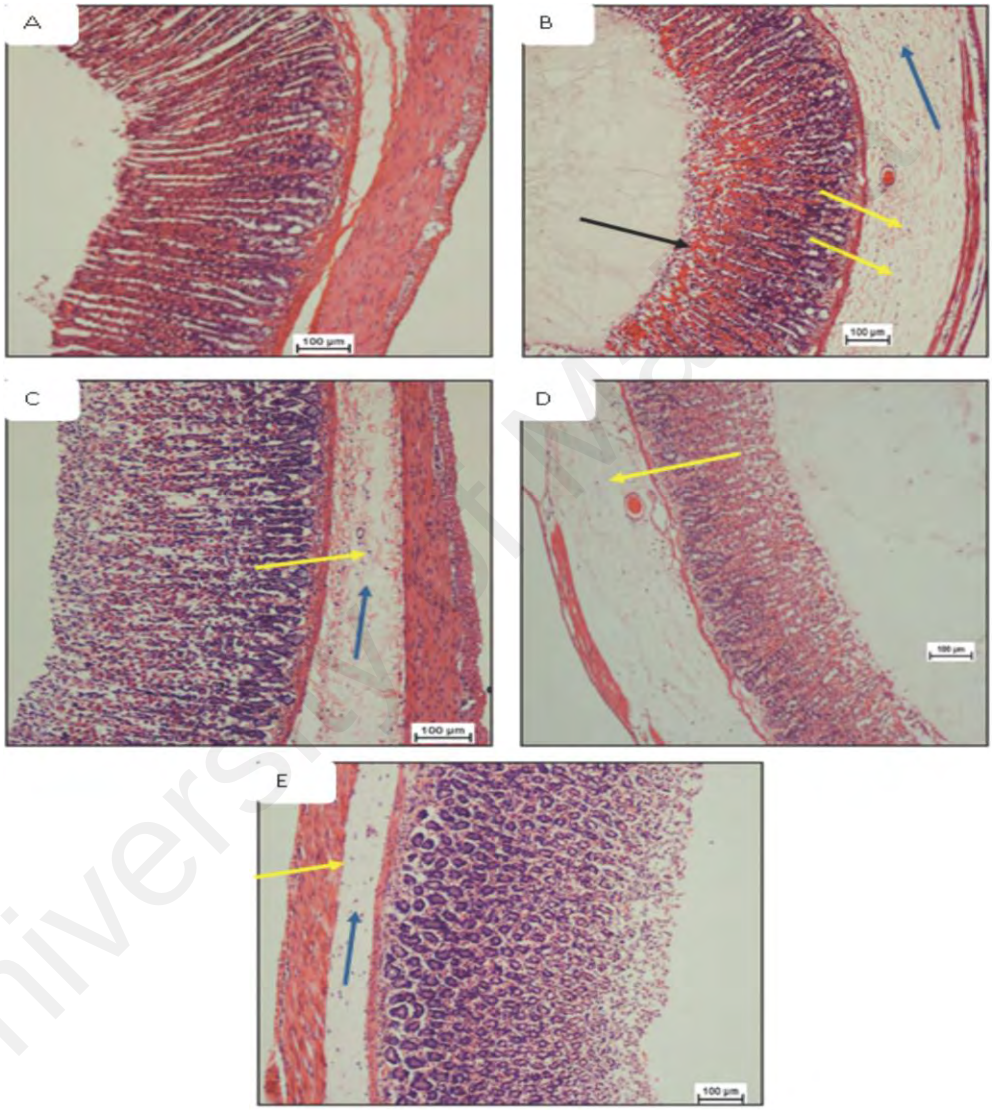


Figure 3.11 Effect of MSAE pre-treatment on histology of stomach mucosal injure in rat's model (10x)
(A) Normal surface epithelium is observed. (B) Severe damage to the exterior epithelium (black arrows) and deep necrosis in the mucosa together with extensive edema (blue arrows) and infiltration of inflammatory cells of sub mucosa (yellow arrows) are noticed. (C) Mild damage of the stomach's wall and reduced edema with inflammatory cells infiltration are observed in Omeprazole-treated (20 mg/kg) group. (D) Moderate surface epithelium disruption together with sub mucosal edema together with infiltration of leukocytes in the rats pre-fed with MSAE 100 mg/kg. (E) Minor gastric mucosal damage and reduced edema with inflammatory cells infiltration in the rats pre-fed by means of MSAE 200 mg/kg.

3.6.2.8 Periodic Acid Schiff (PAS) Staining

As illustrated in Figure 3.11, a significant decrease in the uptake of PAS staining intensity by mucosal glycoprotein in ulcer control group was noticed indicating decrease in mucosal glycoprotein when compared with the rats pre-treated with MSAE or the reference drug Omeprazole.

The accompanying significant increase in mucus production (Figure 3.6) as indicated by the intensive PAS staining in MSAE-treated groups (Figure 3.12) hints that gastro protective effect of *M. speciosa* leaves is attributed partially through the enhanced gastric mucus secretion. Previous studies also showed anti-ulcer activity of plant extracts via induction of gastric wall mucus production [155, 156].

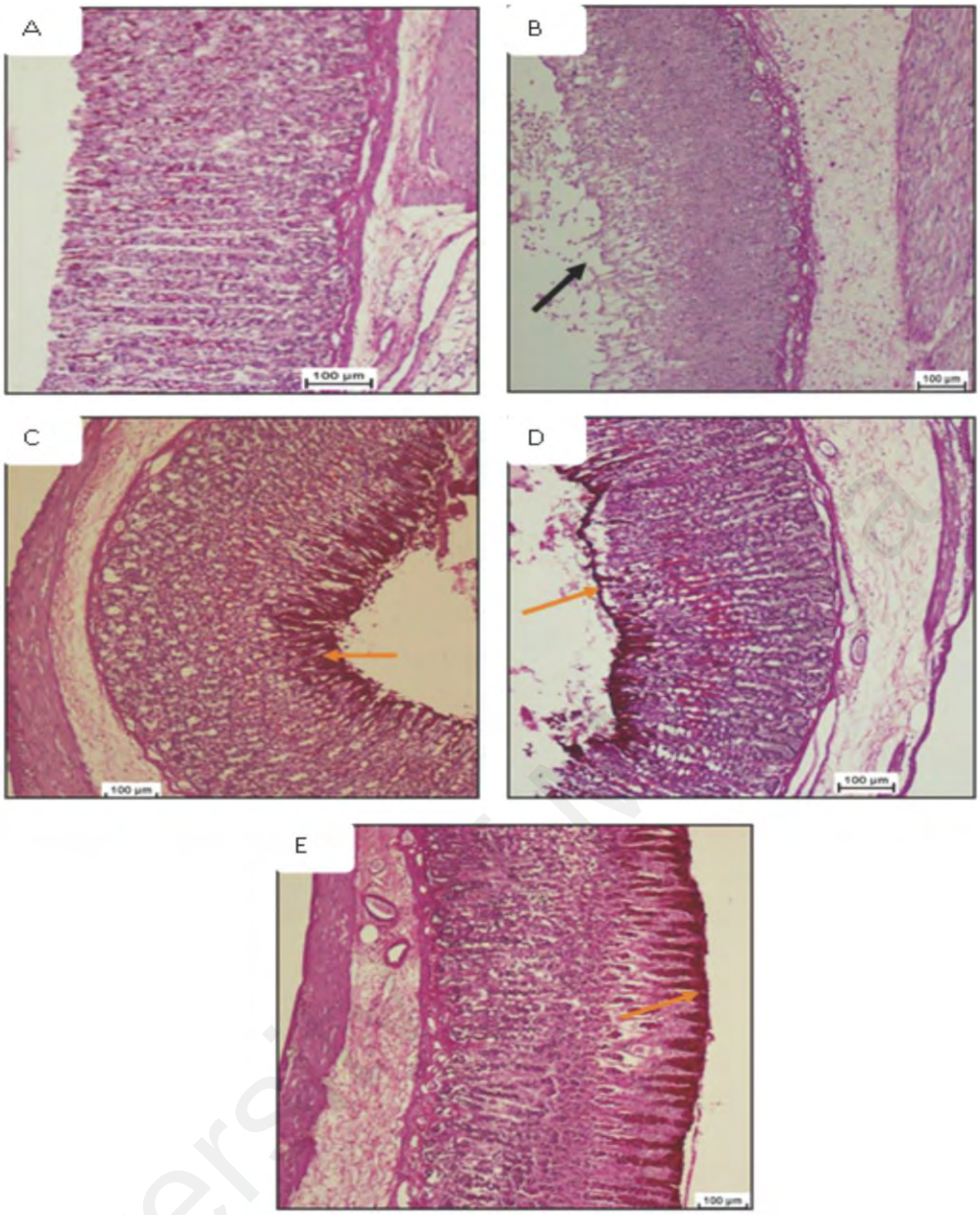


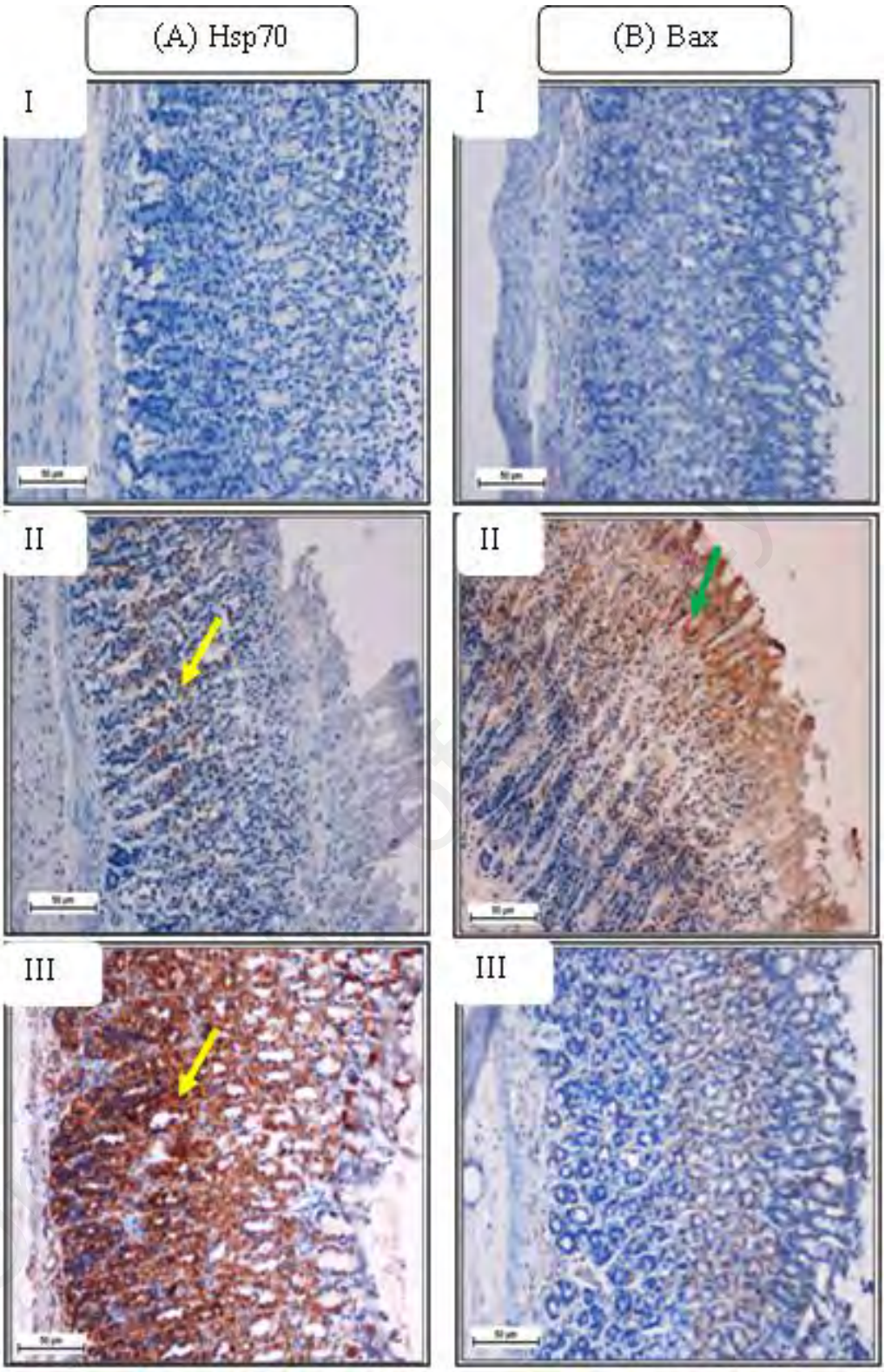
Figure 3.12 Effect of MSAE pre-treatment on gastric tissue glycoprotein as stained by PAS (10x)

(A) Normal group. (B) Ulcer group showed severe destruction of gastric mucosa (black arrow). (C) Omeprazole control group. (D) Rats pre-fed with 100 mg/kg of MSAE (E). Rats pre-fed with 200 mg/kg of MSAE. The apical epithelial cells of the gastric mucosa are stained with magenta colour in the MSAE-treated and Omeprazole-treated groups indicating significant enhancement in mucosal secretion. The orange arrow shows the glycoprotein secretion.

3.6.2.9 Immunohistochemistry

The effect of MSAE pre-treatment on HSP70 and BAX was shown on Figure 3.13A and Figure 3.13B. Rats fed with MSAE reveal remarkable HSP70 protein over-expression and BAX protein down-expression in comparison to ulcer control group. Conversely, ulcer control group showed down-expression of HSP70 and over-expression of BAX protein. These results revealed that MSAE pre-treatment meaningfully regulated the level of HSP70 and BAX proteins in gastric cells of rats after absolute ethanol administration.

Gastric epithelial tissue generates important macromolecule chaperones such as heat shock proteins (HSP70) [162]. When the gastric mucosa is subjected to oxidative stress, these proteins play crucial role in maintaining the structure and function of the gastric tissue through defending necessary protein against denaturation [163]. In addition, peptic ulcer may result from an imbalance between the anti-apoptotic protein BCL-2 family, and pro-apoptotic BAX proteins [126]. Many researchers examined the overall role of HSP70 and BAX on gastric mucosal security and harm, correspondingly [123, 158, 164]. Recent studies reported that ethanol reduces HSP70 protein level and increases BAX protein [157, 165, 166]. In our present study, MSAE pre-treatment reduced the toxic effect of ethanol by over-expression of HSP70 protein and down-expression of BAX protein to provide its gastroprotective activity. Anti-ulcer property of the aqueous plant extracts through up-regulation of HSP70 proteins have been proven recently in *Mucuna pruriens* and *Polygonum chinense* aqueous extracts [165, 166].


(A) Hsp70
(B) Bax
I
I
II
II
III
III

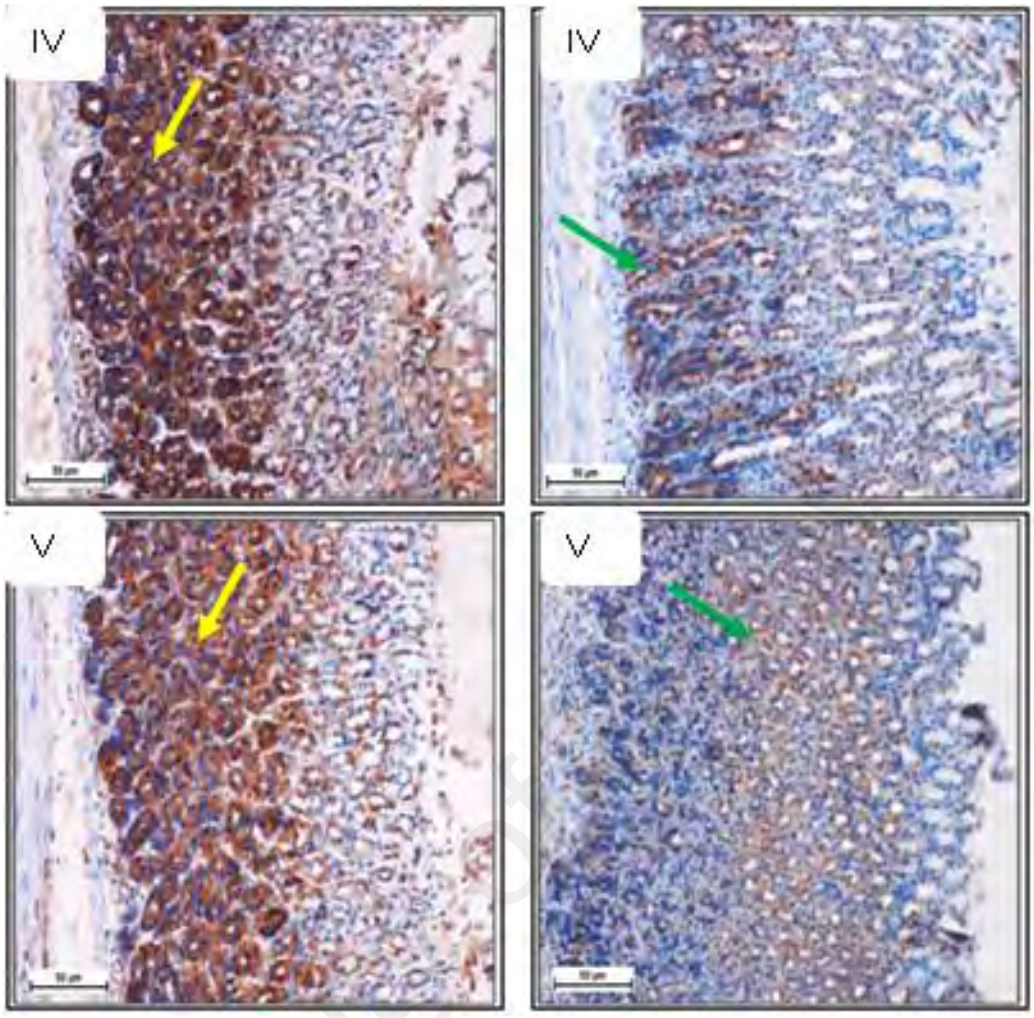


Figure 3.13 Effect of MSAE pre-treatment on the immunohistochemistry staining of (A) Heat shock protein 70 (HSP70) and (B) pro-apoptotic protein BAX (20x)
(I) Normal control group. (II) Ulcer control group showed massive destruction of stomach mucosa (green arrow). (III) The Omeprazole control group. (IV) Rats pre-fed by 100 mg/kg of MSAE. (V) Rats pre-fed by 200 mg/kg of MSAE. Over-expression of HSP70 is more obvious in the Omeprazole and MSAE-treated groups (III, IV and V) compared to ulcer group (II). On the contrary, down-expression of BAX protein is more noticeable in the Omeprazole and MSAE-treated groups compared to ulcer control group.

3.7 Conclusion

In conclusion, this study has identified the NO inhibitory activity of 19 selected extracts of *M. speciosa*, which include the hexane (MSH), ethyl acetate (MSEA), methanol (MSM), aqueous (MSAE) and carbon dioxide crudes (M1S1, M1S2, M2S2, M4S1,

M4S2, M5S1, M5S2, M6S1, M6S2, M7S1, M7S2, M8S1, M8S2, M9S1, M9S2). Among these extracts, M5S1 possessed the strongest activity without cytotoxic effect (NO inhibitory activity at 60.08 ± 10.02% and cell viability, 91.98 ± 5.58%). It is noteworthy that M5S1 was dominated by fatty acid, in particular palmitic acid (34.90%) that has been reported as an anti-inflammatory compound. Parameters for the extraction of M5S1 are determined as the optimal SFE condition (extraction under pure CO_2, pressure: 3000 psi, temperature: 60 ^oC) to furnish extract that possesses the highest anti-inflammatory activity without any toxicity. The inference from the observed NO inhibitory activity supports the traditional use of *M. speciosa* in the treatment of muscle pain and fever. The anti-inflammatory property of *M. speciosa* was further supported by the consistent results in the anti-ulcer test.

The present study revealed that MSAE pre-treatment is capable of suppressing the gastric lesions caused by ethanol in rats. Despite the lethality and toxicity shown by the methanolic, alkaloid extract and mitragynine, the aqueous extract (MSAE) of *M. speciosa* is not toxic. In the acute toxicity study, the MSAE was found to be safe even at a concentration of 1000 mg/kg. MSAE significantly protected the stomach in contrary to gastric lesions persuade via absolute ethanol. The SOD activity was significantly high in the stomach tissue homogenate, accompanied by significant decrease of MDA. The gastroprotective effect of the extract in the experimental animals was established through the elevated stomach wall mucus secretion, decrease in the gastric lesion, edema and reduced neutrophils infiltration of the sub mucosa. Immunohistochemistry of HSP70 and BAX proteins demonstrated over-appearance of HSP70 protein and down-appearance of BAX protein in MSAE treated rats. Increased PAS staining intensity of gastric mucosa of fed animals indicated the increase of glycoprotein content. This study provides histological and immunohistochemical evidences on the gastroprotective role of MSAE.

CHAPTER 4: PHYTOCHEMICAL AND ANALYTICAL STUDIES

The Chelsea College Pharmacognosy Research Laboratories collected thirty samples of *Mitragyna speciosa* from Thailand, Malaysia and Papua New Guinea between 1961 and 1970. All contained mitragynine, but also proved to have considerable variation in the alkaloid makeup. In the current studies, the leaves of Malaysian *M. speciosa* were subjected to three types of extraction with different types of solvents. The crudes were then evaluated for nitric oxide inhibition activity via Griess test. The most active crude without showing any toxicity effect, M5S1 was further analysed for chemical constituents with gas chromatography. In addition, the aqueous extract was also investigated for anti-ulcer and toxicity properties. The crude was column chromatographed to isolate pure compounds and chemical crude profiling was performed using HPLC. In this chapter, the phytochemical and analytical studies of the aqueous extract and SFE (M5S1) will be briefly discussed.

4.1 Plant Material

The plant materials of *M. speciosa* were collected from the forest of Sungai Setong, Jelawang, Kelantan, Malaysia in November 2006. The phytochemical group of Department of Chemistry, Faculty of Science, University of Malaya accomplished the identification and authentication work of the plant. Voucher specimen labelled as KL5321 was deposited at Herbarium of the Department of Chemistry, University of Malaya, Kuala Lumpur, Malaysia.

KL5321 is the one with the red veins in the leaf. The leaves of the sample were dried under the shade, placed in special design boxes that fixed with a few electric light bulbs as a heat source of 35-40 ºC to assist the drying process for a period of one week. The dried leaves were ground to coarse powder of approximately 5 mm size particle using

an electrical blender.

4.2 Chemicals

4.2.1 Solvent

All solvents involved in bulk extraction were of analytical reagent (AR) grade and were distilled prior to use (hexane, ethyl acetate and methanol). Carbon dioxide (99.995% purity, SFE grade), contained in a cylinder with an eductor tube, was purchased from MOX (Selangor, Malaysia). Acetonitrile and methanol of the HPLC grade were purchased from Friendeman Scientific. All other common solvents and chemicals were of AR or HPLC grade and were purchased from Merck. The standard mixture of C_5-C_{30} and other homologous series of *n*-alkanes used in the gas chromatography analysis were obtained from SIGMA Chemical Co., USA.

4.2.2 Reagents

Mayer's Reagent (Potassium mercuric iodide) was used for screening the alkaloids in natural products. A positive result was indicated when white or cream-colored precipitate was formed under acidic conditions. The Mayer's Reagent was prepared as follows:

1.4 g mercuric iodide, dissolved in 60 mL distilled water was mixed with a solution of 5.0 g of potassium iodide in 10 mL of distilled water.

Dragendorff's reagent (Potassium bismuth iodide) was also used to detect the presence of alkaloids spotting on TLC. A positive result is indicated by the formation of orange spots on the developed TLC. The Dragendorff's Reagent was prepared as follows:

Solution A: Bismuth (III) nitrate (1.7 g) was dissolved in a mixture of 20 mL glacial acetic acid and 80 mL of distilled water.

Solution B: Potassium iodide (16 g) was dissolved in 40 mL of distilled water.

Solution A and B were mixed together to give a dark orange stock solution. Then, the final spray reagent was prepared by diluting stock solution (40 mL) with 40 mL glacial acetic acid in 120 mL distilled water.

4.3 General Spectroscopic Methods

The structural elucidations of pure compounds, constituents' characterization of the most active SFE extract and profiling of the aqueous crude extract were accomplished by spectroscopic methods. The details of the instruments and parameters used are given below.

4.3.1 NMR Spectra

NMR spectra of 1D (^{1}H, ^{13}C and DEPT) and 2D (COSY, HSQC, HMBC and NOESY) were recorded in deuterated chloroform ($CDCl_3$) as solvent on JEOL Lambda 400MHz, JEOL ECA 400MHz, Bruker Avance II 400 MHz and Bruker Avance III 600MHz. Chemical shifts (δ) were expressed in ppm relative to TMS and the coupling constants are given in Hz. The $CDCl_3$ peaks were referenced to δ 7.26 ppm (^{1}H NMR) and δ 77.2 ppm (^{13}C NMR).

4.3.2 Ultra Violet-Visible (UV)

The UV spectra were recorded on a Shimadzu UV-1601 spectrophotometer, with HPLC grade methanol as solvent.

4.3.3 Fourier Transform Infrared (FTIR)

The IR spectra were obtained through Perkin Elmer FTIR Spectrometer RX1 using $CHCl_3$ as solvent for sample dilution.

4.3.4 High Performance Liquid Chromatography (HPLC) and Liquid Chromatography-Mass Spectra (LC-MS)

The analytical HPLC Shimadzu LC-20AT system was employed for profiling and the high-resolution electro spray ionization mass spectra (HRESIMS) were measured on Agilent 1200 SL series HPLC system and a 6530 Accurate-Mass Q-TOF LC/MS instrument, equipped with a mass detector QTOF-MS instrument. Liquid Chromatography-Electro Spray Ionization-Time of Flight-Mass Spectrometry (LC-ESI-QTOF-MS).

4.3.5 Gas Chromatography (GC) and Gas Chromatography-Mass Spectrometry (GC-MS)

GC analysis was performed using a Shimadzu GC-2010 gas chromatograph equipped with a FID while GC-MS analysis was carried out by using a Shimadzu GC-2010 system coupled to a quadrupole mass spectrometer, MS-QP2010 Plus.

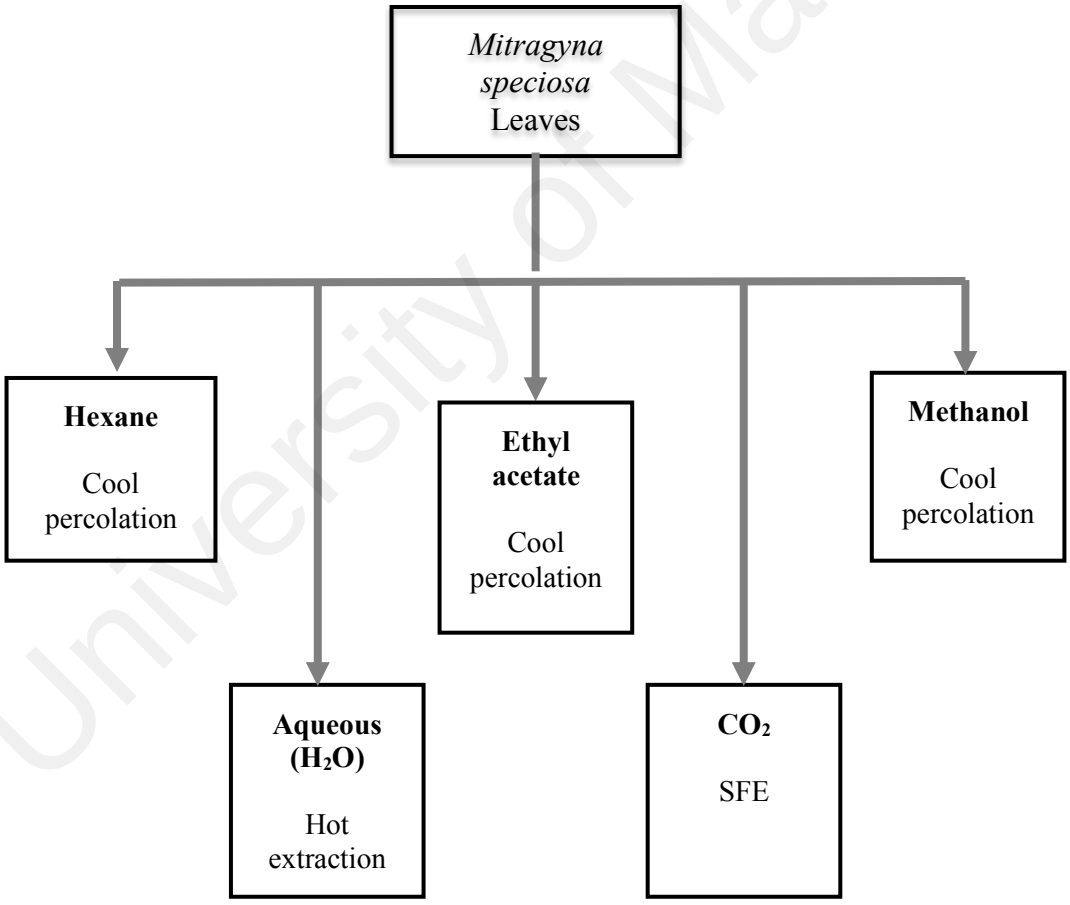
4.3.6 Column Chromatography (CC) and Thin Layer Chromatography (TLC)

Colum chromatography were prepared by using Silica Gel 60F, 70-230 mesh ASTM (Merck 7734); Silica Gel 60F, 230-400 mesh ASTM (Merck 9385); Silica gel 40-63μm (Silicycle R12030B); Silica gel 60-200μm (Silicycle R10040B); Silica Gel $60GF_{254}$, (Merck 1.07730.1000). Analytical Thin Layer Chromatography (TLC) was performed

on commercially Aluminium supported silica gel $60F_{254}$ TLC sheets (Merck 1.05554.0001); Glass supported silica gel $60F_{254}$ TLC plates (Merck 1.05715.0001); Glass backed TLC Amino plates (Silicycle TLG-R52030B-203).

4.4 Extraction of *Mitragyna spesiosa*

In this study, different organic (hexane, ethyl acetate and methanol) and in organic (water and carbon dioxide) solvents have been used in the extraction of the leaves of *M. speciosa*. Several methods of extraction have been applied as depicted in Scheme 4.1 below.



Scheme 4.1 Different methods in preparation of crude extracts from the leaves of *Mitragyna speciosa* (KL5321).

4.4.1 Organic Solvent Extraction

Extraction was carried out by cold percolation, which is a simple maceration method. One kilogram of the plant leaves was first sprinkled with 25% ammonia (NH_3) solution and left for an hour. Later it was soaked with the organic solvent for a specified period of time at room temperature. Periodically stirred manually throughout the duration to enhance the extraction yield and then filtered. Thereafter, the solvent was evaporated to dryness under reduced pressure using rotary evaporator.

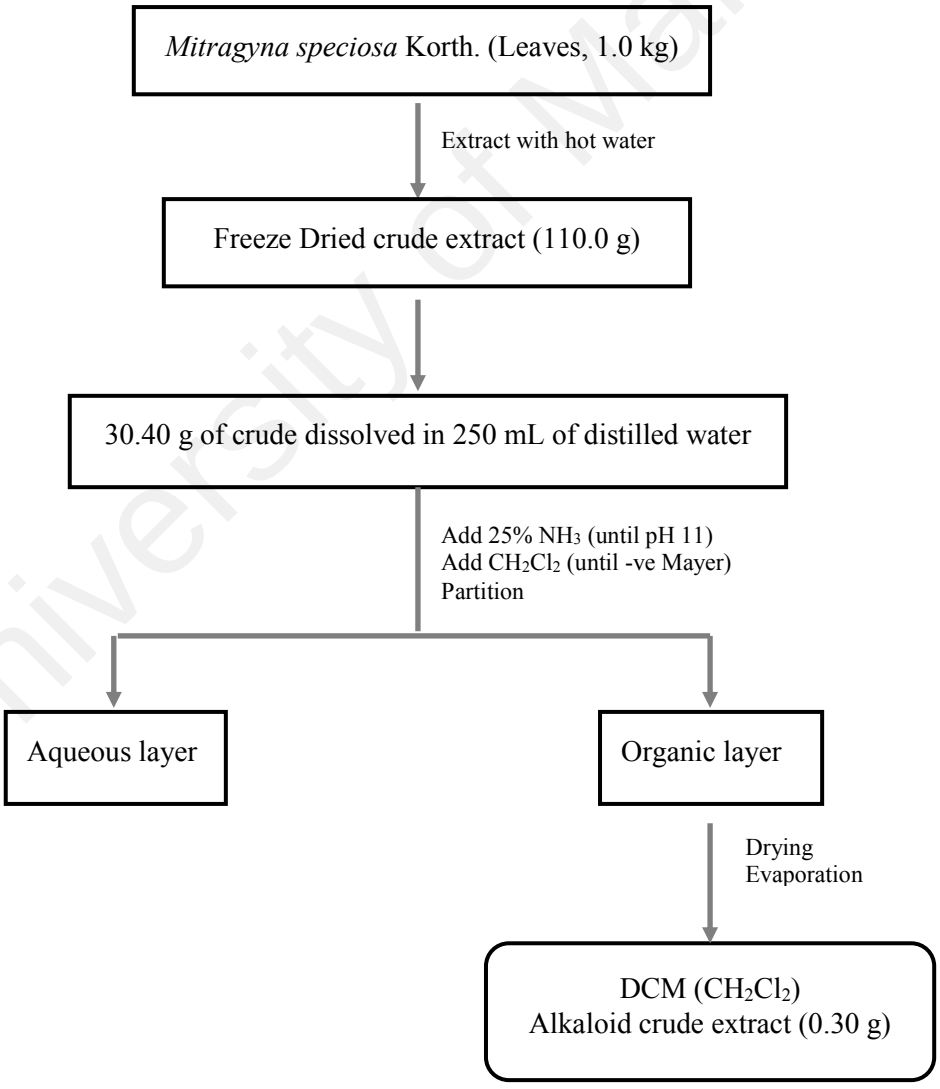
The samples were extracted with hexane for 48 h, ethyl acetate and methanol for 96 h according to the above procedure (work on new batch of sample for each extraction). After removal of the solvents, the crude extracts of hexane (MSH, 0.07%), ethyl acetate (MSEA, 6.6%) and methanol (MSM, 0.35%) were obtained. Each of afforded crude was of different shade of dark green coloured sludge. All crude extracts were sent for ^1H NMR experiment and checked with thin layer chromatographic for composition profiling.

4.4.2 Aqueous Extraction

Three 5L round bottom flask (RBF) were each filled with about 3 litres of distilled water. One kilogram of the dried ground leaves was separated equally into each RBF and boiled for 3 h. Then the water was drained and filtered, combined and stored in freezer (-20 ^oC). Finally the frozen extract underwent freeze-drying process to yield 110.0 g (11.0%) of dark brown powder (MSAE) and kept at -20 ^oC prior to use.

4.4.3 Alkaloid Crude Extraction

30.40 g of the aqueous freeze-dried crude was dissolved in 250 mL distilled water and was basified up to pH 11 with 25% NH_3 solution. Then dichloromethane (CH_2Cl_2) was added to extract out the free base alkaloidal compounds until a negative Mayer's test was obtained. Later the combined organic layer was washed with distilled water and sodium chloride solution (NaCl) and dried with sodium sulphate anhydrous. Finally it was evaporated to dryness to give 0.30 g (0.99%) of alkaloid crude extract. The extraction procedure was shown in Scheme 4.2.



Scheme 4.2 The extraction procedure of alkaloid crude extract from the leaves of *Mitragyna speciosa* (KL5321)

4.4.4 Supercritical Fluid Extraction with Carbon Dioxide

Approximately 25 g of the plant's dried ground leaves was loaded into a 100 mL stainless steel extraction vessel of the analytical-scale SFT 100 XW SFE system (illustrated in Figure 4.1). In order to determine a suitable extraction condition in a wide range with a minimum number of trials, an orthogonal test design $L_9(3)^3$ was employed whereby the temperature, pressure and the percentage of modifier (95% ethanol) were considered to be three major factors for effective extraction. The combinations of three different levels of each factor are listed in Table 4.1. In each test, two-step extractions were performed where at first, the plant material was pre-extracted with pure CO_2 (Step 1; S1) and afterwards the residue was re-extracted with CO_2 modified with aqueous ethanol solution (Step 2; S2). CO_2 was delivered by the SFT-10 Constant Pressure pump at the rate of 24 mL/min. For studies involving the modifier, ethanol solution was added directly to the plant material in the extraction vessel using an additional High Performance Liquid Chromatography (HPLC) pump (Series II Isocratic HPLC pump) prior to the extraction by supercritical CO_2.

In both steps, after 60 minutes of static extraction (no liquid flow), the sample material was subjected to dynamic extraction by flowing the CO_2 liquid (depressurized). The extraction of the crude was terminated when it was visually observed that no more analyte was being trapped in the collection vial and was termed as exhaustive extraction. The crude extracts obtained were evaporated to dryness at 40 ^{o}C under reduced pressure (yielding dark green residue) before their final mass was weighed (Table 4.2) and then checked by TLC for their individual profile.

TLCs were developed with a mobile phase dichloromethane: methanol (96:4 v/v). The extracts were dissolved in dichloromethane and applied to silica gel plates. TLC plates

were then visualized under UV light (254 and 365 nm) and further sprayed with Dragendorff's reagent to detect the presence of alkaloidal compounds.

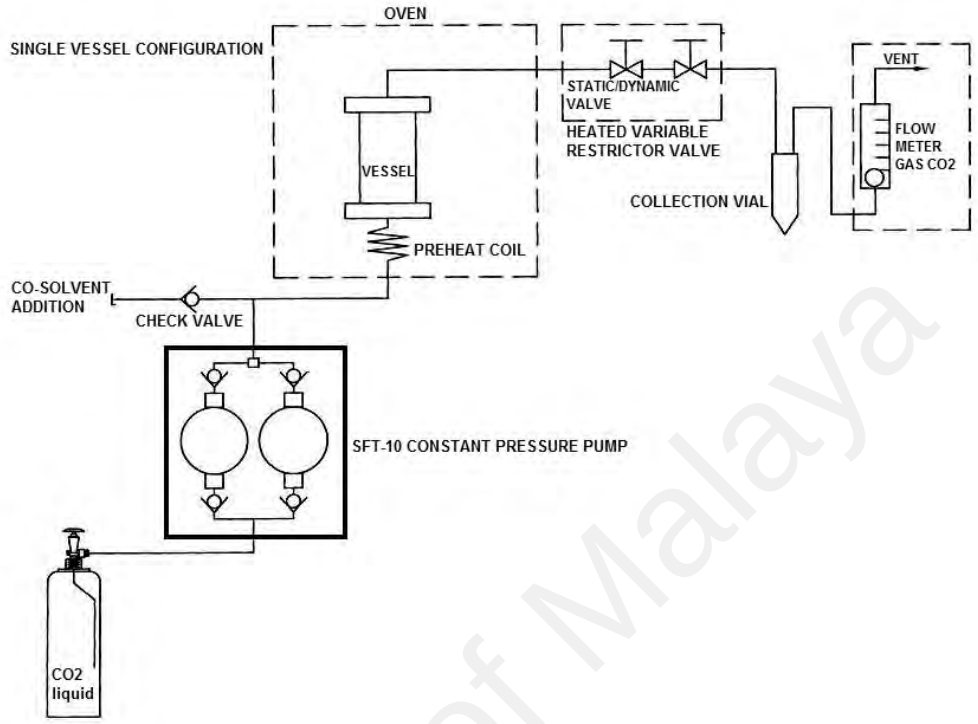


Figure 4.1 Flowsheet of the analytical-scale SFT 100 XW SFE system

Table 4.1 The L_9 $(3)^3$ Orthogonal Test design

Test no.	M: Matrix	Factors		
		A: Pressure (psi)	B: Temperature (°C)	C: Modifier (ethanol, %)[a]
1	M1	A1 1300	B1 40	C1 20
2	M2	A1 1300	B2 60	C2 40
3	M3	A1 1300	B3 80	C3 60
4	M4	A2 3000	B1 40	C2 40
5	M5	A2 3000	B2 60	C3 60
6	M6	A2 3000	B3 80	C1 20
7	M7	A3 5000	B1 40	C3 60
8	M8	A3 5000	B2 60	C1 20
9	M9	A3 5000	B3 80	C2 40

[a]Modifier (ethanol, %) = volume of added ethanol /sample mass (g) x 100

Table 4.2 The yields of L_9 $(3)^3$ Orthogonal Test crudes in the two-step extraction

M	Factors			Extraction yield (%)[a]				
	A	B	C	S1: Step 1 (100% CO_2)		S2: Step 2 (CO_2 + modifier)		Total: S1 + S2
				Crude		Crude		
M1	A1	B1	C1	M1S1	0.18	M1S2	0.10	0.28
M2	A1	B2	C2	M2S1	0.11	M2S2	0.12	0.23
M3	A1	B3	C3	M3S1	0.10	M3S2	0.01	0.11
M4	A2	B1	C2	M4S1	0.69	M4S2	0.27	0.96
M5	A2	B2	C3	M5S1	0.96	M5S2	0.12	1.08
M6	A2	B3	C1	M6S1	0.93	M6S2	0.06	0.99
M7	A3	B1	C3	M7S1	1.08	M7S2	0.48	1.56
M8	A3	B2	C1	M8S1	1.36	M8S2	0.14	1.50
M9	A3	B3	C2	M9S1	1.26	M9S2	0.19	1.45

[a] Extraction yield (%) = amount of extract/sample mass x 100

4.5 Isolation, Purification and Identification

Crude extracts using hexane, ethyl acetate, methanol, aqueous and carbon dioxide were tested for nitric oxide (NO) inhibitory activity. M5S1, which was obtained from SFE, showed the highest inhibitory activity without any sign of toxicity. Thus, M5S1 was examined by chromatographic analysis to identify the chemical constituents. The aqueous crude, even though showed low NO inhibition property, was further investigated for anti-ulcer test as it gave the least toxic effect towards the tested cells. Interestingly, the aqueous crude significantly exhibited the anti-ulcer potential. Therefore, the aqueous crude extract was subjected for further isolation, purification and chemical profiling.

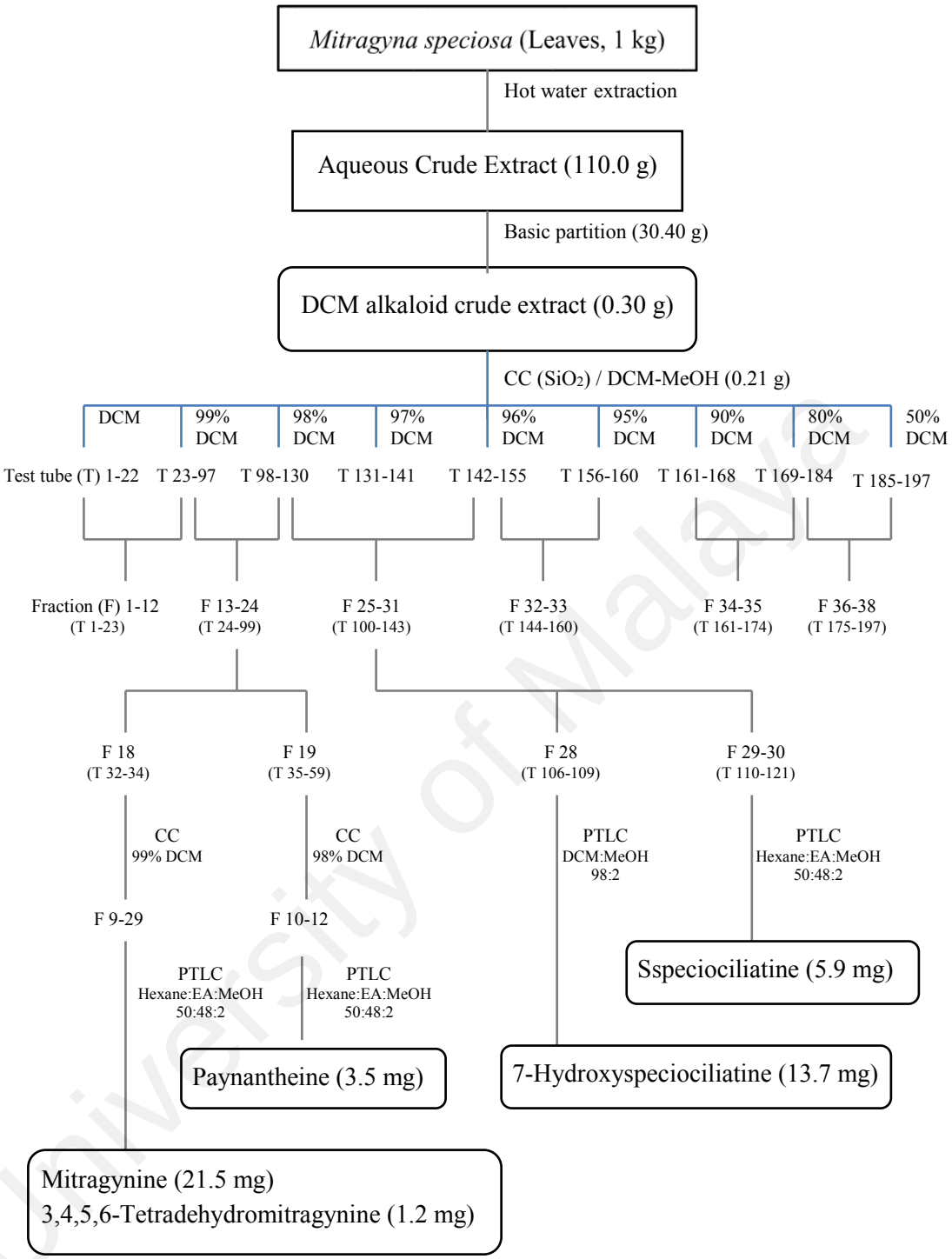
4.5.1 Isolation and Purification of Aqueous Extract

210 mg of the DCM alkaloidal crude from the aqueous extract was subjected to column chromatography over silica gel. All separations were carried out under gravity and the column was eluted with CH_2Cl_2, CH_2Cl_2: MeOH and MeOH on increasing polarity basis. The eluents were collected in the test tubes (10 mL) and monitored by TLC.

Those with the same TLC profile were grouped and combined into series of fraction. Each series of fraction was then treated separately by extensive CC and preparative TLC (PTLC) in order to isolate and purify compounds. The separation process by extensive CC or PTLC was repeated until a single spot on the TLC was obtained.

The PTLCs were developed using the same solvent combinations as above. The plates were placed in closed chambers and under the influence of gravity. Some plates were redeveloped twice or more to achieve a more desirable level of separation. NH_3 vapour was also used to aid for better separation of the alkaloid compounds by placing a small volume of NH_3 solution (in 5 mL conical flask) into the developing chamber. The developed TLCs and PTLCs were examined under an ultraviolet lamp with dual wavelengths; 254 nm and 365 nm, to detect any UV-active compounds. The TLC spots were also visualized for the position (Rf) of separated compounds by spraying the plate with Dragendorff's reagent.

The isolation and purification procedures were summarized in a flow chart diagram as shown in Scheme 4.3. The structures of the isolated compounds were elucidated with the aid of spectroscopic methods such as 1D NMR (^{1}H, ^{13}C, DEPT), 2D NMR (COSY, HSQC, HMBC, NOESY), UV, IR and LC-ESI-MS.



Scheme 4.3 Isolation and purification of compounds from the alkaloid extract of leaves of *Mitragyna speciosa* (KL5321)

4.5.1.1 High Performance Liquid Chromatography (HPLC) Profiling

Separate standard solution at 1 mg/mL for each marker compounds (mitragynine and speciociliatine) were prepared in 100% methanol and stored in darkness at -20 ^{o}C. The solution was further diluted with methanol for preparation of working standard solutions at lower concentrations. Water crude extract was reconstituted in water to give a concentration of 10000 ppm.

Standard solution of the markers and the crude extract were subjected to analytical HPLC Shimadzu LC-20AT system using a GraceSmart RP 18 column (4.6 mm i.d. x 250 mm, 5 μm) at 40 ^{o}C. The extract was eluted at a flow rate of 1.00 mL/min over 30 minutes. The gradient started at 15% mobile phase B (acetonitrile with 0.1% formic acid) and 85% mobile phase A (water with 0.1% formic acid) from 0.01 min to 8 minutes. Later, the gradient was changed from 15% to 32% (B) over 5 minutes and hold for 10 minutes. Then, it was increased from 32% to 50% (B) in 2 minutes, followed by 50-100% (B) for 2 minutes. Finally, there was an isocratic elution of 100% (B) from 27 to 30 minutes. The injection volume was 10 μL.

For the qualitative and quantitative analyses of the markers, LC-ESI-MS consisting of an Agilent 1200 SL series high-performance liquid chromatography (HPLC) system and a 6530 accurate-mass Q-TOF LC/MS instrument (Agilent Technologies, Santa Clara, USA) was used. The Agilent 1200 SL series HPLC was equipped with a degasser, a thermostated HiP-ALS auto sampler, a Bin Pump SL binary pump and a TCC SL column oven. A 1.8-μm Agilent ZORBAX Eclipse XDB-C18 column (100×2.1 mm) was operated at 40 °C. The isocratic elution conditions employed used 35% mobile phase A (water) and 65% mobile phase B (acetonitrile) delivered at 0.25 mL/min. The injection volume was 10 μL of 50 ppm standard solutions. For the detection system, a mass detector was adopted. MS analysis by electrospray ion source ESI + Dual Agilent

Jet Stream AJS was conducted by QTOF-MS instrument in the positive mode. Nitrogen gas was used for nebulization and was delivered at a flow rate of 8 L/min at 300°C. Other conditions were: nebulizer pressure, 35 psi; sheath gas temperature, 350°C; sheath gas flow, 11 L/min; VCap voltage, 3500 V; nozzle voltage, 1000 V; fragmentor voltage, 125 V; mass range (MS and MS-MS), m/z 100 to 1000; scan rate 1.39 Hz; reference ions for mass calibration, purine 121.050873 and HP-921 = hexakis (1H, 1H, 3H-tetrafluropropoxy) phosphazine, 922.009798, $[M+H]^+$. Quantitative analysis of mitragynine and speciociliatine was carried out by monitoring areas of the protonated molecular peaks ($[M + H]^+$) of target compounds in the scan mode. The monitoring ion was m/z 399 for both mitragynine and speciociliatine. Chromatographic peaks were detected and integrated by the MassHunter Acquisition B.04.00 for the Agilent TOF and QTOF and MassHunter Qualitative Analysis B.04.00.

Ultra violet-Visible spectroscopy analyses using Shimadzu UV-1601 spectrophotometer were performed on 5 ppm standard marker solutions in methanol. All solutions were scanned 500-190 nm using blank (methanol).

4.5.2 Identification of CO_2 Extract

The CO_2 extracts of SFE were subjected to gas chromatography analyses for chemical compositions characterization. Extensive identification was performed on the extract that exhibited the strongest nitric oxide inhibitory activity without showing any toxicity effect (M5S1).

4.5.2.1 Gas Chromatography

GC analysis was carried out using a Shimadzu GC-2010 gas chromatograph equipped with a FID. HP-5 fused silica capillary column of 5% phenyl 95% dimethylpolysiloxane

(30 m × 0.32 mm i.d., 0.25 μm film thickness) was employed. The oven temperature was programmed at 60 ^{o}C for 1 min, then increased to 150 ^{o}C at a rate of 4 ^{o}C/min and finally to 280 ^{o}C at 3.5 ^{o}C/min, followed by 20 min under isothermal condition. Injector and detector temperature were 250 and 300 ^{o}C, respectively. The carrier gas, helium (99.999%), was adjusted to a linear velocity of 30 cm/s and column flow of 1.21 mL/min. Two μL of the diluted SFE extract (4000 ppm) was injected in split less mode.

4.5.2.2 Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS analysis was accomplished by using a Shimadzu GC-2010 system coupled to a quadrupole mass spectrometer, MS-QP2010 Plus with the same capillary GC conditions as described above. Significant MS operating parameters were 70 eV of ionization energy, 0.5 s of scan time, ion source temperature of 200 ^{o}C and a mass range of 40-800 amu.

4.5.2.3 Identification of Constituents

The components of the extracts were identified by comparison using the NIST08s.LIB mass spectral database. The identifications were further confirmed through comparison of Kovats indices (*I*) [167-169]. The Kovats index was determined with a standard mixture of *n*-alkanes under chromatographic conditions, consistent with those of the chromatographic conditions of the SFE extract analysed. Relative amounts of individual components were calculated by the area normalization method without response factor correction.

4.5.2.4 Kovats Retention Index

Kovats retention index or simply Kovats index or retention index of a sample

component is a number, method of quantifying the relative elution times of components in such a way as to help determination of unknown constituents in GC. Retention index is obtained from GC by interpolation (usually logarithmic), relating the adjusted retention volume (time) of the sample component to the adjusted retention volumes (times) of two standards eluted before and after the peak of the sample component. In Kovats index, *n*-alkanes serve as the standards and logarithmic scale is utilized. The Kovats index's value is usually represented by the symbol *I* and is given by the equation [170]:

$$I = 100\,(N\text{-}n)\,\frac{\log t'_r(\text{unknown}) - \log t'_r(n)}{\log t'_r(N) - \log t'_r(n)} + 100n$$

Where;

I = Kovats index

N = the number of carbon atoms in the larger alkane (the alkane eluted after the peak of interest)

n = the number of carbon atoms in the smaller alkane (the alkane eluted before the peak of interest)

t'_r = the adjusted retention time (measured time minus the time of the undelayed methane or small compound, t_o)

Example:

			Retention time
unknown (linalool)	$t_{unknown}$	=	12.687 min
n-decane (C_{10})	t_{10}	=	8.918 min
n-undecane (C_{11})	t_{11}	=	13.130 min
void time	t_o	=	1.673 min

Reference alkanes:

$$\text{alpha}_R = \frac{\text{adjusted retention time of } t_{11}}{\text{adjusted retention time of } t_{10}}$$

$$= \frac{t_{11} - t_o}{t_{10} - t_o} = \frac{13.130 - 1.673}{8.918 - 1.673} = \frac{11.457}{7.245}$$

$$= 1.581$$

Unknown:

$$\text{alpha}_{\text{unknown}} = \frac{\text{adjusted retention time of } t_{unknown}}{\text{adjusted retention time of } t_{10}}$$

$$= \frac{t_{unknown} - t_o}{t_{10} - t_o} = \frac{12.687 - 1.673}{8.918 - 1.673} = \frac{11.014}{7.245}$$

$$= 1.520$$

Kovats Index of unknown compound:

$$I = 100\,(\text{N-n}) \frac{\log\ \text{alpha}_{\text{unknown}}}{\log\ \text{alpha}_R} + 100\text{n}$$

$$I = 100\,(11\text{-}10) \frac{\log\ 1.520}{\log\ 1.581} + 100\,(10)$$

$$I = 100 \frac{\log_{10}\ 1.520}{\log_{10}\ 1.581} + 1000$$

$$= 1091$$

4.5.2.5 Precision of the SFE

The precision of SFE was studied with three replicate analyses of the crude M5S1 (crude with the strongest anti-inflammatory activity without toxicity effect) of *M. speciosa*. The precision was expressed as the relative standard deviation (RSD) of the peak areas.

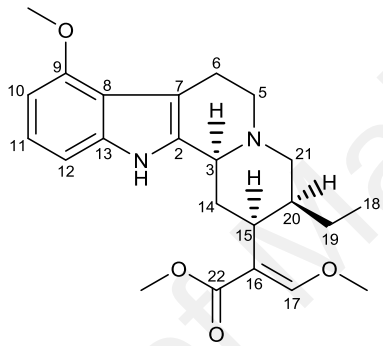
4.6 Results and Discussion

The present work deals with the isolation and structural elucidation of compounds from *Mitragyna speciosa* (leaves) of the Rubiaceae family. Interest has long been focused on the organic solvent extracts of *M. speciosa*, especially the methanol extract of the leaves. A huge number of reports have been published on the chemical composition of the organic solvent crudes. Information on the scientific research of the aqueous and SFE extracts of the leaves is still scarce. In this study, the chemical constituents of the CO_2 SFE and aqueous extracts from the leaves of *M. speciosa* were investigated as both extracts exhibited the inhibition of NO production and anti-ulcer property, respectively. Various chromatographic techniques such as column chromatography and preparative thin layer chromatography have been used in this study to afford the pure compounds from the aqueous extract. The structures were deduced from combination of spectroscopic data, i.e. 1D-NMR (1H, 13C, DEPT), 2D-NMR (COSY, HSQC, HMBC and NOESY), UV, IR and LCMS-TOF. The structures of the compounds were also confirmed by comparison with previous reported data. Characterization of the volatile constituents of the most active NO inhibition, SFE extract (M5S1) was conducted using GC and GC-MS analyses.

4.6.1 Alkaloids from the Aqueous Extract of *Mitragyna speciosa* Leaves

The investigations on the alkaloidal crude from the leaves of *Mitragyna speciosa* have resulted in the isolation of five known Corynanthe-type indole compounds, i.e. mitragynine **1**, specioliatine **4**, paynantheine **5**, 3, 4, 5, 6-tetradehydromitragynine **59** and 7-hydroxyspeciociliatine **10**.

Compound 1: Mitragynine



1

Mitragynine **1** was isolated as a yellow amorphous solid. The full-scan mass spectrum of ESI-QTOF-LCMS of compound **1** showed a strong signal for the ion at m/z 399.2281 corresponding to the protonated molecular ion peak $[M+H]^+$. This is consistent with the molecular formula of $C_{23}H_{30}N_2O_4$ (calcd. for $C_{23}H_{31}N_2O_4$, m/z 399.2284) with 10 degrees of unsaturation.

The UV spectrum of the methanol solution of mitragynine showed a maximum at 225 nm with shoulders at 247, 285 and 290 nm, which is characteristic of the indole chromophore. The IR spectrum exhibited a broadband absorption due to amine group at 3358 cm^{-1}, an intense bands which can be attributed to carbonyl group at 1697 cm^{-1}, C=C stretching at 1630 cm^{-1} and C-O stretching at 1241 cm^{-1}.

The ^{1}H NMR spectrum of mitragynine **1** (Figure 4.2) indicated the presence of an ABX pattern for the three aromatic protons at δ_H 6.98 (dd, J = 7.8, 7.8 Hz, H-11), δ_H 6.89 (d, J = 7.8 Hz, H-12) and δ_H 6.44 (d, J = 7.8 Hz, H-10). Proton H-11 resonated at the lowest field whereas proton H-10 which was shifted up field suggesting that the proton H-11 is at meta position and that H-10 is at ortho position of the methoxy group (-OMe). The methoxy group strongly affects the π cloud of the aromatic ring as a mesomeric electron donor by resonance, more so than as an inductive electron withdrawing group despite the electronegativity of the oxygen. Only meta substituent contributes inductive effects, whereas para and ortho substituents contribute both inductive and resonance effects. Overall, the electron donating resonance effects dominate over the electron withdrawing inductive effect in an aryl ring. Therefore, H-10 that is ortho to the electron donating methoxyl at C-9 is the most shielded aromatic proton. A distinct singlet of methoxy group on an aromatic ring appeared at δ_H 3.86 (9-OCH_3) and another two singlets of two other methoxy protons present at δ_H 3.72 (C-17) and δ_H 3.70 (C-22). As the methoxy group that is attached to the aryl ring (9-OMe) is electron donating through resonance effect, then the methoxy protons are more deshielded compared to those of the methoxy groups attached to C-17 and C-22. At C-17 and C-22, the methoxy groups act as an electron-withdrawing group by inductive effects of the electronegative oxygen atom. Therefore, the protons are more shielded and resonated at upper field. Seven methylene proton signals (H-5$_\alpha$, H-5$_\beta$, H-6$_\alpha$, H-14$_\alpha$, H-14$_\beta$, H-19$_b$ and H-21$_\alpha$) appeared as three overlapped multiplet sets at δ_H 2.93-2.89 (H-5$_\beta$ and H-6$_\alpha$; overlapped), δ_H 2.55-2.42 (H-5$_\alpha$, H-14$_\beta$ and H-21$_\alpha$; overlapped) and δ_H 1.80-1.73 (H-14$_\alpha$ and H-19$_b$; overlapped). Another two methylene protons (H-6$_\beta$ and H-19$_a$) appeared as multiplet at δ_H 3.09 (H-6$_\beta$) and δ_H 1.20 (H-19$_a$), while H-21$_\beta$ and H-15 (proton of the chiral carbon C-15) gave overlapping signals at δ_H 3.04-2.97. Two other methine protons of chiral carbons C-3 and C-20 appeared at δ_H 3.14 (br-d, J = 11.0 Hz, H-3) and δ_H 1.61 (br-d, J

= 11.0 Hz, H-20), respectively. The olefinic proton H-17 signal appeared downfield at δ_H 7.42 as a singlet. It resonated more downfield compared to the normal olefinic proton (δ_H 4.5-6.5) because it experienced an *α*, *β*-unsaturated effect due to its *β*-position. Meanwhile, the typical signal at δ_H 0.86 confirmed the existence of one methyl group C-18 attached to C-19. This signal appeared as a triplet with J value 7.5 Hz. A very broad downfield signal at δ_H 7.71 was assignable to N_a-H. This is supported by the existence of a broad signal at 3358 cm^{-1} in the IR spectrum.

The ^{13}C and DEPT NMR spectra (Figure 4.3) showed the presence of eleven sp^2 and twelve aliphatic carbons, compatible with the proposed structure. The three aromatic carbons, C-10, C-11 and C-12 resonated at δ_C 99.8, 121.9 and 104.3, respectively. The sp^3 methines appeared at δ_C 61.4 (C-3), δ_C 40.0 (C-15) and δ_C 40.8 (C-20). C-3 is more deshielded than C-15 and C-20 because of the electronegativity of the nitrogen atom, N_b that is adjacent to it. The same reason applies for C-5 and C-21 that caused resonation at lower field (δ_C 53.9 and 57.8) compared to other methylene carbons C-6, C-14 and C-19, which showed signals at δ_C 24.0, 30.0 and 19.2, respectively. The methoxy group adjacent to the carbonyl carbon (C-22) appeared at δ_C 51.4, while the one attached to carbon of the aromatic ring (C-9) and the olefinic group (C-17) gave signal at δ_C 55.4 and δ_C 61.6, respectively. The appearance of signal at δ_C 169.3 indicated the presence of carbonyl carbon of an ester (C-22), which experienced an electron withdrawing effect by oxygen atom. A thorough analysis of ^{13}C NMR spectrum revealed the presence of another six quaternary carbons (C-2, C-7, C-8, C-9, C-13 and C-16), which gave signals at δ_C 133.7, 107.9, 117.7, 154.6, 137.3 and 111.6, respectively.

The COSY spectrum (Figure 4.5) gave rise to an off-diagonal signal between the CH_2-6 and CH_2-5 (H-6/H-5) thus indicating that they are vicinal to each other. The cross peaks

corresponding to H-3$_\alpha$/H-14$_\alpha$, H-15$_\alpha$/H-14$_\alpha$, H-15$_\alpha$/H-20$_\alpha$, H-19$_a$/H-20$_\alpha$, H-19$_a$/H-18, H-19$_b$/H-18 and H-21$_\alpha$/H-20$_\alpha$ coupling designated the monoterpenoid unit of the Corynanthe skeleton.

The long-range correlations in the HMBC spectrum confirmed the findings in 1D NMR spectra. Cross peaks that linked H-11 to C-9 and C-13, N_a-H to C-2, C-7 and C-8 were attributed to the five quaternary carbons of the indoline ring (C-2, C-7, C-8, C-9 and C-13). Correlation between H-17 with C-17-OMe, C-15, C-16 and C-22 depicted the position of $\Delta^{16,17}$-double bond. The connections established between 9-OMe with C-9, 17-OMe with C-17 and 22-OMe with C-22 confirmed the location of all the methoxy groups. In addition, the presence of C-22 as carbonyl carbon was further supported with the occurrence of a low field peak at δ_C 169.3, which was seen to correlate only with H-17 and H-15 in the HMBC spectrum. H-5$_\alpha$ revealed correlation with C-3 while H-5$_\beta$ showed connections to C-3, C-6 and C-7. Finally, the ethyl substitution at C-20 position was supported by cross peaks between H-18 to C-19 (J_2) and C-20 (J_3). Other significant correlations were shown in Figure 4.7.

The H-3 and H-20 were shown to be α-oriented since both gave NOESY coupling to H-15, which is also α-oriented [32, 33]. Thorough analyses of all methylene protons' signals in NOESY enable the assignment of all the H$_\alpha$ and H$_\beta$ of carbons 5, 6, 14 and 21.

The comparison between observed data and literature value confirmed that alkaloid **1** is mitragynine. Complete ^{1}H and ^{13}C NMR spectral assignments were given inTable 4.3.

Table 4.3 ^{1}H NMR [400 MHz, δ_H (J, Hz)] and ^{13}C NMR [100 MHz, δ_C] of Mitragynine **1** in $CDCl_3$

Position	^{1}H (J, Hz)	^{1}H (500 MHz, $CDCl_3$) [82]	^{13}C	^{13}C (125 MHz, $CDCl_3$) [82]
1	7.71 (1H, br-s, N_a-H)	7.80 (1H, br-s, N_a-H)	-	-
2	-	-	133.7	133.7
3_α	3.14 (1H, br-d, J = 11.0)	3.13 (1H, br-d, J = 9.8)	61.4	61.2
4	-	-	-	-
5_α	2.55-2.42 (1H, m o/lap H-14_β, H-21_α)	2.55-2.42 (1H, o/lap H-14, H-21)	53.9	53.8
5_β	2.93-2.89 (1H, m o/lap H-6_α)	2.95-2.89 (1H, o/lap H-6)		
6_α	2.93-2.89 (1H, m o/lap H-5_β)	2.95-2.89 (1H, o/lap H-5)	24.0	23.9
6_β	3.09 (1H, m)	3.09 (1H, m)		
7	-	-	107.9	107.9
8	-	-	117.7	117.7
9	-	-	154.6	154.5
10	6.44 (1H, d, J = 7.8)	6.44 (1H, d, J = 7.9)	99.8	99.8
11	6.98 (1H, t, J = 7.8)	6.98 (1H, dd, J = 7.9, 7.9)	121.9	121.8
12	6.89 (1H, d, J = 7.8)	6.89 (1H, d, J = 7.9)	104.3	104.1
13	-	-	137.3	137.2
14_α	1.80-1.73 (1H, m o/lap H-19b)	1.80-1.73 (1H, o/lap H-19)	30.0	30.0
14_β	2.55-2.42 (1H, m o/lap H-5_α, H-21_α)	2.55-2.42 (1H, o/lap H-5, H-21)		
15_α	3.04-2.97 (1H, m o/lap H-21_β)	3.04-2.97 (1H, o/lap H-21)	40.0	39.9
16	-	-	111.6	111.5
17	7.42 (1H, s)	7.42 (1H, s)	160.6	160.5
18	0.86 (3H, t, J = 7.5)	0.86 (3H, dd, J = 7.3, 7.3)	12.9	12.9
19_a	1.20 (1H, m)	1.19 (1H, m)	19.2	19.1
19_b	1.80 and 1.73 (1H, m o/lap H-14_α)	1.80 and 1.73 (1H, o/lap H-14)		
20_α	1.61(1H, br-d, J = 11.0)	1.61(1H, br-d, J = 11.3)	40.8	40.7
21_α	2.55-2.42 (1H, m o/lap H-5_α, H-14_β)	2.55-2.42 (1H, o/lap H-5, H-14)	57.8	57.8
21_β	3.04-2.97 (1H, m o/lap H-15_α)	3.04-2.97 (1H, o/lap H-15)		
9-OMe	3.86 (3H, s)	3.87 (3H, s)	55.4	55.3
17-OMe	3.72 (3H, s)	3.71 (3H, s)	61.6	61.5
COOMe	3.70 (3H, s)	3.70 (3H, s)	51.4	51.3
COO	-	-	169.3	169.2

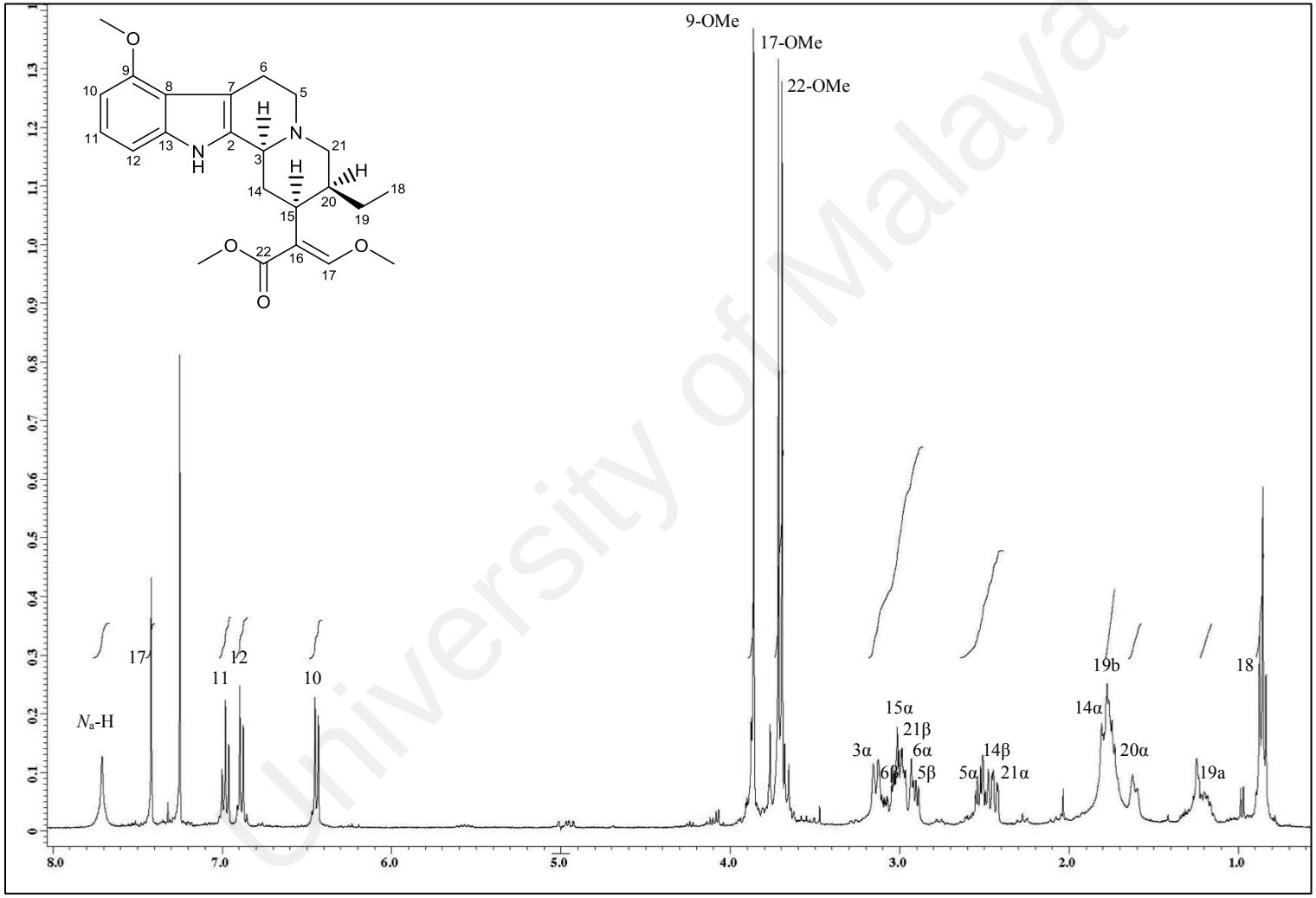


Figure 4.2 ^{1}H NMR spectrum of Mitragynine **1** on JEOL ECA 400 MHz

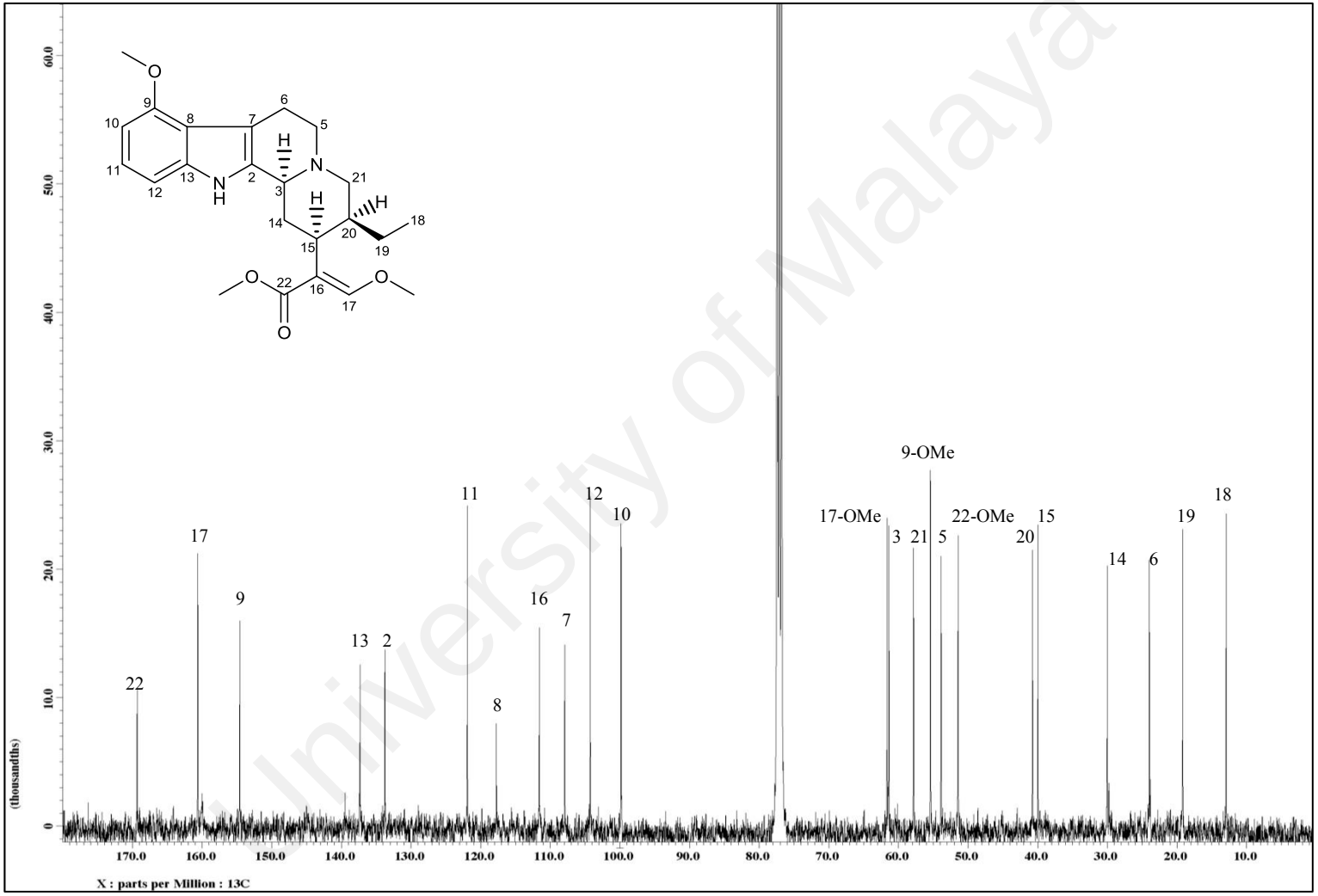


Figure 4.3 ^{13}C NMR spectrum of Mitragynine **1** on JEOL ECA 400 MHz

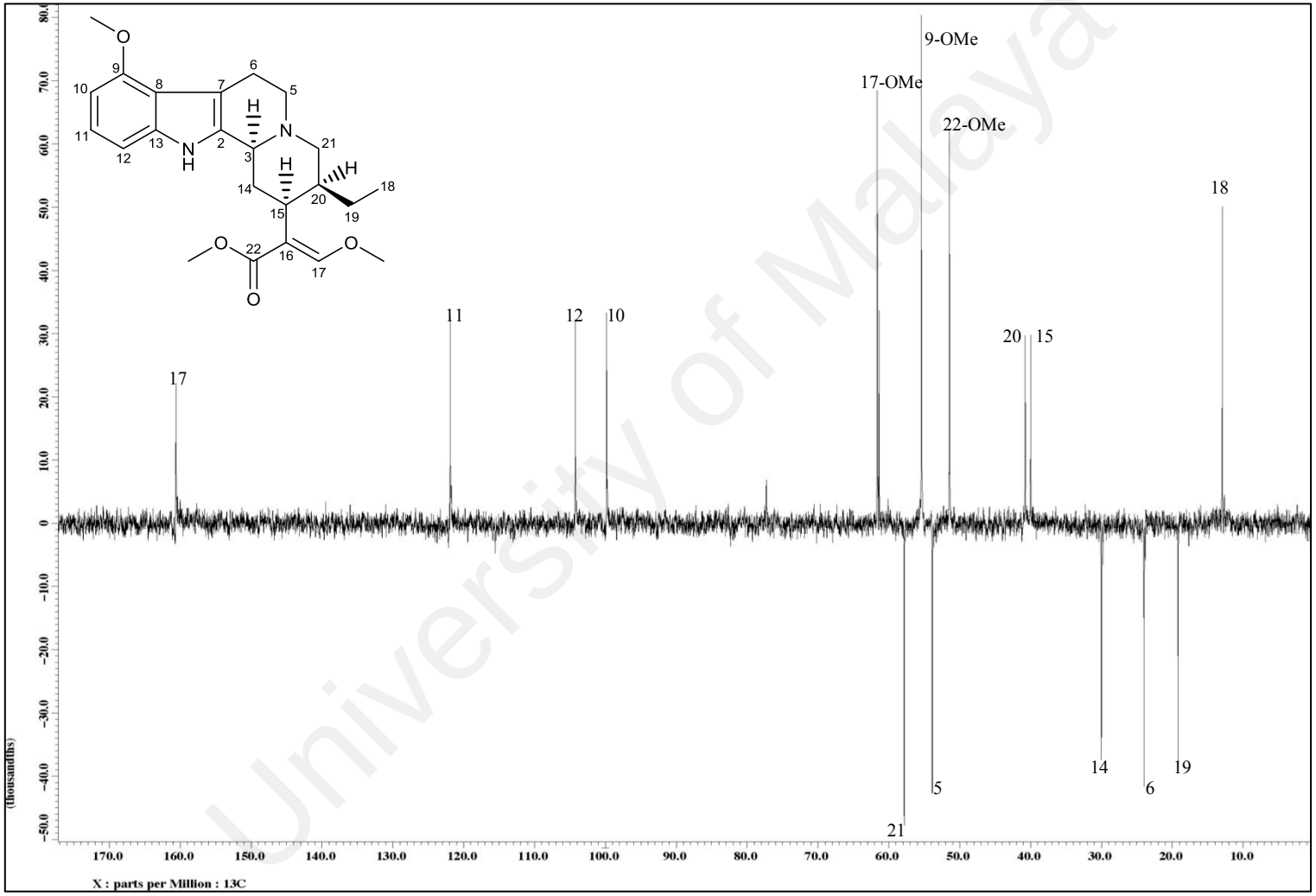


Figure 4.4 DEPT NMR spectrum of Mitragynine **1** on JEOL ECA 400 MHz

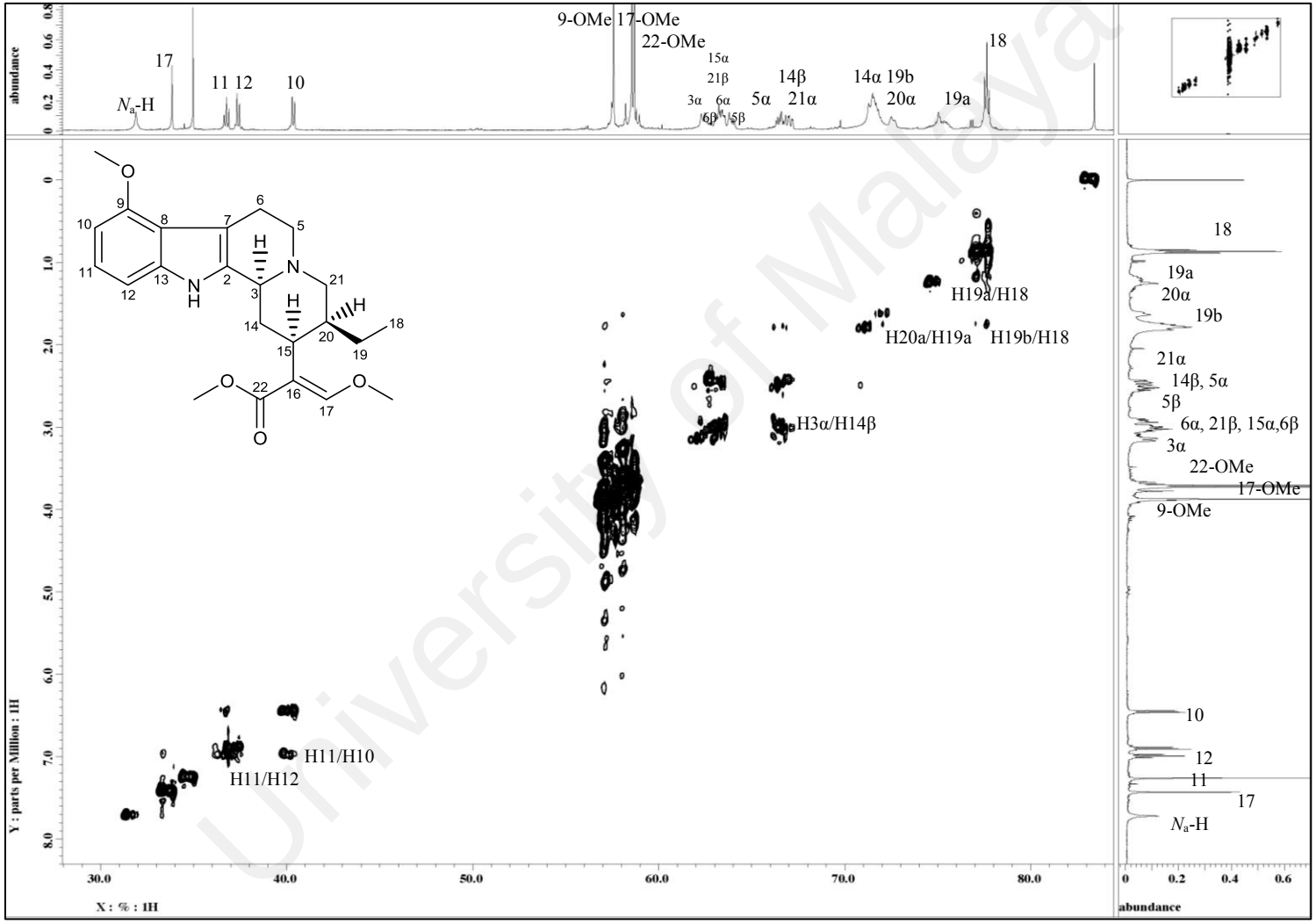


Figure 4.5 COSY NMR spectrum of Mitragynine **1** on JEOL ECA 400 MHz

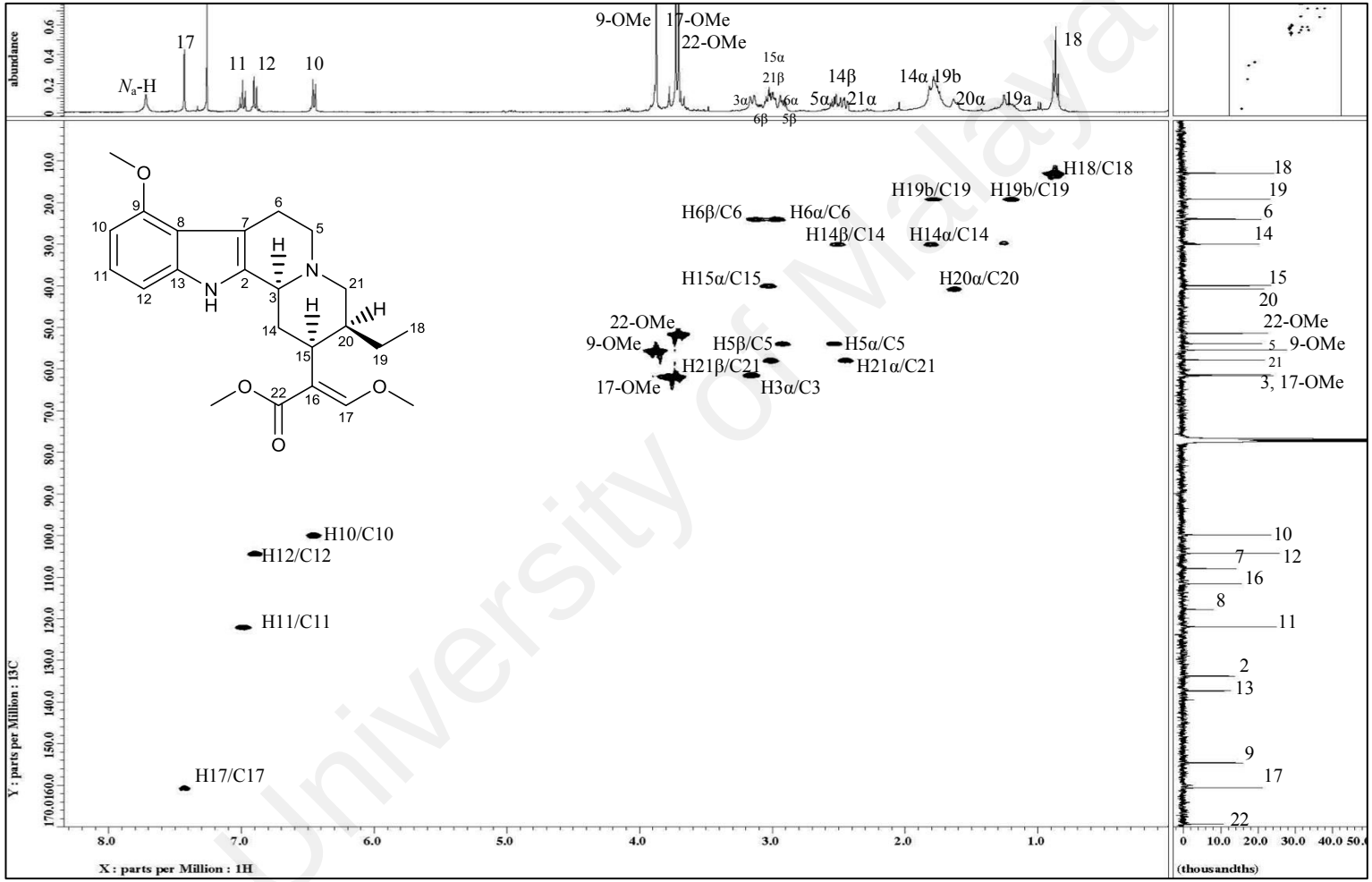


Figure 4.6 HSQC NMR spectrum of Mitragynine **1** on JEOL ECA 400 MHz

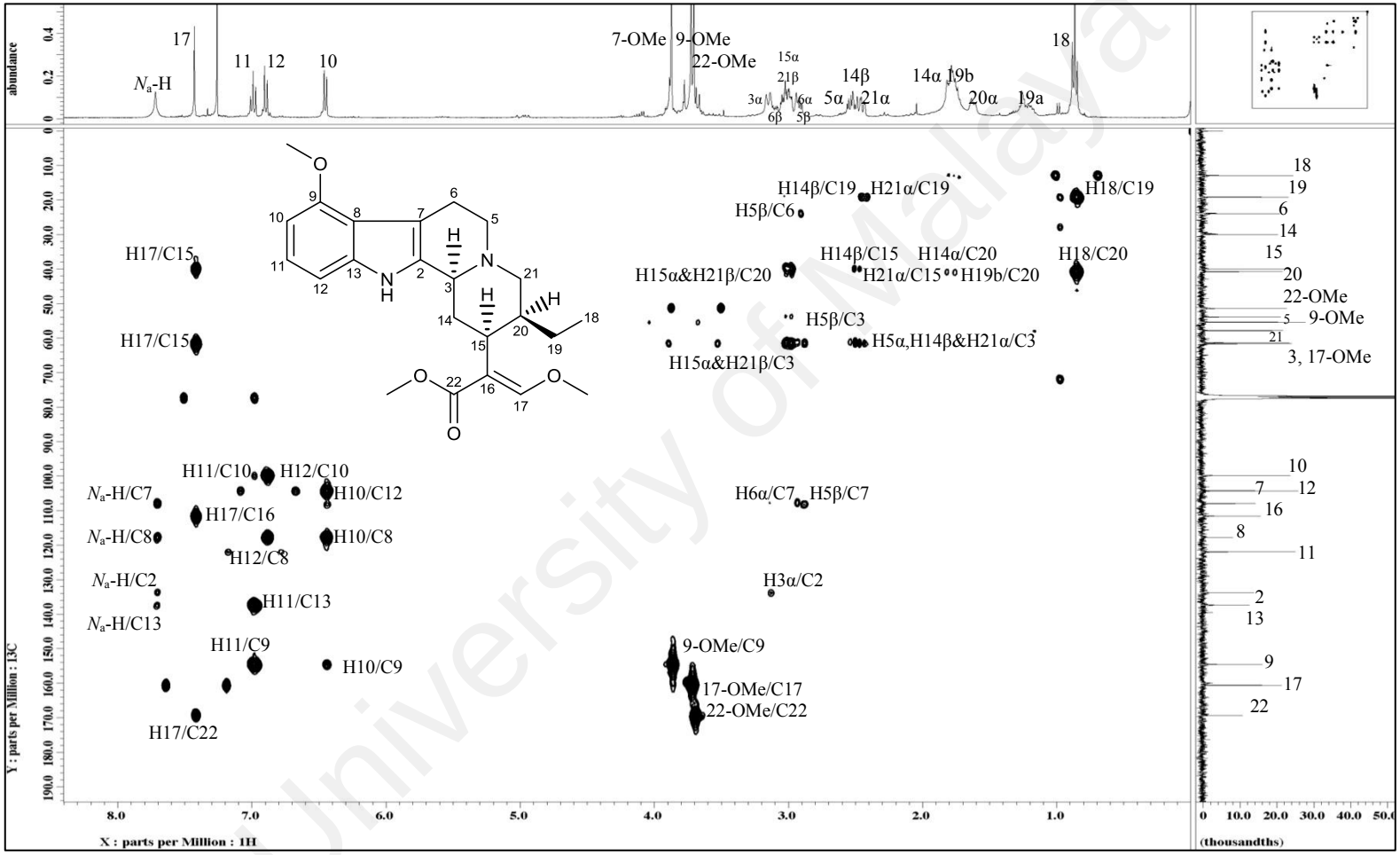
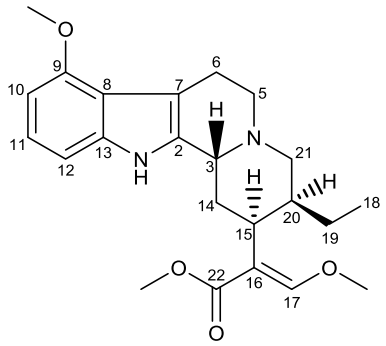


Figure 4.7 HMBC NMR spectrum of Mitragynine **1** on JEOL ECA 400 MHz

Compound 4: Speciociliatine



4

Speciociliatine **4** was obtained as yellow amorphous powder. It was assigned a molecular formula of $C_{23}H_{30}N_2O_4$ based on the quasi-molecular ion peak $[M+H]^+$ at 399.2279 (calcd. for $C_{23}H_{31}N_2O_4$, m/z 399.2284) in the HRESIMS spectrum with 10 degrees of unsaturation.

The UV spectrum gave a maximum absorption at 225 nm with shoulders at 247, 285 and 290 nm. The IR spectrum revealed important peaks at 1241, 1625 and 1680 cm^{-1} characteristic of C-O stretching, C=C stretching and conjugated ester carbonyl functional groups, respectively. A broad band at 3350 cm^{-1} indicated the presence of amine group.

The ^1H NMR spectral patterns of speciociliatine (Figure 4.8) were very reminiscent to that of **1** except for the signals associated with two methoxy groups (17-OMe and 22-OMe) and also the H-3. Unlike mitragynine, of which the two methoxy group signals resonated closely at δ_H 3.72 (17-OMe) and δ_H 3.70 (22-OMe), anisotropic effect was more significant in speciociliatine **4**. In comparison to mitragynine, proton signal of 22-OMe of speciociliatine **4** was resonated particular up field at δ_H 3.66 due to anisotropic shielding effect from neighbouring carbonyl group whereas signal from 17-OMe was more downfield at δ_H 3.77 caused by deshielding effect from anisotropy of the $\Delta^{16,17}$ -

double bond. A broad singlet at δ_H 4.22 was ascribable to *β*–orientation of H-3 as it was shifted more downfield than that of mitragynine (H-3$_\alpha$).

The ^{13}C and DEPT NMR spectra (Figure 4.9) were evident for twenty-three carbons. These values were in complete agreement with that recorded previously for speciociliatine 4, a diastereomer of the main alkaloid mitragynine [82]. The peak for C-21 could not be observed clearly in the ^{13}C NMR spectrum. However, from the DEPT spectrum it was obvious that the peak for C-21 existed at around δ_C 50.8. Furthermore, the chemical shifts of C-18 and C-19 of the ethyl group that was attached to C-20 in 4 were similar to those in 1, of which possessed a *β*-ethyl group at C-20.

The HMBC (Figure 4.12) correlation patterns largely resemble that of **1**. Of particular importance was a cross peak that linked H-3*β* to C-14. This correlation supported the assignment of *β*-orientation of H-3, as it resonated at lower field than *α*-hydrogen of mitragynine.

In depth analyses of 1D and 2D NMR spectra and further comparison with the published data [82, 103, 111] confirmed that **4** is speciociliatine, a diastereoimer and indeed an epimer of mitragynine. The chemical shift of the two-methoxyl protons (17-OMe and 22-COOMe) is one of the characteristic features that can be used to differentiate the compound from its other stereoisomer. Another characteristic peak for this compound is the one of H-3, which appear at δ_H 4.22. The NMR data of **4** were presented in Table 4.4.

Table 4.4 ^{1}H NMR [400 MHz, δ_H (*J*, Hz)] and ^{13}C NMR [100 MHz, δ_C] of Speciociliatine **4** in $CDCl_3$

Position	^{1}H (*J*, Hz)	^{1}H (500 MHz, $CDCl_3$) [82]	^{13}C	^{13}C (125 MHz, $CDCl_3$) [82]
1	7.91 (1H, br-s, N_a-H)	7.77 (1H, br-s, N_a-H)	-	-
2	-	-	132.5	132.1
3_β	4.22 (1H, br-s)	4.12 (1H, br-s)	54.8	54.6
4	-	-	-	-
5_α	3.03 (1H, m)	3.01 (1H, m)	52.5	52.5
5_β	3.21-3.16 (1H, m o/lap H-6_β)	3.12 (1H, dd, *J* = 12.5, 5.8)		
6_α	2.92 (1H, m)	2.89 (1H, m)	21.1	20.8 br
6_β	3.21-3.16 (1H, m o/lap H-5_β)	3.19 (1H, m)		
7	-	-	107.9	108.0
8	-	-	117.8	117.8
9	-	-	154.4	154.3
10	6.46 (1H, d, *J* = 8)	6.47 (1H, d, *J* = 7.9)	99.8	99.6
11	7.00 (1H, t, *J* = 8)	7.00 (1H, dd, *J* = 7.9, 7.9)	122.0	121.7
12	6.91 (1H, d, *J* = 8)	6.91 (1H, d, *J* = 7.9)	104.4	104.3
13	-	-	137.3	137.0
14_α	1.93 (1H, m)	1.90 (1H, ddd, *J* = 13.9, 5.3, 5.3)	29.8 br	30.3 br
14_β	2.62 (1H, m)	2.62 (1H, m)		
15_α	2.94 (1H, m)	2.97 (1H, m)	33.5	33.7
16	-	-	111.2	111.5
17	7.42 (1H, s)	7.42 (1H, s)	160.3	160.0
18	0.88 (3H, t, *J* = 7.3)	0.89 (3H, dd, *J* = 7.3, 7.3)	12.6	12.6
19_a	1.25 (1H, m)	1.25 (1H, m)	20.6 br	20.5 br
19_b	1.62 (1H, m)	1.63 (1H, m)		
20_α	1.77 (1H, br-s)	1.74 (1H, br-s)	39.4	39.7
21_α	2.74 (1H, m)	2.76 (1H, dd, *J* = 11.3, 5.8)	50.8	50.8 br
21_β	2.85 (1H, m)	2.89 (2H, o/lap H-6)		
9-OMe	3.88 (3H, s)	3.89 (3H, s)	55.3	55.2
17-OMe	3.77 (3H, s)	3.77 (3H, s)	61.5	61.3
COOMe	3.66 (3H, s)	3.66 (3H, s)	51.5	51.4
COO	-	-	169.5	169.4

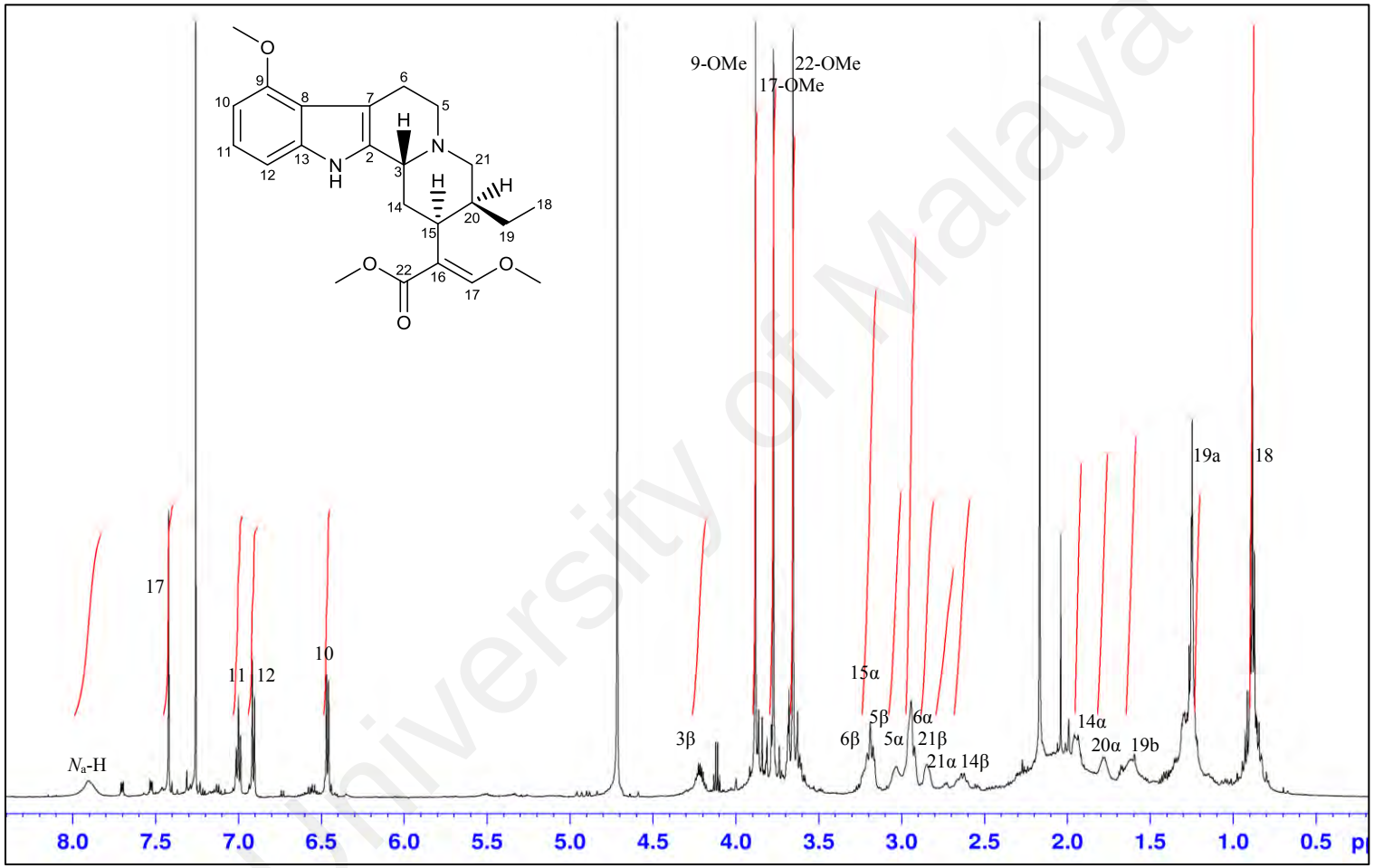


Figure 4.8 ^{1}H NMR spectrum of Speciociliatine **4** on Bruker Avance III 600 MHz

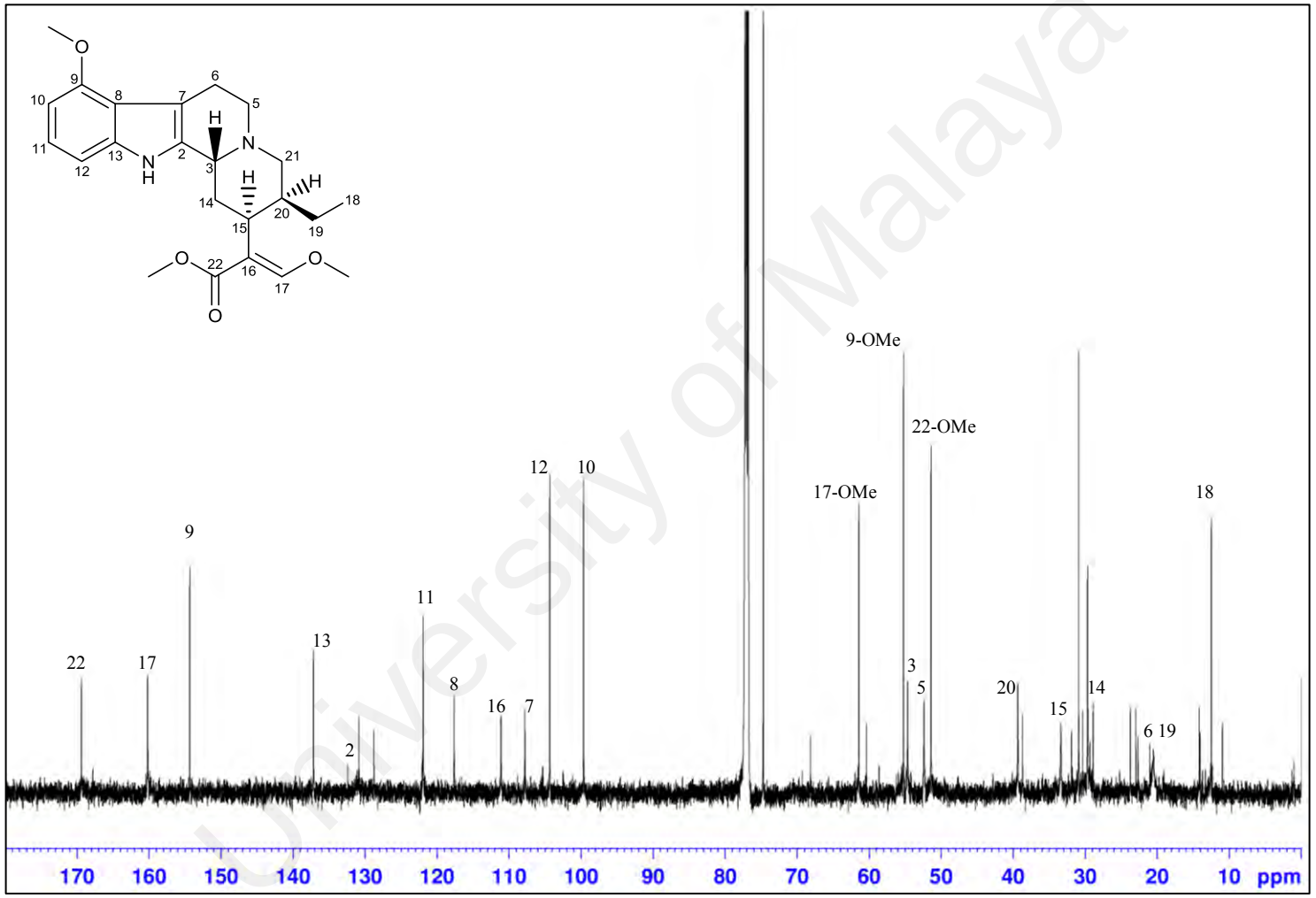


Figure 4.9 ^{13}C NMR spectrum of Speciociliatine **4** on Bruker Avance III 600 MHz

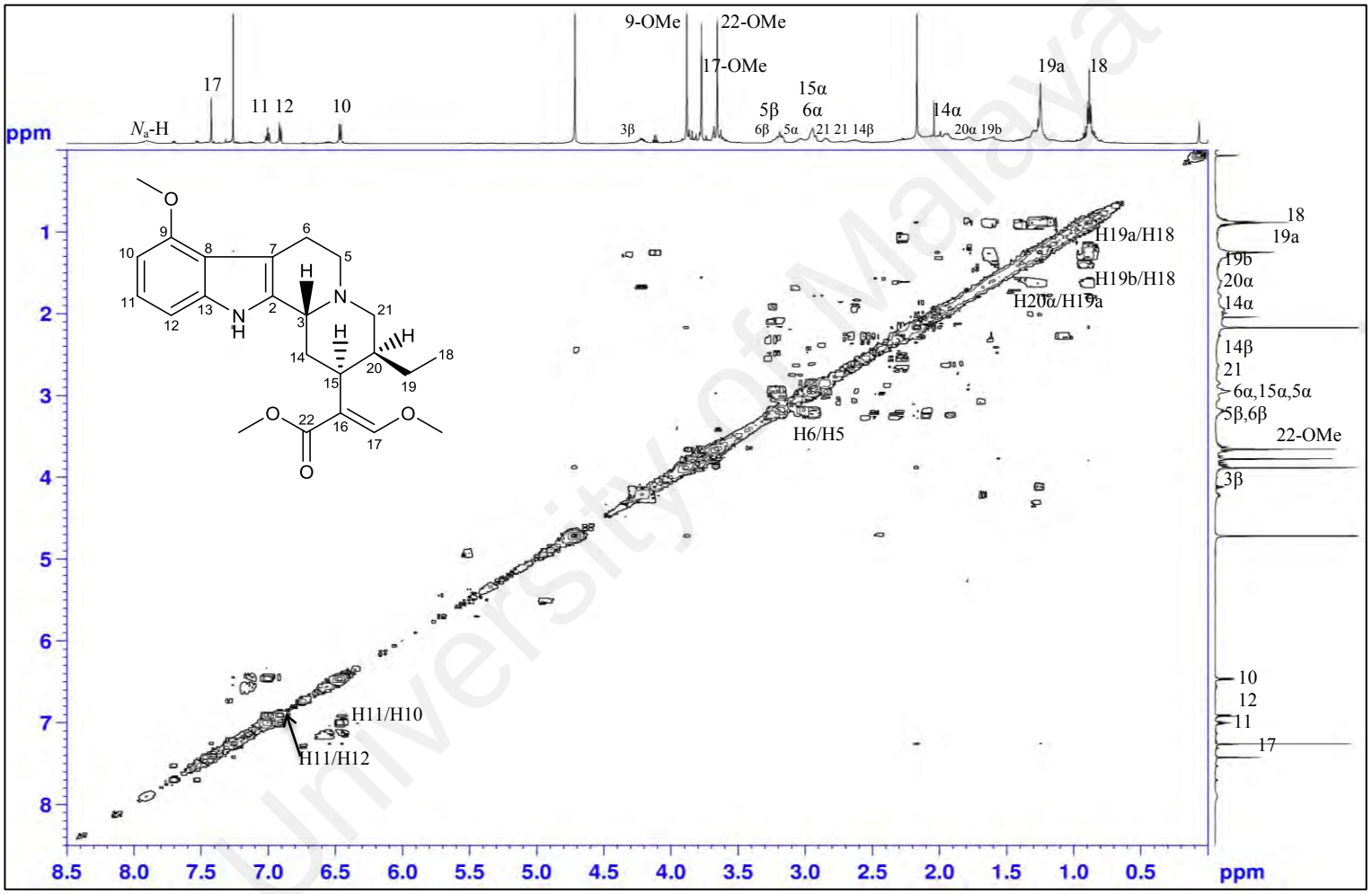


Figure 4.10 COSY NMR spectrum of Speciociliatine **4** on Bruker Avance III 600 MHz

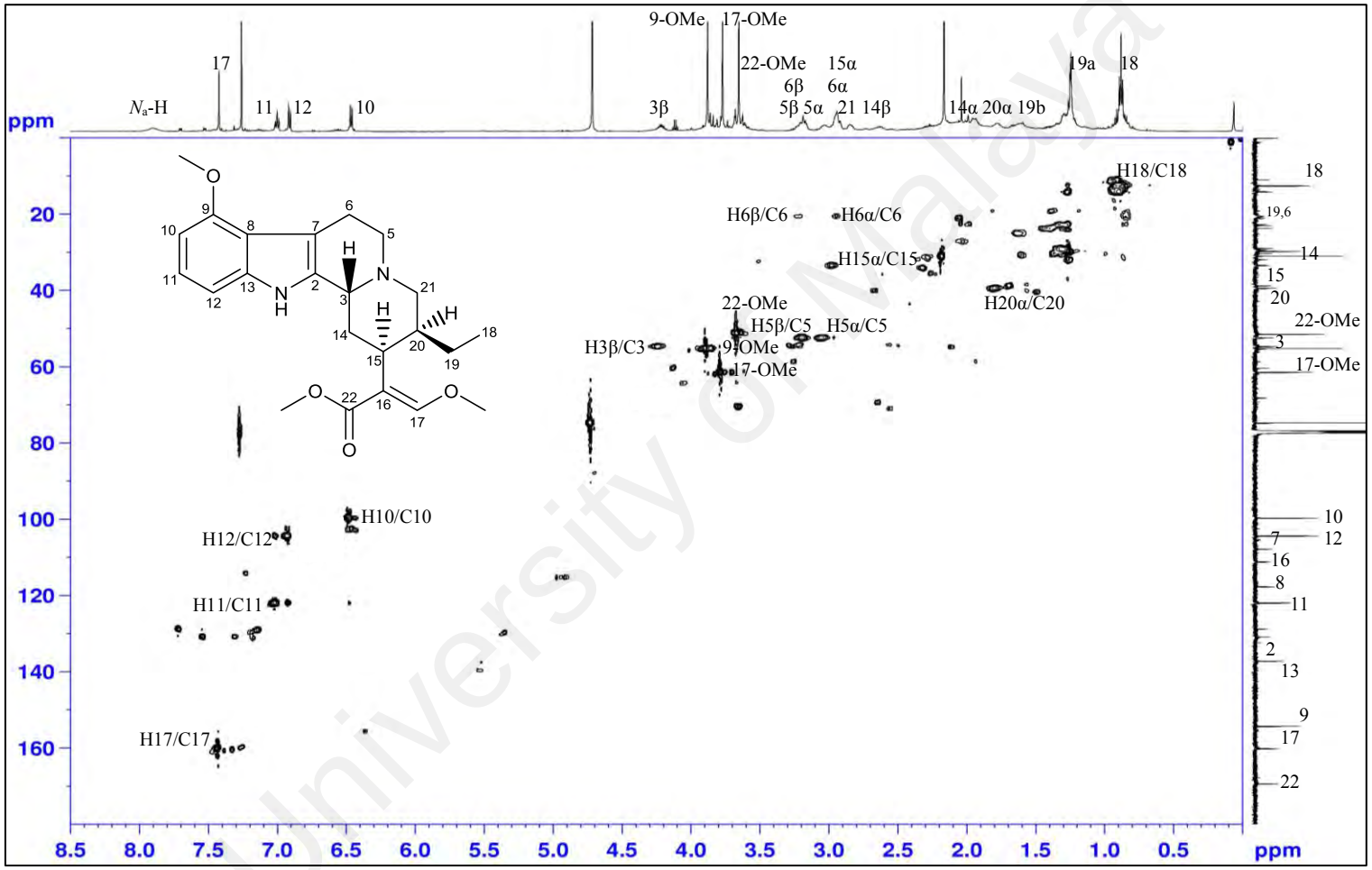


Figure 4.11 HSQC NMR spectrum of Speciociliatine **4** on Bruker Avance III 600 MHz

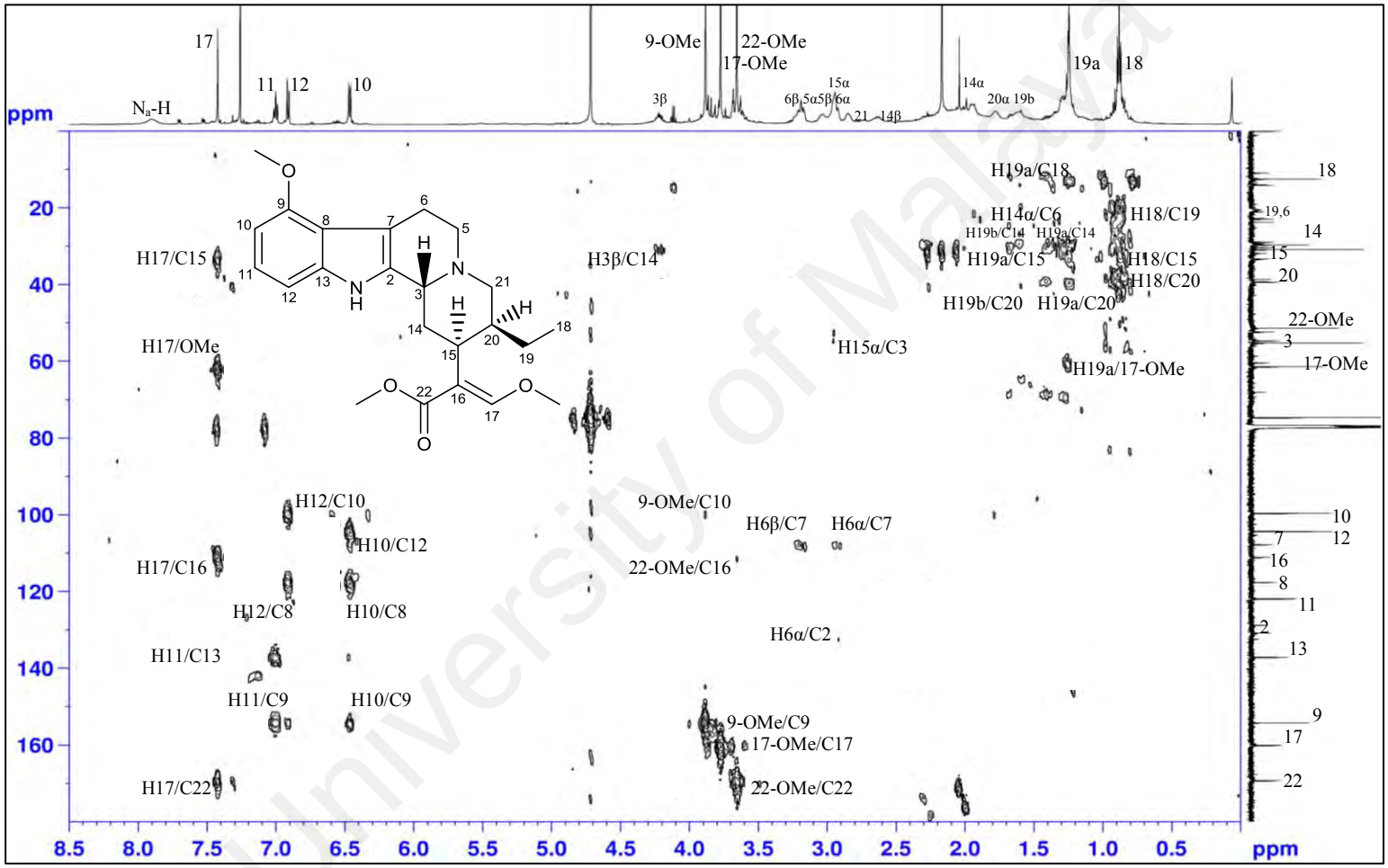
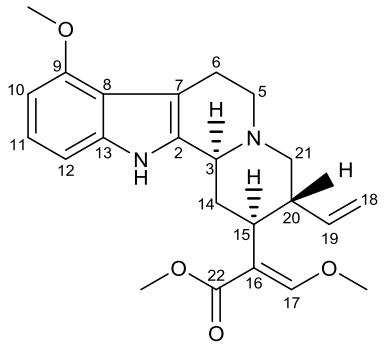


Figure 4.12 HMBC NMR spectrum of Speciociliatine **4** on Bruker Avance III 600 MHz

Compound 5: Paynantheine



5

Paynantheine **5** was afforded as yellowish amorphous powder. The HRESIMS indicated a molecular formula of $C_{23}H_{29}O_4N_2$, derived from the quasi-molecular ion peak $[M+H]^+$ at m/z 397.2126 (calcd. for $C_{23}H_{29}O_4N_2$, m/z 397.2127). Consequently, **5** possesses a molecular formula of $C_{23}H_{28}O_4N_2$ and 11 degrees of unsaturation; therefore it has one more unsaturation degree than mitragynine **1** and speciociliatine **4**.

The UV spectrum gave a maximum at 243 nm with shoulder at 291 nm, which characterized the indole chromophore. The IR spectrum showed a broad amine-stretching band at 3358 cm^{-1} and two bands associated with carbonyl stretches at 1697 cm^{-1} and alkene stretches at 1630 cm^{-1}.

^1H NMR (Figure 4.13) and ^{13}C NMR (Figure 4.14) spectrum for paynantheine **5** are close to those of mitragynine **1** and specioliatine **4**, revealing the presence of a 9-methoxy analogue of the corynantheine-type alkaloid. In place of aliphatic proton signals due to the ethyl side chain C-18/C-19 in **1** and **4**, **5** exhibited the signals of a mono-substituted double bond (δ_H 4.95, dd, *J*=10.5 and 2 Hz, H-18_a; 5.00, dd, *J*=17.4 and 2 Hz, H-18_b; 5.58, m, H-19; δ_C 115.52, C-18; 139.39, C-19). The stereochemistry at C-3, C-15 and C-20 could be deduced from the coupling values of the corresponding protons. H-3 (δ_H 3.28, br-d, *J*=11.4 Hz) showed a diaxial coupling to H-4_{ax} and H-15

(δ_H 2.75, ddd, *J*=12.2, 12.2, 3.8 Hz) presents two diaxial couplings to H-14_{ax} and H-20. Thus, the monitored protons are all in axial positions, indicating the so-called normal configuration for 9-methoxycorynantheines with H-3_α, H-15_α and H-20_β [171].

The DEPT spectrum (Figure 4.15) proved informative as it supports the existence of the vinyl group. Peak at δ_C 115.52 (C-18) and δ_C 139.39 (C-19) were confirmed to be sp^2 methine and methylene, respectively. In addition, the HMBC spectrum (Figure 4.18) also substantiated the presence of the vinyl group as it gave correlation between H-18 and C-20.

Detailed analyses of 2D NMR spectroscopic data, in addition to extensive literature research have led to the full assignments of the ^1H and ^{13}C data that concluded **5** is paynantheine. The observed data was in good agreement with data of paynantheine **5** produced by Philipp *et al.* (2010) [151]. The NMR data of **5** were presented in Table 4.5.

Table 4.5 ^{1}H NMR [400 MHz, δ_H (*J*, Hz)] and ^{13}C NMR [100 MHz, δ_C] of Paynantheine **5** in $CDCl_3$

Position	^{1}H (*J*, Hz)	^{1}H (500 MHz, $CDCl_3$) [151]	^{13}C	^{13}C (125 MHz, $CDCl_3$) [151]
1	7.69 (1H, br-*s,* N_a-H)	-	-	-
2	-	-	133.10	133.7[b]
3_α	3.28 (1H, br-d, *J* = 11.4)	3.27 (1H, br-d, *J* = 11)	60.07	60.05
4	-	-	-	-
5_α	2.58 (1H, ddd, *J* = 11.4, 11.4, 4.1)	2.55 (1H, *ddd*, *J* = 11.5, 11.5, 4.5)	53.25	53.24
5_β	3.07 (1H, m)	3.06 (1H, m)		
6_α	2.98 (1H, m)	2.99 (1H, m)	23.74	23.72
6_β	3.18 (1H, m)	3.17 (1H, m)		
7	-	-	107.91	107.88
8	-	-	117.55	117.54
9	-	-	154.53	154.51
10	6.45 (1H, br-d, *J* = 7.8)	6.44 (1H, br-d, *J* = 8)	99.87	99.79
11	7.00 (1H, t, *J* = 8.0)	6.98 (1H, t, *J* = 8)	121.98	121.96
12	6.87 (1H, br-d, *J* = 7.8)	6.86 (1H, br-d, *J* = 8)	104.19	104.18
13	-	-	137.33	137.31
14_α	1.95 (1H, m)	1.94 (1H, m)	33.43	33.44
14_β	2.11 (1H, m)	2.10 (1H, m)		
15_α	2.75 (1H, ddd, *J* = 12.2, 12.2, 3.8,)	2.74 (1H, ddd, *J* = 12, 12, 3.5)	38.90	38.5[a]
16	-	-	111.60	111.5[b]
17	7.33 (1H, s)	7.31 (1H, s)	159.91	159.82
18_a	4.95 (1H, dd, J = 10.5, 2)	4.93 (1H, dd, *J* = 10.5, 2)	115.52	115.47
18_b	5.00 (1H, dd, *J* = 17.4, 2)	4.98 (1H, dd, *J* = 17.5, 2)		
19	5.58 (1H, m)	5.56 (1H, m)	139.39	139.1[a]
20_β	3.04 (1H, m)	3.03 (1H, m)	42.90	42.87
21_α	2.29 (1H, m)	2.27 (1H, m)	61.45	61.34
21_β	3.02 (1H, m)	3.01 (1H, m)		
9-OMe	3.87 (3H, s)	3.85 (3H, s)	55.40	55.31
17-OMe	3.77 (3H, s)	3.76 (3H, s)	61.65	61.56
COOMe	3.69 (3H, s)	3.67 (3H, s)	51.39	51.29
COO	-	-	169.0[b]	172.2[b]

[a] Estimated from observed HSQC crosspeak

[b] Estimated from observed HMBC crosspeak

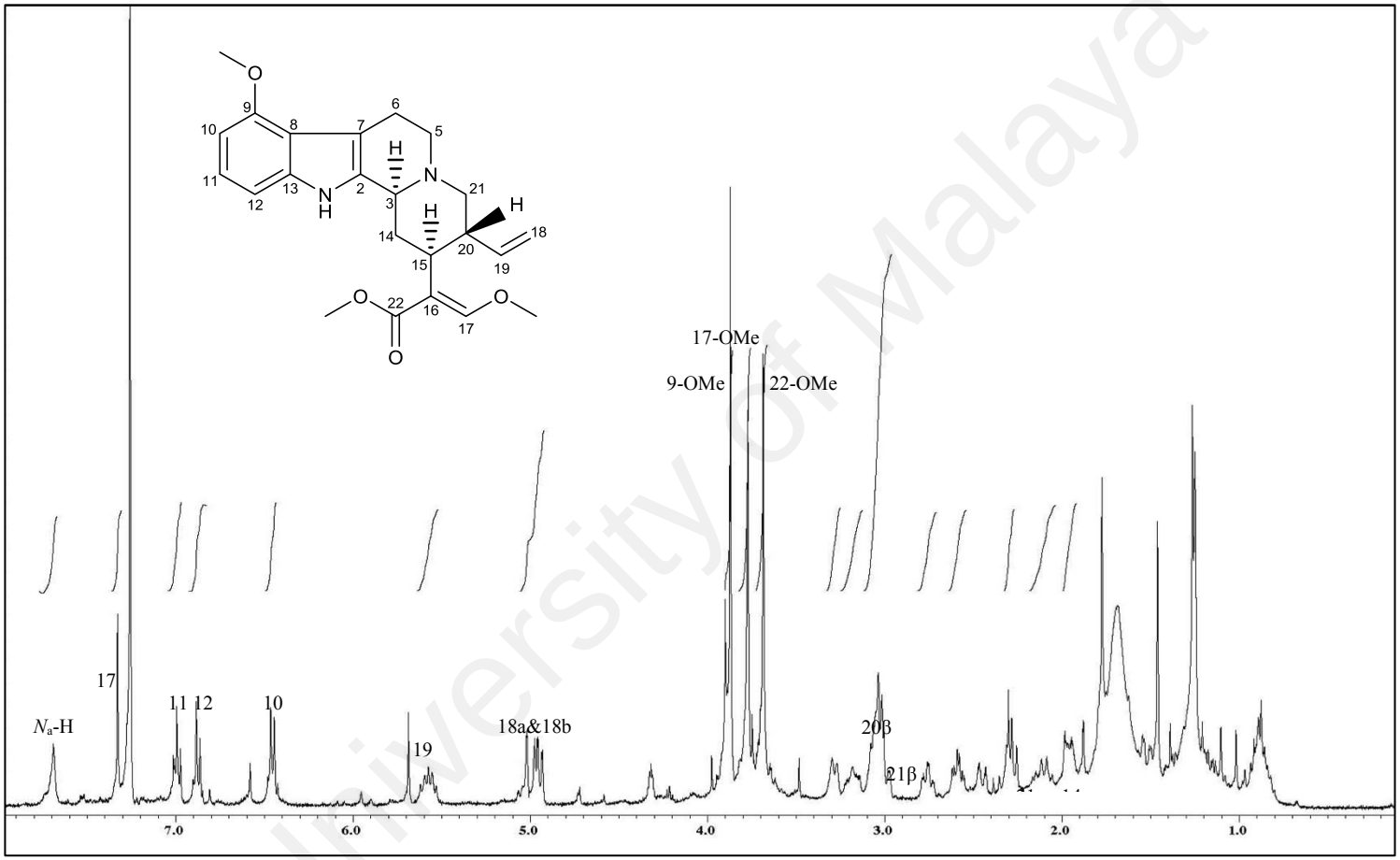


Figure 4.13 ^{1}H NMR spectrum of Paynantheine **5** on JEOL ECA 400 MHz

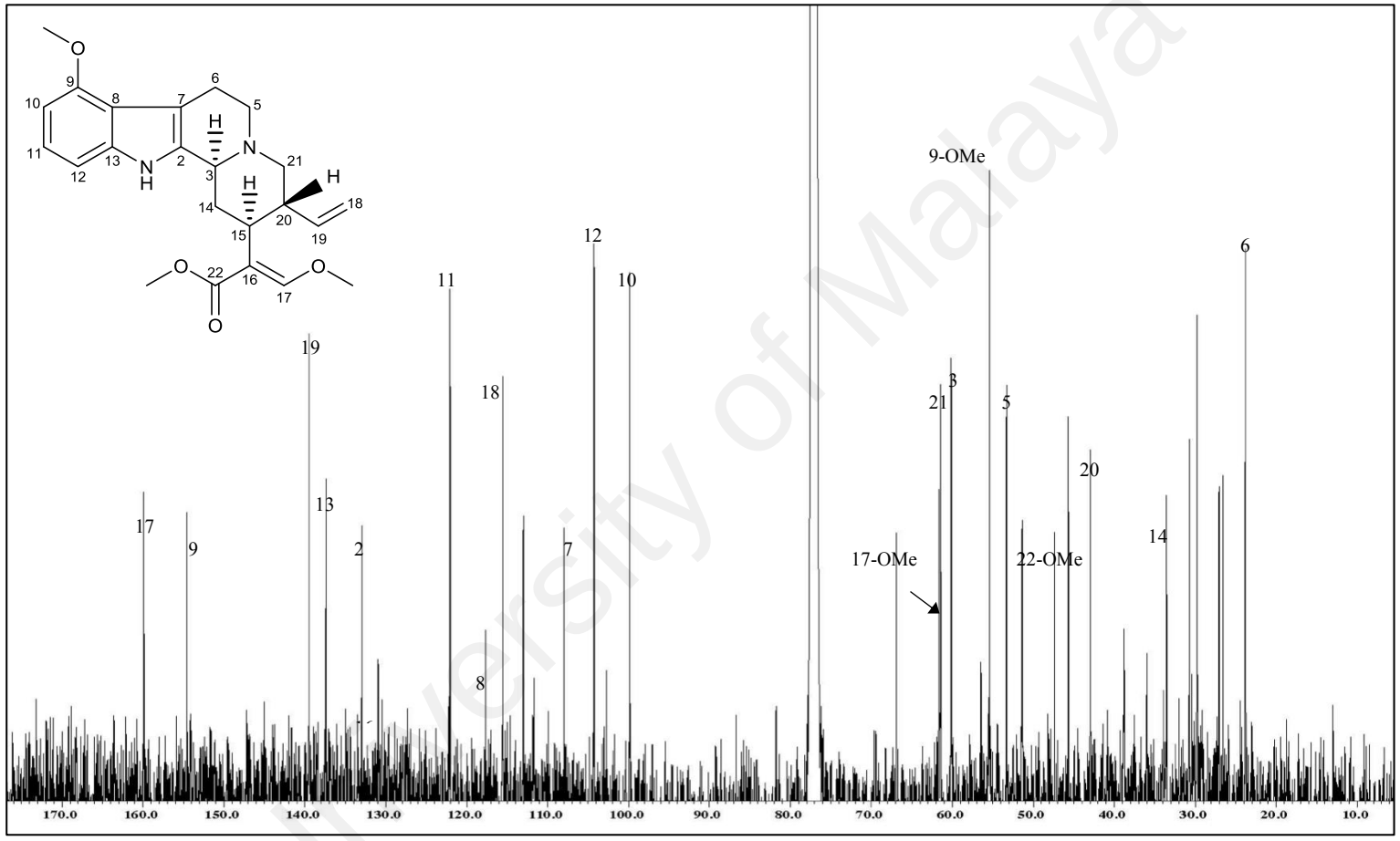


Figure 4.14 ^{13}C NMR spectrum of Paynantheine **5** on JEOL ECA 400 MHz

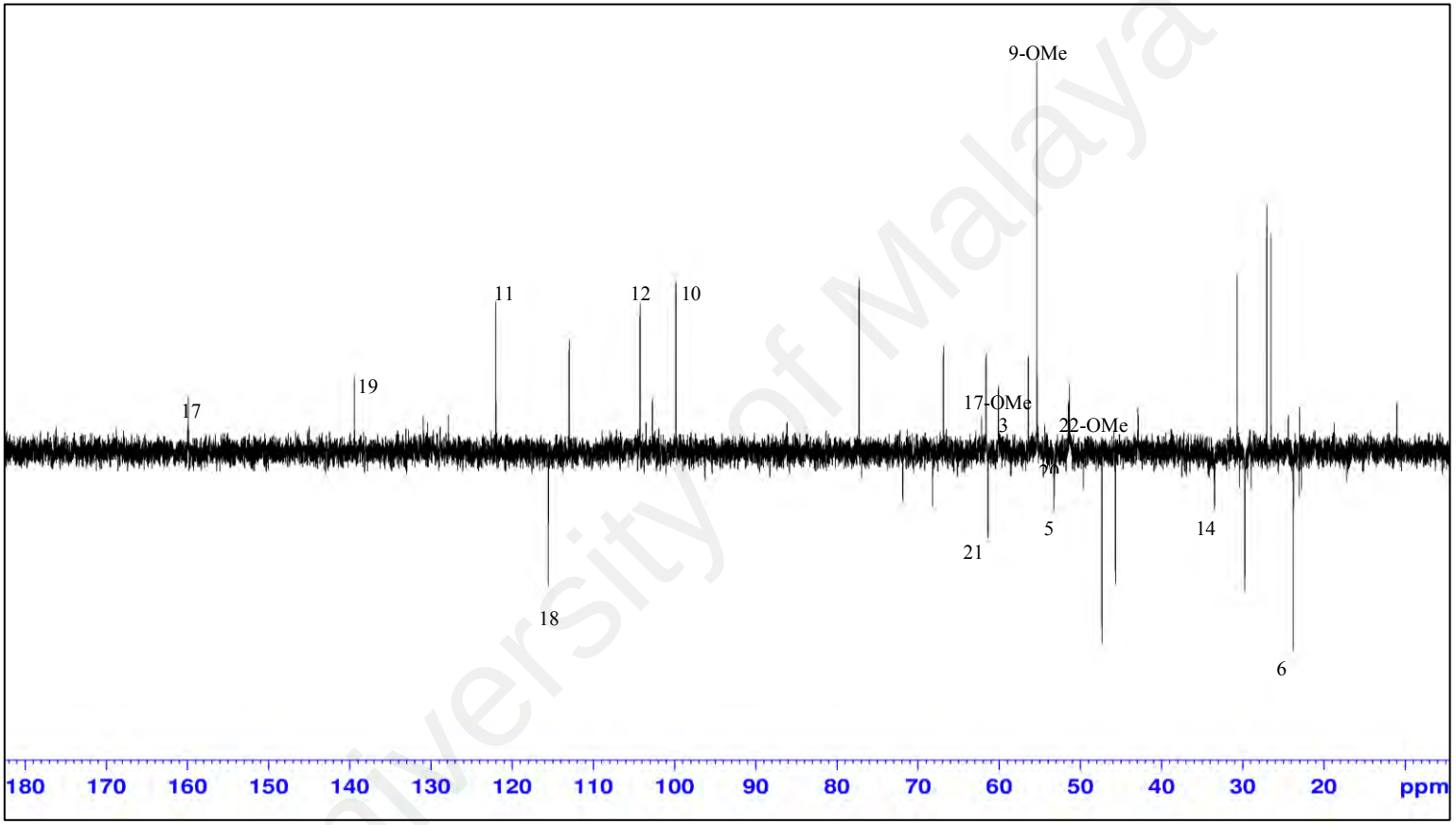


Figure 4.15 DEPT-135 NMR spectrum of Paynantheine **5** on Bruker Avance III 600 MHz

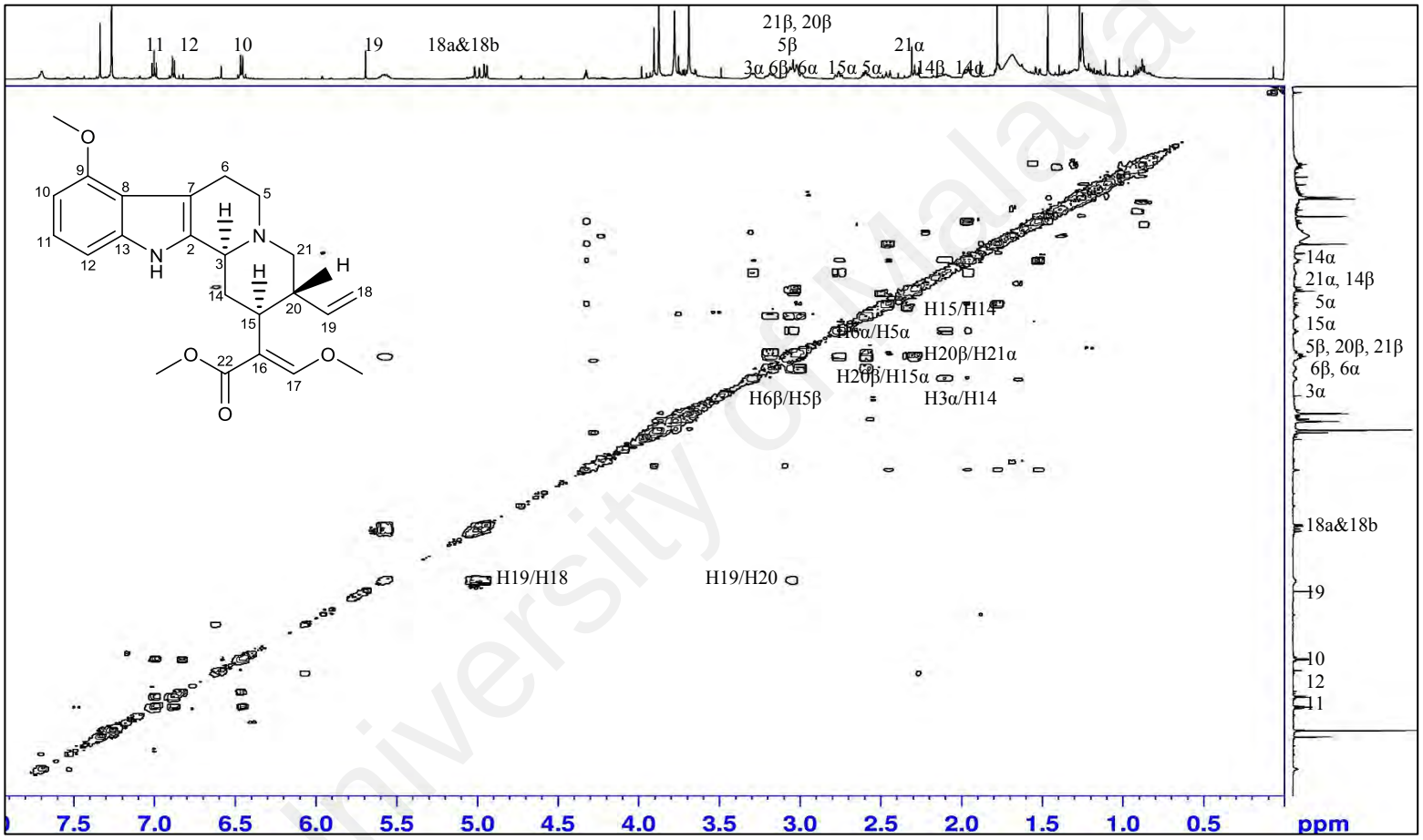


Figure 4.16 COSY NMR spectrum of Paynantheine **5** on Bruker Avance III 600 MHz

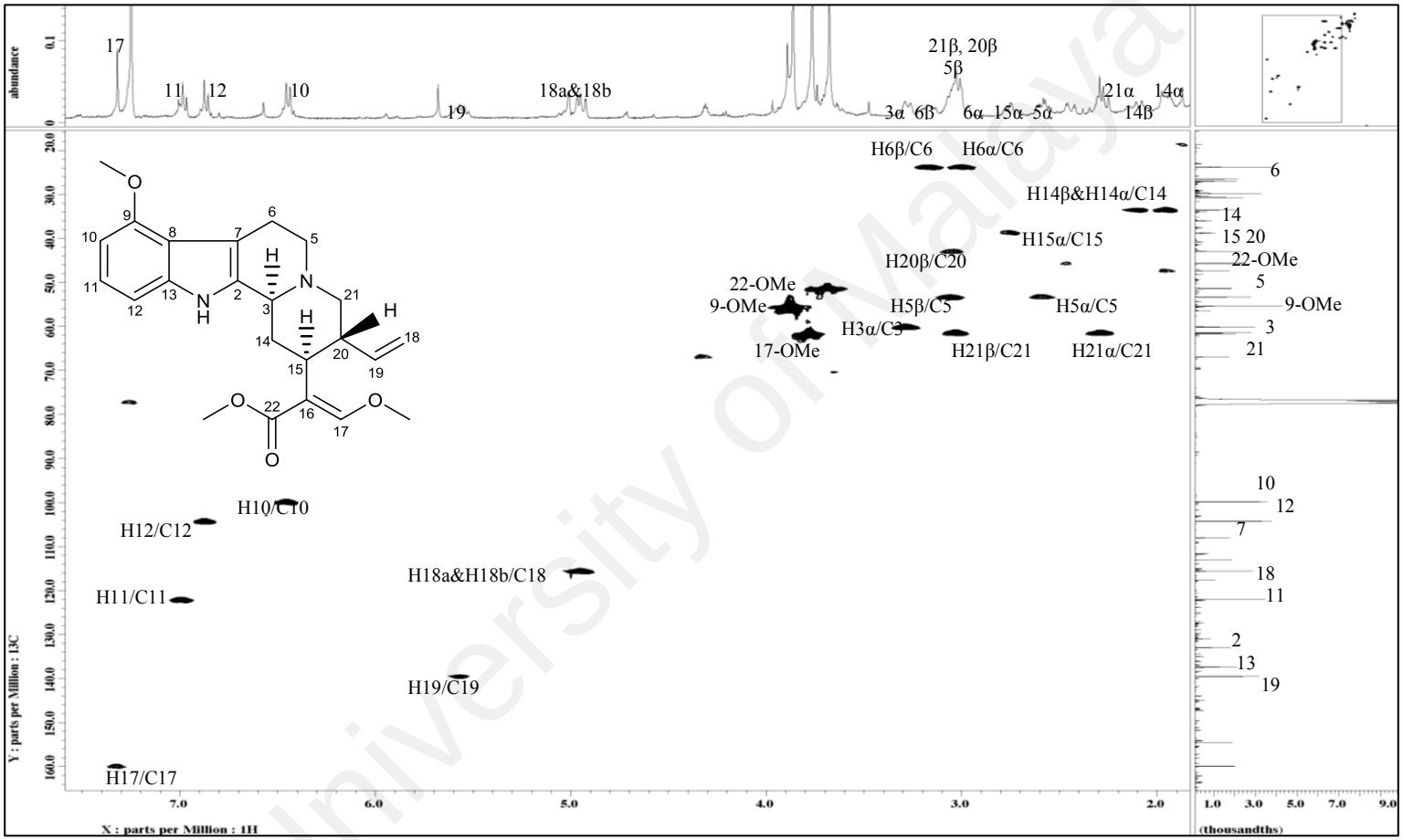


Figure 4.17 HSQC NMR spectrum of Paynantheine **5** on JEOL ECA 400 MHz

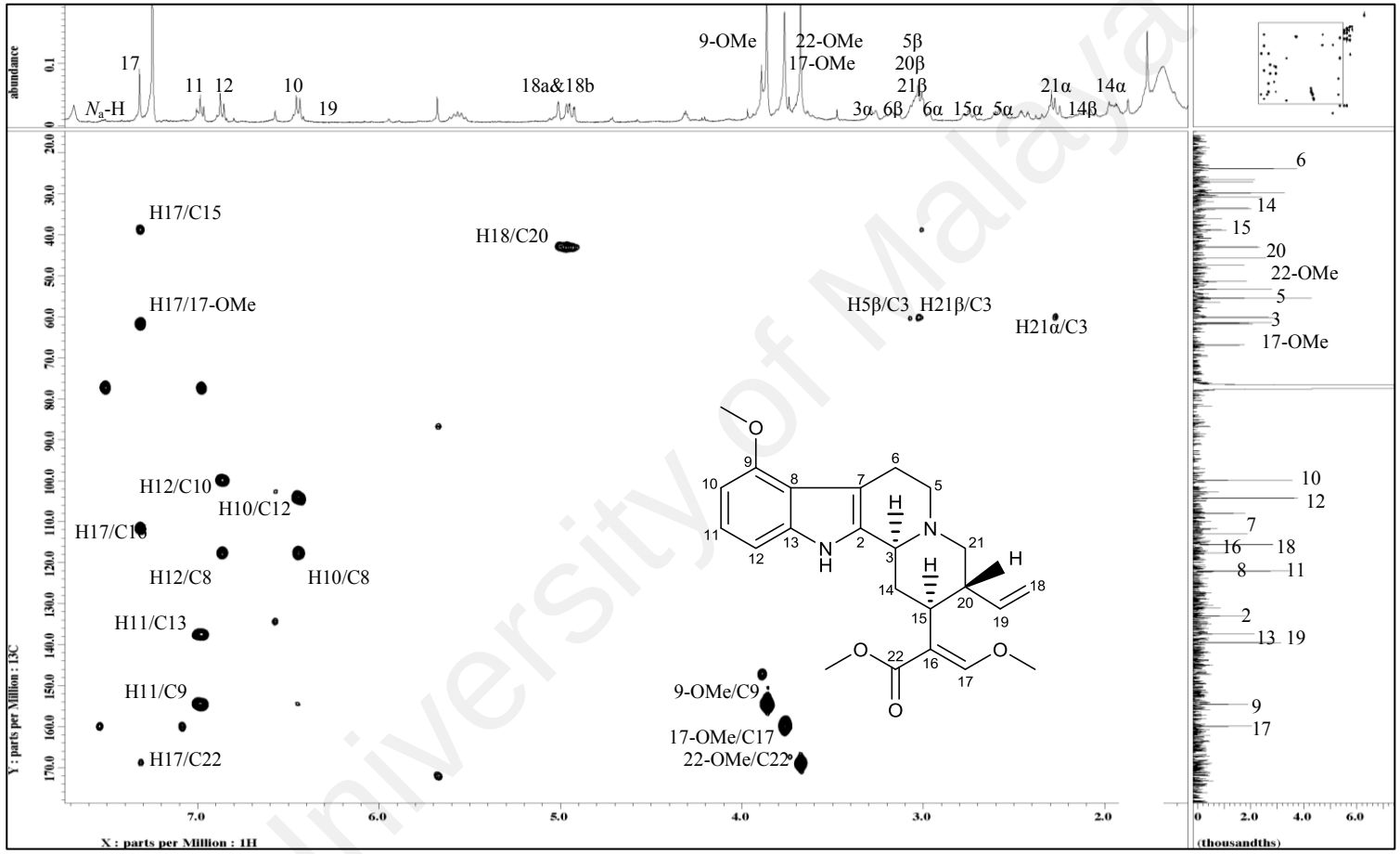
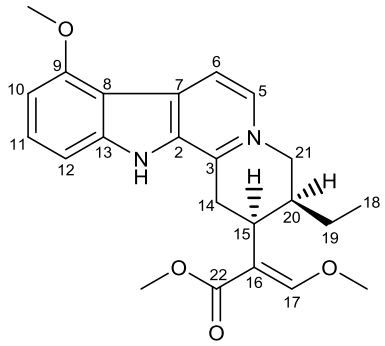


Figure 4.18 HMBC NMR spectrum of Paynantheine **5** on JEOL ECA 400 MHz

Compound 59: 3, 4, 5, 6-Tetradehydromitragynine



59

3, 4, 5, 6-Tetradehydromitragynine **59** was isolated as an orange amorphous powder and was identified using HRESIMS. The mass spectrum showed a quasi-molecular ion peak $[M]^+$ at m/z 395.1045 corresponding to the molecular formula of $C_{23}H_{27}N_2O_4$ (calcd. m/z 395.1971). This led to 12 degrees of unsaturation in **59**, which differ from paynantheine by one extra unsaturation degree and two more than that of mitragynine and speciociliatine.

The UV spectrum exhibited maxima at 366, 315, 258 and 220 nm, indicating the presence of a highly conjugated system in **59**. The IR spectrum showed an absorption peak at 1625 cm^{-1}, indicative of aromatic indole moiety whereas the intense band at 1735 cm^{-1} was attributed to carbonyl stretch of an ester [172].

The ^1H- (Figure 4.19) and ^{13}C-NMR (Figure 4.20) spectra of **59** showed the existence of the fundamental structural units in the common Corynanthe-type indole alkaloids, which are a *β*-methoxyacrylic acid methyl ester at C-15 and an ethyl group at C-20. However, instead of resonances for the aliphatic proton signals due to an ethane-bridge at C5-C6 and a methine proton at C3 positions in the usual monoterpenoid indole alkaloids, two characteristic signals were observed at δ_H 8.26 and δ_H 7.79 in the ^1H-

NMR spectrum. Consequently, the occurrence of a *β*-carbolinium chromophore in **59** was revealed.

The dehydrogenation was also demonstrated in ^{13}C and DEPT NMR (Figure 4.21) spectrum as the quarternary signal at δ_C 142.19 (C-3), the methine signals at δ_C 130.03 (C-5) and δ_C 116.45 (C-6). The relatively downfield shift of C-5 and C-3 in comparison to C-6 was caused by the removal of electron density by the adjacent electronegative nitrogen atom.

The COSY (Figure 4.22) spectrum established the positioning of $\Delta^{5,6}$ double bond by showing an off-diagonal signal that connected H-5 with H-6.

The distinction between C-5 and C-6 sp^2 methine carbons was clear from the one-bond coupling system in the HSQC spectrum (Figure 4.23). Cross-peaks associated with H-5 and H-6 indicated that the C-5 and C-6 signal was located at δ_C 130.03 and δ_C 116.45, respectively.

Along with literature research, extensive spectroscopic data analyses have identified compound **59** as 3, 4, 5, 6-tetradehydromitragynine, a derivative of mitragynine which was isolated previously from Malaysian *Mitragyna speciosa* [81]. The NMR data of **59** were provided in Table 4.6.

Table 4.6 ^{1}H NMR [400 MHz, δ_H (J, Hz)] and ^{13}C NMR [100 MHz, δ_C] of 3, 4, 5, 6-Tetradehydromitragynine **59** in $CDCl_3$

Position	^{1}H (J, Hz)	^{1}H (500 MHz, $CDCl_3$) [81]	^{13}C	^{13}C (125 MHz, $CDCl_3$) [81]
1	Not observed	13.80 (1H, br-s, N_a-H)	-	-
2	-	-	133.50	133.38
3	-	-	142.19	141.79
4	-	-	-	-
5	7.78 (1H, d, J = 6.4)	7.78 (1H, d, J = 6.6)	130.03	130.04
6	8.26 (1H, d, J = 6.4)	8.27 (1H, d, J = 6.4)	116.45	116.39
7	-	-	128.89	129.83
8	-	-	110.77	110.54
9	-	-	157.19	156.96
10	6.70 (1H, d, J = 8)	6.71 (1H, d, J = 7.8)	101.06	101.06
11	7.59 (1H, t, J = 8)	7.61 (1H, dd, J = 8.1, 8.1)	132.72	132.71
12	7.69 (1H, d, J = 8.2)	7.67 (1H, d, J = 8.3)	107.48	107.29
13	-	-	146.06	145.95
14_α	4.04-4.08 (1H, o/lap 9-OMe)	4.04 (1H, dd, J = 19.9, 7.7)	33.43	29.79
14_β	4.09-4.15 (1H, o/lap 9-OMe)	4.11 (1H, dd, J = 19.5, 2.8)		
15	3.81 (1H, m)	3.81 (1H, m)	29.79	28.89
16	-	-	108.36	108.31
17	7.52 (1H, s)	7.52 (1H, s)	162.53	162.44
18	1.04 (3H, t, J = 7.3)	1.05 (3H, dd, J = 7.4, 7.4)	11.98	11.85
19_a	1.25 (1H, m)	1.25 (1H, m)	22.69	22.56
19_b	1.60 (1H, m)	1.60 (1H, m)		
20	2.32 (1H, m)	2.32 (1H, m)	39.60	39.37
21_α	4.32 (1H, t, J= 13)	4.32 (1H, dd, J = 12.1, 12.1)	56.81	56.74
21_β	4.42 (1H, dd, J = 13, 5.0)	4.43 (1H, dd, J = 12.8, 4.4)		
9-OMe	4.08 (3H, s)	4.09 (3H, s)	55.71	55.63
17-OMe	3.69 (3H, s)	3.70 (3H, s)	62.06	61.97
COOMe	3.56 (3H, s)	3.57 (3H, s)	51.55	51.46
COO	-	-	168.53	168.42

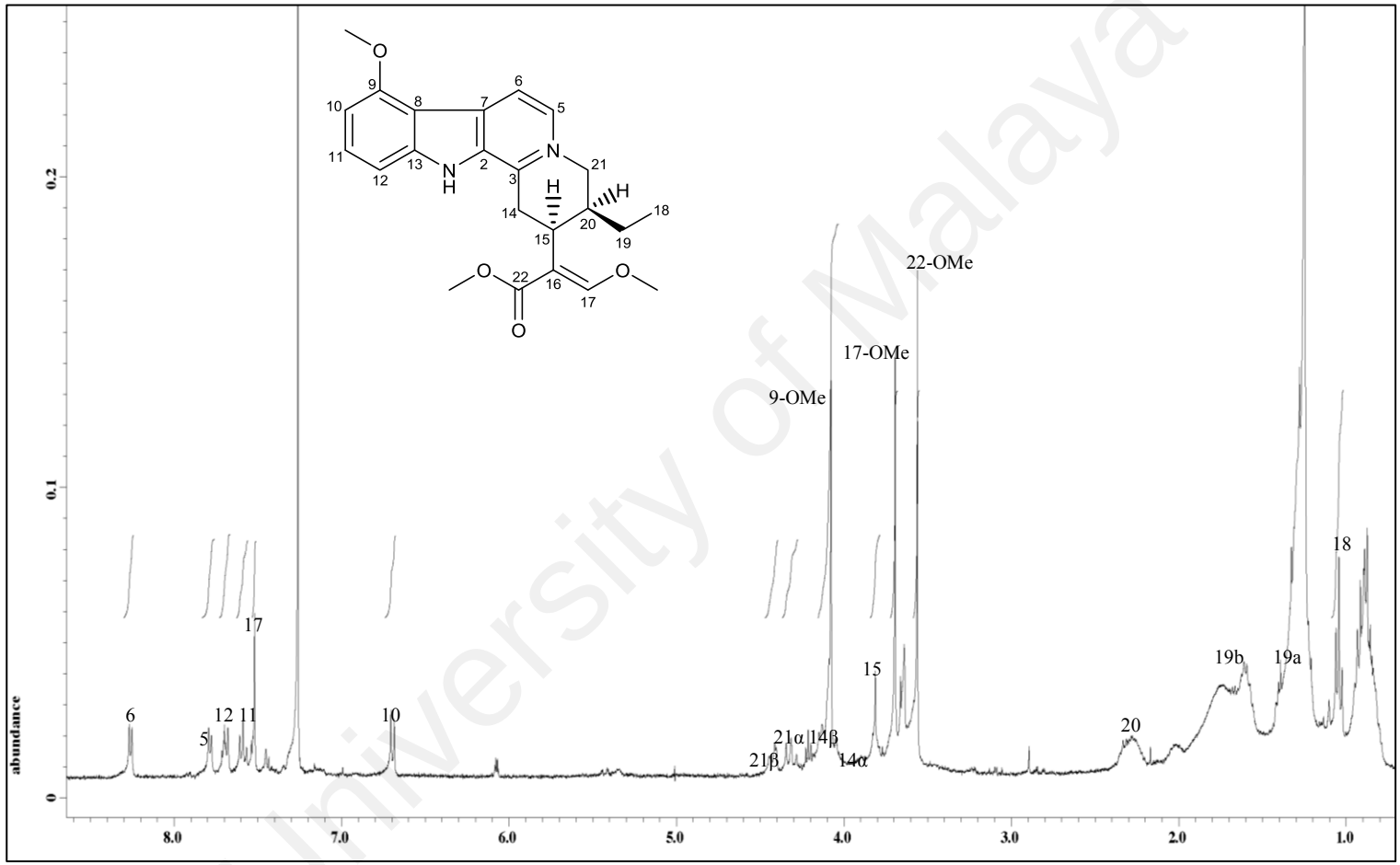


Figure 4.19 ^{1}H NMR spectrum of 3, 4, 5, 6-Tetradehydromitragynine **59** on JEOL ECA 400 MHz

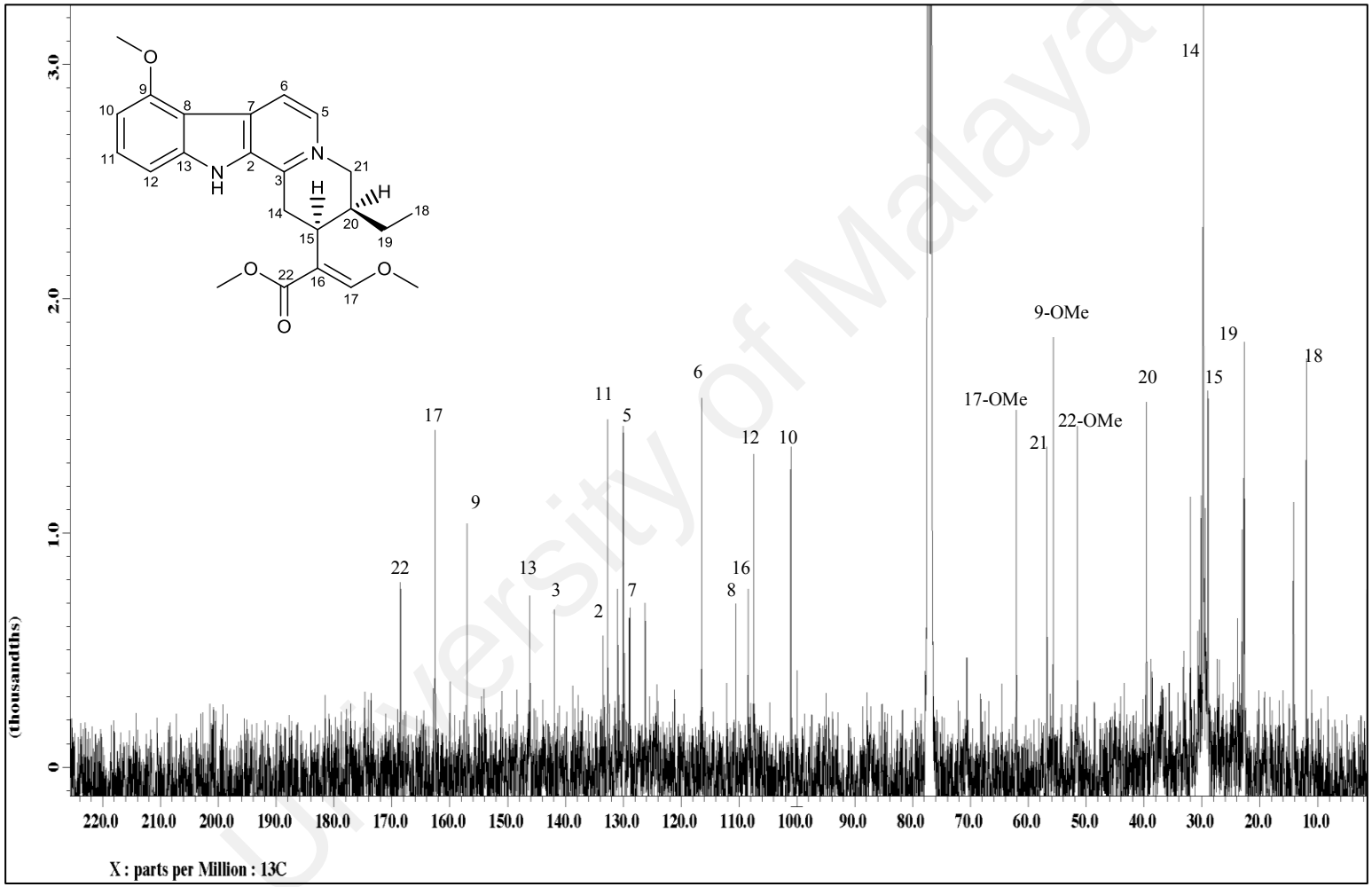


Figure 4.20 ^{13}C NMR spectrum of 3, 4, 5, 6-Tetradehydromitragynine **59** on JEOL ECA 400 MHz

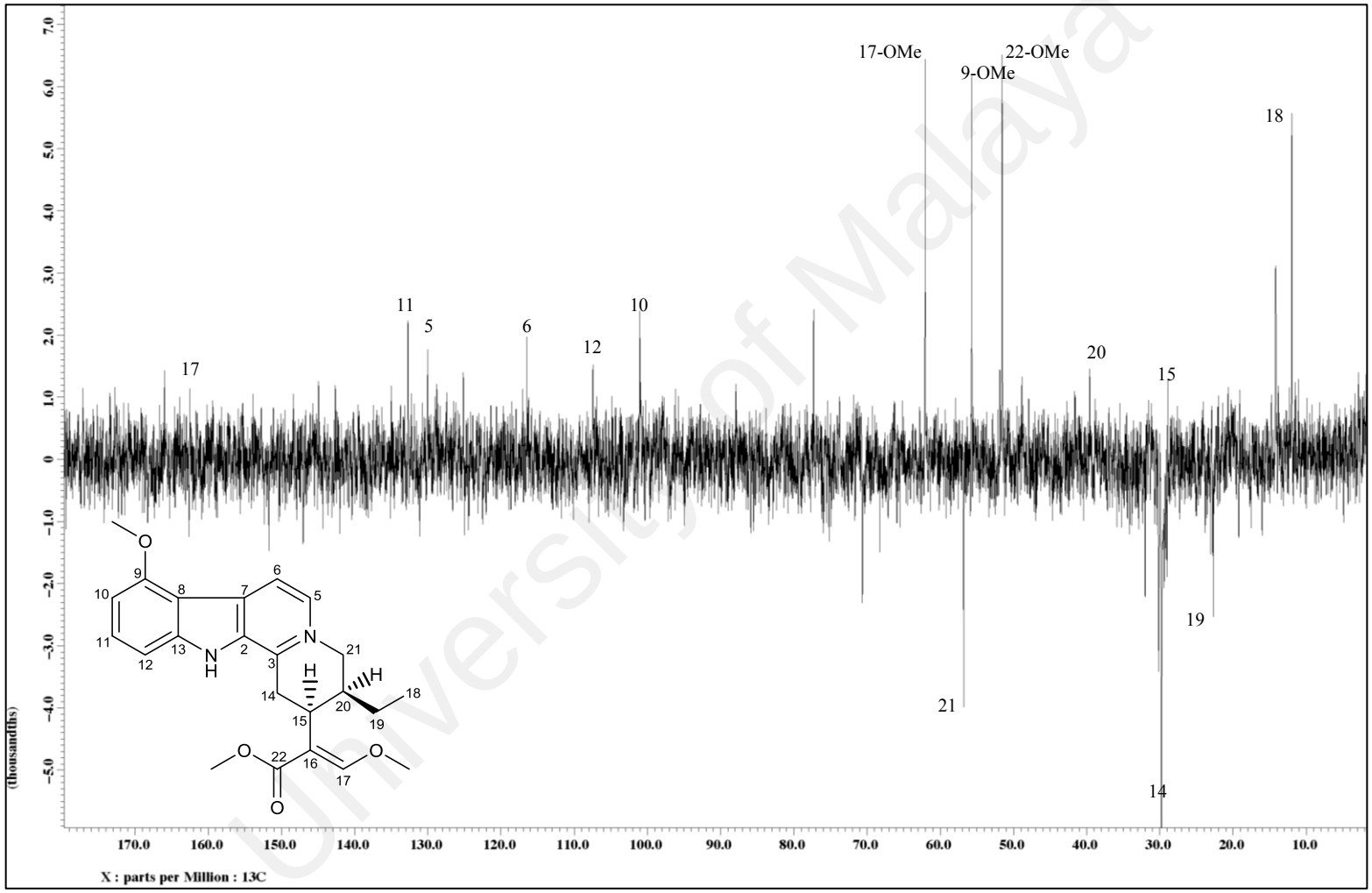


Figure 4.21 DEPT NMR spectrum of 3, 4, 5, 6-Tetradehydromitragynine **59** on JEOL ECA 400 MHz

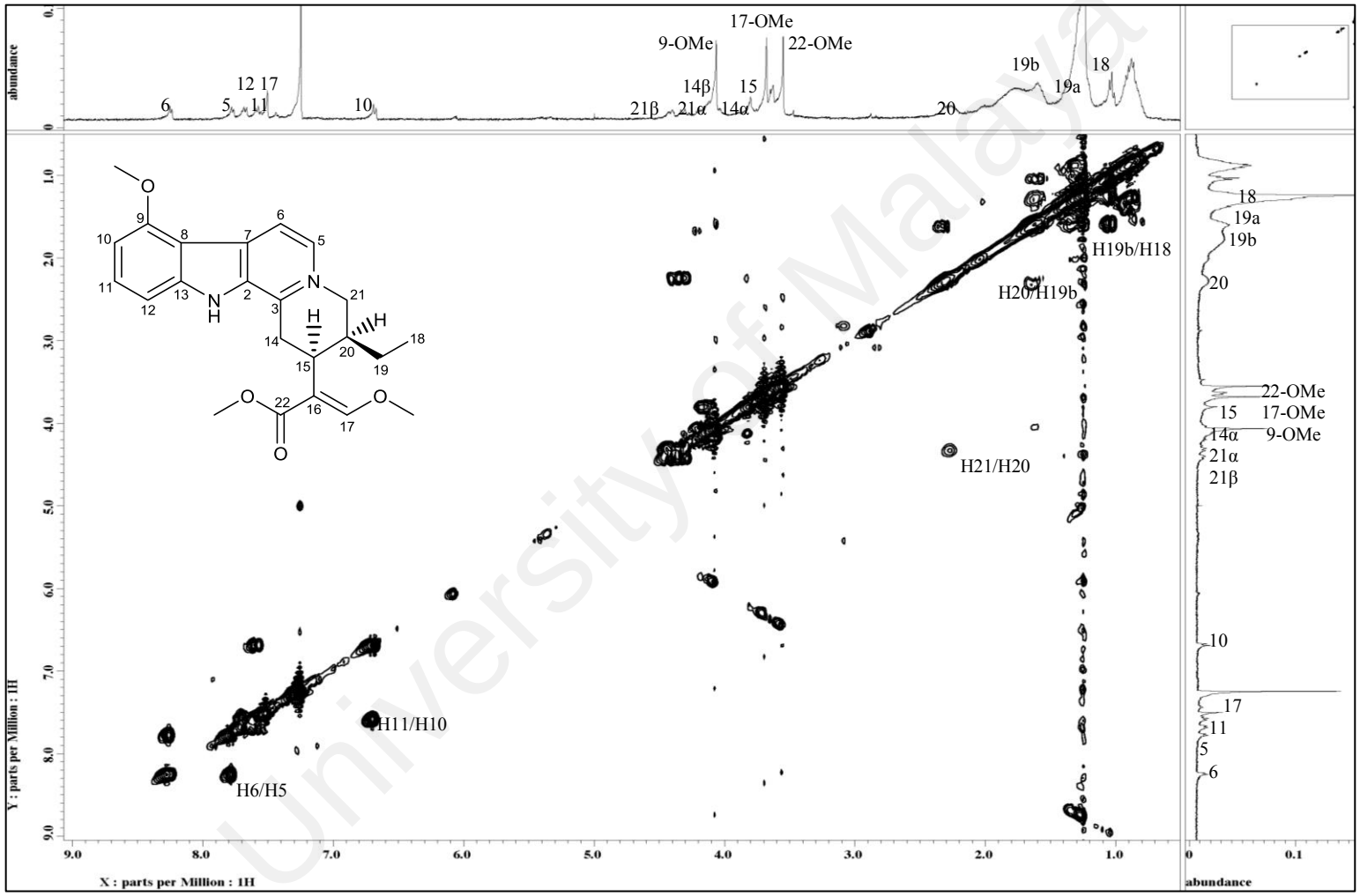


Figure 4.22 COSY NMR spectrum of 3, 4, 5, 6-Tetradehydromitragynine **59** on JEOL ECA 400 MHz

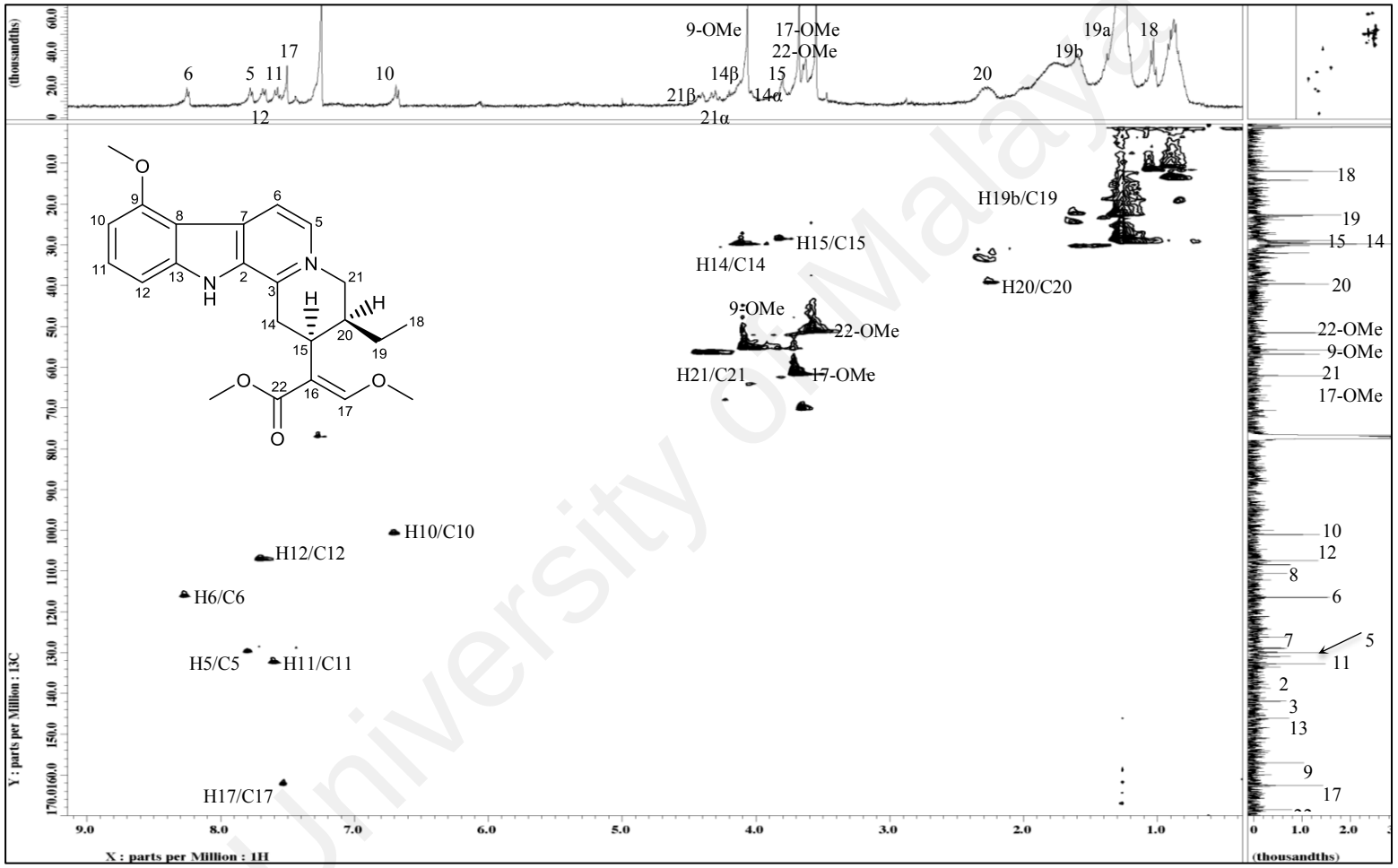
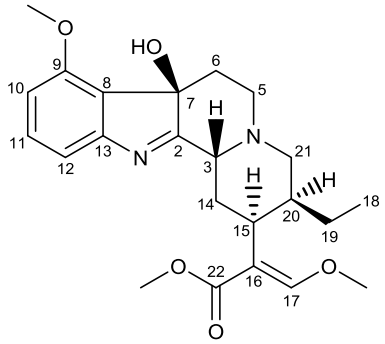


Figure 4.23 HSQC NMR spectrum of 3, 4, 5, 6-Tetradehydromitragynine **59** on JEOL ECA 400 MHz

Compound 10: 7-Hydroxyspeciociliatine



10

The HRESIMS of 7-hydroxyspeciociliatine **10** displayed a protonated molecular ion peak at m/z 415.2253 $[M+H]^+$ (calcd. for $C_{23}H_{31}N_2O_5$, m/z 415.2233), indicating a molecular formula of $C_{23}H_{30}N_2O_5$ with 10 degrees of unsaturation. Compound **10**, thus, contains an additional of one oxygen atom in comparison to the major alkaloid, mitragynine **1** and its diastereomer, speciociliatine.

The UV spectrum of 7-hydroxyspeciociliatine in methanol showed bands at 221, 294 and shoulder at 240 nm. These absorptions were similar to those of 7-hydroxymitragynine, which has a 4-methoxyindolenine nucleus [83]. The IR spectrum exhibited characteristic absorption due to NH/OH group (3 200 cm^{-1}), C-H aromatic (2 940 cm^{-1}), C=O ester (1696 cm^{-1}), C-O bonding (1245 cm^{-1}) and substituted benzene ring (774 cm^{-1}).

Although the ^1H NMR (Figure 4.24) spectral pattern of alkaloid **10** was very similar to that of speciociliatine, the UV spectrum was different from the 4-methoxyindole chromophore in speciociliatine. The typical signals of ABX pattern for the three vicinal aromatic protons can be observed at δ_H 6.44 (d, J = 8.0 Hz, H-10), δ_H 6.91 (d, J = 8.0 Hz, H-12) and δ_H 6.99 (t, J = 8.0 Hz, H-11), a methoxy group on an aromatic ring at δ_H

3.86 (9-OMe), methyl *β*–acrylic ester residue at δ_H 7.41 (H-17), δ_H 3.72 (3H, 17-OMe) and δ_H 3.70 (3H, 22-OMe) and an ethyl group at δ 1.60 and 1.25 (each 1H, m, H_2-19) and 0.88 (3H, t, J = 7.3 Hz). Key structural information was also derived from the appearance of a peak at δ_H 4.00, which was assigned to H-3, indicated a *cis*-quinolizidine-type C/D ring junction [173].

The ^{13}C NMR (Figure 4.25) spectrum of **10** indicated the presence of 23 carbons, including an imine carbon at δ_C 176.0 (C-2) and an oxygenated sp^3 quaternary carbon at δ_C 81.0 (C-7). The signal of C-2 in **10** was observed at lower field by 43.5 ppm as compared with the corresponding signals of speciociliatine, conversely, C-7 in **10** was shifted to upper field (δ_C = 81.0). These data indicated that **10** was a 7-hydroxyindolenine derivative of speciociliatine. The presence of *cis*-quinolizidine C/D ring junction was also supported by the similarity of the chemical shifts of C-3, C-14, C-15, C-20 and C-21 in the D ring of **10** to those of speciociliatine **4** of which had the *cis*-quinolizidine ring junction. Furthermore, the ^{13}C chemical shifts of C-18 and C-19 of the ethyl group that was attached to C-20 in **10** were similar to those in mitragynine and speciociliatine **4**, both of which possessed an ethyl group at C-20.

Unambiguous assignments of all the protons and carbons were obtained by using COSY, DEPT, HSQC and HMBC spectra. In the HMBC spectrum (Figure 4.26), H-11 gave rise to a cross-peak signal that linked to C-13, thus, this long-range correlation confirmed the positioning of C-13 at δ_C 137.4.

Detailed analysis of the spectral data and also data comparison with the literature value (Table 4.7) confirmed that alkaloid **10** is 7-hydroxyspeciociliatine, which was isolated from the fruits of *M. speciosa* Korth. originated from Malaysia [82].

Table 4.7 ^{1}H NMR [400 MHz, δ_H (J, Hz)] and ^{13}C NMR [100 MHz, δ_C] of 7-Hydroxyspeciociliatine **10** in $CDCl_3$

Position	^{1}H (J, Hz)	^{1}H (500 MHz, $CDCl_3$) [82]	^{13}C	^{13}C (125 MHz, $CDCl_3$) [82]
1	-	-	-	-
2	-	-	176.0[a]	183.9
3	4.06 (1H, br-s)	3.90 (1H, br-s)	55.0	55.1
4	-	-	-	-
5_α	2.97 (1H, o/lap H_2-21)	2.71 (1H, o/lap H_2-21)	48.0	49.6
5_β	3.21 (1H, br-d, J=10.2)	3.19 (1H, br-dd, J = 12.6, 12.6)		
6_α	1.97 (1H, m)	1.85 (1H, m)	36.1	36.0
6_β	2.64 (1H, m)	2.61 (1H, br-d, J = 14.4)		
7	-	-	81.0	81.7
8	-	-	117.6	126.5
9	-	-	154.3	155.9
10	6.44 (1H, d, J = 8)	6.73 (1H, d, J = 7.9)	99.7	108.8
11	6.99 (1H, t, J = 8)	7.30 (1H, dd, J = 7.9, 7.9)	122.1	130.8
12	6.91 (1H, d, J = 8)	7.21 (1H, d, J = 7.9)	104.6	114.2
13	-	-	137.4	155.0
14	2.30 – 2.20 (2H, m)	2.41 and 2.20 (each 1H, m)	29.5 br	29.1 br
15	3.55 (1H, m)	3.50 (1H, ddd, J = 5.9, 5.9, 5.9)	33.2	32.4
16	-	-	111.0	111.8
17	7.41 (1H, s)	7.41 (1H, s)	160.5	159.7
18	0.88 (3H, t, J = 7.3)	0.85 (3H, dd, J = 7.4, 7.4)	12.5	12.4
19	1.60 and 1.25 (each 1H, m)	1.45 and 1.24 (each 1H, m)	20.6 br	21.8 br
20	1.79 (1H, m)	1.78 (1H, m)	39.2	39.8
21	2.97 (2H, o/lap, H-5_α,)	2.71 (2H, o/lap, H-5)	52.5	53.5 br
9-OMe	3.86 (3H, s)	3.86 (3H, s)	55.3	55.4
17-OMe	3.76 (3H, s)	3.80 (3H, s)	61.6	61.2
COOMe	3.64 (3H, s)	3.69 (3H, s)	51.5	51.3
COO	-	-	169.4	169.6

[a] Estimated from observed HMBC crosspeak

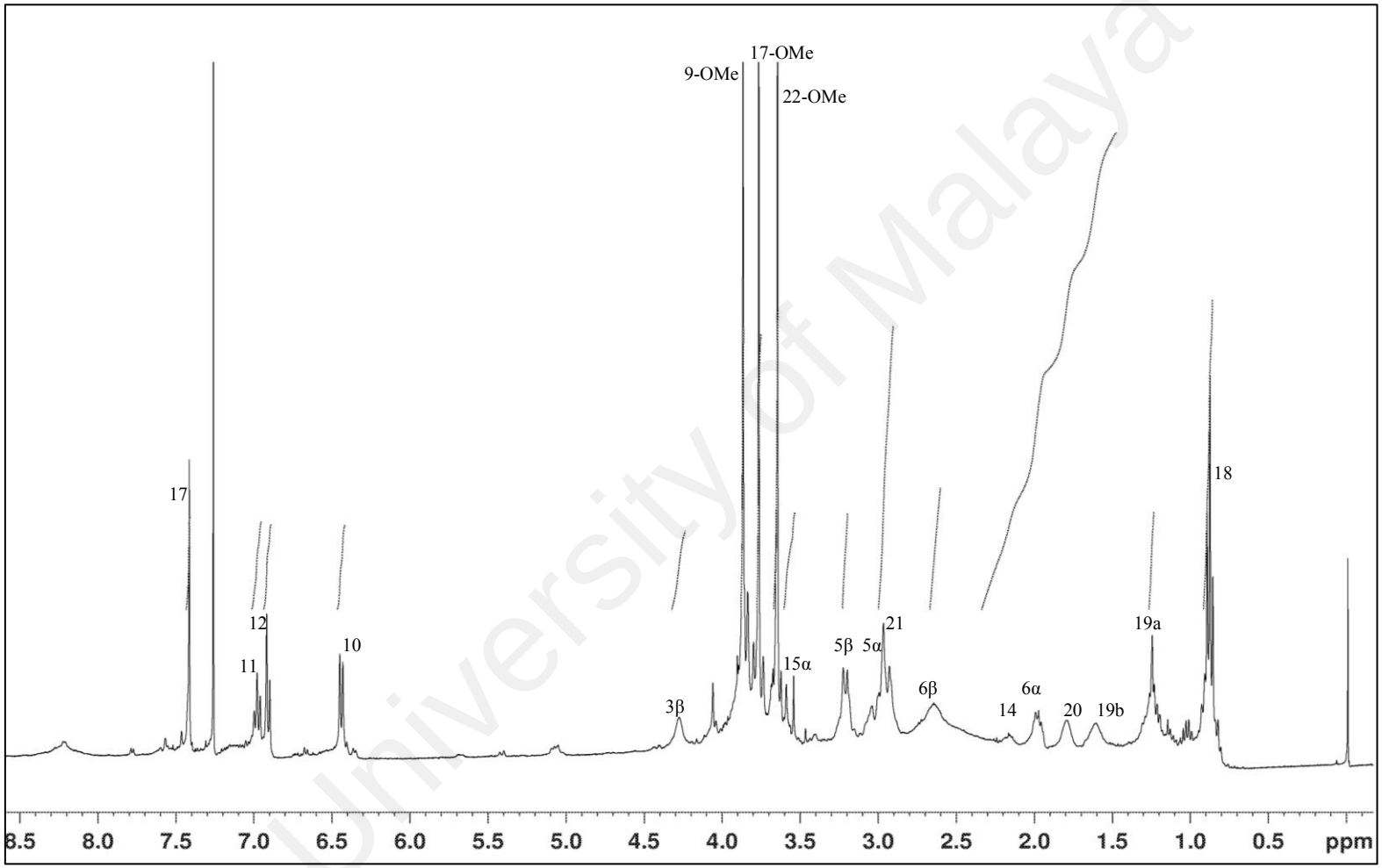


Figure 4.24 ^{1}H NMR spectrum 7-Hydroxyspeciociliatine **10** on Bruker Avance II 400 MHz

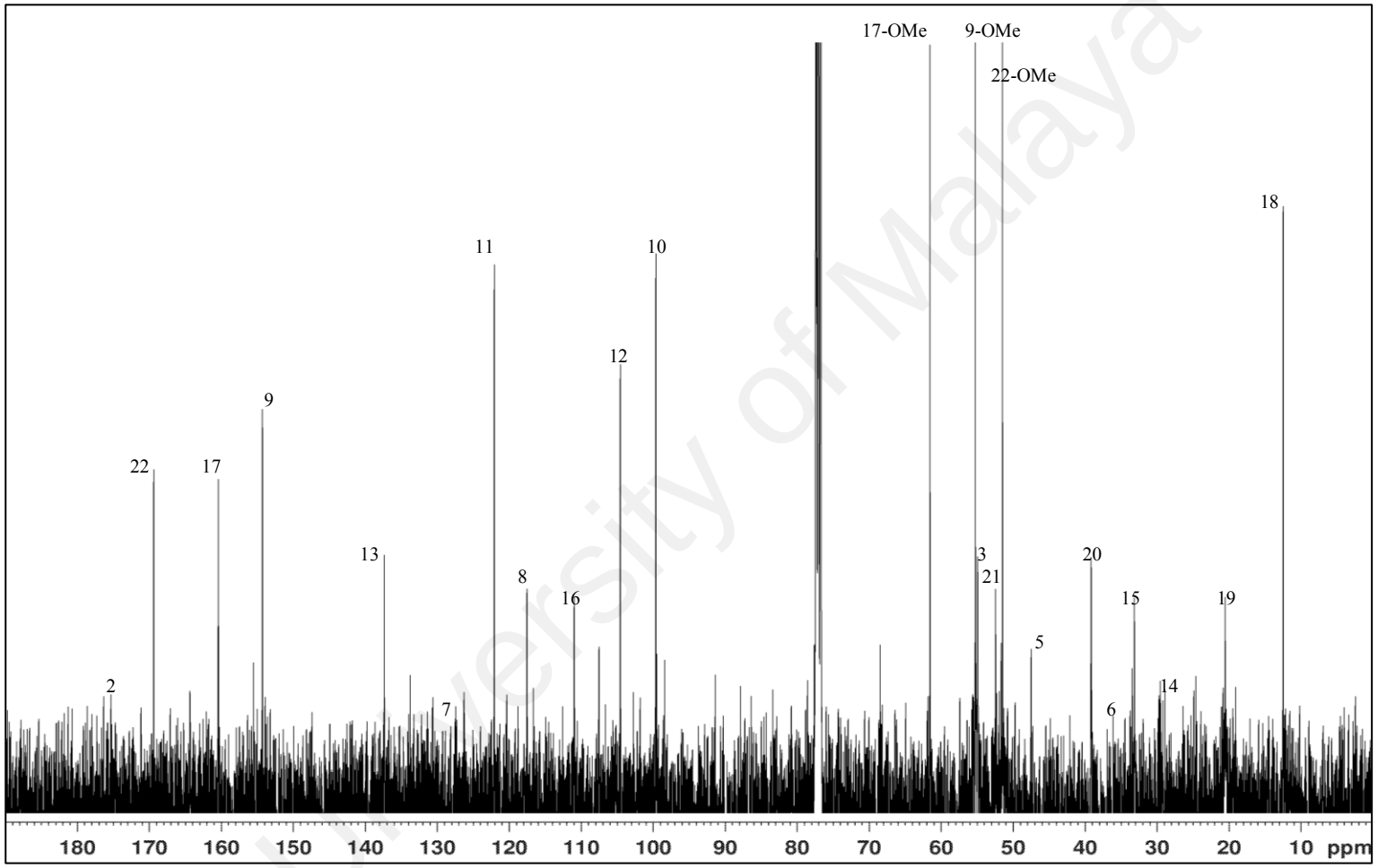


Figure 4.25 ^{13}C NMR spectrum 7-Hydroxyspeciociliatine **10** on Bruker Avance II 400 MHz

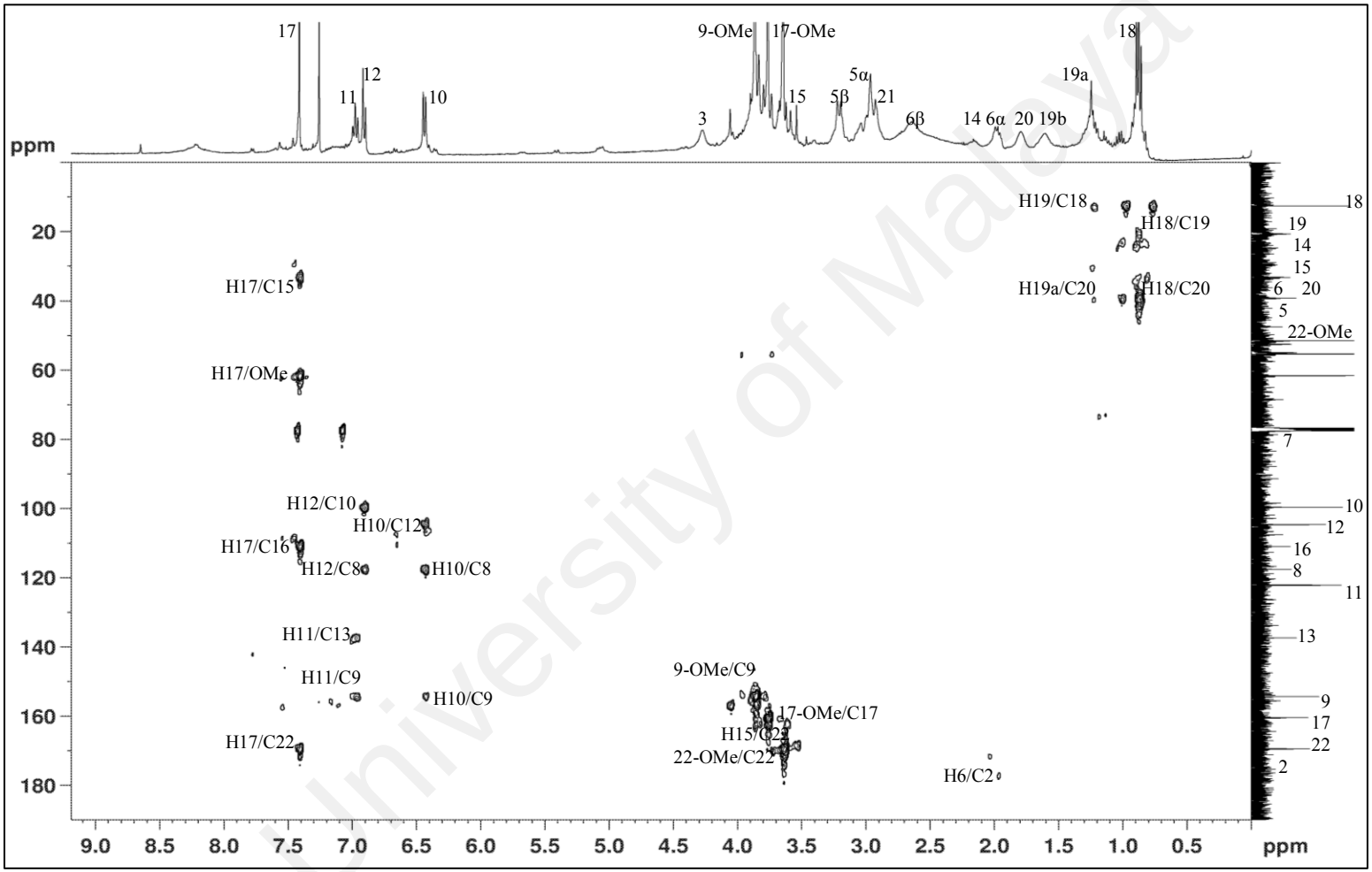


Figure 4.26 HMBC NMR spectrum 7-Hydroxyspeciociliatine **10** on Bruker Avance II 600 MHz

4.6.1.1 Physical and Spectral Data of the Isolated Compounds

Mitragynine 1

Physical appearance : Yellow amorphous solid

Molecular formula : $C_{23}H_{30}N_2O_4$

UV λ_{max} (MeOH), nm : 290, 285, 247 and 225

IR ν_{max} ($CHCl_3$), cm^{-1} : 3358, 1697, 1630 and 1241.

Mass spectrum, m/z : 399.2281 $[M + H]^+$; calc. 399.2284

NMR : Refer Table 4.3 (pg.109) and Figure 4.2- 4.7 pg. (110-115)

Speciociliatine 4

Physical appearance : Yellow amorphous powder

Molecular formula : $C_{23}H_{30}N_2O_4$

UV λ_{max} (MeOH), nm : 290, 285, 247 and 225

IR ν_{max} ($CHCl_3$), cm^{-1} : 3350, 1680, 1625 and 1241

Mass spectrum, m/z : 399.2279 $[M + H]^+$; calcd. 399.2284

NMR : Refer Table 4.4 (pg. 118) and Figure 4.8-4.12 (pg. 119-123)

Paynantheine 5

Physical appearance : Yellowish amorphous powder

Molecular formula : $C_{23}H_{28}N_2O_4$

UV λ_{max} (MeOH), nm : 291 and 243

IR ν_{max} ($CHCl_3$), cm^{-1} : 3358, 1697 and 1630

Mass spectrum, m/z : 397.2126 $[M + H]^+$; calcd. 397.2127

NMR : Refer Table 4.5 (pg.126) and Figure 4.13-4.18 (pg. 127 -132)

3, 4, 5, 6-Dehydromitragynine 59

Physical appearance : orange amorphous powder

Molecular formula : $C_{23}H_{27}N_2O_4$

UV λ_{max} (MeOH), nm : 366, 315, 258 and 220

IR ν_{max} ($CHCl_3$), cm^{-1} : 1735 and 1625

Mass spectrum, m/z : 395.1045 $[M]^+$; calcd. 395.1971

NMR : Refer Table 4.6 (pg.135) and Figure 4.19-4.23 (pg. 136 to 140)

7-hydroxyspeciociliatine 10

Physical appearance : yellowish amorphous powder

Molecular formula : $C_{23}H_{30}N_2O_5$

UV λ_{max} (MeOH), nm : 294, 240 and 221

IR ν_{max} ($CHCl_3$), cm^{-1} : 3200, 2940, 1696, 1245 and 774

Mass spectrum, m/z : 415.2253 $[M+H]^+$; calcd. 415.2233

NMR : Refer Table 4.7 (pg.143) and Figure 4.24-4.26 (pg. 144 -146)

4.6.1.2 HPLC Chemical Profiling of the Aqueous Extract

For successful chromatographic separation of standard solutions and crude extract solution, the stationary phases from different column manufacturers were tested. The best result was obtained with a GraceSmart RP 18 column, which is a reversed-phase HPLC column. With UV detection (monitored at 254 nm), a relatively good separation was confirmed in 30 min. The optimum conditions were determined as described in the Materials and Methods section. The peaks of mitragynine and speciociliatine of the crude extract were identified by comparing the retention time against the markers (Figure 4.27A and B) and also by spiking a small amount of speciociliatine to the extract solution (Figure 4.27C). As shown by the chromatogram of the crude extract,

peaks eluted at 19.877 and 21.245 min were identified as mitragynine and speciociliatine, respectively (Figure 4.27D).

UV spectra and LC-ESI-MS mass spectra of the standard solutions of the two marker compounds are as depicted in Figure 4.28. The protonated molecular ions ($[M + H]^+$) were observed as based peaks for both compounds. Mitragynine and speciociliatine are isomer; molecular formula of $C_{23}H_{30}N_2O_4$ with exact molecular mass is 398.2207.

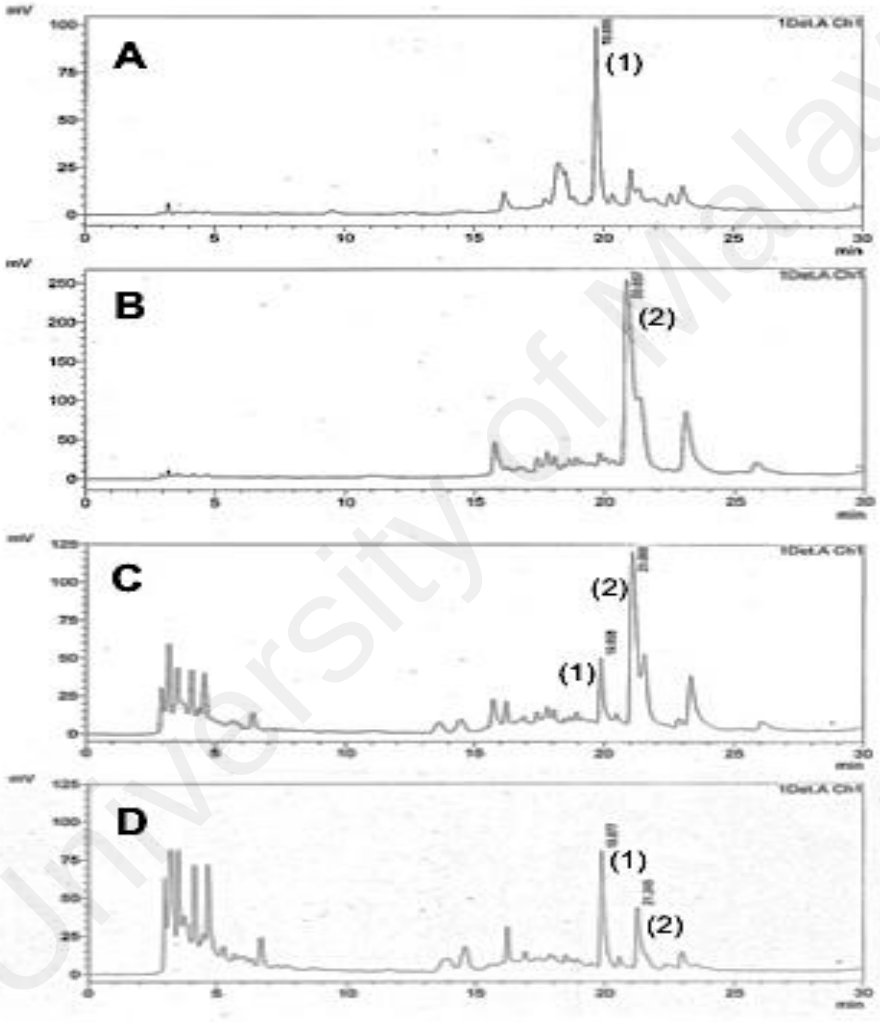


Figure 4.27 Hplc profiles
(A) Standard solution of mitragynine, (B) Standard solution of speciociliatine, (C) Spiking of speciociliatine to the water extract solution, (D) Water crude extract solution. (1) mitragynine, (2) speciociliatine. The retention times of mitragynine and speciociliatine of the water crude extract were 19.877 and 21.245 min, respectively.

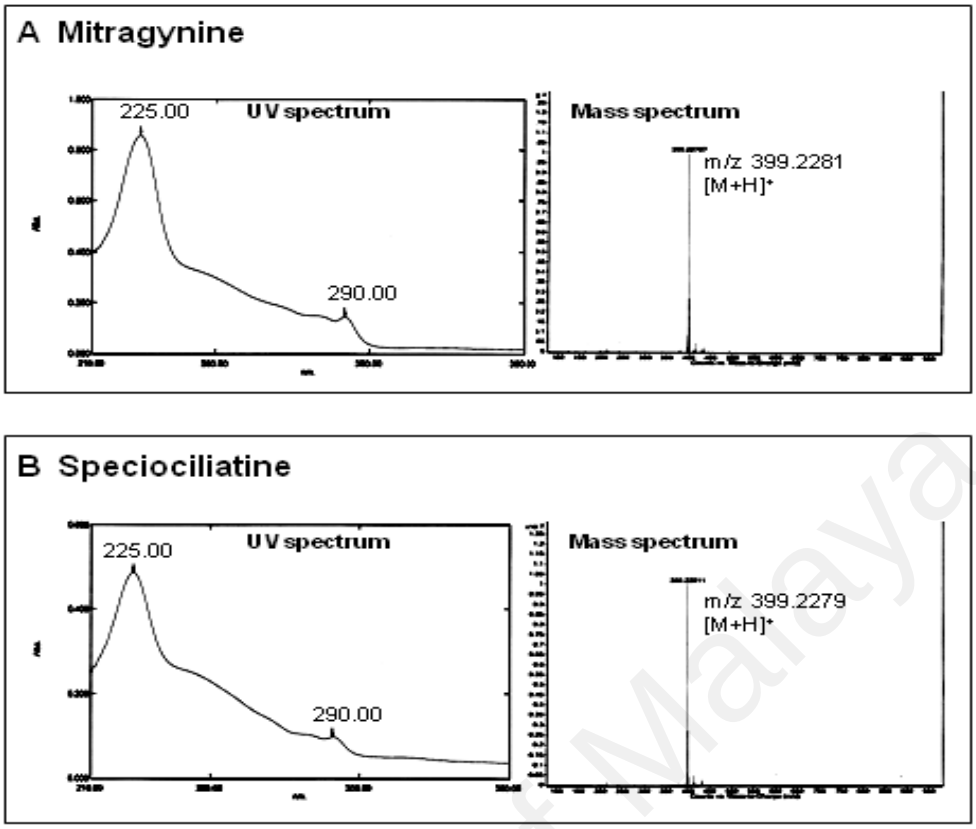


Figure 4.28 Mass and UV spectra of standard compounds of two alkaloids in *M. speciosa*
(A) Mitragynine, (B) Speciociliatine.

4.6.2 Crude Extracts from the CO_2 SFE of *Mitragyna speciosa* Leaves

Optimization of the experimental conditions represents a critical step in the development of an efficient SFE method through the adjustment of certain parameters that potentially affects the extraction process. Optimization of the method can be carried out step-by-step or by using an experimental design. Table 4.2 showed the results of the SFE of *M. speciosa* carried out under different conditions. Pressure and temperature of the fluid and percentage of the modifier were the selected factors examined using a three-level orthogonal array design with L_9 (3^3) matrix. Thus, a complete evaluation of the three factors will require three level tests, i.e., 27 tests. In order to reduce the number of tests, an L_9 (3^3) orthogonal design graph was used, which reduced the total number of tests to 9 (refer to Table 4.1). A two-step extraction process was designed for

this study, which included pre-extraction with pure CO_2, makes it a total of 18 tests to be performed.

In general, results presented in Table 4.2 indicate the percentage of total yield (S1+S2) increased with the increment in pressure. At the static pressure of 3000 psi, the yield increased as the temperature was raised from 40 to 60 ^oC but dropped slightly in range of 60 to 80 ^oC. As at 1300 psi and 5000 psi, a rise in temperature resulted in lower yield.

The yield of extraction by pure CO_2 also followed the same trend of increased yield for an increase in the pressure. A rise in the pressure can increase the density of the fluid and thereby resulting in an increased solvating power-giving rise to more yields. At static pressure of 1500 psi, the yield gradually decreased as the temperature increased. Within the pressure range of 3000 to 5000 psi, the yield increased between the temperatures of 40 to 60 ^oC and somehow decreased from 60 to 80 ^oC. Supercritical CO_2 has a low polarity, making the extraction of polar analytes difficult. This limitation can usually be overcome by adding small amounts of polar modifier, such as ethanol, to the supercritical CO_2 in order to increase its solubilizing power and thus increase the extraction efficiency. In our experiment, the second step of each test in which the modifier was added in succession was also meant to be the exhaustive process for completing the recovery of the high-molecular mass components. Anyhow, the results revealed that the addition of modifier negligibly improved the yield that of pure CO_2. The increments in the yield were only by 0.01-0.27% except in M7S2, where it was 0.48%.

Step-1 (extraction by pure CO_2) and Step-2 (extraction by CO_2 + modifier) process, which includes the pressure factor set to 1300 psi, resulted in low yields and the total yields were not more than 0.3%. A change in the pressure from 3000 psi to 5000 psi resulted in a satisfactory improve in the yield (> 0.9%), except for M4S1 (0.69%) for

Step-1 process. Overall, the total yields at 3000 psi and 5000 psi, were significant (> 0.9%).

4.6.2.1 TLC Profile of the Extracts

All crudes were examined to point out possible interesting differences in the chemical composition obtained by different extraction conditions. TLC sprayed with Dragendorff's reagent presented several orange spots for all crudes extracted at 5000 psi but not for those extracted at 1300 and 3000 psi. This indicated that alkaloidal components can only be furnished at relatively high pressure and the addition of a polar solvent as modifier did not give any effect in this aspect as shown by crudes extracted at 1300 and 3000 psi. Nevertheless, the spots of the alkaloid were more intense in M7S2, M8S2 and M9S2 compared to M7S1, M8S1 and M9S1 respectively, thus suggesting that the presence of modifier might enhance the yield of the alkaloid components.

4.6.2.2 GC and GC-MS Analyses of M5S1

Figure 4.29 depicted the GC-MS profile of the SFE extract (M5S1) that possesses the strongest nitric oxide (NO) inhibitory activity without showing toxicity towards the test cells. Table 4.8 lists the constituents identified in M5S1, the relative GC peak areas of these constituents and their experimental Kovats index (*I*). The gas chromatographic profile reveals 15 identifiable constituents that represented 93.53% of the total peak area. The prominent components were fatty acids, accumulated to 39.01% of the total yield. The rest of the crude was made up of four hydrocarbons (36.67%), three phytosterols (9.32%), two esters (3.45%), two alcohols (2.30%) and last but not least, a tocochromanol (2.78%). Palmitic acid, at 34.90% was the most abundant compound in the crude matrix followed by heptacosane (18.56%) and nonacosane (11.00%).

Fatty acids are involved in the formation of healthy cell membranes, proper development and functioning of the brain and nervous system, regulating blood pressure and viscosity as well as immune and inflammatory responses [140, 141]. This may explain the anti-inflammatory potential exhibited by M5S1, as it is rich in palmitic acid.

In addition, the presence of phytosterols may also enhance the nitric oxide inhibition activity exhibited by M5S1 as they have been claimed to possess anti-inflammatory [143], anti-oxidant [144] and angiogenic [145] activities. *β*-Sitosterol, stigmasterol and campesterol were present at an appreciable amount, *i.e.*, 6.67%, 0.99% and 1.66% respectively. Along with these phytosterols, the appearance of tocopherol that constituted 2.78% of the extract, although present at a low concentration, might contribute towards the observed nitric oxide inhibition activity through its anti-oxidant properties, especially against lipid peroxidation in biological membranes [146, 147].

4.6.2.3 Precision of the SFE

To obtain the method precision, three replicate analyses of M5S1 crudes were performed. The results are presented in Table 4.8. RSD values less than 10% was considered good precision for a method.

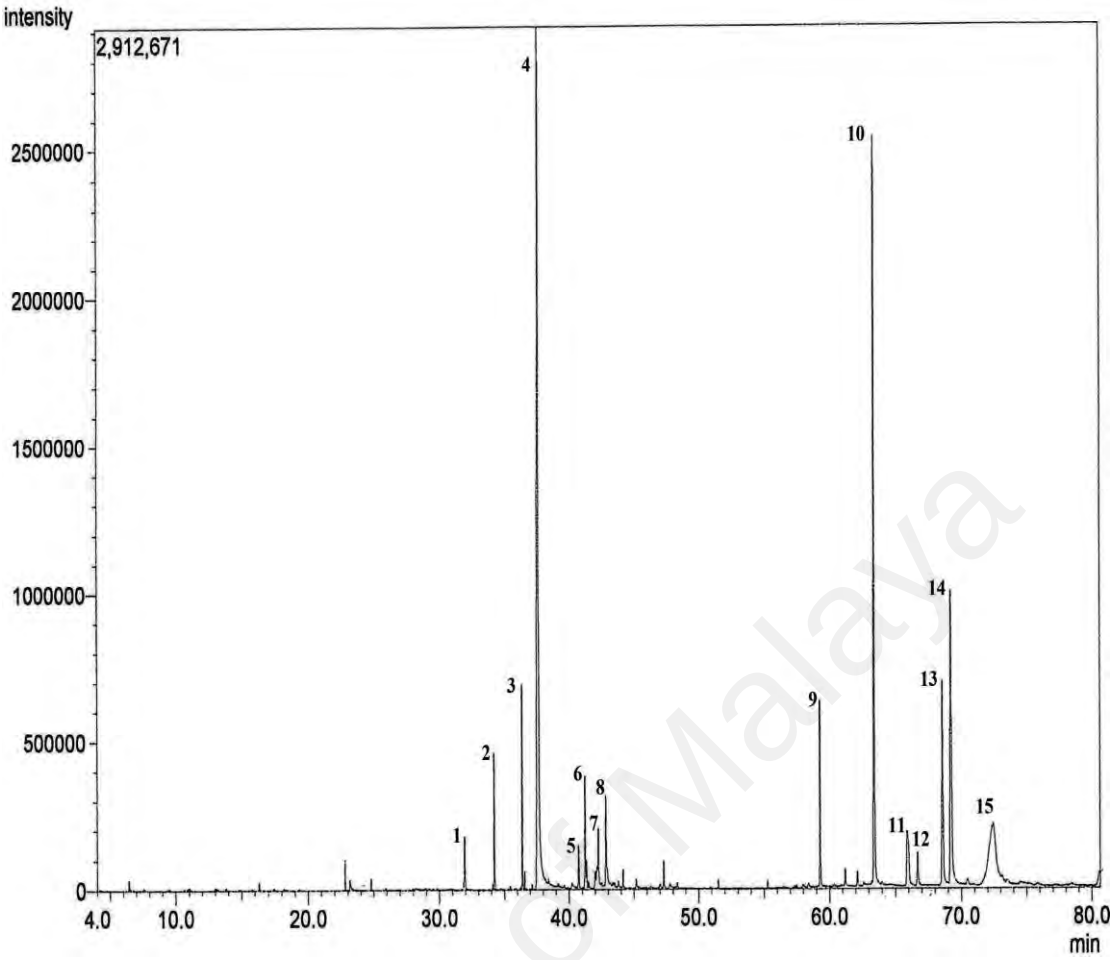


Figure 4.29 The GCMS total ion chromatogram of M5S1 from *M. speciosa*
The extract obtained by SFE that possesses the strongest nitric oxide inhibitory activity without cytotoxicity effect.

Table 4.8 Constituents identified in the leaves of *M. speciosa* extract obtained by SFE that possesses the strongest nitric oxide inhibitory activity without cytotoxicity effect, M5S1

NO.	Identified compounds	*I*	Relative Content (%)[a]	RSD (%)	ID
1	α-Bisabolol	1687	1.15	9.1	RI
2	Z-Nuciferol acetate	1832	2.35	2.3	RI
3	9 (11),15-diene-Isopimara	1907	3.54	7.5	RI
4	Palmitic acid	1984	34.90	1.5	RI, MS, CO
5	2,3-Dimethoxypropyl tetradecanoate	2073	1.10	1.3	RI
6	Methyl ester oleic acid	2090	2.20	5.7	RI, MS
7	Nezukol	2132	1.15	9.5	RI
8	Stearic acid	2166	1.91	9.0	RI, MS,
9	2,10-Dimethyloctacosane	2649	3.57	2.3	RI, MS
10	Heptacosane	2702	18.56	5.7	RI, MS
11	Campesterol	2723	1.66	3.7	RI, MS
12	β-/γ-Stigmasterol*	2731	0.99	6.0	RI, MS
13	β-/γ-Sitosterol*	2739	6.67	3.5	RI, MS
14	Nonacosane	2904	11.00	2.5	RI, MS
15	β-/γ-Tocopherol*	3036	2.78	9.5	RI, MS

I; Kovats index calculated on HP-5 column, RSD; relative standard deviation, ID; means of identification, RI; comparison of Kovats indices with literature values, MS; mass spectral data, CO; co-injection with standard

[a] Percentage of total FID peak area obtained on HP-5 column.

* Correct isomer not identified.

4.7 Conclusion

In summary, it has been observed that M5S1 from the SFE was found to exhibit the strongest NO inhibitory property and thus, further investigation was embarked on the chemical constituents of the crude. It is noteworthy that M5S1 was dominated by fatty acid, in particular palmitic acid (34.90%) that has been reported as an anti-inflammatory compound. Parameters for the extraction of M5S1 are determined as the optimal SFE condition (extraction under pure CO_2, pressure: 3000 psi, temperature: 60 ^oC) to furnish extract that possesses the highest anti-inflammatory activity without any toxicity.

Majority of the previous biological studies were focused on the alkaloidal crude extract and its major alkaloid component, mitragynine with regard to its anti-depressant, anti-nociceptive and psychostimulant action. There is lack of studies on the non-alkaloid crude. The present review highlighted the effect of the non-alkaloid crude, which may be of important potential in anti-inflammatory treatment. This is also the first attempt to explain the biological activity, in particularly the anti-inflammatory activity from a non-alkaloidal crude, which provide support to the traditional utilization of this plant in pain and inflammation.

This study also demonstrated a good ulcer inhibitory activity of the aqueous extract and the crude was further subjected to isolation of pure compounds. Five Corynanthe type indole alkaloids have been successfully isolated and identified, namely mitragynine (21.5 mg), speciociliatine (5.9 mg), paynantheine 3.5 mg), 3,4,5,6-tetradehydromitragynine (1.2 mg) and 7-hydroxyspeciociliatine (13.7 mg). Mitragynine at 10% abundancy dominated the alkaloid constituent of the leaves. It has been documented that in both Thai and Malay leaf samples, mitragynine was the most abundant alkaloid, it made up about half (66%) of the total alkaloid in the Thai originated sample, yet it made up only 12% of the alkaloids from the Malaysian sample. *Mitragyna speciosa* alkaloid content varies quantitatively from geographical location, and from month to month, at different leaf harvest times, which has led some teams (Shellard *et al.* in the 1970s) to conclude that there may be different geographical variants within the same species.

CHAPTER 5: PLANT VOLATILE ORGANIC COMPOUNDS

The World Health Organization (WHO) defines Volatile Organic Compounds (VOCs) as organic compounds with boiling points between 50 °C and 260 °C, excluding pesticides. In general, VOCs are organic chemicals that have a high vapour pressure at ordinary room temperature (approximately 25 ± 3 °C for Malaysia). Their high vapour pressure resulted from a low boiling point, which causes large numbers of molecules to evaporate or sublimate from the liquid or solid form of the compound and enter the surrounding air, a trait known as volatility.

5.1 General Introduction

VOCs are numerous, varied, and ubiquitous. They include both man-made and naturally occurring chemical compounds. Man-made volatile organic compound emissions primarily come from transportation, solvent use, industrial processes and gasoline evaporation. Whereas, fossil fuel deposits, including oil sands, volcanoes, vegetation, bacteria and trees are natural sources of volatile organic compounds. Some VOCs are dangerous to human health or cause harm to the environment. Anthropogenic VOCs are regulated by law, especially indoors, where concentrations are the highest. Harmful VOCs typically are not acutely toxic, but have compounding long-term health effects. It is quite a misnomer that all VOCs are bad. In fact many VOCs are found naturally within the environment as such those being produced by the plants.

Plants emit an amazing number of different chemical compounds that can disperse in the air at ambient temperature. For example, deciduous trees produced great amounts of the compound isoprene during a hot day; coniferous trees emit the volatile organic compound pinene day and night. VOCs in plants are secondary metabolites that play an important role in communication between plants and messages from plants to animals.

In nature, these compounds attract pollinators and seed dispersers, protect plants through repulsion or intoxication of attacking herbivores, entice predator or parasitoid insects that prey on herbivores, prime defences of neighbouring plants against imminent attack, confer anti-microbial properties critical to defence against pathogens, and mitigate oxidative stresses. These volatiles have served mankind, perhaps since pre-Neolithic times, as perfumes and natural flavour compounds [174, 175].

5.2 Plant Volatile Organic Compounds

Plant volatiles organic compounds (PVOCs) are typically classified into four major categories based on their origin, which are terpenoids, fatty acid derivatives, amino acid derivatives and phenylpropanoid/benzenoid compounds, though a number of species- or genus-specific volatile compounds, such as those found in selected species of Alliaceae and Brassicaceae, fall outside these categories [174, 175].

The volatiles derived from some part of a plant, for example the leaf, stem, flower or peel, and usually carry the odour or flavour of the plant is in general named as essential oil. Essential oils are usually lipophilic compounds and therefore usually not miscible with water. Some essential oils are nearly pure single compounds, for example oil of wintergreen. However, most are mixtures of many chemicals [174, 175].

Essential oils are generally extracted by distillation, including steam distillation and hydro distillation. Other processes include cold pressing, for example for citrus peel oils, solvent extraction and supercritical fluid extraction (with CO_2). In some cases, extracted essential oils are further processed to remove undesirable components, for example rectification of peppermint oil to remove dimethyl sulphide [174, 175].

Most importantly, the process used for extraction needs to be compared with the allowable processes in the definition of a naturally-occurring chemical, and any likelihood of change in chemical composition during the extractive process needs to be examined. Numerous studies have indicated differences in chemical composition between the natural plant oil and the commercial oil. Also, the scientific literature contains numerous studies citing the variations in chemical composition between oils extracted by different means.

In connection with our interest in *Mitragyna speciosa* and particularly in its potential as an anti-inflammatory agent, we had occasion to examine the volatile constituents from the leaves of this plant. This chapter will discuss on the volatile compositions that have been afforded by means of hydro distillation (HD) and supercritical fluid extraction (SFE) method, which has not been performed by previous researchers.

5.3 Material and Methods

Leaves from the same sample plant KL5321, as described in Chapter 4, sub-heading 4.1 were used to collect the volatile components in the present study of *M. speciosa*. In this study, plant sample was subjected to hydro distillation (HD) method and supercritical fluid extraction (SFE) for the isolation of the volatile fraction. The constituents of the volatiles were then analysed by capillary gas chromatography and gas chromatography coupled to mass spectrometry (GC-MS).

5.3.1 Hydro Distillation

Volatile fraction achieved by the hydro distil extraction is classified as an essential oil. The essential oil was isolated from 200 g of the dried leaves by using a Clevenger-type apparatus (Figure 5.1). Ground material was placed in a 5L RBF and 2335 mL of

distilled water was added. Water-distilled vigorously for 5 h by means of heating mantle. Experiment was carried out in triplicate.

Distillate was let to cool at room temperature after completing the hydro distillation process. The oil from the distillate was extracted with *n*-hexane and dried over anhydrous sodium sulphate. Then the solvent was removed under vacuum at a low temperature to obtain the essential oil. Content (%) was calculated as weight (g) of oil per 200 g of dry plant material and the yields were averaged over three experiments. The concentrated essential oils were stored at 4 ^{o}C until further analysis.

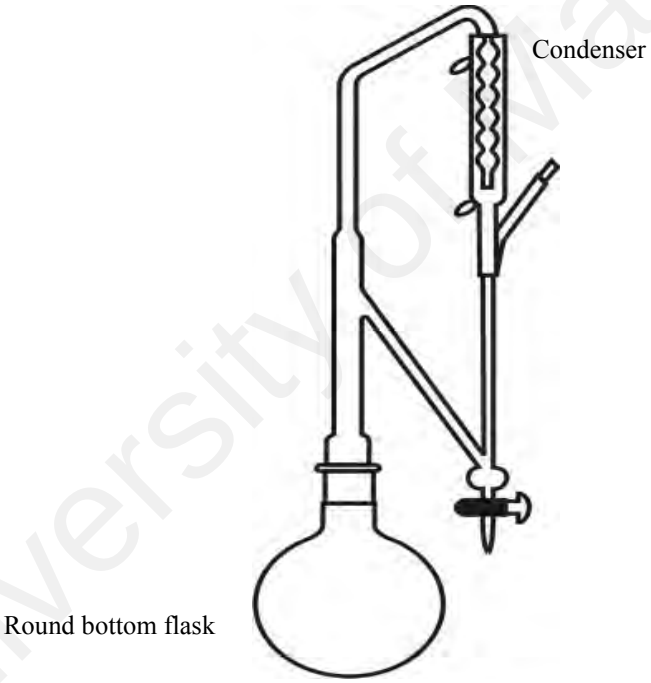


Figure 5.1 Clevenger type apparatus

5.3.2 Supercritical Fluid Extraction (SFE)

Procedure of volatile components collected via SFE method was as mentioned in Chapter 4, sub-heading 4.4.4 (pg. 94). Analysis of the SFE volatile components was focused on the crudes that were obtained under extraction at 1300 psi as this is the most

similar and common condition used in previously published reports when comparison with hydro distillation was in concern [176, 177].

5.3.3 Gas Chromatography (GC)

The GC analyses of the essential oils obtained from HD were carried out with an Agilent 7890A GC system (Agilent Technologies, AT, Palo Alto, CA, USA) equipped with a flame ionization detector (FID). The non-polar column was HP-5 (J&W Scientific, Folsom, CA, USA) of 30 m x 0.32 mm i.d. x 0.25 μm film thickness, coated with 5% phenyl 95% dimethylpolysiloxane. The temperature program for column was 70 ^{o}C for 1 min, then increased up to 180 ^{o}C at a rate of 2 ^{o}C min^{-1} and finally programmed to 250 ^{o}C at 4 ^{o}C min^{-1}, kept constant at 250 ^{o}C for 10 min. A volume of 2 μL of diluted oil (8000 ppm) was injected into the split mode (20:1) GC inlet, held at 250 ^{o}C. The temperature of the FID was 300 ^{o}C and nitrogen as the carrier gas (1 mL/min).

GC analyses for the SFE extracts were carried out using the same Agilent 7890A GC system and column. Appropriate operational condition to obtain satisfied GC chromatogram was to keep the oven temperature at 60 ^{o}C for 1 min, then increased up to 150 ^{o}C at a rate of 4 ^{o}C min^{-1} and finally programmed to 280 ^{o}C at 3.5 ^{o}C min^{-1}, kept constant at 280 ^{o}C for 25 min. The rest of the operational conditions were the same as the analysis for HD essence.

5.3.4 Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS analyses for HD and SFE extracts were accomplished by using a Shimadzu GC-2010 system with the same columns and temperature programme as GC to obtain

the same elution order. The GC was fitted with a quadrupole mass spectrometer, MS-QP2010 Plus with ionization energy of 70 eV, a scan time of 0.5 s and a mass range of 40-800 amu. The components of the crude were identified by comparison of their mass spectra with those of the spectrometer database using the NIST08s.LIB mass spectral database.

5.3.5 Identification of Constituents

The constituents were identified by co-chromatography with authentic samples and by comparison of their mass spectral fragmentation patterns with those stored in the Wiley/ NIST08s.LIB database. Further confirmation was established by comparing their calculated Kovats indices with those of the published index values.

5.3.5.1 Kovats Index

The identifications were confirmed by comparison of Kovats index (*I*) with those reported in the literature (Table 5.9). The homologous *n*-alkanes series C_5-C_{40} injected in GC under the same conditions as the volatile extracts were used to calculate the Kovats index as explained in Chapter 4 (sub-heading 4.5.2.4, pg. 101). Relative amounts of individual constituent were based on the peak areas obtained without FID response factor correction.

5.3.6 The Precision of HD and SFE

The precision of HD and SFE was studied with three replicate analyses of each sample. Experimental results represented as mean ± standard deviation (SD) of the peak areas of each identified component.

5.4 Results and Discussion

The GC and GCMS conditions described in section 5.3.3 and 5.3.4 were the optimum parameters chosen to analyse the constituents for the *M. speciosa* volatile extracts from HD and SFE. Besides HP-5, other equivalent apolar column such as DB-5, BP-5, OV-3, OV-5, OV-73, SE 52, SE 54 and HP-5MS are also suitable due the similarity in McReynolds' constant. Regardless of the trade names, McReynolds' constant of a column should be examined first before making any choice, thus selectivity of the column is determined by those constants [178, 179]. Assignment of the compounds are often cannot be made solely on the basis of mass spectrometric data only, as many terpenes have essentially identical mass spectra. This can be due to the initial similarity of structures, which led to same mass fragmentation patterns or due to various fragmentations and rearrangements after ionization. Hence some knowledge of retention characteristics such as Kovats retention index is often required to complement mass spectral data [180].

5.4.1 Essential Oil Constituents from the Hydro Distillation

For the first time, the essential oils obtained from *M. speciosa* were investigated for their chemical components. The oils recovered from hydro distillation of the leaves of this species were light yellow in colour and have a sweet, tea like with a mild rosy odour. The oils were isolated in low yields, 0.011 ± 0.002 % (w/w) when averaged over three experiments from dry plant material. Table 5.1 listed the oil constituents identified in the leaves of 'ketum', the relative GC peak areas of these constituents and their experimental retention indices on the HP-5 column. Thirty-nine compounds, constituting 85.29 ± 2.26% of the leaf oil of 'ketum' were identified (Figure 5.2). The major families of detected volatiles were oxygenated monoterpenes (10.5% consisting

of eleven compounds), sesquiterpene hydrocarbons (20.5% consisting of twelve compounds), oxygenated sesquiterpenes (15.21% consisting of five compounds), oxygenated diterpenes (33.6% consisting of three compounds) and the non-terpenoids (5.5% consisting of eight compounds). The leaf oil of 'ketum' is dominated the most by oxygenated diterpenes, which include the most abundant compound, isophytol at 23.51 ± 4.19% and the second major constituent, (*E*)-phytol at 9.17 ± 2.52% of the total yield. Other compositions with significant amounts are (2*E*, 6*E*)-farnesyl acetate (8.96 ± 2.54%) followed by *α*-curcumene (4.74 ± 0.78%), *β*-bisabolene (3.48 ± 0.49%) and (+)-*δ*-cadinene (2.87 ± 0.34%).

Phytol is one part of the chlorophyll and important in plant biosynthesis. It is a key acyclic diterpene alcohol that is a precursor in the formation of both vitamins E and K_1. When humans as well as rodents are fed with free phytol, a high proportion is absorbed and converted *in vivo* to phytonic acid. This conversion is a natural rexinoid, which shows anti-diabetic activity in type-II diabetic patients. Furthermore, this phytochemical was also observed to possess promising anti-microbial, anti-schistosomal, anti-cancer, anti-inflammatory and diuretic properties [181]. Unlike many terpenes, phytol has very little odour.

Damascenones are a series of closely related chemical compounds that are components of a variety of essential oils. The damascenones belong to a family of chemicals known as rose ketones, which also includes damascones and ionones. *β*-Damascenone is a major contributor to the aroma of roses and is an important fragrance chemical used in perfumery [182]. This may explain the mild rosy fragrance of the HD essence, despite its low concentration (0.46 ± 0.10% for (*Z*)-*β*-damascenone and 1.09 ± 0.25% for (*E*)-*β*-damascenone).

Nerol is a monoterpene found in many essential oils such as lemongrass and hops. It was originally isolated from neroli oil, hence its name. This colourless liquid is also commonly used in perfumery. Like geraniol, nerol has a sweet rose odour but it is considered to be fresher.

Terpineol is a naturally occurring monoterpene alcohol that has been isolated from a variety of sources such as cajuput oil, pine oil and petitgrain oil. There are four isomers, *α*-, *β*-, *γ*-terpineol, and terpinen-4-ol. *β*- and *γ*-terpineol differ only by the location of the double bond (Table 5.9). Terpineol is usually a mixture of these isomers with *α*-terpineol as the major constituent which is in agreement with the obtained results of which *α*-terpineol constituted 1.5 ± 0.70%, *β*-terpineol constituted 0.22 ± 0.02%, *γ*-terpineol constituted 0.36 ± 0.20% and terpinen-4-ol constituted 1.5 ± 0.70% of the yield. Terpineol has a pleasant odour similar to lilac and is a common ingredient in perfumes, cosmetics, and flavours.

Table 5.1 Chemical composition of essential oil from leaves of *Mitragyna speciosa* obtained by hydro distillation (HD)

Peak No.	Compound[a]	*I*[b]	Area, %[c] (± SD, n = 3)	Identification method
	Oxygenated monoterpene			
	Alcohol			
1	*β*-Linalool	1101	2.57 ± 0.63	*I*, MS
2	Trans-*β*-terpineol	1163	0.22 ± 0.02	*I*, MS
3	Terpinen-4-ol	1173	0.53 ± 0.30	*I*, MS
4	*α*-Terpineol	1192	1.5 ± 0.70	*I*, MS
5	*γ*-Terpineol	1200	0.36 ± 0.20	*I*, MS
6	Nerol	1231	0.95 ± 0.37	*I*, MS
7	(*E*)-Geraniol	1258	0.99 ± 0.45	*I*, MS
	Ether			
8	(*E*)-Linalool oxide	1077	0.34 ± 0.17	*I*, MS

Table 5.1 Continued

	Aldehyde			
9	(*E*)-Geranial	1272	0.52 ± 0.17	*I*, MS
	Ketone			
10	(*E*)-*α*-Geranyl acetone	1455	2.39 ± 0.91	*I*, MS
	Ester			
11	Isopulegyl acetate	1281	0.16 ± 0.03	*I*, MS,
	Sesquiterpene hydrocarbon			
12	*α*-Copaene	1375	0.57 ± 0.20	*I*, MS
13	(*Z*)-Caryophyllene	1406	0.61 ± 0.16	*I*, MS
14	(*Z*, *E*)/(*Z*, *Z*)-*α*-Farnesene*	1417	0.78 ± 0.13	MS
15	(*E*)-*β*-Farnesene	1459	0.63 ± 0.14	*I*, MS
16	*γ*-Muurolene	1476	1.37 ± 0.11	*I*, MS
17	*α*-Curcumene	1483	4.74 ± 0.78	*I*, MS
18	*β*-Selinene	1492	0.82 ± 0.19	*I*, MS
19	*α*-Muurolene	1497	0.99 ± 0.16	*I*, MS
20	*β*-Bisabolene	1508	3.48 ± 0.49	*I*, MS
21	(*E*)-*γ*-Bisabolene	1511	2.33 ± 0.15	*I*, MS
22	(+)-*δ*-Cadinene	1522	2.87 ± 0.34	*I*, MS
23	Cadalene	1671	1.30 ± 0.19	*I*, MS
	Oxygenated sesquiterpene			
	Alcohol			
24	(*E*)-Nerolidol	1566	0.98 ± 0.29	*I*, MS
25	Spathulenol	1579	0.75 ± 0.16	*I*, MS
26	*β*-Bisabolene-12-ol	1764	2.16 ± 0.32	*I*, MS
	Ketone			
27	(5*E*, 9*E*)-Farnesyl acetone	1918	2.36 ±0.22	*I*, MS
	Ester			
28	(2*E*, 6*E*)-Farnesyl acetate	1846	8.96 ± 2.54	*I*, MS
	Oxygenated diterpene			
	Alcohol			
29	Isophytol	1950	23.51 ± 4.19	*I*, MS
30	(*E*)-Phytol*	2113	9.17 ± 2.52	MS
	Ester			
31	(*E*)-Phytol acetate*	2222	0.89 ± 0.20	*I*, MS

Table 5.1 Continued

	Non-terpene			
	Ketone			
32	(*Z*)-*β*-Damascenone	1370	0.46 ± 0.10	*I*, MS
33	(*E*)-*β*-Damascenone	1385	1.09 ± 0.25	*I*, MS
34	(*Z*)-*β*-Damascone	1388	0.3 ± 0.07	*I*, MS
35	(*E*)-*α*-Damascone	1391	0.38 ± 0.12	*I*, MS
36	(*E*)-)-*α*-Ionone	1428	1.17 ± 0.30	*I*, MS
	Ester			
37	Methyl palmitate	1929	0.80 ± 0.22	*I*, MS
38	Methyl linoleate	2093	0.40 ± 0.03	*I*, MS
39	Methyl linolenate	2099	0.89 ± 0.09	*I*, MS
	Total identified volatile compounds		85.29 ± 2.26	
	Oxygenated monoterpenes		10.53	
	Sesquiterpene hydrocarbons		20.49	
	Oxygenated sesquiterpenes		15.21	
	Oxygenated diterpenes		33.57	
	Non-terpenes		5.49	

[a] Identified compounds are reported according to their families on HP-5^{TM} column. [b] Kovats index with respect to C_5-C_{40} *n*-alkanes calculated on non-polar HP-5^{TM} capillary column. [c] Percentage calculated by GC-FID on non-polar HP-5^{TM} capillary column. *Correct isomer not identified. *I*-comparison of Kovats indices with literature values. MS-mass spectral data.

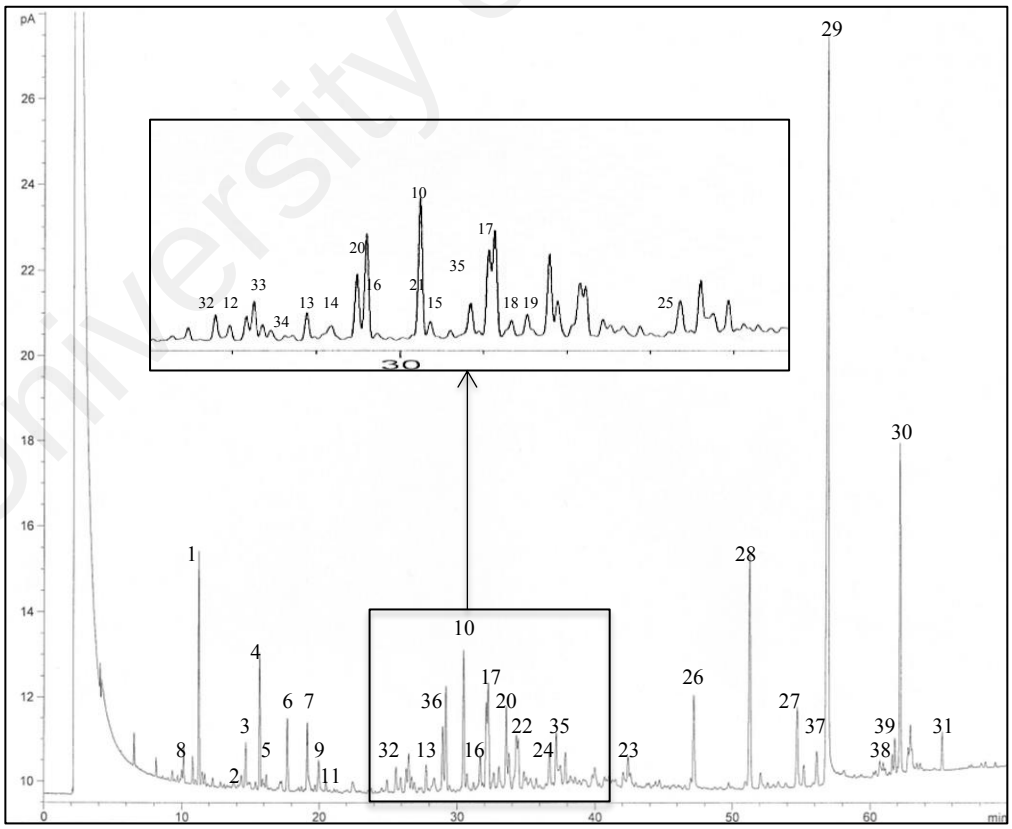


Figure 5.2 Chromatogram of essential oil of *Mitragyna speciosa* leaves obtained by hydro distillation (HD) on HP-5 column

5.4.2 Volatile Constituents from the SFE

The analysis of volatile constituents from the SFE extracts was performed on M1S1, M1S2, M2S1, M2S2, M3S1 and M3S2.

5.4.2.1 Volatile Constituents of M1S1

M1S1 crude was obtained at moderate pressure of 1300 psi, 40 ^{o}C and using CO_2 as supercritical fluid without addition of ethanol as modifier. The extraction yield was 0.18% from 25 g sample leaves extracted as depicted in Table 4.2 (pg. 96). The oily extract had a pleasant herbaceous odour, different from the stronger rosy smell of the essential oil from hydro distillation. The extract was light yellow in colour.

Table 5.2 presented the relative abundances of over eighteen successfully identified components, which represented 98.03 ± 2.06% of the M1S1, obtained from *M. speciosa* leaves. Figure 5.3 showed the simple chromatographic FID profile on HP-5 column. Peak numbers in Table 5.2 correspond to those of chromatogram in Figure 5.3.

(6*E*, 10*E*, 14*E*, 18*E*)-Squalene was the major compound in the M1S1 volatile extract (21.29 ± 2.30%) followed by (*E*)-phytol (15.67 ± 2.62%), methyl palmitate (13.25 ± 1.02%), 4-hexadecyl hexanoate (8.98 ± 0.15%), *n*-hentriacontane (7.44 ± 0.99%), methyl oleate (6.75 ± 0.65%), *β*-stigmasterol (4.53 ± 0.66%) and *β*/*γ*–sitosterol (4.27 ± 0.70%).

Table 5.2 Chemical composition of M1S1 crude from leaves of *Mitragyna speciosa* obtained by supercritical fluid extraction (SFE)

Peak No.	Compound[a]	*I*[b]	Area, %[c] (± SD, n = 3)	Identification method
1	(*Z*)-*γ*-Bisabolene	1516	0.50 ± 0.01	*I*, MS
2	6*S*, 7*R*-Bisabolone	1750	0.70 ± 0.04	*I*, MS
3	Hexahydrofarnesyl acetone	1842	0.92 ± 0.06	MS
4	Methyl palmitate	1961	13.25 ± 1.02	*I*, MS
5	Methyl oleate	2113	6.75 ± 0.65	*I*, MS
6	(*E*)-Phytol*	2136	15.67 ± 2.62	*I*, MS
7	*n*-Docosane	2202	0.52 ± 0.11	*I*, MS
8	(*E*)-Phytol acetate*	2218	0.48 ± 0.43	*I*, MS
9	*n*-Pentadocosane	2496	1.23 ± 0.71	*I*, MS
10	4-Hexadecyl hexanoate	2525	8.98 ± 0.15	*I*, MS
11	(6*E*, 10*E*, 14*E*, 18*E*)-Squalene	2819	21.29 ± 2.30	*I*, MS
12	*n*-Nonacosane	2899	3.53 ± 0.86	*I*, MS
13	*n*-Hentriacontane	3107	7.44 ± 0.99	*I*, MS
14	*n*-Dotriacontane	3199	4.03 ± 0.13	*I*, MS
15	*n*-Tritriacontane	3298	1.77 ± 0.74	*I*, MS
16	*β*-Stigmasterol	3343	4.53 ± 0.66	*I*, MS
17	*β*/*γ*-Sitosterol*	3460	4.27 ± 0.95	*I*, MS
18	*n*-Pentatriacontane	3502	2.20 ± 0.04	*I*, MS
	Total volatile compounds (%)		98.03 ± 2.06	

[a] Identified compounds are reported according to their elution order on HP-5^{TM} column. [b] Kovats index with respect to C_5-C_{40} *n*-alkanes calculated on non-polar HP-5^{TM} capillary column. [c] Percentage calculated by GC-FID on non-polar HP-5^{TM} capillary column. *Correct isomer not identified. *I*-comparison of Kovats indices with literature values. MS-mass spectral data. CO-co-injection with standard compound.

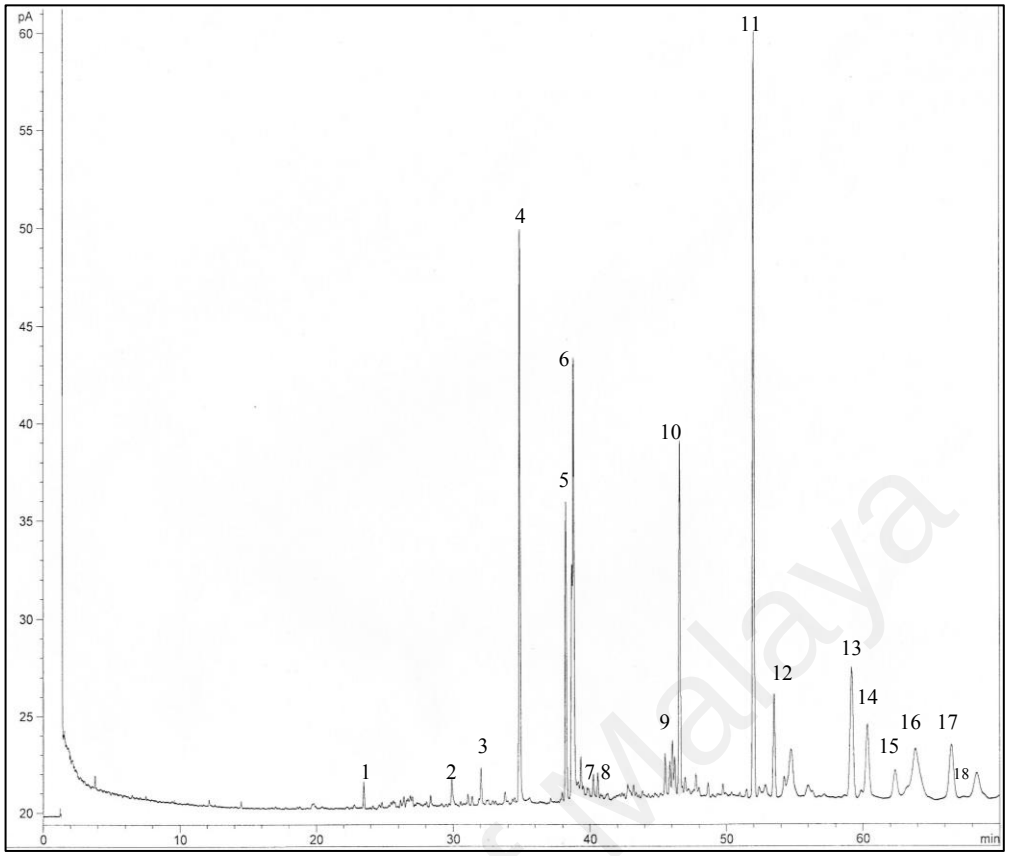


Figure 5.3 Chromatogram of M1S1 volatile crude of *Mitragyna speciosa* leaves obtained by super critical fluid extraction (SFE) on HP-5 column

5.4.2.2 Volatile Constituents of M1S2

M1S2, was yielded from the same batch of sample and under the same SFE conditions as extraction of M1S1 but with an addition of 20% ethanol. The extraction yield was 0.10% (Table 4.2). The light yellow coloured extract has the similar odour as M1S1.

Eighteen volatile compounds identified in M1S2 were presented in Table 5.3, which represented 90.08 ± 1.16% of the extract. Typical chromatographic FID profile on HP-5 column was as depicted in Figure 5.4. Peak numbers in Table 5.3 correspond to those of chromatogram in Figure 5.4.

The main compounds characterized were (*E*)-phytol (21.80 ± 1.52%), followed by methyl palmitate (19.31 ± 1.22%), (6*E*, 10*E*, 14*E*, 18*E*)-squalene (10.25 ± 1.35%), *n*-

hentriacontane (8.85 ± 0.69%), methyl oleate (5.57 ± 0.15%), *β*/*γ*–sitosterol (5.44 ± 0.31%) and *β*-stigmasterol (1.27 ± 0.06%).

Table 5.3 Chemical composition of M1S2 crude from leaves of *Mitragyna speciosa* obtained by supercritical fluid extraction (SFE)

Peak No.	Compound[a]	*I*[b]	Area, %[c] (± SD, n = 3)	Identification method
1	(*Z*)-*γ*-Bisabolene	1515	0.54 ± 0.01	*I*, MS
2	6*S*, 7*R*-Bisabolone	1750	1.39 ± 0.02	*I*, MS
3	Hexahydrofarnesyl acetone	1842	0.92 ± 0.02	MS
4	Methyl palmitate	1964	19.31 ± 1.22	*I*, MS
5	Ethyl palmitate	1993	0.72 ± 0.03	*I*, MS
6	Methyl oleate	2114	5.57 ± 0.15	*I*, MS
7	(*E*)-Phytol*	2142	21.80 ± 1.52	*I*, MS
8	(*E*)-Phytol acetate*	2219	0.30 ± 0.12	*I*, MS
9	4-Hexadecyl hexanoate	2525	1.25 ± 0.05	*I*, MS
10	(6*E*, 10*E*, 14*E*, 18*E*)-Squalene	2819	10.25 ± 1.35	*I*, MS
11	*n*-nonacosane	2887	2.94 ± 0.23	*I*, MS
12	*n*-Hentriacontane	3107	8.85 ± 0.69	*I*, MS
13	*n*-Dotriacontane	3197	2.91 ± 0.05	*I*, MS
14	*n*-Tritriacontane	3298	2.24 ± 0.04	*I*, MS
15	Campesterol	3305	0.65 ± 0.03	*I*, MS
16	*β*-Stigmasterol	3343	1.27 ± 0.06	*I*, MS
17	*β*/*γ*-Sitosterol*	3461	5.44 ± 0.31	*I*, MS
18	*n*-Pentatriacontane	3502	3.75 ± 0.07	*I*, MS
	Total volatile compounds (%)		90.08 ± 1.16	

[a] Compounds identified according to their families on HP-5^{TM} column. [b] Kovats index with respect to C_5-C_{40} *n*-alkanes calculated on non-polar HP-5^{TM} capillary column. [c] Percentage calculated by GC-FID on non-polar HP-5^{TM} capillary column. *Correct isomer not identified. *I*-comparison of Kovats indices with literature values. MS-mass spectral data. CO-co-injection with standard compound.

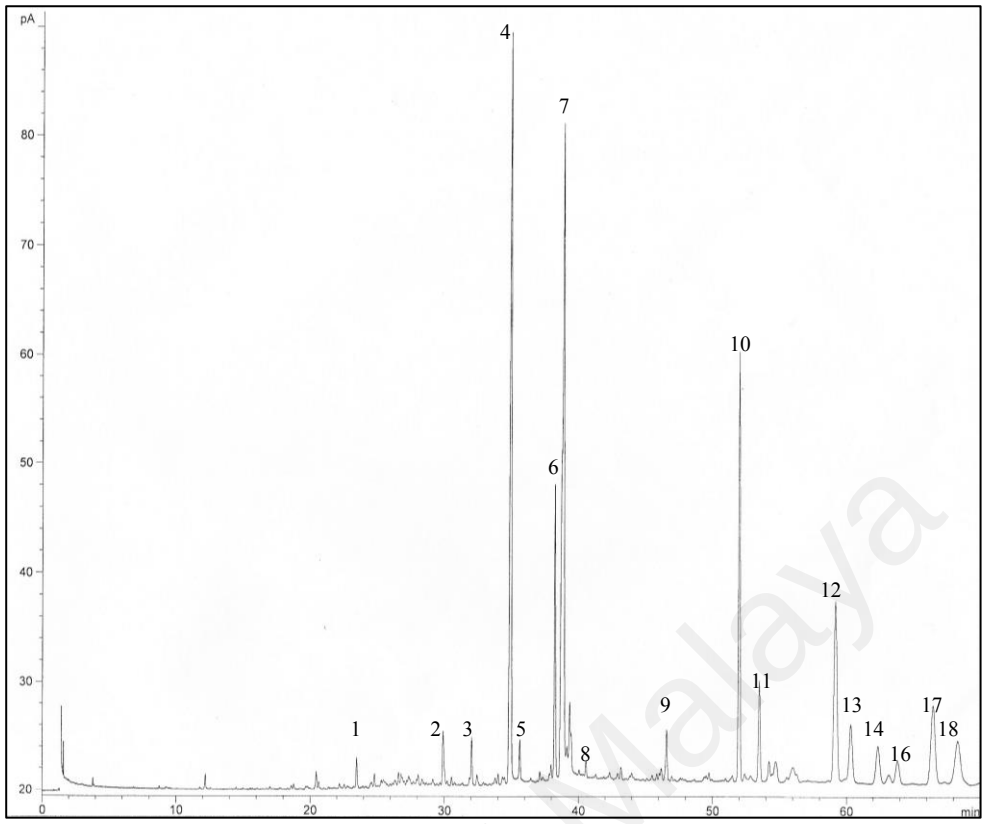


Figure 5.4 Chromatogram of M1S2 volatile crude of *Mitragyna speciosa* leaves obtained by super critical fluid extraction (SFE) on HP-5 column

5.4.2.3 Volatile Constituents of M2S1

M2S1 crude was extracted under similar SFE condition as those for M1S1 but at higher temperature, which was at 60 ^{o}C. The yield of M2S1 was 0.11% from the mass of the dry sample leaves (Table 4.2, pg. 96). Similar to M1S1, the yellowish M2S1 extract also give out the same herbaceous odour.

The relative abundances of seventeen identified components were presented in Table 5.4. This represented 91.70 ± 1.67% of the total peak area of FID chromatographic profile (Figure 5.5). Peak numbers in Table 5.4 were based on those of chromatogram in Figure 5.5.

In general the volatile fraction of M2S1 was predominated by methyl oleate (13.65 ± 1.93%), methyl palmitate (13.41 ± 2.32%), *n*-hentriacontane (13.10 ± 2.38%),

(6*E*, 10*E*, 14*E*, 18*E*)-squalene (12.56 ± 2.28%), (*E*)-phytol (10.15 ± 2.45%), *n*-pentatriacontane (6.68 1.33%), *n*-nonacosane (4.11 ± 0.83%), *β*/*γ*–sitosterol (2.93 ± 0.80%) and hexahydrofarnesyl acetone (2.74 0.73%).

Table 5.4 Chemical composition of M2S1 crude from leaves of *Mitragyna speciosa* obtained by supercritical fluid extraction (SFE)

Peak No.	Compound[a]	*I*[b]	Area, %[c] (± SD, n = 3)	Identification method
1	6*S*, 7*R*-Bisabolone	1750	1.65 ± 0.21	*I*, MS
2	Hexahydrofarnesyl acetone	1843	2.74 ± 0.73	MS
3	Methyl palmitate	1962	13.41 ± 2.32	*I*, MS
4	Ethyl palmitate	1993	1.47 ± 0.66	*I*, MS
5	Methyl oleate	2114	13.65 ± 1.93	*I*, MS
6	(*E*)-Phytol*	2136	10.15 ± 2.45	*I*, MS
7	(*E*)-Phytol acetate*	2220	1.86 ± 0.73	*I*, MS
8	4-Hexadecyl hexanoate	2526	1.38 ± 0.59	*I*, MS
9	(6*E*, 10*E*, 14*E*, 18*E*)-Squalene	2820	12.56 ± 2.28	*I*, MS
10	*n*-Nonacosane	2898	4.11 ± 0.83	*I*, MS
11	*n*-Hentriacontane	3108	13.10 ± 2.38	*I*, MS
12	*n*-Dotriacontane	3197	1.98 ± 0.87	*I*, MS
13	*n*-Tritriacontane	3299	1.52 ± 0.63	*I*, MS
14	Campesterol	3318	1.38 ± 0.64	*I*, MS
15	*β*-Stigmasterol	3343	1.13 ± 0.47	*I*, MS
16	*β*/*γ*-Sitosterol*	3461	2.93 ± 0.80	*I*, MS
17	*n*-Pentatriacontane	3503	6.68 ± 1.33	*I*, MS
	Total volatile compounds (%)		91.70 ± 1.67	

[a] Compounds identified according to their families on HP-5^{TM} column. [b] Kovats index with respect to C_5-C_{40} *n*-alkanes calculated on non-polar HP-5^{TM} capillary column. [c] Percentage calculated by GC-FID on non-polar HP-5^{TM} capillary column. *Correct isomer not identified. *I*-comparison of Kovats indices with literature values. MS-mass spectral data. CO-co-injection with standard compound.

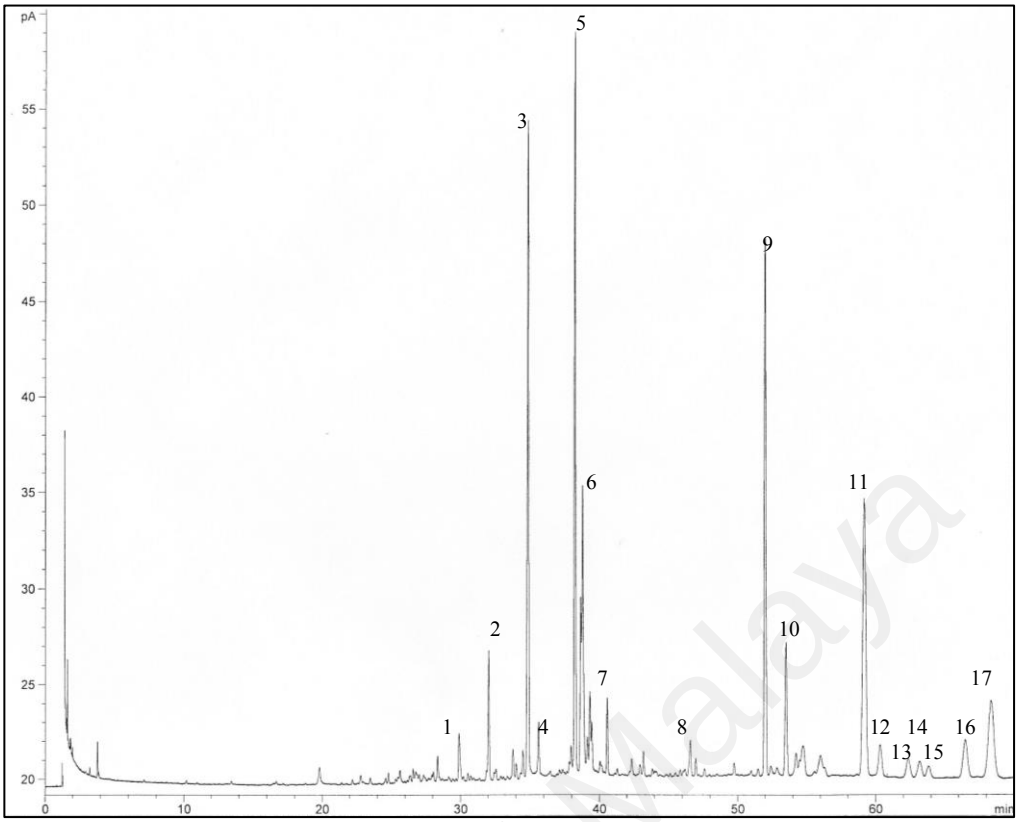


Figure 5.5 Chromatogram of M2S1 volatile crude of *Mitragyna speciosa* leaves obtained by super critical fluid extraction (SFE) on HP-5 column

5.4.2.4 Volatile Constituents of M2S2

M2S2 was extracted under the same SFE operational parameter as for M2S1 but with an addition of ethanol. The polarity of supercritical CO_2 was modified with 40% ethanol. The extraction yield was 0.12% as depicted in Table 4.2 (pg. 96). The oily extract had a pleasant herbaceous odour, different from the stronger rosy smell of the essential oil from hydro distillation. The extract was light yellow in colour.

Table 5.5 reported the volatile content (expressed as relative area percentage) of M2S2 over seventeen identifiable components, which represented 90.47 ± 1.02% of the extract. The chromatographic GC profile on HP-5 column was as depicted in Figure 5.6. Peak numbers in Table 5.5 were according to the elution time of each component as shown by Figure 5.6.

Although both M2S1 and M2S2 possessed basically the same components, they differed in the quantitative compositional. (*E*)-Phytol (21.52 ± 3.25%), was the most dominant compound in the M2S2 volatile extract followed by methyl palmitate (18.53 ± 1.82 %), (6*E*,10*E*,14*E*,18*E*)-squalene (15.20 ± 1.98%), *n*-hentriacontane (9.38 ± 2.49%), methyl oleate (5.38 ± 0.82%), *n*-pentatriacontane (4.89 ± 1.12%), n-dotriacontane (3.11 ± 0.73%), and *β*/*γ*–sitosterol (2.93 ± 0.80%) .

Table 5.5 Chemical composition of M2S2 crude from leaves of *Mitragyna speciosa* obtained by supercritical fluid extraction (SFE)

NO.	Compound[a]	*I*[b]	Area, %[c] (± SD, n = 3)	Identification method
1	(*Z*)-*γ*-Bisabolene	1516	0.50 ± 0.01	*I*, MS
2	6*S*, 7*R*-Bisabolone	1751	0.83 ± 0.07	*I*, MS
3	Hexahydrofarnesyl acetone	1842	1.13 ± 0.11	MS
4	Methyl palmitate	1965	18.53 ± 1.82	*I*, MS
5	Methyl oleate	2057	5.38 ± 0.82	*I*, MS
6	(*E*)-Phytol*	2071	21.52 ± 3.25	*I*, MS
7	(*E*)-Phytol acetate*	2219	0.37 ± 0.33	*I*, MS
8	4-Hexadecyl hexanoate	2525	0.41 ± 0.15	*I*, MS
9	(6*E*, 10*E*, 14*E*, 18*E*)-Squalene	2819	15.20 ± 1.98	*I*, MS
10	*n*-Nonacosane	2901	2.85 ± 0.86	*I*, MS
11	*n*-Hentriacontane	3109	9.38 ± 2.49	*I*, MS
12	*n*-Dotriacontane	3198	3.11 ± 0.73	*I*, MS
13	*n*-Tritriacontane	3299	1.21 ± 0.52	*I*, MS
14	Campesterol	3306	0.98 ± 0.44	*I*, MS
15	*β*-Stigmasterol	3344	1.20 ± 0.51	*I*, MS
16	*β*/*γ*-Sitosterol*	3461	2.98 ± 0.89	*I*, MS
17	*n*-Pentatriacontane	3503	4.89 ± 1.12	*I*, MS
	Total volatile compounds (%)		90.47 ± 1.02	

[a] Compounds identified according to their families on HP-5^{TM} column. [b] Kovats index with respect to C_5-C_{40} *n*-alkanes calculated on non-polar HP-5^{TM} capillary column. [c] Percentage calculated by GC-FID on non-polar HP-5^{TM} capillary column. *Correct isomer not identified. *I*-comparison of Kovats indices with literature values. MS-mass spectral data. CO-co-injection with standard compound.

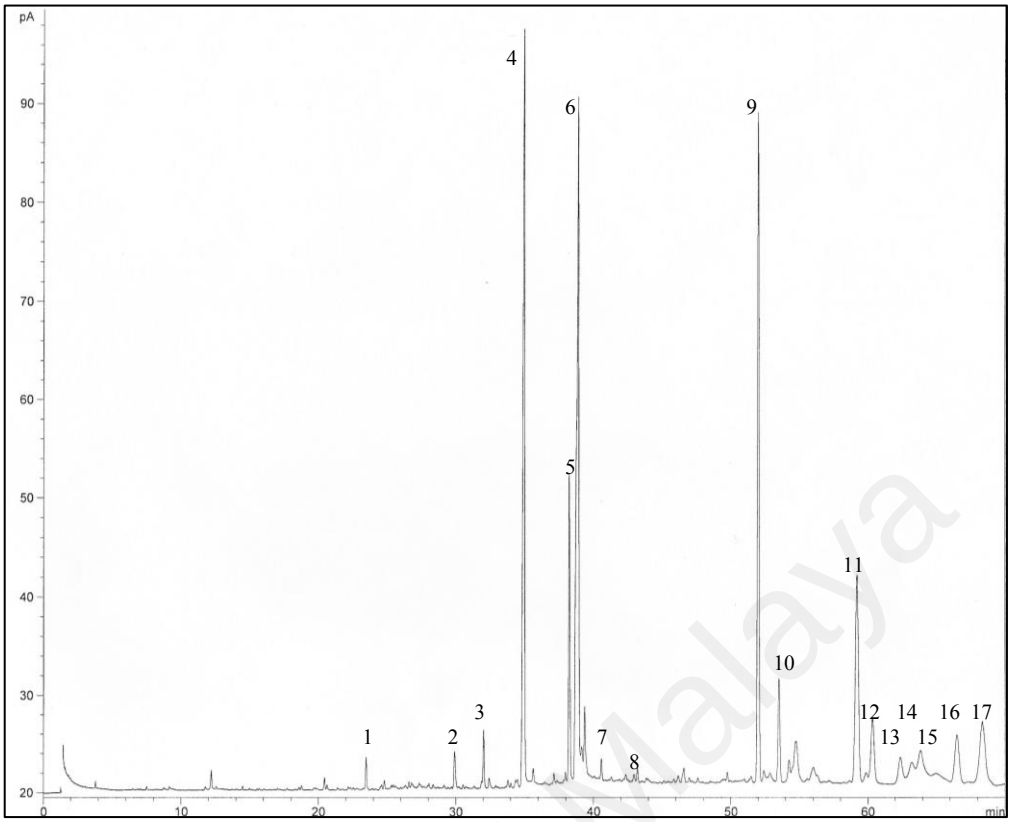


Figure 5.6 Chromatogram of M2S2 volatile crude of *Mitragyna speciosa* leaves obtained by super critical fluid extraction (SFE) on HP-5 column

5.4.2.5 Volatile Constituents of M3S1

The SFE conditions used to extract M3S1 crude were 1300 psi, 80 ^{o}C and supercritical CO_2 fluid without addition of ethanol. The extraction yield was 0.10% from 25 g sample leaves extracted as depicted in Table 4.2 (pg. 96). The oily extract had a pleasant herbaceous odour, different from the stronger rosy smell of the essential oil from hydro distillation. The extract was light yellow in colour.

The analysis of the volatile compositions of M3S1 was reported in Table 5.6 as well as chromatographic FID profile (Figure 5.7). Thirteen compounds were identified, which represented 92.34 ± 1.73% of the extract. Figure 5.7 showed the sample chromatogram on HP-5 column. Peak numbers in Table 5.6 correspond to those of chromatogram in Figure 5.7.

Methyl palmitate (27.73 ± 1.22 %), *n*-hentriacontane (25.29 ± 2.69%), pentatriacontane (11.97 ± 2.33%), *n*-nonacosane (7.31 ± 1.03%), hexahydrofarnesyl acetone (4.20 ± 0.81), (*E*)-phytol (2.74 ± 0.65%), campesterol ((2.38 ± 0.54%) and (*E*)-phytol acetate (1.91 ± 0.43%) were the main constituents in M3S1.

Table 5.6 Chemical composition of M3S1 crude from leaves of *Mitragyna speciosa* obtained by supercritical fluid extraction (SFE)

Peak No.	Compound[a]	*I*[b]	Area, %[c] (± SD, n = 3)	Identification method
1	6*S*, 7*R*-Bisabolone	1752	1.54 ± 0.87	*I*, MS
2	Hexahydrofarnesyl acetone	1843	4.20 ± 0.81	MS
3	Methyl palmitate	1964	27.73 ± 1.22	*I*, MS
4	Ethyl palmitate	1994	1.13 ± 0.44	*I*, MS
5	Methyl oleate	2114	3.40 ± 0.77	*I*, MS
6	(*E*)-Phytol*	2137	2.74 ± 0.65	*I*, MS
7	*n*-Docosane	2202	1.88 ± 0.66	*I*, MS
8	(*E*)-Phytol acetate*	2220	1.91 ± 0.43	*I*, MS
9	4-Hexadecyl hexanoate	2526	0.86 ± 0.06	*I*, MS
10	*n*-Nonacosane	2899	7.31 ± 1.03	*I*, MS
11	*n*-Hentriacontane	3109	25.29 ± 2.69	*I*, MS
12	Campesterol	3316	2.38 ± 0.54	*I*, MS
13	*n*-Pentatriacontane	3503	11.97 ± 2.33	*I*, MS
	Total volatile compounds (%)		92.34 ± 1.73	

[a] Compounds identified according to their families on HP-5^{TM} column. [b] Kovats index with respect to C_5-C_{40} *n*-alkanes calculated on non-polar HP-5^{TM} capillary column. [c] Percentage calculated by GC-FID on non-polar HP-5^{TM} capillary column. *Correct isomer not identified. *I*-comparison of Kovats indices with literature values. MS-mass spectral data. CO-co-injection with standard compound.

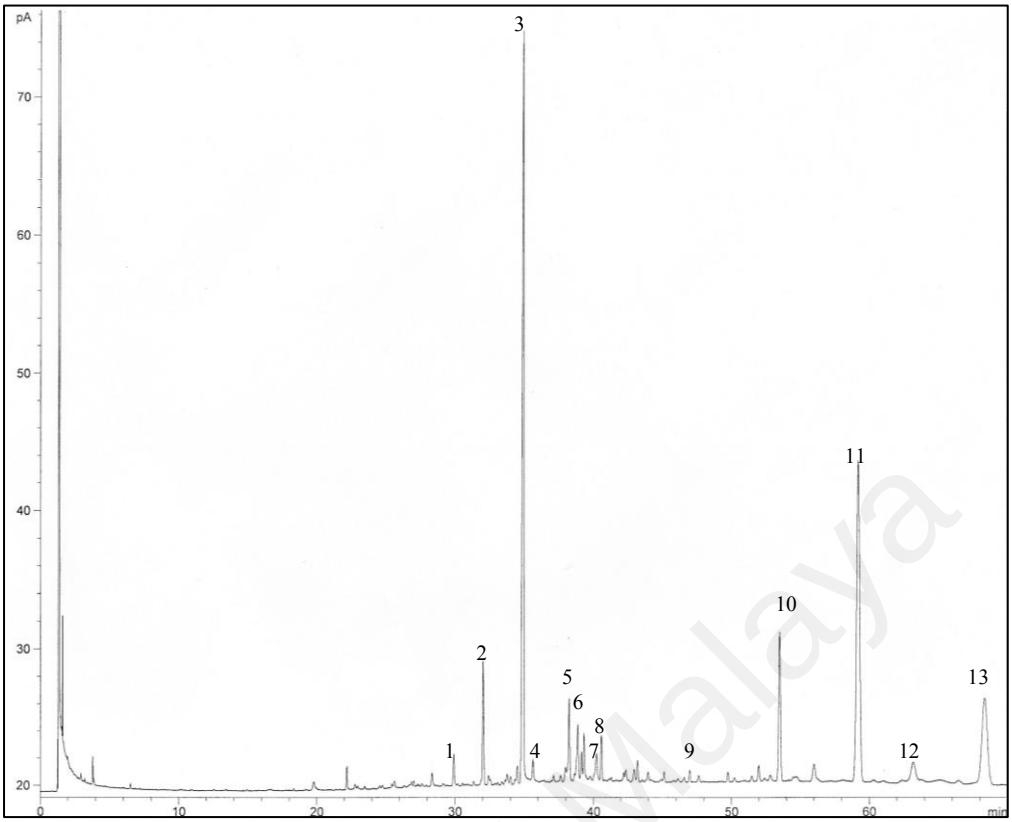


Figure 5.7 Chromatogram of M3S1 volatile crude of *Mitragyna speciosa* leaves obtained by super critical fluid extraction (SFE) on HP-5 column

5.4.2.6 Volatile Constituents of M3S2

M3S2 crude was afforded with the same SFE conditions as for M3S1 but modified with 60% ethanol. Under this parameter the extraction yield was 0.01% of the sample leaves used (Table 4.2, pg. 96). Like the other SFE extracts, M3S2 also was in the form of light yellow oily extract with a mild herbaceous odour.

Table 5.7 listed the extract constituents identified in M3S2, the relative GC peak areas of these constituents and their experimental retention indices on HP-5 column. Mainly fifteen prominent compounds with their corresponding peaks were identified (Figure 5.8), which constituted 94.37 ± 2.36% of the extract.

Major comprised compounds were *n*-hentriacontane (20.99 ± 2.19%), methyl palmitate (16.46 ± 2.02 %), (*E*)-phytol (14.23 ± 2.05%), (6*E*, 10*E*, 14*E*, 18*E*)-squalene (12.84 ±

1.55%), pentatriacontane (12.29 ± 2.19%), methyl oleate (8.36 ± 0.85%), *n*-nonacosane (4.57 ± 0.73%) and campesterol (2.01 ± 0.34%).

Table 5.7 Chemical composition of M3S2 crude from leaves of *Mitragyna speciosa* obtained by supercritical fluid extraction (SFE)

Peak No.	Compound[a]	*I*[b]	Area, %[c] (± SD, n = 3)	Identification method
1	*α*-Bisabolol	1684	1.01± 0.37	
2	6*S*, 7*R*-Bisabolone	1752	1.01 ± 0.35	*I*, MS
3	Hexahydrofarnesyl acetone	1843	1.40 ± 0.52	MS
4	Methyl palmitate	1964	16.46 ± 2.02	*I*, MS
5	Ethyl palmitate	1993	0.86 ± 0.37	*I*, MS
6	Methyl oleate	2114	8.36 ± 0.85	*I*, MS
7	(*E*)-Phytol	2138	14.23 ± 2.05	*I*, MS
8	(*E*)-Phytol acetate	2219	1.28 ± 0.33	*I*, MS
9	4-Hexadecyl hexanoate	2547	0.91 ± 0.11	*I*, MS
10	(6*E*, 10*E*, 14*E*, 18*E*)-Squalene	2819	12.84 ± 1.55	*I*, MS
11	*n*-Nonacosane	2899	4.57 ± 0.73	*I*, MS
12	*n*-Hentriacontane	3107	20.99 ± 2.19	*I*, MS
13	*n*-Dotriacontane	3197	0.96 ± 0.19	*I*, MS
14	Campesterol	3315	2.01 ± 0.34	*I*, MS
15	*n*-Pentatriacontane	3503	12.29 ± 2.19	*I*, MS
	Total identified volatile compounds		94.37 ± 2.36	

[a] Compounds identified according to their families on HP-5^{TM} column. [b] Kovats index with respect to C_5-C_{40} *n*-alkanes calculated on non-polar HP-5^{TM} capillary column. [c] Percentage calculated by GC-FID on non-polar HP-5^{TM} capillary column. *Correct isomer not identified. *I*-comparison of Kovats indices with literature values. MS-mass spectral data. CO-co-injection with standard compound.

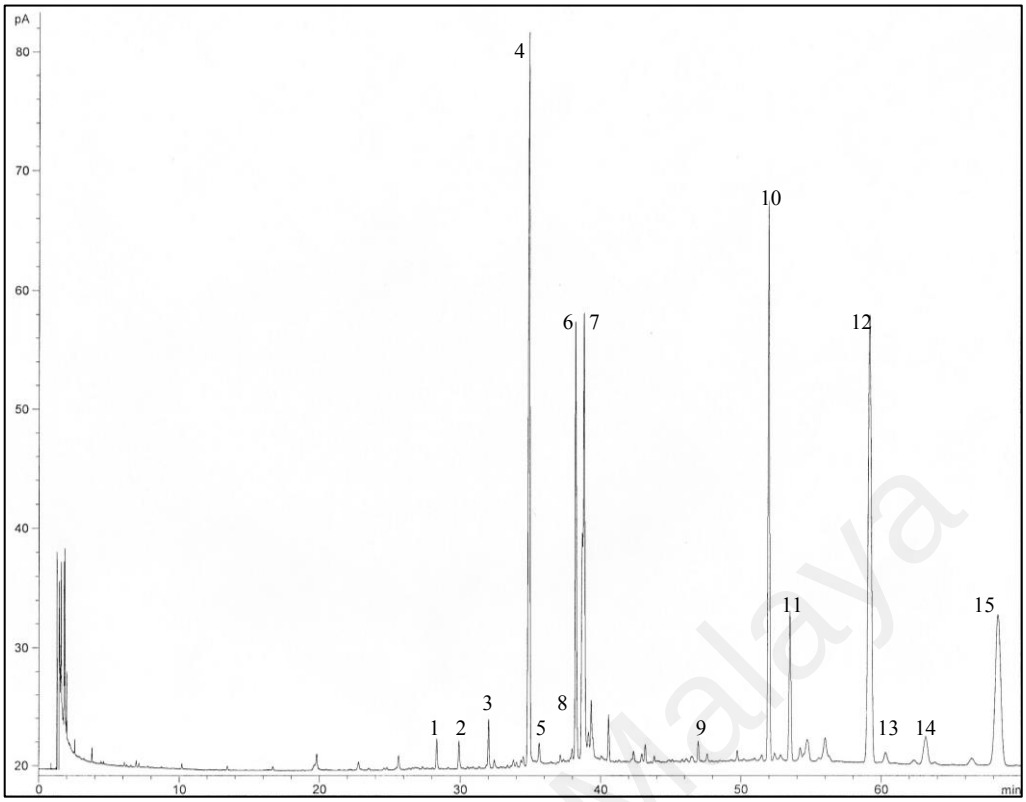


Figure 5.8 Chromatogram of M3S2 volatile crude of *Mitragyna speciosa* leaves obtained by super critical fluid extraction (SFE) on HP-5 column

Although all of the SFE crudes possessed basically the same components, their different chromatogram profiles show large quantitative compositional differences. Table 5.8 clearly showed that non-terpene compounds dominated the composition of all the extracts from SFE.

Triterpene hydrocarbon constituting 21.3% of the total peak area were the second most abundant constituent of M1S1 followed by oxygenated diterpenes at 16.2%. In regard to the addition of ethanol as polarity modifier, a converse situation was observed for M1S2. Increasing in polarity of the supercritical fluid increased the extraction of oxygenated diterpenes to 22.1% but decreased in the amount in triterpene hydrocarbons to 10.3%. This pattern was also repeated by M2S1 and M2S2 as illustrated in Table 5.8. It is worth noting that squalene was the only triterpene hydrocarbon extracted and oxygenated diterpene was composed of phytol and phytol acetate.

As of extracts that were obtained at 1300 psi and 80 ^{o}C, M3S1 (using only CO_2 as supercritical fluid) accumulated the highest non-terpenoids at 79.6% followed by 65.4 % in M3S2 (using ethanol as modifier). Meanwhile, the composition of terpenes was the lowest in M3S1. This limitation can be overcome by the addition of small amount of ethanol as showed by increment of terpenoids in M3S2.

Somehow, the total yield of phytosterols did not show any large different between crudes being extracted with and without addition of modifier (between M1S1 and M1S2, M2S1 and M2S2, M3S1 and M3S2). Stigmasterol and sitosterol were extracted out the most in M1S1 (1300 psi, 40 ^{o}C and 100% CO_2) but campesterol had not been eluted under the same condition. Interestingly, M3S1 and M3S2 that did not contain stigmasterol or sitosterol yielded the highest quantity of campesterol.

The anti-inflammatory composition, (*E*)-Phytol, (6*E*, 10*E*, 14*E*, 18*E*)-squalene and methyl palmitate (the three most abundant components of *M. speciosa* extracts of SFE) had a combined relative amount of over 43.5% in M1S1, M1S2, M2S2 and M3S2 but it was just above 30.5% in M3S1. But interestingly, M3S1 contained the highest amount of methyl palmitate (27.7%) among all the SFE extracts.

Table 5.8 Variability in the chemical composition of SFE crudes from leaves of *Mitragyna speciosa*

Compound[a]	**Area, %**[b]					
	M1S1	M1S2	M2S1	M2S2	M3S1	M3S2
Sesquiterpene hydrocarbon						
(*Z*)-*γ*-Bisabolene	0.50	0.54	-	0.50	-	1.01

Table 5.8 Continued

Oxygenated sesquiterpene						
Ketone						
6*S*, 7*R*-Bisabolone	0.70	1.39	1.65	0.83	1.54	1.01
Hexahydrofarnesyl acetone	0.92	0.92	2.74	1.13	4.20	1.40
Oxygenated diterpene						
Alcohol						
(*E*)-Phytol*	15.67	21.80	10.15	21.52	2.74	14.23
Ester						
(*E*)-Phytol acetate*	0.48	0.30	1.86	0.37	1.91	1.28
Triterpene hydrocarbon						
(6*E*, 10*E*, 14*E*, 18*E*)-Squalene	21.29	10.25	12.56	15.20	-	12.84
Other terpene						
Steroid						
Campesterol	-	0.65	1.38	0.98	2.38	2.01
β-Stigmasterol	4.53	1.27	1.13	1.20	-	-
β/*γ*-Sitosterol*	4.27	5.44	2.93	2.98	-	-
Non-terpene						
Hydrocarbon						
n-Docosane	0.52	-	-	-	1.88	-
n-Pentacosane	1.23	-	-	-	-	-
n-Nonacosane	3.53	2.94	4.11	2.85	7.31	4.57
n-Hentriacontane	7.44	8.85	13.10	9.38	25.29	20.99
n-Dotriacontane	4.03	2.91	1.98	3.11	-	0.96
n-Tritriacontane	1.77	2.24	1.52	1.21	-	-
n-Pentatriacontane	2.20	3.75	6.68	4.89	11.97	12.29
Ester						
Methyl palmitate	13.25	19.31	13.41	18.53	27.73	16.46
Ethyl palmitate	-	0.72	1.47	-	1.13	0.86
Methyl oleate	6.75	5.57	13.65	5.38	3.40	8.36
4-Hexadecyl hexanoate	8.98	1.25	1.38	0.41	0.86	0.91

Table 5.8 Continued

Sesquiterpene hydrocarbons	0.50	0.54	-	0.50	-	1.01
Oxygenated sesquiterpenes	1.62	2.31	4.39	1.96	5.74	2.41
Oxygenated diterpenes	16.15	22.10	12.01	21.89	4.65	15.51
Triterpene hydrocarbons	21.29	10.25	12.56	15.20	-	12.84
Other terpenes	8.80	7.36	5.44	5.16	2.38	2.01
Non-terpenes	49.70	47.54	57.30	45.76	79.57	65.40

[a] Identified compounds are reported according to their families on HP-5^{TM} column. [b] Area percentage expressed as mean values over three replicates calculated by GC-FID on non-polar HP-5^{TM} capillary column. *Correct isomer not identified.

Table 5.9 Odour impact (aroma descriptor), medicinal values and other uses of identified compounds obtained from hydro distillation and supercritical fluid extraction from leaves of *Mitragyna speciosa*

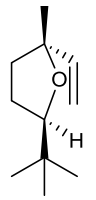
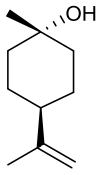
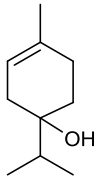
Compound	*I*	Odour [174]	Activities/Uses
Oxygenated monoterpene			
 (*E*)-Linalool oxide	1073 [183]	Fresh, floral, lemon	-
 *β*-Linalool	1097 [167]	Fresh, floral, clean, sweet, lemon notes	- Inhibiting a wide range of *Candida albicans* isolates. - display pro-oxidant activity [174]
 (*E*)-*β*-Terpineol	1163 [183]	Liliac-like	- Flavor and fragrance [174]
 Terpinen-4-ol	1177 [167]	Nutmeg-like, spicy, woody-earthy, liliac-like	- Flavor and Fragrance [174]

Table 5.9 Continued

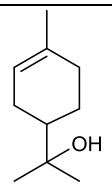
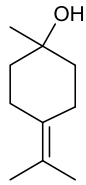
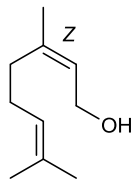
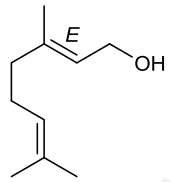
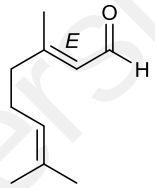
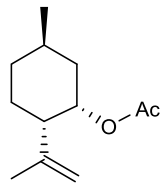
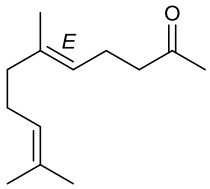
 α-Terpineol	1189 [167]	Floral, liliac-like	- perfume - cosmetic [174]
 γ-Terpineol	1199 [167]	Liliac-like	- fragrance
 Nerol	1230 [167]	Rose, sweet, fresh	- perfume [182]
 (*E*)-Geraniol	1253 [167]	Floral, lemon-like, minty	-
 (*E*)-Geranial	1267 [167]	Lemon, sweet	-
 Isopulegyl acetate	1278 [167]	-	-
 (*E*)-α-Geranyl acetone	1455 [167]	-	-

Table 5.9 Continued

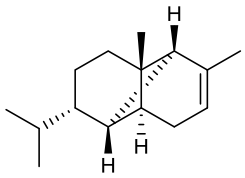
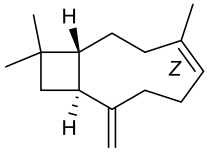
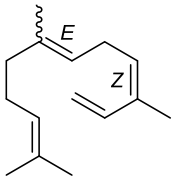
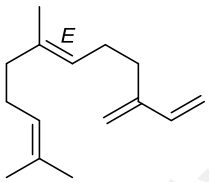
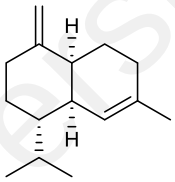
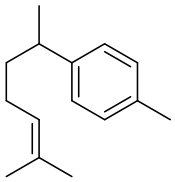
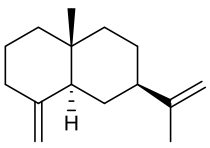
Sesquiterpene hydrocarbon			
 *α*-Copaene	1377 [167]	Vegetable-like, hops-like, woody	-
 (*Z*)-Caryophyllene	1409 [167]	Woody, spicy, terpene notes	-
 (*Z, E*)/(*Z, Z*)-*α*-Farnesene	-	Mild, warm, sweet	-
 (*E*)-*β*-Farnesene	1457 [167]	Mild, warm, sweet	-
 *γ*-Muurolene	1480 [167]	Weak spicy, weak herbal, woody	-
 *α*-Curcumene	1487 [167]	-	-
 *β*-Selinene	1490 [167]	-	-

Table 5.9 Continued

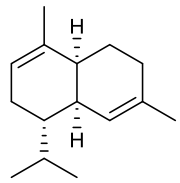
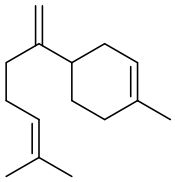
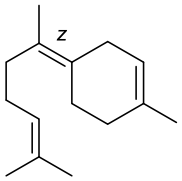
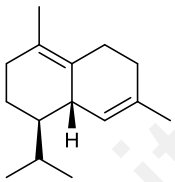
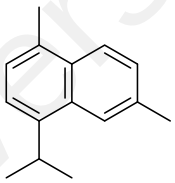
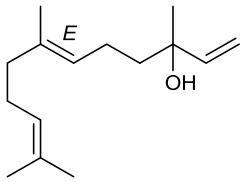
 *α*-Muurolene	1500 [167]	-	-
 *β*-Bisabolene	1506 [167]	Sweet, warm, balsamic, woody, spicy	-
 (*Z*)-*γ*-Bisabolene	1515 [167]	-	-
 (+)-*δ*-Cadinene	1523 [167]	Dry-woody, weak medicinal	-
 Cadalene	1677 [167]	-	-
Oxygenated sesquiterpene			
 (*E*)-Nerolidol	1563 [167]	Woody-floral, weak green	- insect repellent [184] - facilitates absorption of medicaments through skin - anti-fungal - anti-malaria - anti-Leishmania - anti-parasites - active against larvae of *Anisakis* genus - display pro-oxidant activity [174]

Table 5.9 Continued

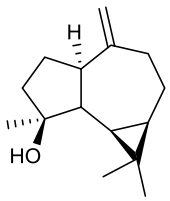
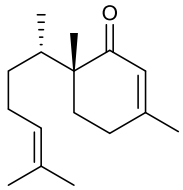
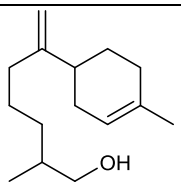
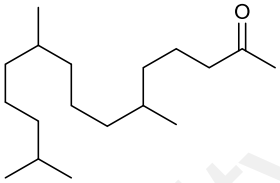
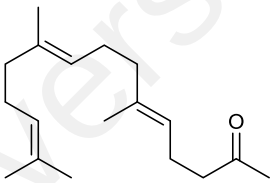
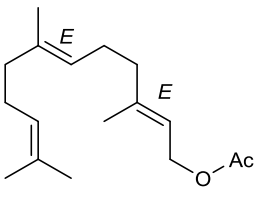
 Spathulenol	1578 [183]	Weak fruity, weak herbal	-
 6*S*, 7*R*-Bisabolone	1750 [167]	-	-
 *β*-Bisabolene-12-ol	1762 [167]	-	-
 Hexahydrofarnesyl acetone	-	-	-
 (5*E*, 9*E*)-Farnesyl acetone	1913 [167]	-	-
 (2*E*, 6*E*)-Farnesyl acetate	1726 [183]	-	-

Table 5.9 Continued

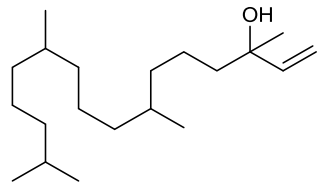
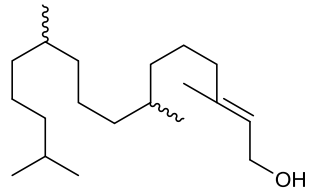
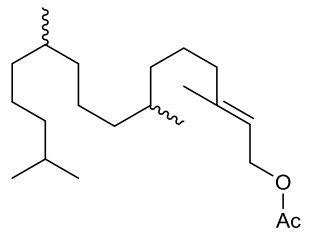
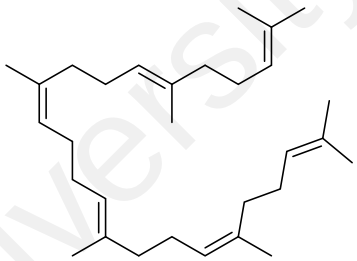
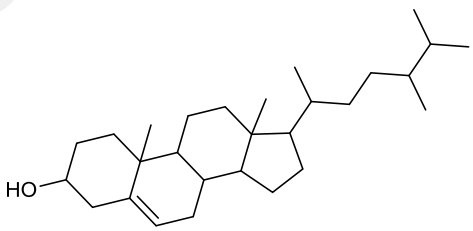
Oxygenated diterpene			
 Isophytol	1948 [183]	-	-
 (*E*)-Phytol*	1943 [167] 1942 [183] 2066 2116 [185]	-	- Anti-microbial - Anti-chistomal - Anti-cancer - Anti-inflammatory - Diuretic [181]
 (*E*)-Phytol acetate*	2218 [183]	-	-
Triterpene hydrocarbon			
 (6*E*,10*E*,14*E*,18*E*)-Squalene	2790 [186]	-	- Anti-inflammatory [143]
Other terpene			
 Campesterol	3305 [187]	-	- Anti-oxidant [144] - Anti-inflammatory [142]

Table 5.9 Continued

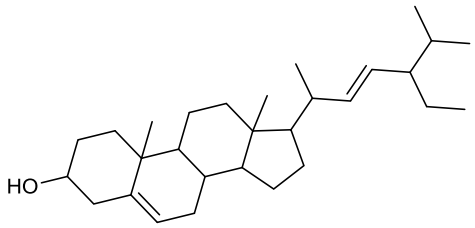
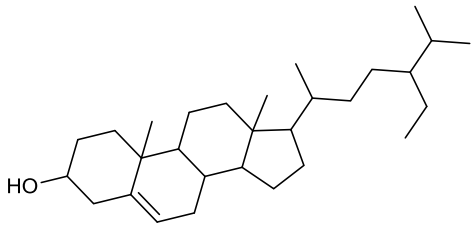
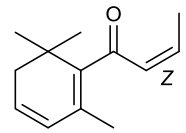
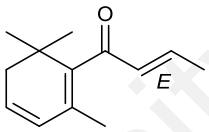
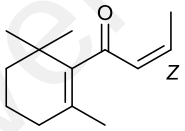
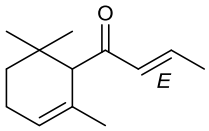
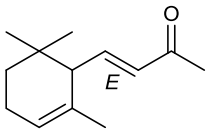
 *β*-Stigmasterol	3332 [187]	-	- Anti-oxidant [144] - Anti-inflammatory [142]
 *β*/*γ*-Sitosterol*	3408 [187]	-	- Anti-oxidant [144] - Angiogenic property [145] - Anti-inflammatory [142]
Non-terpene			
 (*Z*)-*β*-Damascenone	1364 [167]	Sweet, rosy	- Perfume [182]
 (*E*)-*β*-Damascenone	1385 [167]	Sweet, rosy	- Perfume [182]
 (*Z*)-*β*-Damascone	1388 [167]	Sweet, rosy	- Perfume [182]
 (*E*)-*α*-Damascone	1393 [167]	Sweet, rosy	- Perfume [182]
 (*E*)-*α*-Ionone	1428 [167]	Sweet, rosy	- Perfume [182]

Table 5.9 Continued

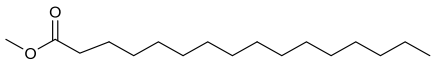
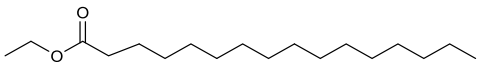
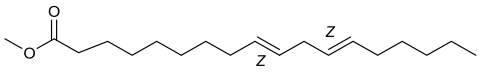
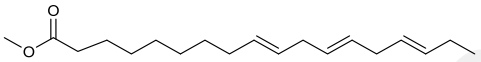
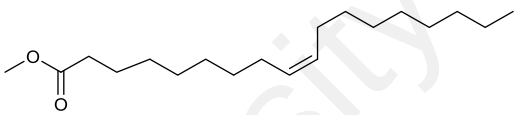
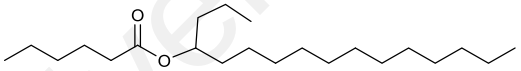
 Methyl palmitate	1922 167] 1921 [183] 1926 [188] 1928 [189]	-	- Anti-inflammatory [142, 143]
 Ethyl palmitate	1993 [167] 1991 [188]	-	-
 Methyl linoleate	2096 [167]	-	-
 Methyl linolenate	2098 [190]	-	-
 Methyl oleate	2106 [191]	-	-
 4-Hexadecyl hexanoate	-	-	-
CH_3-(-CH_2-)$_{18}$-CH_3 *n*-Docosane	2200 [167]	-	-
CH_3-(-CH_2-)$_{23}$-CH_3 *n*-Pentacosane	2500 [183]	-	-
CH_3-(-CH_2-)$_{27}$-CH_3 *n*-Nonacosane	2900 [183]	-	-
CH_3-(-CH_2-)$_{29}$-CH_3 *n*-Hentriacontane	3100 [192]	-	-
CH_3-(-CH_2-)$_{30}$-CH_3 *n*-Dotriacontane	3200 [183]	-	-

Table 5.9 Continued

CH_3-(-CH_2-)$_{31}$-CH_3 *n*-Tritriacontane	3300 [193]	-	-
CH_3-(-CH_2-)$_{33}$-CH_3 *n*-Pentatriacontane	3500 [193]	-	-

I – Kovats index from published scientific reports calculated on HP-5^{TM} or equivalent columns. *Correct isomer not identified.

5.5 Conclusion

The accumulated mass of essential oil extracted from ketum plant leaves by means of hydro distillation and supercritical CO_2 extraction, are shown in the following Table 5.10. In general, the recovery of oily extract by SFE method was higher than that of HD. Both extracts had the same light-yellow colour.

Constituents of the essential oil from the HD were found to be markedly different from the corresponding SFE extracts. The major differences between HD and SFE oils are the presence of a small percentage of oxygenated monoterpenes (10.5%) and the non-terpene compounds (5.5%) in the essential oil from HD. Even though present at low concentration, nerol, geraniol, terpineol and damascenone may justify the characteristic and sweet fresh rose fragrance of the essential oil from HD. These phytochemicals were absent in all of the CO_2 extracts. Conversely, SFE extracts were characterized by large amounts of non-terpenes, especially by methyl palmitate and methyl oleate. Additionally, the phytosterol such as campesterol, stigmasterol and sitosterol that constituted most of the SFE extract in significant amounts, were not detected in the HD essence.

This investigation highlights the advantages of the SFE technique in order to recover the most anti-inflammatory compounds like phytol, squalene, campesterol, stigmasterol, sitosterol and methyl palmitate. Compared with SFE, HD was more efficient for the

extraction of sesquiterpenoids and overall oxygenated components. The results suggested that each volatile extract would have a profit.

Table 5.10 Hydro distillation (HD) and supercritical fluid extraction (SFE) from leaves of *Mitragyna speciosa*

	Essential Oil (HD)	**M1S1**	**M1S2**	**M2S1**	**M2S2**	**M3S1**	**M3S2**
Extraction Yield (%)	0.011	0.18	0.10	0.11	0.12	0.10	0.01
Extract Colour	Light yellow	Light yellow	Light yellow	Light yellow	Light yellow	Light yellow	Light yellow
Total identified compounds (%)[a]	85.29	98.03	90.08	91.70	90.47	92.34	94.37
				Peak Area, %[a]			
Oxygenated monoterpenes	10.53	-	-	-	-	-	-
Sesquiterpene hydrocarbons	20.49	0.50	0.54	-	0.50	-	1.01
Oxygenated sesquiterpenes	15.21	1.62	2.31	4.39	1.96	5.74	2.41
Oxygenated diterpenes	33.57	16.15	22.10	12.01	21.89	4.65	15.51
Triterpene hydrocarbons	-	21.29	10.25	12.56	15.20	-	12.84
Other terpenes	-	8.80	7.36	5.44	5.16	2.38	2.01
Non-terpenes	5.49	49.70	47.54	57.30	45.76	79.57	65.40

[a] Percentage calculated by GC-FID on non-polar HP-5^{TM} capillary column.

CHAPTER 6: CONCLUSION

6.1 General Conclusion

The world is endowed with a rich heritage of medicinal plants. Medicinal plants play an important role in the lives of rural people, particularly in remote parts of developing countries with few health facilities. *Mitragyna* genus has a history of use as a medicinal plant especially in the tropical and sub-tropical areas, such as Africa, India, Sri Lanka, Bangladesh, Myanmar, Thailand and Malaysia. Pharmacological studies reported in the literature (Chapter 2) support the therapeutic value of *Mitragyna speciosa*. The plant has been studied for their vast pharmacological activities like anti-nociceptive, anti-inflammatory, anti-cancer, anti-diarrheal, anti-oxidant, anti-bacterial, anti-diabetic and so on. Then it is necessary to exploit its maximum potential in the field of medicinal and pharmaceutical sciences for novel and fruitful application.

The present results have demonstrated *M. speciosa* therapeutic effects on inflammation through its nitric oxide (NO) inhibition activity and anti-ulcerogenic property. A total of 19-selected *M. speciosa* leaves extracts, which include the hexane, ethyl acetate, methanol, aqueous and carbon dioxide crudes (15 SFE extracts from various conditions) have been investigated to elucidate the effect *M. speciosa* on the production of inflammatory mediator in macrophages. The results revealed that M5S1 crude, which was obtained via SFE technique, was effective inhibitor of LPS-induced NO in RAW 264.7 cells. M5S1 possessed the strongest activity without cytotoxic effect (NO inhibitory activity at 60.08 ± 10.02% and cell viability, 91.98 ± 5.58%). Thus, the extraction conditions of M5S1 (extraction under pure CO_2, pressure: 3000 psi, temperature: 60 ^{o}C) have been determined as the optimal SFE parameter to yield extract that possesses the highest anti-inflammatory activity without any toxicity.

Supportive results from the anti-ulcer test accentuated the anti-inflammatory property of *M. speciosa*. When orally administered to rats, in comparison to the ulcer control group, treatment with the aqueous extract (MSAE) indicates increased protection of the gastric mucosa by increased gastric wall mucus, pH level of gastric contents and SOD activity, and a decrease in MDA level. It also produced a reduction in necrotic lesion, flattening of gastric mucosa, reduction of edema and leucocyte infiltration of the sub mucosal layer. There was an observed increase in PAS staining, up-regulation of HSP70 protein and suppression of BAX protein expression. This study provides first evidence that MSAE could significantly prevent gastric ulcer. Furthermore, the aqueous extract of *M. speciosa* has shown no symptoms of toxicity or mortality during the acute toxicity study. MSAE was proven to be safe even at a concentration of 1000 mg/kg. In conclusion, MSAE was clearly demonstrated to function as an anti-ulcer agent without toxicity effect.

The chemical composition of the most active extract to inhibit the production of NO without toxicity effect, M5S1 was dominated by fatty acid, amounting to 39.01% of the total yield. This figure was largely due to palmitic acid (34.90%). Four hydrocarbons (36.67%), three phytosterols (9.32%), two esters (3.45%), two alcohols (2.30%) and a tocochromanol (2.78%) significantly constitute the rest of the extract. Palmitic acid was the major compound among the 15 identified constituents followed by heptacosane (18.56%) and nonacosane (11.00%). The anti-inflammatory potential exhibited by M5S1 is perhaps due to the high composition of palmitic acid that has been claimed to possess anti-inflammatory and anti-oxidant property by previous documented reports. Other established anti-inflammatory and anti-oxidant constituents such as *β*-sitosterol (6.67%), stigmasterol (0.99%), campesterol (1.66%) and phytosterols (2.78%), helped account for the nitric oxide inhibition activity exhibited in M5S1.

The phytochemical study on the aqueous extract of leaves of *M. speciosa* has led to isolation of five pure compounds; mitragynine, speciociliatine, paynantheine, 3, 4, 5, 6-tetradehydromitragynine and 7-hydroxyspeciociliatine. All five compounds are of the Corynanthe indole alkaloid. Mitragynine and speciocilatine are diasteromers while the rest are of their derivatives. The detailed spectroscopic discussion on each of the isolated compounds were presented in Chapter 4 (pg. 105-146)

Investigation on the volatile constituents of the leaves of *M. speciosa* has been made on the essential oil obtained through hydro distillation (HD) and extracts from SFE method (M1S1, M1S2, M2S1, M2S2, M3S1 and M3S2). Percentages of the yields of the SFE extracts were significantly higher (0.01% to 0.18%) than that of the HD (0.011%). Both extracts had the same light-yellow colour but differ in the odour. HD essential oil and SFE extracts differed in chemical composition, the former comprising mainly sesquiterpene hydrocarbons, oxygenated sesquiterpenes and oxygenated diterpenes, while triterpenes and the non-terpene compounds dominated the latter. Oxygenated monoterpenoids (10.5%) that characterized the sweet rosy odour of the HD essence were diminished in all of the SFE extracts. Whereas, phytosterol such as campesterol, stigmasterol and sitosterol that constituted most of the SFE extract in significant amounts, were not detected in the HD essence. Nevertheless, both volatile extract compositions obtained from the two processes would imply great interest in investigating their effect on human health, as a source of different bioactive compounds.

The results of this study have shown that the leaves of *M. speciosa* have demonstrated significant level of anti-inflammatory, which established the ethno pharmacological basis for its use in local traditional medicine for the treatment of inflammation caused diseases. The inference from the observed NO inhibitory activity supports the

traditional use of *M. speciosa* in the treatment of muscle pain and fever whereas the gastroprotective property support folk use for stomach disorder and wound healing.

6.2 Suggestions for Further Works

Even though in this study the aqueous extract was found to be safe in the acute toxicity test, it is worthy to note upon previous published studies that have communicated on the toxicity of various types of *M. speciosa* preparations. It is currently unclear which substances at what dose ranges may be responsible for these effect. Further studies are clearly warranted here.

This investigation highlights the advantages of the SFE technique in affording the anti-inflammatory compounds like phytol, squalene, campesterol, stigmasterol, sitosterol and methyl palmitate. Meanwhile, HD was more efficient in extracting off sesquiterpenoids and overall oxygenated components. Thus, it is commendable that further analysis should be carried out on anti-inflammatory potential of the volatile constituents.

Lastly, the regulatory law put in place to control 'ketum' addiction by banning the cultivation of the tree has not been a major success, since it is a local plant that can be grown everywhere. Instead of outright ban through legal means, a better solution would be to raise public awareness of the dangers of using medicinal plant such as ketum as recreational drug. It is hoped that drug education for the youth in areas where 'ketum' can be grown will be a more effective step towards its control. Then only, the advantages of this plant can be extensively exploited.

These present and past findings of the usefulness of the plant named *Mitragyna speciosa* or so called 'ketum', hopefully could alleviate the misconceptions of this

unfortunate beautiful plant and its true capabilities could be put into beneficial use for humanity.

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