CHAPTER 1

INTRODUCTION

1.1 GENERAL

Phytochemistry is a study of the chemical composition of plants and explanation of the various plant processes in which chemical phenomena are concerned. It is concerned with enormous variety of organic substances, their biosynthesis, turnover and metabolism, their norm distribution and their biological function [1]. Phytochemistry is also a branch of chemistry that deals with the study of plant secondary metabolites which usually occur in plants in a high structural diversity. Biochemical and physiological features of secondary metabolism are strongly correlated with its function which are secondary metabolism are not useless waste products but important means of plants for defence against herbivores, microbes (bacteria, fungi) and viruses [2]. Study about the phytochemistry involves chemistry, botany and medical aspects. In other words, botany contributes to chemistry and vice versa, and so does chemistry contributes to medicine and vice versa.

Once a new compound has been isolated from the plant material, its molecular structure and properties are determined included all aspect of chemistry from extraction and separations of plant constituents to structural elucidations using modern spectroscopic techniques. Nevertheless, chemistry contributes towards the methods of extraction and isolation and to determine the plant components qualitatively or quantitatively by providing scientific explanations of the plant responses to the surroundings. Normally, the same plant species but different locations produce different

type of compounds. Plant material contains a different group of chemical compounds, both organic and inorganic. The organic and mineral components of plants can be classified according to the species even though the compounds can be commonly found present in a great variety of different kinds of plants.

Moreover, the search for new drugs is an important way to find a better treatment for many diseases such as cancer, heart disease, AIDS, ulcer, infectious disease and others. According to Singh *et al.,* (2011), they reported that there is no specific medication that can treat critical diseases such as cancer, AIDS, HIV. If a patient has a critical illness, the choice of treatment is either to delay or prevent the disease from spreading to other areas [3]. Therefore, research of natural products should be continued as to find alternative new drugs which can treat critical illness.

As such, new drugs can be discovered in various ways, for instance, using the synthesis of combinatorial libraries of compounds, the rational synthesis of compounds based on a particular molecule target, and computer modelling design. Standard medicinal chemistry approaches and the discovery of new bioactive compounds from nature are also used to find new drugs.

So, the search for a new drug from nature is based on a biological and ecological rationale, since natural sources such as plants, microorganisms, and marine species produce bioactive compounds as their defence substances and for other purposes. For instance, plants that must coexist with animals and microorganisms have developed

defence strategies to assist in their survival in a competitive environment, and one of the common strategies is the production of toxic and other bioactive compounds.

From the previous studies and reports, natural products have provided many effective drugs. These include the old drugs such as quinine **1** [4] and morphine **2** [5] and latest drugs such as pactitaxel (Taxol) **3** [6], camptothecin **4** [7], etoposide **6** [8], mevastatin **8**, and artemisinin **9** [9]. For example, the medical plants that have valuable and clinically used as antimalarial drugs are quinine **1** and artemisinin **7** [10]. Another example, camptothecin **4** was reported since 1966 and it was isolated from *Camptotheca acuminata* [7, 11]. It was exhibited potent antiluekemic, anticancer agent for lung, colorectal, ovarian, and cervical cancers [12], antiviral (HIV and herpes) [13], antifungal [14] and antipsoriasis [13]drugs.

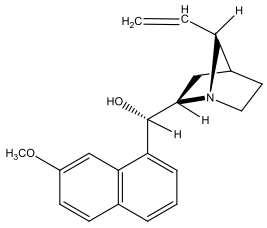
Natural products have thus played an important role in drug discovery in the past and will continue to provide more drugs in the future. This significance is supported by a report that natural compounds, their derivatives, and their analogs represent over 50% of all drugs in clinical use, with higher plant-derived natural substances representing about 25% of the total [15]. A recent review has listed 32 natural products or their analogs which are in clinical use or in clinical trials as antitumor agents over the last few years [16]. Another survey indicates that 39% of new drugs approved between 1983 and 1994 are of natural origin, consisting of original natural products, semisynthetic and synthetic drugs based on natural product models [17].

So, the examples for the semisynthetic compound were modified and gave good results for the bioactivities are two semisynthetic glycosides, etoposide **6** and teniposide **7**. These compounds are from a semi-synthetic derivative of 4'-demethylpipodophyllotoxin, a naturally occurring compound synthesized by the North American May apple (*Podophyllum peltatum*) [18] and the Indian species *Podophyllum emodi* Wallich [19]. The reason to do modification of podophyllotoxin **5**, as it has anticancer activity but has more side effects. The modification of podophyllotoxin **5** at various sites are due to make different mode of actions. So, glycosidic derivatives of podophyllotoxin reduces the side effect. As such, modifications at the C-4 position in C ring are mostly acceptable and bulky groups at this position enhance anticancer and topoisomerase activities [20]. Podophyllotoxin **5** have been widely used in traditional herbal medicine of many diverse cultures such as remedies for purgative, snakebites, periodontitis, skin disorders, coughs, various intestinal worm disease, veneral wart condyloma acuminatum, lymphadenopathy and certain tumours [20]. But its glycosidic derivatives; etoposide **6** and teniposide **7** showed bioactivities in several human neoplasms, including leukemia, testicular tumor, Hodgkin's disease and large cell lymphomas [21]. They form a ternary DNA breaks, leading to cell death.

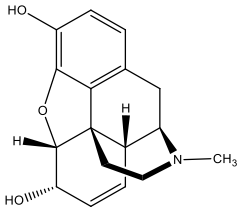
Several compounds containing unique structural composition have been isolated and characterised from natural sources. The discovery of novel drugs from nature is also important because many isolated molecules are unique complex, and would not be obtained by simple synthetic approach. These natural products have exhibited promising bioactivities *in vitro* and *in vivo*. However, limitations such as toxicity, low

bioavailability and poor solubility have restricted the use of several natural products in humans.

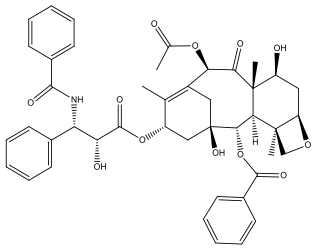
As for the initial discovery of one new drug, sometimes need more than even twenty years. For example, pactitaxel (Taxol) **3**, Wall and Wani (1971) had discovered this drug more than twenty-five years ago. Pactitaxel (Taxol) **3** is a novel natural product with excellent activity in a number of animal models and has reached the stage where the pactitaxel (Taxol) **3** is now available in adequate quantity for therapeutic use [6]. Therefore, in conclusion, there are other highly active natural products from plant, marine, and fungal sources as yet unknown which, when discovered, will have therapeutic utility. Disease is not one, but several hundred diseases and will require many different types of agents.



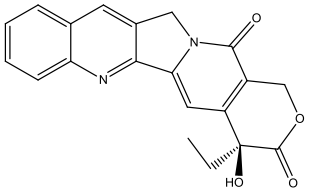
quinine **1**



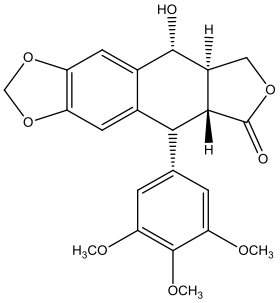
morphine **2**



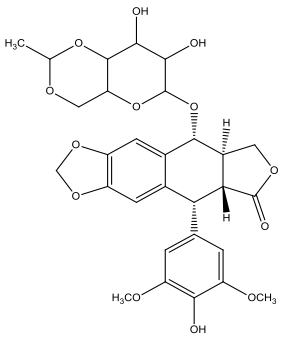
paclitaxel (Taxol) **3**



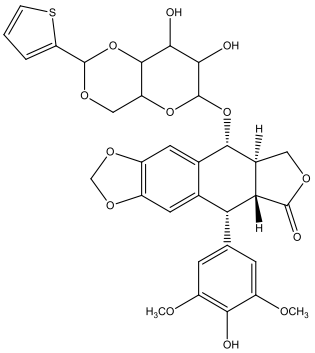
camptothecin **4**



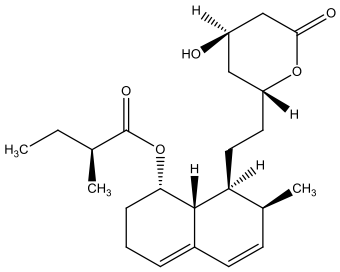
podophyllotoxin **5**



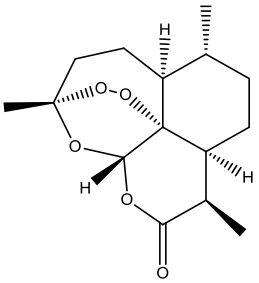
etoposide **6**



teniposide **7**



mevastatin **8**

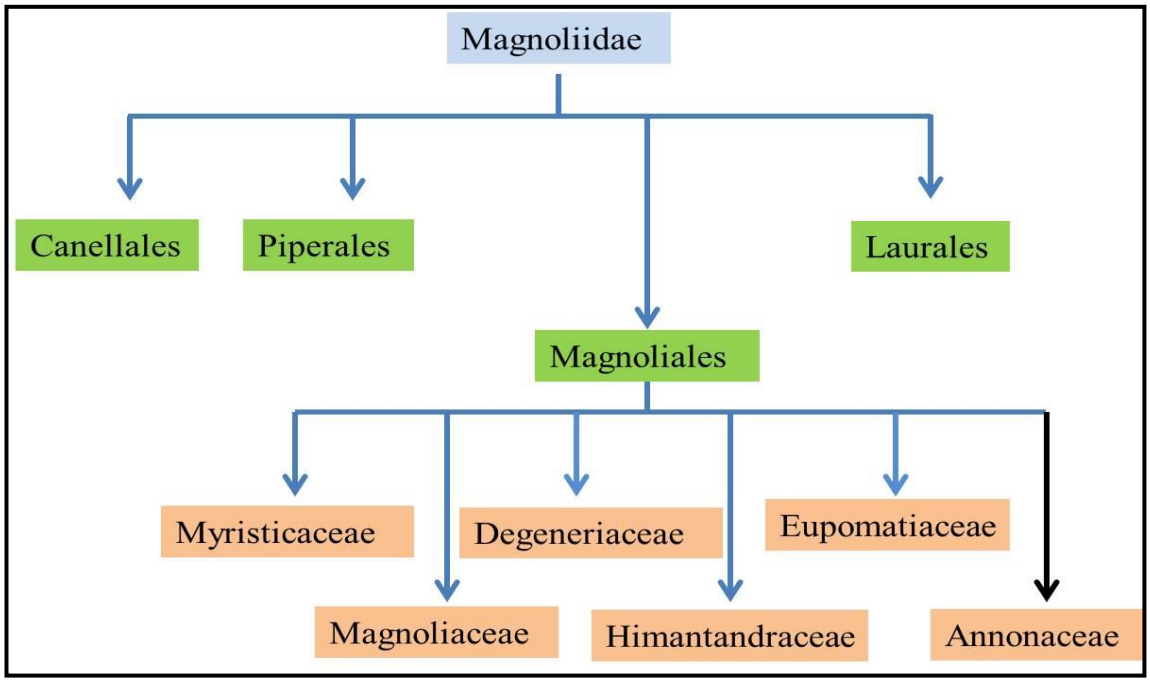


artemisinin **9**

1.2 ANNONACEAE: DISTRIBUTION AND HABITAT

The order Magnoliales consist of six families (Annonaceae, Degeneriaceae, Eupomatiaceae, Himantandraceae, Magnoliaceae and Myristicaceae), approximately 154 genera, and about more than 3,000 species [22]. Among all these families, the Annonaceae and Magnoliaceae have been the most studied. The Annonaceae also called the custard apple family is the largest family in the Magnoliales order, with about 135 genera and 2,500 species. The family is mainly concentrated in the tropical zones, with few species found in temperate regions [23]. Scheme 1.1 showed the order of Manoliales.

One of the most important reasons for focusing on Annonaceae is that there are different categories of chemicals such as alkaloids, non-alkaloids constituents, and acetogenins, which have displayed a broad array of pharmacological effect [24, 25]. It is because Annonaceae family is rich in compounds with unique structural features. This family has attracted the attention of scientists world-wide because the compounds produced from these plants possess medical values that can benefit the human life. Extracts from these species are known to be widely used in traditional medicine and previous studies have demonstrated their anticancer, anti-microbial and other pharmacological activities.



Scheme 1.1: The order of Magnoliidae

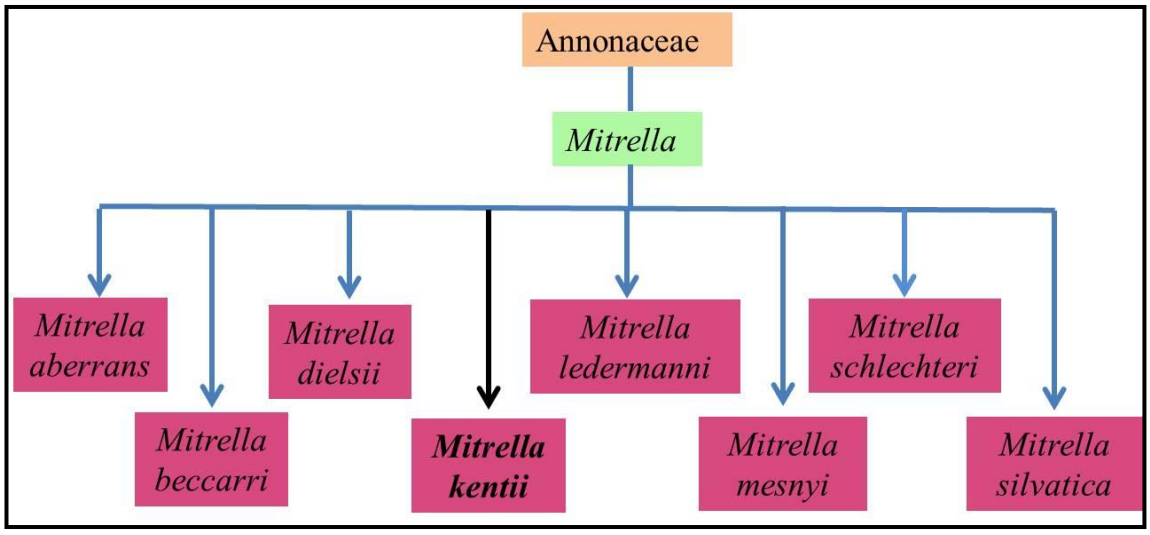
1.3 BOTANICAL ASPECT OF ANNONACEAE

Annonaceae family is evergreen or deciduous trees, shrubs, subshrubs and lianes. It is stipules absent and indumentum of simple, stellate or lepidote hirs. The leaves are simple, alternate, entire, typically distichous and often flexuose and with conspicuous oil glands. The flowers are bisexual, rarely unisexual, hypogynous. Annonaceae is pantropical family with 120-130 genera and 2,100-2,300 species. Some species of this family, which is often referred to as the custard Apple family provide edible fruits which are also used in medicinal applications, or cultivated as ornamentals. Members include Custard and Sugar apples (*Annona reticulata, A. squamosa*), Soursop (*A. muricata*), Biriba (*Rollinia mucosa*) and Calabash Nutmeg (*Monodora myristica*), the latter used for flavouring foods and for relieving headaches and toothaches.

1.4 CLASSIFICATION OF GENUS

1.4.1 Genus *Mitrella*

Mitrella is a genus of plants in the Annonaceae family. It has climbing shrubs, indumentum of simple hairs. It is also in florescences axillary, fasiculate or solitary. The flowers are bisexual. Genus recorded from the Malaysian Peninsula to New Guine and Australia and containing six or seven species. Genus *Mitrella* contains 8 species but only *Mitrella kentii* was studied for their phytochemistry. Scheme 1.2 showed the species in *Mitrella*.



Scheme 1.2: Species in Genus *Mitrella*

1.4.2 *Mitrella kentii* (Bl) Miq (KL 4139)

Mitrella kentii (Bl) Miq. (KL 4139) was collected at Mersing, Johor. It is a tree-climbing liana belonging to the custard apple family found in the Malaysian Peninsula and Islands of Sumatra, Borneo, and New Guinea [26]. Its synonyms are *Melodorum pisocarpum* Hk. f. et Thoms, and *Melodorum elegans* Hk. f. et Thoms, while the common (Indonesian) name is 'kiawi'. The plant is found in the tropics, especially in the Asia-Pacific regions. It is used as a drink in the form of the roots of *Mitrella kentii* (Bl.) Miq decoction to treat fever in Malaysia [27].

1.4.3 Physical Description of *Mitrella kentii* (Bl) Miq

It is a woody climber of peat swamp forest, coastal beach and seashores of the Asia-Pacific. The pedicels, calyx, outer petals and young leaves are covered with a golden tomentum. The leaves are simple, alternate and exstipulate. The petiole is 1cm long. The blade is oblong-elliptic, leathery, dark green above and grey green below. The

flowers are axillary. The calyx consists of 3-angled connate cups, and the petals are ovate, valvate, small, the inner ones being smaller. The fruits are dull-orange ripe carpels. Scheme 1.3 showed the example of *Mitrella kentii*.

Scheme 1.3: Example of *Mitrella kentii* (Bl) Miq (KL 4139)

1.5 OBJECTIVES OF THE STUDY

1. To extract and isolate the phytochemical compounds from *Mitrella kentii* (Bl.) Miquel (Annonaceae).

2. To elucidate the structure of the isolated compounds by using spectroscopic technique such as UV, IR and MS, 1D-NMR, (^{1}H, ^{13}C, DEPT), 2D-NMR, (COSY, HSQC, HMBC).

3. To classify the types of compounds isolated from *Mitrella kentii* (Bl.) Miquel.

4. To determine biological activities on the major compounds; anti-oxidant and anti-ulcer activities.

CHAPTER 2

GENERAL CHEMICAL ASPECTS AND BIOLOGICAL ACTIVITIES

2.1 GENERAL

Annonaceae is a tropical family of trees, shrubs and lianas belong to Magnoliales. It is a diverse family comprising of 135 genera and ca. 2500 species [23]. Annonaceae is almost entirely tropical, with three main centres of distribution of South-East Asia, Australia and the Pacific islands are the richest in genera (ca. 60) and species (ca. 1100). Annonaceae is also the one of the most exciting families of medicinal plants starting with prospect of the flora of the Asia-Pacific for drugs [28]. In the Asia-Pacific, approximately 50 species from this family have medicinal values, but not for clinical uses. Since from that, some evidence had been presented that this family have potential for the treatment of cancer, bacterial infection, hypertension and brain dysfunction [29].

Plants produce a variety of organic compounds as a response to environmental stresses like microbial attack, insect or animal predation and ultraviolet radiations. The role of the metabolites is to increase plants resistance to these stresses. They can be classified into four major groups according to bisynthetic route and structural features which are acetogenins, alkaloids, terpenoids and phenolic compounds [30]. From the previous phytochemical studies on the Annonaceae species, it has been reported that several plants from this species have different sources of the secondary metabolites which is very important for the pharmacological activities such as cytotoxic (flavanones), antifungal (terpenes), anti-microbial (alkaloids) and antitumor (diterpenes, alkaloids and acetogenins) [31-33].

2.2 ALKALOIDS

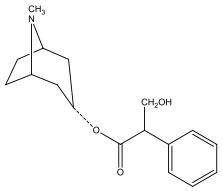
The alkaloids represent a very extensive group of secondary metabolites which are diverse structures, distribution in nature and important for the biological activities. Alkaloids is generally defined as nitrogen-containing natural molecules which are abundant as secondary metabolites in plants and represent one of the most widespread classes of compounds and has varied pharmacological properties. Among alkaloids, the aporphinoid groups constitute a subgroup of benzylisoquinoline compounds with more than 500 alkaloids were isolated until now.

The majority of alkaloids found were derived from amino acids such as tyrosine, phenylalanine and anthranilic acid. However, alkaloids may be derived from other precursors such as purines in case of caffeine, terpenoids, which become "aminated" after the main skeleton has been synthesized, for example aconitine or the steroidal alkaloids. Alkaloids may also form from acetate-derived polyketides where the amino nitrogen is introduced as the hemlock alkaloid such as coiine. Depending on the ring of the structures, alkaloids are subdivided into pyrrolidine, piperidine, pyrrolizidine, quinolizidine, isoquinoline, protoberberine, aporphine, morphinane, quinoline, aridone, indole, monoterpene indole, diterpene or steroid alkaloids [2]. Most alkaloids are classified according to the nitrogen-containing in the ring system. There are 170 alkaloids of the types isoquinoline, protoberberine, aporphines and others are structurally similar to each other which were isolated from different genera of Annonaceae [34]. Alkaloids are one of the most important classes of natural products in drug development. Alkaloids are the physiologically-active nitrogenous bases derived from biogenetic precursors. A number of alkaloids are well known because of their

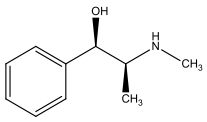
toxicity or use as psychedelic drugs such as cocaine, morphine **2** or the semisynthetic LSD.

2.2.1 Classification of Alkaloids

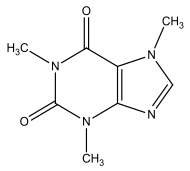
1. True (Typical) alkaloids that are derived from amino acids and have nitrogen in a heterocyclic ring. e.g: Atropine **10**
2. Protoalkaloids that are derived from amino acids and do not have nitrogen in a heterocyclic ring. e.g: Ephedrine **11**
3. Pseudo alkaloids that are not derived from amino acids but have nitrogen in a heterocyclic ring. e.g: Caffeine **12**
4. False alkaloids are non-alkaloids give false positive reaction with alkaloidal reagents.



atropine **10**



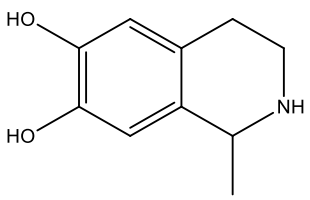
ephedrine **11**



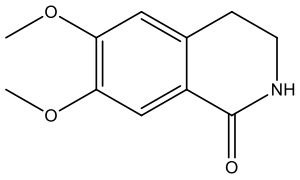
caffeine **12**

2.2.2 Simple isoquinolines

The isoquinolines are the largest groups of alkaloids. The isoquinoline skeleton is a basic building block of various types of alkaloids including benzylisoquinoline. The most simple isoquinolines were isolated are salsolinol **13** [35] from the leaf and stem of *Annona reticulata* and corydaldine **14** from the stem bark of *Enantia polycarpa* [36].



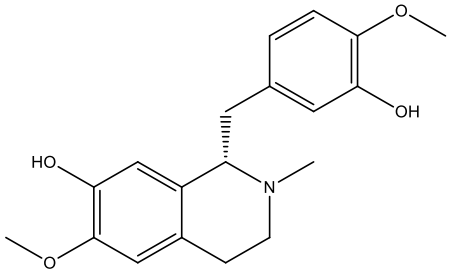
salsolinol **13**



corydaldine **14**

2.2.3 Tetrahydrobenzylisoquinolines

Most of tetrahydrobenzylisoquinoline alkaloids were isolated from some species of the genera *Annona* and *Xylopia*. One of the compound is reticuline **15**, which can reduce heart rate and blood pressure in rats, inhibits phenylephrine- and KCl-elicited contractions of rat aortic rings and antagonizes Ca^{2+}- induced contractions [37, 38].



reticuline **15**

2.2.4 Aporphine Alkaloids

Aporphine is one of a class of isoquinoline alkaloids widely distributed in the plants of Annonaceae family. The aporphine alkaloids are the largest group for the present time and about 50 alkaloids were isolated from plants of 20 families. Mostly, plants from the families of Annonaceae and Lauraceae are rich in the aporphine alkaloids. In addition, aporphine alkaloids are mostly discovered from Annonaceae

family other than Lauraceae family. Besides that, they possess various pharmacological activities such as antiplatelet, antitumor, cytotoxic and antibacterial activities. All aporphine alkaloids are based on the skeleton I [39] and consist of di-, tri-, tetra-, penta-, and hexasubstituted derivatives, the substituent being hydroxyl, methoxyl, or methylenedioxyl groups, sugar residues and others [40]. Figure 2.2.1 illustrate the general structure of tetrahydroaporphine alkaloids [41, 42].

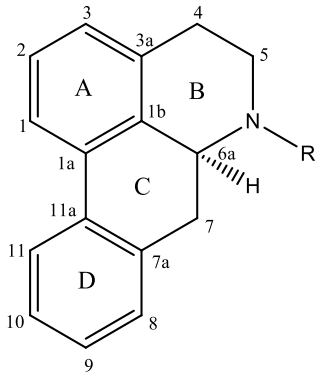
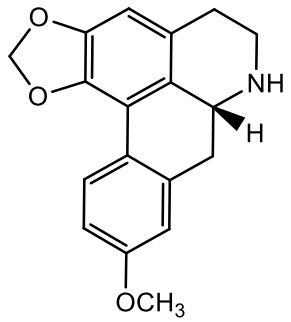
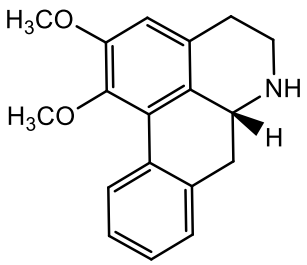


Figure 2.2.1: Skeleton I: General structure of tetrahydroaporphine alkaloids

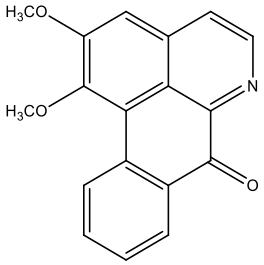
Some of aporphine alkaloids isolated from the leaves of *Guatteria amplifolia,* were xylopine **16**, nornuciferine **17**, lysicamine **18** and laudanosine **19**. Xylopine **16** showed significant activity (LD_{50}=3μM) against *Leishmania mexicana* and *L. panamensis.*



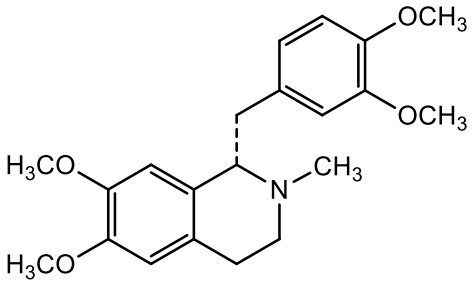
xylopine **16**



nornuciferine **17**



lysicamine **18**

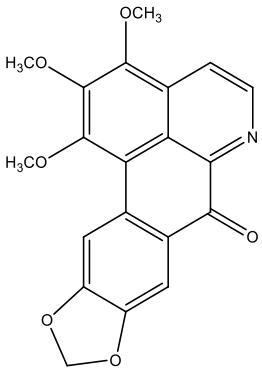


laudanosine **19**

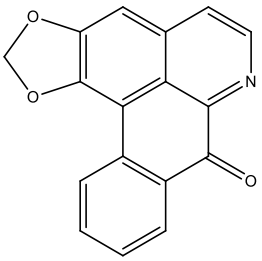
2.2.5 Oxoaporphine Alkaloids

Oxoaporhine alkaloids represent another subclass of aporphine alkaloids peculiar to the Annonaceae family and a few other alkaloid-bearing plant families. The oxoaporphine alkaloids such as oxophoebine **20** and liriodenine **21** have been isolated from *Xylopia aethiopica* (Annonaceae). Both showed selective toxicity against DNA repair and recombination deficient mutants of the yeast *Saccharomyces cerevisae*. Three related compounds isolated from this plant are inactive and they are oxoglaucine **22**, *O*-methylmoschatoline **23**, and lysicamine **18** [5]. The selective toxicity against DNA repair and recombination deficient mutants of the yeast *Saccharomyces cerevisae* was observed to 10-methoxyliriodenine (lauterine) **24** and 10-hydroxyliriodenine **25** which

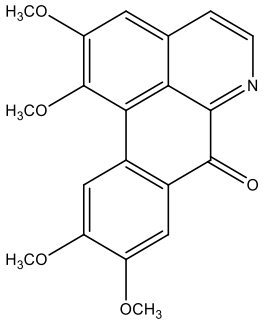
are novel oxoaporphines. These two oxoaporphine alkaloids were isolated from *Miliusa cf. banacea* (Annonaceae). The role of the bioactive oxoaporphine alkaloids as DNA topoisomerase inhibitors were also determined [43].



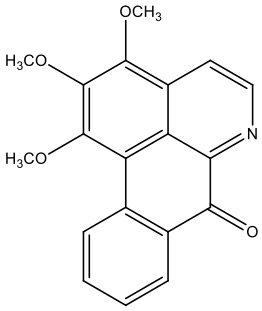
oxophoebine **20**



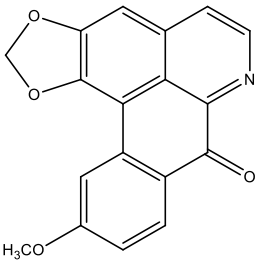
liriodenine **21**



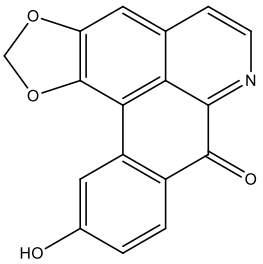
oxoglaucine **22**



O-Methylmoschatoline **23**



lauterine **24**



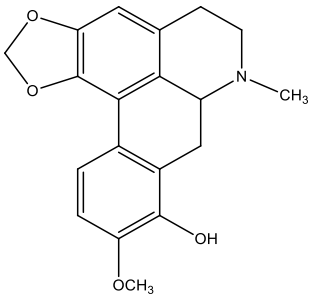
10-Hydroxyliriodenine **25**

2.2.6 Example of alkaloids extracted from Annonaceae species

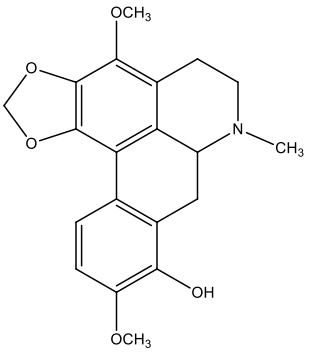
2.2.6.1 Aporphine Alkaloids of *Fissistigma poilanei*

Four new aporphine alkaloids were isolated from *Fissistigma poilanei* species and these are 8-hydroxy-9-methoxy-1,2-methylenedioxyaporphine **26** and 8-hydroxy-3,9-dimethoxy-1,2-methylenedioxyaporphine **27**. These alkaloids exhibited a moderate cytotoxic activity against four human cancer cell lines (KB, Hep-G2, MCF-7, LU) as

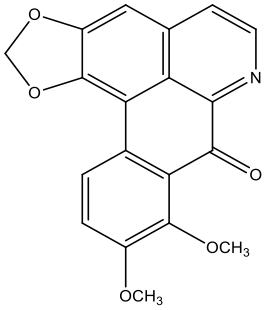
well as antimicrobial activity against *Lactobacillus fermentum*, *Enterococcus feacium*, *Staphylococcus aureus* and *Bacillus subtillis*. Another two known aporphine alkaloids compounds are oxocrebanine **28** and kuafumine **29**.



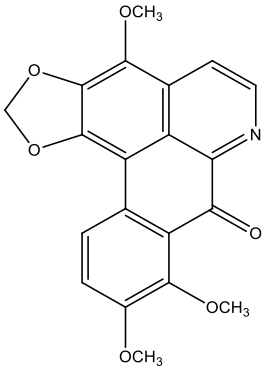
8-hydroxy-9-methoxy-
1,2-methylenedioxyaporphine **26**



8-hydroxy-3,9-dimethoxy-
1,2-methylenedioxyaporphine **27**



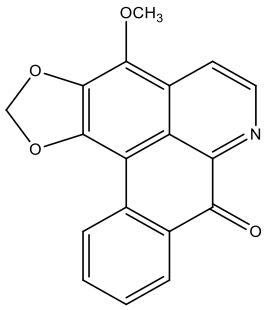
oxocrebanine **28**



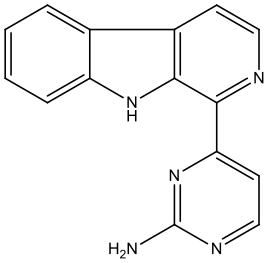
kuafumine **29**

2.2.6.2 Oxoaporphine and pyrimidine-β-Carboline Alkaloids of *Annona foetida*

Four alkaloids isolated from *Annona foetida* in which three are oxoaporhine alkaloids; atherospermidine **30**, liriodenine **21**, *O*-methylmoschatoline **23** and one is indole alkaloids, annomontine **31**. These compounds showed potent trypanocidal effect when evaluated against epimastogote and trypomastigote from *Trypanosoma cruzi*.



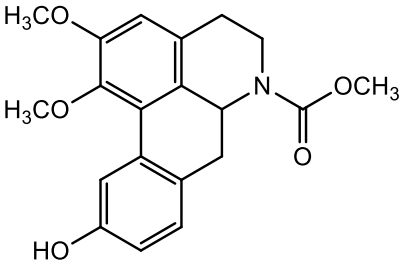
atherospermidine **30**



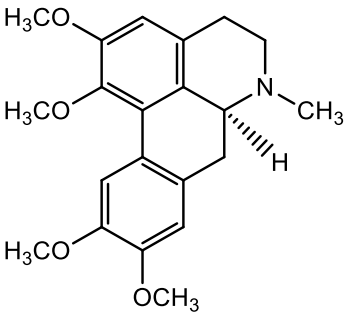
annomontine **31**

2.2.6.3 Isoquinoline Alkaloids from *Rollinia mucosa*

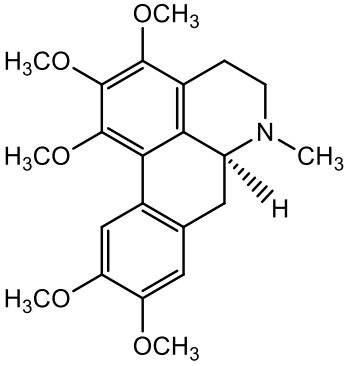
The fresh unripe fruit of *Rollinia mucosa* afforded one novel aporphinoid alkaloid, romucosine **32** and twelve known compounds including eight isoquinoline alkaloids; anonaine **33**, glaucine **34**, purpureine **35**, liriodenine **21**, oxoglaucine **36**, oxopurpureine **37**, berberine **38** and tetrahydroberberine **39** [44]. In this study liriodenine **21** might act as a muscarinic receptor antagonist in isolated trachea ileum and cardiac tissue of guinea-pigs and as a selective M3 receptor antagonist in canine tracheal smooth muscle [45].



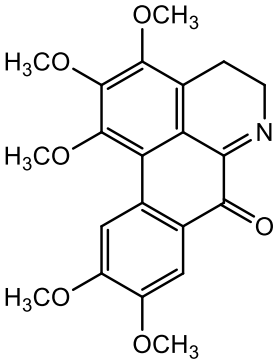
romucosine **32**



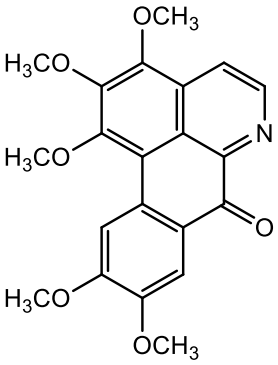
glaucine **34**



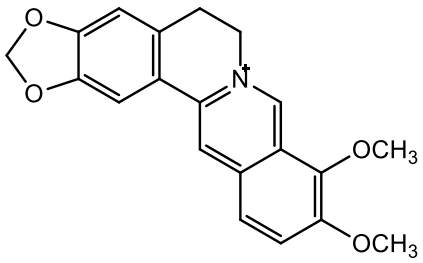
purpureine **35**



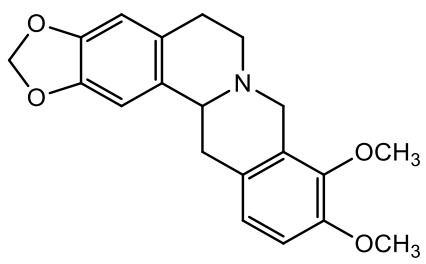
oxoglaucine **36**



oxopurpureine **37**



berberine **38**



tetrahydroberberine **39**

2.3 TERPENOIDS

The terpenoids constitute the largest and have varied classes of natural products. Terpenoids are volatile substances which give fragrance to the plants and flowers. They occur widely in the leaves and fruits as pigments, hormones and signalling molecules. It also serves a wide range of biological functions such as antibiotics, anti-feedants or pollinator attractants. Terpenoids are found abundance in higher plants. The terpenoids content of the plant can be used as a taxonomic tool upon increasing isolation of the compounds. For example the terpenoids; limonoids, quassinoids and ridoids have been particularly used in the field of chemosystematics.

2.3.1 Classification of Terpenoids

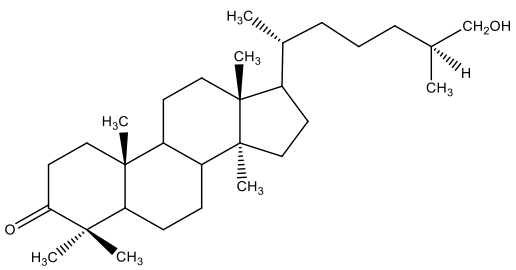
The natural terpenoid hydrocarbons have the general formula $(C_5H_8)_n$. They can be classified on the values of n or number of carbon atoms present in the structure. Table 2.3.1 showed the classification of terpenoid compounds.

Table 2.3.1: Classification of Terpenoids

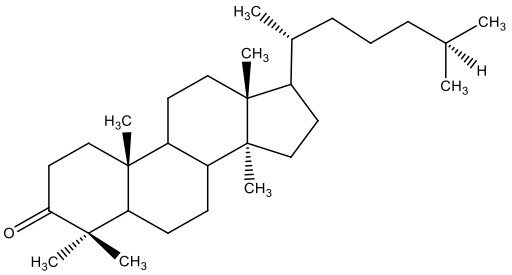
Number of carbon atoms	Value of n	Class
10	2	Monoterpenoids ($C_{10}H_{16}$)
15	3	Sesquiterpenoids ($C_{15}H_{24}$)
20	4	Diterpenoids ($C_{20}H_{32}$)
25	5	Sesterpenoids ($C_{25}H_{40}$)
30	6	Triterpenoids ($C_{30}H_{48}$)
40	8	Tetraterpenoids ($C_{40}H_{64}$)
>40	>8	Polyterpenoids $(C_5H_8)_n$

2.3.2 Example of Terpenoids from *Mangifera indica*

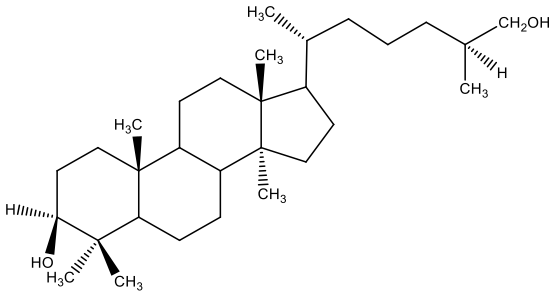
Two new triterpenoids, 25(*R*)-3-oxo-24-methylene cycloartan-26-ol **37** and taraxastanonol **42** have been isolated from the neutral fraction n-hexane extract of the stem bark of *Mangifera indica*. The acidic fraction of the same extract has yielded three new tetracyclic triterpenoids, 3-oxo-23(*R*or*S*)-hydroxy cycloart-24-en-26-oic acid **49**, 3β,23-dihydroxy cycloart-24-en-26-oic acid **51** and 23-epimeric of 3β,23-dihydroxy cycloart-24-en-26-oic acid **52**. Besides that, several known triterpenoids compounds were isolated.



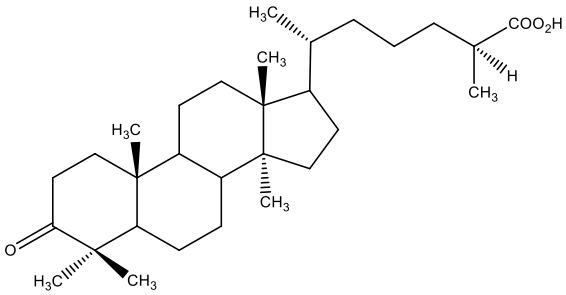
25(*R*)-3-oxo-24-methylenecycloartan-26-ol **40**



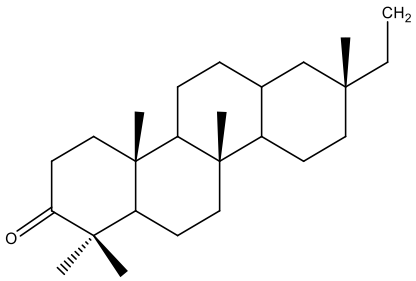
24-methyl-enecycloartanone **41**



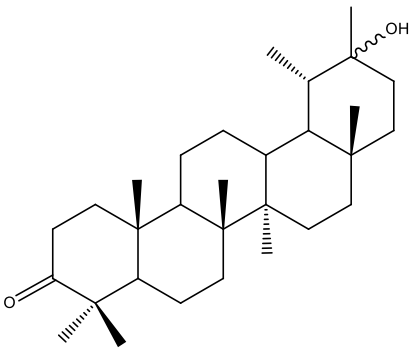
24-methylenecycloartane-3β,26-diol **42**



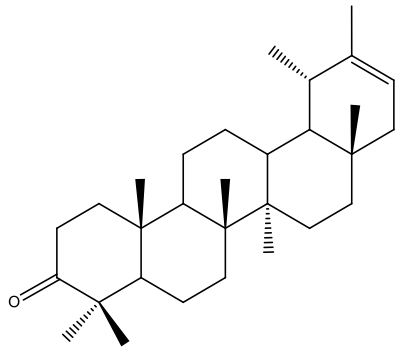
ambonic acid **43**



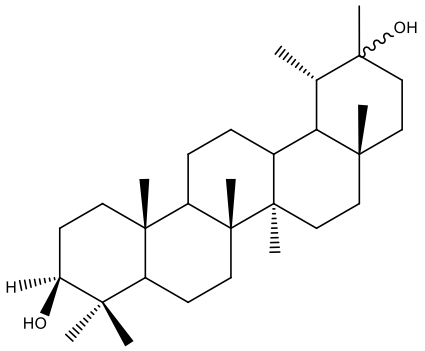
taraxastanone **44**



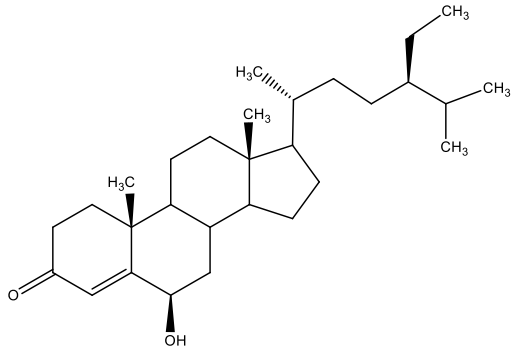
taraxastanonol **45**



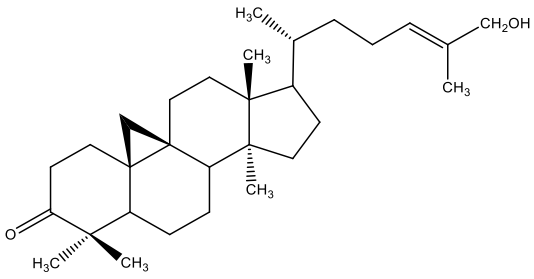
taraxastenone **46**



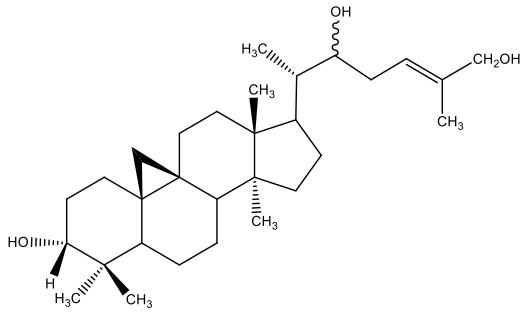
taraxastane-3β, 20-diol **47**



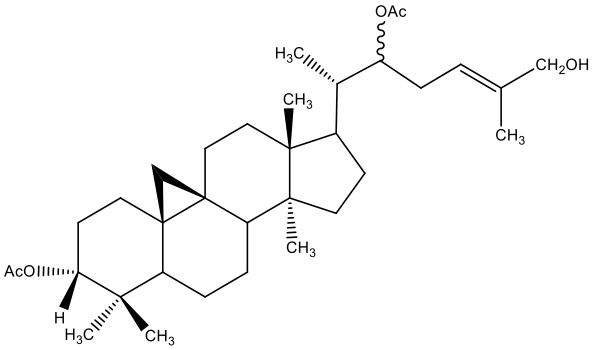
6β-hydroxy stigmast-4-en-3-one **48**



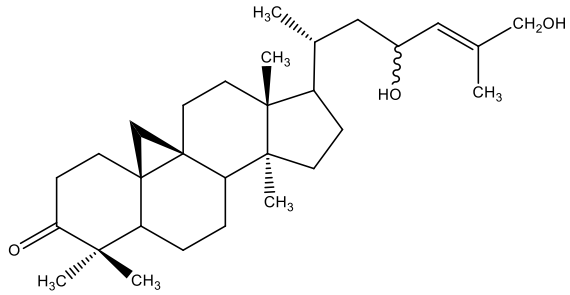
mangiferonic acid **49**



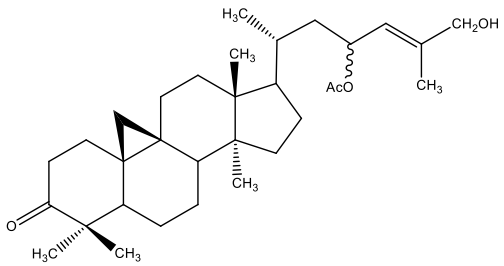
3α,22(*R*or*S*)-dihydroxy cycloart-24-en-26-oic acid **50**



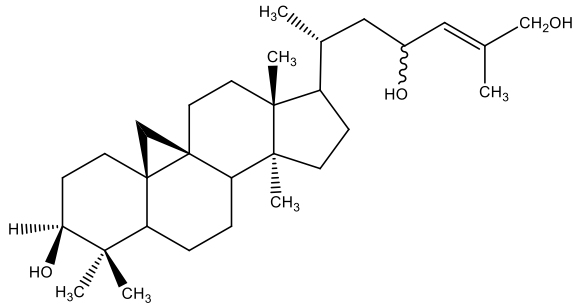
3α,22(*R*or*S*)-acetyl oxide cycloart-24-en-26-oic acid **51**



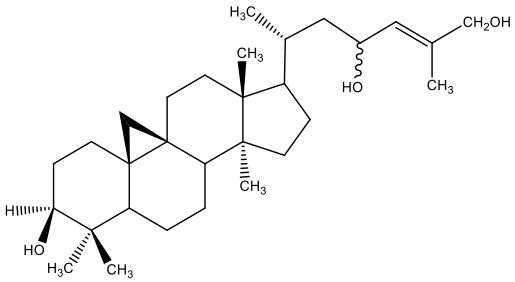
3-oxo-23(*R*or*S*)-dihydroxy cycloart-24-en-26-oic acid **52**



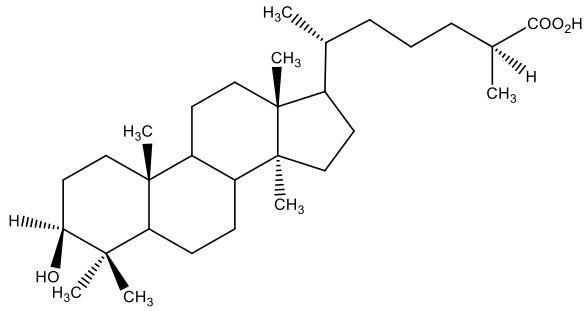
3-oxo-23(*R*or*S*)-acetyl oxide cycloart-24-en-26-oic acid **53**



3β,23(*R*or*S*)-dihydroxy cycloart-24-en-26-oic acid **54**



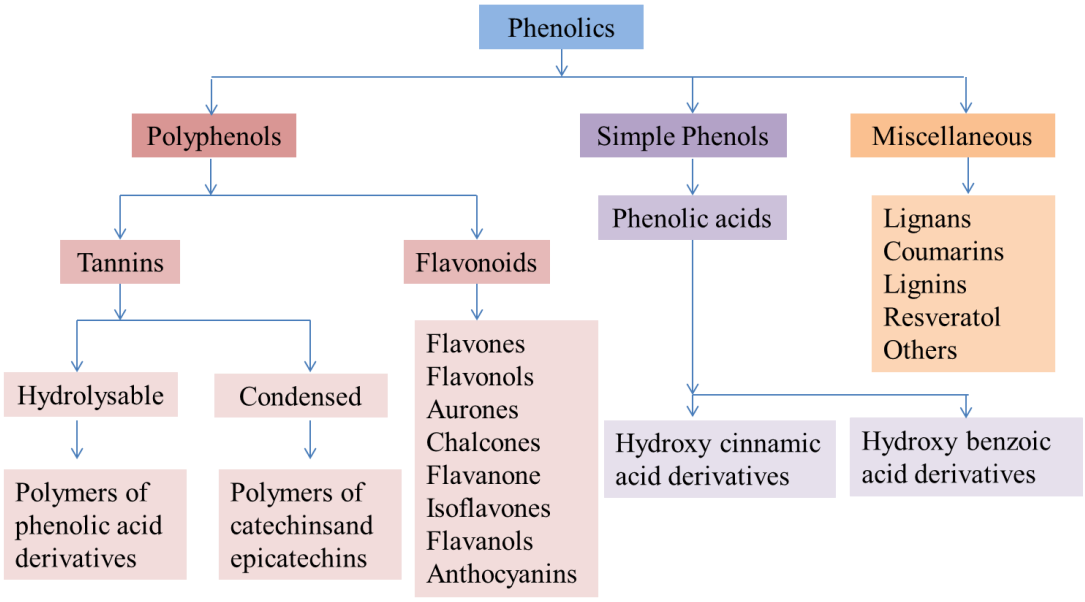
23-epimeric 3β,23(*R*or*S*)-dihydroxy cycloart-24-en-26-oic acid **55**



ambolic acid **56**

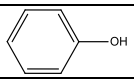
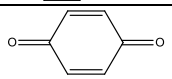
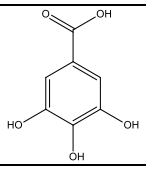
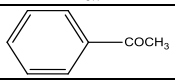
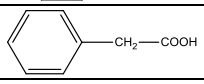
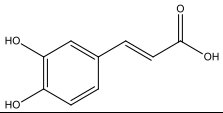
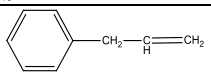
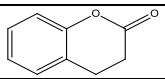
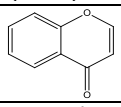
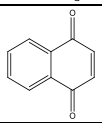
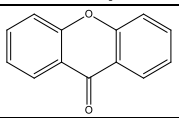
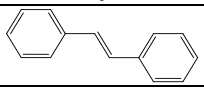
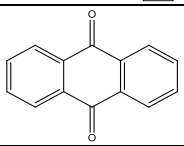
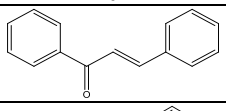
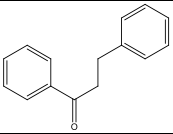
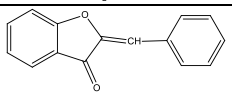
2.4 PHENOLIC COMPOUNDS

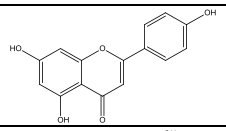
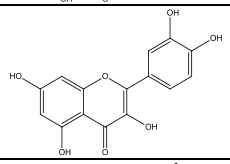
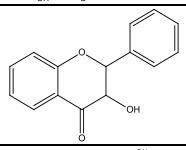
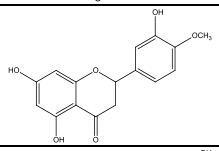
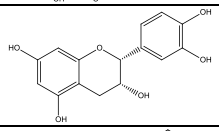
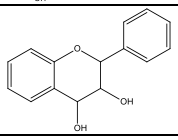
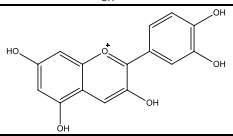
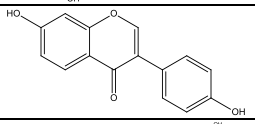
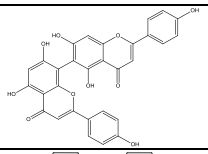
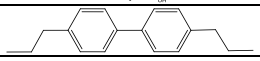
The four major secondary metabolites in plants are terpenoids, alkaloids, sulphur-containing compounds and phenolic compounds. The phenolic phytochemicals have unique areas in the natural products due to their distribution throughout the plant kingdom and in products such as fruits, vegetables, beverages, herbs, cosmetics and nutraceuticals and also consumed by the general population [46]. The phenolic compound family is huge and comprises a complex group of compounds. About 8,000 phenolics with wide structural diversity and polarities have been isolated from plants [47]. Phenolics can be chemically grouped into three broad categories: Polyphenols (tannins and flavonoids), simple phenols (phenolics acid) and a miscellaneous group. Scheme 2.4.1 showed classification of the phenolic groups [48]. Phenolic compounds can be divided into several classes depending on the structure of the aglycone in Table 2.4.



Scheme 2.4.1: General classification of plant phenolics, modified from [48]

Table 2.4: Classification of phenolic compounds

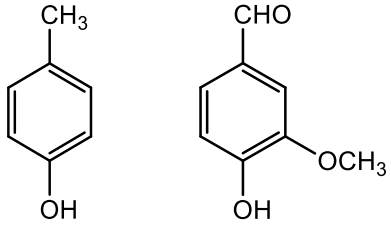
Basic Skeleton	Class	Basic structure	Examples
C_6	Simple phenols		Phenol, cresol, resorcinol
	Benzoquinone		Benzoquinone
C_6-C_1	Hydroxybenzoic acids Condensed tannins		Gallic acid, vanillic acid Gallotannins, ellagitannins
C_6-C_2	Acetophenones		Annphenone
	Phenyl acetic acids		*p*-Hydroxypheylacetic acid
C_6-C_3	Hydroxycinnamic acid		Caffeic acid, ferulic acid
	Phenylpropenes		Eugenol, myristicin
	Coumarins,		Umbilliferone, scopoletin
	Chromones		Eugenin
C_6-C_4	Naphthoquinones		Juglone
C_6-C_1-C_6	Xanthones		Mangostin, mangiferin
C_6-C_2-C_6	Stilbenes		Resveratrol
	Anthraquinones		Emodin
C_6-C_3-C_6	Chalcones		Phloridzin, arbutin
	Dihydrochalcones		Phloretin
	Aurones		Sulferetol

	Flavones		Apigenin, luteolin
	Flavonols		Quercetin, myricetin
	Dihydroflavonol		Taxifolin
	Flavanones		Hesperitin, naringenin
	Flavanol		(epi)Catechin
	Flavandiol		(+)-Leucocyanidin
	Anthocyanidins		Cyanidin, pelargonidin
	Isoflavonoids		Daidzein, genistein
$(C_6\text{-}C_3\text{-}C_6)_2$	Biflavonoids		Agathisflavone
$(C_6\text{-}C_3)_2$	Lignans, neolignans		Procyanidins

2.4.1 Definition of Phenolic Compounds

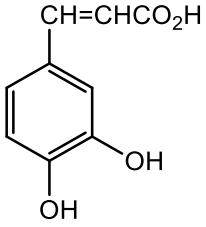
Phenolics are defined as a class of polyphenols which are important secondary metabolites present in plants [49] and also responsible for their antioxidant action and various beneficial effects in a multitude of diseases [50, 51]. Scheme 2.4.2 showed biosynthesis of phenolic compounds. The phenolic compounds, include simple phenols

(C_6) (e.g. cresol **57** and vanillin **58**), phenolic acids (e.g. gallic acid **59**), hydroxycinnamic acid derivatives (e.g. caffeic **60** and ferulic acid **61** derivatives). The more complex and diverse polyphenolic flavonoids occurring widely in food plants are bioactive compounds [52].

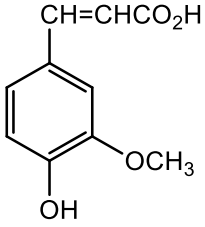


cresol **57**

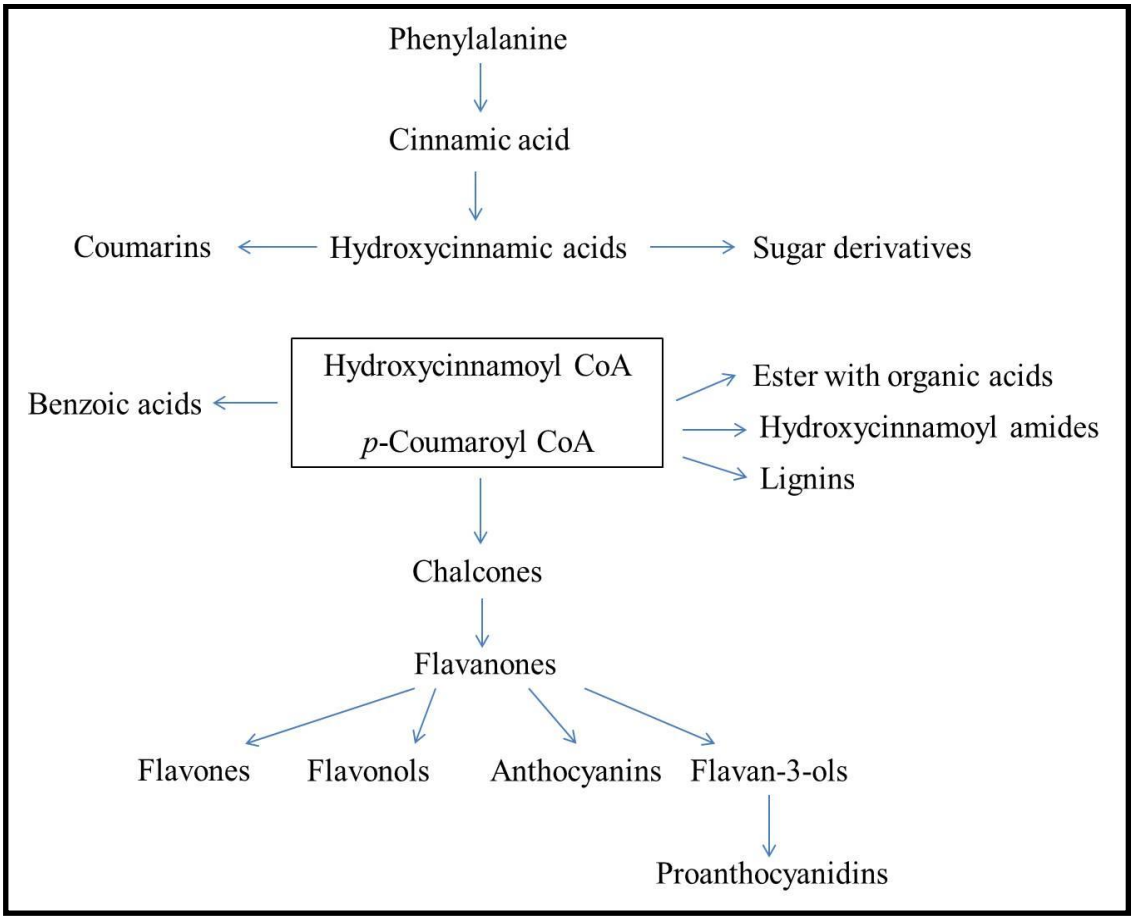
vanillin **58**



caffeic acid **60**



ferulic acid **61**



Scheme 2.4.2: Phenolic compounds biosynthesis[53].

2.4.2 Flavonoids

Flavonoids are a group of phenolic compounds which are widely distributed throughout the plant kingdom. Flavonoids are also a diverse group from natural products with encompassing more than 5000 compounds. Basically, flavonoids contain a common phenylchromanone structure (C_6-C_3-C_6) with at least one hydroxyl group substituent or a hydroxyl derivative such as a methoxy group. Flavonoids are classified based on the level of oxidation and substitution pattern of the C-ring, whereas members

within a class differ in the pattern of substitution on the A-and B-rings. Plants often synthesize O-linked or C-linked glycosides compounds [54].

Flavonoid is generally named based on the phytochemicals of 15 carbon (C6-C3-C6) skeleton and over 4,500 different flavonoids have been isolated and identified from plants [55]. Flavonoids are ubiquitous in photosynthesis cells, therefore occurs widely in the plant kingdom [56]. They are found in fruits, vegetables, nuts, seeds, stems and flowers as well as tea, wine [57], propolis and honey [58]. More than 70 types of flavonoids have been reported from Annonaceae. For example flavanol types, taxifolin **62** had been reported frequently [59].

Mostly, the function of flavonoids in flowers is to provide colours attractive to plant pollinators [57, 60] and as the pigments responsible for plant colour, ranging from red, orange and yellow to violet in flowering plants. In leaves, these compounds are increasingly believed to promote physiological survival of the plant, protecting it from, fungal pathogens and UV-B radiation [60, 61]. In addition, flavonoids are also involved in photosensitisation, energy transfer, the action of plant growth hormones and growth regulators, control of respiration, photosynthesis, morphogenesis and sex determination [57, 61].

The flavonoids constitute one of the most characteristic classes of compounds in higher plants, generally categorized as phenols or polyphenols, with low molecular weight. Their chemical structures are based on a C15 skeleton with a chromane ring bearing a second aromatic ring B in position 2, 3, or 4. The skeleton can be represented

as a C6-C3-C6 system. Figure 2.4.3 showed the general structure and the numbering system used to distinguish the carbon positions around the molecule [62]. Flavonoids can be classified according to biosynthetic origin.

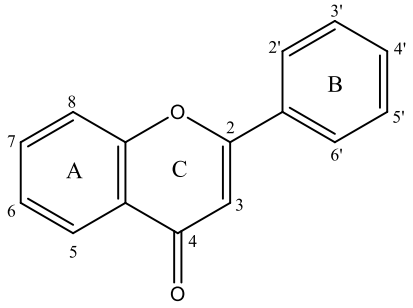
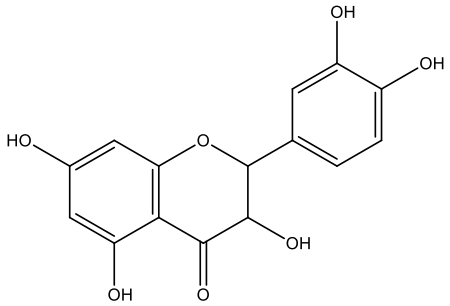


Figure 2.4.3: The skeleton structure of the flavones (a class of flavonoids), with rings named and positions numbered [61]



Taxifoline **62**

In some cases, the six-membered hetercyclic ring C occurs in an isomeric open form or is replaced by a five-membered ring, for example, chalcone **63** and aurone **64**. The carbon numbering under these conditions is different compared to the basic structure.

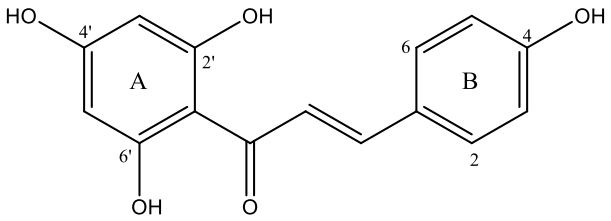
This nucleus can undergo many modifications such as hydroxylation, alkylation or glycosylation. Depending on these modifications, the flavonoids are classified into 8 groups - flavones **65**, flavonols **66**, flavanones **67**, isoflavones **68**, flavanols **69**, anthocyanins **70**, chalcones **63**, aurones **64**. Compounds belonging to the same group differ between them by the degree and the position of hydroxylation, the presence of substituents on the nucleus and the state of their polymerization.

Anthocyanins **70** were the first group of flavonoids to be documented. The pigments of anthocyanins **70** were recently noticed as antioxidant, antitumor, astringents and others [63]. The original "flavonoid" research apparently began in 1936, when Hungarian scientist Albert Szent-Gyorgi was uncovering a synergy between pure vitamin C and as yet unidentified co-factors from the peels of lemons, which he first called "citrin", and later, "vitamin P" [64]. The isolation and identification, structures, distribution and biosynthesis of the flavonoids in plants were reviewed by many authors [65-69]. The flavonoids as medical resources were also reviewed.

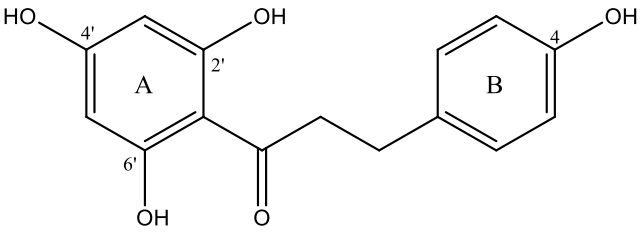
2.4.3 Major Sub-Classes of Flavonoids

Flavonoids are a very large group of polyphenolic natural products. There are different ways to classify flavonoids, for example, according to their biosynthetic origin, according to whether the central heterocyclic ring is unsaturated or not, according to their molecular size [70]. The most common way is according to the variation of the heterocyclic C ring. From the flavonoid basic structure, a heterocyclic pyrone C ring can be derivatised to flavones **65**, flavonols **66**, flavanones **67** and

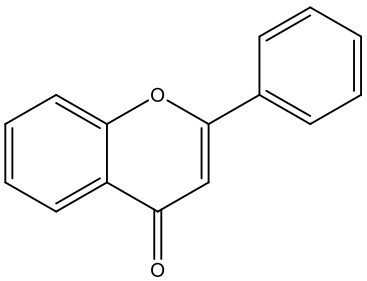
isoflavones **68**; a pyran C ring produces the flavanols **69** and anthocyanins **70** [71]. The basic structures of each sub-class are shown in figure 3.



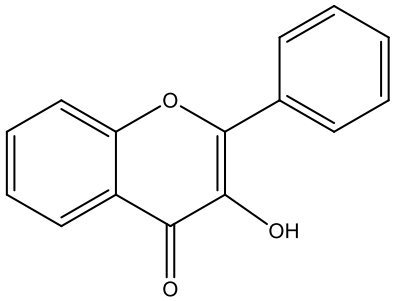
chalcone **63**



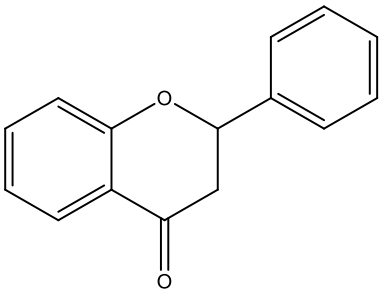
dihydrochalcone **71**



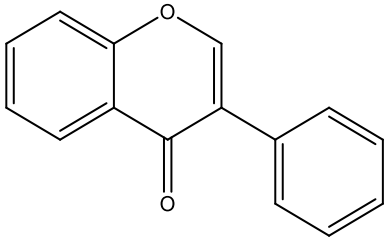
flavones **65**



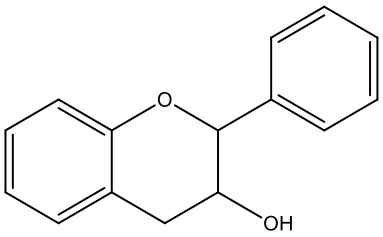
flavonols **66**



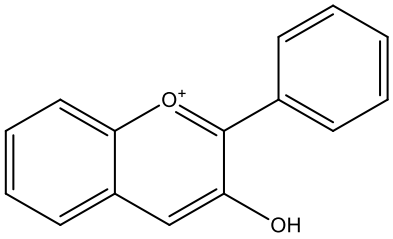
flavanones **67**



isoflavones **68**



flavanols **69**



anthocyanidins **70**

Figure 2.5.2: Major subclasses of flavonoids. Classification is based on variations in the heterocyclic C-ring.

2.4.4 Minor Sub-Classes of Flavonoids

Besides the major sub-classes, there are other sub-classes of flavonoids, which have skeleton structures differing form the flavonoid basic structure. Chalcones **63**, dihydrochalcones **71** and aurones **64** are biochemically related compounds of restricted occurrence and for this reason they are described as minor flavonoids [54]. Chalcones as well as dihydrochalcones have an open structure and a carbon skeleton numbered in a different way than other flavonoids.

2.4.4.1 Chalcone

Chalcones **63**, and the closely related dihydrochalcones **71**, are unique in the flavonoid family in lacking a heterocyclic C ring [60]. They are open-chain C_6-C_3-C_6 compounds which are the first C15 intermediates in flavonoid biosynthesis. The important attributes is the unique feature that distinguishes chalcones **63** and dihydrochalcones **71** from other flavonoids, the open-chain three-carbon structure linking the A- and B-rings in place of a heterocyclic C-ring. The numbering system of chalcones differs from those ring-closed flavonoids, which has been shown in figure 2.4.3.

Chalcones **63** have a diverse array of substituents on the two aromatic rings of 1,3-diphenyl-2-propen-1-one, which was derived by the cleavage of the C ring in flavonoids. Chemically they are open-chain of flavonoids in which the two aromatic rings are joined by a three carbon α, β-unsaturated carbonyl system. Chalcones **63** are abundantly present in nature starting from ferns to higher plants and a number of them are polyhydroxylated in the aryl rings.

The chalcones **63** and dihydrochalcones **71** are two distinctive classes of compounds that comprise more than 900 of all the naturally occurring flavonoids have been reported. The chalcones **63** are structurally one of most diverse groups of flavonoids, as witnessed by the formation of a wide range of dimers, oligomers, Diels-Alder adducts, and conjugates of various kinds.

The common structures of chalcones **63** and dihydrochalcones **71** are all hydroxylated to varying degrees and many are also *O*-methylated as well. Chemically chalcones **63** can be classified into two groups. The first are chalcones **63** with varying hydroxylation. They may be partly *O*-methylated and some have substituted phenyl. They may occur as glycosides but glycosidic variation is limited with glucose being the common sugar. The second group are those with complex structures involving many cases extra furano or pyrano rings fused to either the A or B ring [72].

Chalcones **63** have a limited but scattered occurrence. They are abundant in the leguminosae, for example being present in heartwood of trees or flowers of gorse, and in the Compositae, where they provide yellow colour in *Coreopsis* and related species. They are also present in the crystalline deposit of fronds of certain fern species. Apart from providing their yellow colour, no other clear cut functions have yet been elucidated in plants for the chalcones **63**. However, they are of medicinal interest and some structures have anti-peptic or anti-hepatotoxic activities [72] and recently, chalcones **63** have been found with anti-angiogenic effect [73].

The compounds with chalcone **63** as backbone have been reported to possess varied biological and pharmacological activities, including antimicrobial, anti-inflammatory, analgesic, cytotoxic, antitumor, antimalarial, antibercular, antiviral, anti-HIV, antiulcerative, antileishmanial, antioxidant, antiprotozoal, antihistaminic, antifedent, immunomodulatory, anticonvulsant, antihyperglycemic, antihyperlipidemic and antiplatelet activities. Thus chalcones **63** continue to attract considerable scientific attention because of their association with varieties of biological activities.

2.4.5 Pharmacology of Flavonoids

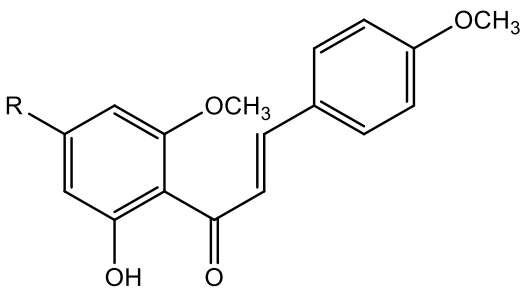
Flavonoids have become the subject of medical research. They have been reported to possess many useful properties, including anti-flammatory, anti-allergic, antimicrobial [74], antibacterial, antiviral, anti-allergic, anti-ischemic, anti-platelet, immunomodulatory, treatment of neurodegenerative diseases, vasodilatory action, anti-tumoral and anti-ulcer [75] activities. They have also been shown to inhibit some enzymes, including lipoxygenases and cyclooxygenases, mono-oxygenases, xanthine oxidase, mitochondrial succinoxidase, reduces nicotinamide-adenine dinucleotide (NADH) oxidase, phospholipase A_2, topoisomerases and protein kinases. The biological activities of flavonoids were thought to be mainly due to their antioxidant properties, which are displayed by limiting the production of reactive oxygen species (ROS) and scavenging them. Now it is thought that inhibition of enzymes has at least an important role.

2.4.6 Example of flavonoids extracted from Annonaceae species

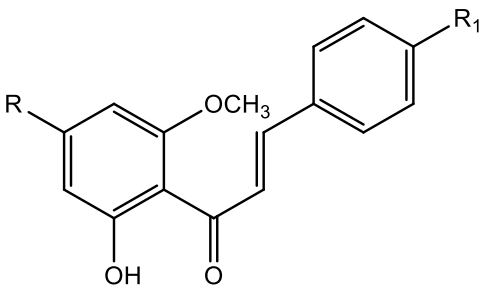
2.4.6.1 Flavonoids from *Goniothalamus gardneri* and *Goniothalamus thawaitesii*

The aerial part of *Goniothalamus gardneri* has yielded the known flavonoids 2'-hydroxy-4,4',6'-trimethoxychalcone (flavokawain A) **72**, 2',4'-dihydroxy-4,6'-dimethoxydihydrochalcone **73**, 4,2',4'-trihydroxy-6'-methoxydihydrochalcone **74**, 5,7,4'-trimethoxyflavanone (naringenin trimethyl ether) **75** and 7-hydroxy-5,4'-dimethoxyflavanone (tsugafolin) **76** together with three novel compounds, the dimer is characterised as (*rel*)-1β, 2α-di-(2,4-dihydroxy-6-methoxybenzoyl)-3β,4α-di-(4-methoxyphenyl)-cyclobutane, 2',4'-dihydroxy-4,6'-dimethoxychalcone **77** and 2'-hydroxy-4,4',6'-trimethoxydihydrochalcone **78**. The aerial parts of *G. thwaitesii* have

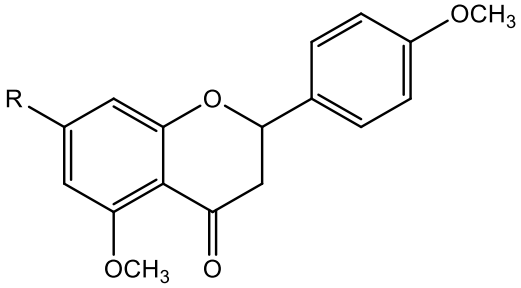
led only to isolation of the known flavonoids myricetin 4'-*O*-methyl ether-3-*O*-α-L-rhamnopyranoside (mearnsitrin) **79** and myricetin-3-*O*-methyl ether (annulatin) **80**, together with the triterpenes [76].



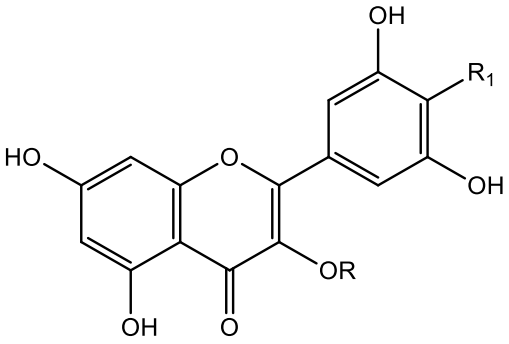
72 R = OCH_3
73 R = OH



78 R = R_1 = OCH_3
77 R = OH; R = OCH_3
74 R = R_1 = OH



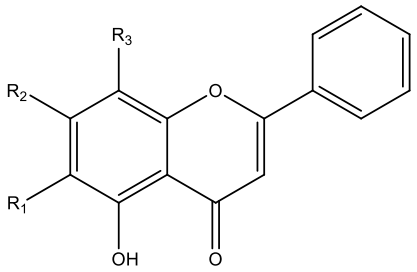
75 R = OCH_3
76 R = OH



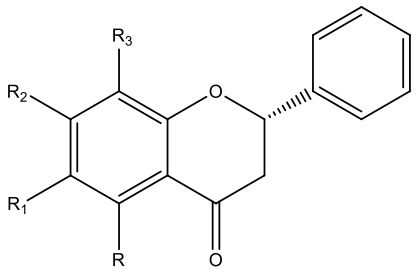
79 R = rhamnose; R_1 = OCH_3
80 R = CH_3; R_1 = OH

2.4.6.2 Flavonoids from genus *Desmos*

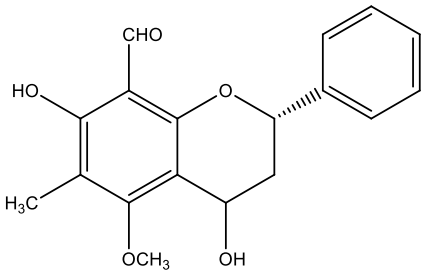
The inhibitory effects of the organic extracts of four *Desmos* Lour. species which are *D. chinensis, D. grangifolius, D. dumosus,* and *D. yunnanensis* have been examined against HIV-1 replication in acutely infected H9 lymphocytic cells. According to bioactivity-directed fractionation, they have isolated and characterized 18 flavonoids and their derivatives from these four species. They were categorized structurally into five groups, including flavones (**81-85**), flavanones (**86-90**), flavan (**91**), chalcones (**92-93**), and other flavonoid derivatives with unique structures (**94-96**). These compounds were evaluated for inhibition of HIV replication in H9 lymphocyte cells. 2-methoxy-3-methyl-4,6-dihydroxy-5-(3'-hydroxy)cinnamoylbenzaldehyde (**92**) and lawinal (**86**) demonstrated potent anti-HIV activity with EC_{50} values of 0.022 and 2.30μg/mL and therapeutic indexes of 489 and 45.2, respectively [77].



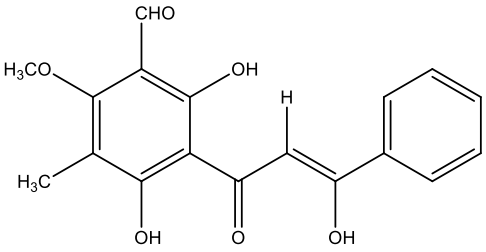
	R_1	R_2	R_3
81	OCH_3	OCH_3	H
82	OH	OCH_3	H
83	CH_3	OH	CHO
84	CHO	OH	CH_3
85	CH_3	OCH_3	CH_3



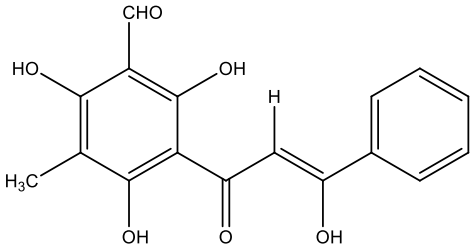
	R	R_1	R_2	R_3
86	OH	CHO	OH	CH_3
87	OH	CH_3	OH	CHO
88	OH	CH_3	OH	CH_3
89	OH	CH_3	OCH_3	CH_3
90	OCH_3	CH_3	OH	CHO



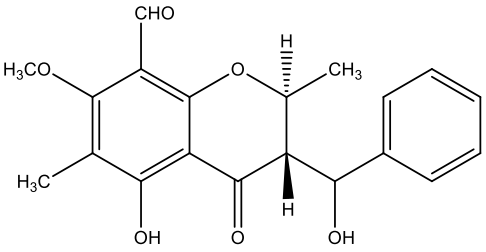
91



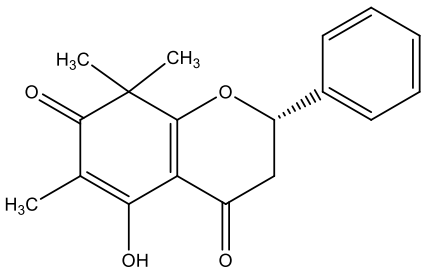
92



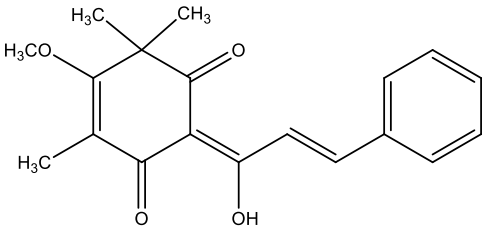
93



94



95



96

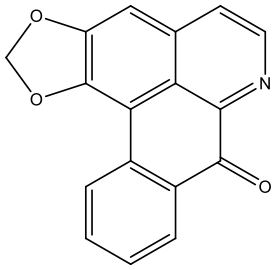
2.5 CHEMICAL CONSTITUENTS OF *MITRELLA KENTII* SPECIES

Previous phytochemical studies on this species described the isolation and identification on the bark and leaves of the plant. In 1972, Ellis J., *et al.,* [78] found four known compounds of type oxoaporphine and isoquinoline alkaloids, liriodenine **21**, anonaine **33**, asimilobine **97** and aequaline **98** from the bark of *M. kentii*.

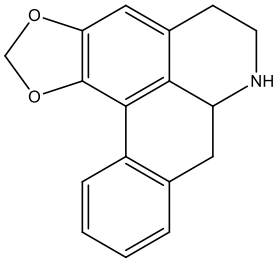
In 1997, Benosman A., *et al.,* [79] isolated two new compounds, terpenylated dihydrochalcones; (-)-neolinderatin **99**, (-)-linderatin **100**, also from the stem bark of *M. kentii* in ethanolic extraction, together with known compounds 2',6'-dihydroxy-4'-methoxydihydrochalcone **101** and (+)-catechin **102**, which was isolated for the first time from this plant. In 2012, Saadawi S., *et al.,* [80] had reported six known compounds, benzoic acid **103**, acetymelodorinol **104**, chrysin **105**, benzoquinone **106**, stigmasterol **107** and polycarpol **108** which were isolated from the methanol extract of the leaves of *M. kentii*. The structures were elucidated by spectroscopic analysis and comparison of their spectral data was in agreement with those reported previously. These compounds were isolated for the first time from this plant.

The compounds were evaluated for their ability to inhibit prostaglandin E_2 (PGE_2) and thromboxane B_2 (TXB_2) production in human whole blood using a radioimmunoassay technique. Their inhibitory effect on platelet activating factor (PAF) receptor binding to rabbit platelet was determined using ^{3}H-PAF as a ligand. Among the compounds tested, chrysin showed a strong dose-dependent inhibitory activity on PGE_2 production (IC_{50} value of 25.5 µM), which might be due to direct inhibition of cyclooxygenase-2 (COX-2) enzymatic activity. Polycarpol, acetylmelodorinol and

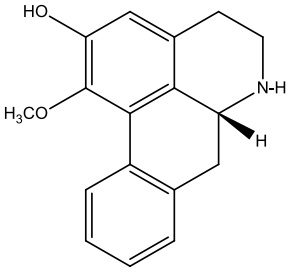
stigmasterol exhibited significant and concentration-dependent inhibitory effects on TXB_2 production with IC_{50} values of 15.6, 19.1 and 19.4 µM, respectively, suggesting that they strongly inhibited COX-1 activity. Polycarpol and acetylmelodorinol showed strong dose-dependent inhibitory effects on PAF receptor binding with IC_{50} values of 24.3 and 24.5 µM, respectively [80].



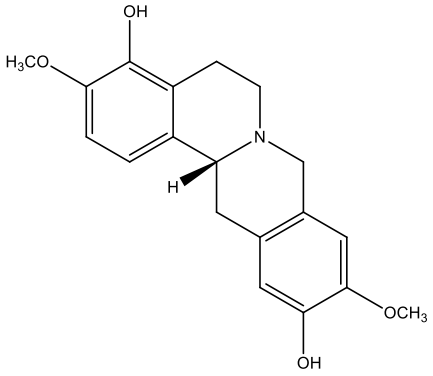
liriodenine **21**



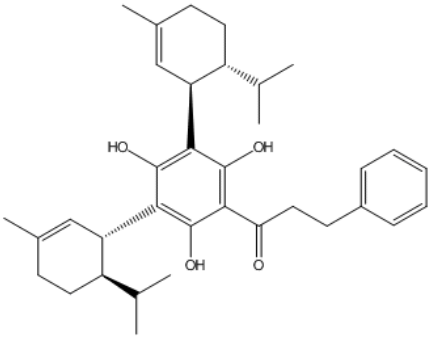
anonaine **33**



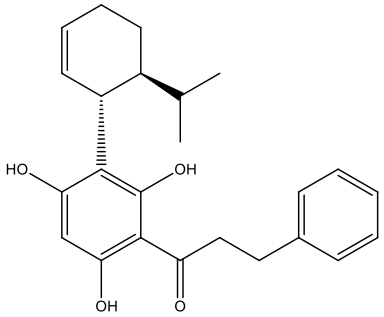
asimilobine **97**



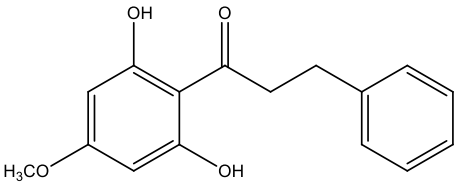
aequaline **98**



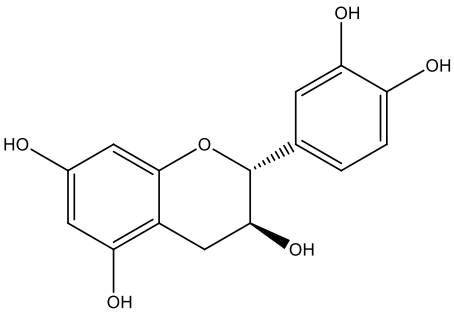
(-)-neolinderatin **99**



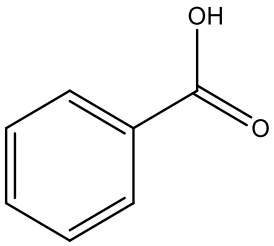
(-)-linderatin **100**



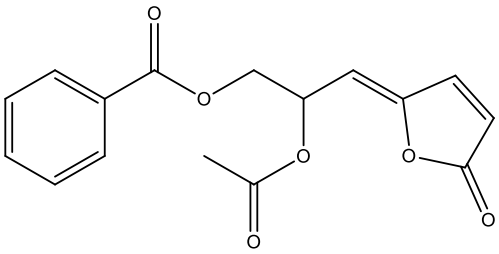
2', 6'-dihydroxy-4'-methoxydihydrochalcone **101**



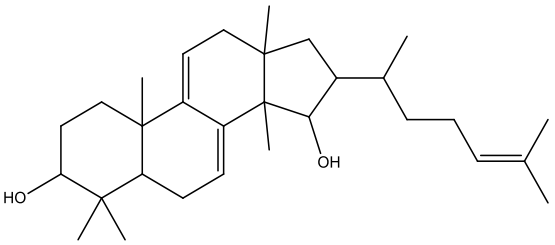
(+)-catechin **102**



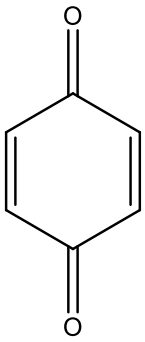
benzoic acid **103**



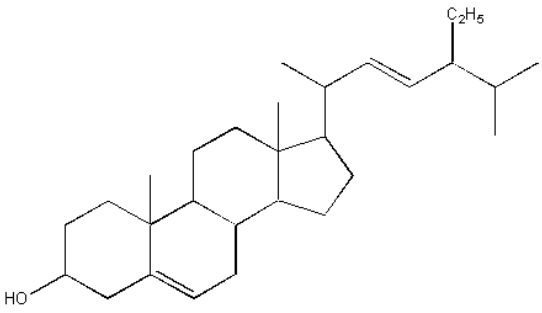
acetylmelodorinol **104**



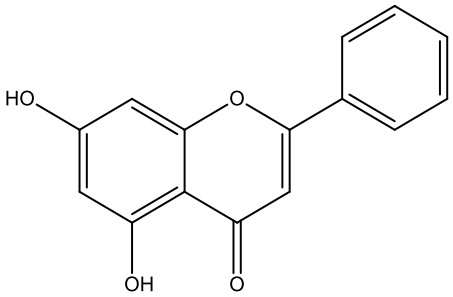
chrysin **105**



benzoquinone **106**



stigmasterol **107**



polycarpol **108**

2.6 Biological Activities of Annonaceae Plants

2.6.1 Antiulcer

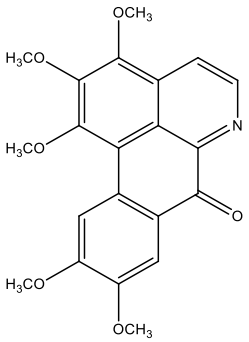
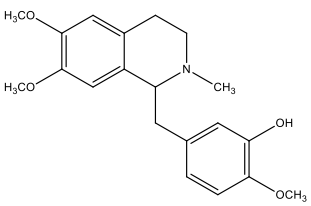
Peptic ulcer disease (encircling gastric ulcer and duodenal ulcers) affects a large population of the world. It is now generally agreed that gastric lesions develop when the delicate balance between some gastroprotective (mucin, prostaglandin, bicarbonate, nitric oxide and growth factors) and aggressive factors (acid, pepsin, and *Helicobacter pylori*) is lost [81].

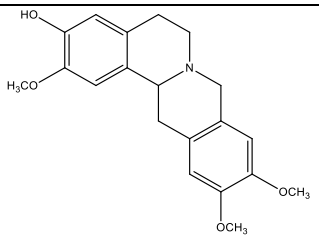
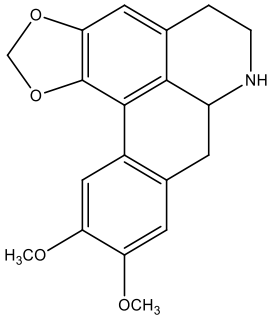
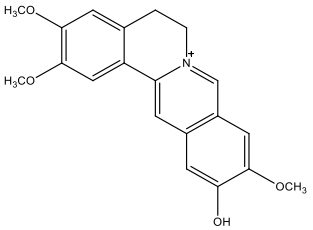
Alcohol is known as a necrotizing substance and its excessive ingestion may result in gastritis, cahracterized by mocous membrane edema, sub epithelial haemorrhages, cell exfoliation and inflammatory cells infiltration [82]. Thus, gastrointestinal tract diseases related to excessive alcohol use play an important role in the clinical gastroenterology [83].

Many chemical compounds have been isolated from medicinal plants with anti-ulcer activity [84]. This is an important reason to study the antiulcerogenic effects of plants traditionally used in popular medicine for the treatment of gastric diseases. Among the main classes of chemical bioactive components capable of offering gastroprotection are the alkaloids, saponins, xanthones, triterpenes and tannins [85-89].

2.6.2 Antioxidant

Potentially harmful reactive oxygen species (ROS) are produced as a consequence of normal aerobic metabolism [90, 91]. These "free radicals" are usually removed or inactivated *in vivo* by a team of antioxidants [90-94]. Individual members of the antioxidant defense team are deployed to prevent generation of ROS, to destroy potential oxidants, and to scavenge ROS. Thus, oxidative stress-induced tissue damage is minimized. However, an absolute or relative deficiency of antioxidant defenses may lead to a situation of increased oxidative stress, and this may be associated with both the causes and consequences of variety of disorders, including coronary heart disease and cancer [95].

Species	Type of compounds	Chemical constituents	Bioactivity
Xylopia championii	Alkaloids	 oxopurpureine **109**  (+)-laudanidine **110**	Puvanendran *et al.*, (2008) were found oxopurpureine **109**, (+)-laudanidine **110**, (-)-discretine **111**, nordicentrine **112** and dehydrocorytenchine **113**, isolated from the stem bark and stem of *Xylopia championii*. The alkaloids (+)-laudanidine and (-)-discretine, showed exhibited exceptionally high antioxidant activity at a concentration of 0.5 mg/mL whereas nordicentrine and dehydrocorytenchine showed moderate activity as compared with the standard antioxidant DL-α-tocopherol in the DPPH assay[96].

		 (-)-discretine **111**  nordicentrine **112**  dehydrocorytenchine **113**	

CHAPTER 3

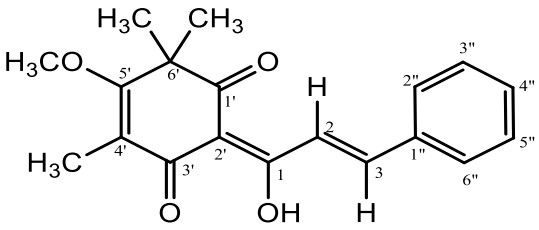
RESULTS AND DISCUSSION

PART A: PHYTOCHEMICALS

The hexane, dichloromethane (CH_2Cl_2) and acid-base extracts from the stem bark of *Mitrella kentii*, were subjected to column chromatography (CC). Then, the fractions of the compounds were purified by Preparative Thin Layer Chromatography (PTLC) and Thin Layer Choromatography (TLC) to isolate the pure compounds. The isolated compounds were elucidated using spectroscopic techniques such as UV, IR, MS, 1D-NMR (^{1}H-NMR, ^{13}C-NMR and DEPT), 2D-NMR (COSY, HSQC, and HMBC) and by comparison their spectral data with those previously reported in the literatures.

The compounds isolated from the stem bark of *M. kentii* were: desmosdumotin C **MK1**, desmosdumotin C tautomer, 2'-cinnamoyl-3'-hydroxy-5'-methoxy-4',6',6'-trimethylcyclohexa-1',1-dienone **MK2(B)**, stigmasta-4-en-3-one **MK3**, stigmasta-4,22-diene-3,25-dione **MK4**, 6-hydroxy-5,7-dimethoxy-2-phenylchroman-4-one **MK5**, benzyl benzoate **MK6**, oxybis(ethane-2,1-diyl) dibenzoate **MK7**, liriodenine **MK8** and atherospermidine **MK9**.

3.1 Structure Elucidation of Compound MK1



MK1

Chalcone desmosdumotin C, **MK1** was separated from the n-hexane extract and was isolated as a yellow needle crystal with m.p. 93-94 ^{o}C (93-94 ^{o}C) [97]. The UV spectrum showed the absorption maxima λ_{max} at 191, 232 and 376 nm which indicated the existence of the conjugated double system and an extended benzyl chromophore [97]. Its IR spectrum showed the absorption peaks at 1657 cm^{-1} and 1624 cm^{-1} indicating the presence of conjugated carbonyl and the absorption peaks for the alkene groups and aromatic rings at 1514 and 1576 cm^{-1}, respectively [97]. The high-resolution electron impact mass spectroscopy (HREI-MS) spectrum gave molecular ion peak $[M+H]^{+}$ at *m/z* 313.1435 corresponding to molecular formula $C_{19}H_{20}O_4$ [97].

The ^{1}H-NMR spectrum of **MK1** (Table 3.1, Figure 3.1.3) showed signals of unsubstituted aromatic ring protons which were resonated at δ 7.66-7.37 as a multiplet (H-2", H-3", H-4", H-5" and H-6"). Methoxy protons appeared at δ 3.93 as a singlet assigned to OMe-5', a methyl group appeared at δ 2.02 as a singlet (H-4') and two geminal methyl protons appeared at δ 1.36 as a singlet with six protons assigned to H-

6’and two *trans*-oriented methine protons were resonated at δ 8.32 as a doublet with *J* value 16.0 Hz and δ 7.92 as a doublet with *J* value 16.0 Hz which were assigned as H-2 and H-3, respectively.

The ^{13}C-NMR spectrum of **MK1** (Table 3.1, Figure 3.1.4) showed the presence of nineteen signals; six quaternary carbons, two carbonyl groups, two methine groups, three methyl groups, one methoxy group and five aromatic carbons. The two very low-field carbons at δ 198.1 and δ 192.5 seem to be attributable to carbonyl groups assigned to C-1' and C-3'. The C4'-CH_3 appeared at δ 9.9, C6'-2CH_3 was resonated at δ 24.2 and OCH_3 appeared at δ 62.2. The *trans* methine group, C-2 and C-3 were resonated at δ 130.7 and 130.7, respectively. The five aromatic carbons were resonated at δ 128.9, 130.7 and 130.7 which were assigned to C-2",6", C-3",5" and C-4", respectively. The other six quaternary carbons appeared at δ 113.7, 106.7, 176.7, 50.5, 187.3 and 135.3 assigned to C-2’, C-4’, C-5’, C-6’, C-1 and C-1”, respectively. DEPT-135 spectrum (Figure 3.1.4) didn’t reveal any negative peak for sp^2 methylene group. Therefore, the data from the 1D-NMR spectrums are correlated with the predicted structure.

In the HMBC spectrum (Table 3.1, Figure 3.1.9) measurements of compound **MK1**, as in Figure 3.1.1, revealed the positions of substituents; the three methyl groups, one methoxy group and carbonyl groups, respectively to their carbon.

From these 1D and 2D-NMR measurements (COSY, HSQC and HMBC), the structure of C_6-C_3 moiety was assigned in Figure 3.1.1. Based on the spectral data and comparison with the literature value [97], it was confirmed that **MK1** was desmosdumotin C. Table 3.1 showed the ^{1}H-NMR, ^{13}C-NMR and HMBC data of **MK1** comparison with literature value. The structure of **MK1** was reconfirming by the X-ray crystallography in Figure 3.1.2. This compound may exist as an equilibrated mixture of keto- and enol-tautomers.

Table 3.1: ^{1}H-NMR (400 MHz),^{13}C-NMR (100 MHz) and HMBC data of **MK1** in $CDCl_3$

Position	**δ_H (mult.; *J*(Hz))**	**Reference[97]**	**δ_C**	**Reference[97]**	**HMBC**
1'	-	-	198.1	198.0	-
2'	-	-	113.7	113.5	-
3'	-	-	192.5	192.3	-
4'	-	-	106.7	106.1	2',5',3'
5'	-	-	176.7	175.6	-
6'	-	-	50.5	50.4	-
1	-	-	187.3	187.1	-
2	8.3,d,16.0	8.32,d,14.7	123.3	144.8	1",1
3	7.9,d,16.0	7.93,d,14.7	144.9	144.8	2",6",1,
1"	-	-	135.3	135.2	-
2", 6"	7.7,m	7.66,m	128.9	123.2	3",4",5"
4"	-	-	130.7	128.3	2",6",1"
3", 5"	7.4,m	7.39,m	130.7	130.5	2",6",1"
C4'-CH_3	2.0,s	2.00,s	9.9	9.7	-
C5'-OCH_3	3.9,s	3.95,s	62.2	62.1	5'
C6'-$2CH_3$	1.7,s	1.38,s	24.4	24.3	6',5',1'

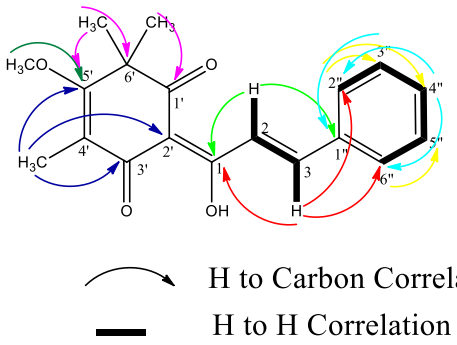


Figure 3.1.1: Important Correlations on COSY and HMBC of **MK1**

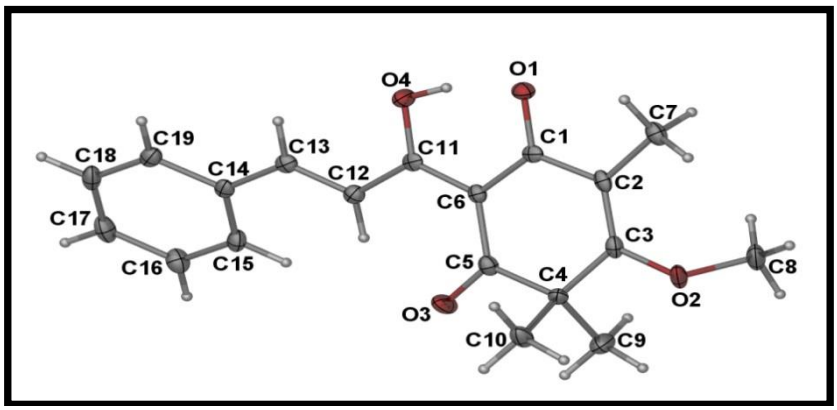


Figure 3.1.2: X-ray crystallography of **MK1**

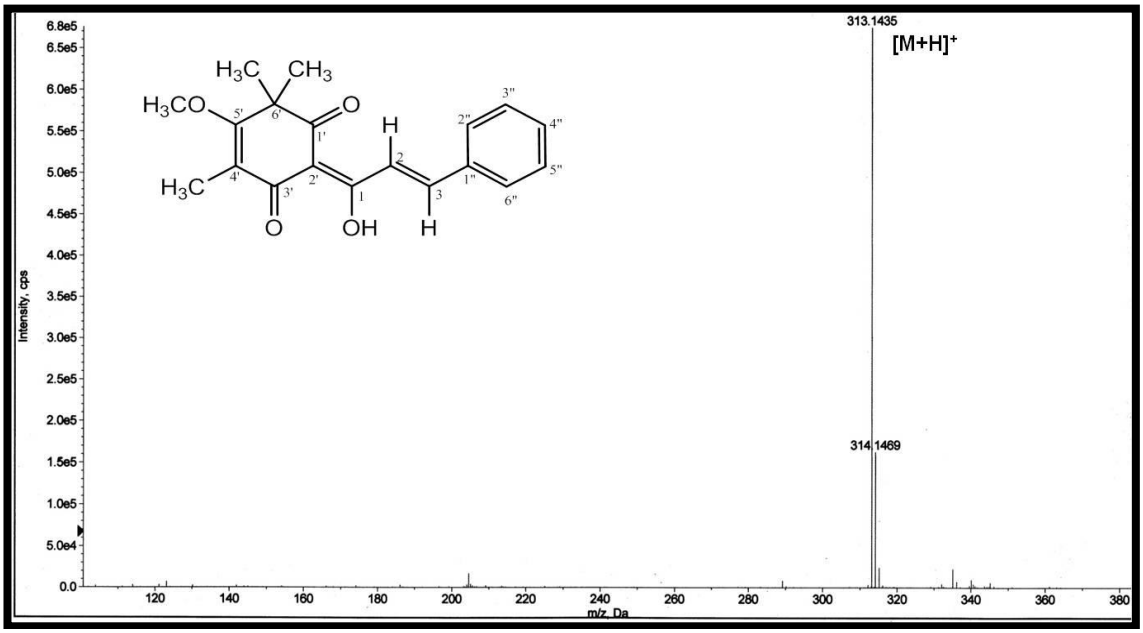


Figure 3.1.3: The MS Spectrum of **MK1**

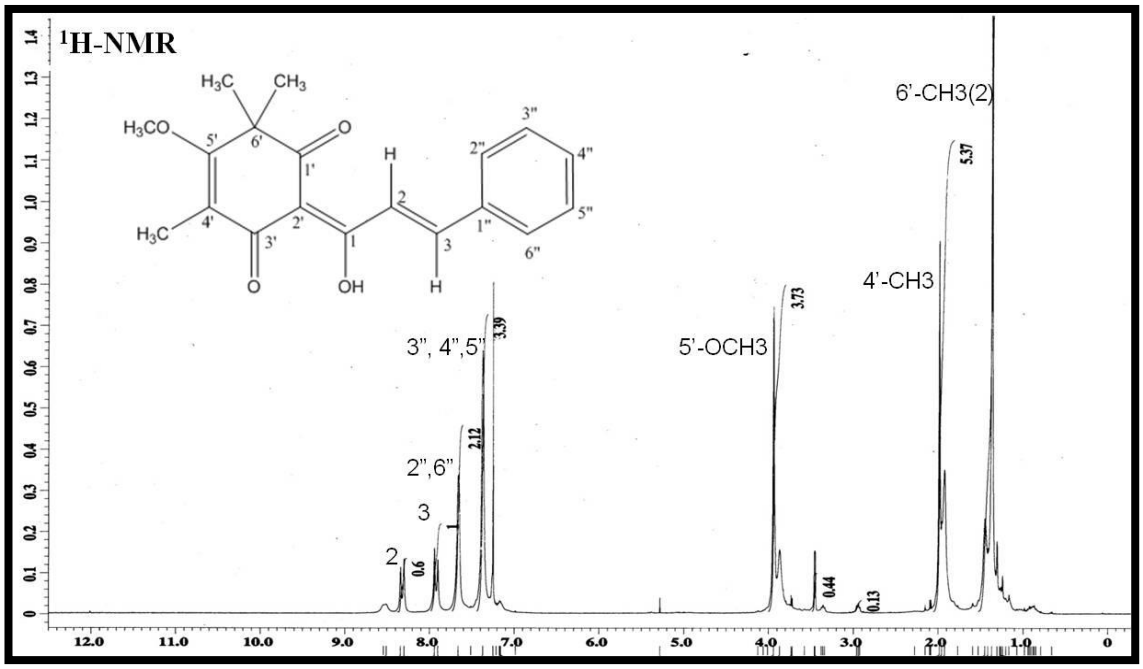


Figure 3.1.4: ^{1}H-NMR Spectrum of **MK1**

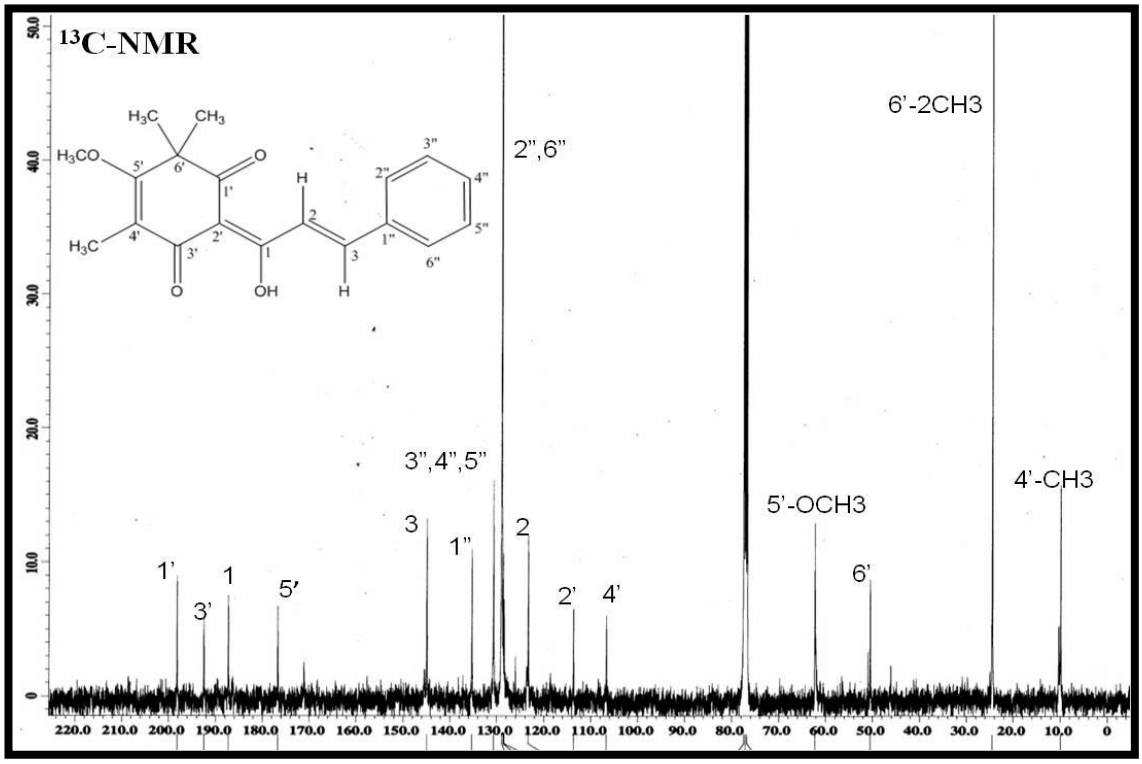


Figure 3.1.5: ^{13}C-NMR Spectrum of **MK1**

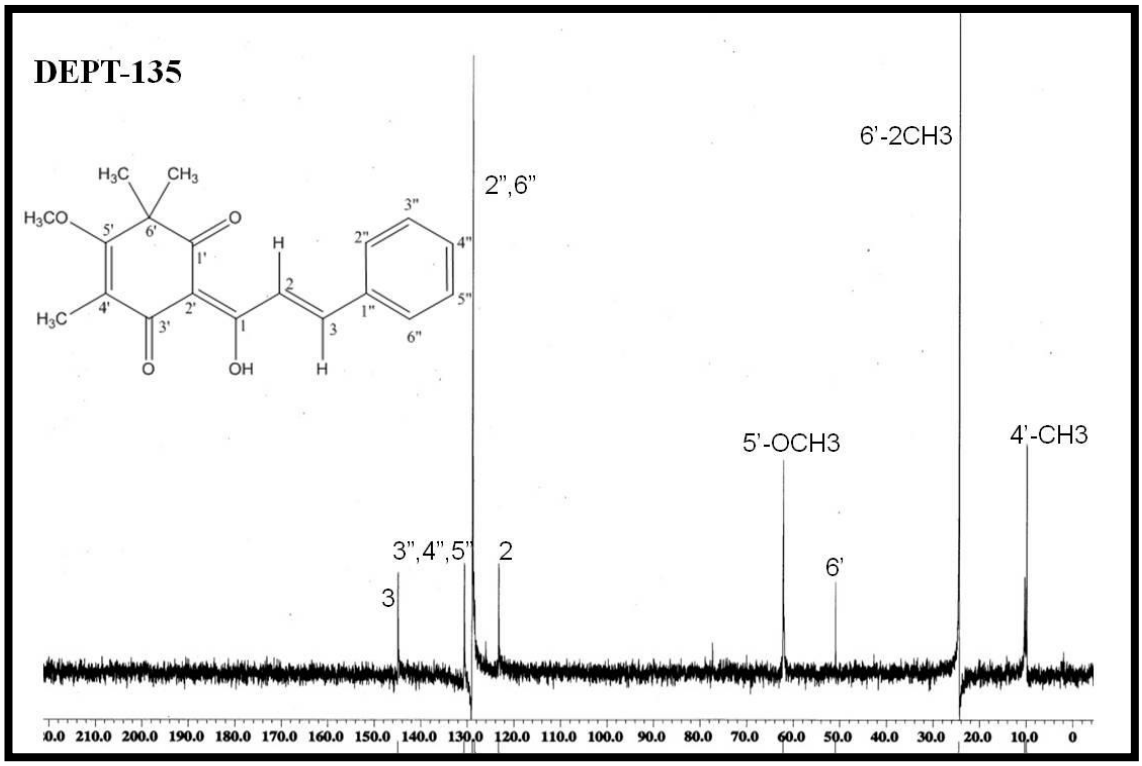


Figure 3.1.6: DEPT-135 Spectrum of **MK1**

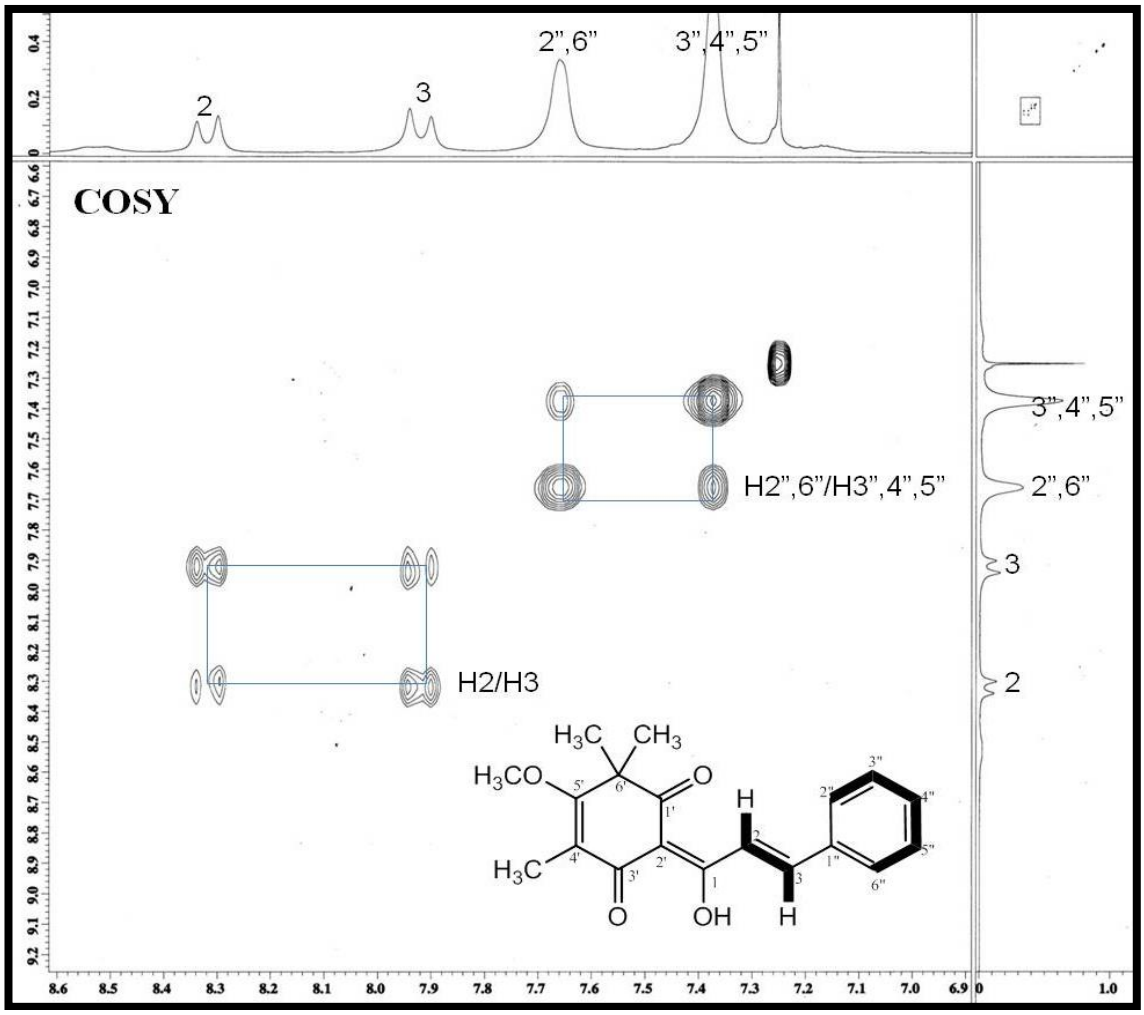


Figure 3.1.7: COSY Spectrum of **MK1**

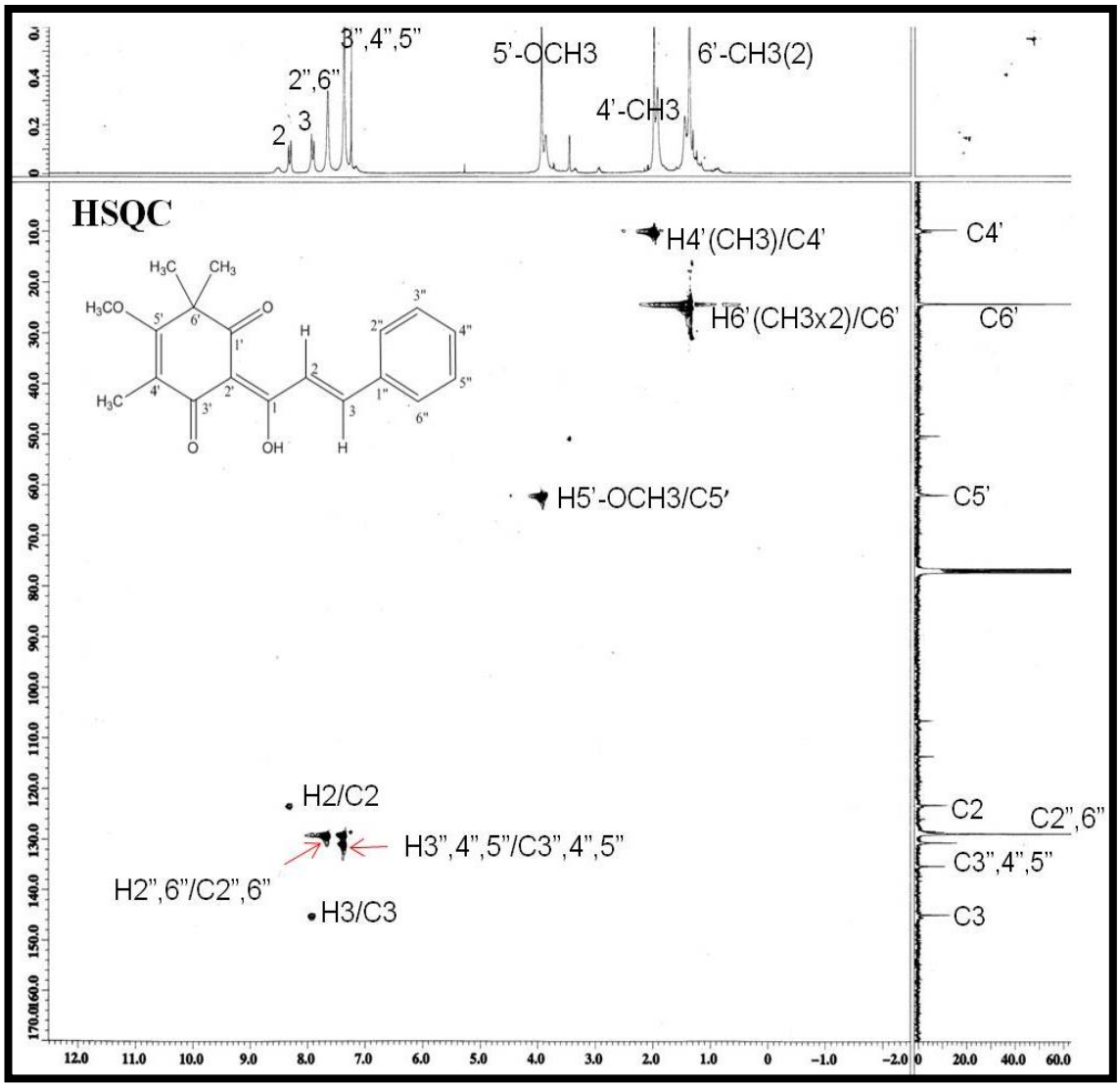


Figure 3.1.8: HSQC Spectrum of **MK1**

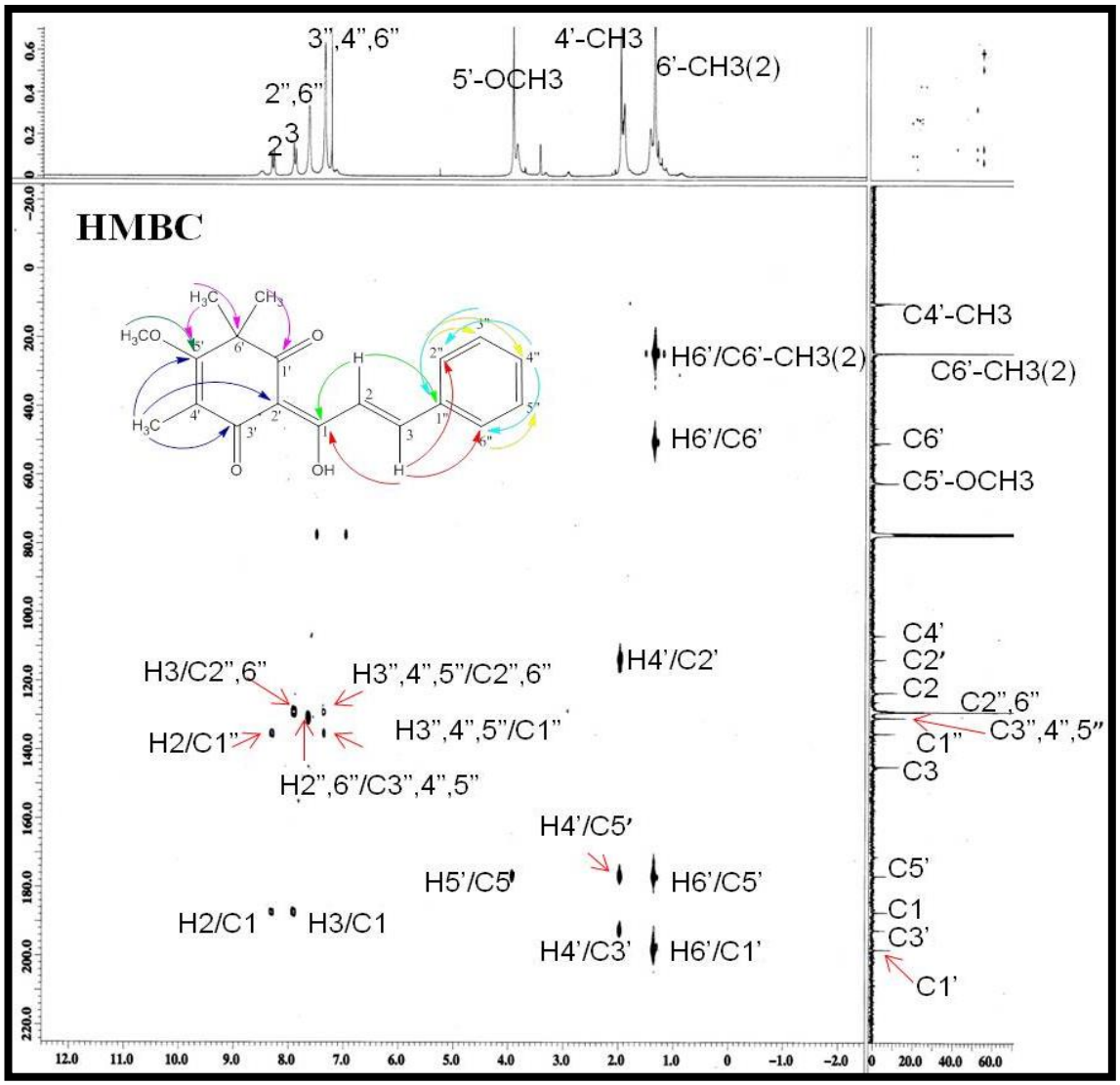
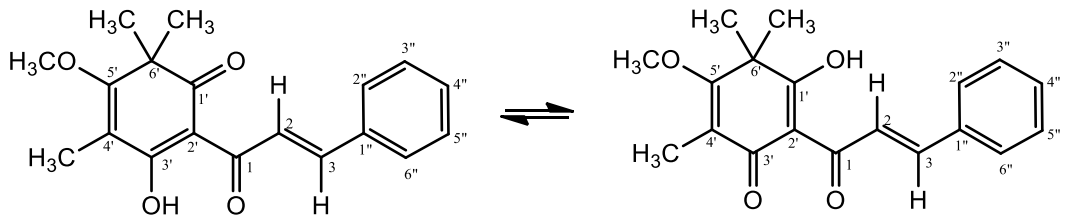


Figure 3.1.9: HMBC Spectrum of **MK1**

3.2 Structure Elucidation of Compound MK2



MK2(B) **MK2(C)**

Chalcone 2'-cinnamoyl-3'-hydroxy-5'-methoxy-4',6',6'-trimethylcyclohexa-1',1-dienone **MK2(B)** is tautomer of **MK1,** has been previously reported from *Desmos dumosus* [97] and *Campomanesia lineatifolia* [98] as a single tautomer. Furthermore, **MK1** also was isolated from *Desmos rostrata* [99] but it was isolated in a tautomeric mixture of the two isomers, **MK1** and 2'-cinnamoyl-1'-hydroxy-5'-methoxy-4',6',6'-trimethylcyclohexa-3',1-dienone **MK2(C)**. The ^{1}H and ^{13}C -NMR of the β-triketone part of compounds **MK1** and **MK2(B)** resemble to each other.

MK2(B) was isolated as a yellow needle crystal with m.p 93-94 ^{o}C (lit 93-94 ^{o}C)[99]. The UV spectrum showed the absorption maxima at 242, 320 and 485 nm and its IR spectrum showed the absorption peaks at 1657 cm^{-1} indicating the presence of conjugated carbonyl group, the alkene group absorbed at 1624 cm^{-1} and aromatic rings at 1576 and 1514 cm^{-1} [99]. The HREI-MS spectrum of this compound gave the molecular ion peak $[M+H]^{+}$ at *m/z* 313.1435 corresponding to molecular formula $C_{19}H_{20}O_4$ (Figure 3.2.2) [99]. All the characteristics of this compound were same as its

tautomer, **MK1**. The movement bonding of the hydroxyl group depend on the acidity of the solvent that was used to run NMR. For example, if the compound dissolves in the chloroform-d and acetone-d the percentage of the tautomer was about 33%. However, if dissolves in the methanol-d the percentage was 50%. Therefore, this compound is not stable in the solvent that has high acidity.

The ^{1}H-NMR spectrum **MK2(B)** (Table 3.2, Figure 3.2.3) showed the mixture of **MK1** and **MK2(B)** and the percentage of **MK2(B)** in the mixture was 33%. This mixture could not be separated using normal chromatography technique including HPLC. It may be separated by HPLC using chiral column. The ^{1}H-NMR pattern of **MK2(B)** is similar to the spectrum of **MK1**. A methoxy proton was resonated at δ 3.87 as a singlet; a methyl proton appeared at δ 1.94 as a singlet and two geminal methyl groups appeared at δ 1.45 as a singlet with six protons. The aromatic protons were resonated at δ 7.65-7.26 as multiplet and two *trans*-oriented olefinic protons were resonated at δ 8.53 as a doublet with *J* value 16.0 Hz and δ 7.94 as a doublet with *J* value 16.0 Hz which were assigned for H-2 and H-3, respectively. The difference between two tautomeric was shown in the ^{1}H-NMR spectrum. The olefinic proton for H-2 of **MK2(B)** was de-shielded more than **MK1**.

The ^{13}C-NMR spectrum **MK2(B)** (Table 3.2, Figure 3.2.4) showed the presence of nineteen signals carbons which belongs to six quaternary carbons, two carbonyl groups, two methine groups, one methoxy group, three methyl groups and five aromatic carbons. The two carbonyl groups were resonated at δ 201.7 and 189.5 assigned to C1'

and C-1. Thus, the C-3' bearing OH group was resonated at δ 186.3 and sp^2 carbon C-2 and C-3 were resonated at the same position that is at δ 145.5. The three methyl groups appeared at δ 10.31 assigned to C-4' whereas the C-6'-2CH_3 was resonated at δ 24.4 and OCH_3 appeared at δ 62.0 assigned to C-5'. The five aromatic sp^2 carbons appeared at δ 123.7 and 130.8 which assigned to C-2",6" and C-3",4",5", respectively. The other five quaternary carbons were resonated at δ 108.4, 118.6, 171.1, 46.2 and 135.3 assigned to C-2', C-4', C-5', C-6' and C-1", respectively.

MK2(B) found to be tautomeric with compound **MK1** from its elemental analysis. Its IR and UV data indicated **MK2(B)** is a chalcones. From these results, the structure of **MK2(B)** was determined. Several possible formulas could exist for **MK2(B)**, which should change mutually by keto-enol tautomerization as depicted in Figure 3.2.1. From these facts, structures **MK2 (B)** and **MK2 (C)** in Figure 3.2.1 are plausible structures, similar to **MK1**. Of the two possible tautomers shown in Figure 3.2.1(B and C), it is not clear which tautomer is predominant in $CDCl_3$ because the hydrogen-bonded enol-proton exihibited no correlations in the HMBC spectrum. Based on the spectral data and comparison with 2'-cinnamoyl-1'-hydroxy-5'-methoxy-4',6',6'-trimethylcyclohexa-3',1-dienone **MK2(C)** [99] it was confirmed that **MK2(B)** was 2'-cinnamoyl-3'-hydroxy-5'-methoxy-4',6',6'-trimethylcyclohexa-1',1-dienone and it was a tautomeric structure of **MK1** in Figure 3.2.1 of **MK1**. Table 3.2 showed the ^{1}H NMR and ^{13}C-NMR data for **MK2(B)** and comparison with the literature [99].

Table 3.2: ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) of **MK2(B)** in $CDCl_3$

Position	**δ_H (mult.; *J*(Hz))**	**References[99]**	**δ_C**	**References[99]**
1'	-	-	201.7	201.6
2'	-	-	108.4	108.4
3'	-	-	186.3	186.2
4'	-	-	118.6	118.5
5'	-	-	171.1	171.0
6'	-	-	46.2	46.1
1	-	-	189.5	189.5
2	8.5,d,16.0	8.47,d,16.0	145.5	123.7
3	7.9,d,16.0	7.88,d,16.0	145.5	145.4
1"	-	-	135.3	135.2
2", 6"	7.7,m	7.32,m	123.7	129.0
3",4", 5"	7.3,m	7.32,m	130.8	130.7
4”	-	7.61,m	-	-
C4'-CH_3	1.9,s	1.88,s	10.3	10.2
C5'-OCH_3	3.9,s	3.82,s	62.1	61.9
C6'-$2CH_3$	1.5,s	1.40,s	24.4	24.4

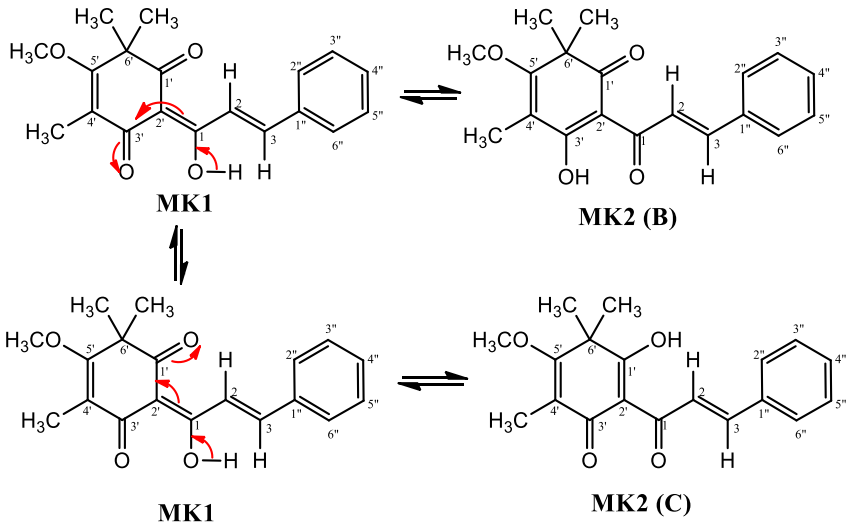


Figure 3.2.1: Possible tautomers of **MK1**, **MK2(B)** and **MK2(C)**

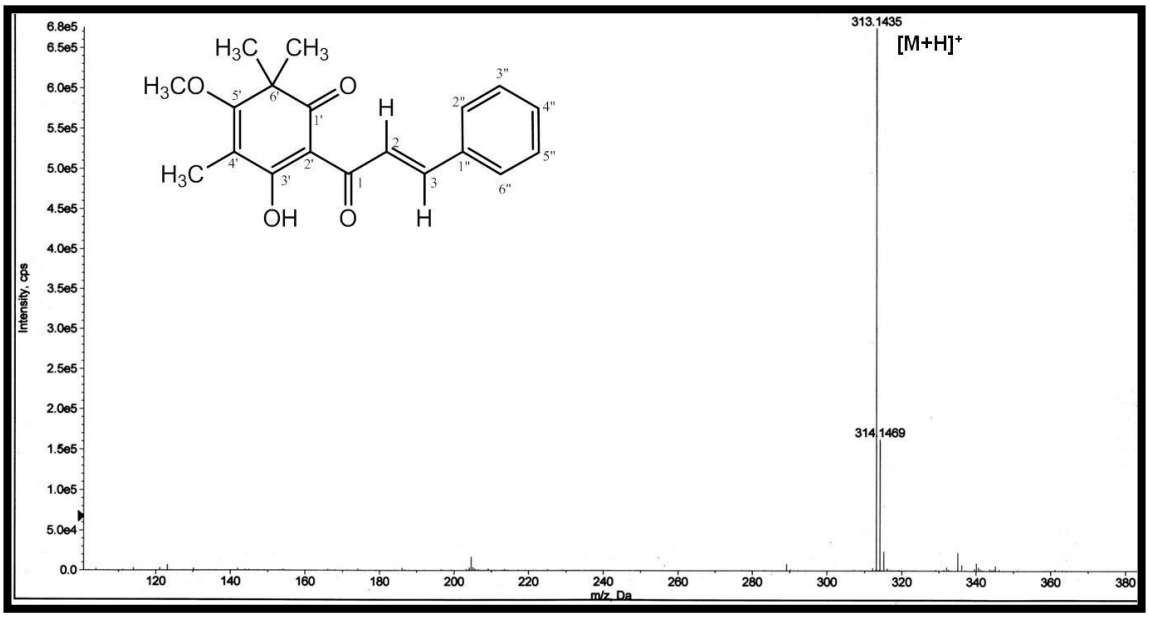


Figure 3.2.2: The MS Spectrum of **MK2**

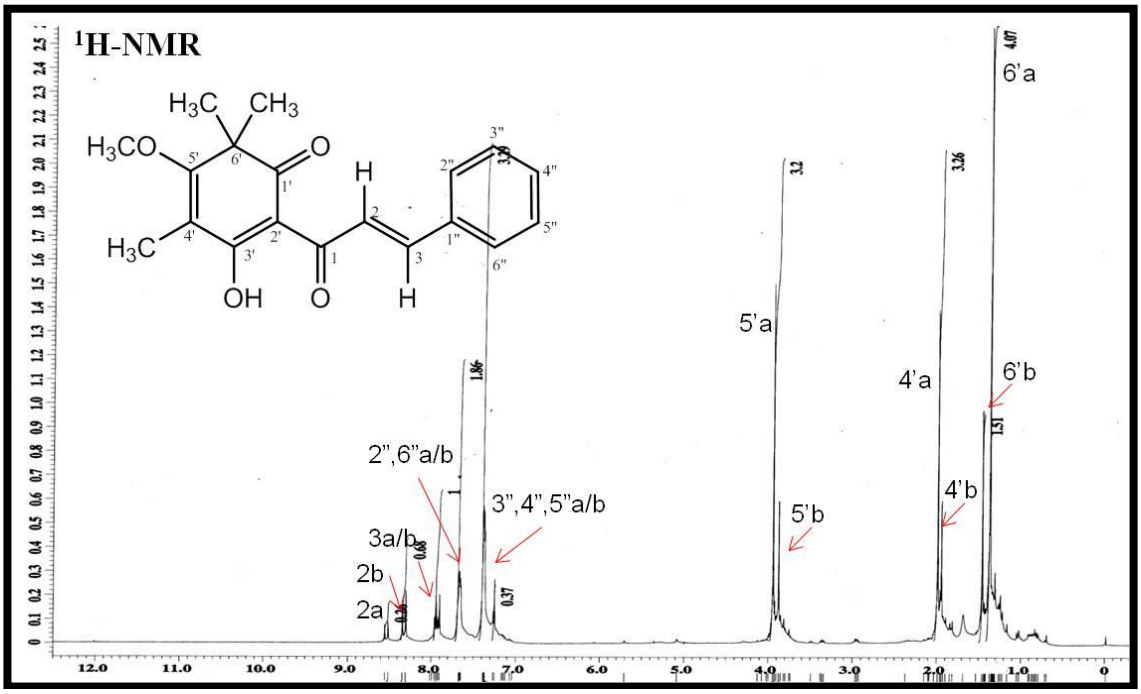


Figure 3.2.3: ^{1}H-NMR Spectrum of **MK2**

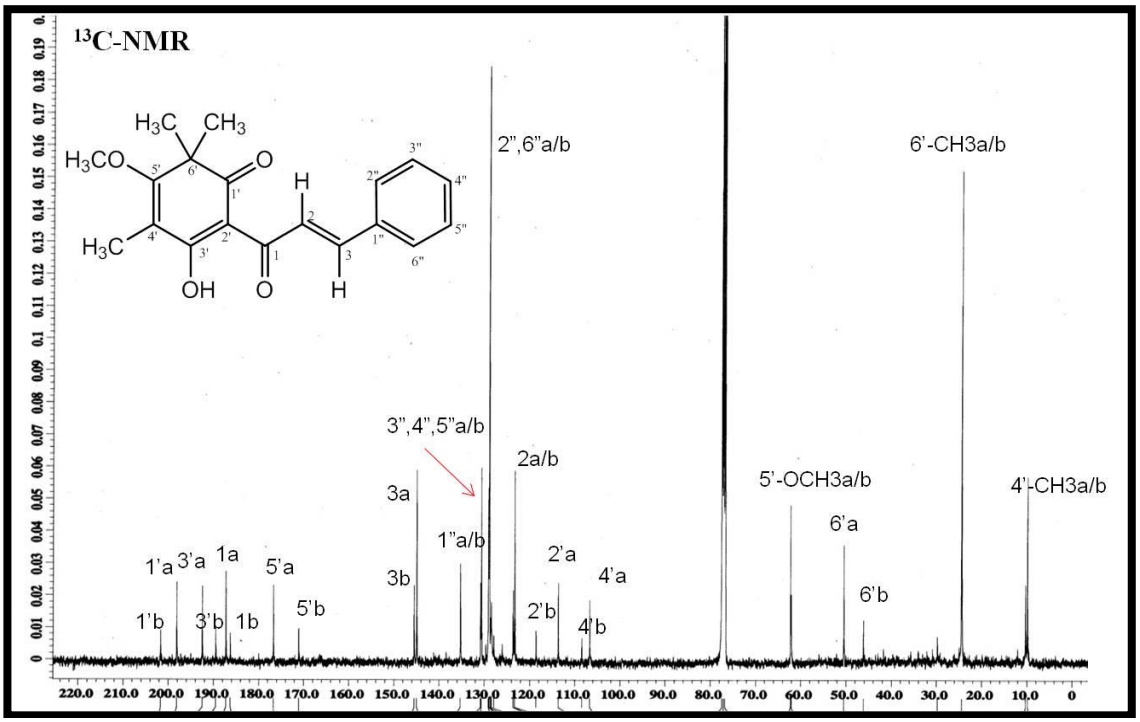


Figure 3.2.4: ^{13}C-NMR Spectrum of **MK2**

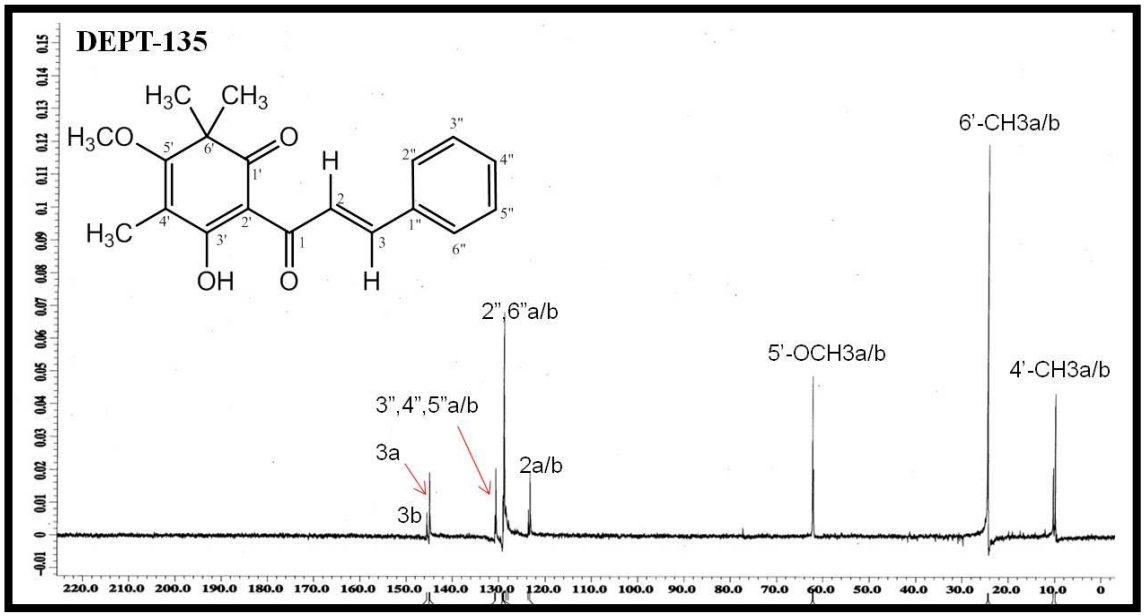
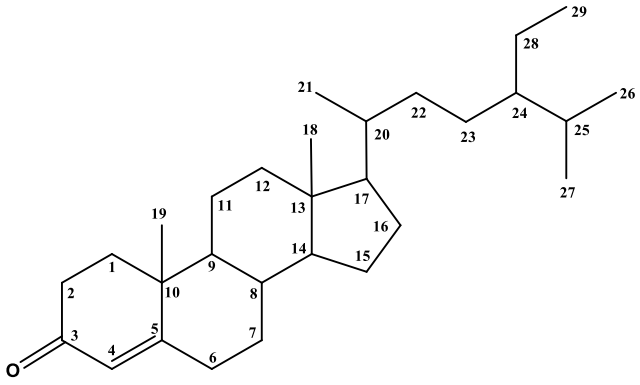


Figure 3.2.5: DEPT-135 Spectrum of **MK2**

3.3 Structure Elucidation of Compound MK3



MK3

Terpenoids stigmasta-4-en-3-one **MK3**, was separated from hexane extract and was isolated as white amorphous. The UV spectrum showed absorption, λ_{max} at 282nm which is characteristic of terpenoids [100]. The IR spectrum showed absorptions for the carbonyl group and carbon-carbon double bond at 1677 cm^{-1} and 1618 cm^{-1} respectively [100]. The GC-TOFMS spectrum revealed a molecular ion peak $[M]^+$ at *m/z* 412 (parent ion) corresponding to the molecular formula of $C_{29}H_{48}O$ (Figure 3.3.1) [100].

The ^{1}H-NMR spectrum of **MK3** (Table 3.3, Figure 3.3.2) indicated six methyl protons at δ 0.78, 0.68, 0.82, 0.83, 0.80 and 0.70 assigned to H-18, H-19, H-21, H-26, H-27 and H-29, respectively. The eleven methylene protons were resonated at δ 1.99, 2.24, 2.40, 1.80, 1.49, 2.02, 1.58, 1.82, 2.34, 1.23, and 1.23 assigned to H-1, H-2, H-6, H-7, H-11, H-12, H-15, H-16, H-22, H-23 and H-28, respectively. The other eight methine protons appeared at δ 1.41, 0.88, 1.00, 1.10, 1.64, 1.42 and 1.69 assigned to H-8, H-9, H-14, H-17, H-20, H-24 and H-25 and one olefinic proton at H-4 was resonated

at δ 5.69 as a singlet. From the ^{1}H-NMR spectra of compound **MK3** were identical to the compound stigmasta-4-en-3-one [100].

The ^{13}C-NMR spectrum of **MK3** (Table 3.3, Figure 3.3.3) showed the presence of the twenty-nine signals total carbons due to three quaternary carbons, one carbonyl group, six methyl groups, eight methine groups and eleven methylene groups. The carbonyl group appeared at δ 199.8 assigned to C-3, six methyl groups were resonated at δ 12.2, 17.5, 18.8, 19.9, 19.0 and 12.0 assigned to C-18, C-19, C-21, C-26, C-27 and C-29, respectively. Thus, eight methine groups appeared at δ 35.7, 53.9, 55.9, 56.1, 36.2, 45.9 and 29.2 assigned to C-8, C-9, C-14, C-17, C-20, C-24 and C-25, respectively while C-4 appeared at δ 123.8 more de-shielded than the other methine group due to existing a double bond and a carbonyl group. The eleven methylene groups were resonated at δ 35.8, 33.9, 33.0, 32.1, 21.1, 39.7, 24.3, 28.3, 34.1, 26.1 and 23.1 assigned to C-1, C-2, C-6, C-7, C-11, C-12, C-15, C-16, C-22, C-23 and C-28, respectively. The quaternary carbons were C-5, C-10 and C-13 showed the signals at δ 171.9, 38.7 and 42.5.

Furthermore, the evidence from COSY, HSQC and HMBC spectra also confirmed the planar structure of **MK3**. The carbon resonances in Table 3.3 of compound **MK3** was in close agreement to stigmasta-4-en-3-one which was isolated from *Schizonepeta tenuifolia* Briquet [100].

The HMBC cross peaks between H-2 and C-3, and CH_3-28 and C-3 indicated the carbonyl group was located at C-3, the COSY correlations from H-15 to H-23 and HMBC cross peaks between CH_3-20 and C-17/C-20/C-22, CH_2CH_3-28/29 and C-23/C-24/C-25, and CH_3-26 and C-24/C-25/C-27 revealed the side chain was situated at C-17.

According to the aforementioned evidence, analyses of 1D and 2D NMR spectra and comparison with literature of stigmasta-4-en-3-one confirmed that the structure of **MK3** was stigmasta-4-en-3-one [100]. Table 3.3 showed the ^{1}H NMR, ^{13}C-NMR data and comparison with literature value of **MK3**.

Table 3.3: ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) of **MK3** in $CDCl_3$

Position	δ_H (mult.; *J*(Hz))	Reference[100]	δ_C	Reference[100]
1	1.99	-	35.8	35.9
2	2.24	-	33.9	34.1
3	-	-	199.8	199.9
4	5.69,s	5.74,s	123.8	124.0
5	-	-	171.9	171.9
6	2.40	-	33.0	33.2
7	1.80	-	32.1	32.3
8	1.41	-	35.7	35.8
9	0.88	-	53.9	54.0
10	1.15	-	38.7	38.8
11	1.49	-	21.1	21.2
12	2.02	-	39.7	39.8
13	-	-	42.5	42.6
14	1.00	-	55.9	56.1
15	1.58	-	24.3	24.4
16	1.82	-	28.3	28.4
17	1.10	-	56.1	56.2
18	0.78	0.73,s	12.2	12.2
19	0.68	1.20,s	17.5	17.6
20	1.64	-	36.2	36.3
21	0.82	0.93,d,6.5	18.8	18.9
22	2.34	-	34.1	34.2
23	1.23	-	26.1	26.3
24	1.42	-	45.9	46.0
25	1.69	-	29.2	29.4
26	0.83	0.84,d,6.5	19.9	20.0
27	0.80	0.83,d, 6.5	19.0	19.2
28	1.23	-	23.1	23.3
29	0.70	0.85,t,7.5	12.0	12.1

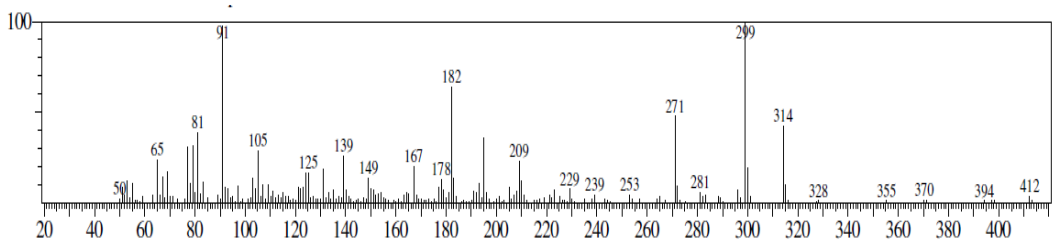


Figure 3.3.1: GC-TOFMS Spectrum of **MK3**

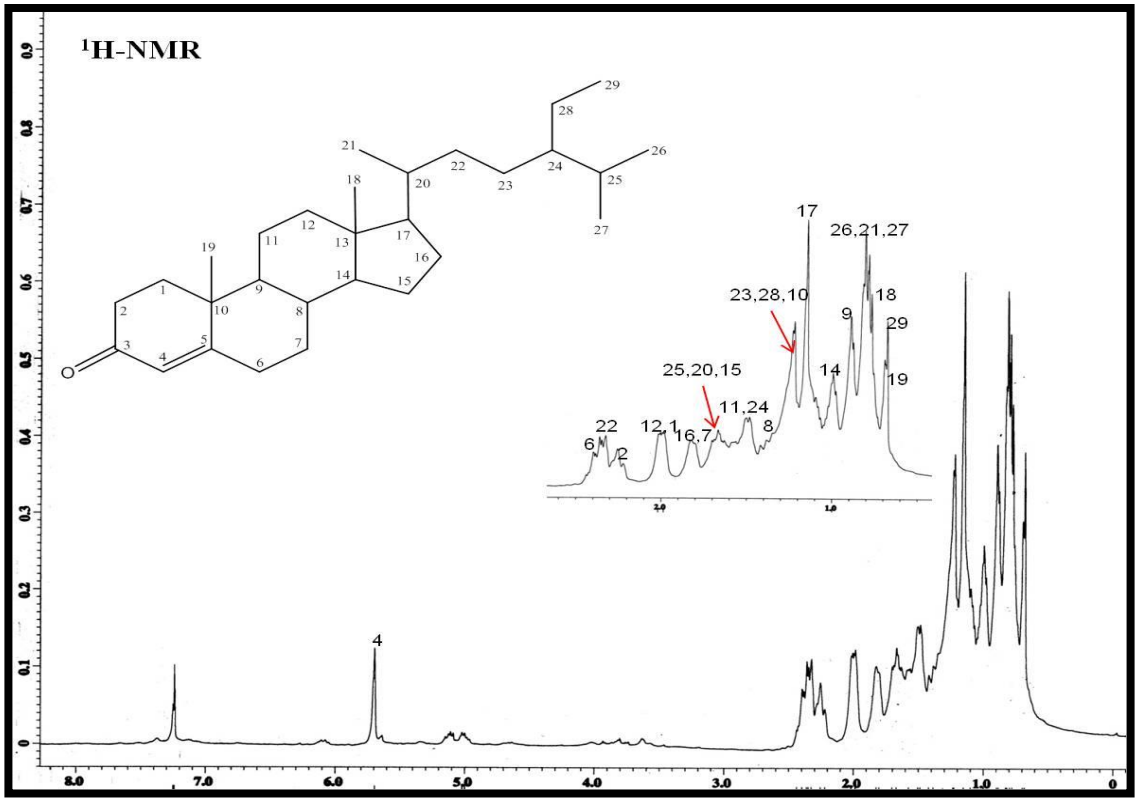


Figure 3.3.2: ^{1}H-NMR Spectrum of **MK3**

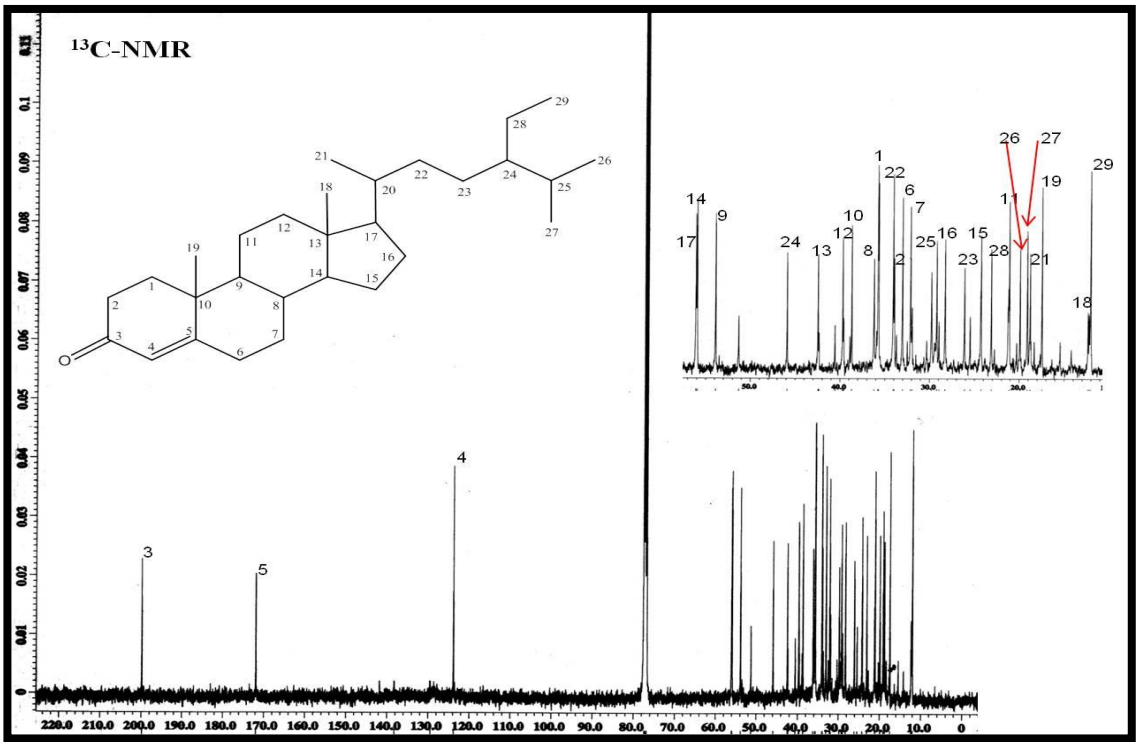


Figure 3.3.3: ^{13}C-NMR Spectrum of **MK3**

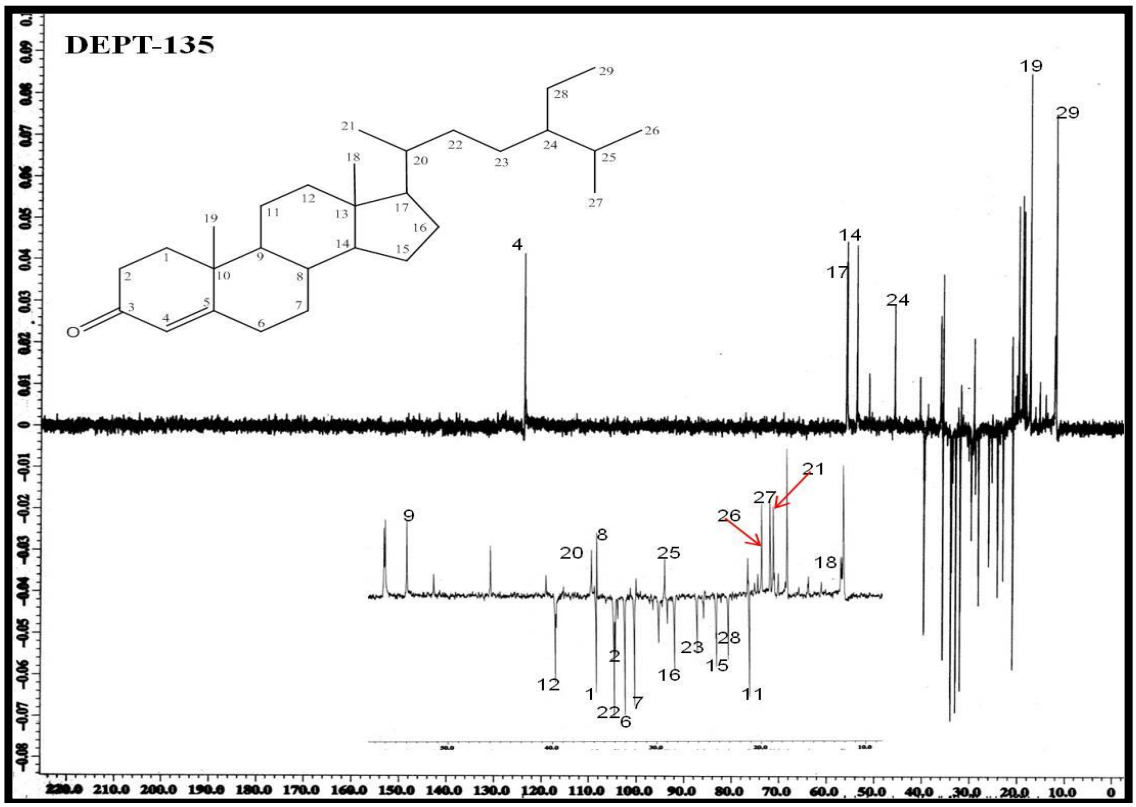
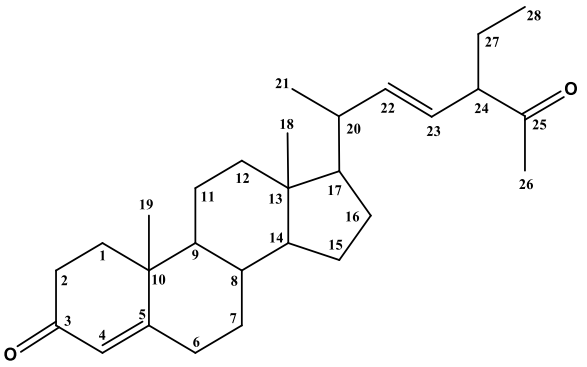


Figure 3.3.4: DEPT-135 Spectrum of **MK3**

3.4 Structure Elucidation of Compound MK4



MK4

Terpenoids stigmasta-4,22-diene-3,25-dione **MK4**, separated from n-hexane extract and was isolated as white amorphous. The UV spectrum showed absorption, λ_{max} at 282 nm. Its IR spectrum indicated the presence of carbonyl group at 1730 cm^{-1} and carbon-carbon double bond at 1618 and 1677 cm^{-1}, respectively. The GC-TOFMS spectrum revealed a molecular ion peak $[M]^+$ at *m/z* 410 (parent ion) corresponding to a molecular formula of $C_{28}H_{42}O_2$ (Figure 3.4.1).

The ^{1}H-NMR spectrum of **MK4** (Table 3.4, Figure 3.4.2) was showed closely resemble to compound **MK3** [100] and stigmasterol [101]. However, the ^{13}C-NMR spectrum showed a slightly difference due to the presence of two signals carbonyl groups. From the ^{1}H-NMR spectrum of **MK4** indicated five methyl protons at δ 1.17, 1.17, 1.17, 0.80 and 0.82 attached to H-18, H-19, H-21, H-26 and H-28, respectively.

The nine methylene protons were resonated at δ 2.50, 2.47, 5.40, 1.95, 1.52, 1.97, 1.25, 1.14 and 0.81 attached to H-1, H-2, H-6, H-7, H-11, H-12, H-15, H-16 and H-27, respectively. The other six methine protons appeared at δ 2.57, 1.38, 1.01, 1.13, 2.14 and 2.68 attached to H-8, H-9, H-14, H-17, H-20 and H-24. The three olefinic protons attached to H-4 as a singlet at δ 6.17, H-22 and H-23 as a doublet at δ 5.15 and 5.04.

The ^{13}C-NMR spectrum of **MK4** (Table 3.4, Figure 3.4.3) showed the presence of the twenty-eight signals of total carbons including three quaternary carbons, two carbonyl groups, five methyl groups, nine methine groups and nine methylene groups. In ^{13}C-NMR spectra of compound **MK4**, the two very down-field carbons were attributable to carbonyl group at C-3 and C-25 at δ 199.5 and 202.3, five methyl groups appeared at δ 11.9, 17.5, 18.7, 19.8 and 20.9 assigned to C-18, C-19, C-21, C-26 and C-27, respectively. The six methine groups were resonated at δ 34.2, 51.0, 55.9, 56.6, 36.0 and 45.8 assigned to C-8, C-9, C-14, C-17, C-20 and C-24 while the other three methine groups were resonated at δ 125.5, 137.8 and 129.8 assigned to C-4, C-22 and C-23 was more de-shielded because of the double bond. The nine methylene groups appeared at δ 34.0, 33.9, 29.7, 28.0, 20.9, 39.1, 23.1, 24.0, and 20.9 assigned to C-1, C-2, C-6, C-7, C-11, C-12, C-15, C-16, and C-27, respectively. Lastly, the other three quaternary carbons were resonated at δ 161.0, 39.2 and 42.6 assigned to C-5, C-10 and C-13.

Furthermore, the evidence from COSY, HSQC and HMBC spectra also confirmed the planar structure of **MK4**. The carbon resonances (Table 3.4) of compound **MK4** was in close agreement to **MK3** [100] and stigmasterol [101] except

for C-25 of the side chain. One set of carbon resonances are in close agreement with those of known **MK3** [100] and stigmasterol [101].

According to the analyses data of 1D and 2D NMR spectra and comparison with the data of **MK3** [100] and stigmasterol [101], it is confirmed that the structure of **MK4** was stigmasta-4,22-diene-3,25-dione. Table 3.4 showed the ^{1}H NMR, ^{13}C-NMR data and comparison to the literature values of **MK4** [100, 101].

Table 3.4: ^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz) of **MK4** in $CDCl_3$

Position	δ_H (mult.; *J*(Hz))	Reference [100]	Reference Stigmasterol[101]	δ_C	Reference [100]	Reference Stigmasterol[101]
1	2.50	-	1.81	34.0	35.9	37.3
2	2.47	-	1.79	33.9	34.1	31.6
3	-	-	3.52,m	199.5	199.9	71.8
4	6.17,s	5.74,s	2.27	125.5	124.0	42.3
5	-	-	-	161.0	171.9	140.8
6	5.40,m	-	5.36,m	29.7	33.2	121.7
7	1.95	-	1.93	28.0	32.3	31.9
8	2.57	-	1.45	34.2	35.8	31.9
9	1.38	-	0.92	51.0	54.0	51.2
10	-	-	-	39.2	38.8	36.5
11	1.52	-	1.50	20.9	21.2	21.1
12	1.97	-	1.95	39.1	39.8	39.7
13	-	-	-	42.6	42.6	42.3
14	1.01	-	1.00	55.9	56.1	56.9
15	1.25	-	1.54	23.1	24.4	24.4
16	1.14	-	1.65	24.0	28.4	28.4
17	1.13	-	1.12	56.6	56.2	56.1
18	1.17	0.73,s	0.70,s	11.9	12.2	11.0
19	1.17	1.20,s	1.01,s	17.5	17.6	21.2
20	2.14	-	2.00	36.0	36.3	40.5
21	1.17	0.93,d,6.5	1.02,d,7.5	18.7	18.9	21.2
22	5.15	-	5.09	137.8	34.2	138.3
23	5.04	-	4.96	129.8	26.3	129.2
24	2.68	-	1.52	45.8	46.0	51.2
25	-	-	1.53	202.3	29.4	31.9
26	0.80	0.84,d,6.5	0.795,d,6.5	19.8	20.0	21.2
27	0.81	0.83,d, 6.5	0.85,d,6.5	20.9	19.2	19.0
28	0.82	-	1.43	21.1	23.3	25.4
29	-	-	0.804,t,7.5	-	-	12.1

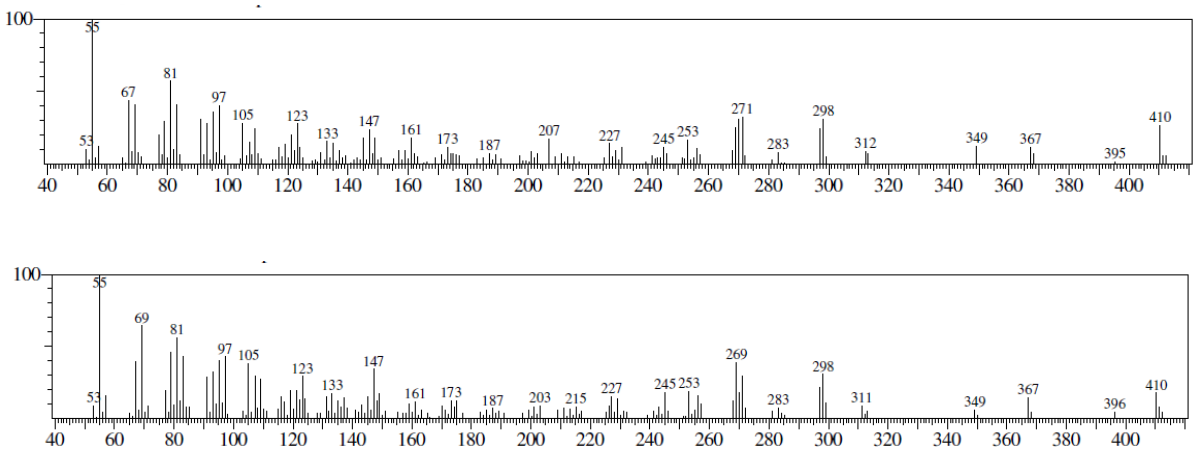


Figure 3.4.1: GC-TOFMS Spectrum of **MK4**

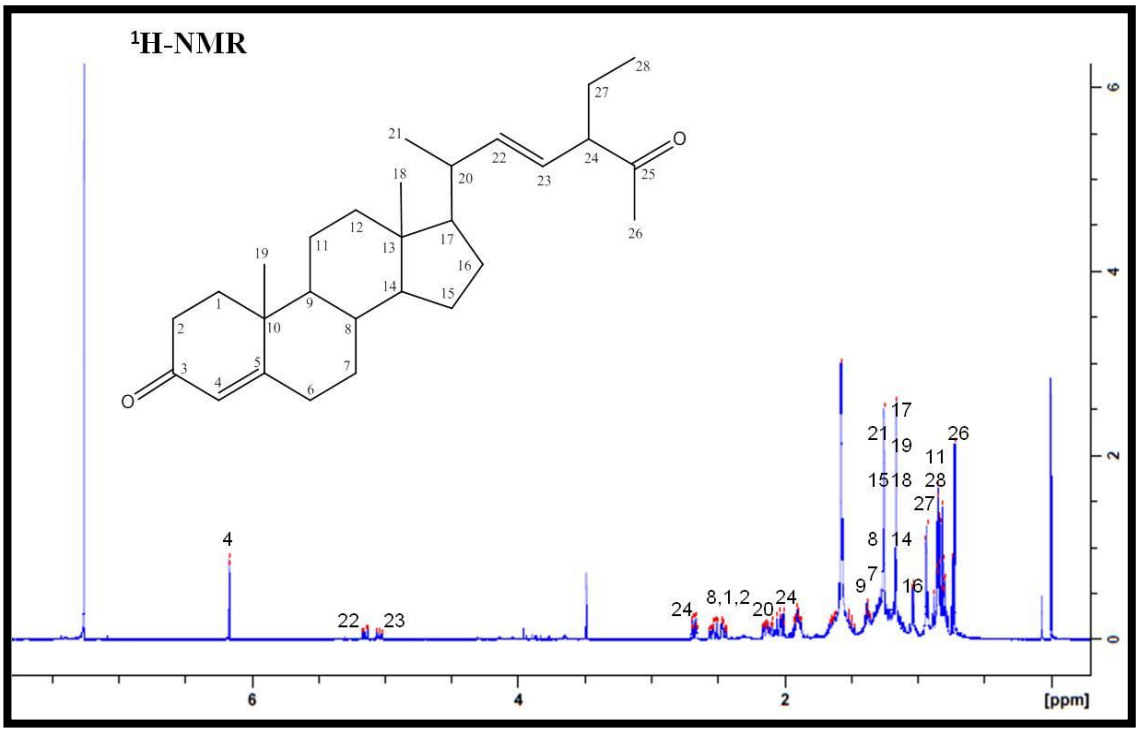


Figure 3.4.2: ^{1}H-NMR Spectrum of **MK4**

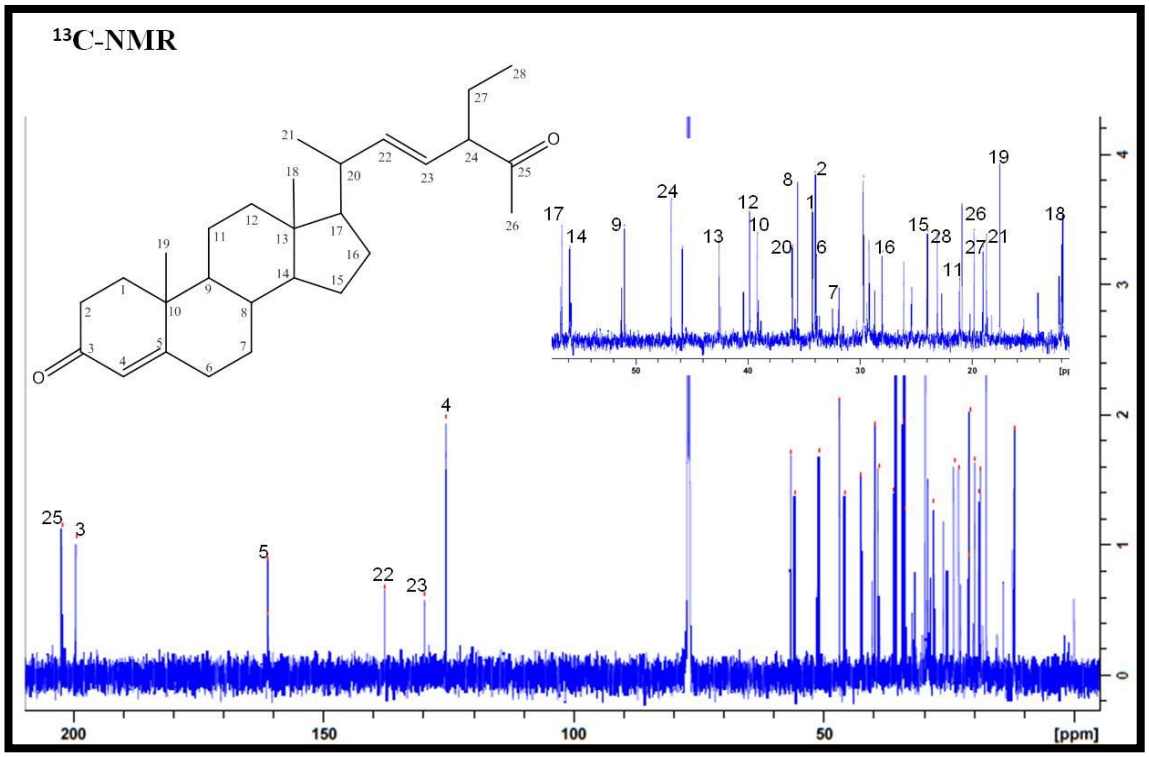


Figure 3.4.3: ^{13}C-NMR Spectrum of **MK4**

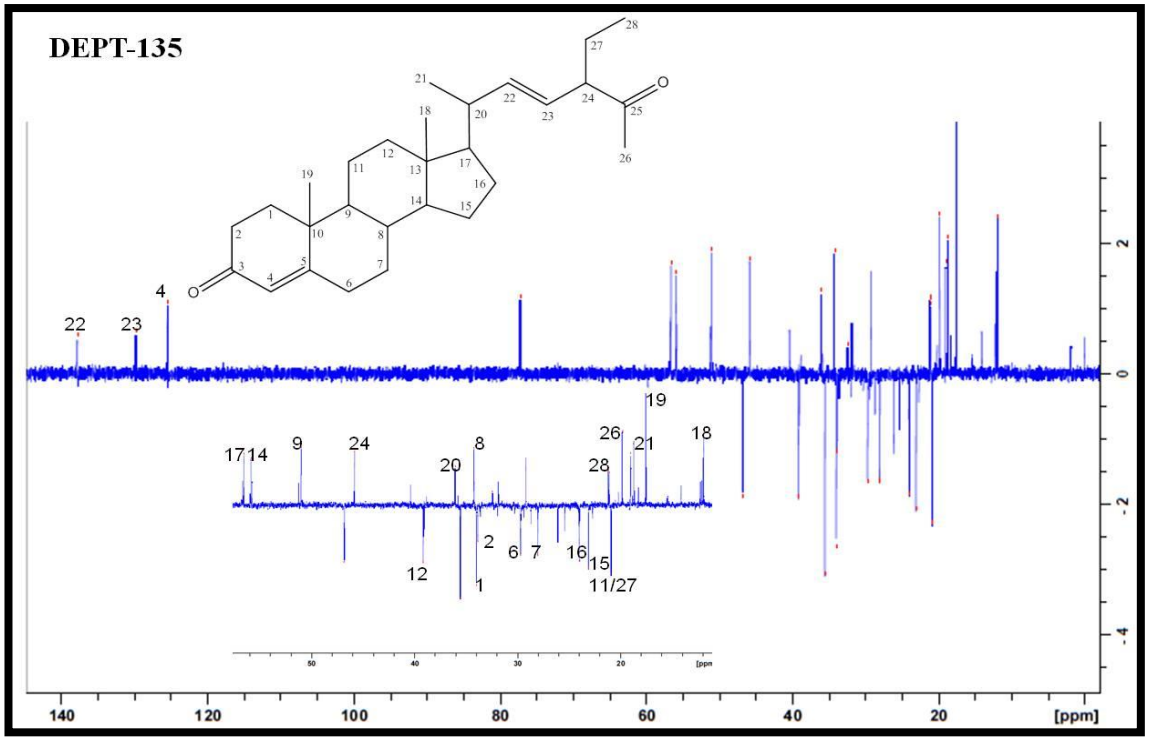
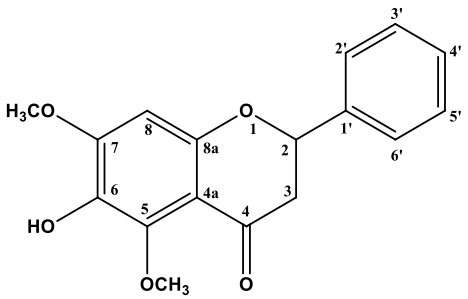


Figure 3.4.4: DEPT-135 Spectrum of **MK4**

3.5 Structure Elucidation of Compound MK5



MK5

Flavanone 6-hydroxy-5,7-dimethoxy-2-phenylchroman-4-one **MK5**, was obtained as brown powder. The UV spectrum showed absorption at λ_{max} 243, 288 and 348, suggesting that **MK5** has a flavanone skeleton [102]. Its IR spectrum showed the presence of OH group at 3371 cm^{-1}, carbonyl group at 1660 cm^{-1} and aromatic group at 1613 cm^{-1} [102]. The positive ion HREIMS of **MK5** showed a molecular ion peak $[M+H]^+$ at *m/z* 301.1063 corresponding to molecular formula $C_{17}H_{16}O_5$ (Figure 3.5.2).

The ^{1}H-NMR spectrum of **MK5** (Table 3.5, Figure 3.5.3) showed a typical system of flavanones and an unsubstituted ring B. From the ^{1}H-NMR spectrum indicated the presence of two methoxy groups which were resonated at δ 3.88 and 3.94 H-5 and H-7. The aromatic proton appeared at δ 6.16 as a singlet assigned to H-8 and the other aromatic protons in the unsubstituted phenyl group in ring B were resonated at δ 7.37 - 7.39 as multiplet, integrated to five protons. The methylene group (H-3α and H-

3β) was resonated at δ 2.79 and 3.05 as a triplet with coupling constant, *J* value 12.8 Hz. The methine group (H-2) was appeared at δ 5.43 as a doublet with *J* value 12.8 Hz while the hydroxyl group (OH-6) appeared at δ 5.28 as a singlet.

The ^{13}C-NMR spectrum of **MK5** (Table 3.5, Figure 3.5.4) indicated the presence of seventeen carbon signals which consist of six quaternary carbon, one carbonyl group, two methoxyl groups, six aromatic carbons, one methine group and one methylene group. The carbonyl group was resonated at δ 189.44 assigned to C-4 while the two methoxy groups appeared at δ 56.3 and 56.4 assigned to C5-OCH_3 and C7-OCH_3. The methine and methylene carbons appeared at δ 80.0 and δ 45.9 assigned to C-2 and C-3. The aromatic carbons were resonated at δ 89.8, 126.4 assigned to C-8, C-2' and C-6', whereas at δ 128.9 assigned to C-3', C4' and C5', respectively. The other six quaternary carbons were resonated at δ 105.9, 155.0, 149.5, 152.6, 127.8 and 138.5, assigned to C4a, C5, C6, C7, C8a and C1', respectively. The carbon resonances of compound **MK5** were assigned in Table 3.5 by comparing the carbon resonances of 6-hydroxy-5,7,8-dimethoxy-2-phenylchroman-4-one [102] and also taking into consideration that the hydroxyl group substitution at C-6.

In the HMBC spectrum of **MK5** the typical carbonyl function was confirmed by correlation between H-3α/C=O and H-3β/C=O. HMBC correlations between H-8/C-4a, H-8/C-8a, H-8/C-6, H-8/C-7 and H-8/C-5 and correlations between H-5-OCH_3/C-5 and H-7-OCH_3/C-7 demonstrated that the methoxy groups located at C-5 and C-7, respectively. The above HMBC correlations and the presence of one downfield carbonyl

group in the ^{13}C-NMR spectrum suggested that **MK5** has a flavanone skeleton rather than a flavone [102]. The positions of the two methoxy groups at C-5 and C-7 were established from the connectivity indicated in the 2D HMBC experiments (Figure 3.5.8). The methoxy protons at δ 3.88 and 3.94 showed the long-range HMBC correlations to the signals of C-5 and C-7, respectively.

All assignments were supported by COSY, HSQC and HMBC spectra. Table 3.5 showed the ^{1}H-NMR, ^{13}C NMR data and HMBC correlations. Therefore, it can be concluded that **MK5** is established as a new flavanone and elucidated as 6-hydroxy-5,7-dimethoxy-2-phenylchroman-4-one. This is the first isolation of such compound with the absence of methoxy group at C-8.

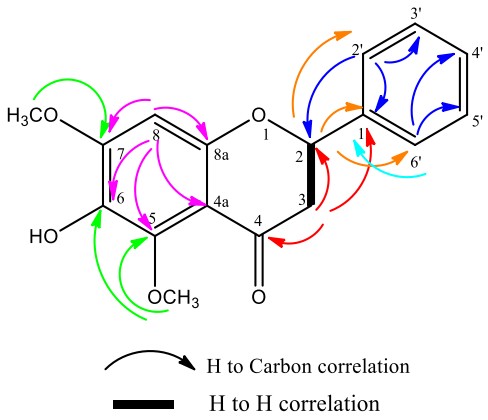


Figure 3.5.1: Important Correlations on COSY and HMBC of **MK5**

Table 3.5: ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) of **MK5** in $CDCl_3$

Position	**δ_H (mult.; *J*(Hz))**	**δ_C**	**HMBC**
2	5.43,d,12.8	80.0	1', 2', 6'
3α	2.79,dd,12.8	45.9	2, 1', 4
3β	3.05,dd,12.8	-	-
4	-	189.4	-
4a	-	105.9	-
5	-	155.0	-
5-OCH_3	3.88,s	56.3	5, 6
6	-	149.5	-
6-OH	5.28,s	-	-
7-OCH_3	3.94,s	56.4	7
7	-	152.6	-
8	6.16,s	89.8	4a, 8a, 6, 7, 5
8a	-	127.8	-
1'	-	138.5	-
2',6’	7.37	126.4	2', 6', 3', 4', 5'
3',5’	7.39	128.9	1'
4'	7.37	128.9	-

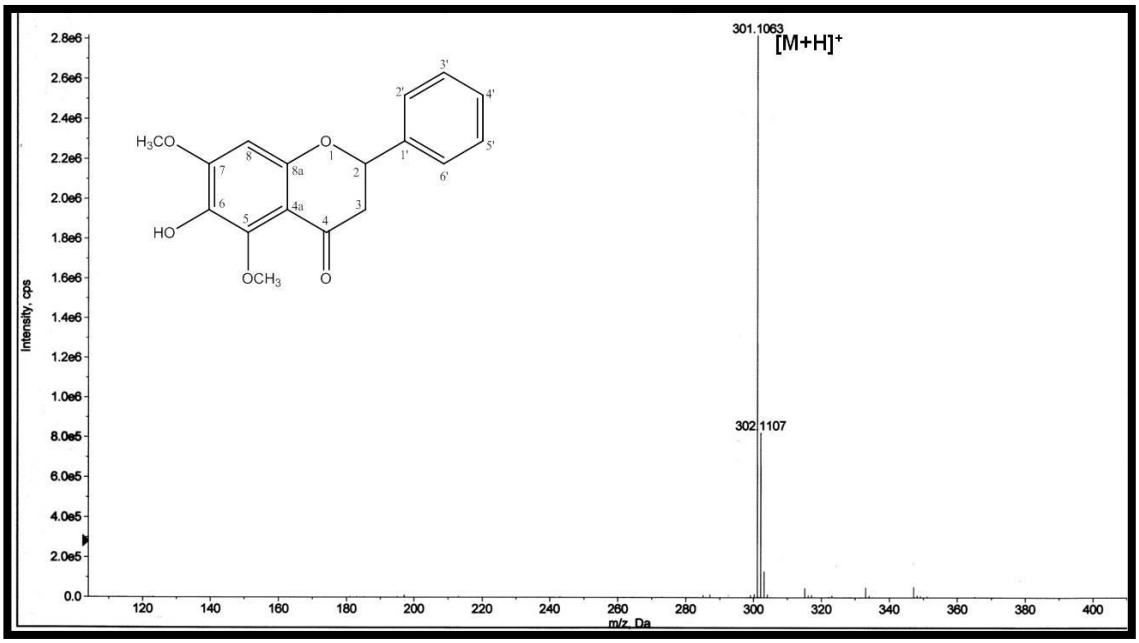


Figure 3.5.2: The MS Spectrum of **MK5**

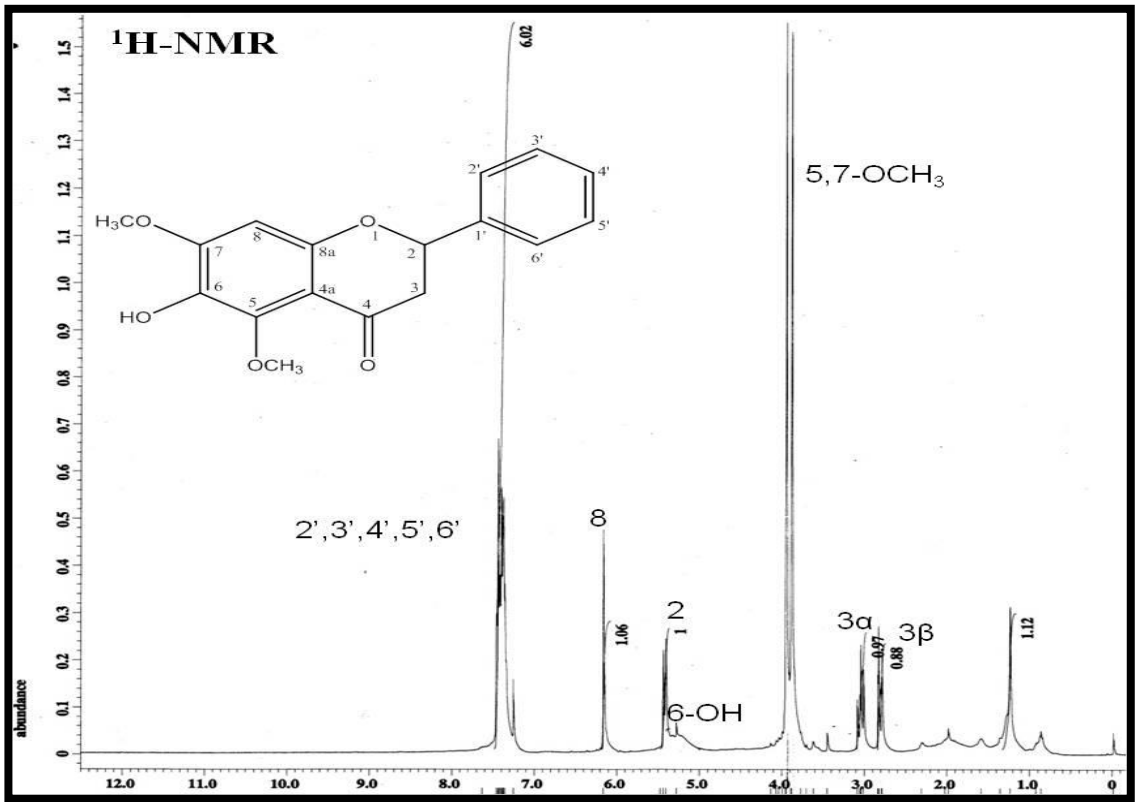


Figure 3.5.3: ^{1}H-NMR Spectrum of **MK5**

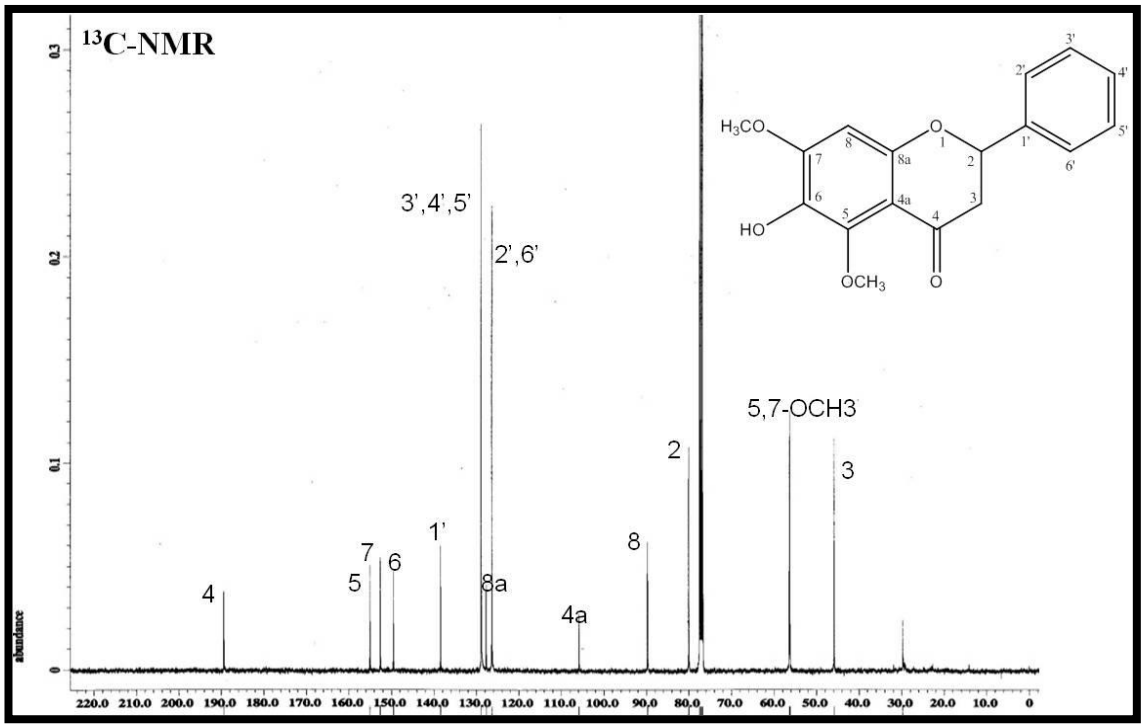


Figure 3.5.4: ^{13}C-NMR Spectrum of **MK5**

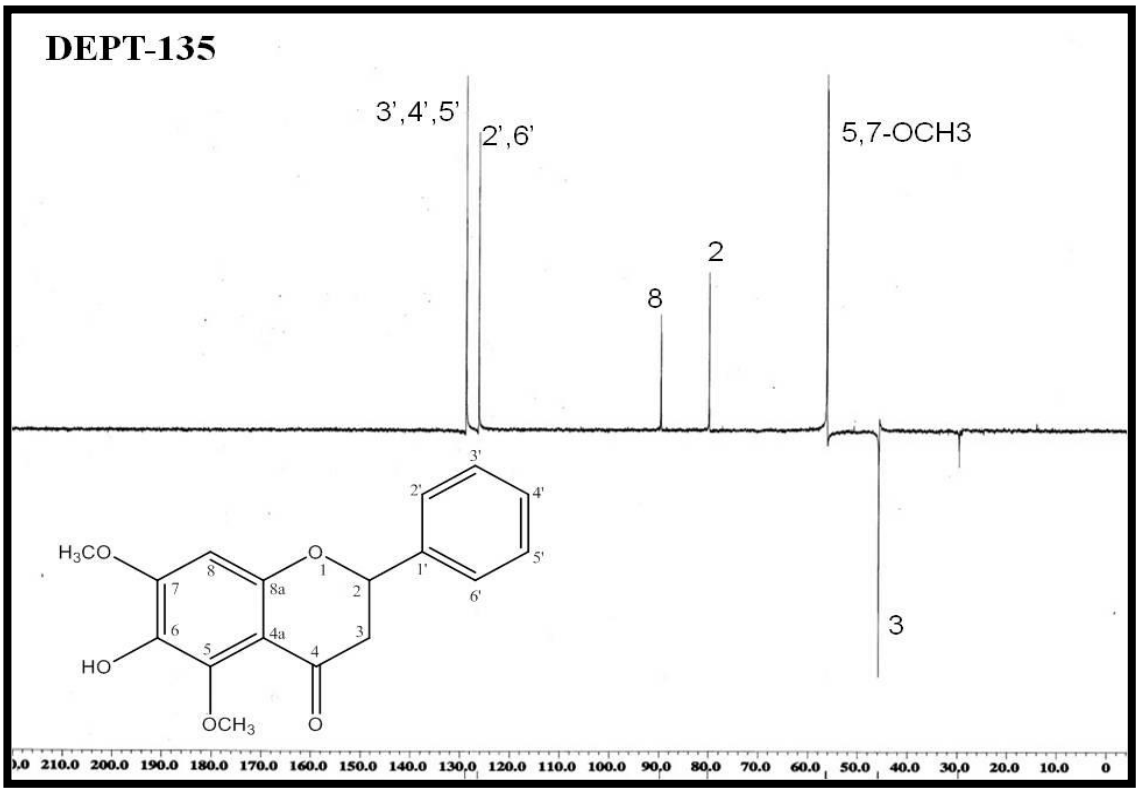


Figure 3.5.5: DEPT-135 Spectrum of **MK5**

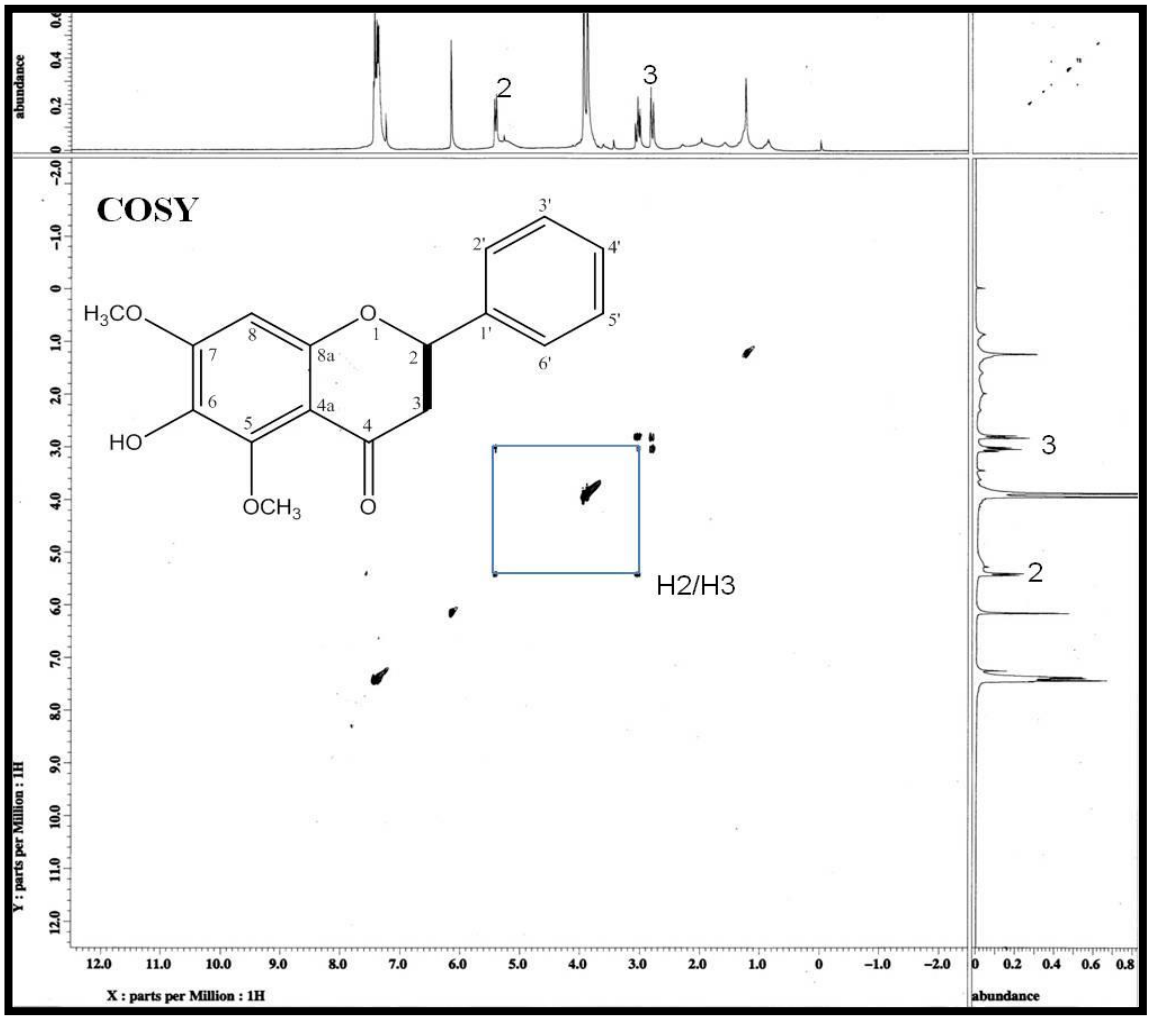


Figure 3.5.6: COSY Spectrum of **MK5**

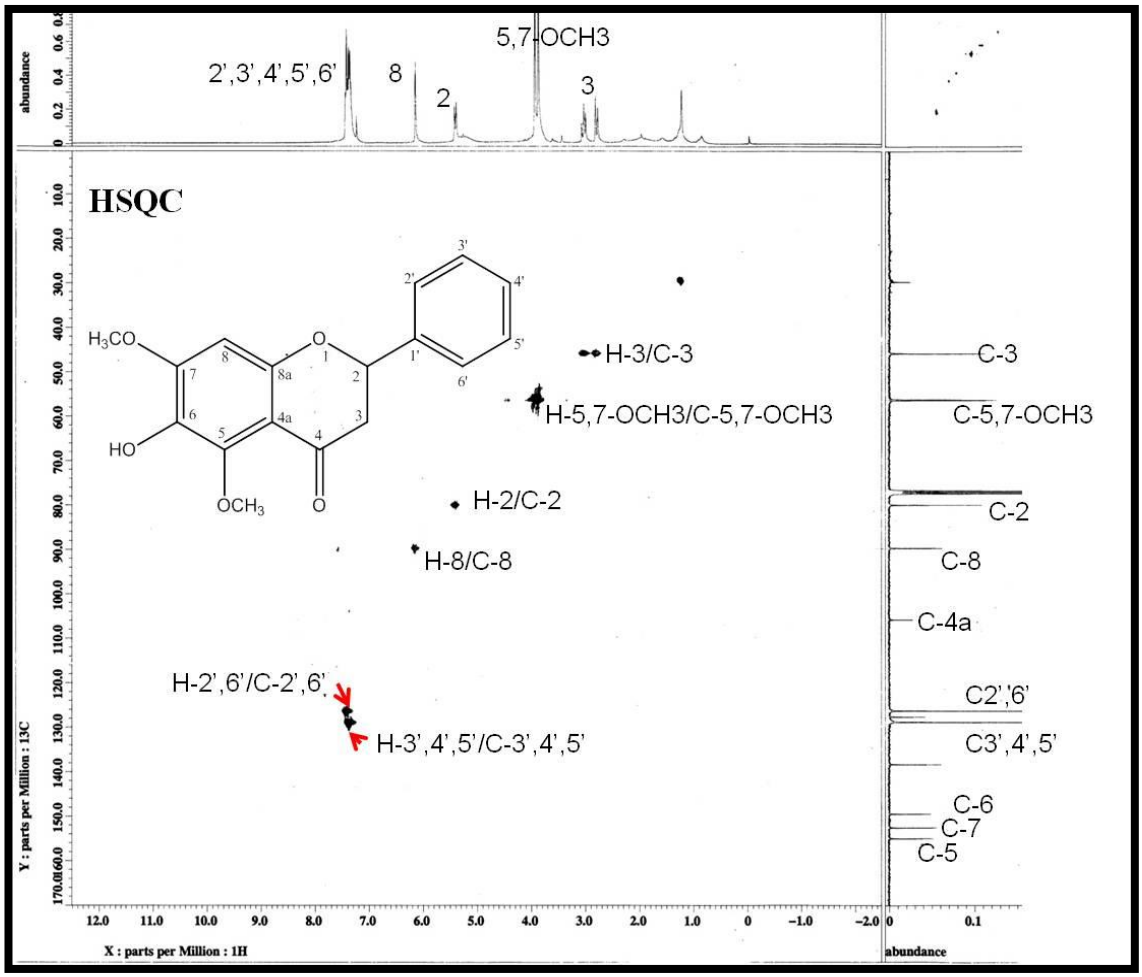


Figure 3.5.7: HSQC Spectrum of **MK5**

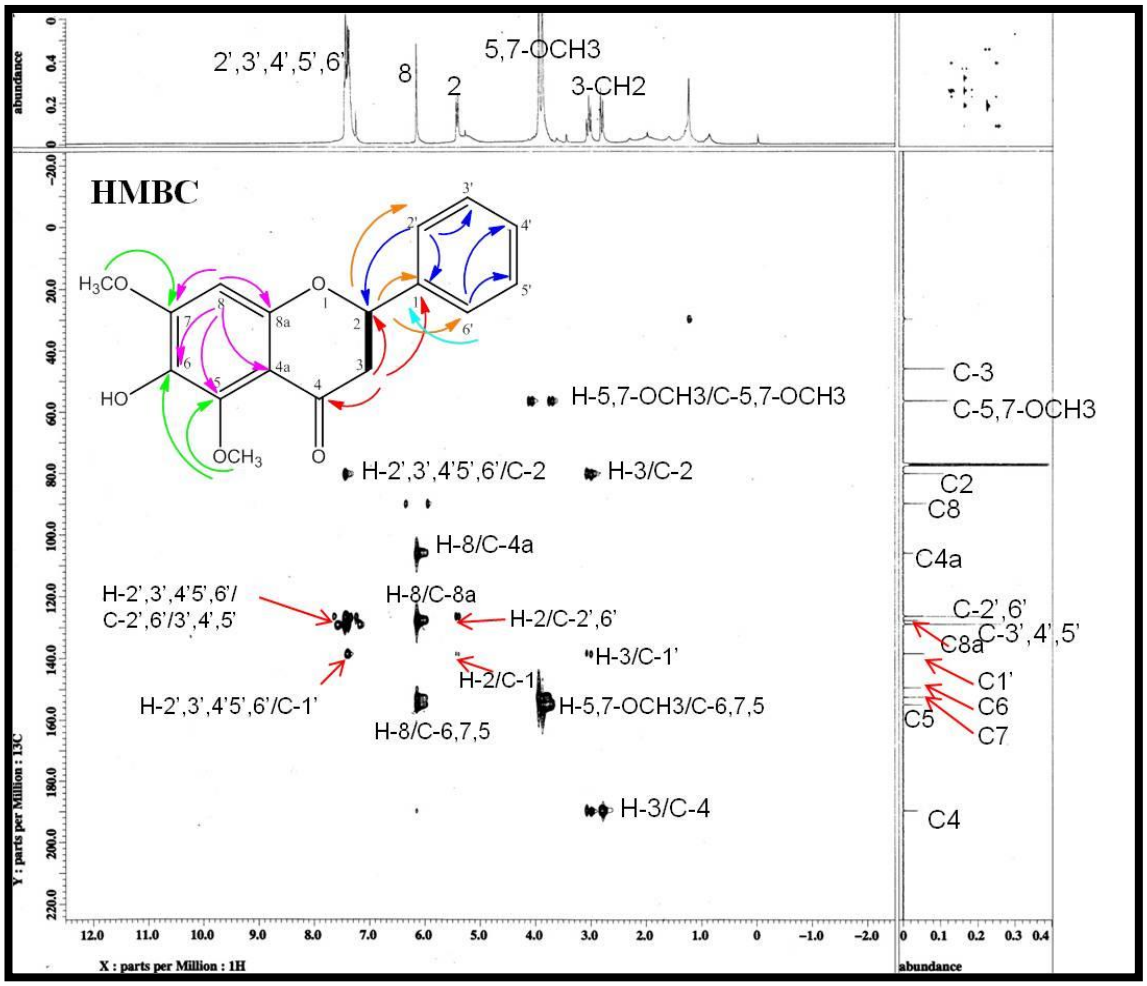
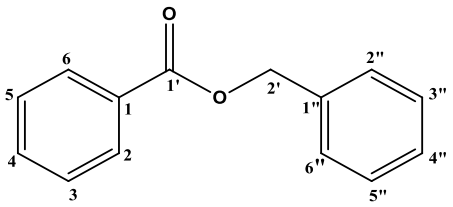


Figure 3.5.8: HMBC Spectrum of **MK5**

3.6 Structure Elucidation of Compound MK6



MK6

Compound benzyl benzoate, **MK6** was separated from dichloromethane extract and was obtained as colourless liquid with m.p 122^{o}C. The UV spectrum showed absorptions, at λ_{max} 228, 273 and 300 nm which indicated the occurrence of an aromatic ring [103]. The IR spectrum displayed the absorption of carbonyl group at 2924, 1722, 1270, 1109 and 711cm^{-1} while the benzene ring group at 3066 and 3033cm^{-1} [103]. The GC-TOFMS spectrum showed the molecular ion peak [M^{+}] at m/z 212 corresponding to the molecular formula of $C_{14}H_{12}O_2$ (Figure 3.6.1) [103].

The ^{1}H spectrum of **MK6** (Table 3.6, Figure 3.6.2) showed the existence of the methylene group was resonated at δ 5.36 as a singlet assigned to H-2'. The aromatic protons appeared at δ 7.39 and 7.35 assigned to H-2",6" and H-3",4",5", respectively. The other aromatic protons resonated at δ 8.07, 7.44 and 7.55 were assigned to H-2,6, H-3,5, and H-4, respectively.

The ^{13}C spectrum of **MK6** (Table 3.6, Figure 3.6.3) indicated the presence of fourteen signals of total carbons which consists to two quaternary carbons, one carbonyl group, one methylene group and ten aromatic carbons. The carbonyl group resonated at δ 166.5 was assigned to C-1' while the methylene group appeared at δ 66.8, was assigned to C-2'. The aromatic carbons were resonated at δ 129.8, 128.4 and 133.1 and assigned to C-2,6, C-3,5, and C-4, respectively whereas the other aromatic protons appeared at δ 128.2, 128.7 and 128.3 were assigned to C-2",6", C-3",5" and C-4", respectively. The other two quaternary carbons as C-1 and C-1" were resonated at δ 130.2 and 136.1, respectively.

The long-range HMBC correlations spectrum of **MK6** (Figure 3.6.) showed the cross peaks between H-2' and C-1', and H-2' and C-1" indicating the carbonyl group was located at C-1'. The COSY showed correlations from H-2 to H-6 and H-2" to H-6", respectively.

From the previous study, **MK6** has also been isolated from the stem bark of *Artabotrys odoratissimus* [104]. The carbon resonances of compound **MK6** are assigned in Table 3.6. The spectral data and the literature values confirmed the occurrence and structure of the **MK6** [104]. All assignments were supported by DEPT-135, COSY and HMBC spectra.

Table 3.6: ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) of **MK6** in $CDCl_3$

Position	δ_H (mult.; *J*(Hz))	δ_C	HMBC
1	-	130.2	-
2,6	8.07,m	129.8	3,4,5,1'
3,5	7.44,m	128.4	-
4	7.55,m	133.1	2,3,4,5,1'
1'	-	166.5	-
2'	5.36,s	66.8	2",6",3",5",1",1'
1"	-	136.1	-
2",6"	7.39,m	128.2	2',2",3",4",5",6"
3",5"	7.35,m	128.7	-
4"	7.35,m	128.3	-

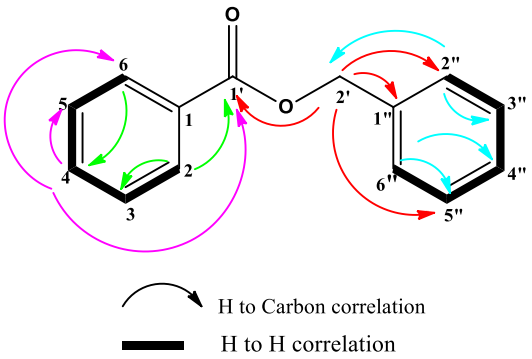


Figure 3.6.1: Important Correlations on COSY and HMBC of **MK6**

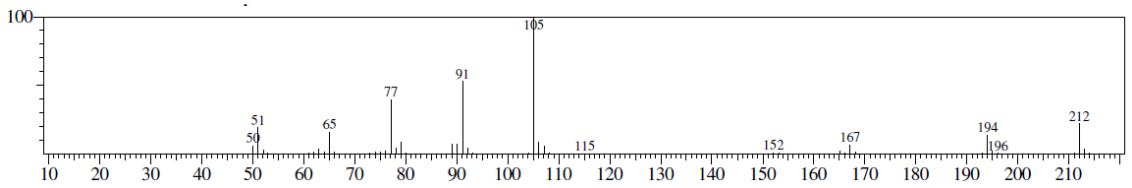


Figure 3.6.2: GC-MS Spectrum of **MK6**

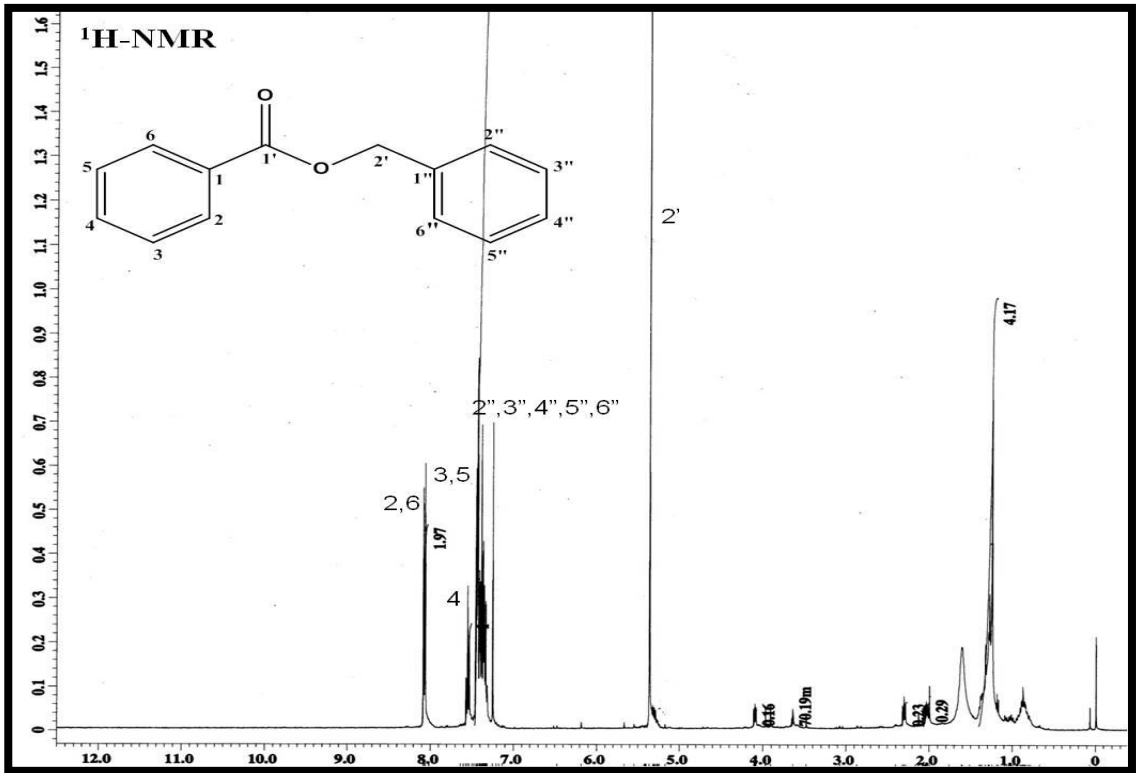


Figure 3.6.3: ^{1}H-NMR Spectrum of **MK6**

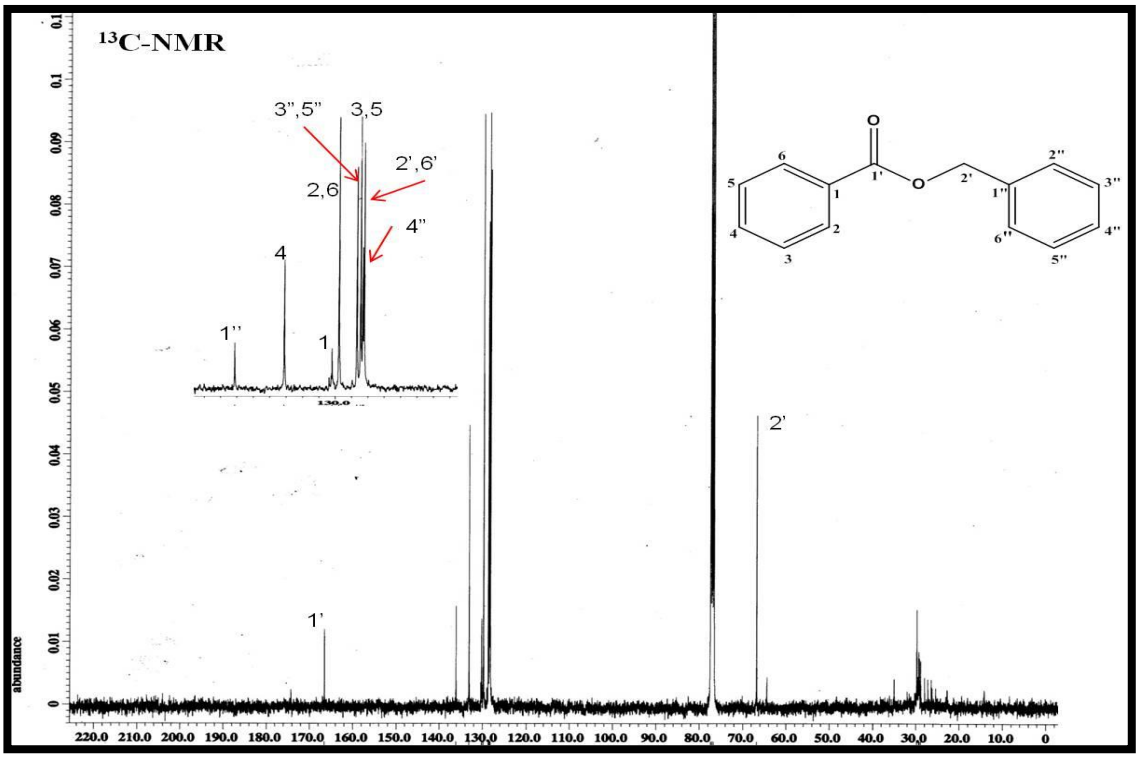


Figure 3.6.4: ^{13}C-NMR Spectrum of **MK6**

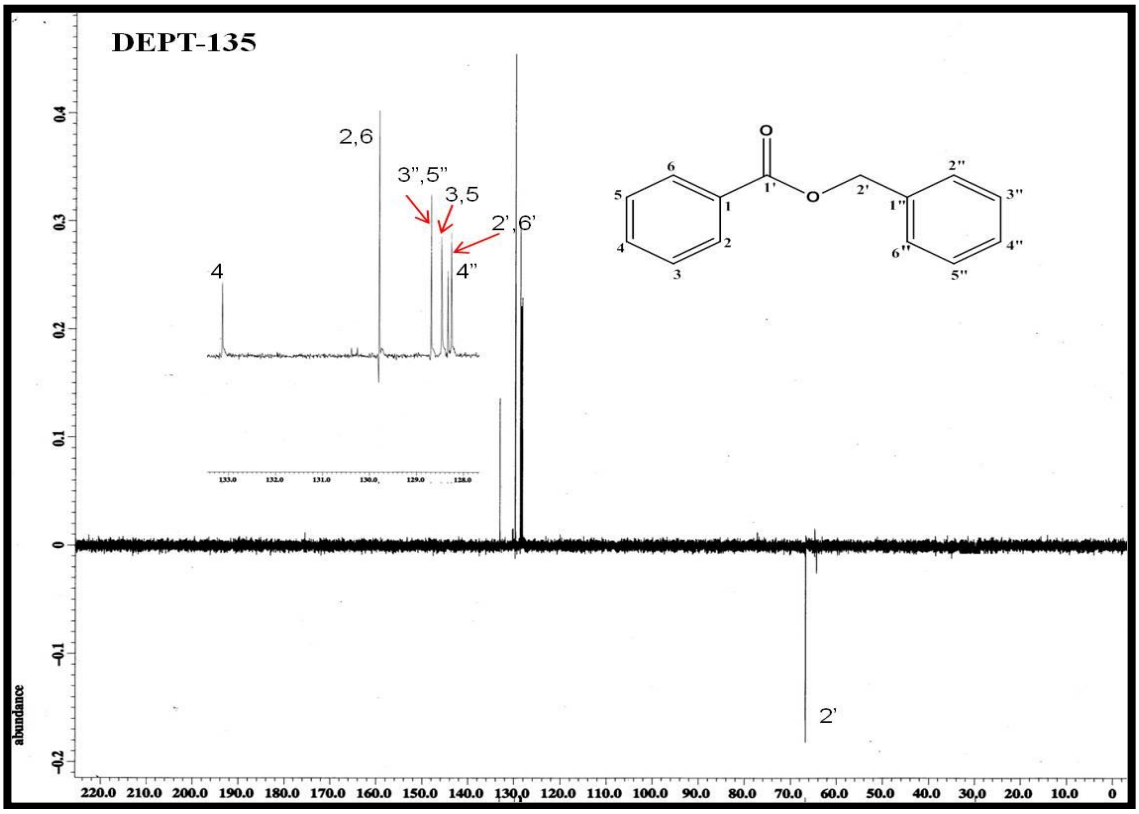


Figure 3.6.5: DEPT-135 Spectrum of **MK6**

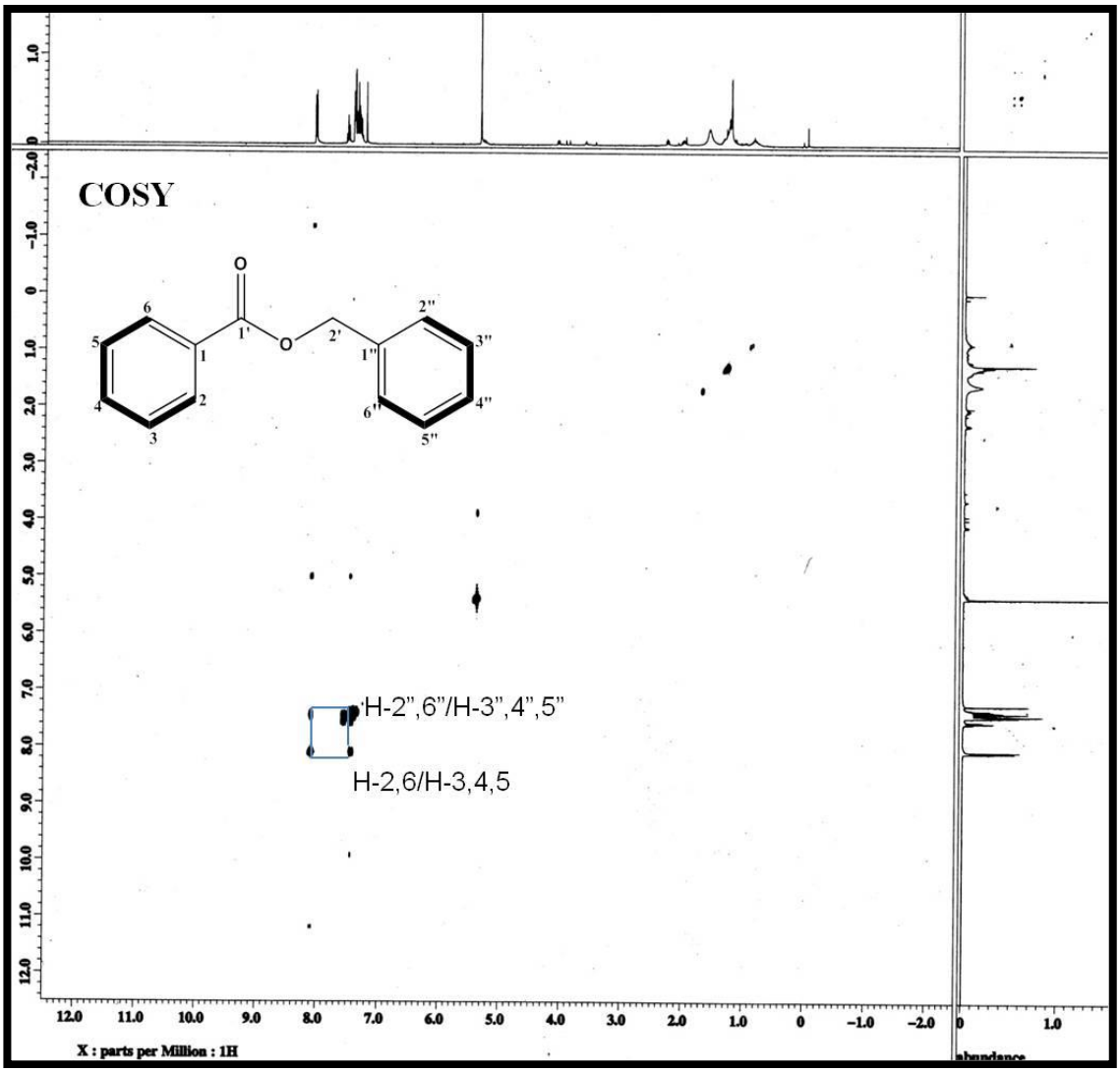


Figure 3.6.6: COSY Spectrum of **MK6**

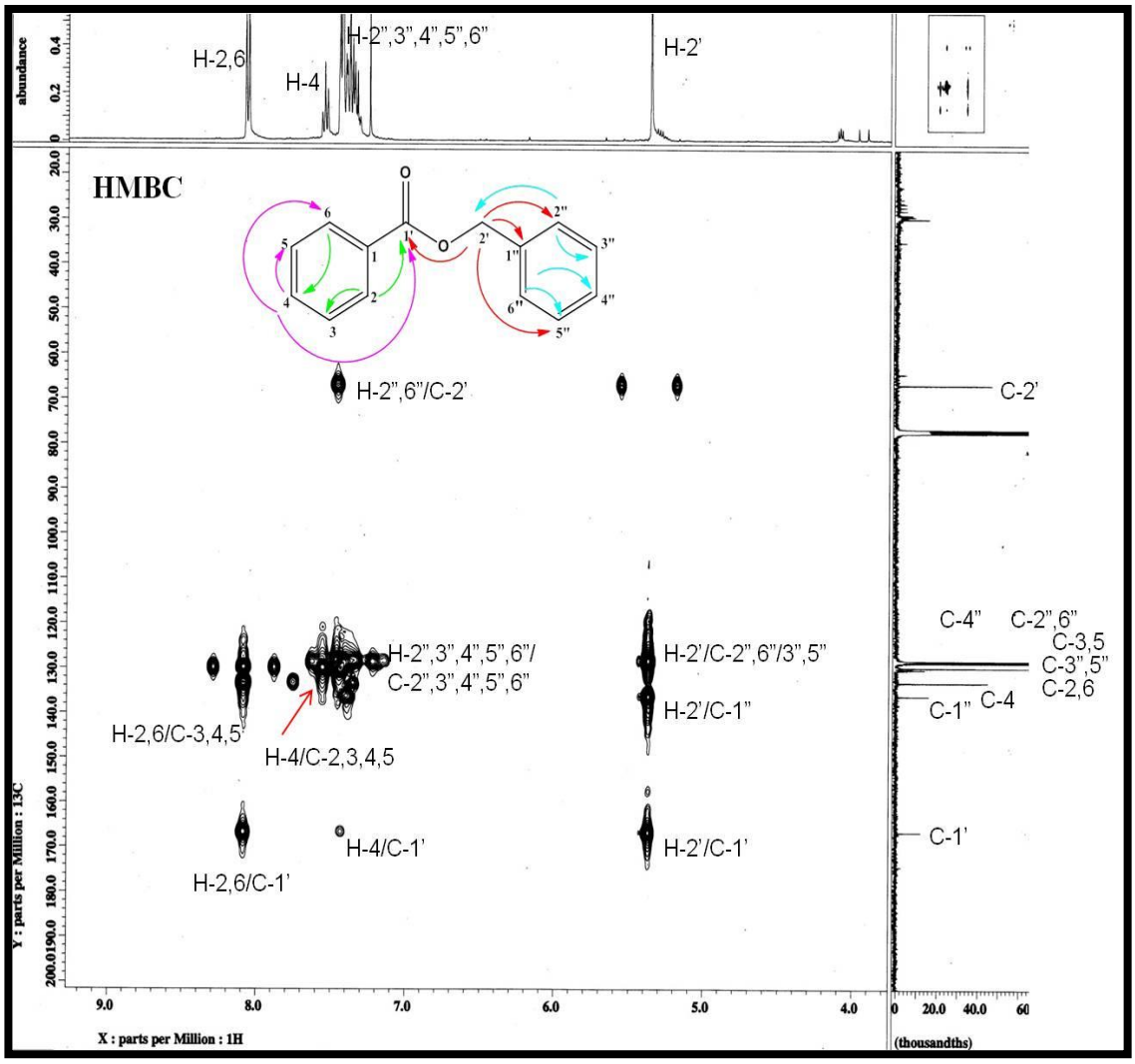
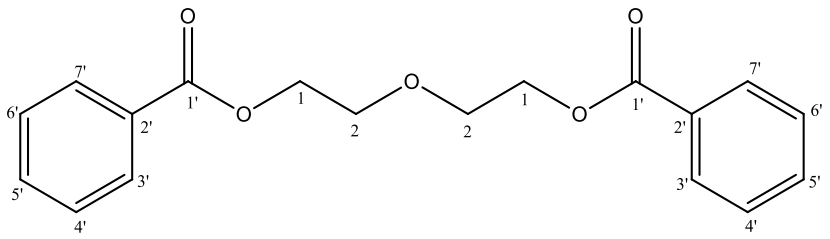


Figure 3.6.7: HMBC Spectrum of **MK6**

3.7 Structure Elucidation of Compound MK7



MK7

Compound oxybis(ethane-2,1-diyl) dibenzoate, **MK7**, was separated from dichloromethane extract by using PLTC after fractionation by column chromatography (CC). The UV spectrum of **MK7** exhibited absorptions at λ_{max} 197, 229, 275, 331, 346, 392, 405, 429, 463 and 485 nm. Its IR spectrum showed bands for carbonyl at 1667 cm^{-1} and aromatic at 1615cm^{-1}. The MS spectrum of **MK7** gave the molecular ion peak $[M+H]^+$ at *m/z* 315.1231 corresponding to the molecular formula $C_{18}H_{18}O_5$ (Figure 3.7.2).

The ^{1}H-NMR spectrum of **MK7** (Table 3.7, Figure 3.7.3) revealed the characteristic of unsubstituted aromatic protons signal appeared at δ 7.42-8.05, and were assigned to C-3', C-4', C-5', C-6' and C-7'. The methylene protons were resonated at δ 4.50 and 3.90 as a triplet, assigned to H-1 and H-2, respectively.

The ^{13}C-NMR spectrum of **MK7** (Table 3.7, Figure 3.7.4) indicated the presence of eighteen signals of carbons including two quaternary carbon, two carbonyl groups, four methylene groups and ten aromatic carbons. The two carbonyl groups were resonated at δ 166.6 and assigned to C-1'. The four methylene groups appeared at δ 64.0 and 69.2 were assigned to C-1 and C-2. The regions of aromatic carbons were resonated at δ 128.3-133.0, assigned to C-3', C-4', C-5', C-6' and C-7'. And the other two quaternary carbons appeared at δ 130.0, assigned to C-2'.

This compound is a symmetrical molecule and has similar position in ^{1}H- and ^{13}C-NMR spectra. From the LC-MS showed that more than 90% the molecular ion mass (*m/z*) of **MK7** was matched with the library.

The long-range HMBC and COSY correlations of **MK7** showed the position of the substituents of the two phenyl groups at C-2'. From COSY correlation indicated H-2 correlated with H-1 and H-3',7' correlated with H-4',6' and H-5' correlated with H-4',6' (Figure 3.7.6). The HMBC correlation showed that the methylene group (H-1) was correlated with methylene group (C-2). Another correlation indicated that the two carbonyl groups were positioned at C-1', as shown from correlations between H-1 and H3',7' with C-1' in ^{3}J (Figure 3.7.8). These are important correlations to prove that the two carbonyl groups are substituted at C-1'.

MK7 is confirmed to be oxybis(ethane-2,1-diyl) dibenzoate based on the 1D and 2D-NMR spectra data and comparison with the literature values. Table 3.7 showed the ^{1}H NMR, ^{13}C-NMR and HMBC data of **MK7**.

Table 3.7: ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) of **MK7** in $CDCl_3$

Position	δ_H (mult.; *J*(Hz))	δ_C	HMBC
1	4.50,t	64.0	2,1'
2	3.90,t	69.2	2
1'	-	166.6	-
2'	-	130.0	-
3',7'	8.05	129.7	4',6',5',1'
4', 6'	7.42	128.3	3',7'
5'	7.56	133.0	3',5'

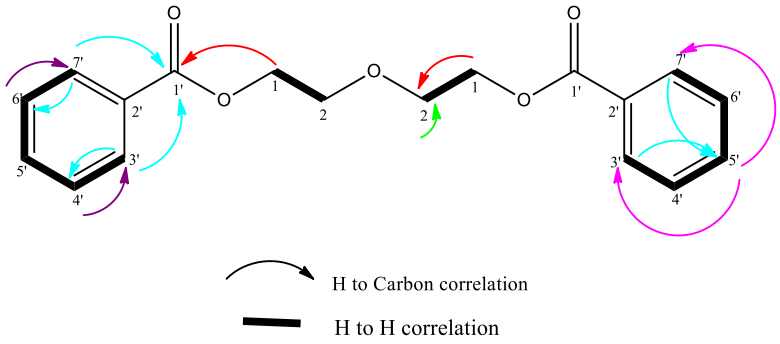


Figure 3.7.1: Important Correlations on COSY and HMBC of **MK7**

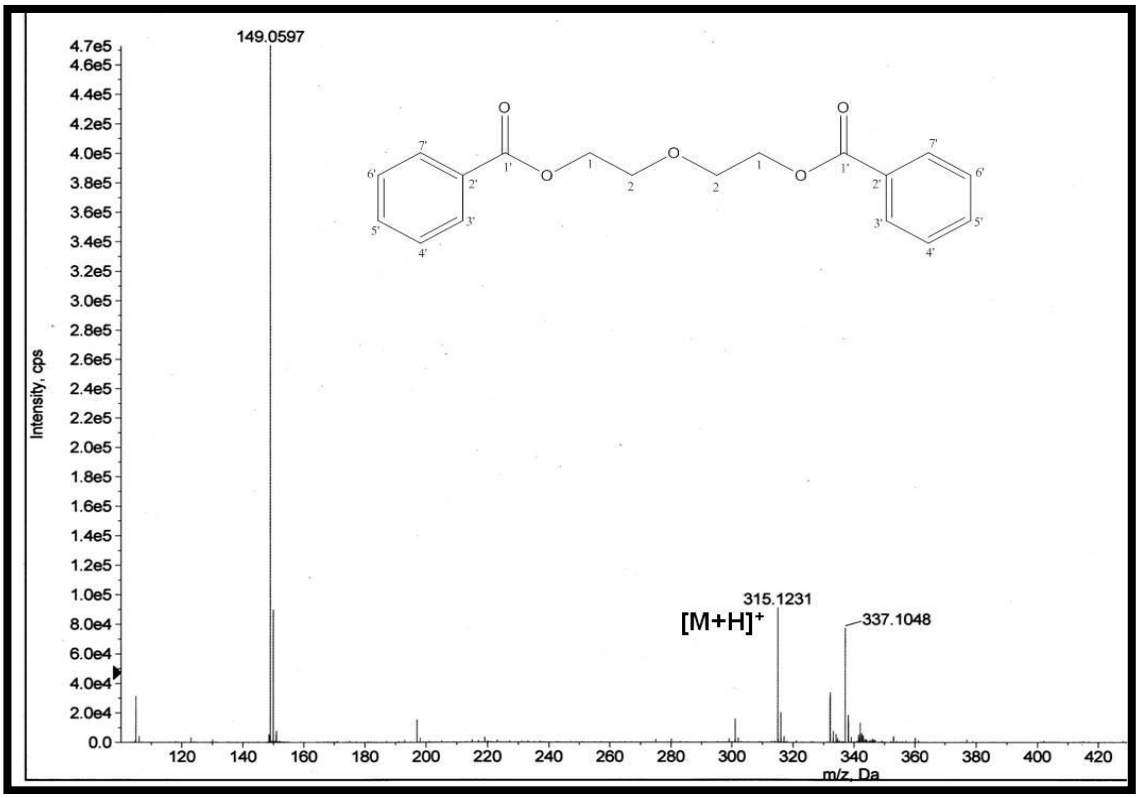


Figure 3.7.2: The MS Spectrum of **MK7**

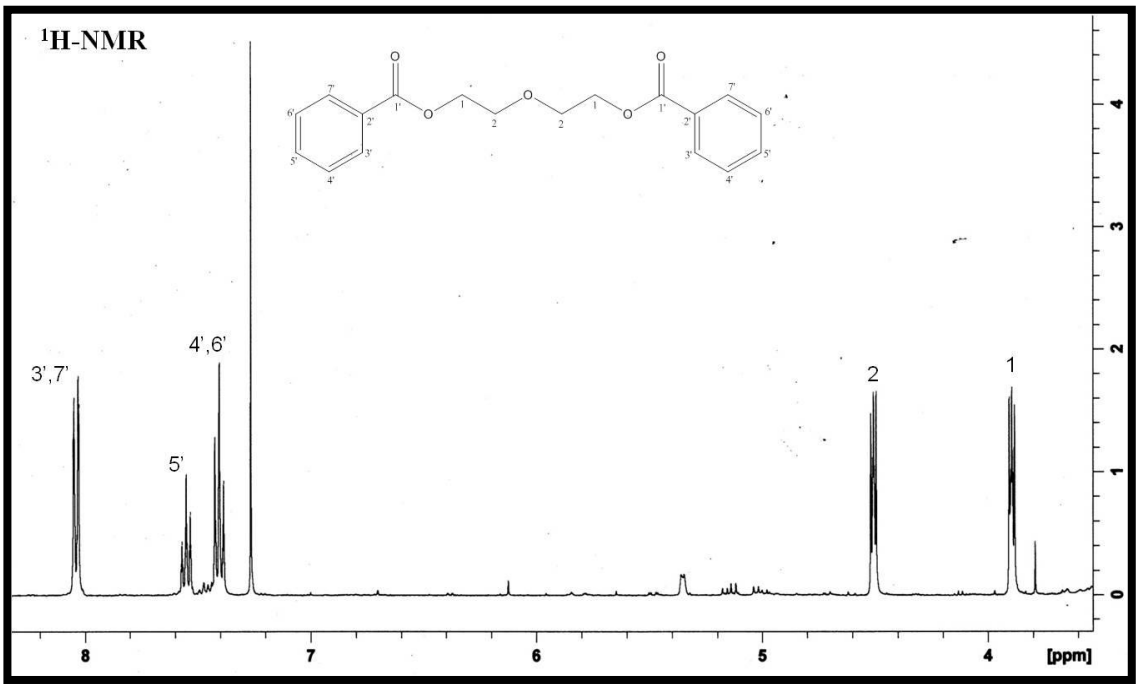


Figure 3.7.3: ^{1}H-NMR Spectrum of **MK7**

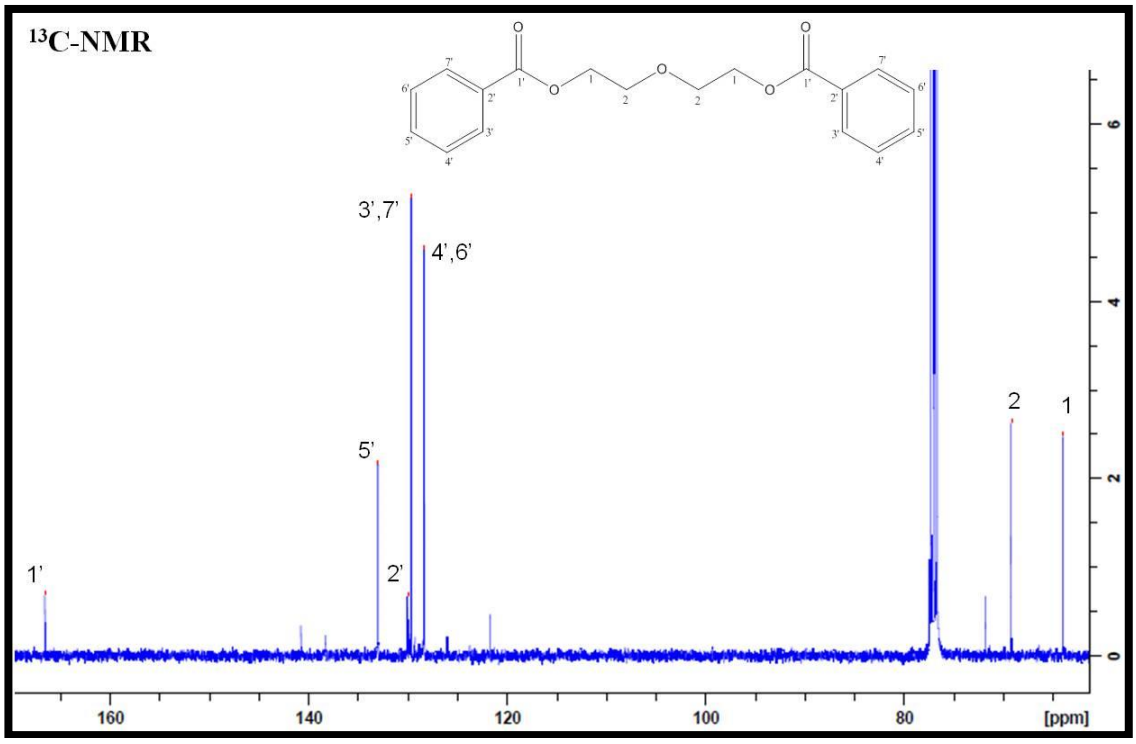


Figure 3.7.4: ^{13}C-NMR Spectrum of **MK7**

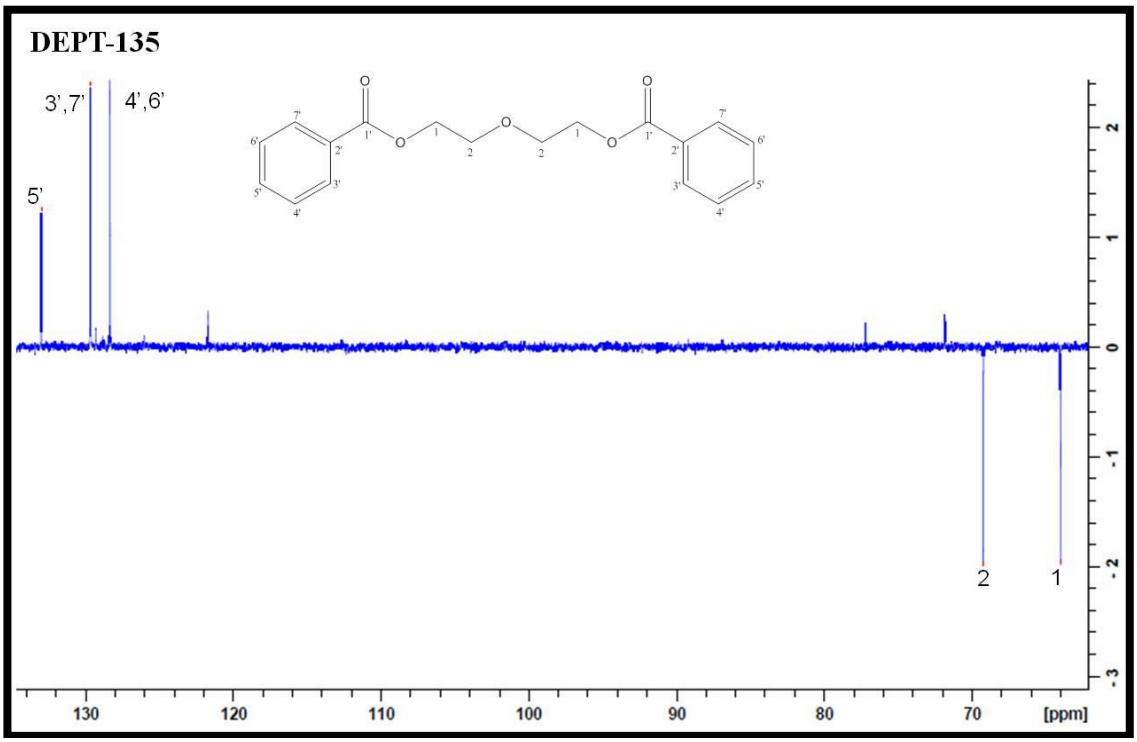


Figure 3.7.5: DEPT-135 Spectrum of **MK7**

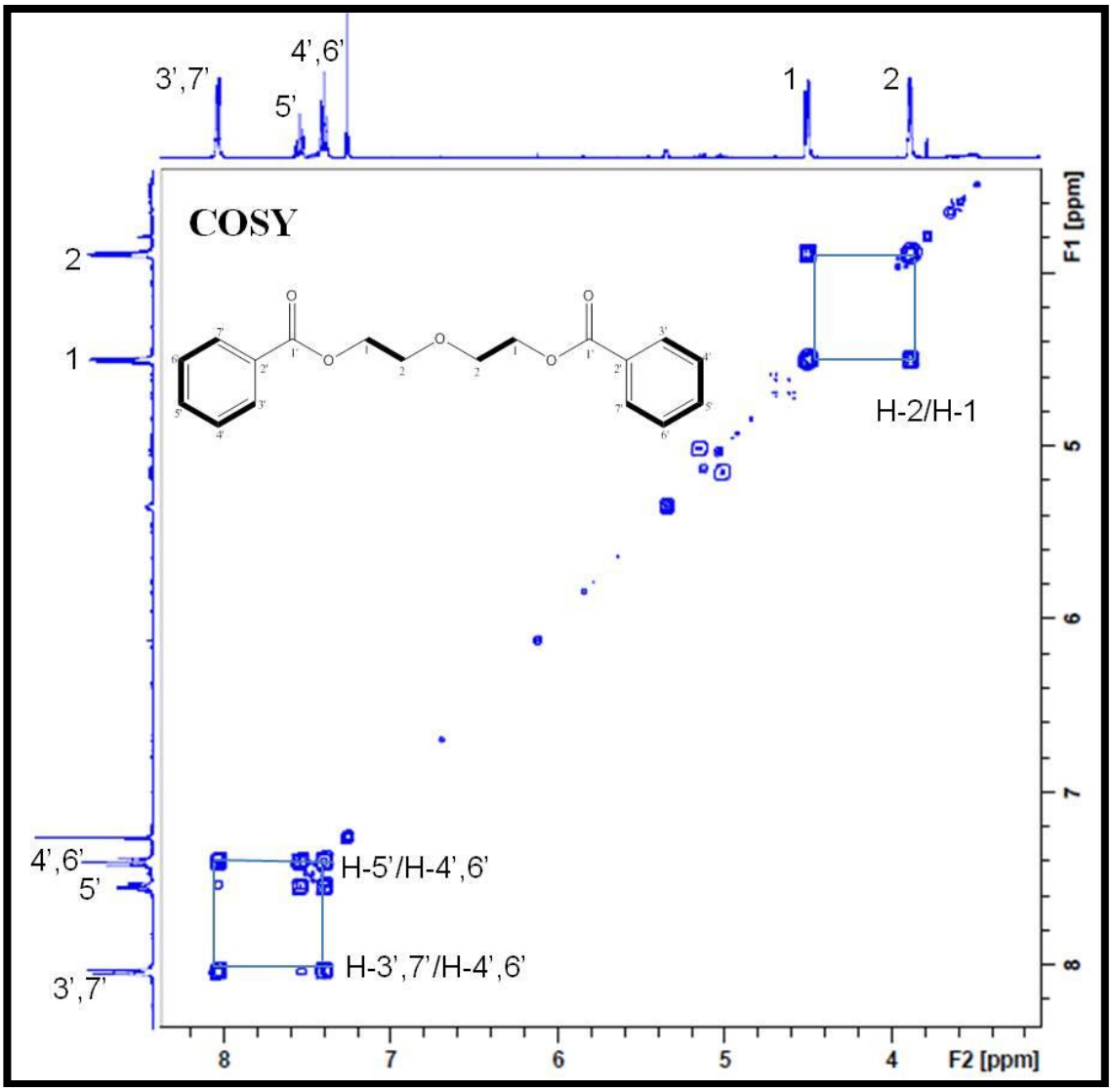


Figure 3.7.6: COSY Spectrum of **MK7**

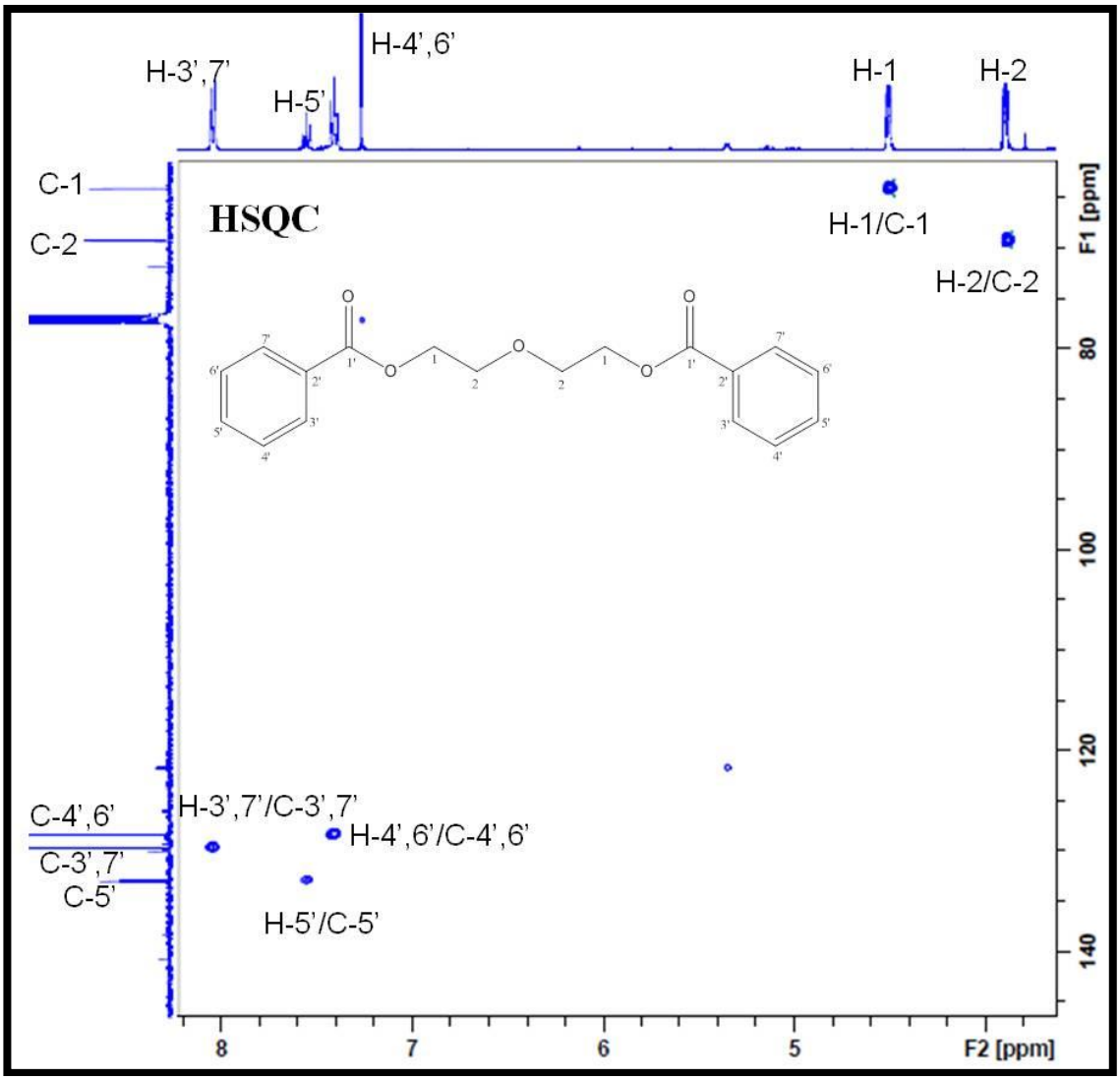


Figure 3.7.7: HSQC Spectrum of **MK7**

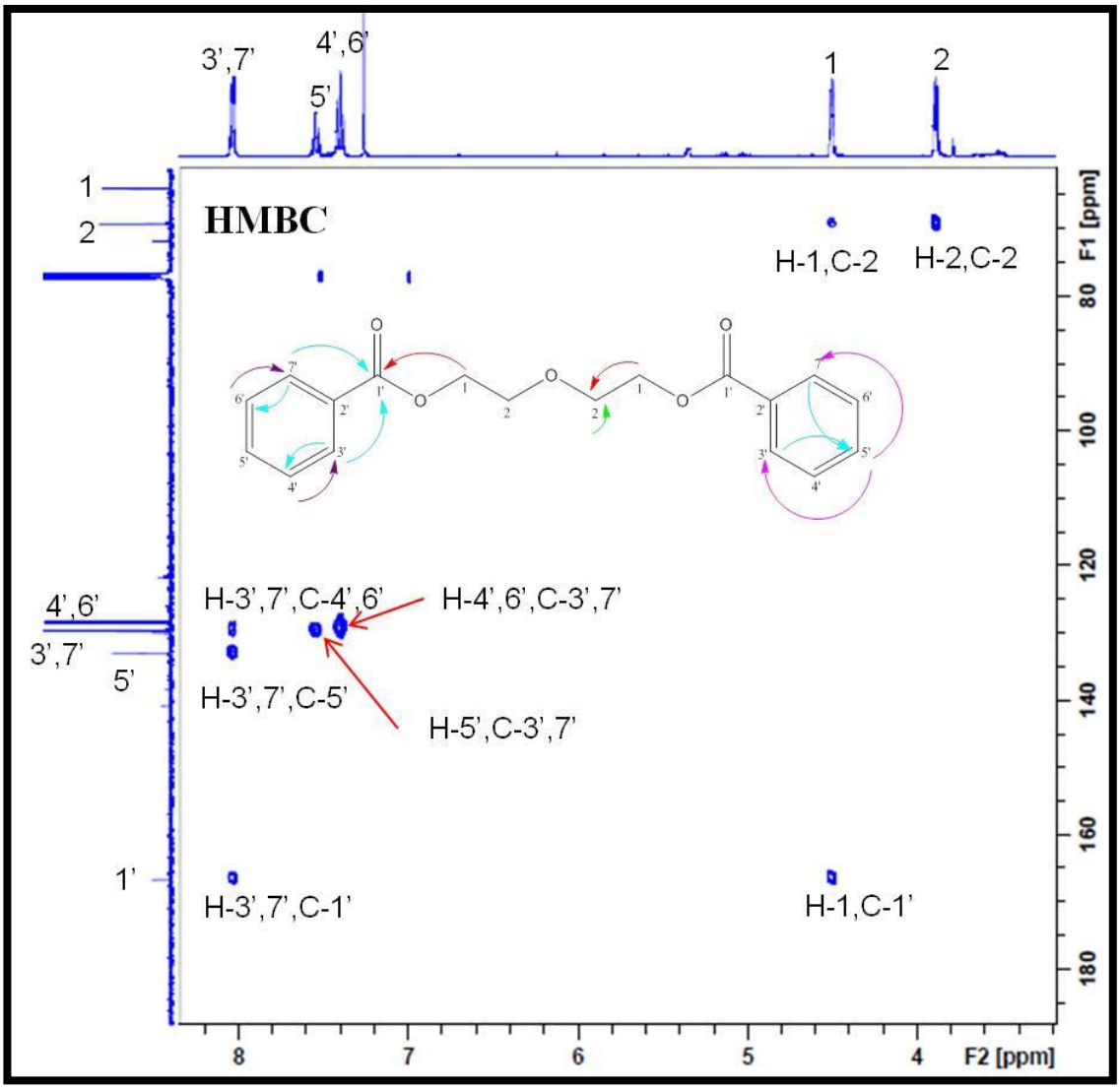
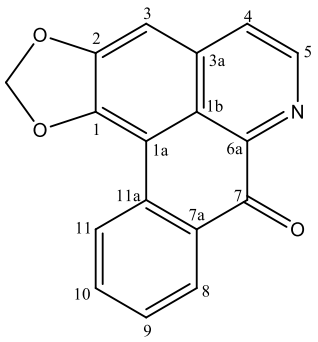


Figure 3.7.8: HMBC Spectrum of **MK7**

3.8 Structure Elucidation of Compound MK8



MK8

Oxoaporphine alkaloid, liriodenine, **MK8** separated from dichloromethane extract and isolated as yellow needles, gave positive result to Dragendorff's test. The characterization of intense yellow fluorescent colouration in solution, indicates the characteristic of an oxoaporphine chromophore with m.p 275-276^{o}C (lit 279-281^{o}C) [105]. The UV spectrum showed absorptions at λ_{max} 205, 248, 269, 309 and 413 nm, typical of an aporphine skeleton [106]. The IR spectrum showed an absorption band at 1737cm^{-1} indicating of carbonyl group [107]. Its molecular formula was determined as $C_{17}H_9NO_3$ based on the HREI-MS spectrum in positive mode and the molecular ion peak $[M+H]^+$ was at *m/z* 276.0658 (figure 3.8.1) [106].

The ^{1}H-NMR spectra of **MK8** (Table 3.8,Figure 3.8.2) showed seven aromatic protons, three doublets at δ 8.6 (*J* value of 6.3 Hz), δ 7.76 (*J* value of 6.6 Hz) and δ 8.68 (*J* value of 8.0 Hz) were assigned to H-8, H-10 and H-11 protons, while at δ 7.59 (*J* value of 16.3 Hz) as triplet was assigned as H-9, at δ 7.79 (*J* value of 5.1 Hz) as a

doublet and δ 8.90 (*J* value 5.1 Hz) as a doublet showed the presence of H-4 and H-5, respectively. The methylene proton resonated as a singlet at δ 7.22 was assigned to H-3 and the methylenedioxy group (O-CH_2-O) was appeared as a singlet at δ 6.39. The ^{1}H-NMR pattern was consistent with substitution of methylenedioxy group at the 1, 2-position of an aporphine skeleton.

The ^{13}C-NMR spectrum of **MK8** (Table 3.8, Figure 3.8.3) showed the presence of seventeen of the total carbons which consist of eight quaternary carbons, one carbonyl group, seven aromatic carbons and one methylenedioxy group. The down field signal at δ 182.4 had confirmed that the presence of the carbonyl group which was assigned to C-7. The seven aromatic carbons were resonated at δ 103.6, 124.3, 145.0, 128.5, 128.8, 134.0, 127.4 assigned to C-3, C-4, C-5, C-8, C-9, C-10 and C-11, respectively. One methylenedioxy group carbon appeared at δ 102.4 and eight quaternary carbons were resonated at δ 148.7, 106.4, 122.8, 151.9, 145.0, 135.8, 131.4 and 133.1, assigned to C-1, C-1a, C-1b, C-2, C-3a, C-6a, C-7a and C-11a, respectively.

MK8 was confirmed to be liriodenine based on the spectroscopic properties and comparison with the literature values [105]. Table 3.8 showed the ^{1}H-NMR, ^{13}C-NMR data and literature was obtained in the same solvent $CDCl_3$.

Table 3.8 : ^{1}H NMR (400 MHz) and ^{13}C NMR spectral data (100 MHz) of **MK8** in $CDCl_3$

Position	**δ_H (mult.; *J*(Hz)**	**Reference[105]**	**δ_C**	**Reference[105]**
1	-	-	148.7	148.6
1a	-	-	106.4	106.1
1b	-	-	122.8	122.6
2	-	-	151.9	151.7
3	7.22,s	7.16,s	103.6	103.3
3a	-	-	145.0	144.4
4	7.79,d,5.1	7.74,d,5.2	124.3	124.6
5	8.90,d,5.1	8.87,d,5.2	145.0	144.4
6a	-	-	135.8	135.4
7	-	-	182.7	181.2
7a	-	-	131.4	130.8
8	8.60,dd,6.3	8.57, dd,7.9, 1.2	128.5	127.8
9	7.59,dt,16.4	7.57,dt,7.9, 1.2	128.8	128.6
10	7.76,dt,6.6	7.72,dt,7.9, 1.2	134.0	134.2
11	8.68,d,8.0	8.60,d,7.9	127.4	127.0
11a	-	-	133.1	132.5
CH_2O_2	6.39,s	6.36,s	102.4	103.3

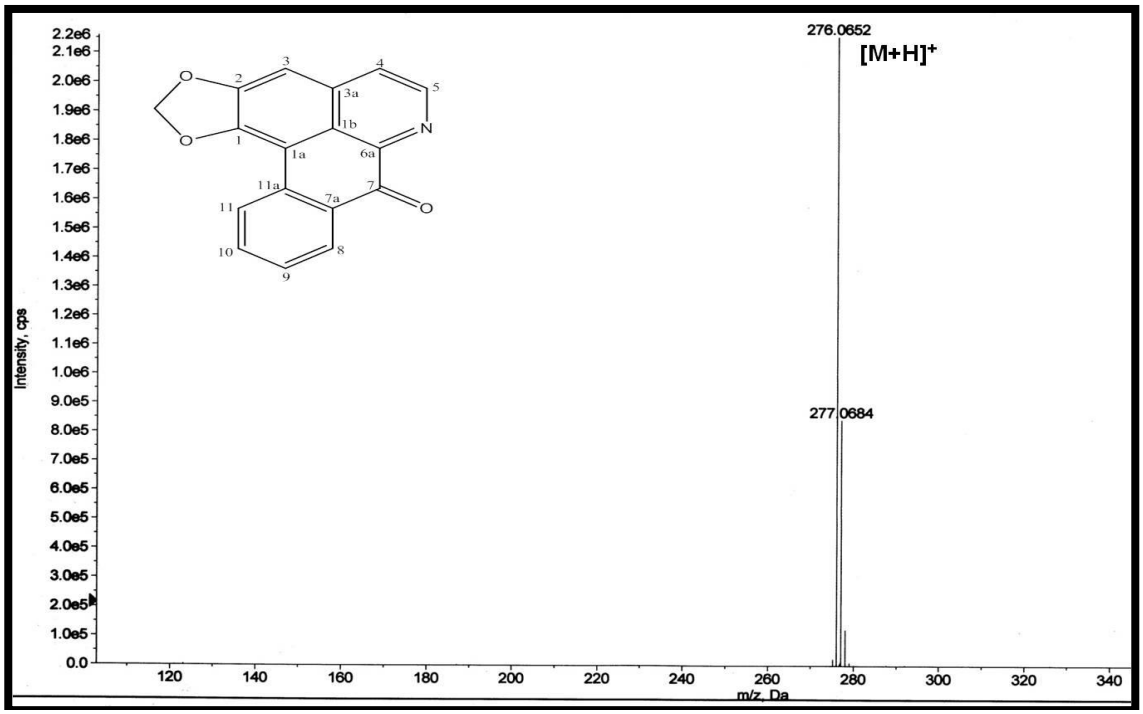


Figure 3.8.1 : The MS Spectrum of **MK8**

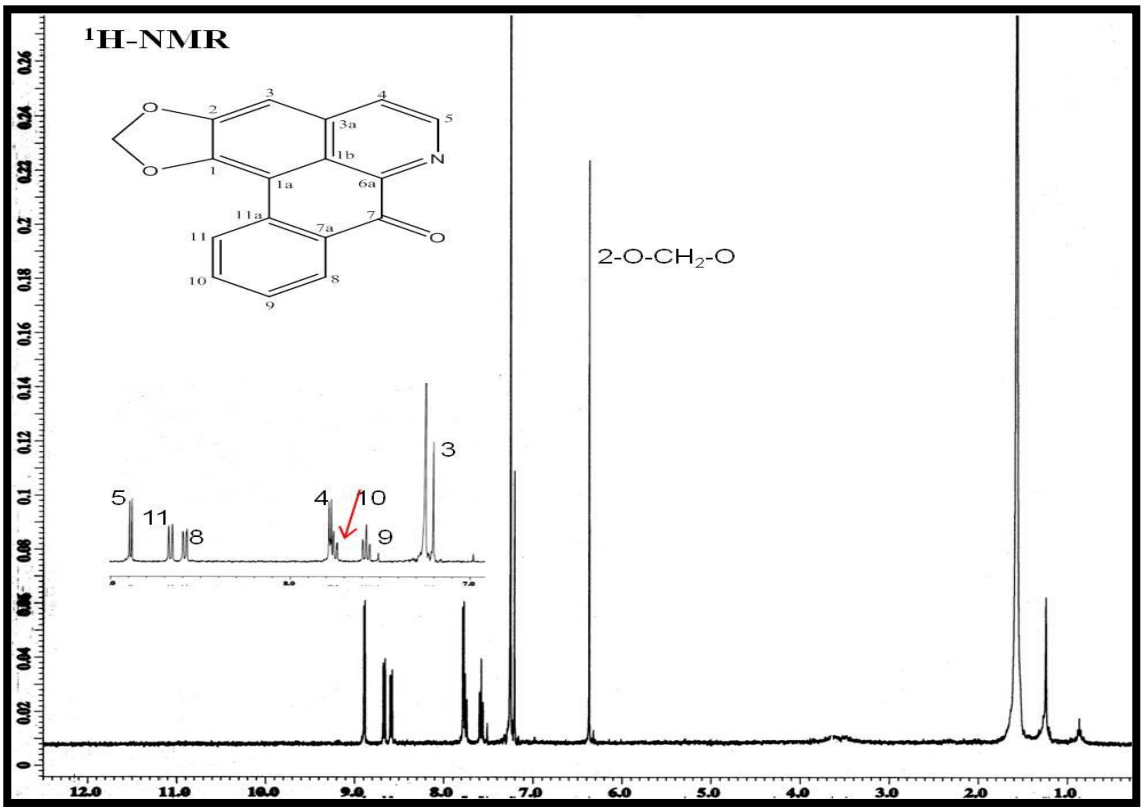


Figure 3.8.2: ^{1}H-NMR Spectrum of **MK8**

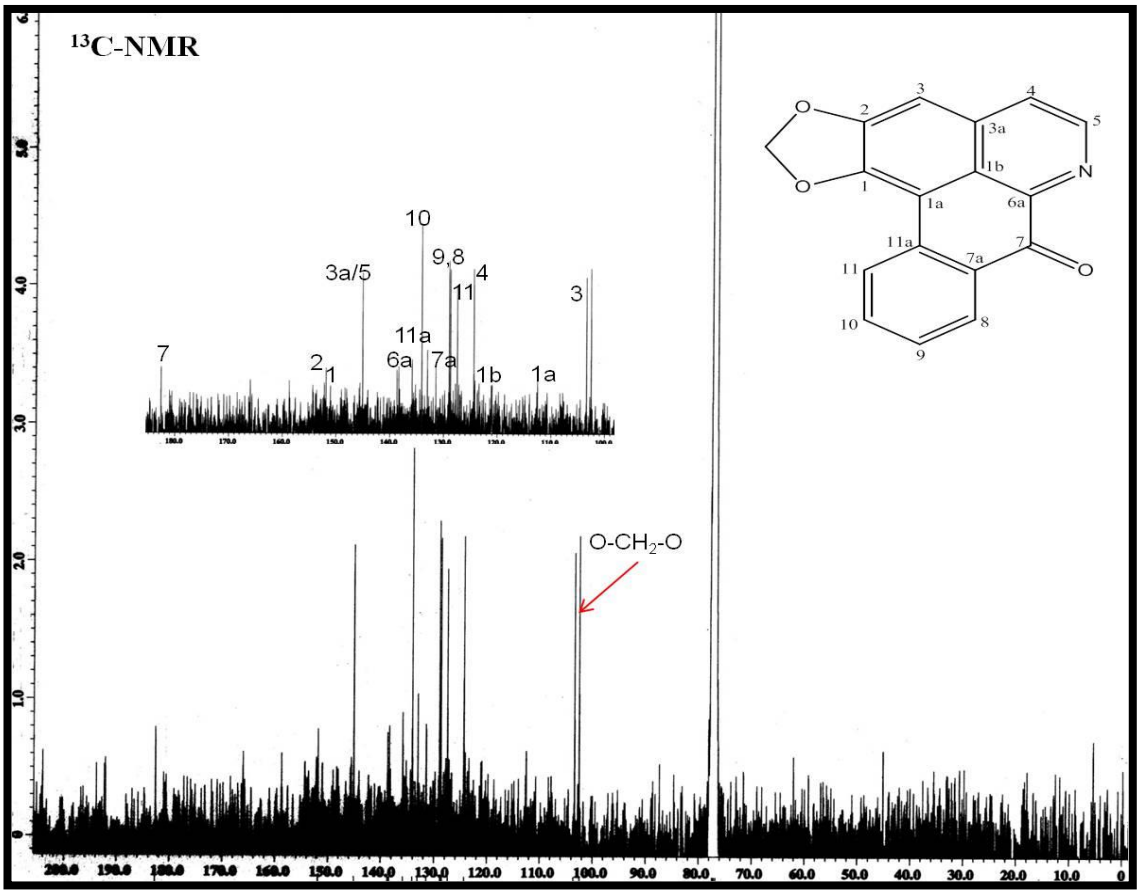
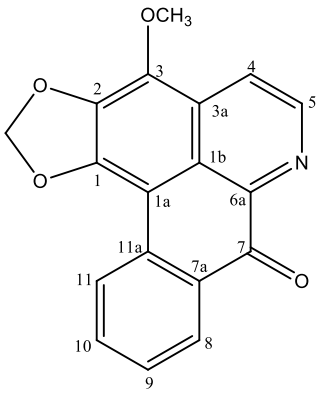


Figure 3.8.3: ^{13}C-NMR Spectrum of **MK8**

3.9 Structure Elucidation of Compound MK9



MK9

Oxoporphine alkaloid atherospermidine, **MK9** was separated from dichloromethane extract and was isolated as orange powder and gave yellow fluorescent colouration in solution. Thus, **MK9** gave similar characteristic of **MK8** as the oxoaporphine chromophore with m.p 282-284^{o}C (lit285-288^{o}C) [105]. The UV spectrum showed the absorption bands at λ_{max} 248, 280, 440 and 485 nm and were characteristic of an oxoaporphine skeleton [108]. This compound was isolated together with **MK8**, which is under UV light **MK9** is at the upper band under the long-wave (365 nm) absorption gave the orange colour while **MK8** was at the lower band under the long-wave (365 nm) absorption gave the yellow colour. Therefore, from the difference colour under the long-wave absorption, these two compounds, **MK9** and **MK8** were isolated by preparative TLC. The IR spectrum showed an absorption band at 1737cm^{-1} indicating a carbonyl group [107]. **MK9** displayed the HREI-MS in positive

mode of the molecular ion peak $[M+H]^+$ at *m/z* 306.0759, corresponding to the molecular formula $C_{18}H_{11}NO_4$ (figure 3.9.1) [109].

The ^1H and ^{13}C-NMR spectra showed signals of one methoxy group (Table 3.9 and Figure 3.9.2 and 3.9.3). Thus, the molecular ion peak (*m/z*) of **MK9** exhibited one methoxy group more than in **MK8**. In combination of the MS spectra, the ^1H-NMR and ^{13}C-NMR spectra, the molecular formula of **MK9** was determined and revealed one methoxyl group rather than in **MK8**.

The ^1H-NMR spectra of **MK9** (Table 3.9, Figure 3.9.2) showed the presence of one methylenedioxy, appeared as a singlet at δ 6.39 and assigned to positions H-1 and H-2, whereas one methoxy group was identified at δ 4.31, characteristic position at H-3. The aromatic protons region of the spectrum revealed the presence of one pair of doublets at δ 8.20 and 8.92 (*J* value 5.30 Hz), assigned to H-4 and H-5, respectively. The other pair of aromatic protons region at δ 8.58 (*J* value of 9.0 Hz) as doublets of doublets, δ 7.53 (*J* value of 8.1 Hz) as triplet of doublet and δ 8.63 (*J* value 7.6 Hz) as a doublet were assigned to H-8, H-10 and H-11 protons, respectively. The aromatic proton at δ 7.74 (*J* value 8.5 Hz) appeared as triplet of doublet was assigned as H-9.

The ^{13}C-NMR spectrum of **MK9** (Table 3.9, Figure 3.9.3) showed the presence of eighteen signals of the total carbons which consists of nine quaternary carbons, one carbonyl group, six aromatic carbons, one methylenedioxy group and one methoxyl group. The low field signal at δ 182.6 showed the presence of carbonyl group at C-7.

The six aromatic carbons resonated at δ 119.3, 144.5, 127.5, 126.5, 134.0 and 128.5 were assigned to C-4, C-5, C-8, C-9, C-10 and C-11, respectively. Thus, a methylenedioxy group was appeared at δ 101.8, a methoxy group was resonated at δ 59.6 assigned to C-3 and nine quaternary carbons appeared at δ 144.5, 122.4, 127.5, 135.5, 16.3, 129.2, 146.7, 130.8 and 133.0 assigned to C-1, C-1a, C-1b, C-2, C-3, C-3a, C-6a, C-7a and C-11a, respectively.

Based on the spectral data and comparison with the literature values [110], it was confirmed that **MK9** was atherospermidine. Table 3.9 showed the ^{1}H-NMR, ^{13}C-NMR spectra of **MK9** in the same solvent $CDCl_3$ as compared to the literature values. It was clear from these data that **MK9** is closely related to **MK8**. However, **MK8** has fully substituted in ring A.

Since the plant Annonaceae family is known to possess aporphine alkaloids, the compounds, **MK8** and **MK9** were considered and most likely to be an oxoaporphine alkaloid. These two compounds are widely found in almost all genera of Annonaceae, and **MK8** is considered to be a chemotaxonomic marker [111]. The ^{1}H-NMR spectrum for both compounds showed similar absorption peaks as those of oxoaporphine alkaloids. They also showed a Dragendorff's positive spot on TLC, indicating alkaloidal compounds.

Table 3.9: ^{1}H NMR (400 MHz) and ^{13}C-NMR spectral data (100 MHz) of **MK9** in $CDCl_3$

Position	**δ_H (mult.; *J*(Hz)**	**Reference[110]**	**δ_C**	**Reference[110]**
1	-	-	144.5	143.8
1a	-	-	122.4	122.3
1b	-	-	127.5	127.4
2	-	-	135.5	135.8
3	-	-	136.3	136.8
3a	-	-	129.2	129.6
4	8.20,d,5.3	8.78,d,5.4	119.3	119.6
5	8.92,d,5.3	8.09,d,5.4	144.5	144.5
6a	-	-	146.7	150.0
7	-	-	182.6	182.4
7a	-	-	130.8	130.5
8	8.58,dd,9.0	8.52,dd,8.0	127.5	127.3
9	7.74,dt,8.5	7.65,dt,7.6	126.5	126.3
10	7.53,dt,8.1	7.45,dt,7.6	134.0	134.0
11	8.63,d,7.6	8.46,d,8.0	128.5	128.0
11a	-	-	133.0	132.9
CH_2O_2	6.39,s	6.28,s	101.8	102.3
OCH_3	4.31,s	4.27,s	59.6	59.7

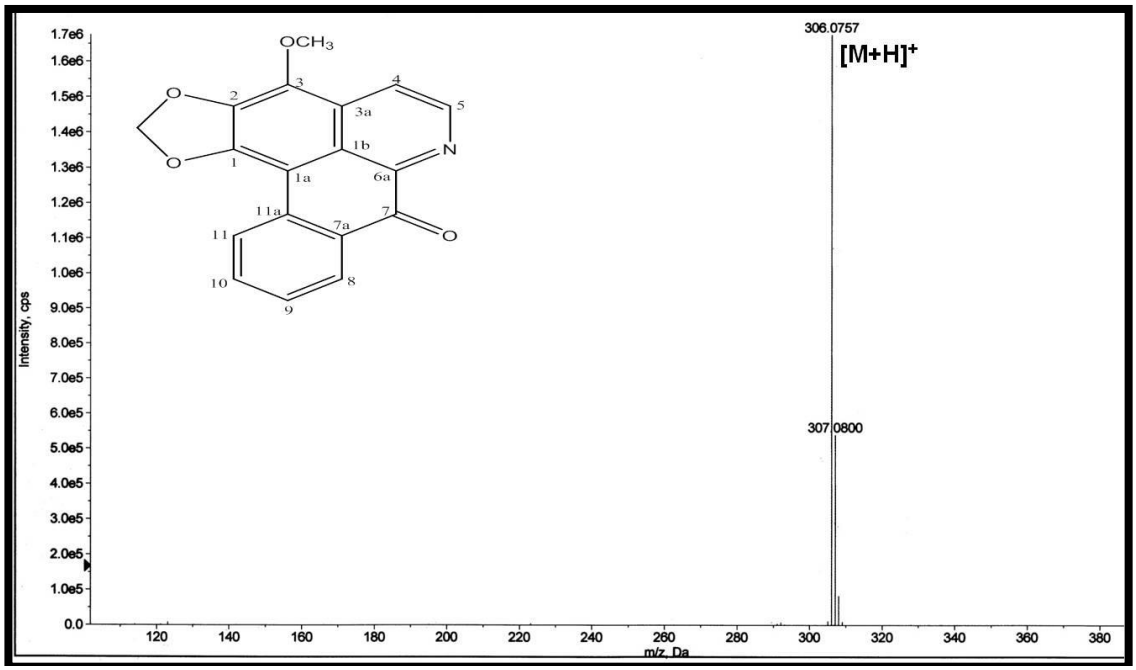


Figure 3.9.1 : The MS Spectrum of **MK9**

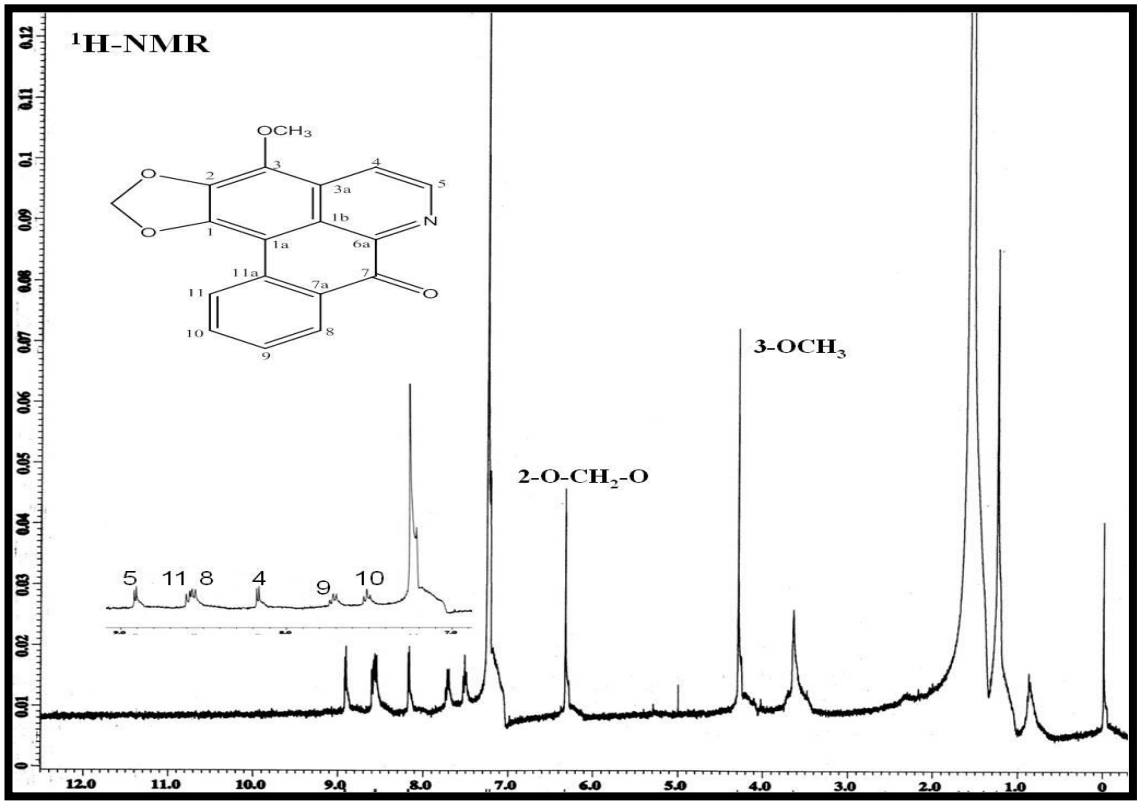


Figure 3.9.2: ^{1}H-NMR Spectrum of **MK9**

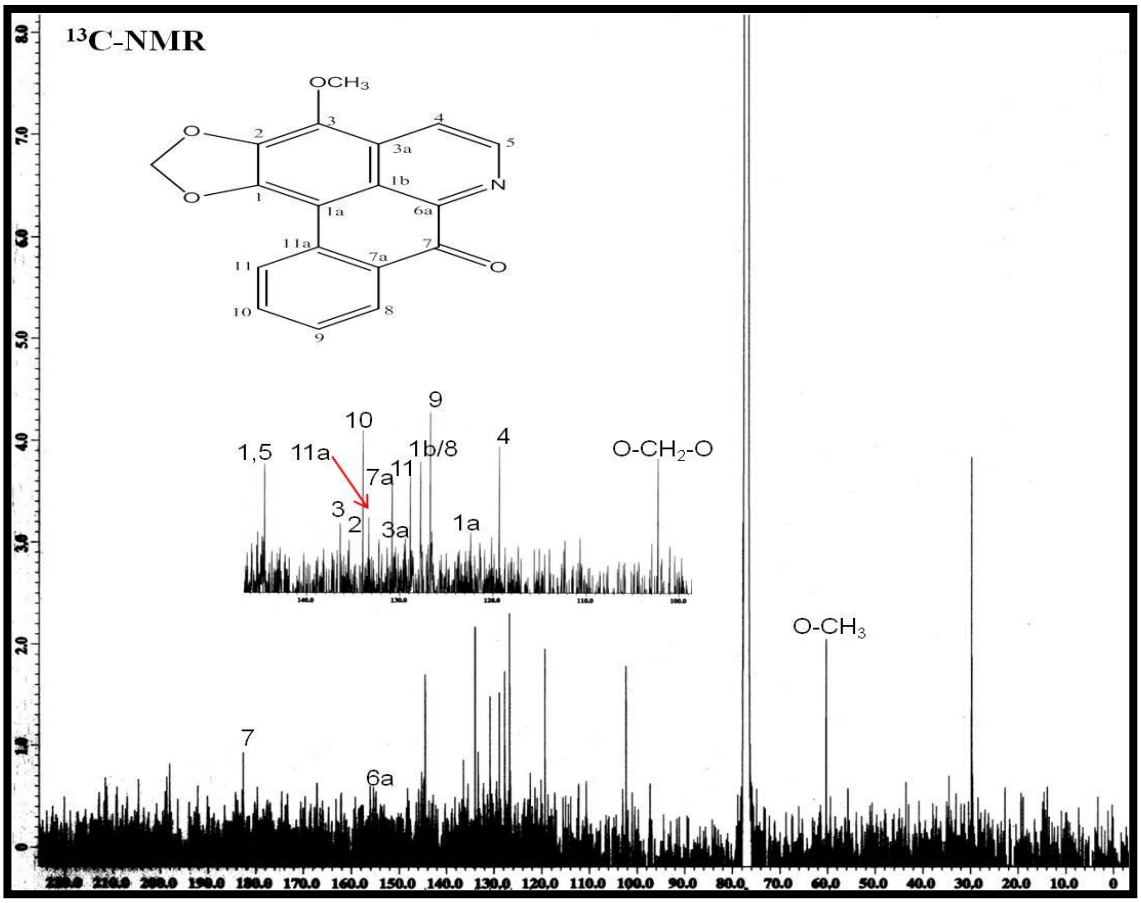


Figure 3.9.3: ^{13}C-NMR Spectrum of **MK9**

CHAPTER 3

PART B: BIOACTIVITY

3B.1 INTRODUCTION

Peptic ulcer disease (PUD) is the most common gastrointestinal tract disease of multiple causes which occur when the offensive factors overcome the defensive one.

Peptic ulcer disease (PUD) is the most common gastrointestinal tract disease of multiple causes hinders a lot of people worldwide [83] which occur as when the offensive factors overcome the defensive one [112]. There are many noxious agents such as *H. pylori*, attack the stomach and result in mucosal ulceration. Excessive non-steroidal anti-inflammatory drugs (NSAIDs), psychological stress as well as cigarette smoking. On the other hand, the stomach protects itself by many defensive mechanisms, mainly the entire mucosal layers which act as a barrier against inflammatory and cytotoxic agents [113]. It is known that there are several endogenous factors play a role in gastroprotection mechanism such as prostaglandin E_2, nitric oxide (NO) and non-protein sulphhydryl (NP-SH) compounds. Alcohol is known as a necrotizing substance and its excessive ingestion may result in gastritis, characterized by mucous membrane edema, sub epithelial hemorrhages, cell exfoliation and inflammatory cells infiltration [83].

It is reported that accumulation of reactive oxygen species (ROS) are in charge of peptic ulcer etiology in various gastric ulcer model suggesting that participation of antioxidant enzymes and therefore it is a trend to explore antioxidant agent [112].The

reciprocal antiulcer medication to treat the peptic ulcer is either by inhibiting gastric acid secretion or by enhancing mucus layer protection. However, majority of them possess adverse side effects that may limit their usage. Therefore, there is a need to discover new antiulcer drug [114].

Ethanol induced experimental gastric ulcer in animal is the common ulcer model since it's easily and rapidly penetrate into the gastric mucosa activating some pathological events result in ulcer formation. It believes that oxygen free radicals participate in gastric damage caused by ethanol [115].

A large number of medicinal plants with anti-ulcer properties have been reported by gastric ulcer researchers [116-119]. Many chemical compounds from plant kingdom have been isolated with antiulcer activity [84]. **MK1** is a novel cytotoxic principle from *Desmos dumosus* root of significant and selective *in vitro* cytotoxicity against bone (HOS), breast (MCF-7), and ovarian (IA9) cancer cell lines [97]. However, no report or study about this compound in anti-ulcer activity. Therefore, this study was conducted to evaluate the probable gastroprotection and possible mechanisms of **MK1** against ethanol ulcer model in experimental animal.

From the previous study, **MK1** was isolated from the roots of *Desmos dumosus* and has a distinctive chalcone skeleton with an unusual non-aromatic A-ring possessing a *gem*-dimethyl group on C-6' and methyl group on C-4'. This compound showed significant and selective antiproliferative activity against 1A9 (ovarian cancer) and A549 (human lung carcinoma) cell lines with ED_{50} values of 3.5µg/mL (11.2µM). In

addition, it was more active against drug resistant KB-VIN [vincristine-resistant KB, overexpressing P-glycoprotein (P-gp)] cells than against the parent KB (epidermoid nasopharyngeal carcinoma) cell line [97].

Gastric ulcer is a common disease affecting a lot of people worldwide. Some factors are contributed in the etiology of this disorder such as stress, cigarette smoking, alcohol consumption, nutritional deficiencies and infections [120]. However, the over ingestion of non-steroidal anti-inflammatory drugs (NSAIDs) and *Helicobacter pylori* (*H. pylori*) infection remain the predominant causes of peptic ulcer disease [121]. The gastric ulcer disease was observed to correlate with changes in some physiological parameters such as free radical oxygen species (ROS), nitric oxide (NO), lipid peroxidation and gastric acid over secretion [115].

Treatment of gastric ulcer considers clinical problem because of the increasingly widespread use in non-steroidal anti-inflammatory drugs (NSAIDs) and low-dose aspirin [122]. Despite the effectiveness of the reciprocal antiulcer drugs such as the antiacids, anticholinergics, proton pump inhibitors and histamine H_2 receptor antagonists, majority of them possess adverse effects that limit their use [123]. Nowadays, discovering alternative therapies to treat gastric ulcer is of high concern to many researchers [124].

Large numbers of natural antiulcer compounds have been isolated from medicinal plants. Among of the common chemical classes of bioactive compounds with antiulcer activity are alkaloids, saponins, xanthones, triterpenes and tannins [83]. From

previous studies on *M. kentii*, the chemical constituents isolated were isoquinoline alkaloids [78], terpenylated dihydrochalcones [79] and these compounds showed inhibitory activity on TXB_2 production and strong PAF antagonistic effect [80]. In continuation to our research for biological active compounds for the treatment of gastric ulcer from the Malaysian flora, the hexane extract of the stem bark of this plant was selected for phytochemical investigations. **MK1** was isolated for first time from *Mitrella kentii*. It's a known compound which was previously isolated from the roots of *Desmos dumosus* [97] and *Uvaria scheffleri* [125].

It is known that ethanol induce gastric mucosa lesions and petechial bleeding in humans [126], where ethanol found to be easily and rapidly penetrates into the gastric mucosa causing membrane damage, exfoliation of cells, erosion, and ulcer formation [127]. It's claimed that ROS are involved in ulcer formation caused by ethanol [128]. Ethanol-induced gastric ulcer models are commonly used to study both pathogenesis of and therapy for human ulcerative disease [120].

3B.2 RESULTS

3B.2.1 Toxicity study

The toxicity study showed no toxic symptoms or mortality and there were no abnormal physiological or behavioral changes, body weight alteration at any time of observation up to 300mg/kg during the experimental period (14 days). Histological examination to the liver and kidney and the serum biochemical analysis didn't show any differences in comparison to the control group.

3B.2.2 Gross evaluation

Pretreament with **MK1** at doses of 5, 10, 20 mg/kg b.w, was observed to inhibit ulcer formation by 69.77%, 90.18% and 86.56%, respectively compared to ulcer control. The **MK1** at doses 10, 20 mg/kg had protected stomach from ulceration at significantly ($p<0.05$) higher than that obtained by omeprazole at dose 20 mg/kg with 79.07%, shown in Table 3B.1. Gross observation showed that **MK1** in pretreated groups in Figure 3B.1: 2C, 2D and 2E or omeprazole group in Figure 3B.1: 2B were considerably less gastric lesion in compare to ulcer control group in Figure 3B.1: 2A.

3B.2.3 Gastric mucus content, pH and biochemical analysis

Ulcer control group produced the lowest content of gastric mucosa, while the pretreated **MK1** group significantly ($p<0.05$) increased the mucus production in compare with the ulcer control group. On the other hand, the pH of the gastric contents was increased ($p<0.05$) in animal pretreated with **MK1**. Serum analysis that rats induced ulceration with ethanol had increased level of the liver enzymes AST and ALT compared to normal and **MK1** treated animal in Table 3B.1.

3B.2.4 Gastric tolerability

Animal's stomach pretreated with **MK1** didn't exhibit any significant gastric lesions. The changes were observed in the range of 0-1 according to Adami scoring scale but only few petechiae were scored in rat stomach regardless of given dose.

Table 3B.1: Gastroprotective effect of **MK1** against ethanol induced ulceration and observed liver function test

Animal group	Pretreatment 5ml/kg	pH of gastric juice	Mucus weight	Ulcer area	Inhibition (%)	ALT (IU/L)	AST (IU/L)
A	Tween 80 (ulcer control)	2.77±0.06 [a]	0.99±0.3 [a]	557.28±6.2 [a]	-	55.4±2.71 [a]	292.0±2.15 [a]
B	Omeprazole (20 mg/kg)	6.74±0.05 [b]	1.56±0.2 [b]	108±7.7 [b]	79.07 [b]	53.0±1.47 [b]	274.7±6.01 [b]
C	**MK1** (5 mg/kg)	5.92±0.12 [b]	1.32±0.5 [b]	168.48±9 [c]	69.77	47.2±2.48 [b]	280.6±4.39 [b]
D	**MK1** (10 mg/kg)	6.96±0.04 [b]	2.01±0.1 [b]	54.72±3.8 [b]	90.18	34.0±2.08 [b]	238.04 [b]
E	**MK1** (20 mg/kg)	6.81±0.05 [c]	1.49±0.4 [c]	74.88±10.3 [b]	86.56	32.19±1.58 [b]	259.4±9.22 [b]
F	Normal group	7.01±0.011 [b]	2.89±0.2 [b]	0.00	0.00	35.67±1.67 [b]	227.0±9.81 [b]

Groups sign by different alphabets (^{a,b,c}) are significantly different at ($P < 0.05$)

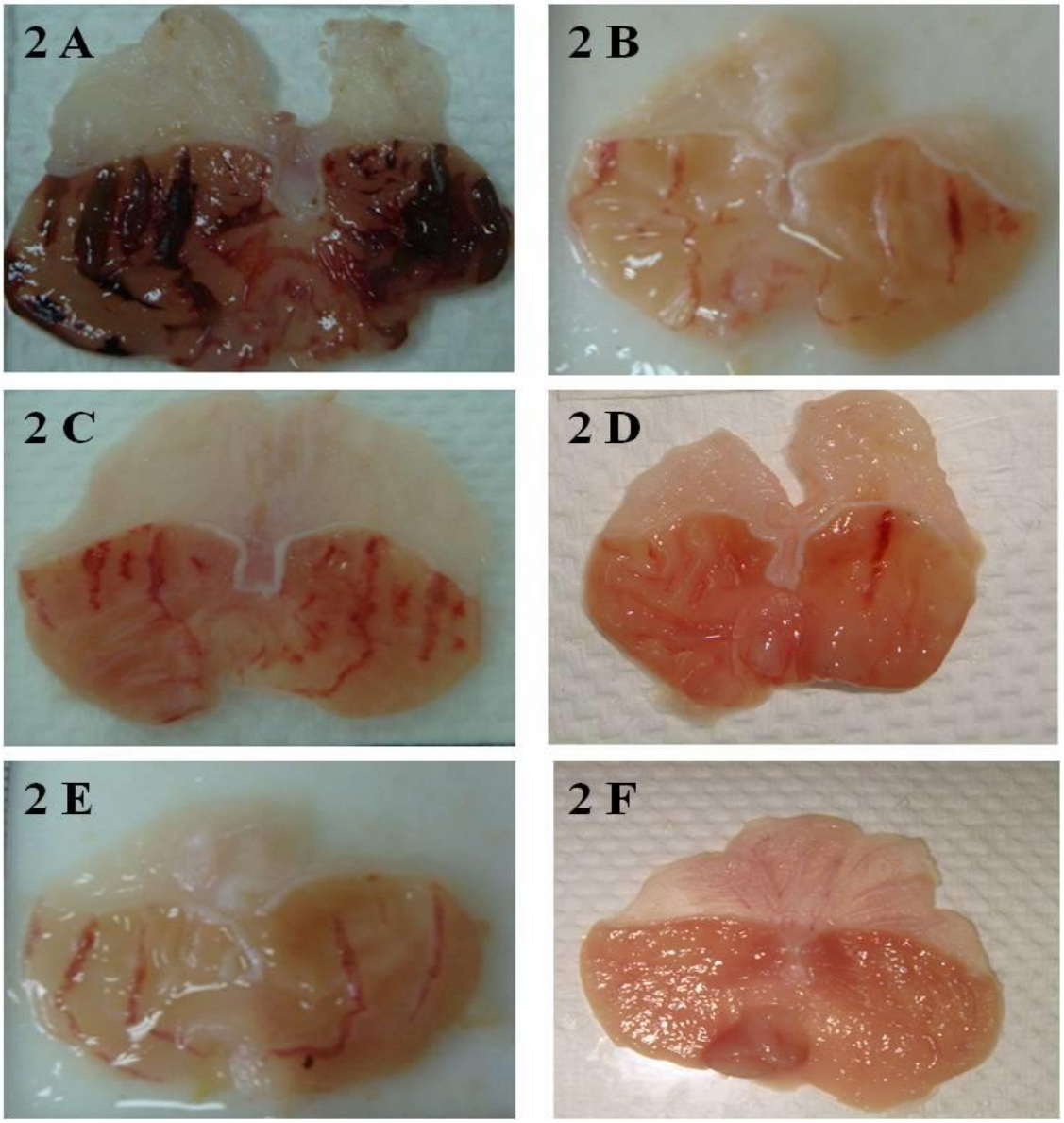


Figure 3B.1: Macroscopic appearance of the gastric mucosa of the rats pretreated with **MK1** at doses 5, 10, 20 mg/kg (2C, 2D and 2E) or omeprazole 20mg/kg (2B) showed reduced lesion formation in compare with the ulcer control rats (2A).

3B.2.5 Histological evaluation

Histological observation using H&E staining further confirmed the ability of **MK1** to prevent ethanol induce damage in the superficial layer of the gastric mucosa. When compared to the normal group (Figure 3B.2 : 3F), the ulcer control group (Figure 3B.2 : 3A), showed highly extensive gastric lesion, submucosal edema and leucocytes infiltration. Pretreatment with **MK1** (Figure 3B.2 : 3C, 3D and 3E) and omeprazole (standard) (Figure 3B.2 : 3B), had relatively better protection as seen by decreasing ulcer area, reduction or complete absence of edema and leucocytes infiltration and flattening of mucosal fold were also observed. Furthermore, **MK1** had resulted into the expansion of a substantial continuous PAS-positive mucous gel layer that lining the entire gastric mucosal surface observed as a magenta colour in Figure 3B.3. However stomachs of animals in ulcer control group didn't exhibit this magenta staining of PAS.

3B.2.6 HSP-70 and Bax immunohistochemistry

The immunostained area of HSP-70 in all experimental animals was larger in Figure 3B.4 than that of the ulcer control group indicating participation of this protein in antiulcer effect of **MK1** while the immunostained area of Bax in all experimental animals was less showed in Figure 3B.5, to compare with ulcer group, hence HSP-70 expression and BAX suppression in treatment group may contribute in gastroprotection role of **MK1**. The antigen site appears as a brown-coloured.

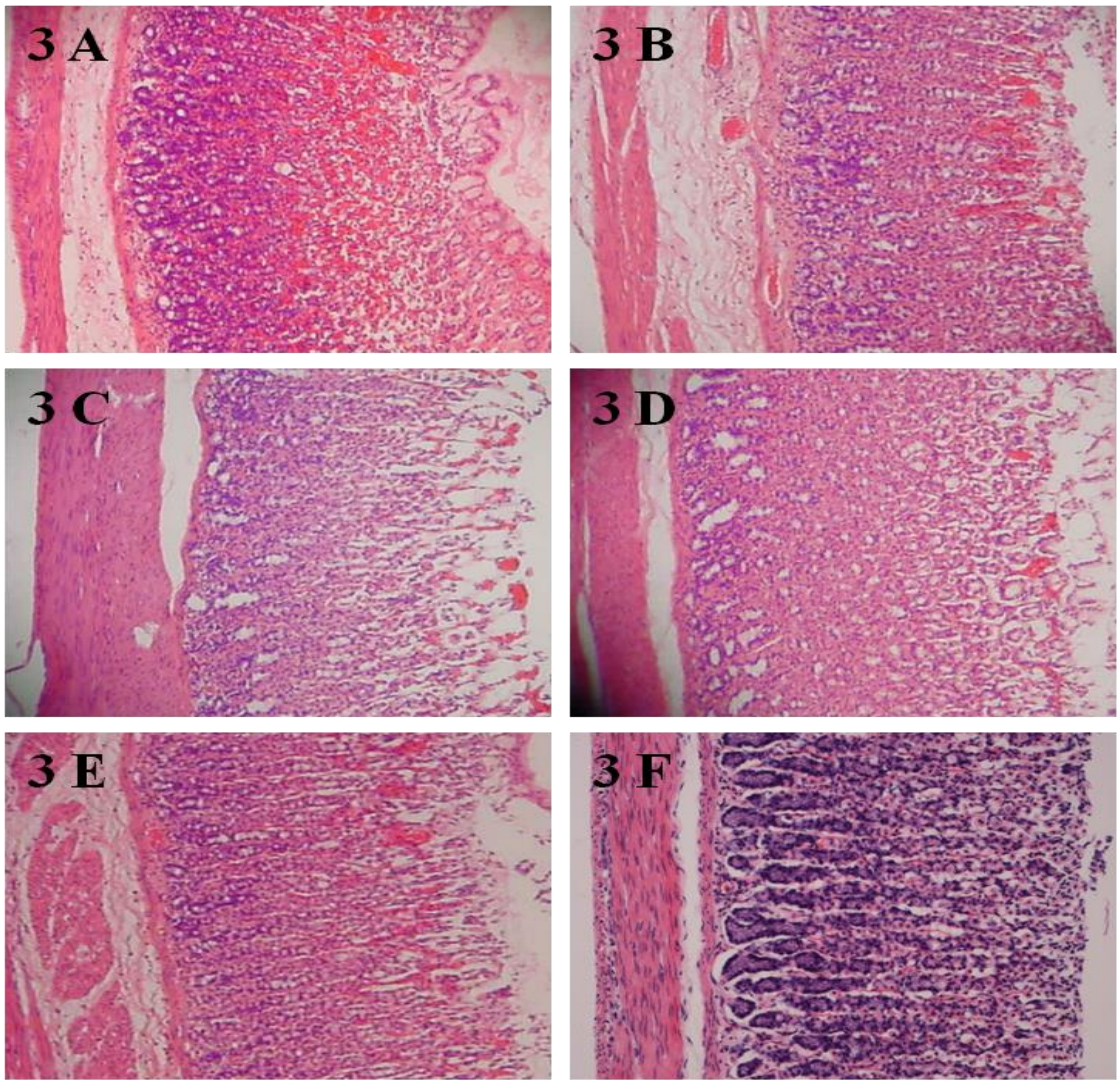


Figure 3B.2: Macroscopic evaluation of the gastric mucosa of the rats pretreated with **MK1** at doses 5, 10, 20 mg/kg (C-E) or omeprazole (B) showed improved histological appearance compared to ulcer control rats (A) which have extensive visible hemorrhagic necrosis of the gastric mucosa with edema and leucocytes infiltration of submucosa. (H & E stain 20x).

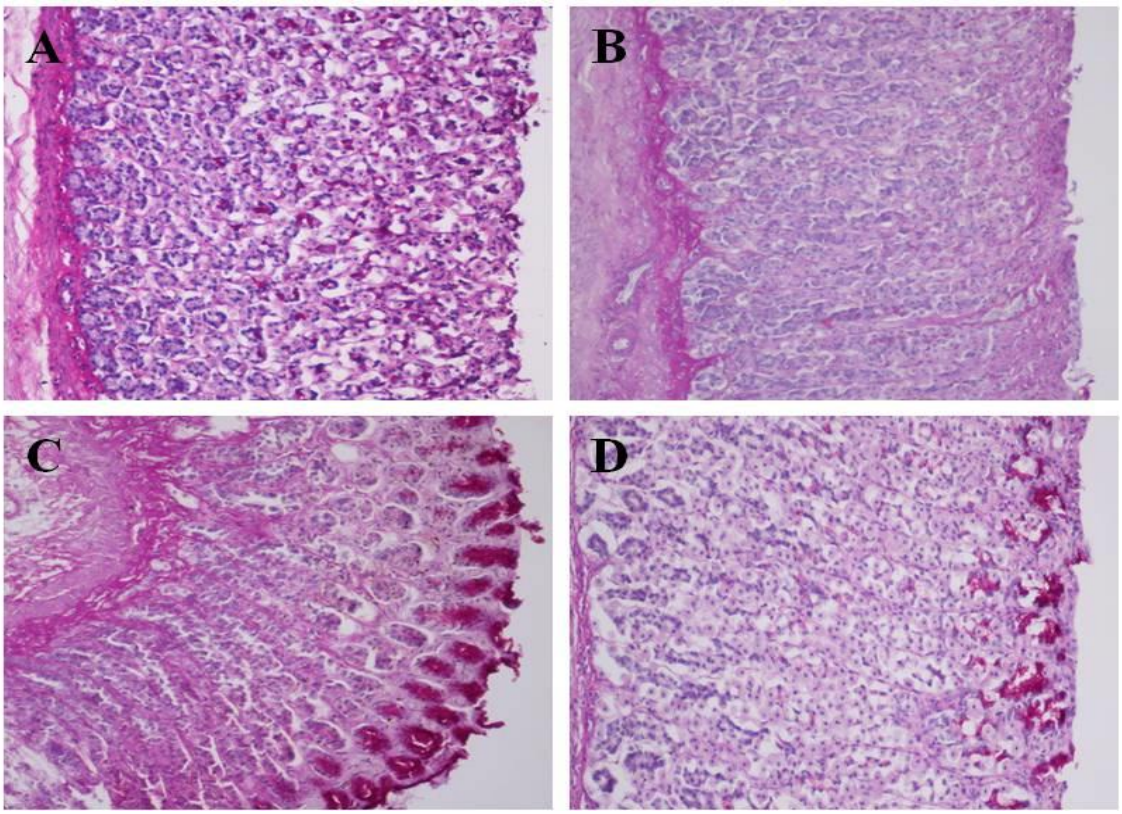


Figure 3B.3: Effect of **MK1** gastric tissue glycoprotein-PAS staining in ethanol-induced gastric ulcer in rats. (A) Normal group, (B) Ulcer group, (C, D) treated **MK1** group at doses 10, 20 mg/kg respectively (PAS stain 20x).

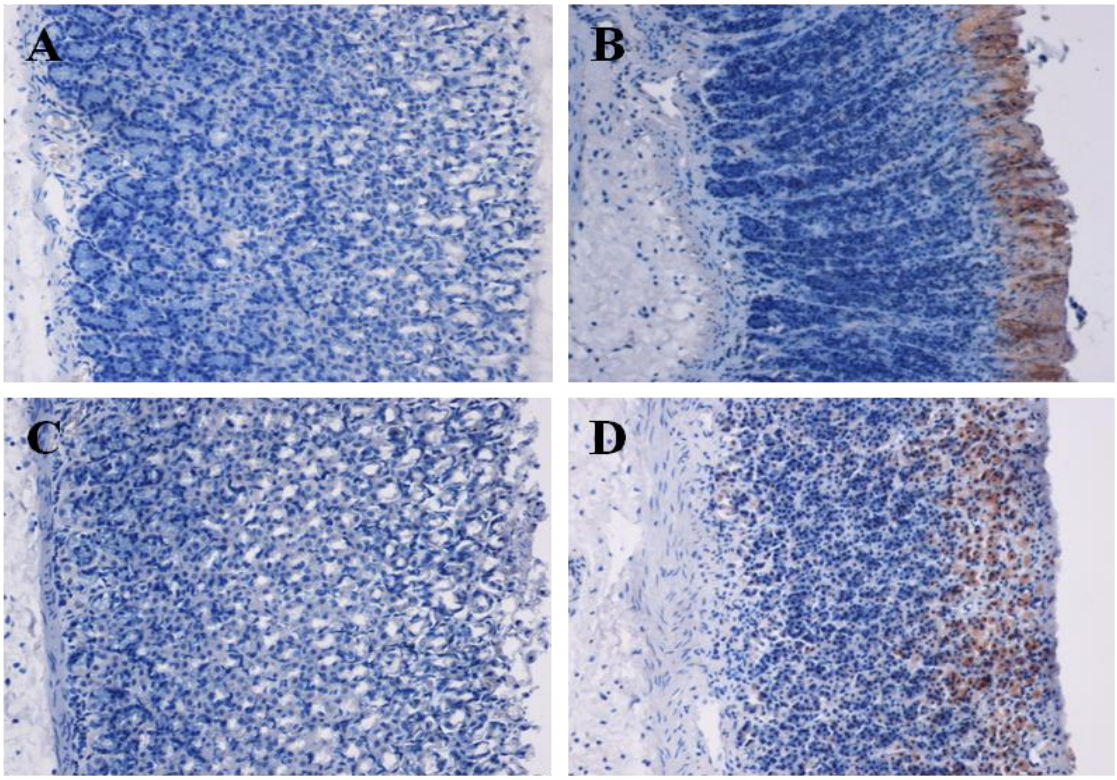


Figure 3B.4: Immunohistochemical analysis of Bax protein expression in the stomachs of rats with ethanol-induced gastric mucosal lesions (A) normal control group, ulcer control group (B) and the treated group with **MK1** at 10 and 20 mg/kg (C, D), respectively (20x).

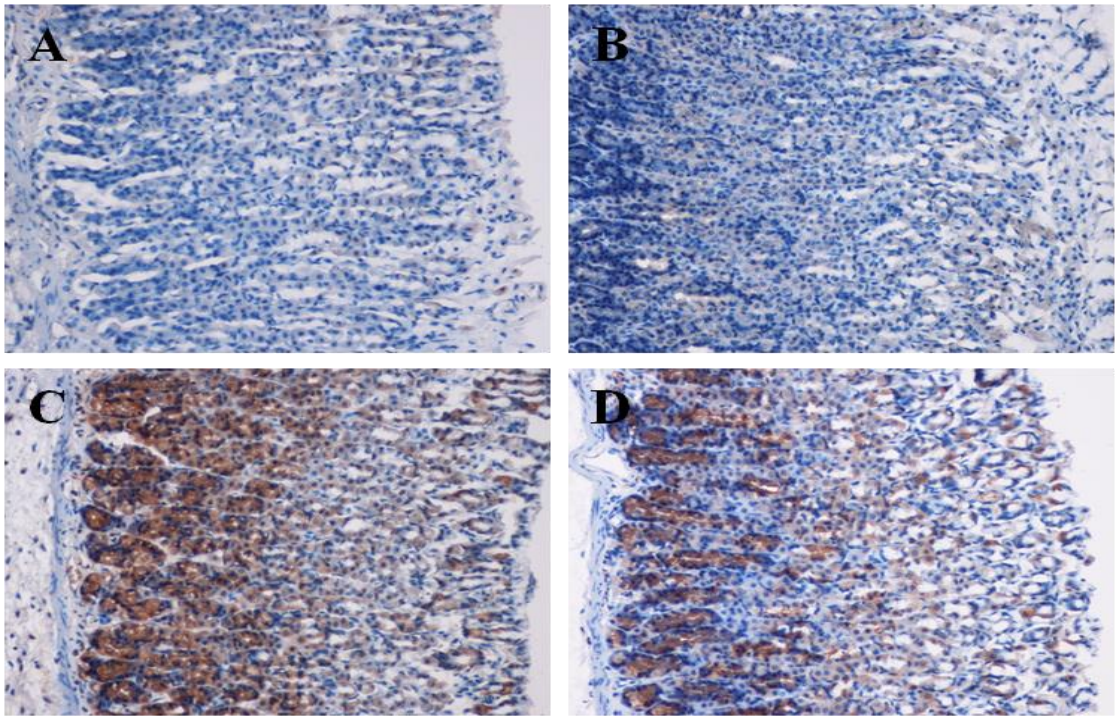


Figure 3B.5: Immunohistochemical analysis of HSP-70 protein expression in the stomachs of rats with ethanol-induced gastric mucosal lesions. (A) Normal control group, ulcer control group (B), the treated groups with **MK1** at doses 10 and 20 mg/kg, (C, D) respectively (20x).

3B.2.7 Effect of MK1 on GSH level in gastric homogenate

GSH levels were significantly ($p<0.05$) lower in ulcer control group than the normal group. The treatment of animals with the **MK1** restored the GSH levels depletion after ethanol administration in Table 3B.2.

3B.2.8 Effects of MK1 on lipid peroxidation

MDA was used as indicator for lipid peroxidation. The ulcer control group showed ($p<0.05$) higher MDA level than the other groups. Gastric MDA level significantly ($p<0.05$) decreased after **MK1** administration in Table 3B.2.

3B.2.9 *In vitro* effect on NP-SH compounds

To investigate **MK1** gastroprotection mechanism, nonprotein sulphydryl group (NP-SH) level was estimated. The fundus parts of animals in ulcer control group showed the lower NP-SH, while, the compound significantly ($p<0.05$) elevate NP-SH level in pretreated animal in compare to animals in normal group showed in Table 3B.2.

3B.2.10Nitric oxide (NO) and COX 2 inhibitory activity

None of the treatment was able to maintain its NO level near to the normal group which is dropped due to ethanol administration. Moreover, no involvement of COX-2 inhibitory activity in Table 3B.2.

Table 3B.2: Effects of **MK1** on the level of malondialdehyde (MDA), glutathione (GSH), nonprotein sulfhydryl (NP-SH) group, nitric oxide (NO) and COX-2 inhibition.

Animal group	Pretreatment 5ml/kg	MDA µmol/g tissue	GSH nmol/g tissue	NP-SH µmol/g of tissue	NO µmol/g tissue	COX-2 Inhibitory (%)
A	Tween 80 (ulcer control)	32.01 ±0.4 [a]	1.00±0.12 [a]	0.88±.031 [a]	4.9±0.2 [a]	-
B	Omeprazole (20 mg/kg)	14.90±0.6 [b]	1.40±0.1 [b]	1.33±.0.04 [b]	8.9±1.1 [b]	-
C	**MK1** (5 mg/kg)	16.00±0.09 [b]	1.37±0.4 [b]	0.99±0.03 [b]	6.6±0.2 [b]	-
D	**MK1** (10 mg/kg)	8.80±0.03 [c]	1.56±0.13 [c]	1.82±0.02 [c]	8.0±0.31 [b]	34.8% [a]
E	**MK1** (20 mg/kg)	11.80±0.05 [b]	1.49±0.09 [b]	154.00±0.03 [b]	7.5±0.9 [b]	-
F	Normal group	9.09±0.03 [c]	1.50±0.15 [c]	1.19±0.032 [c]	9.3±1 [c]	90.4 % [b]

Groups sign alphabetically (^{a,b,c}) are significantly different at ($p<0.05$)

3B.2.11 In vitro anti-*Helicobacter pylori* activity

MK1 represents minimum inhibitory concentration MIC of 125 µg/ml against *H. pylori* J99.

3B.2.12 Antioxidant evaluation of MK1

GSH as endogenous antioxidant, its level was significantly ($p<0.05$) lower in ulcer control group than the normal group. The treatment of animals with the **MK1** restore the GSH levels depletion after ethanol administration in Table 3B.2. MDA was used as indicator for lipid peroxidation. The ulcer control group showed ($p<0.05$) higher than the other groups. Gastric MDA level significantly ($p<0.05$) decrease after **MK1** administration in Table 3B.2. **MK1** exhibited FRAP value of 120.7 ± 0.001 significantly ($p<0.05$) lower than the positive controls used in this study those exhibiting for quercetin, 2046.7± 0.024, gallic acid, 2562.7± 0.024 and ascorbic acid, 879.3 ± 0.005, respectively. Meanwhile the DPPH assay couldn't show any significant inhibition in the dose dependant manner used in the study. Therefore, it can be concluded that **MK1** exert its antiulcer effect through indirect antioxidant mechanism. Furthermore, the immunostained localization of HSP-70 was up regulated in all treated animals in Figure 3B.5 more than the ulcer control group indicating participation of this protein in antiulcer effect of **MK1**.

3B.3 Discussions

In this study, the anti-ulcer effect of **MK1** was evaluated in ethanol ulcer model in animal. In addition, the effect of **MK1** on antioxidant system, COX-2 inhibitory activity and the anti *H. pylori* effect were also assessed. The ethanol model is widely

used to evaluate gastroprotection activity, since ethanol was found easily and rapidly penetrates into the gastric mucosa, causing membrane damage, exfoliation of cells, and erosion, subsequently increase mucosal permeability together with the release of vasoactive products result in gastric lesions and gastric ulcer formation [129]. In the present study, **MK1** pretreatment was found to be significantly prevented ethanol induced stomach ulceration. The following discussion is to evaluate the possible mechanisms involved in its gastroprotective effect.

To define the side effect of **MK1** on overall physiological function, serum biochemical parameters were evaluated. In our study, ulcer animal group in comparison to normal group showed increase serum level of AST and ALT as indicator of hepatic injury since the high level of hepatic enzymes is a sign of alcoholic tissue damage due to ethanol administration [128]. However, **MK1** pretreatment significantly showed decreased in the elevated level of serum liver enzymes near to the normal control level. This finding indicates high efficacy of the compound against ethanol induced tissue injures.

Reactive oxygen species (ROS) are final products generated from normal cellular metabolic process [130]. Excessive production of ROS affects cell integrity in oxidative stress condition when the antioxidant system was not able to overcome them [131]. Gastrointestinal mucosal lesions can be as a result of oxidative stress [132]. Antioxidants have been observed to protect gastric mucosa from ulceration [130]. Antioxidant is those compounds have the ability to protect tissue damage by radical scavenging mechanism [133]. Previous study proved that ethanol induced gastric tissue

injured by increasing reactive species formation [134]. ROS accumulation leads to glutathione depletion, and lipid peroxidation [131]. GSH is intracellular antioxidants inhibit oxidative stress [135]. Mutoh *et al.,* (1990) report that intracellular GSH protection against ethanol induced gastric cell injury [136]. It was observed that aggressive effect of ethanol on gastric mucosa is associated with reduced GSH level [137]. Apart from GSH, ethanol exerts its ulcerogenic effect on gastric tissue by increasing lipid peroxidation [113]. MDA is the main products of lipid peroxidation, therefore, MDA is considered marker of ROS mediate gastric lesions [138]. The present study showed pretreatment with **MK1** was significantly protect gastric mucosa from ethanol induced ulceration by restoring the depleted GSH level and reducing the elevated MDA level when compare to ulcer control group. Those results together indicate the ability of **MK1** to reduce oxidative stress. Hence, to evaluate further this antioxidant property, **MK1** was examined for *in vitro* radical scavenging activity. However, the compound showed no significant antioxidant activity either against FRAP or DPPH assay. It can be conclude that **MK1** inhibit oxidative stress via indirect antioxidant effect.

HSPs are stress proteins maintain cellular homeostasis against stress factors [139]. HSP-70 over expression was observed to be as a result of various stimulus such as heat, drug exposure or oxidative stress [140]. Acute and chronic gastric ulcers in rats were observed to be associated with HSP-70 induction [139]. HSP-70 expression promotes cellular protection-tolerances against high concentration of alcohol [141]. In experimental ulcerated rat stomachs, there is a correlation between HSP induction and mucosal protection [142]. Many compounds have been reported to protect tissue from

oxidative damage remarkable by their effect on HSPs over expression [143,144]. Our study indicates that **MK1** pretreatment followed by ethanol administration increased HSP-70 expressions in rat gastric mucosa, suggesting that induction of HSP-70 might contribute to the protective effect of **MK1** against ethanol-induced gastric injuries. Again this result supports the hypothesis of the antioxidant activity of **MK1** against oxidative stress.

Many researchers were reported earlier that, apoptosis or programmed cell death was believed to be one of the main factors that result in gastric ulcer formation [145-148]. Blocking of apoptotic cell death is among mechanisms that implicated to control gastric lesions [145]. Omeprazole proved to exert its antiulcer action by offering antiapoptotic effect [149]. Ethanol was reported to induce gastric mucosal lesion by increasing apoptotic cell death [134, 150]. In many experimental ulcer models, apoptosis result from alteration of Bcl-2 antiapoptotic and Bax apoptotic proteins expression [145, 151]. Bcl-2 Protein inhibits most types of apoptotic cell death [152], while Bax proteins promote this process [145]. In the results presented herein, IHC assay showed that **MK1** was able to suppress Bax protein expression when compare to ulcer control group. This result can be suggested that **MK1** offered antiapoptotic effect which may involve in its gastroprotective effect against ethanol induced gastric tissue injured.

Gastric defensive mechanisms based mainly on the delicate balance between aggressive and protective factors [153]. Several studies suggest that mucus gel layer is the first defensive mechanisms of the mucosa against internal and external aggressive

factors [154]. Ethanol tends to disrupt gastric mucosal layer and lowers the level of tissue proteins [155]. Hence, the compound that has the ability to increase mucus production might be expected to have gastroprotective activity [156]. To evaluate this effect, **MK1** was subjected to PAS staining and the result revealed the capability of **MK1** to maintain gastric mucus integrity from depletion by ethanol administration.

NP-SH are considered to take a role in protecting gastric mucosa from aggressive agents [157]. Various ulcerogenic agents have been reported to induce tissue damage through decreasing the Endogenous NP-SH level [158]. It's known that ethanol exert its aggressive effect on gastric mucosa through diminishing Endogenous NP-SH content [159, 160]. NP-SH takes a role in controlling the production and the mucus nature to protect the gastric mucosa from noxious effect of ROS formed due to ethanol administration [161]. Our study showed that the **MK1** pretreatment significantly inhibit ethanol induced NP-SH depletion when compare to normal control group. So, it can be conclude that the replenishment of the endogenous NP-SH may contribute in gastroprotective activity of **MK1**.

The role of COX-2 enzymes and NO in gastric ulcer are controversial. It is known that the participation of COX-2 and NO in maintenance of mucosal integrity [162, 163]. COX enzymes: COX-1 and COX-2 are responsible of PGs synthesized [164]. Ethanol was reported to induce gastric mucosal damage through reduction of NO level [115] and inhibition of prostaglandin synthesis [113]. On the other hand, stimulation of the COX-2 enzyme mediates the inflammatory process in the gastric tissue during ulcerogenesis [165]. Again, NO stimulate COX-2 enzyme activity [166].

Hence, control of stomach ulceration is achieved by suppression of inflammatory mediators. Many anti-inflammatory natural compounds were proven to mediate their effect by inhibiting COX -2 and NO production [116]. However, the present study showed **MK1** didn't inhibit COX-2 activity or affect NO level. Therefore, the anti-inflammatory activity is not involved in gastroprotective effect of **MK1**.

The microaerophilic bacterium *Helicobacter pylori* is a gram negative bacilliform consider one of the main etiologic factor in developing peptic ulcer disease [167, 168]. The bacterium infection result from its induction effect on inflammatory cells to the gastric mucosa [169], without invading to the gastric epithelium [170]. Currently, common anti *H. pylori* regimen therapy posses' side effects. Therefore, to discover a new agent with potential anti *H. pylori* activity of high are concern [171]. A potent antibacterial compound will be shown MIC value of less than or equal to 250 [172]. To evaluate the antimicrobial activity of **MK1**, the compound examined against *H. pylori* strains. In this study showed that **MK1** has displayed good result for MIC value against *H. pylori* J99 of 125µg/ml.

CHAPTER 4

CONCLUSION

The studies on the chemical constituents and bioactivity of *Mitrella kentii* have been performed. The results of this study showed that this plant contains alkaloids, terpenoids and flavonoid. These compounds are chalcones, desmosdumotin C **MK1** and its tautomer, 2'-cinnamoyl-3'-hydroxy-5'-methoxy-4',6',6'-trimethylcyclohexa-1',1-dienone **MK2(B)**, terpenoids, stigmasta-4-en-3-one **MK3** and stigmasta-4,22-diene-3,25-dione **MK4**, flavanone, 6-hydroxy-5,7-dimethoxy-2-phenylchroman-4-one **MK5**, benzyl benzoate **MK6**, oxybis(ethane-2,1-diyl) dibenzoate **MK7**, oxoaporphine alkaloids, liriodenine **MK8** and atherospermidine **MK9**. All these compounds were isolated for the first time from *Mitrella kentii* species except for **MK8**.

The current study offered **MK1,** was isolated from *M. kentii* for the first time and the **MK1** had demonstrated the gastroprotective activity against ethanol induced gastric ulcer. The possible gastroprotective mechanisms of **MK1** include indirect antioxidant effect revealed by lowering MDA level and restored GSH level beside HSP-70 up-regulation. In addition, **MK1** also maintained NP-SH content, GSH level, decreased MDA level but didn't affect NO level or COX-2 activity. **MK1** also showed increased HSP-70 activity and decrease control Bax proteins expression in ulcerated tissue. Lastly, **MK1** also displayed good result for MIC against *H Pylori* bacterium.

CHAPTER 5

EXPERIMENTAL

PART A: PHYTOCHEMICAL

5.1 General Experiment Procedures

The ^{1}H- NMR and ^{13}C-NMR experiments were performed on a JOEL 400 MHz spectrometer, a Bruker 400 MHz and 600MHz, respectively. The 2-D NMR experiments were performed on the JOEL and Bruker spectrometer using appropriate pulse sequence programs. The melting point recorded by Stuart SMP30. The infrared spectra (IR) were taken on a Perkin Elmer 1600 Double-Beam recording spectrometer, using chloroform as solvent. The molecular mass spectra were obtained in MeOH on Agilent Technologies 6530 Accurate-Mass Q-TOF LC/MS. The ultraviolet (UV) spectra were obtained in MeOH on a Shimadzu UV-160A ultraviolet-visible spectrometer.

Chromatography was performed using Silica gel 60, 70-230 mesh ASTM (Merck 7734) were used for column and flash chromatography, respectively. All chromatography solvents were analytical grade and HPLC grade. Solvents used for HPLC were filtered through a PTFE membrane 47mm 0.45µm.

5.2 Chromatography

5.2.1 Column Chromatography (CC)

From the beginning of the crude, the column chromatography was used to separate the compounds to the small fractions. It is the first and important method that

must be followed first before doing the next separation such as TLC and PTLC to isolate the compounds. The ratio of the silica gel 60 to the weight of crude (30:1 w/w) usually used to load into the column.

5.2.2 Thin Layer Chromatography (TLC)

Aluminium supported silica gel 60 F_{254} plate was used to observe the spots of the isolated compounds. UV light (254nm-short wave and 365nm-long wave) was used to examine spots or bands on the TLC after subjected to mobile systems.

5.2.3 Preparative Thin Layer Chromatography (PTLC)

PTLC silica gel 60 F_{254} glass plate of size 20 cm x 20 cm was used for the separation of the fractions after CC. UV light (254nm-short wave and 365nm-long wave) was used to examine spots or bands on the TLC after subjected to mobile systems.

5.3 Reagents

For the detection of alkaloids, several different methods have been described. The methods that are used for the detection of all kind of organic compounds in TLC is called non-selective methods like quenching of UV light on fluorescent plates, iodide spray reagents and concentrated sulphuric acid, are usually fairly sensitive, allowing a detection limit of less than 1μg. However, because of lack of specificity of these reagents, other methods are usually preferred for the detection of alkaloids. Methods by means of which alkaloids can be selectively detected are particularly important. Selective and specific alkaloids reagents are various modifications of Dragendorff's

reagent and potassium iodoplatinate. Both reagents react with tertiary and quaternary nitrogen atoms.

5.3.1 Mayer's Reagent

The detection of alkaloids in natural products is by using the Mayer's reagent. It is freshly prepared by dissolving a mixture of mercuric chloride (1.36g) and of potassium iodide (5.00g) in water (100mL). Most alkaloids are precipitated from neutral or slightly acidic solution by Mayer's reagent (potassium mercuric iodide) solution to give a cream coloured precipitate. The Mayer's test is a positive result in indicated by formation of white precipitate (under acidic conditions).

5.3.2 Dragendorff's Reagent

The modifications of this reagent were originally described for the detection of alkaloids on paper chromatograms. The first spray reagent described for this purpose was the modifications proposed by Munier and Macheboeuf. Both modifications are aqueous solutions, the difference being the acid used to dissolved the bismuth salt (acetic and tartaric acid) respectively. The sensitivity of the reagent for alkaloids ranges from 0.01-1µg.

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

Solution B :Potassium iodide (8.00g) in distilled water (20mL)

Stock solution :The mixture of equal volumes of solution A and solution B. Spray reagent the stock solution (20mL) was diluted in a mixture of glacial acetic acid (20mL) and distilled water (60mL)

Dragendorff's test showed positive result by the formation of orange precipitate/spots.

5.4 Plant Material

The stem bark of *Mitrella kentii* (B1.) Miq. was collected in Mersing, Johor. The plant was identified by the phytochemistry group of the Chemistry Department, University of Malaya. A voucher specimen (KL 4139) is deposited at the Herbarium of Department of Chemistry, University of Malaya, Kuala Lumpur, Malaysia.

5.5 Extraction of the stem bark of *M. kentii*

Plant extraction was carried out by exhaustive extraction using the Soxhlet extractor for the hexane extraction, and cold percolation for dichloromethane and methanol extraction.

5.5.1 Hexane extraction

The dried and ground stem bark of *M. kentii* (2.2kg) were extracted with hexane solvent using Soxhlet extractor for 18 hours and the procedure was repeated for three times. Then, the hexane extract was filtered and evaporated under reduced pressure to give the hexane crude.

5.5.2 Acid-base extraction of alkaloids

After the plant material was dried, half (1.0kg) of the samples were soak with 15% ammonia (NH_3) solution and left overnight. Then, they were extracted with dichloromethane (CH_2Cl_2) solvent by cold percolation for three days of each extraction and it was repeated for three times. The CH_2Cl_2 extract were concentrated to about

500ml using the rotary evaporator. After that, the CH_2Cl_2 extract was extracted with 5% hydrochloric acid (HCl) using a separating funnel by liquid-liquid extraction method. The extraction was repeated until Mayer's test is negative. The combined extracts were then basified with concentrated ammonia solution to pH 10-11 and re-extracted with CH_2Cl_2. The CH_2Cl_2 extract was dried with anhydrous sodium sulphate. The solvent was evaporated to dryness. The crude alkaloid was obtained as a dark gummy residue.

5.5.3 Dichloromethane extraction

Another half (1.2kg) of the plant material after the hexane partition was extracted with CH_2Cl_2 solvent by cold percolation without soaking with 15% ammonia (NH_3) solution which means the plant material was only extracted with CH_2Cl_2 solvent. The sample was extracted with CH_2Cl_2 solvent for three days of each extraction and it was repeated for three times. The solvent was evaporated to dryness, to give CH_2Cl_2 crude.

5.5.4 Methanol Extraction

Finally, all the plant material (2.2kg) was extracted with methanol (CH_3OH) solvent. The same procedure as CH_2Cl_2 extraction was followed to give methanol crude.

5.6 Isolation of the crudes

5.6.1 Hexane crude

The hexane crude (3.0g) was subjected to a silica gel column (230-400 mesh). Solvents (n-hexane/CH_2Cl_2/Acetone/CH_3OH) were used to separate the compounds. Eighty-three fractions were collected. After that, each fraction was monitored with TLC

silica gel 60 F_{254} to see the spots of the isolated compounds and if the one fraction is same with another fraction, it is can be combined. Then, the combined fractions were separated using Preparative Thin Layer Chromatography (PTLC). For this hexane crude, fractions 1 to 6 were combined together, and the PTLC method was used to isolate **MK1**, **MK2**, **MK3** and **MK4**. The mobile systems used for these separations were (n-hexane/CH_2Cl_2/Acetone, 8:1.5: 0.5, v/v).

5.6.2 Alkaloid crude

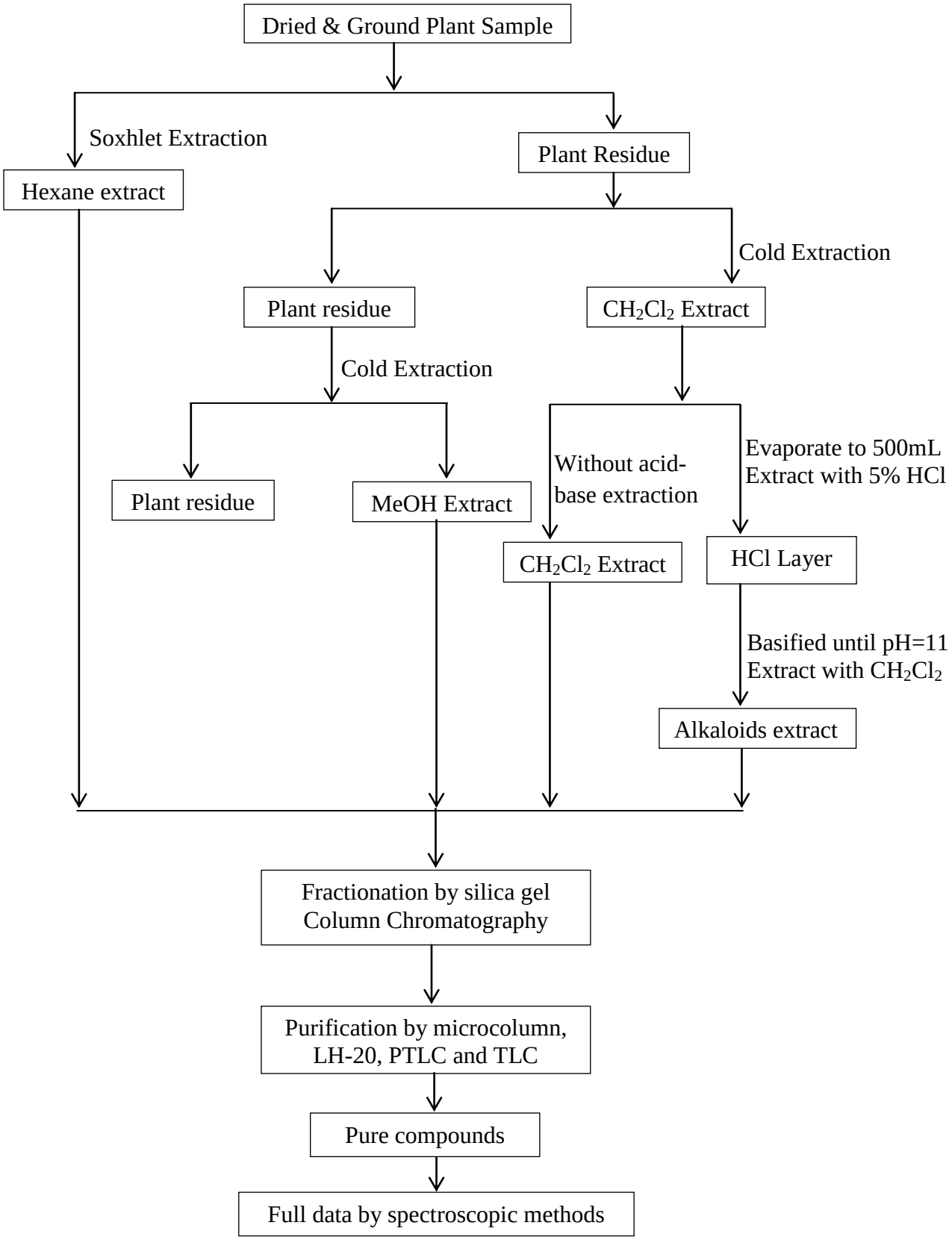
The alkaloid crude (500mg) was subjected to a silica gel column (230-400 mesh). Solvents (n-hexane/CH_2Cl_2/Acetone/CH_3OH) were used to separate the compounds. Fifty-four fractions were collected. Next, the fractions were monitored with TLC. Only fraction 20 gave the good amount to continue the preparative TLC to isolate flavanone compound **MK5**. The mobile systems used for this separation were (CH_2Cl_2/CH_3OH, 98:0.2, v/v)

5.6.3 Dichloromethane crude

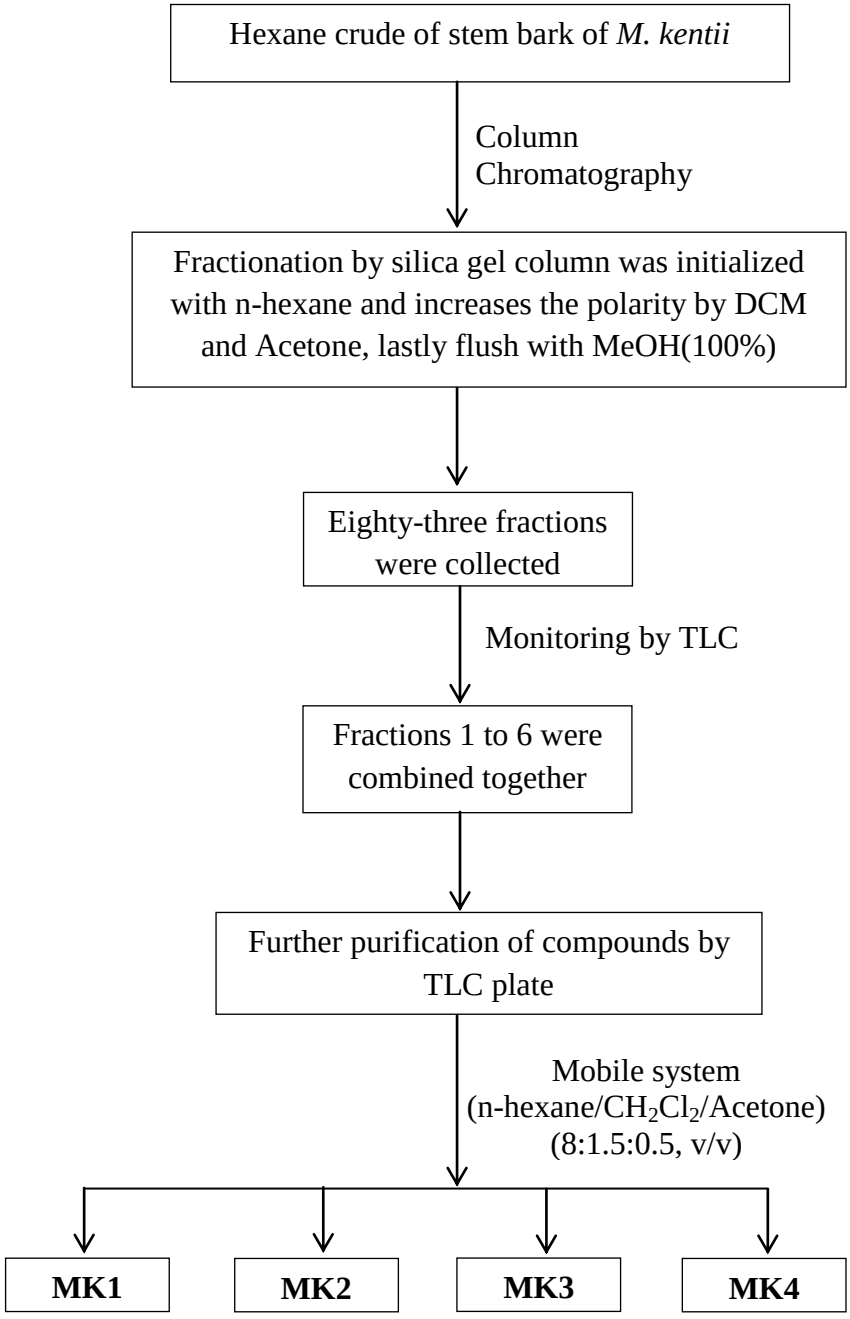
The dichloromethane crude (3.0g) was subjected to a silica gel column (230-400 mesh). Solvents (n-hexane/CH_2Cl_2/CH_3OH) were used to separate the compounds. After fractionating the crude, the fractions were monitored using TLC and the TLC plate was sprayed by Dragendorff's reagent, to determine the alkaloid compounds. Then, the positive fractions with Dragendorff's reagent were selected to isolate the compounds. Fractions 1 to 3 were pooled together and the fractions were isolated with TLC plate by the mobile system (n-hexane/EtOAc,7:3,v/v) to get **MK6**. Fractions 7 to 8 were combined and again proceed to the column chromatography, CC. After that, the

collected fractions were monitored with TLC plate. From these fractions, fraction 2 was separated using TLC plate with mobile system (n-hexane/ EtOAc, 7:3, v/v) to get the **MK7**.

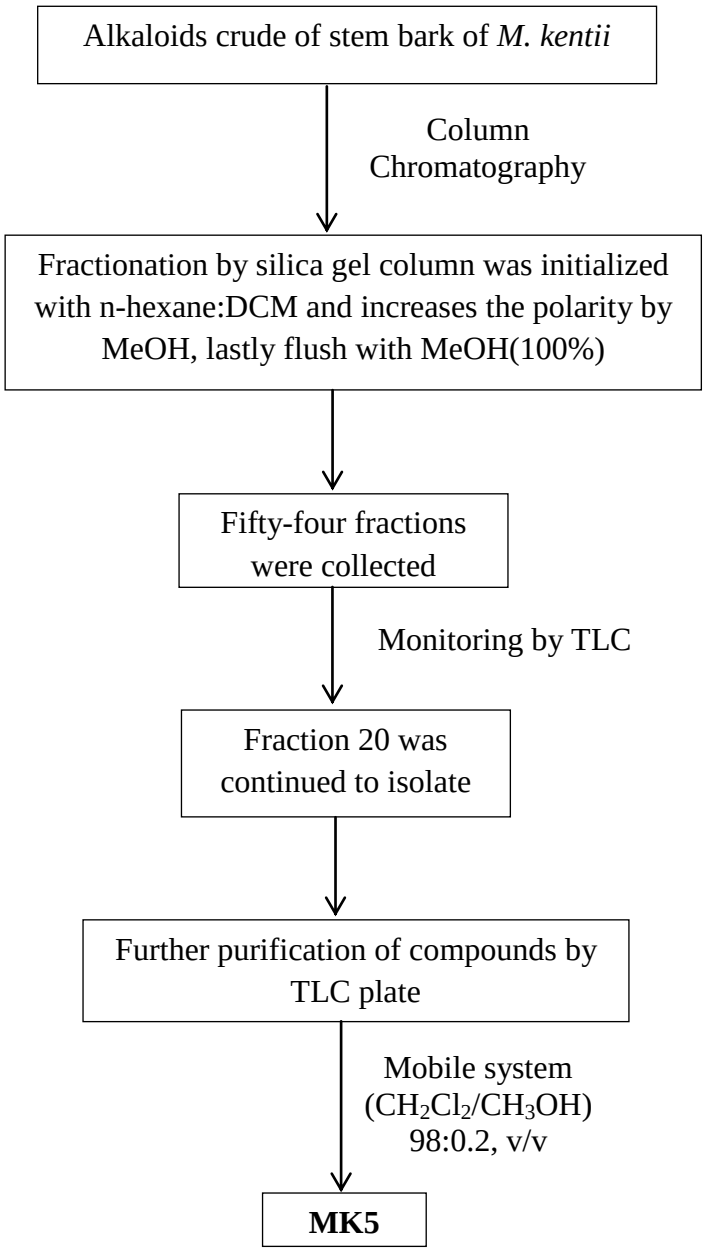
Other fractions with the same compounds were combined and proceed to the isolation using the LH-20 silica. It was used to separate the compounds based on the difference of molecular weight of the compounds. After that, the fractions were monitored with TLC and the fractions with the same compound inside will be combined together and separated using preparative TLC. The mobile systems were used for this separation is (n-hexane/ CH_2Cl_2/Acetone, 1:8.5:0.5,v/v).



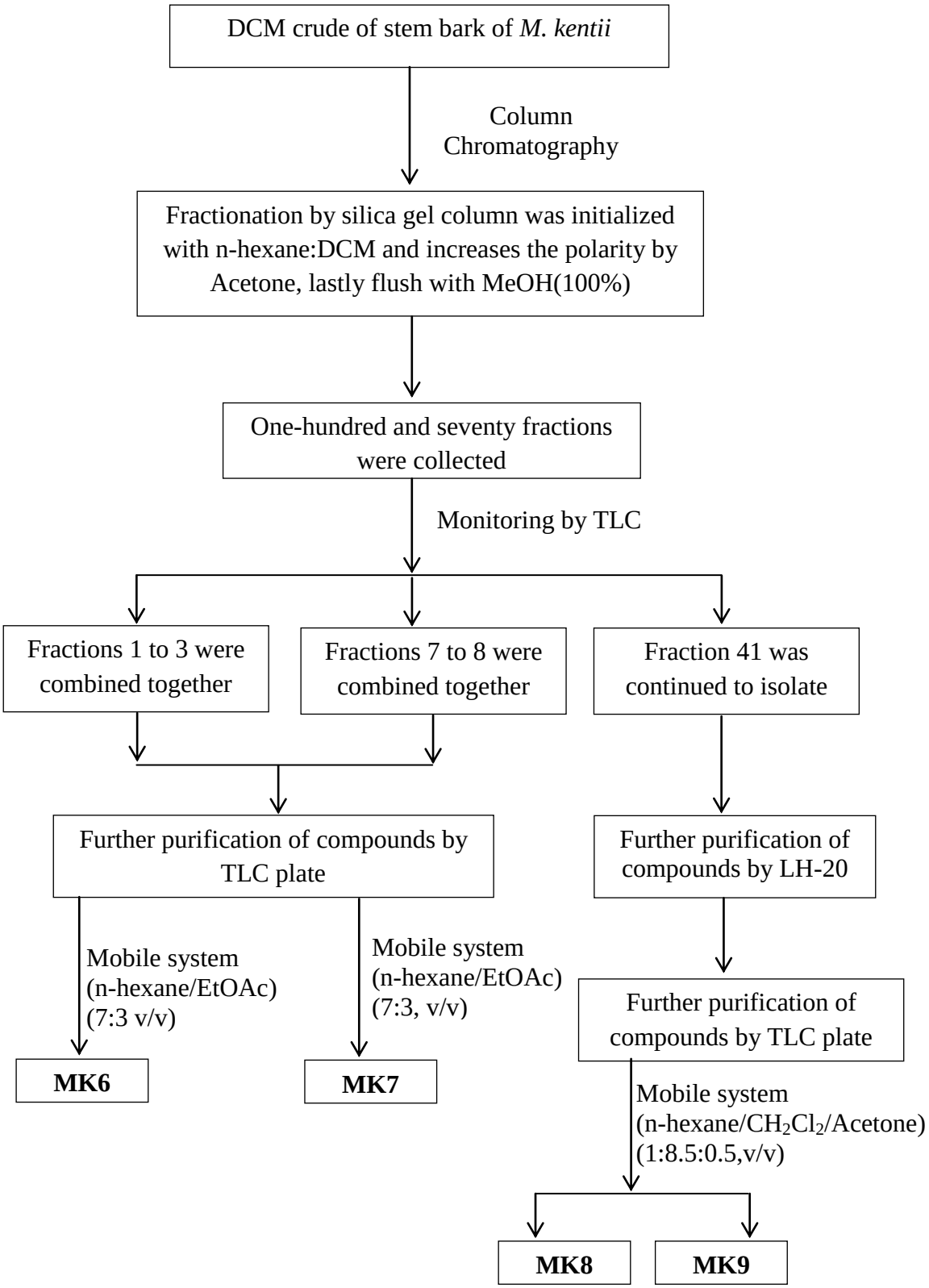
Scheme 5.1: Extraction and isolation of chemical constituents from *M. kentii*



Scheme 5.2: Isolation and purification of chemical constituents from *M. kentii*



Scheme 5.3: Isolation and purification of chemical constituents from *M. kentii*



Scheme 5.4: Isolation and purification of chemical constituents from *M. kentii*.

PART B: BIOACTIVITIES

5B.1 MATERIALS AND METHODS

5B.1.1 Drugs and chemicals

Griess reagent, TPTZ and DTNB were purchased from Sigma-Aldrich Chemical Co. Kuala Lumpur, Malaysia. Indomethacin and Omeprazole were obtained from University Malaya Medical Center and other chemicals and reagents were used as analytical grade (AR grade).

5B.1.2 Animals

Healthy adult male and female ICR mice (6-8 weeks old: 20-30 g) and Sprague Dawley rats (200-220 g) were obtained from the Experimental Animal House [Ethic No FAR/29/06/2012/HMAS(R)] from Faculty of Medicine, University of Malaya. The animals were fed with standard pellets and free access to water *ad libitum*. All animals were received human care according to the criteria outlined in the "Guide for the Care and use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institute of Health.

5B.1.3 Acute toxicity study

Thirty-six mice (18 males and 18 females) were assigned equally into three groups each. The animals were fasted for 24 h, and prior for orally administration of **MK1** at doses of (30, 300 mg/kg) b.w, and 5% Tween 80 v/v served as control group OECD [173]. The food was withheld for further 3 to 4 h after dosing. During 14 days

the animals were been under oversight for any mortality or physiological changes, particularly changes in skin, eyes, mucus membrane, autonomic and central nervous system. The food and water were provided throughout the experiment. At the end, animals were fasted overnight to sacrifice on the day 15th, and the blood was collected for biochemical parameters analysis, the liver and the kidney were excised for organ weight variation and histology study. In addition, to calculate variation of body weight based on the standard method [174].

5B.2.4 Induction of acute gastric lesion

To avoid coprophagy, each rat was kept individually in cages with raised floors of wide mish and all animals divided randomly into six groups (n=6). According to the method by Potrich *et al.,* (2010) and Rozza *et al.,* (2011) was followed[112, 113]. The animals were fasted for 24 h prior for an oral dosing and treatment as follows: groups (A, B) treated with vehicle (5% v/v Tween 80 (5ml/kg b.w.) as normal group and ulcer control group, group (C) (20 mg/kg omeprazole in vehicle (5ml/kg) as positive group control and groups (D, E, F) was treated with **MK1** (5, 10 and 20 mg/kg (5ml/kg), respectively. One hour after this pre-treatment, the rats except group (A) were orally treated with absolute ethanol (5ml/ kg). One hour later, all experimental animal were sacrificed under anesthesia (ketamine & xylazil), the blood was collected from the jugular vein and their stomachs were removed.

5B.2.5 Measurement of gastric juice acidity, mucus content and the biochemical parameters.

Each stomach of the experimental animals were opened along the greater curvature and the gastric contents of each animal was collected and centrifuge to measure the pH of the gastric juice from the supernatant using the pH meter, and to weight the gastric mucosa from the sedimentation using precise balance. The animal blood samples were analyzed at University Malaya Medical Centre to evaluate changes in biochemical parameters [175].

5B.2.6 Gastroprotective Assessments

Gastric ulcer appears as elongated bands of hemorrhagic lesions. The length (mm) and the width (mm) of each bands were measured using planimeter [(10 × 10 mm 2 = ulcer area) under dissecting microscope (1.8×)]. The area of each ulcer lesion was measured by counting the number of small squares, 2 mm × 2 mm, covering the length and width of each ulcer band. The summation of the areas of all lesions for each stomach was applied in the calculation of the ulcer area (UA) wherein the sum of small squares × 4 × 1.8 = UA mm^2. The inhibition percentage (I %) was calculated by the following formula described in Njar *et al.*, (1995) [176] with slight modifications:

Inhibition percentage (I %) = [(UAcontrol − UAtreated) / UAcontrol] × 100%.

5B.2.7 Gastric tolerability test

Each experimental stomachs were observed under an illuminated magnifier (3x) to evaluate the gastric lesions according to the modified scoring system of Adami *et al.*, (1964) [177] (0 : no lesions; 0.5: slight hyperaemia or ≤5 petechiae; 1 : ≤5 erosions ≤ 5

mm in length; 1.5 : ≤5 erosions ≤5 mm in length and many petechiae; 2 : 6-10 erosions ≤5 mm in length; 2.5 : 1-5 erosions > 55 mm in length; 3 : 5-10 erosions >5 mm in length; 3.5 : >10 erosions > 5 mm in length; 4 : 1-3 erosions ≤ 5 mm in length and 0.5-1 mm in width; 4.5 : 4-5 erosions ≤ 5 mm in length and 0.5-1 mm in width; 5 : 1-3 erosions > 5 mm in length and 0.5-1 mm in width; 6 : 4 or 5 grade 5 lesions; 7: ≥ 6 grade 5 lesions; 8 : complete lesion of the mucosa with hemorrhage.

5B.2.8 Histological evaluation

A small fragment of each stomach was fixed in 10% buffered formalin solution, followed by tissue dehydrated with alcohol and xylene. Then, each sample was embedded in paraffin wax, sectioned at 5µm in slides prior for staining. Hematoxylin and Eosin (H&E) stain was used for light microscopy [178]. Moreover, to evaluate mucus production, the slides also stained by periodic acid Schiff base (PAS) following the manufacture instruction (Sigma Periodic Acid-Schiff (PAS) Kit). For further analysis the slides underwent for immunohistochemistry staining using Dako ARK™ (Animal Research Kit), to observed immunohistochemical localization of HSP-70 (1:100) and Bax (1:50) proteins. Both proteins were purchase from Santa Cruz Biotechnology, Inc., Carlifornia, USA.

5B.2.9 Effect of MK1 on tissue homogenate contents

A specimen from each stomach was homogenized in ice cold in 0.1mol/l PBS at a concentration of 10%. The homogenate then centrifuged at 10000 for 15 min at 4^{o}C. The pure supernatant was used to quantify the gastric level of glutathione (GSH), malondialdehyde (MDA), non-protein sulfhydryl group (NP-SH) and nitric oxide (NO).

5B.2.9.1Glutathione levels

Total GSH content (nmol GSH/g tissue) was estimated by interaction with DTNB (5,5-ditiobis-2-nitrobenzoic acid) and the absorbance was read in a spectrophotometer at 412 nm [179].

5B.2.10Thiobarbituric Acid reactive substance assay

Thiobarbituric Acid reactive substance (TBARS) assay was used to estimate malondialdehyde (MDA) content. According to Hodges *et al.,* (1999) [180] the stomach homogenate was added to a 0.126 ml solution containing 26 Mm thiobarbituric, 0.26 M HCl, 15% trichloroacetic acid and 0.02% butaylated hydroxytoluene. The mixture was incubated in a water bath at 95^{o}C for 1h. After cooling, the mixture was centrifuged at 3000 g for 10min. The absorbance was determined in a spectrophotometer at 532 nm and the results were expressed in µmol/g tissue MDA. Tetramthoxy propane was used as standard.

5B.2.11Determination of nitric oxide

Nitric oxide content (µmol/g tissue) in tissue was quantified by measuring nitrite/nitrate concentration using Griess assay [181]. In brief, stomach homogenates were deproteinated with absolute ethanol for 48h at 4°C, and then centrifuged at 12000g for 15min at 4°C. To an aliquot of the supernatant, vanadium trichloride 0.8% (w/v) in 1M HCl was added for the reduction of nitrate to nitrite, followed by the rapid addition of Griess reagent (Sigma Aldrich, Malaysia) and the absorbance was measured at 540nm. Sodium nitrite was used as standard.

5B.2.12Estimation of nonprotein sulfhydryls (NP-SH)

Gastric mucosal nonprotein sulfhydryls (NP-SH) (µmol/g of tissue) were measured according to the method of Sedlak and Lindsay (1968) [182]. In briefly, aliquots of 5ml of the stomach homogenates were mixed with a solution containing 4ml of distilled water and 1ml of 50% trichloroacetic acid. The mixture was vortex for 15 min and centrifuged at 3000× g. 2ml of supernatant was mixed with 4ml of 0.4M Tris Buffer at pH 8.9; 0.1ml of DTNB [5,5 dithio-bis-(2-nitrobenzoic acid)] was added and the sample was shaken. The absorbance was recorded within 5 min of addition of DTNB at 412nm against a reagent blank with no homogenate.

5B.2.13*In vitro* evaluation of COX- 2 inhibitory activity

The COX-2 inhibitory activity of MK1was estimated using a COX-inhibitor screening Kit (Cayman Chemical, USA). According to the manufactures an instruction, MK1 was dissolved in DMSO at final concentration was 0-100µg/ml. The inhibition was calculated by the comparison of compound treated to control incubations. Indomethacin COX-2 inhibitor was used as standard reference.

5B.2.14*In vitro* anti-*Helicobacter pylori* activity

Helicobacter pylori strains J99 (*ATCC* 700824) was cultured with brain heart infusion broth (BHI; Oxoid) supplemented with 10% horse serum (Invitrogen) incubated at 37^{o}C in a humidified CO_2 incubator (Forma Steri-Cycle) for 3days. Minimum inhibitory concentration (MIC) was determined by a modified microtiter broth dilution method on sterile 96-well Polypropylene microtitre plates with round-bottom wells (Eppendorf). Briefly, **MK1** was dissolved and diluted in 5% DMSO to

give a 10x working stock solution *H. pylori* was diluted to a final concentration of 2x 10^6 CFU/mL in culture medium. Aliquots of 10µL of **MK1** were added to 90µL of *H. pylori* in a well of the microtitre plate. Concentration of **MK1** ranged from 31.25 to 250 µg/mL. The microtiter plate was incubated for 3 days in a CO_2 incubator. The plate was examined visually and measured using a microplate reader (Varioskan Flash) at 600nm to determine the lowest concentration showing complete growth inhibition, which was recorded as the MIC. Wells containing *H. pylori* with 10µL of 5% DMSO and BHI medium containing 250µg/mL **MK1** was used as control and blank, respectively. The result was recorded in accordance with the Clinical and Laboratory Standards Institute [183].

5B.2.15Ferric-reducing antioxidant power (FRAP) assay

To describe total antioxidant activity of **MK1**, the FRAP assay was estimated according to the method of Benzie and Strain (1996) with slightly modification [184]. In briefly, FRAP reagent was prepared freshly from acetate buffer (pH 3.6), 10mM TPTZ [2,4,6-Tri(2-pyridyl)-s-triazine] solution in 40mM HCl and 20mM iron (III) chloride solution in proportions of 10:1:1 (v/v), respectively. 50µl of the compound were added to 1.5ml of the FRAP reagent in the dark, 4 min later the absorbance was then recorded at 593nm. The standard curve was constructed linear (R^2 = 0.9723) using iron (II) sulfate solution (100–1000µM), and the results were expressed as µM Fe (II)/g dry weight of the compound.

5B.2.16DPPH assay method

The scavenging activity of the **MK1** was evaluated according to the recommended method by Michielin *et al.,* (2011) [185]. Briefly, the compound was mixed with 0.3 Mm DPPH/ethanol solutions to give final concentration s of **MK1** (50, 25, 12.5, 6.25µg/ml in ethanol. After 30 min, the absorbance was observed at 518nm then converted into percentage of antioxidant activity expressed as the inhibition concentration at 50% (IC_{50}), the meaning is the concentration of the compound in µg/ml required to inhibit the production of radicals by 50%.

5B.2.17Statistical analysis

All tests were performed at least in triplicates and the values were represented as mean ± S.E.M (standard error mean). The Statistical differences between groups were determined according to SPSS version 16.0 and Graph Pad prism using ordinary one-way ANOVA followed by Dunnetts multiple comparison tests. A value of $p<0.05$ was considered significant.

5.3 Physical and Spectral Data of Isolated Compounds

5.3.1 Desmosdumotin C MK1

Molecular formula : $C_{19}H_{20}O_4$

UV λ_{max} nm : 191, 232, 376

IR (NaCl) ν_{max} cm^{-1} : 1514, 1576, 1624, 1657

Mass Spectrum $[M+H]^+$: 313.1435

^{1}H-NMR ($CDCl_3$) ppm : Refer Table 3.1

^{13}C-NMR ($CDCl_3$) ppm : Refer Table 3.1

5.3.2 2'-cinnamoyl-3'-hydroxy-5'-methoxy-4',6',6'-trimethylcyclohexa-1',1-dienone MK2(B)

Molecular formula : $C_{19}H_{20}O_4$

UV λ_{max} nm : 242, 320, 485

IR (NaCl) ν_{max} cm^{-1} : 1514, 1576, 1624, 1657

Mass Spectrum $[M+H]^+$: 313.1435

^{1}H-NMR ($CDCl_3$) ppm : Refer Table 3.2

^{13}C-NMR ($CDCl_3$) ppm : Refer Table 3.2

5.3.3 Stigmasta-4-en-3-one MK3

Molecular formula : $C_{29}H_{48}O$

UV λ_{max} nm : 282

IR (NaCl) ν_{max} cm^{-1} : 1618, 1677

Mass Spectrum $[M]^+$: 412

^{1}H-NMR ($CDCl_3$) ppm : Refer Table 3.3

^{13}C-NMR ($CDCl_3$) ppm : Refer Table 3.3

5.3.4 Stigmasta-4,22-diene-3,25-dione MK4

Molecular formula : $C_{28}H_{42}O_2$

UV λ_{max} nm : 282

IR (NaCl) ν_{max} cm^{-1} : 1618, 1677, 1730

Mass Spectrum $[M]^+$: 410

^{1}H-NMR ($CDCl_3$) ppm : Refer Table 3.4

^{13}C-NMR ($CDCl_3$) ppm : Refer Table 3.4

5.3.5 6-hydroxy-5,7-dimethoxy-2-phenylchroman-4-one MK5

Molecular formula : $C_{17}H_{16}O_5$

UV λ_{max} nm : 243, 288, 348

IR (NaCl) ν_{max} cm^{-1} : 1613, 1660, 3371

Mass Spectrum $[M+H]^+$: 301.1063

^{1}H-NMR ($CDCl_3$) ppm : Refer Table 3.5

^{13}C-NMR ($CDCl_3$) ppm : Refer Table 3.5

5.3.6 Benzyl benzoate MK6

Molecular formula : $C_{14}H_{12}O_2$

UV λ_{max} nm : 228, 273, 300

IR (NaCl) ν_{max} cm^{-1} : 711, 1109, 1270, 1722, 2924, 3033, 3066

Mass Spectrum $[M]^+$: 212

^{1}H-NMR ($CDCl_3$) ppm : Refer Table 3.6

^{13}C-NMR ($CDCl_3$) ppm : Refer Table 3.6

5.3.7 Oxybis(ethane-2,1-diyl) dibenzoate MK7

Molecular formula : $C_{18}H_{18}O_5$

UV λ_{max} nm : 197, 229, 275, 331, 346, 392, 405, 429, 463, 485

IR (NaCl) ν_{max} cm^{-1} : 1615, 1667

Mass Spectrum $[M+H]^+$: 315.1231

^{1}H-NMR ($CDCl_3$) ppm : Refer Table 3.7

^{13}C-NMR ($CDCl_3$) ppm : Refer Table 3.7

5.3.8 Liriodenine MK8

Molecular formula : $C_{17}H_9NO_3$

UV λ_{max} nm : 205, 248, 269, 309, 413

IR (NaCl) ν_{max} cm^{-1} : 1737

Mass Spectrum $[M+H]^+$: 276.0658

^{1}H-NMR ($CDCl_3$) ppm : Refer Table 3.8

^{13}C-NMR ($CDCl_3$) ppm : Refer Table 3.8

5.3.9 Atherospermidine MK9

Molecular formula : $C_{18}H_{11}NO_4$

UV λ_{max} nm : 248, 280, 440, 485

IR (NaCl) ν_{max} cm^{-1} : 1737

Mass Spectrum $[M+H]^+$: 306.0759

^{1}H-NMR ($CDCl_3$) ppm : Refer Table 3.9

^{13}C-NMR ($CDCl_3$) ppm : Refer Table 3.9

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