# PHYTOCHEMICAL AND ANTIOXIDANT STUDIES OF *CALOPHYLLUM SCLEROPHYLLUM*

By

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## ABSTRACT

### PHYTOCHEMICAL AND ANTIOXIDANT STUDIES OF *CALOPHYLLUM SCLEROPHYLLUM*

**Yong Fui Lu**

Chemical study on medicinally important plant secondary metabolites had led to the identification of various useful compounds for further study into their pharmacological activities. It involves extraction, isolation, purification and structural characterization of compounds isolated from plants. In this project, the stem bark of *Calophyllum sclerophyllum* was subjected to sequential solvent extraction by using dichloromethane, ethyl acetate and methanol. The chemical compounds in the crude extracts were subsequently separated using column chromatography. From the ethyl acetate crude extract, a triterpenoid and three coumarins were successfully isolated, namely friedelin **[24]**, 5,7-dihydroxy-6-(2-methylbutyryl)-4-phenylcoumarin **[25]**, 5,7-dihydroxy-6-(3-methylbutyryl)-4-phenylcoumarin **[26]** and isodispar B **[27]**. Characterization of these pure compounds was carried out through various spectroscopic analyses, including 1D- and 2D-NMR, UV-Vis, IR, GC-MS and LC-MS. Besides, all the crude extracts of *Calophyllum sclerophyllum* and isolated compounds were investigated for their antioxidant potential via DPPH assay.

# ABSTRAK

Kajian kimia tentang metabolit tumbuhan yang mempunyai nilai perubatan telah membawa pengenalan pelbagai kompaun berguna untuk kajian aktiviti farmakologi masa depan. Ia melibatkan pengekstrakan, pengasingan, pembersihan dan analisis struktur kompaun. Dalam projek ini, kulit kayu batang *Calopyhllum sclerophyllum* telah diekstrak berturutan dengan menggunakan diklorometana, etil asetat dan metanol. Kompaun-kompaun dalam ekstrak mentah seterusnya diasingkan dengan menggunakan kromatografi. Daripada ekstrak etil asetat, triterpenoid dan tiga koumarin telah diasingkan, iaitu friedelin **[24]**, 5,7-dihydroksi-6-(2-metilbutiril)-4-fenilkoumarin **[25]**, 5,7-dihydroksi-6-(3-metilbutiril)-4-fenilkoumarin **[26]** dan isodispar B **[27]**. Struktur-struktur kompaun telah diperolehi melalui pelbagai spektroskopi analisis, termasuk 1D- dan 2D-NMR, UV-Vis, IR, GC-MS dan LC-MS. Selain itu, ekstrak mentah *Calophyllum sclerophyllum* dan kompau-kompaun tulen telah diujikan bagi potensi antioksiden dengan menggunakan kaedah DPPH.

## ACKNOWLEDGEMENTS

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## DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledge. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

_____

(YONG FUI LU)

## APPROVAL SHEET

The project report entitled "**PHYTOCHEMICAL AND ANTIOXIDANT STUDIES OF *CALOPHYLLUM SCLEROPHYLLUM***" was prepared by YONG FUI LU and submitted as partial fulfillment of the requirements for the degree of Bachelor of Science (Hons) Chemistry at Universiti Tunku Abdul Rahman.

Approved by:

_____ Date: _____

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**FACULTY OF SCIENCE**

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**Date: _____**

**PERMISSION SHEET**

It is hereby certified that **YONG FUI LU** (ID No: **12ADB01527**) has completed this final year project entitled "**PHYTOCHEMICAL AND ANTIOXIDANT STUDIES OF *CALOPHYLLUM SCLEROPHYLLUM***" supervised by Dr. Lim Chan Kiang from the Department of Chemical Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

_____

(YONG FUI LU)

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| $^{13}C$ | Carbon-13 |
| $^{1}H$ | Proton |
| DCM | Dichloromethane |
| EtOAc | Ethyl acetate |
| MeOH | Methanol |
| TE | Trolox Equivalent |
| µmol | Micromole |
| µg | Microgram |
| µL | Microliter |
| mL | Mililiter |
| mm | Milimeter |
| cm | Centimeter |
| nm | Nanometer |
| mg | Miligram |
| mol | Mole |
| g | Gram |
| kg | Kilogram |
| $^{o}C$ | Degree in Celsius |
| β | Beta |
| δ | Chemical shift |
| $\delta_C$ | Chemical shift of carbon |
| $\delta_H$ | Chemical shift of proton |
| $\lambda_{max}$ | Maximum wavelength |
| NMR | Nuclear Magnetic Resonance |
| 1D-NMR | One Dimension Nuclear Magnetic Resonance |
| 2D-NMR | Two Dimension Nuclear Magnetic Resonance |

| | |
|---|---|
| FTIR | Fourier-Transform Infrared Spectroscopy |
| IR | Infrared |
| HMQC | Heteronuclear Multiple Quantum Coherence |
| HMBC | Heteronuclear Multiple Bond Coherence |
| HRESIMS | High Resolution Electrospray Ionization Mass Spectrometry |
| HPLC | High Performance Liquid Chromatography |
| LC-MS | Liquid Chromatography-Mass Spectrometry |
| GC-MS | Gas Chromatography-Mass Spectrometry |
| UV-Vis | Ultraviolet-Visible |
| TLC | Thin Layer Chromatography |
| $A_1$ | Absorbance of the test sample |
| $A_o$ | Absorbance of the blank (negative control) |
| c | Concentration of sample in g/mL |
| O-H | Oxygen-Hydrogen (or Hydroxyl) |
| C-O | Carbon-Oxygen (or Carbinol) |
| C=O | Carbon=Oxygen (or Carbonyl) |
| C-H | Carbon-Hydrogen |
| C=C | Carbon=Carbon |
| $CDCl_3$ | Deuterated chloroform |
| Acetone-$d_6$ | Deuterated acetone |
| KBr | Potassium bromide |
| TMS | Tetramethylsilane |
| DPPH | 1,1-diphenyl-2-picrylhydrazyl |
| $IC_{50}$ | Half maximal inhibitory concentration |
| BPX5 | 5% phenyl/ 95% methyl polysilphenylene/ siloxane phase |
| ppm | Part per million |
| $R_f$ | Retention factor |

| | |
|---|---|
| s | Singlet |
| d | Doublet |
| dd | Doublet of doublets |
| dq | Doublet of quartets |
| t | Triplet |
| m | Multiplet |
| Hz | Hertz |
| *J* | Coupling constant in Hertz |
| $J_{CH}$ | Heteronuclear coupling between carbon and proton |
| *m/z* | Mass-to-charge ratio |
| AGEs | Advanced glycation end-product |
| B.C.E. | Before common era (used in timeline) |
| mM | Milimoles |

# CHAPTER 1

# INTRODUCTION

## 1.1 General Introduction

Nature has been a source of medicinal agents for thousands of years. In today's world, application of medicines originated from natural sources is growing worldwide due to its minimal side effects. "Natural products" is generally referred to chemical compounds that are derived from living organisms, including plants, animal, insects, and microorganisms. The compounds that plant produce can be categorized as primary and secondary metabolites. The primary metabolites, including protein, carbohydrates, lipids, nucleic acids and enzymes, play an integral role in vital life functions of plant, like photosynthesis as well as respiration. They are crucially important molecules of life. In contrast to primary metabolites, secondary metabolites are not essential for organism's development and growth. Plants synthesize them in small quantities for the purpose of survival. The secondary compounds perform other functions such as rendering colour to flowers and fragrance to assist in pollination, or toxicity as a deterrent to predators. It is this area of secondary metabolism which provides most of the pharmacologically active natural products (Dewick, 2009).

Natural products have served as an important source of drugs since ancient times. Several thousand years ago, hunter-gatherers collected certain herbs with medicinal values to cure specific ailments. This knowledge was passed down by word of mouth over generations, and the first medicinal plant record written on clay tablets was from Mesopotamia, dates from about 2600 B.C.E. (Wong, 2009). In the history of traditional Chinese medicine, The *Compendium of Materia Medica*, also known as *Pen-Tsao Kang-Mu*, has been extensively documented over the centuries. This compilation consisting of 11096 prescriptions used to treat common illnesses, and is widely spread until today. Likewise, the Indian Ayurvedic medicine also has a long history in pharmaceutical field, dates from 1100 B.C.E. (Cragg and Newman, 2002).

The fundamental methodologies involved in natural product chemistry are extraction, compounds isolation, purification and structural elucidation. Innovations in technology and equipment have often played an important role in the progress of natural product chemistry. Early in the 19th century, plant extraction technology was developed, whereby the bioactive compounds from plants were chromatographically isolated, purified and drugs were produced. Once the structure of compound was known, it was possible to produce them synthetically. Natural compounds are used as templates for chemical synthesis of their analogs which are having different structures but possess common pharmacophores. These synthetic compounds may have superior and more effective biological activities than the original compounds. The medicinal products can be in the form of tablets, capsules, ointments, or liquids. For example, salicin is an anti-inflammatory agent isolated from willow bark,

which is then chemically modified into aspirin by scientists through "Kolbe synthesis" method (Vane, 1971).

It is of significance that secondary metabolites found in plant-derived natural products are proven to be beneficial as therapeutic agents, even though these phytochemicals are non-nutritive, and not essential to life processes. These chemical constituents include alkaloids, flavonoids, coumarins, terpenes, saponins, and steroids (Corrado, 2001). Many of these phytochemicals show antioxidant activity, which are natural antioxidants that human valued for treatment of cancers. Free radicals are generated via oxidation, and they are reactive and unstable, thus, initiate chain reaction and caused oxidative stress in human body. Oxidative stress is caused by unbalance between oxidant and antioxidant, which can increase the risk of developing cancers due to cell damaged and tissue injury. One of the therapies that can reduce the risk of cancer is by intake of antioxidant (Rissyelly, 2014). Antioxidants terminate these radical chain reactions by conversion of free radical intermediates to a relatively stable compound. As a result, antioxidants are termed as "free radical scavengers".

Malaysia is one of the 12 mega biodiversity countries in the world which has about 19.12 milion hectares of rainforest area. It is estimated that there are around 8100 plant species, with 10% of them were reported to show some medicinal values (Wong, 2009). The *Calophyllum* species in Sarawak are of great interest for phytochemical investigations due to their broad-spectrum of

pharmacological properties, particularly with immune protection in HIV disease and AIDS (Ma, et al., 2008). In this project, our works have been focused on the isolation of bioactive secondary metabolites from *Calophyllum sclerophyllum* and to test their antioxidant activity.

## 1.2 Botany of Plant Species Studied

### 1.2.1 Taxonomy

The plant kingdom represents an extraordinary reservoir of novel molecules. Of the estimated 400 000 – 500 000 plant species around the globe, only a small percentage has been investigated phytochemically (Corrado, 2001). The genus *Calophyllum*, previously placed in Clusiaceae (or Guttiferae) family, is currently classified as a member of Calophyllaceae family (APG III, 2009; Wurdack and Davis, 2009). *Calophyllum* is the largest genus in Calophyllaceae that encompasses about 190 variety species (Díaz, 2013). The taxonomy of *Calophyllum sclerophyllum* is shown in the Table 1.1.

**Table 1.1: Taxonomy of *Calophyllum sclerophyllum***

| | | |
|---|---|---|
| Kingdom | : | Plantae |
| Division | : | Tracheophyta |
| Class | : | Spermatopsida |
| Order | : | Malpighiales |
| Family | : | Calophyllaceae |
| Genus | : | *Calophyllum* |
| Species | : | *Calophyllum sclerophyllum* |

### 1.2.2 Morphology

*Calophyllum sclerophyllum* is an evergreen tree, growing up to 40 m in height. It has a thick trunk covered with a rough, greyish-brown, fissured and cracked bark. The bark oozes a yellowish-white resin. Stilt-roots or pneumatophore ("breathing") roots growing up to 4 m high on the trunk is one of the special features of this species (Corner, 1978). The leaves of trees are simple, deep glossy green, elliptic to obovate with a blunt end, and about 8 cm long and 5 to 7 cm wide. There are many parallel lateral veins perpendicular to the midvein of the leaves. The flowers are white, about 2.5 cm wide, and have a very strong sweet fragrance (Wong, 2009). *Calophyllum sclerophyllum* produces spherical drupes fruits and is in green color. They arrange in clusters and attach to the branches (Dweck and Meadows, 2002). The picture of *Calophyllum sclerophyllum* is shown in Figure 1.1.

**Figure 1.1 *Calophyllum sclerophyllum* tree and its fruits**

### 1.2.3 Geographical Distribution and Habitat

*Calophyllum sclerophyllum* is widely distributed in tropical rainforest of Asia regions such as Thailand, Johor and Sarawak of Malaysia, Singapore and Kalimantan of Indonesia (Tjitrosoepomo, 1996). *Calophyllum* is more generally known by indigenous people as *Bintagor jangkang* or *Penaga darat*. It grows naturally and abundant in the swamp-forest, some of which on rocky and sandy sea shores (Corner, 1978).

### 1.2.4 Ethnomedicinal Uses and Pharmacological Studies

Numerous *Calophyllum* species have been used as folk medicine. In Malaysia, the Malays use its seed oil as a remedy for ulcer and rheumatism ailments. Moreover, Dweck and Meadows (2002) reported that there was great improvement in scars after tamanu oil applied on visually obvious scars. An infusion of the leaves is used to treat inflamed eyes. The wood of the tree is hard, thus it is used in timber industry for construction material, ship building, and furniture (Díaz, 2013).

The modern pharmacology research on the genus *Calophyllum* has further revealed a variety of biological activities exhibited by these plants, such as inhibition of HIV, antioxidant, antitumor-promoting, antimalarial, as well as

cytotoxic activity. Various parts of these trees are found to be rich in bioactive phytochemicals, including xanthones, coumarins, chromanones (flavonoids, biflavonoids), terpenes and steroids (Su, et al., 2008). Hence, *Calophyllum* has been gaining interest of the scientific community due to its promising chemical and biological results. Previous studies on *Calophyllum sclerophyllum* has reported the isolation of jacaerubin, 1,3,5,6 tetraoxygenated xanthones and euxanthone, but there was no study about their biological activities (Jackson, 1966). Recently, a research study conducted by Rissyelly and co-workers (2014) on the ethanolic extract of the same species has resulted in the isolation of astilbin which showed antioxidant activity, and may lower the risk of cancer.

## 1.3 Objectives of Study

The purposes of conducting this study are:

- To extract and isolate chemical constituents from the stem bark of *Calophyllum sclerophyllum.*
- To identify and characterize the structures of isolated compounds through modern spectroscopic techniques.
- To investigate the antioxidant activity of the pure isolates and crude extracts of *Calophyllum sclerophyllum* via DPPH assay.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Phytochemical Studies

Phytochemistry, generally termed “plant chemistry”, is a study of chemical constituents that are naturally occurring in plants. The term secondary metabolites is referring to the phytochemicals that are synthesized by specialized plants cells in a trace quantity via secondary metabolism pathway. Each plant family, genus and species produces characteristic phytochemicals. Secondary metabolites have no obvious function in a plant’s primary metabolism as well as in photosynthesis and reproduction. They may possess an ecological role, as pollination attractants, represent chemical adaptions to environmental stresses, or coordinate the development of the whole plant defence mechanisms. For instance, plants produce toxic phytoalexins to kill pathogenic microbes and insects (Monika, Joseph and Teresa, 2008).

These secondary compounds are highly valued by human. Mankind use many of these compounds as spices, flavors, fragrances, insecticides and in many cases as drugs. In fact, a large number of plants used in the traditional medicine have now become a part of the modern world health care system because of their unique ability to synthesize a wide array of compounds with diverse health-related benefits (Naithani, et al., 2010). Natural products are

inexpensive, better compatibility with the human body and minimal side effects. Thus, many research efforts have been devoted for the discovery of therapeutic constituents from plants. A wide variety of active phytochemicals have been identified and found to possess a broad range of biological activities such as antimicrobial, antiviral, antioxidative, anti-malarial and antitumor properties (Su, et al., 2008). Extensive phytochemical studies have shown plant kingdom to be a rich source of secondary metabolites including alkaloids, saponins, triterpenoids, flavonoids, lactones and organic acids.

### 2.1.1 Coumarins

Coumarins (2*H*-1-benzopyran-2-one) are plant-derived natural products with molecular formula of $C_9H_6O_2$. The core structure of coumarin is comprising of fused benzene and $\alpha$-pyrone rings, as shown in Figure 2.1. Dietary exposure to benzopyrones is significant as these compounds are found in vegetables, fruits, seeds, nuts, coffee, tea, and wine. Natural coumarins are mainly classified into six categories based on their chemical structure, including simple coumarins, furanocoumarins, pyranocoumarins, dihydrofuranocoumarins, phenylcoumarin and bicoumarins (Venugopala, Rashmi and Odhav, 2013).

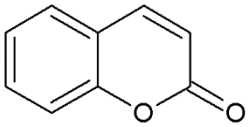


**Figure 2.1: Basic building block of coumarins**

The physicochemical properties and therapeutic applications of natural coumarins depend upon the pattern of substitution. Several compounds belonging to this class have shown a wide range of biological activitives such as potent cytotoxic activity against several tumor cell lines, anti-HIV, anti-inflammatory, antioxidant, anticoagulant, and antifungal properties. Numerous reports have appeared in the literature concerning about the calanolide A, which is a coumarin isolated from *Calophyllum lanigerum* and was identified as an inhibitor of HIV-1 reverse transcriptase as well as treatment for tuberculosis (Saklani and Kutty, 2008). Meanwhile, coumarin A/AA was reported to be cytotoxic towards cervical cancer cells by causing cell shrinkage or apoptosis (Carolina, et al., 2009). Fraxin and esculin extracted from *Actinidia deliciosa* (kiwifruit) showed free radical scavenging effect which protects cells from oxidative stress (Hirsch, Longeon and Guyot, 2002).

### 2.1.2 Xanthones

Xanthones, also known as 9*H*-xanthen-9-ones, are a class of natural polyphenolic compounds with a yellow coloration and a basic molecular formula of $C_{13}H_8O_2$. All of them have dibenzo-γ-pyrone as the nuclear skeleton, in which the two benzene rings bridged across a carbonyl group and an oxygen. Xanthone and its derivatives are principally obtained from higher plants in Gentianaceae, Guttiferae and Polygalaceae families, fungi as well as lichens (Negi, et al., 2013). The activities of xanthones are altered by the types of chemical substituents that attached to the backbone and their positions.

Naturally occurring xanthones have gradually risen to great importance in view of their remarkable pharmacological and other biological activities. Interestingly, xanthones extracted from the plant *Hypericum perforatum* possess antidepressant action and can be used as antidepressive drugs (Zhao, et al., 2014). Besides that, 1,8-dihydroxy-3,5-dimethoxyxanthone (swerchirin) isolated from *Swertia chirayita* was reported to have hypoglycemic effect, thus reducing blood sugar significantly against diabetes (Bajpai, et al., 1991). Basic molecular structure of xanthone is shown in Figure 2.2.

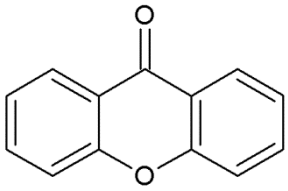


**Figure 2.2: Structure of xanthone core**

### 2.1.3 Terpenes

Terpenes are a large and diverse class of naturally occurring phytochemicals derived from five carbon isoprene units. Since the molecular formula of isoprene is $C_5H_8$, hence, terpenes are generally expressed in the formula of $(C_5H_8)_n$, whereby n indicates the number of isoprene units that linked together (Corrado, 2001). For instance, triterpene is a natural compound containing thirty carbon atoms based on six isoprene units. According to isoprene rule, isoprene units are linked in a head-to-tail manner.

Terpenoids are derivatives of terpenes and their structures consisting of functional groups such as hydroxyl and carbonyl groups. The terpenes are classified into monoterpenoids ($C_{10}$), sesquiterpenoids ($C_{15}$), diterpenoids ($C_{20}$), sesterterpenoids ($C_{25}$), triterpenoids ($C_{30}$) and carotenoids ($C_{40}$). Most of the terpenes exist in cyclic form (Corrado, 2001). Monoterpenoids are major components of the aromas of plants. These volatile natural products, known as essential oils, form the basis of the perfumery and flavouring industries. Menthol, a constituent of mint oil, is obtained commercially from *Mentha arvensis* and possesses useful physiological properties including anaesthetic and refreshing effects. It is used to flavour sweets, tobacco and toothpaste (Wiart, 2013). Figure 2.3 shows the molecular structure of isoprene.

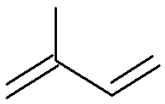


**Figure 2.3: Molecular structure of isoprene**

### 2.1.4 Flavonoids

Flavonoids are compounds that are responsible for the colour of flowers, fruits and leaves. The name refers to the Latin word ‘flavus’ meaning yellow. Flavonoids protect the plant from UV-damaging effects and play a role in pollination by attracting animals by their colours. Flavonoids (or bioflavonoids) are polyphenolic compounds that occurs in nature with the basic structural formula $C_6$—$C_3$—$C_6$. The basic structure of flavonoids is 2-phenyl chromane

or an Ar–$C_3$–Ar skeleton, which involves two aromatic rings bonded to a three carbons aliphatic moiety that form an oxygenated heterocycle. In plant, flavonoids can either occur as aglycones or as O- or C-glycosides. Based on their chemical structure, flavonoids are divided into several subclasses, namely flavone, flavonols, isoflavonoids, chalcones, flavanols and flavanones (Gurib-Fakim, 2006). Figure 2.4 shows the basic molecular structure of flavones.

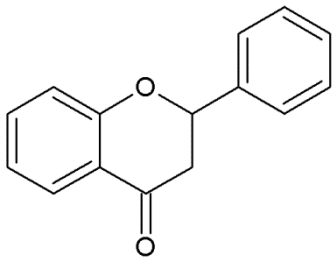


**Figure 2.4: Molecular structure of flavones**

Natural flavonoids are of great interest due to their widespread pharmacological properties, and this attracts many medicinal chemists for further backbone derivatization, and screening them for novel therapeutic agents. Pinocembrin, a flavonoid which occurs naturally in *Alpinia galangal*, has been found to suppress the growth of a range of cancer cell lines including cervical, breast and colon cancer cells. This is because flavonoids are very effective scavengers of hydroxyl and peroxyl radicals as well as quenching superoxide radicals and singlet oxygen (Wiart, 2013). Consequently, they are widely used as antioxidant to reduce the risk of cancers.

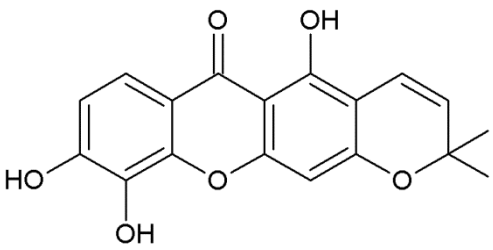
## 2.2 Chemistry of *Calophyllum* Species

*Calophyllum* is a nature's gift that occurs in the ethnopharmacy and folklore of many countries. The important cures for various ailments were carefully passed on verbally from one generation to another. Scientists prove that many members of this genus were not only traditionally used in folklore medicine, but also be applied into modern *in vivo* studies. This genus comprises of about 190 different plant species. Among all these species, *C. lanigerium*, *C. brasiliense*, *C. inophyllum*, *C. teysmanni* and *C. soulattri* have been extensively studied and have led to many important publications. Phytochemical studies have shown *Calophyllum* to be a rich source of secondary metabolites including coumarins, xanthones, triterpenoids, flavonoids and organic acids (Su, et al., 2008).

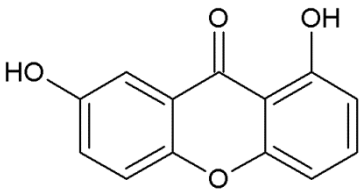
### 2.2.1 *Calophyllum sclerophyllum*

In this project, *C. sclerophyllum* was selected for phytochemical investigation in the search for medicinally important and chemically interesting compounds. This species is less likely to be studied by researchers, probably due to the limiting factor in geographical distribution of the plant. By far, there are only two publications related to *C. sclerophyllum*, reported by Jackson et al. in 1966 and Rissyelly, et al. in 2014.

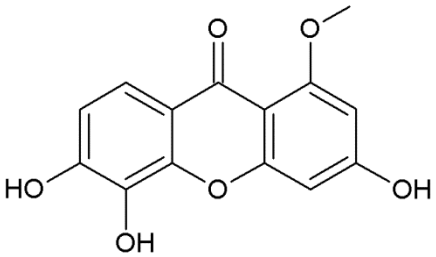
In year 1966, a total of five xanthones was isolated and identified from the heartwood of *Calophyllum sclerophyllum*, namely jacareubin **[1]**, euxanthone **[2]**, 3,5,6-trihydroxy-1-methoxyxanthone **[3]**, 1,3,5,6-tetrahydroxyxanthone **[4]** and 2-(3,3-dimethylallyl)-1,3,5,6-tetrahydroxyxanthone **[5]**. However, no biological studies were reported. In year 2014, Rissyelly, et al. had isolated two pure compounds belonging to the chromanone acid and flavonoid classes, which were isoapetalic acid **[6]** and astilbin **[7],** respectively. The crude extract, fractions and isolated compounds from stem bark of *C. sclerophyllum* were examined for their antioxidant activity via DPPH method. The butanol fraction containing flavonoids and phenolic compounds was found to show the highest antioxidant activity with $IC_{50}$ value of 3.03 ppm. In addition, astilbin also exhibited antioxidant activity with $IC_{50}$ value of 7.24 ppm.



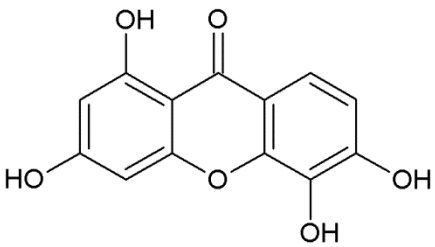

**[1]**




**[2]**

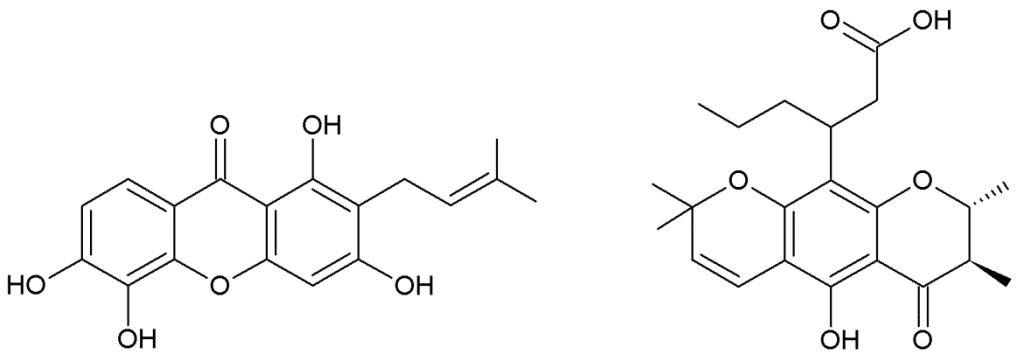



**[3]**

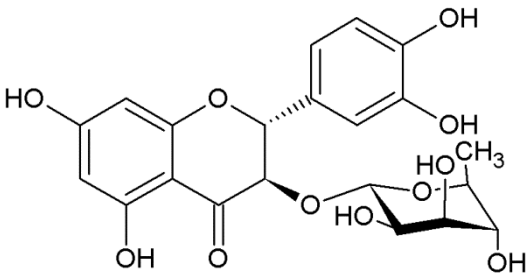



**[4]**

**Figure 2.5: Structures of chemical compounds isolated from *Calophyllum sclerophyllum***



**[5]** **[6]**



**[7]**

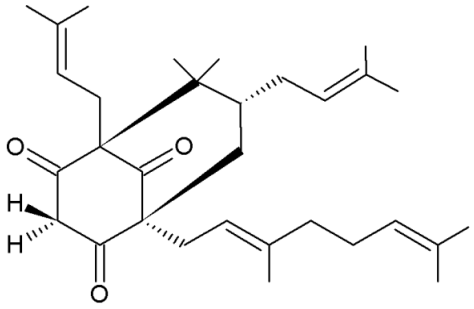
**Figure 2.6: Structures of chemical compounds isolated from *Calophyllum sclerophyllum* (continued)**

### 2.2.2 *Calophyllum enervosum*

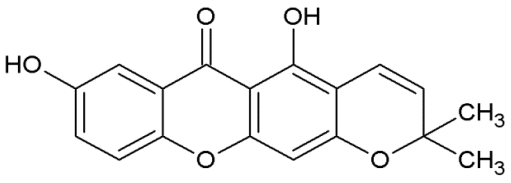
Chemical study on the stem bark of *Calophyllum enervosum* collected from Indonesia has revealed the isolation of a polyisoprenylated ketone named enervosanone **[8]** together with three known compounds, osajaxanthone **[9],** epicatechin **[10]** and cambogin **[11]** (Taher, et al., 2005). All these isolated compounds were tested against microorganisms using the disc diffusion method. Among all, compound **8** in the form of white needles showed the strongest antimicrobial activity against four types of microbes, which were

*Bacillus subtilis, Escherecia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*.

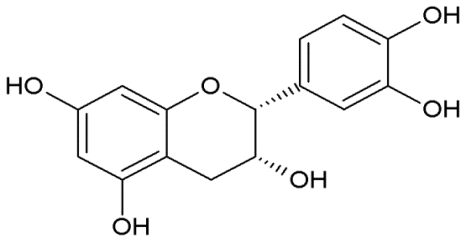
In year 2007, Taher, et al. reported that compound **8** also showed a significant cytotoxic effect ($IC_{50}$ 1.07 μM) against human breast cancer cells. However, the mechanism of action of this compound on the cancer cells is still not understood. Furthermore, there is only little radical scavenging effect exhibited by compound **8** in the antioxidant assay (percent inhibition= 42.7%).




**[8]**

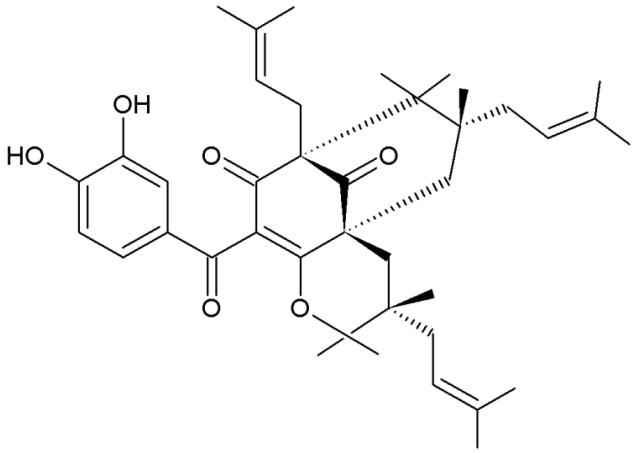



**[9]**




**[10]**

**Figure 2.7: Structures of chemical compounds isolated from *Calophyllum enervosum***




**[11]**

**Figure 2.8: Structures of chemical compounds isolated from *Calophyllum enervosum* (continued)**

### 2.2.3 *Calophyllum flavoramulum*

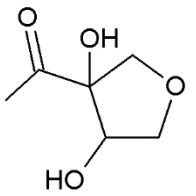
Advanced glycation end-products (AGEs) are associated with many pathogenic disorders such as Alzheimer's disease, diabetes, atherosclerosis, joint diseases and cardiovascular diseases. They are also responsible for aging and tissue damage (Ferchichi, et al., 2012). In order to reduce or inhibit their formation, the bioactive molecules must be α,β-dicarbonyl trapping agents, crosslink breakers or antioxidants (radical scavengers or metal-ion chelators). Thus, in year 2012, phytochemical and biological studies on *Calophyllum flavoramulum*, a native Malaysian plant, were conducted by Ferchichi, et al. in seeking for secondary metabolites as potent anti-AGEs drugs.

Prior to isolation, an anti-AGEs screening on crude leaves extracts was carried out, however, only the MeOH extract showed positive result. Consequently Ferchichi and co-researchers started searching for anti-AGEs natural compounds from this extract via bioguided fractionation, whereas the cyclohexane, DCM and EtOAc extracts were subjected to a systematic phytochemical study.

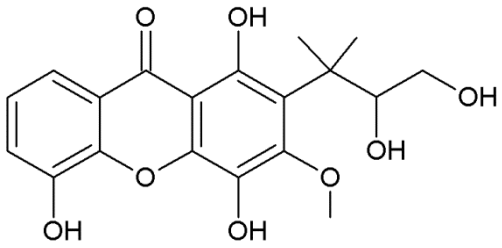
A total of twelve pure chemical constituents were isolated. Two new compounds were 3,4-dihydroxytetrahydrofuran-3-carboxylic acid **[12]** and flavoramulone **[13]**. In addition, the other ten known compounds were also isolated from the leaves extract, namely 3-methoxy-2-hydroxyxanthone **[14]**, quercitrin **[15]**, 3,4-dihydroxybenzoic acid **[16]**, benzoic acid **[17]**, canophyllol **[18]**, apetalactone **[19]**, 6-deoxyjacareubin **[20]**, rheediachromenoxanthone **[21]**, amentoflavone **[22]** and 2,3-dihydroamentoflavone **[23]**.

As a result from this study, a biflavonoid, amentoflavone **[22]** and a xanthone, 3-methoxy-2-hydroxyxanthone **[14]** were reported to be excellent AGEs inhibitors, with $IC_{50}$ values of 0.05 and 0.06 mM, respectively. Besides that, quercitrin **[15]** and 3,4-dihydroxybenzoic acid **[16]** were also found to be moderate AGEs inhibitors, both with $IC_{50}$ value of 0.5 mM. On contrary, the two new compounds, 3,4-dihydroxytetrahydrofuran-3-carboxylic acid **[12]** and flavoramulone **[13]** were both reported to be inactive towards anti-AGEs activities. Apart from that, those compounds with anti-AGEs activity were also

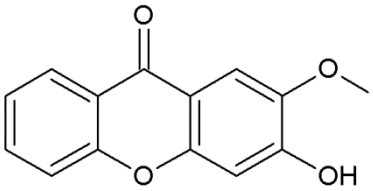
evaluated for their antioxidant potential. Among all, amentoflavone **[22]** has the highest antioxidant activity (DPPH: 0.41 ± 0.03 μmol TE/ μmol).



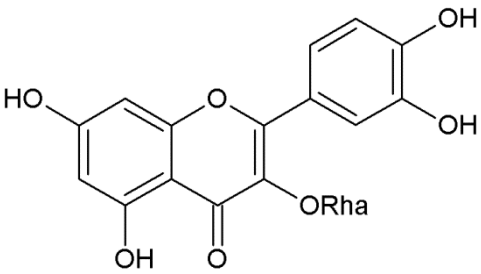

**[12]**



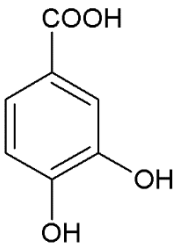

**[13]**



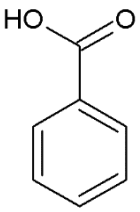

**[14]**




**[15]**

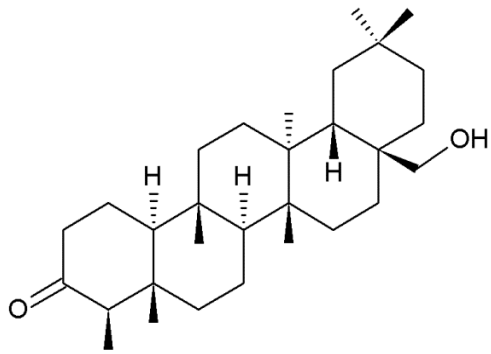



**[16]**

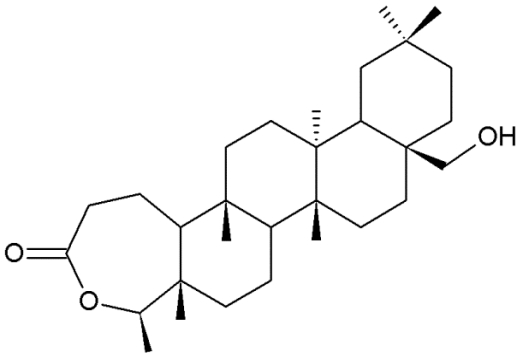



**[17]**

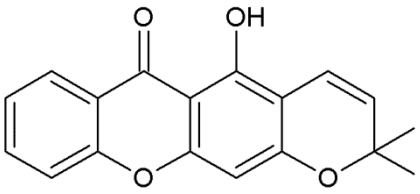
**Figure 2.9: Structures of chemical compounds isolated from *Calophyllum flavoramulum***



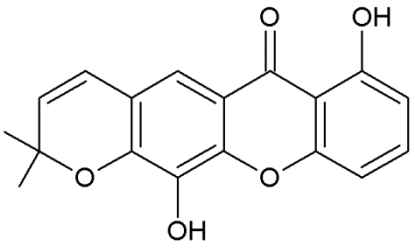

**[18]**



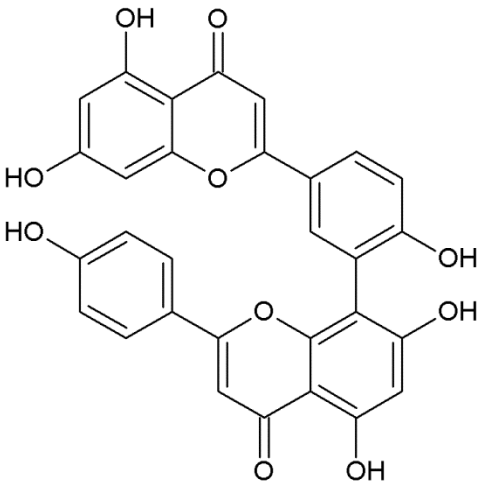

**[19]**



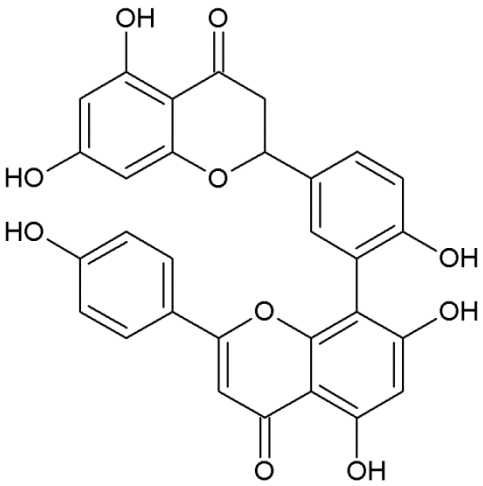

**[20]**




**[21]**




**[22]**




**[23]**

**Figure 2.10: Structures of chemical compounds isolated from *Calophyllum flavoramulum* (continued)**

### 2.2.4 Summary of Literature Review on the Genus *Calophyllum*

The types of secondary metabolites isolated from *Calophyllum* species and their pharmacological importance are summarized in Table 2.1.

**Table 2.1: Summary of literature data on the genus *Calophyllum***

| Plant Species | Classes of Compound | Biological Activities | References |
|---|---|---|---|
| *C. apetalum* | • Chromanone acids<br>• Coumarins<br>• Triterpenes<br>• Xanthones | -- | • Govindachari, Prakash, and Viswanathan, 1968a; 1968b<br>• Inuma, et al., 1997<br>• Nigam and Mitra, 1967 |
| *C. austroindicum* | • Coumarins<br>• Xanthones | -- | • Inuma, et al., 1996 |
| *C. blancoi* | • Chromanones<br>• Coumarins<br>• Xanthones | • Antiviral<br>• Antitumor<br>• Cytotoxic | • Shern, et al., 2004; 2005<br>• Stout and Karl, 1968 |
| *C. brasiliense* | • Chromanones<br>• Coumarins<br>• Flavonoids<br>• Terpenes<br>• Triterpenes<br>• Xanthones | • Antileishmanial<br>• Anti-cancer<br>• Anti-HIV<br>• Antioxidant<br>• Antiulcer<br>• Cyctotoxic<br>• Anti-mycobacterium tuberculosis activity | • Kudo, et al., 2013<br>• Lemos, et al., 2012<br>• Pires, et al., 2014<br>• Brenzan, et al., 2007<br>• Blanco-Ayala, et al., 2013<br>• Kimura, et al., 2004 |
| *C. bracteatum* | • Xanthones | -- | • Dharmaratne, 1986 |
| *C. calaba* | • Biflavonoids<br>• Terpenoids<br>• Xanthones | -- | • Kumar, et al., 1982<br>• Gunatilaka, et al., 1984<br>• Dharmaratne, 1986 |
| *C. caledonicum* | • Xanthones | • Antimalarial | • Hay, et al., |

| | | | |
|---|---|---|---|
| | | | 2004<br>• Morel, et al., 2000; 2002 |
| *C. chapelieri* | • Chromanone acids | -- | • Guerreiro, Kunesch and Polonsky, 1971 |
| *C. cordato-oblongum* | • Chromene acids<br>• Coumarins<br>• Triterpenoids<br>• Xanthones | -- | • Dharmaratne, et al., 1985<br>• Dharmaratne, et al., 1998<br>• Dharmaratne, et al., 1999<br>• Gunasekera and Sultanbawa, 1975 |
| *C. costatum* | • Coumarins | -- | • Stout and Stevens, 1964 |
| *C. decipiens* | • Chromanones<br>• Triterpenes<br>• Xanthones | • Antibacterial<br>• Antioxidant | • Ajithabai, et al., 2012 |
| *C. dispar* | • Coumarins | • Cytotoxic | • Guilet, et al., 2001a; 2001b |
| *C. dryobalonoides* | • Chromanone acids<br>• Xanthones | -- | • Ha, et al., 2012 |
| *C. enervosum* | • Benzophenones<br>• Flavonoids<br>• Ketones<br>• Xanthones | • Antimicrobial<br>• Antioxidant<br>• Cytotoxic | • Taher, et al., 2005; 2007 |
| *C. flavorammulum* | • Flavonoids<br>• Organic acids<br>• Triterpenes<br>• Xanthones | • Anti-AGEs activity<br>• Antioxidant | • Ferchichi, et al., 2012 |
| *C. gracilipes* | • Triterpenes<br>• Xanthones | • Cytotoxic | • Cao, et al., 1997a; 1997b<br>• Nasir, et al., 2013 |
| *C. inophyllum* | • Coumarins<br>• Flavonoids<br>• Triterpenes<br>• Xanthones | • Antimicrobial<br>• Antiproliferative<br>• Anti-tumor<br>• Cytotoxic<br>• Antioxidant<br>• Antidyslipidemic | • Yimdjo, et al., 2004<br>• Itoigawa, et al., 2001<br>• Mah, et al., 2015<br>• Prasad, et al., 2012 |

| | | | |
|---|---|---|---|
| | | | • Zou, et al., 2010 |
| *C. lanigerum* | • Coumarins | • Anti-HIV | • McKee, et al., 1996 |
| *C. lankaensis* (*C. zeylancum*) | • Chromanone acids<br>• Triterpenes<br>• Xanthones | -- | • Dharmaratne, Sotheeswaran and Balasubramaniam, 1984 |
| *C. macrocarpum* | • Neoflavonoids<br>• Steroids<br>• Triterpenoids | -- | • Ampofo and Waterman, 1986 |
| *C. membranaceum* | • Xanthones | • Anti-inflammatory<br>• Cytotoxic | • Chen, et al., 2008<br>• Zou, et al., 2005 |
| *C. moonii* | • Triterpenes<br>• Xanthones | -- | • Dharmaratne and Wijesinghe, 1997 |
| *C. nodusum* | • Xanthones | -- | • Nasir, et al., 2011 |
| *C. neo-ebudicum* | • Triterpenes<br>• Xanthones | -- | • Scheinmann and Sripong, 1970 |
| *C. polyanthum* | • Chromanones<br>• Chromanone acids<br>• Coumarins | -- | • Wang, et al., 2010<br>• Ma, et al., 2004 |
| *C. ramiflorum* | • Xanthones | -- | • Bhanu, Scheinmann and Jefferson, 1975 |
| *C. sclerophyllum* | • Chromanone acids<br>• Flavonoids<br>• Xanthones | • Antioxidant | • Jackson, Locksley and Scheinnman, 1966<br>• Rissyelly, 2014 |
| *C. soulattri* | • Coumarins<br>• Terpenoids<br>• Triterpenes<br>• Xanthones | • Cytotoxic | • Nigam, et al., 1988<br>• Mah, et al., 2015<br>• Ee, et al., 2011 |
| *C. teysmannii* | • Chromanone acids<br>• Coumarins | • Anti-HIV<br>• Cytotoxic | • Cao, et al., 1998<br>• Fuller, et al., |

| | | | |
|---|---|---|---|
| | • Triterpenes<br>• Xanthones | | 1994<br>• Kijjoa, et al., 2000<br>• McKee, et al., 1996<br>• Lim, et al., 2015<br>• Pengsuparp, et al., 1996 |
| *C. thorelii* | • Benzophenones<br>• Triterpenes<br>• Xanthones | • Cytotoxic | • Nguyen, et al., 2012 |
| *C. thwaitesii* | • Neoflavonoids<br>• Terpenoids<br>• Triterpenes<br>• Xanthones | -- | • Dahanayake, et al., 1974.<br>• Dharmaratne, et al., 1986<br>• Dharmaratne and Wanigasekera, 1996<br>• Dharmaratne, Sotheeswaran and Balasubramaniam, 1984 |
| *C. tomentosum* | • Benzopyrans<br>• Coumarins<br>• Xanthones<br>• Triterpenes | -- | • Babu, et al., 1994<br>• Banerji, et al., 1994<br>• Karunayake, et al., 1981<br>• Nigam and Mitra, 1967<br>• Govindachari, Prakash, and Viswanathan, 1968b |
| *C. verticillatum* | • Neoflvonoids<br>• Triterpenoids | • Mulloscicidal acitivity | • Ravelonjato, Kunesch and Poisson, 1987 |
| *C. walker* | • Neoflavonoids<br>• Triterpenes<br>• Terpenoids<br>• Xanthones | -- | • Ampofo and Waterman, 1986<br>• Dahanayake, et al., 1974. |

# CHAPTER 3

# MATERIALS AND METHODOLOGY

## 3.1 Plant Materials

The plant studied in this project was *Calophyllum sclerophyllum*. The stem bark of this plant was collected from the jungle in Landeh, Sarawak. It was identified by Mr. Tinjan Anak Kuda, a botanist from the Forest Department of Sarawak. A voucher specimen (UITM 3008) was deposited at the herbarium of Universiti Teknologi MARA, Sarawak.

## 3.2 Chemical Reagents

The solvents and materials used in this project are summarized in Table 3.1 to Table 3.6.

**Table 3.1: Industrial grade solvents and materials used in the extraction, isolation and purification of chemical constituents**

| Solvents/Materials | Molecular formula | Density, ρ (g $cm^{-3}$) | Source, Country |
|---|---|---|---|
| n-Hexane | $CH_3(CH_2)_4CH_3$ | 0.659 | Merck, Germany |
| Dichloromethane | $CH_2Cl_2$ | 1.325 | Fisher Scientific, UK |
| Ethyl acetate | $CH_3COOC_2H_5$ | 0.902 | Lab Scan, Ireland |
| Acetone | $CH_3COCH_3$ | 0.791 | QReC, Malaysia |
| Methanol | $CH_3OH$ | 0.791 | Mallinckrodit Chemicals, Phillisburg |
| Silica gel (60 Å) | $SiO_2$ | - | Nacalai Tesque, Japan |
| Sodium sulphate anhydrous | $Na_2SO_4$ | 2.66 | John Kollin Corporation, USA |

**Table 3.2: Deuterated solvents used in NMR analysis**

| Solvents | Molecular formula | Source, Country |
|---|---|---|
| Deuterated chloroform | $CDCl_3$ | Acros Organics, Belgium |
| Acetone-$d_6$ | $CD_3COCD_3$ | Acros Organics, Belgium |
| Methanol-$d_4$ | $CD_3OD$ | Acros Organics, Belgium |

**Table 3.3: HPLC grade solvents and materials used in LC- and GC-MS analysis**

| Solvents/Materials | Molecular formula | Density, ρ (g $cm^{-3}$) | Source, Country |
|---|---|---|---|
| Acetonitrile | $CH_3CN$ | 41.05 | Fisher Scientific, UK |
| Methanol | $CH_3OH$ | 32.04 | Fisher Scientific, UK |
| Nylon syringe filter (0.5 μm) | - | - | Membrane solution, USA |

**Table 3.4: Analytical grade solvent and materials used in TLC analysis**

| Solvents/Materials | Molecular formula | Source, Country |
|---|---|---|
| TLC silica gel 60 $F_{254}$ | - | Merck, Germany |
| Iodine | $I_2$ | Fisher Scientific, UK |
| n-Hexane | $CH_3(CH_2)_4CH_3$ | R & M Chemicals, UK |
| Dichloromethane | $CH_2Cl_2$ | QReC (Malaysia) |
| Ethyl acetate | $CH_3COOC_2H_5$ | Fisher Scientific, UK |

**Table 3.5: Analytical grade solvents and materials used in UV-Vis analysis**

| Solvents/Materials | Molecular formula | Source, Country |
|---|---|---|
| Chloroform | $CHCl_3$ | Fisher Scientific, UK |
| Cuvette (quartz) | - | Membrane solution, USA |

**Table 3.6: Chemical reagents and materials used in antioxidant assay**

| Chemical reagents/Materials | Source, Country |
|---|---|
| Kaempferol | Sigma-Aldrich, USA |
| Ascorbic acid (Vitamin C) | Sigma-Aldrich, USA |
| 1,1-Diphenyl-2-picryhydrazyl (DPPH) | Sigma-Aldrich, USA |
| 96-well plate | Techno Plastic, Switzerland |

## 3.3 Methodology

### 3.3.1 Sequential Solvent Extraction, Isolation and Purification of Chemical Constituents from *Calophyllum sclerophyllum*

Prior to any isolation and purification work, natural products have to be extracted from the sample material. In this study, a simple extraction method called maceration was chosen, in which the plant material was placed in a closed container and solvent was added until cover the top of the sample. Approximately 1.5 kg of stem bark of *Calophyllum sclerophyllum* was collected, air dried and finely ground into powder form. This is to ensure powdered plant materials have maximum surface area contact with solvents for an efficient extraction. The powdered stem bark was soaked in dichloromethane for 48 hours at room temperature. It was noted that plant extracts should not be stored in solvent for long periods or in sunlight, because this will increases the risk of artifact formation and decomposition of extract constituents. The dichlormethane crude extract was filtered and solvent was evaporated under reduced pressure via a rotary evaporator to give a dark viscous semisolid extract. The steps were repeated twice and the extracts were combined.

The plant material was then extracted subsequently with increasing polarity solvents, which were ethyl acetate and methanol, twice for each solvents as

described in the first extraction. Meanwhile, in order to minimize solvent wastage, the recovered solvent was used during second time soaking. The dried masses of the dichloromethane, ethyl acetate and methanol crude extracts were 18.6149 g, 49.5728 g and 27.6303 g, resepectively. About 2 g of each crude extract were kept for antioxiadant assay.

The crude extracts of *Calophyllum sclerophyllum* were subjected to gravity column chromatography using gradient elution and thereby separated into a series of fractions. The chemical compositions of fractions were analyzed using TLC. Fractions that showed identical pattern of spots on TLC were combined for further purification using similar chromatographic technique. The effectiveness of separation was monitored by TLC analyses. The process of purification and TLC analysis were repeated until pure compounds were obtained. Compounds that showed a single spot on the TLC plate were sent for spectroscopic analyses such as NMR, IR, UV-Vis and LC-MS for structural investigation.

### 3.3.2 Column Chromatography

Column chromatography is a common technique used to isolate and purify chemical constituents from the plant crude extracts. In column chromatography, silica gel as the stationary phase is held in a glass column, and the mobile phase (solvent) is flown through it by means of gravitational force. The

components distribute themselves between the mobile phase and the stationary phase. Separation is based on affinity of components towards both phases, thus resulting differences in migration rates. Consequently, the components in a mixture separate into different color or colorless bands along the column. The eluents were collected according to separated color bands or volumes.

The sizes of columns used were 25 mm, 30 mm and 80 mm in internal diameter, depending on the amount of sample. The sample was prepared via dry packing method in which the sample was dissolved in a suitable amount of solvent, followed by adding dropwise into silica gel and mixed homogeneously. In a separate beaker, silica gel was mixed with hexane to form slurry. It was then poured into a glass column. To obtain a compact packing of stationary phase, the column was tapped gently to allow bubbles to rise and the silica to settle down gradually. The prepared sample was then subjected to column chromatography using gradient elution in increasing polarity to separate the compounds of different polarities. On top of sample and sintered disc, a thin layer of sea sand or drying agent was added to serve as protection layer, prevent leakage of silica gel as well as to absorb moisture from the mobile phase.

During elution, flow rate of mobile phase is important in controlling the quality of separation. If the flow rate is too slow, diffusion process may lead to band broadening. Conversely, if it is too fast, there may not be enough time for compounds to achieve equilibrium distribution between stationary and mobile

phases and the compounds may be forced down the column without proper separation. For small diameter columns, the optimal rate is lower than that for large diameter columns. Therefore, larger columns can be run with a higher flow rate than smaller columns. Furthermore, to ensure a better isolation, air bubbles should be avoided within the column bed. If this happens, bubbles can be removed by wrapping the column with a layer of tissue wetted methanol. Volatile methanol will give a cold effect on outer wall of column, hence resulting in contraction of column bed. Figure 3.1 shows the apparatus set up for column chromatography.

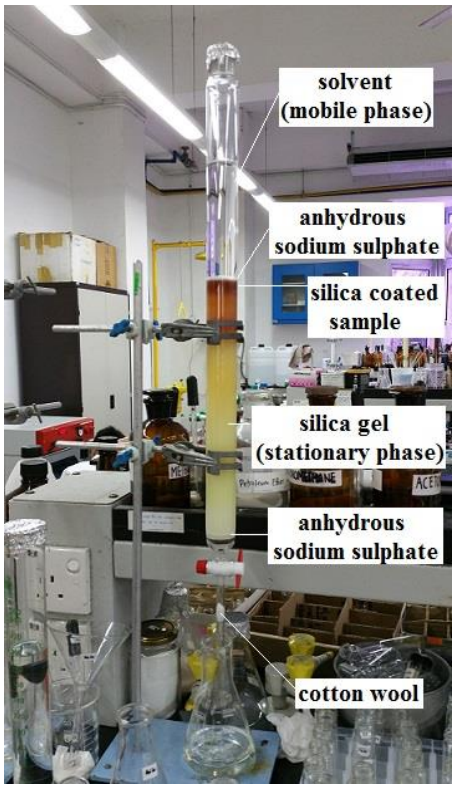



**Figure 3.1 Apparatus set up for column chromatography**

### 3.3.3 Thin Layer Chromatography (TLC)

To examine the purity or chemical composition of fractions, TLC analysis was carried out by using precoated aluminium sheets of silica gel 60 $F_{254}$ Merck. Initially, a baseline was drawn on the TLC plate, suitable amount of sample solution was spotted onto it using a micro capillary tube. This plate was then placed in a developing chamber, which containing solvent mixture and was pre-saturated with solvent vapour. The solvent (mobile phase) migrates up the plate through the silica sorbent (stationary phase) due to capillary action. According to planar adsorption chromatography principle, polar compounds will adsorbed strongly on the stationary phase, thus move slowly up the plate as the solvent migrates. These compounds will therefore have small retention factor, $R_f$. Contrastly, non-polar compounds will have larger $R_f$ values as they show less affinity towards the stationary phase. As a consequence of development, components of a fraction will be separated according to their relative polarities. The spots were visualized under a UV lamp at 254 nm and 356 nm, and iodine vapor. The $R_f$ value of each analyte can be calculated by using the following formula:

$$R_f = \frac{\text{distance travelled by the compound (cm)}}{\text{distance of the solvent front (cm)}}$$

### 3.3.4 TLC Detection Methods

#### 3.3.4.1 UV Detection

TLC plates are usually impregnated with indicator such as manganese-activated zinc silicate, which will emit a pale green light and pale purple blue light under short and long wavelength UV lights, respectively. With effective visualization, the UV active compounds containing aromatic rings and conjugated systems can be detected. At short wavelength (254 nm), compounds will appear as dark grey spots on a bright green background. Compounds that absorb light at long wavelength (365 nm) will appear as fluorescence color spots on a pale purple background when UV light is irradiated onto the plate. Care should be taken not to direct UV light from these lamps to eyes or on skin as UV light is mutagenic.

#### 3.3.4.2 Iodine Vapour Detection

Iodine chamber was prepared by introducing iodine crystals into a close container. The sample TLC plate was then placed into this chamber which was saturated with iodine vapour, for few minutes before it was taken out. Iodine forms dark brown complex with compounds that could not be observed under UV light, such as terpenoids or non-conjugated compounds. The observed

sports were marked down immediately using a pencil as the staining effect of iodine on TLC plate was temporary.

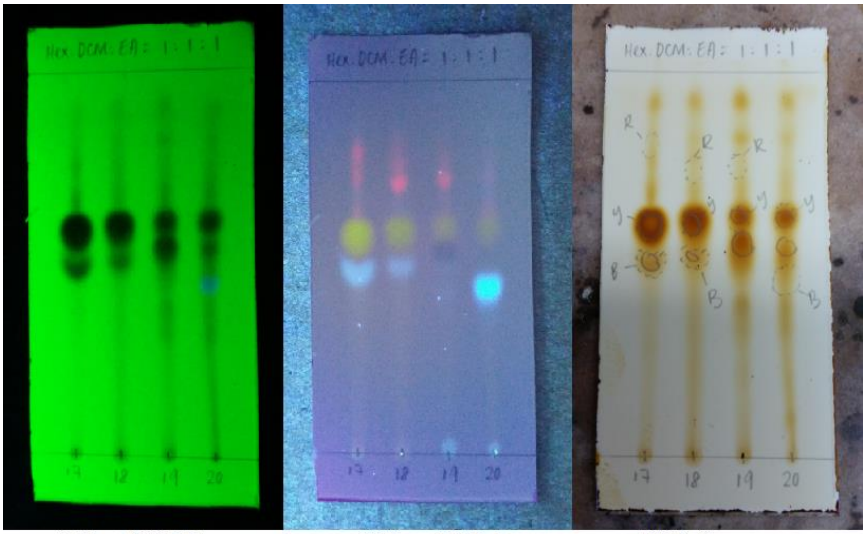



**Figure 3.2 TLC plate under different detection methods**

## 3.4 Instruments

### 3.4.1 Nuclear Magnetic Resonance (NMR)

NMR is a spectroscopic method that provides valuable information on the number and types of protons and carbons present in a molecule, and the structural relationships among these atoms. As each nucleus of an atom has different surrounding chemical environment, each atom experiences distinct magnetic field and hence produces characteristic signal in the chemical shift region which aids in elucidation of structure of a molecule. In this project,

JEOL JNM-ECX 400 MHz spectrometer was used to obtain $^{1}$H-NMR, $^{13}$C-NMR, HMQC, and HMBC spectra for each pure compound.

Samples were prepared by dissolving them in deuterated solvents, such as deuterated dichloromethane, acetone and methanol, and were then transferred to NMR tubes up to 4 cm in height. Selection of solvents depends on the degree of dissolution. The tubes were capped and wrapped with parafilm to prevent solvent evaporation. Tetramethylsilane (TMS) was used as an internal standard during analysis.

### 3.4.2 Infrared (IR) Spectroscopy

Infrared (IR) spectroscopy is used to identify different functional groups present in a molecule. The specific fingerprint region is helpful in structural determination of compounds. Preparation of sample could be done by mixing homogenously the solid sample with potassium bromide (KBr) in a ratio of 1:10, and pressing the mixture under high pressure. This gives KBr pellet and is inserted into a sample holder for IR measurement. Perkin Elmer 2000-FTIR spectrophotometer was used to obtain IR spectrum in the range of 4000 to 400 $cm^{-1}$.

### 3.4.3 Ultraviolet-Visible (UV-Vis) Spectroscopy

UV-Vis spectroscopy gives information about the chromophores present in a molecule by defining its conjugated system. The classes of compounds thus can be primarily identified from characteristic absorption peaks. Perkin Elmer Lambda 35, a double-beam instrument was used in this analysis. In a double-beam spectrophotometer, radiation is split into two compartments with equal radiant energy, one beam passing through the sample and the other through the blank solution. Sample was dissolved and diluted with chloroform. Chloroform was used as blank solvent. For each sample, absorption spectrum was obtained by scanning the sample in the range of 200–400 nm.

### 3.4.4 Liquid Chromatography-Mass Spectrometry (LC-MS)

The accurate molecular mass, molecular formula, and fragmentation pattern of a non-volatile compound can be obtained precisely from a coupled technique, namely liquid chromatography – mass spectrometry (LC-MS). About 2 mg of sample was dissolved in 2 mL HPLC grade solvent and filtered to remove any undissolved solid particles before it was introduced into the LC-MS. 5 µL of sample solution was auto-injected into the column, followed by flushing with 30% water and 70% methanol in a flow rate of 0.6 mL/min. In this project, Agilent Technologies 6520 LC/MS equipped with an electrospray source was used to ionize the sample solutions into free cations for analysis.

### 3.4.5 Gas Chromatography–Mass Spectrometry (GC-MS)

GC-MS is a coupled technique that not only used for determining nominal molecular weight, molecular formula and fragmentation of a compound, but also for the comparison of chromatogram fingerprint. A concentration of 100 ppm of sample was prepared by dissolving in HPLC grade solvent. 5 µL of sample solution was auto-injected into the non-polar BPX5 column, followed by flushing with inert helium gas in a flow rate of 0.6 mL/min. In this project, Shimadzu GC-MS QP2010 equipped with a flame ionization detector (FID) was used to ionize the sample solution into free cations for analysis.

### 3.4.6 Melting Point Apparatus

Melting point measurement is essential in examine purity of a compound. A pure crystalline compound shows a narrow and sharp range of melting point when changing from solid state to liquid state. Conversely, if impurities are present, compound may have a broader range of melting point. In this study, the melting point measurement was carried out by using Stuart SMP 10 melting point apparatus. Trace amount of solid sample was introduced into a hematocrit capillary tube and heated until it completely melted. The temperature range at which the compound starts and entirely melted was recorded.

## 3.5 Antioxidant Assay

For preparation of master stocks at concentration of 1 mg/mL, crude extracts, isolated compounds and standard compounds (kaempferol and ascorbic acid) were dissolved separately in methanol. These master stocks were then sonicated for 5 minutes in order to form homogenous solutions. Similarly, a DPPH solution with concentration of 2 mg/mL was prepared by dissolving DPPH powder in methanol, and then sonicated. Master stocks of test samples and DPPH solution were kept in a 4 $^{o}$C chiller in dark condition to avoid exposure to light which may led to decomposition of chemicals.

Test solutions were prepared through serial dilution of master stocks, at concentrations of 240, 120, 60, 30, 15, 7.5 and 3.75 µg/mL in a 96-well plate, followed by addition of 10 µL DPPH solution and 90 µL methanol. Kaempferol and ascorbic acid were used as positive control in this assay. For the blank or negative control, DPPH solution in methanol without test sample was used.

The plate was immediately wrapped with aluminium foil to avoid solvent evaporation and incubated for 30 minutes. The absorbance of the solutions in each well was measured at 520 nm using a microplate reader. The assay was performed in triplicate and the average absorbance for each concentration was

noted. Finally, the percentage inhibition rates of the test compounds were calculated using the following equation:

$$\text{Inhibition Rate (\%)} = \frac{A_o - A_1}{A_0} \times 100\%$$

where $A_o$ = absorbance of the blank (negative control)

$A_1$ = absorbance of the test sample

The resulting data were presented in a graph of inhibition rate against sample concentration. From the plotted graph, $IC_{50}$ value can be obtained. $IC_{50}$ is defined as the concentration of sample that required to inhibiting 50% of DPPH radical scavenging activity.

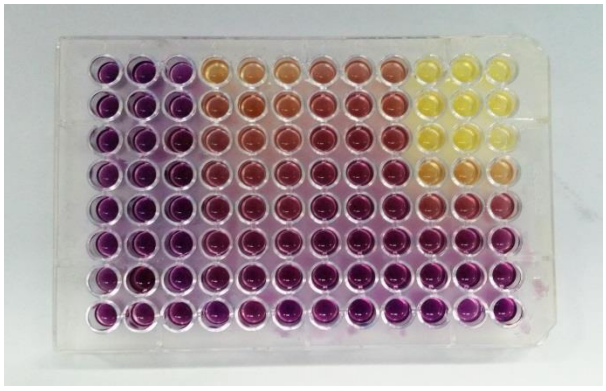


**Figure 3.3 96-well plate used in antioxidant assay**

# CHAPTER 4

# RESULTS AND DISCUSSION

## 4.1 Chemical Constituents Isolated from *Calophyllum sclerophyllum*

Before isolation, selection of solvent system is important for column chromatography. The solvent system that gives the best separation of compounds on TLC plate is generally used as a mobile phase for column chromatography with the same stationary phase. In this study, both hexane: ethyl acetate and ethyl acetate: acetone solvent systems were selected as mobile phase as they showed good separation effect on TLC plates. Whereas methanol was adopted as the last mobile phase to elute out the remaining compounds in the column.

The ethyl acetate crude extract was first subjected to column chromatography using a 80 mm internal diameter glass column. Silica gel was used as column bed and sample was eluted with hexane-ethyl acetate, ethyl acetate-acetone and lastly methanol, in stepwise gradient elution. The separation afforded a total of 19 fractions and they were labeled as A1 to A19. Due to similar migration rate of spots on TLC plate, fractions A11 to A13 were combined for further separation using a smaller internal diameter column (30 mm) to yield subfractions, B1 to B41. Among these subfractions, B3, B17 and B21 showed a single spot on TLC plate. Meanwhile, subfractions B14 and B15 were

combined for further column chromatography. Separation results in a series of fractions, C1 to C19. Similarly, among these fractions, C7 displayed a single spot during TLC analysis. In order to confirm the purity of these single spot isolates, an adequate amount of each sample was taken out from these subfractions and prepared for NMR analysis.

According to spectroscopic data obtained and comparison with literature data, the pure compounds were identified as friedelin **[24]**, 5,7-dihydroxy-6-(3-methylbutyryl)-4-phenylcoumarin **[25]**, 5,7-dihydroxy-6-(2-methylbutyryl)-4-phenylcoumarin **[26]** and isodispar B **[27]**. Besides NMR analysis, other spectroscopic techniques including IR, GC-MS, LC-MS and UV-Vis were also used in structural elucidation for these pure compounds.

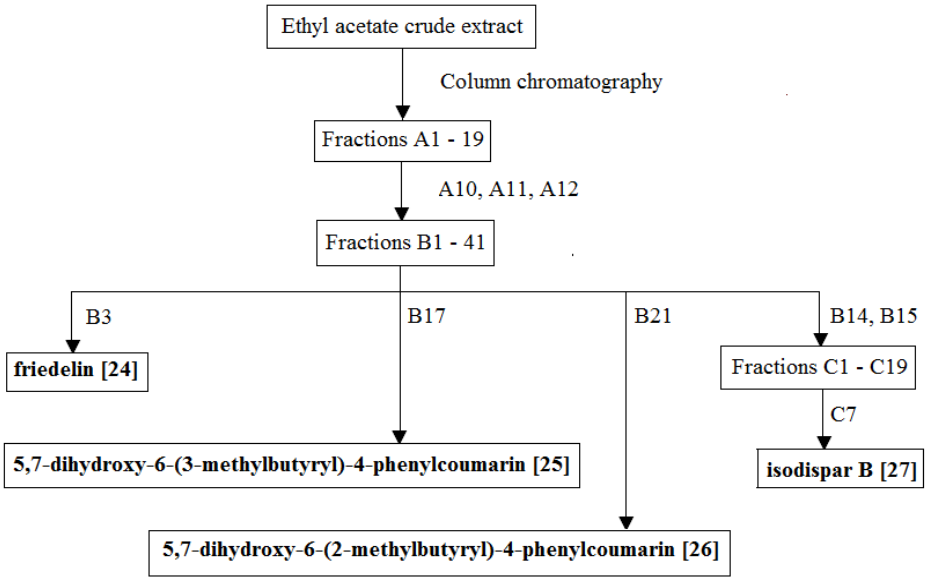



**Figure 4.1 Isolation pathways of pure compounds**

## 4.2 Chemical Identification and Structural Characterization of 5,7-Dihydroxy-6-(2-methylbutyryl)-4-phenylcoumarin [25]

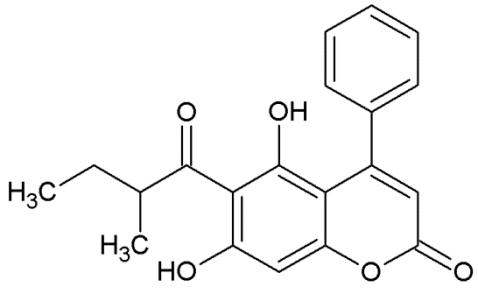



**Figure 4.2: Molecular structure of 5,7-dihydroxy-6-(2-methylbutyryl)-4-phenylcoumarin [25]**

A total of 237 mg of yellow crystals was isolated and identified as a coumarin, namely 5,7-dihydroxy-6-(2-methylbutyryl)-4-phenylcoumarin. It has a melting point of 203-205 $^{o}$C (Lit. 201-202 $^{o}$C, Crombie, et al., 1987). During TLC analysis, compound **25** appeared as a single dark grey spot under short λ UV light at 254 nm, and a dark brown spot when treated with iodine vapour, due to its phenolic nature. The retention factor, $R_f$ value of single spot is 0.61 via a mixture of hexane, dichloromethane and ethyl acetate (1:2:1) as mobile phase.

Meanwhile, the elemental composition of compound **25** was determined by both GC-MS and LC-MS. A molecular ion peak $[M]^+$ with a nominal mass of 338 g $mol^{-1}$ shown in GC-MS spectrum (Figure 4.3) was corresponding to the molecular formula $C_{20}H_{18}O_5$. Besides, the HRESI mass spectrum (Figure 4.4) shows dominant ion at m/z 339.1223, which is consistent with the expected protonated molecular ion, $[M+H]^+$. Protonated molecular ion is expected because the sample was analyzed under positive ionization mode. Hence, the

accurate mass of the molecular ion is deduced to be 338.1154 g $mol^{-1}$, which is 97.21% match with the calculated value of 338.1150 g $mol^{-1}$.

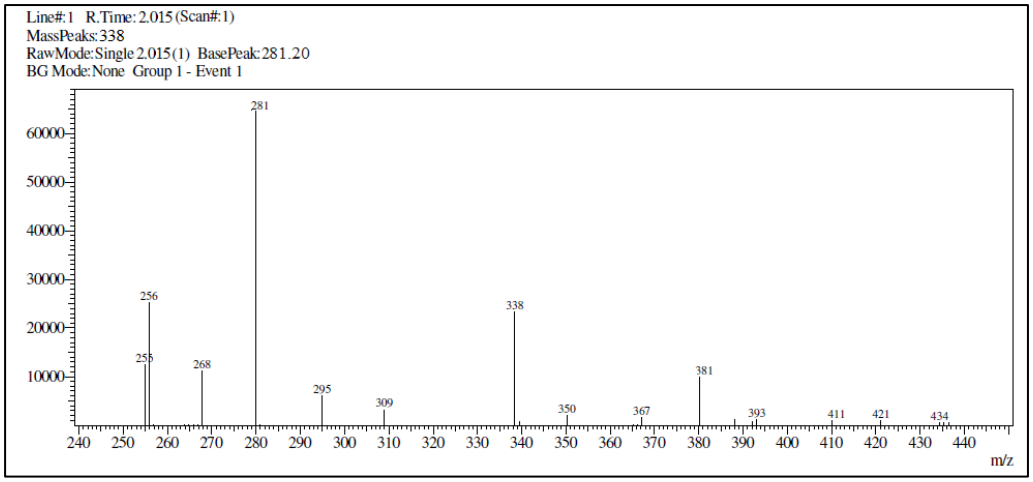
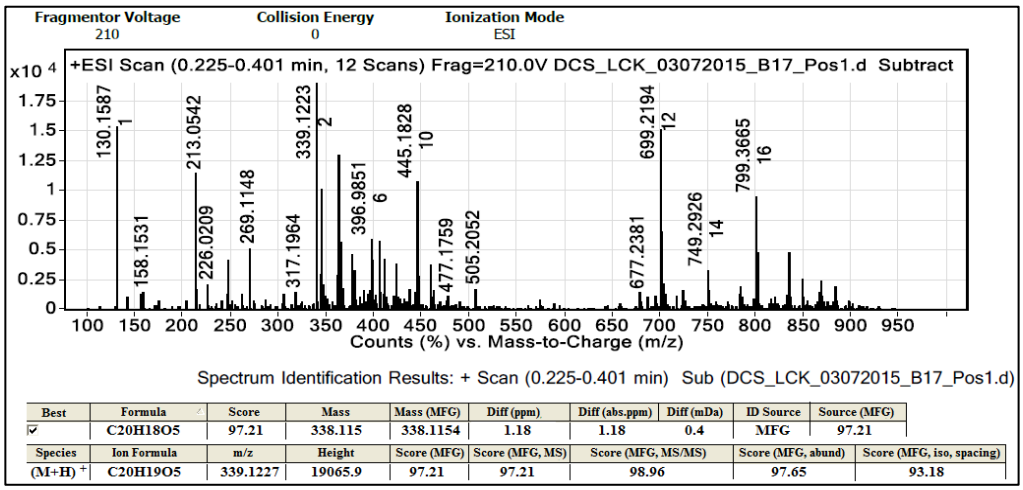



**Figure 4.3: GC-MS spectrum of compound 25**




| Best | Formula | Score | Mass | Mass (MFG) | Diff (ppm) | Diff (abs.ppm) | Diff (mDa) | ID Source | Source (MFG) |
|---|---|---|---|---|---|---|---|---|---|
| ☑ | C20H18O5 | 97.21 | 338.115 | 338.1154 | 1.18 | 1.18 | 0.4 | MFG | 97.21 |

| Species | Ion Formula | m/z | Height | Score (MFG) | Score (MFG, MS) | Score (MFG, MS/MS) | Score (MFG, abund) | Score (MFG, iso, spacing) |
|---|---|---|---|---|---|---|---|---|
| $(M+H)^+$ | C20H19O5 | 339.1227 | 19065.9 | 97.21 | 97.21 | 98.96 | 97.65 | 93.18 |

**Figure 4.4: HRESIMS spectrum of compound 25**

The UV-Vis spectrum is mainly used to deduce the conjugation effect present in the structure of a chemical compound. Coumarins with no substituent group show maximum absorption at 274 and 311 nm, resulting from the benzene ring and pyrone ring in its skeleton, respectively. Compound **25** with a coumarin showed absorption peaks maxima at wavelengths of 278 and 330 nm in Figure

4.5. The introduction of hydroxyl group into the coumarin nucleus at C-5 and -7 and a phenyl group at C-4 causes bathochromic shift of the principal absorption bands at 274 and 311 nm to 278 and 330 nm, respectively.

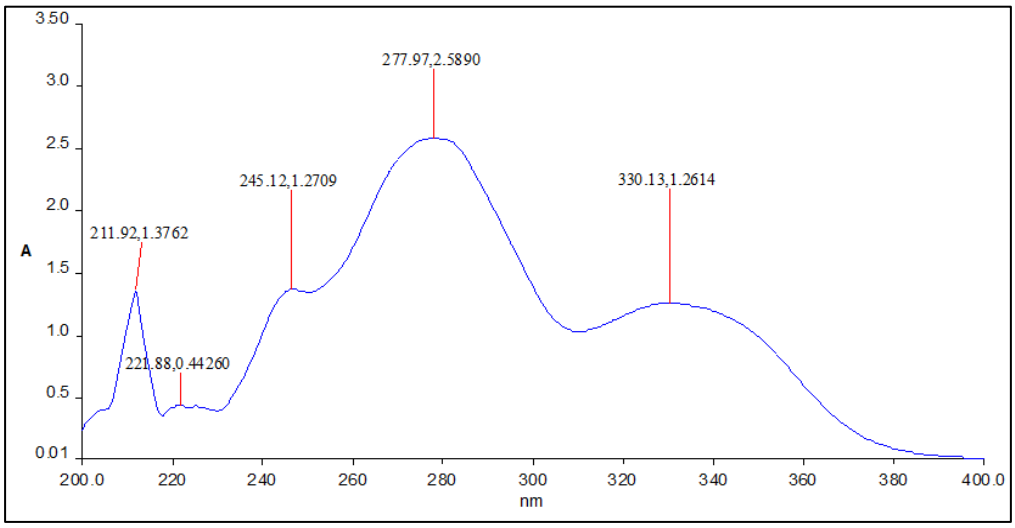



**Figure 4.5: UV-Vis spectrum of compound 25**

The chemical structure of compound **25** was elucidated based on the 1D- and 2D-NMR spectral data. In the $^{1}$H-NMR spectrum (Figure 4.7), the protons in the aryl group displayed multiplet peaks integrated for five protons at $\delta_H$ 7.3. A sharp singlet signal at $\delta_H$ 14.91 was assigned to the chelated hydroxyl proton, 5-OH. This proton is strongly deshielded via intramolecular hydrogen bonding with the keto group. Contrastly, the free hydroxyl proton 7-OH gave a broad singlet at $\delta_H$ 11.06. On the other hand, both aromatic protons H-8 and H-3 appeared as singlet signals at $\delta_H$ 6.41 and $\delta_H$ 5.83, respectively. The methyl protons at C-4" and C-5" are in the upfield regions, each carrying three protons but showed different multiplicity. The signal of H-4" observed at $\delta_H$ 0.87 was a triplet ($J$ = 7.4 Hz) because of the coupling with two adjacent protons H-3". Whereas, a doublet signal at $\delta_H$ 1.11 ($J$ = 6.7 Hz) was assigned to H-5" as there

is only one neighbouring proton found on carbon C-2". Meanwhile, proton H-2" gave a relatively deshielded multiplet at $\delta_H$ 3.87 due to anisotropic effect extended by the adjacent carbonyl group. The methylene protons $H_a$-3" and $H_b$-3" experience a slight different in their magnetic environment due to their close vicinity to the carbonyl group. One of the protons experiences a relatively greater deshielding effect than the other one, thus resulting in non-equivalent methylene protons $H_a$ and $H_b$ which appeared as doublet of quartets separately at $\delta_H$ 1.80 ($J$ = 7.4, 13.4 Hz) and 1.36 ($J$ = 7.3, 14.0 Hz).

A total of 20 carbon signals were observed in $^{13}$C-NMR spectrum (Figure 4.11), indicating that compound **25** to consist of 20 carbons. Generally, the chemical shift for carbonyl group of ketone is in the range of $\delta_c$ 185-220 ppm, while for ester is $\delta_c$ 155-185, due to presence of highly electronegative O atom. In this case, the two very downfield signals at $\delta_c$ 211.8 and 158.9 were attributed to carbonyl groups, which are C-1" and C-2, respectively. The five aromatic carbons resonances at $\delta_c$ 127.3, 127.5 and 128.0 were assigned to C-2'& 6', C-3' & 5' and C-4', respectively. In short, the presence of $sp^2$ hybridized carbons including aromatic and carbonyl carbons were found to give carbon signals in the chemical shift regions above $\delta_c$ 90. On the contrary, signals appeared in the region below $\delta_c$ 90 were assigned to $sp^3$ hybridized carbons, such as carbon C-2", 3", 4" and 5" in the methylbutyryl substituent group.

With HMQC analysis, the types of carbons can be determined by the direct $^1J_{CH}$ coupling between protons and carbons. In HMQC spectrum (Figure 4.14),

the presence of two methyl carbons, one methylene carbons, eight methine carbons and nine quaternary carbons were observed. Proton signals at $\delta_H$ 0.87 (H-4") and $\delta_H$ 1.11 (H-5") were correlated to carbon signals at $\delta_c$ 11.3 (C-4") and $\delta_c$ 15.9 (C-5"), respectively, implying the existence of methyl carbons. Likewise, methylene carbon was assigned to C-3" as both proton signals at $\delta_H$ 1.80 and $\delta_H$ 1.36 intersect with carbon signal $\delta_C$ 26.7 to give two cross peaks in the spectrum. In addition, presence of methine carbons was deduced from the coupling interactions between a carbon and a proton, such as C-2" with H-2", C-3" with H-3" and C-8 with H-8. It was noted that quaternary carbons do not give any cross peaks in HMQC spectrum. Meanwhile, HMBC spectra (Figures 4.15-4.18) provide carbon to proton long-range coupling information, involving $^2J_{CH}$ and $^3J_{CH}$ correlations. From the HMBC spectrum, the hydroxyl proton at C-5 ($\delta_H$ 14.91) showed $^2J$ correlation with an oxygenated quaternary carbon at $\delta_C$ 165.8 (C-5) and $^3J$ correlations with two quaternary carbons at $\delta_C$ 106.2 (C-6) and $\delta_C$ 101.7 (C-4a), which confirmed the position of hydroxyl group at C-5. The remaining correlations are listed in Table 4.1.

Assignment of chemical structure of compound **25** was further supported by FTIR analysis, showing the presence of aromatic $sp^2$ C-H stretch at 3064 $cm^{-1}$, $sp^3$ C-H stretch at 2971 $cm^{-1}$, C=O stretch at 1691 $cm^{-1}$, aromatic C=C stretch at 1591 and 1460 $cm^{-1}$, and C-O stretch at 1227 $cm^{-1}$. Meanwhile, O-H stretch appeared as a broad peak which occurs in the range of 3400- 3200 $cm^{-1}$, thus is overlapping with C-H stretch. Besides stretching vibrations, C-H in-plane bending vibration was observed at 1372 $cm^{-1}$. Whereas, the peaks with medium to weak intensity appeared at 946, 892, 831 and 776 $cm^{-1}$ in IR spectrum

(Figure 4.6) are assigned to C-H out-of-plane bending vibrations. Lastly, C-C out-of-plane vibration occurs at 697 and 462 $cm^{-1}$, and symmetric C-C in-plane vibration appeared at 583 $cm^{-1}$. By combination of all the spectral data, it was deduced that compound **25** was 5,7-dihydroxy-6-(2-methylbutyryl)-4-phenylcoumarin.

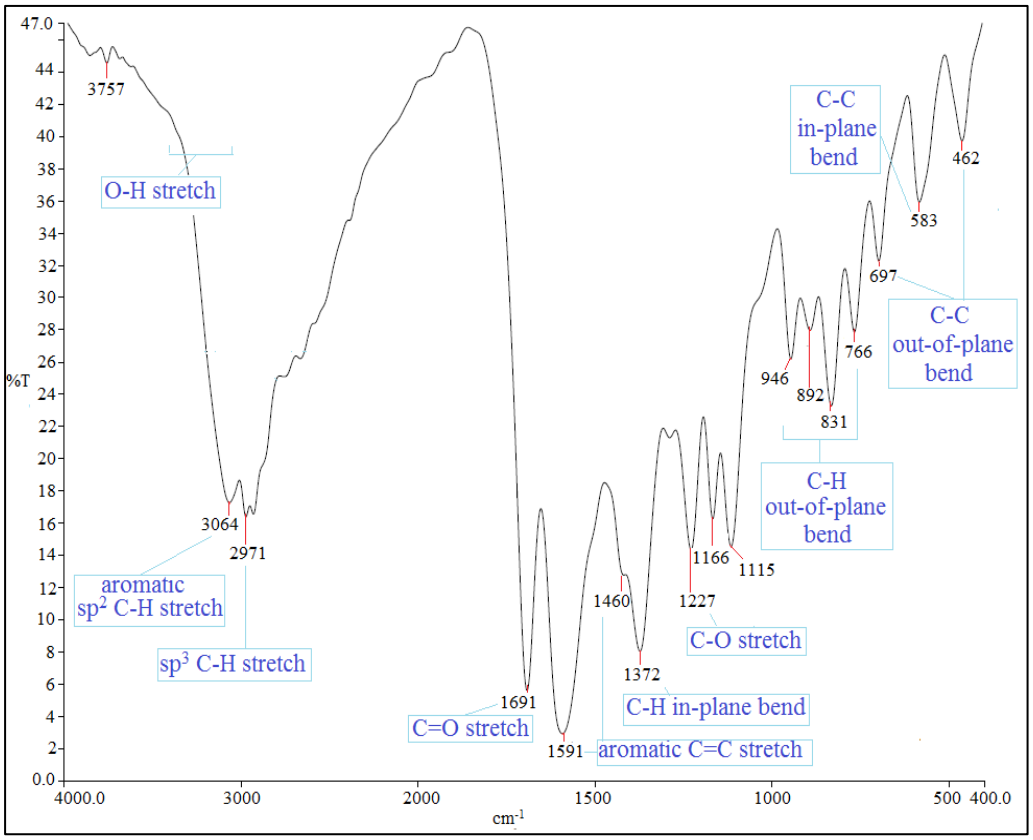



**Figure 4.6: IR spectrum of compound 25**

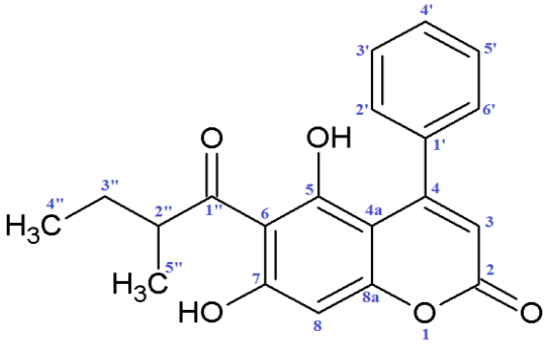
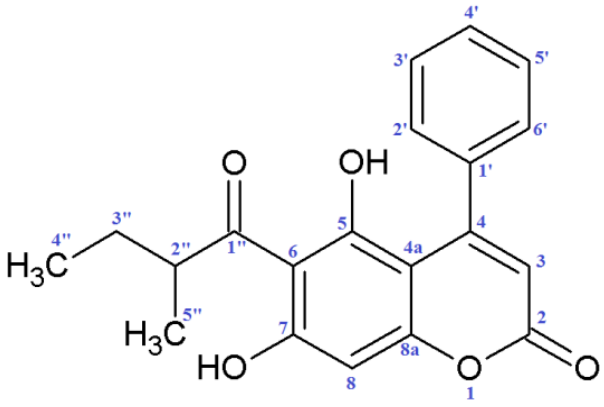



**Table 4.1: Summary of NMR data for 5,7-dihydroxy-6-(2-methylbutyryl)-4-phenylcoumarin [25]**

| Position | $\delta_H$ (ppm), Integration and Multiplicity | $\delta_C$ (ppm) | HMBC |
|---|---|---|---|
| 1 | - | - | - |
| 2 | - | 158.9 | - |
| 3 | 5.83 (1H, s) | 112.1 | C-1', 2, 4a |
| 4 | - | 156.0 | - |
| 4a | - | 101.7 | - |
| 5 | - | 165.8 | - |
| 6 | - | 106.2 | - |
| 7 | - | 163.1 | - |
| 8 | 6.41 (1H, s) | 95.0 | C-4a, 6 , 7, 8a |
| 8a | - | 160.4 | - |
| 1' | - | 139.6 | - |
| 2' & 6' | 7.35 (2H, m) | 127.3 | C-3', 4', 5' |
| 3' & 5' | 7.37 (2H, m) | 127.5 | C-2', 4', 6' |
| 4' | 7.39 (1H, m) | 128.0 | C-2', 3', 5', 6' |
| 1" | - | 211.8 | - |
| 2" | 3.87 (1H, m) | 46.2 | C-3" |
| 3" | 1.80 ($1H_a$, dq, $J$ = 7.4 Hz, 13.4 Hz)<br>1.36 ($1H_b$, dq, $J$ = 7.3 Hz, 14.0 Hz) | 26.7 | C-2", 4" |
| 4" | 0.87 (3H, t, $J$ = 7.4 Hz) | 11.3 | C-3", C-2" |
| 5" | 1.11 (3H, d, $J$ = 6.7 Hz) | 15.9 | C-1", 2", 3" |
| 5-OH | 14.91 (1H, s) | - | C-4a, 5, 6 |
| 7-OH | 11.06 (1H, brs) | - | - |




**5,7-dihydroxy-6-(2-methylbutyryl)-4-phenylcoumarin [25]**

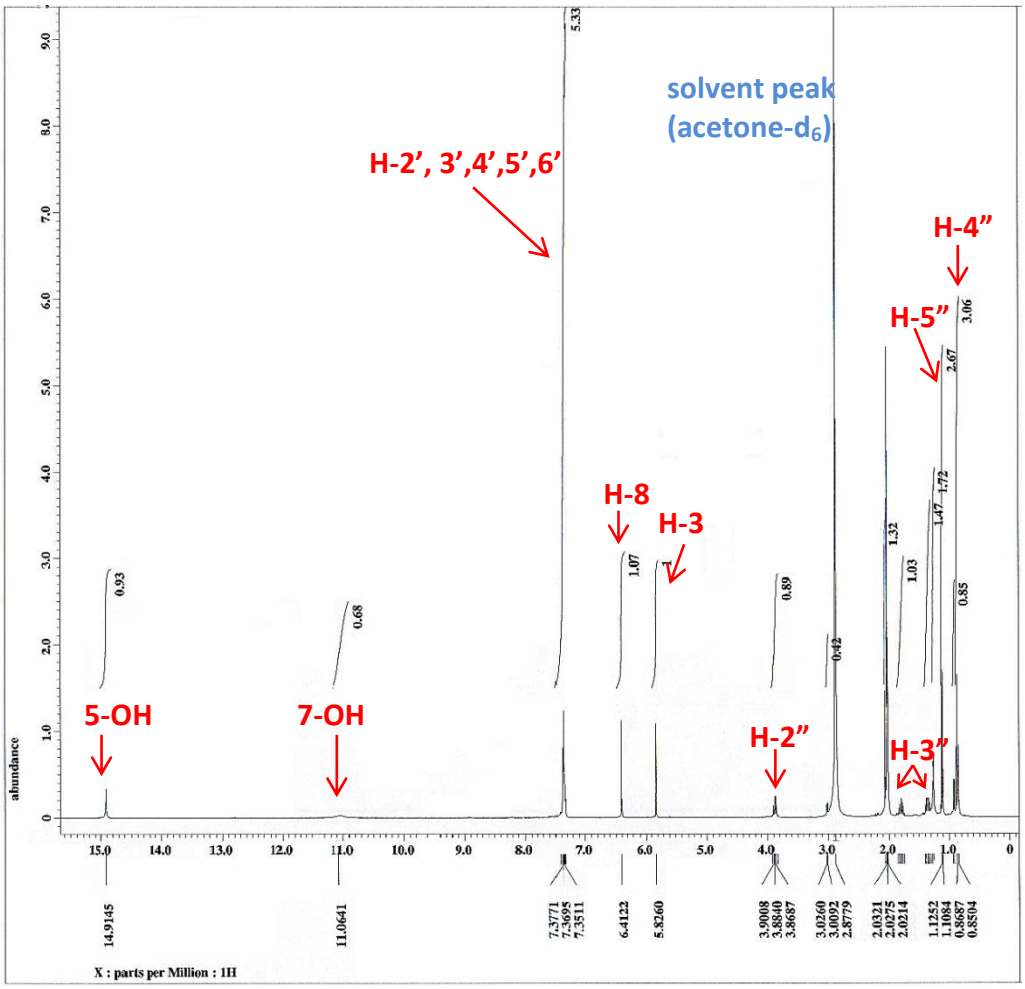
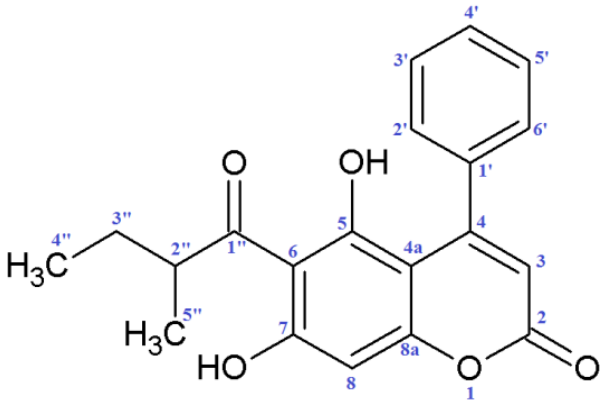



**Figure 4.7: $^{1}$H-NMR spectrum of compound 25**




**5,7-dihydroxy-6-(2-methylbutyryl)-4-phenylcoumarin [25]**

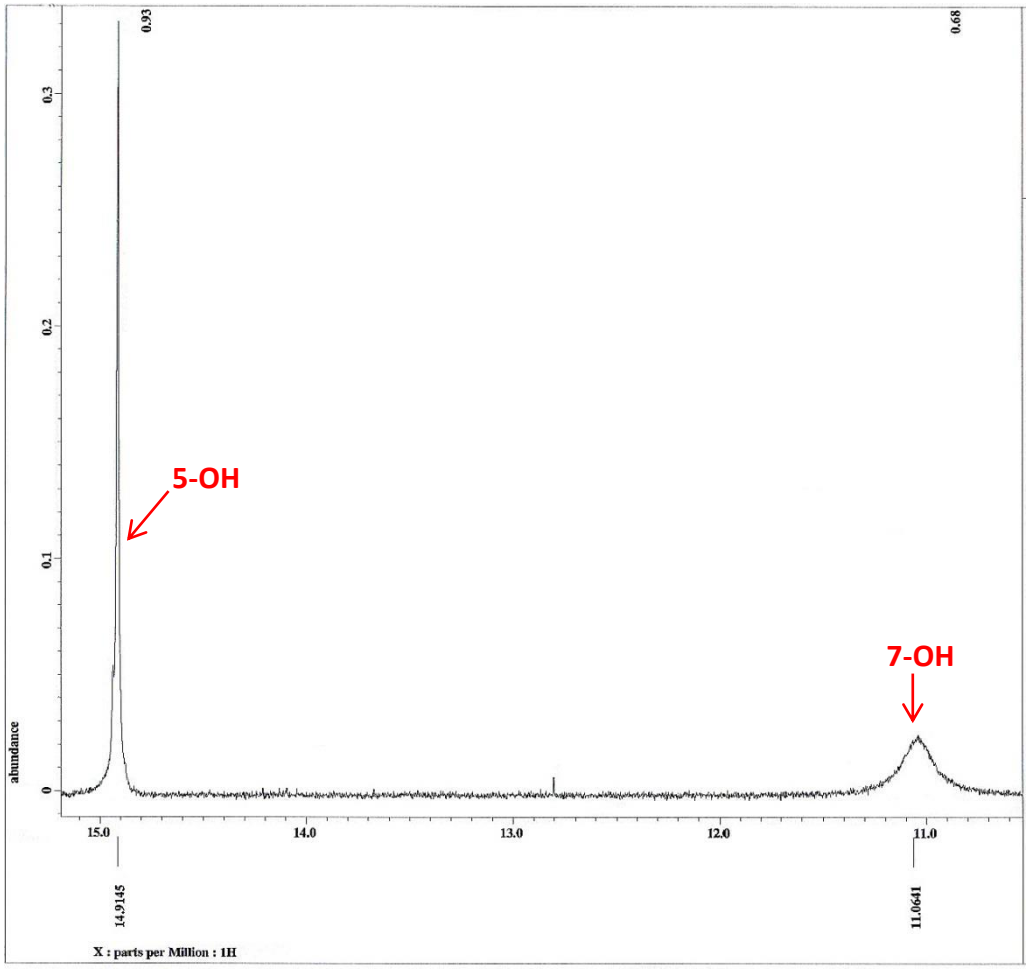
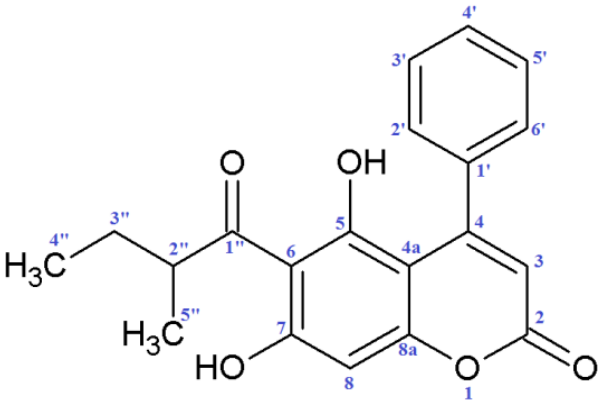



**Figure 4.8: Expanded $^{1}$H-NMR spectrum of compound 25**




**5,7-dihydroxy-6-(2-methylbutyryl)-4-phenylcoumarin [25]**

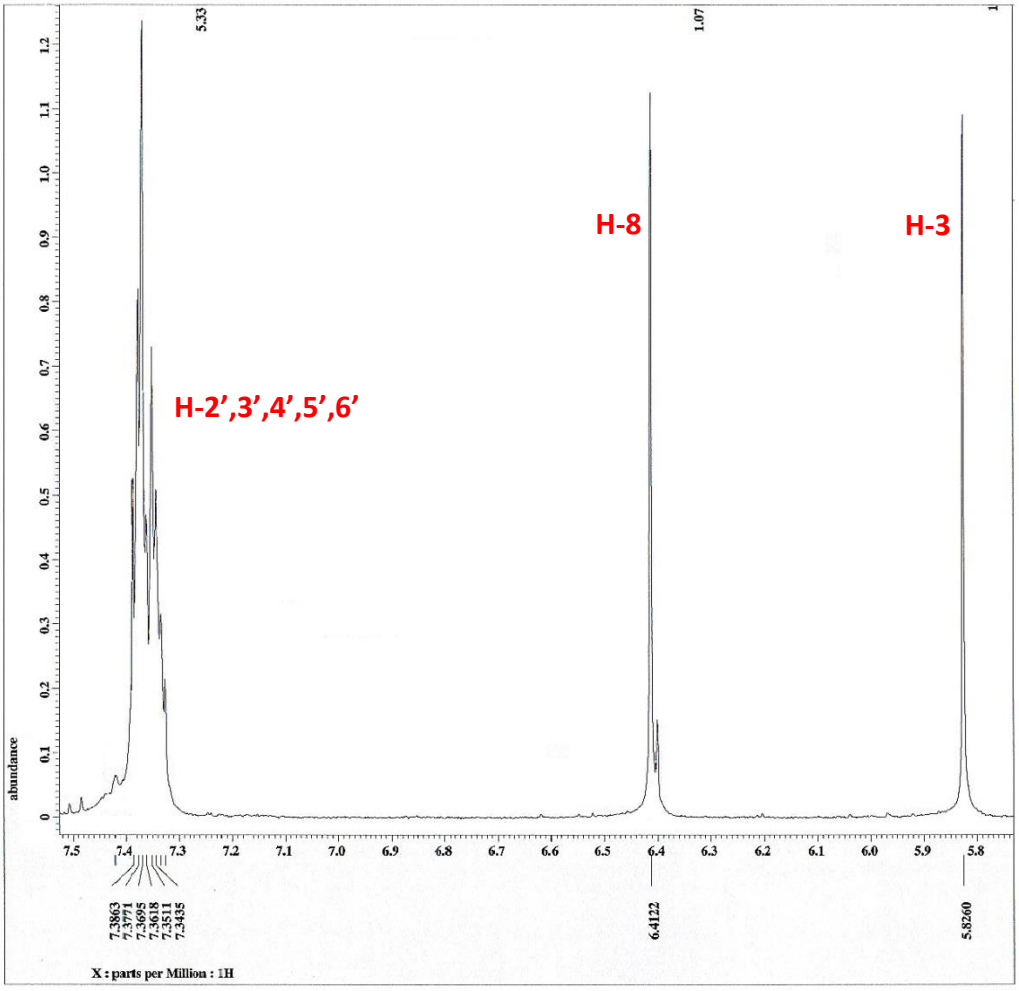
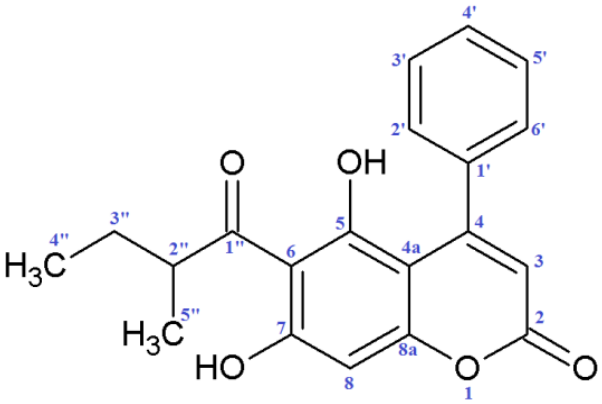



**Figure 4.9: Expanded $^{1}$H-NMR spectrum of compound 25**




**5,7-dihydroxy-6-(2-methylbutyryl)-4-phenylcoumarin [25]**

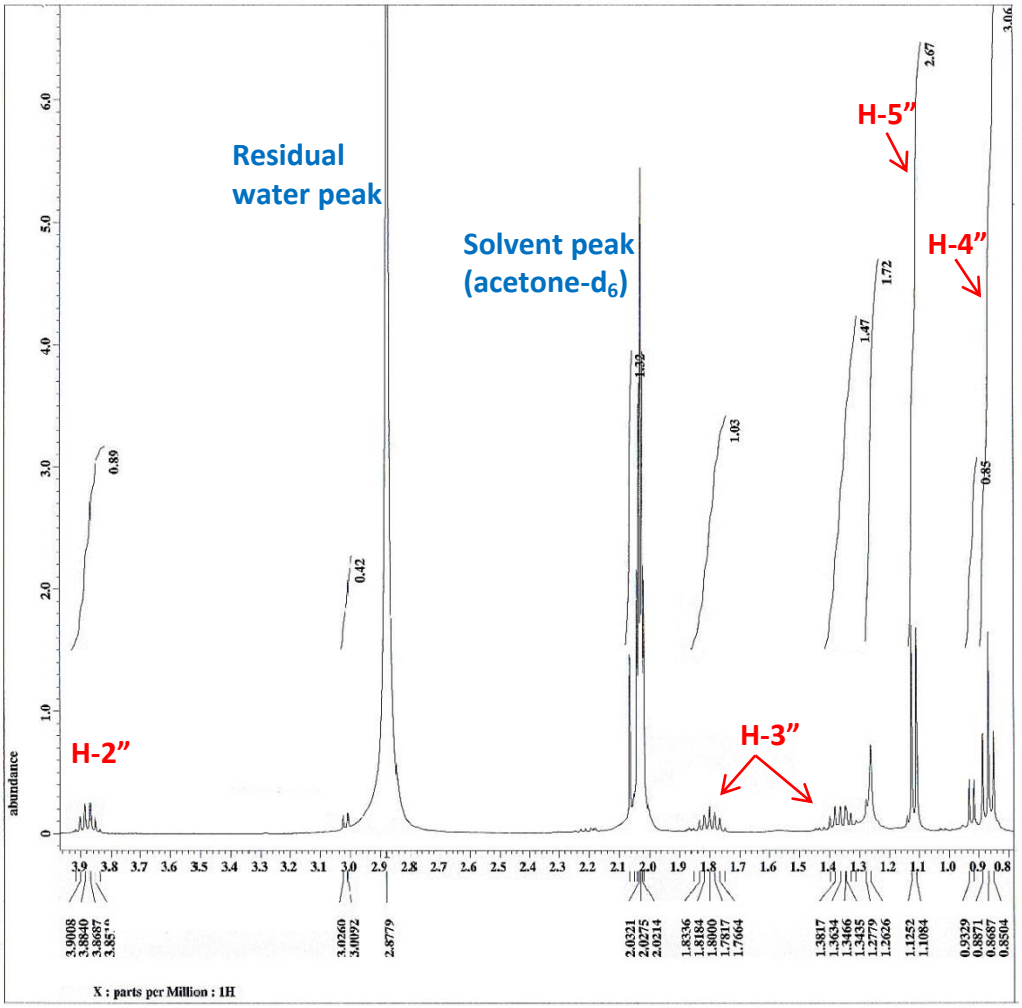
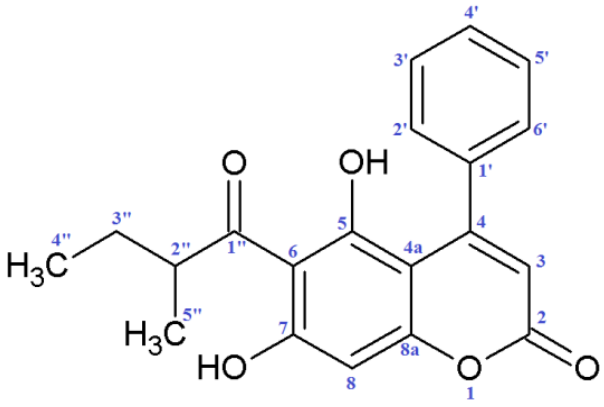



**Figure 4.10: Expanded $^{1}$H-NMR spectrum of compound 25**




**5,7-dihydroxy-6-(2-methylbutyryl)-4-phenylcoumarin [25]**

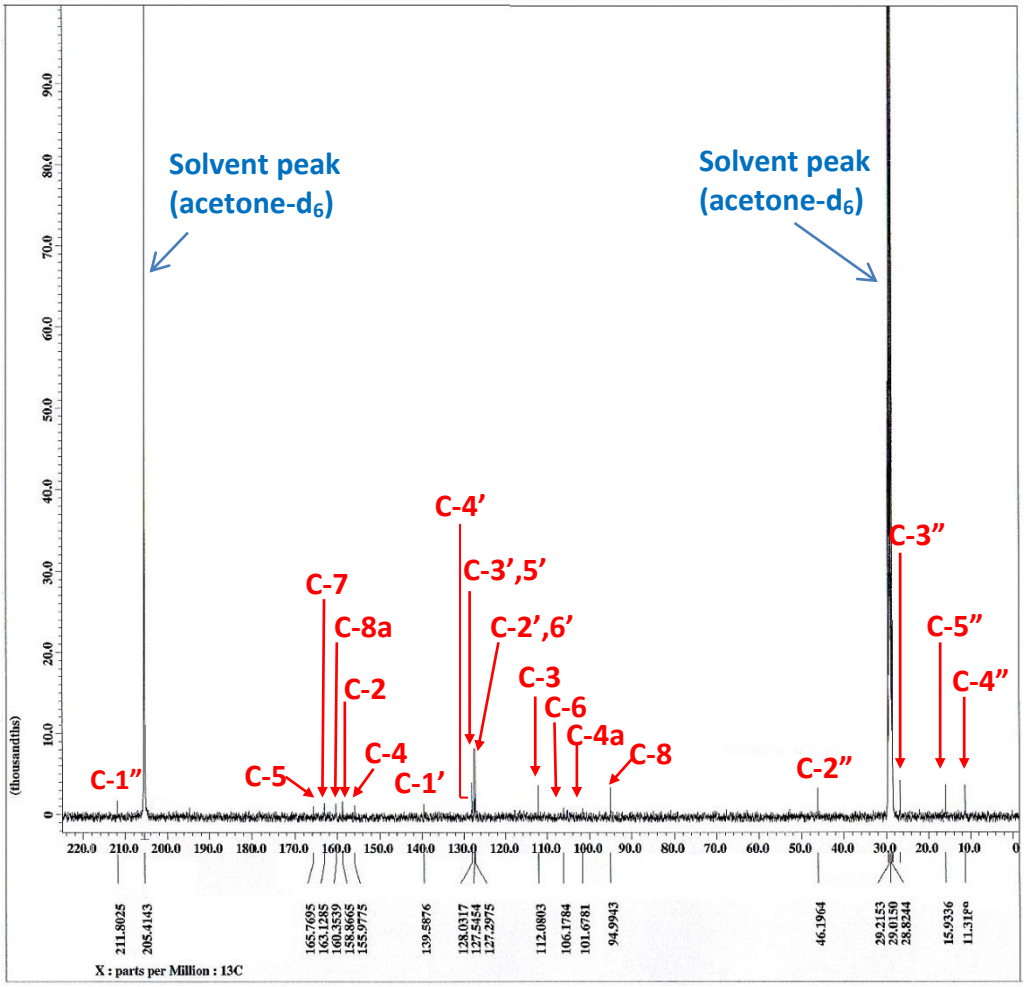
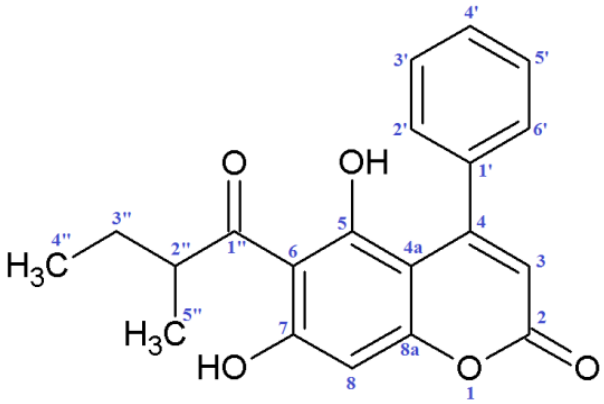



**Figure 4.11: $^{13}$C-NMR spectrum of compound 25**




**5,7-dihydroxy-6-(2-methylbutyryl)-4-phenylcoumarin [25]**

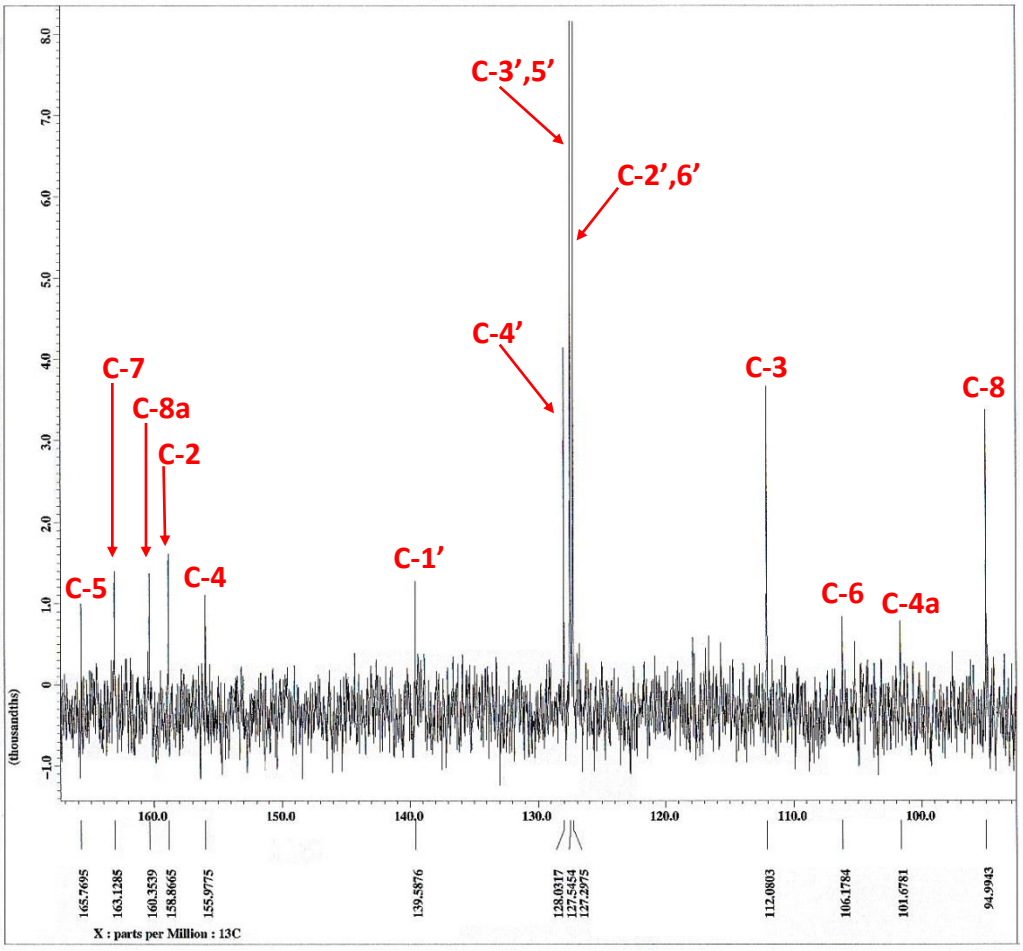
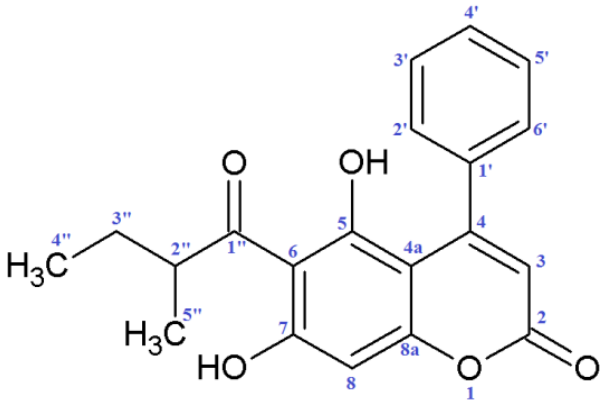



**Figure 4.12: Expanded $^{13}$C-NMR spectrum of compound 25**




**5,7-dihydroxy-6-(2-methylbutyryl)-4-phenylcoumarin [25]**

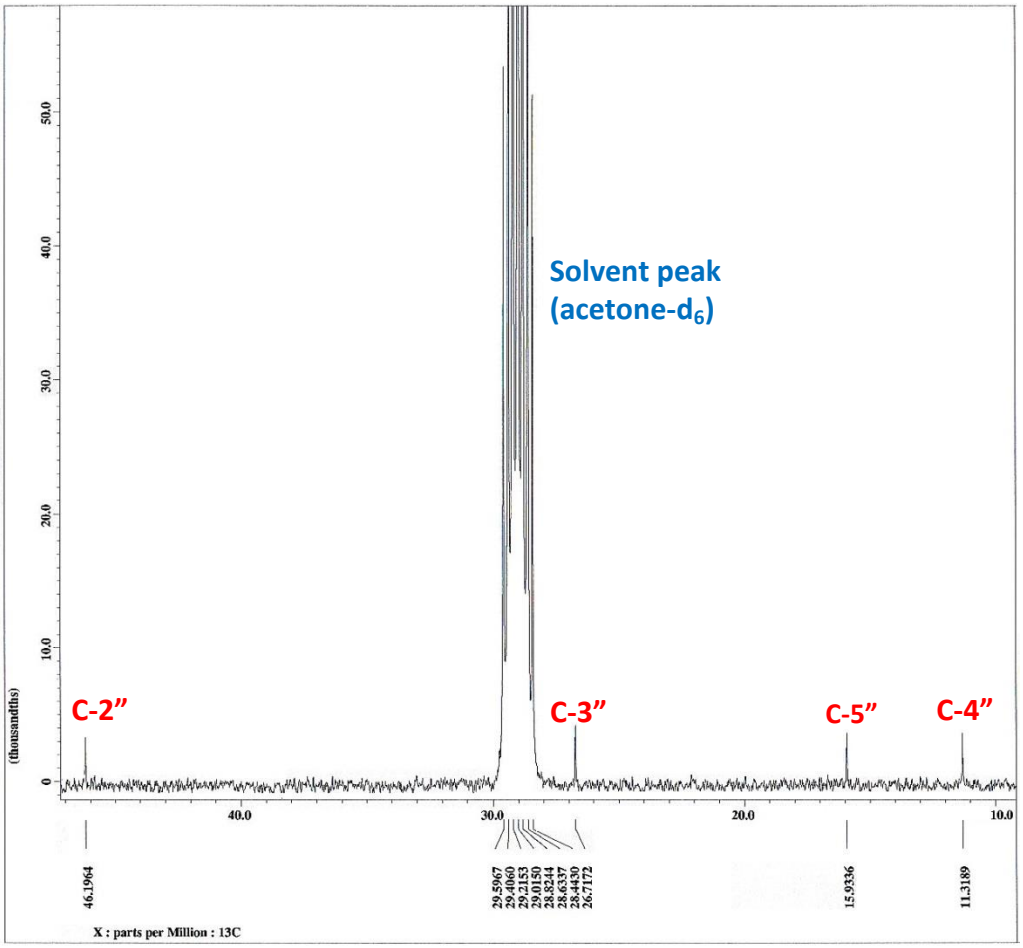
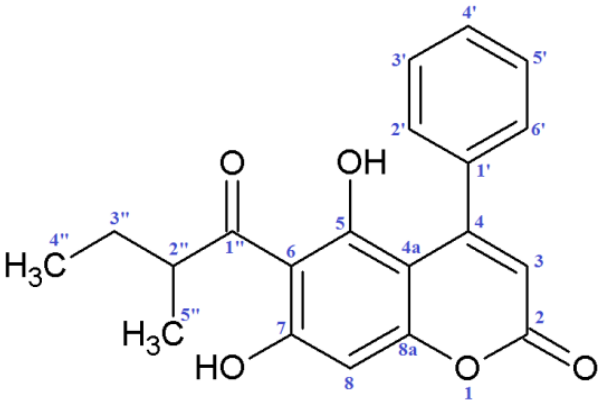



**Figure 4.13: Expanded $^{13}$C-NMR spectrum of compound 25**




**5,7-dihydroxy-6-(2-methylbutyryl)-4-phenylcoumarin [25]**

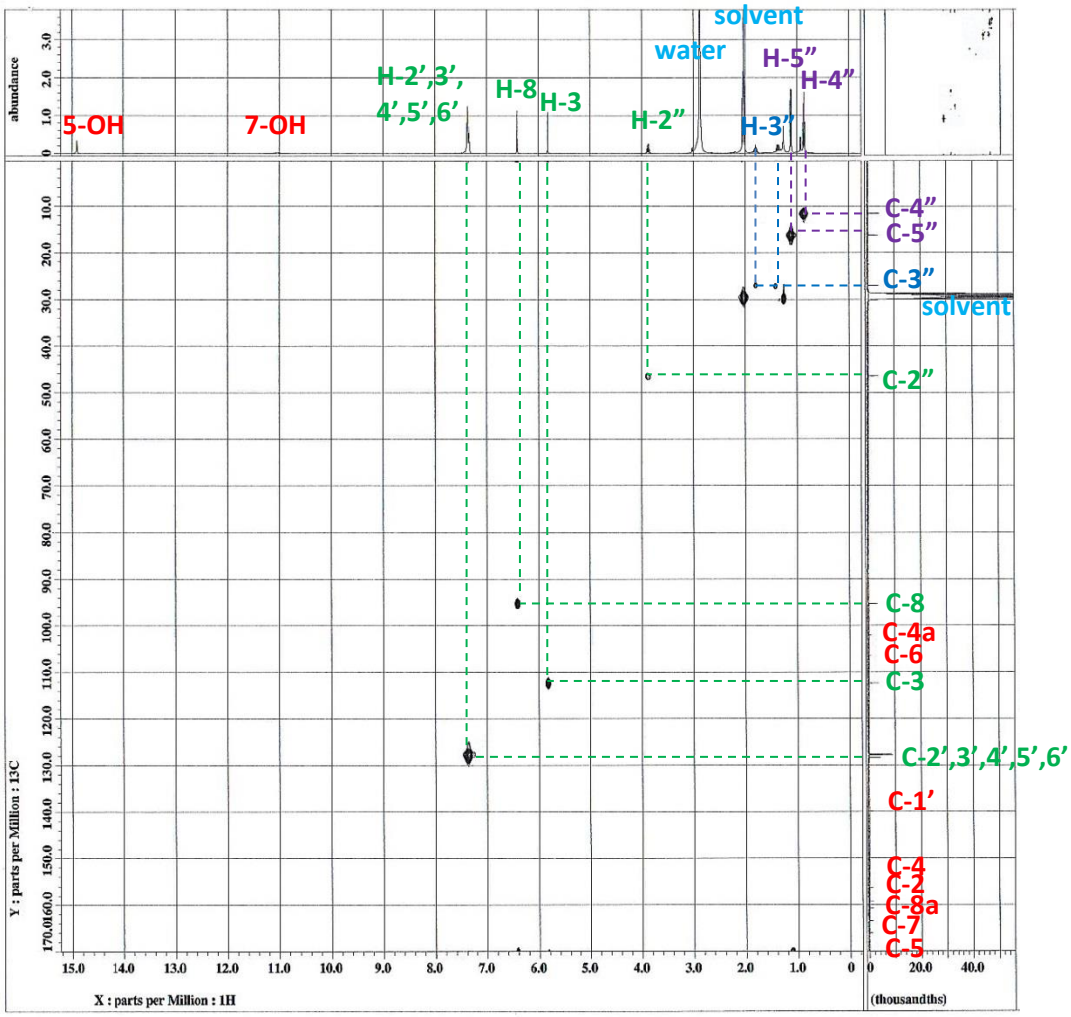
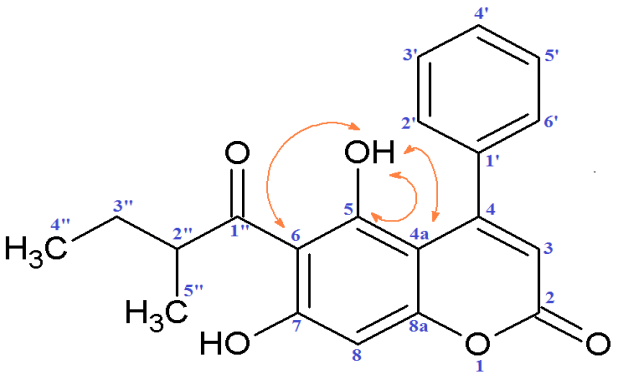



**Figure 4.14: HMQC spectrum of compound 25**




**5,7-dihydroxy-6-(2-methylbutyryl)-4-phenylcoumarin [25]**

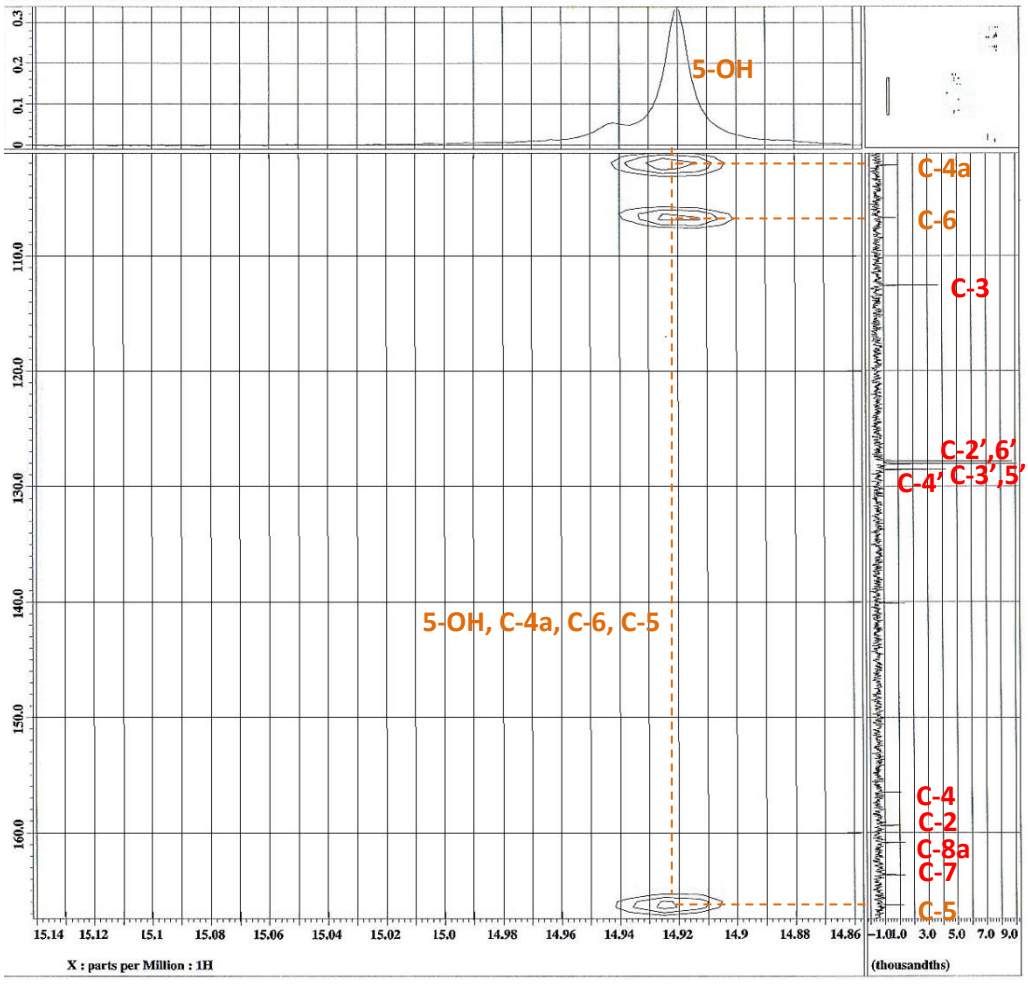
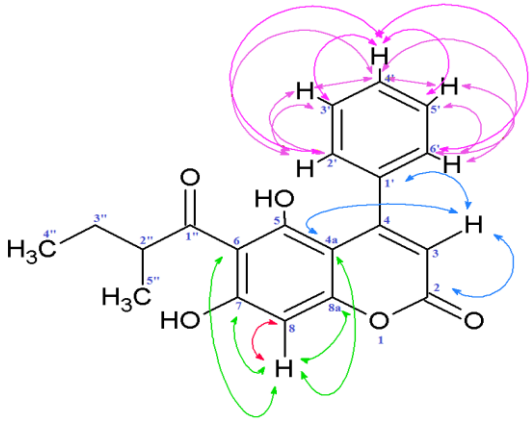



**Figure 4.15: Expanded HMBC spectrum of compound 25**



**5,7-dihydroxy-6-(2-methylbutyryl)-4-phenylcoumarin [25]**

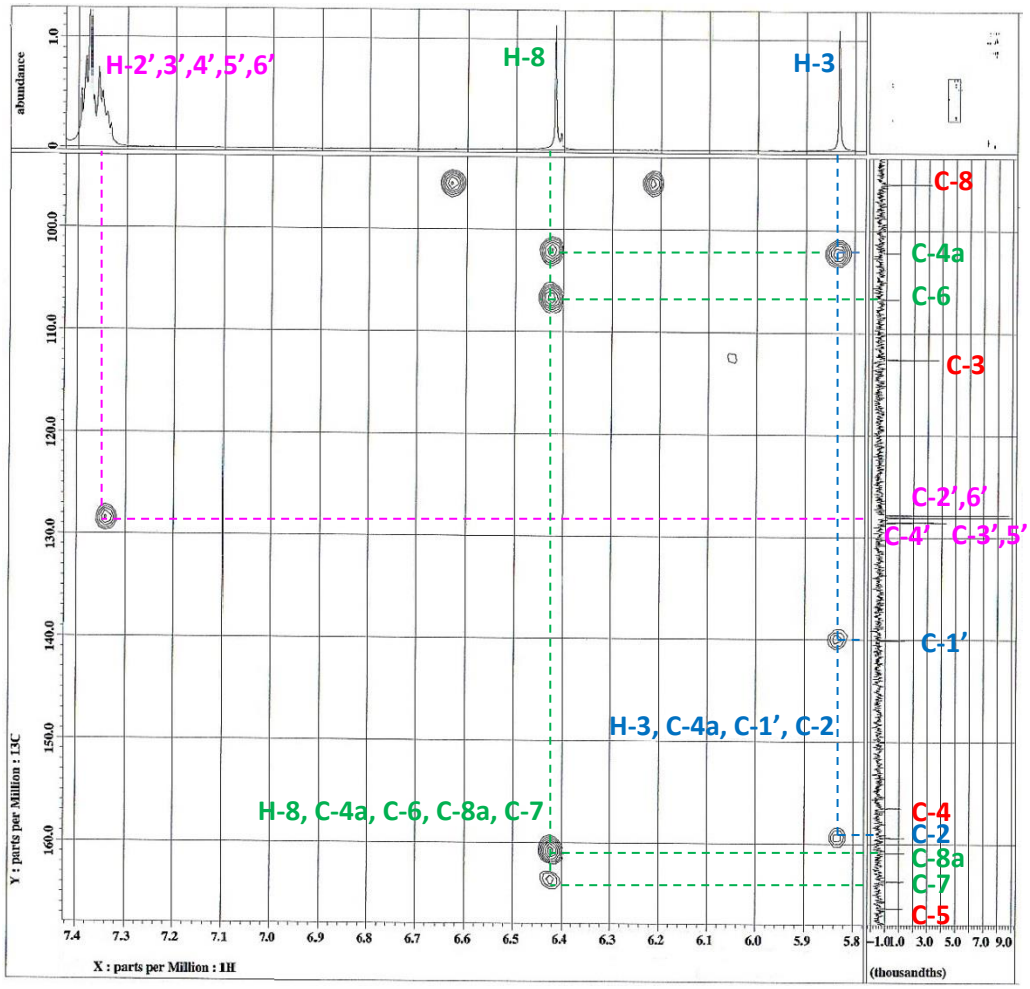
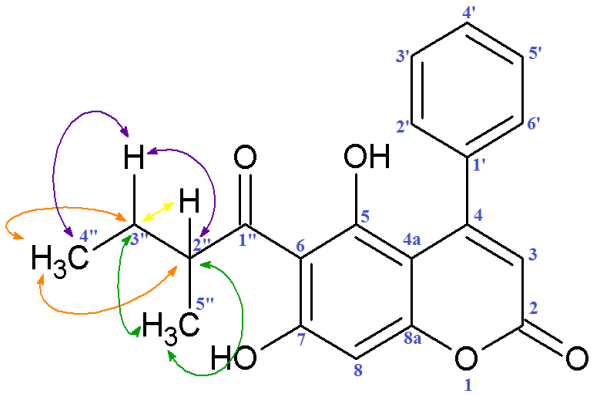



**Figure 4.16: Expanded HMBC spectrum of compound 25**




**5,7-dihydroxy-6-(2-methylbutyryl)-4-phenylcoumarin [25]**

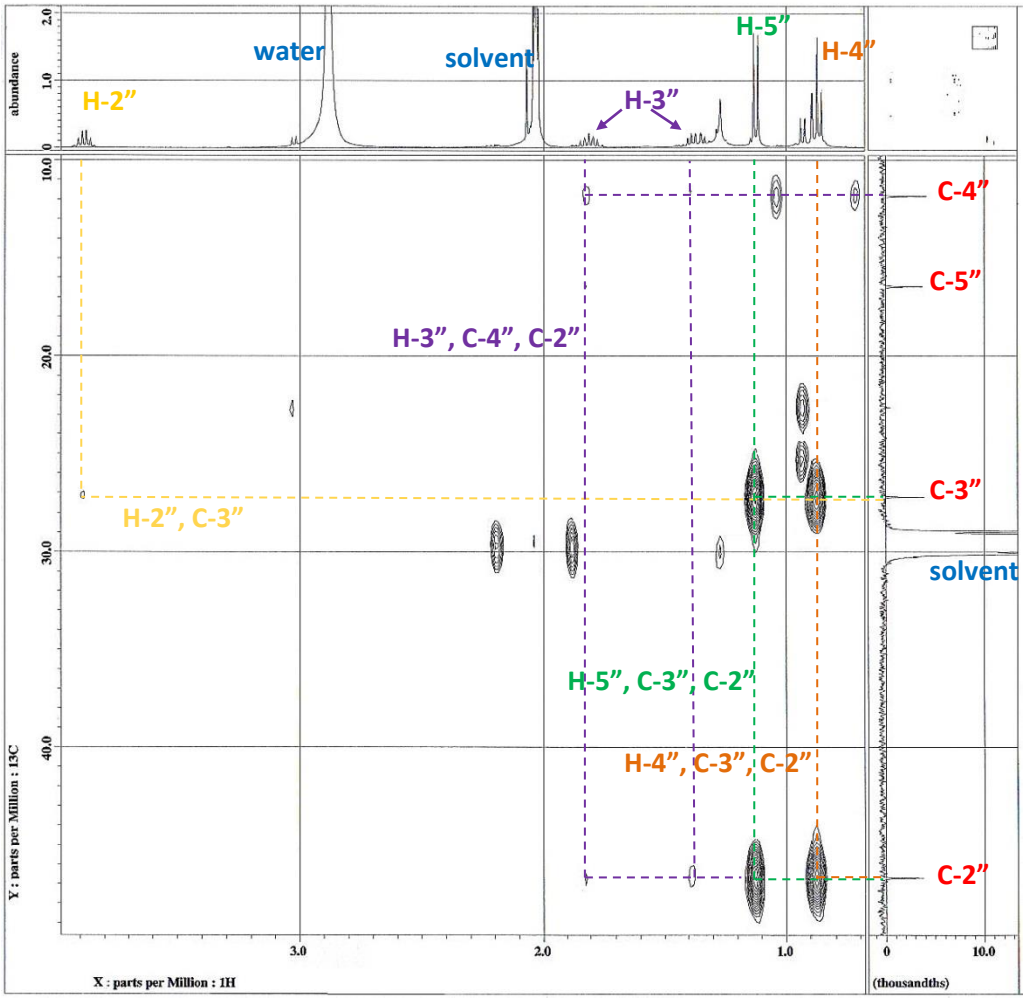
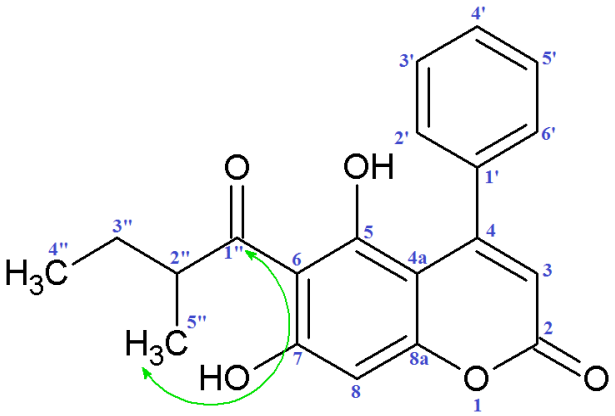



**Figure 4.17: Expanded HMBC spectrum of compound 25**




**5,7-dihydroxy-6-(2-methylbutyryl)-4-phenylcoumarin [25]**

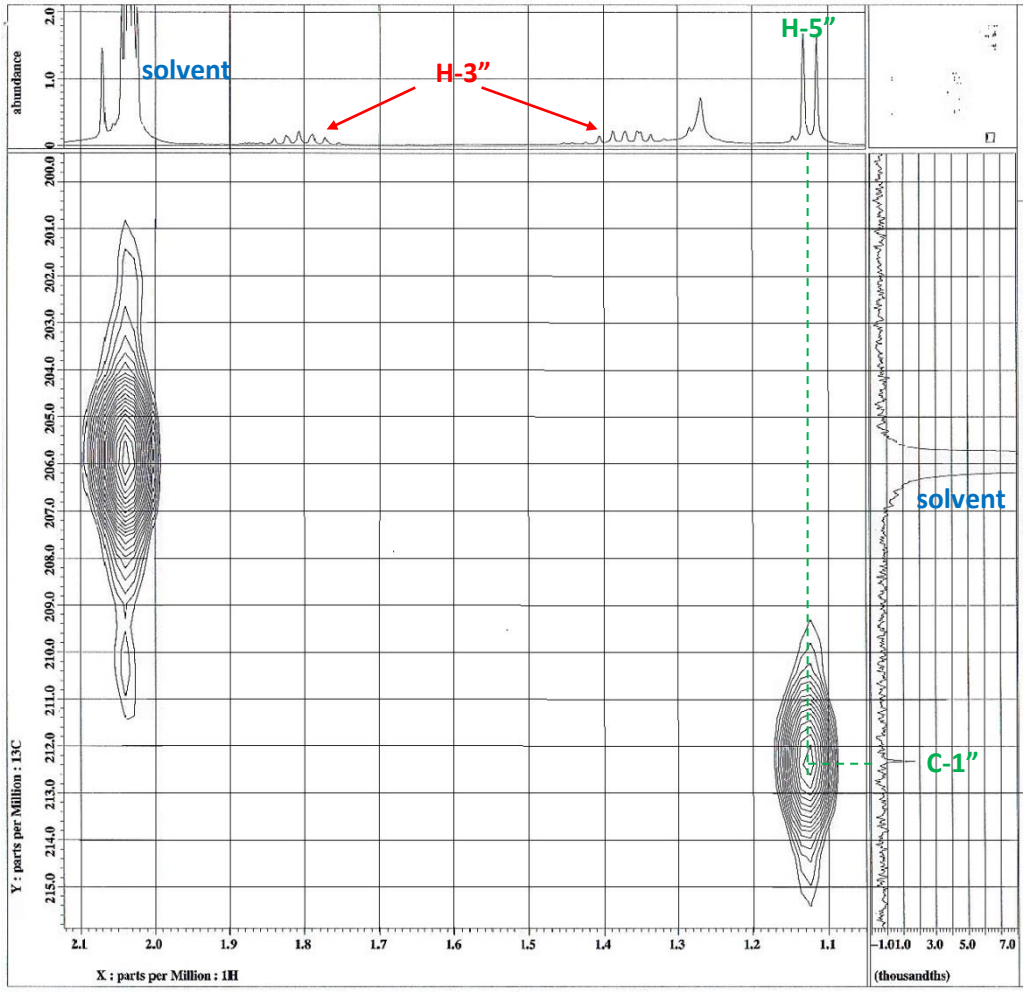



**Figure 4.18: Expanded HMBC spectrum of compound 25**

## 4.3 Chemical Identification and Structural Characterization of 5,7-Dihydroxy-6-(3-methylbutyryl)-4-phenylcoumarin

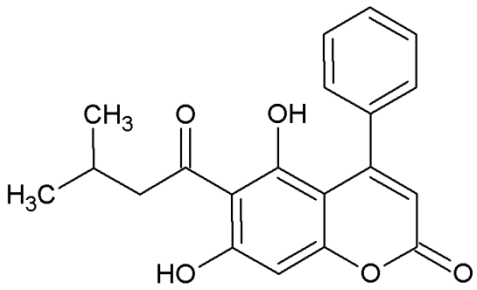



**Figure 4.19: The molecular structure of 5,7-dihydroxy-6-(3-methylbutyryl)-4-phenylcoumarin [26]**

Compound **26** was isolated as a yellow crystalline compound with the amount of 185.4 mg. Since it is a structural isomer of compound **25**, thus it has the same molecular formula $C_{20}H_{18}O_5$ with compound **25**, but with a different acyl substituent group. In GC-MS chromatogram (Figure 4.20), compound **26** gave a molecular ion peak $[M]^+$ with a nominal mass of 338 g $mol^{-1}$. While in HRESI mass spectrum (Figure 4.21), an accurate mass of 338.1149 g $mol^{-1}$ was reported for compound **26**. Both molecular weights obtained are corresponding to the molecular formula $C_{20}H_{18}O_5$.

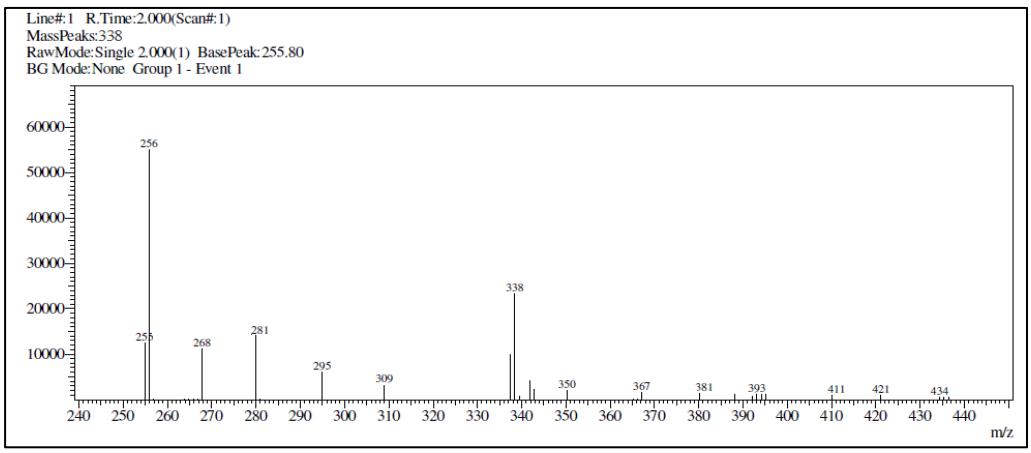



**Figure 4.20: GC-MS spectrum of compound 26**

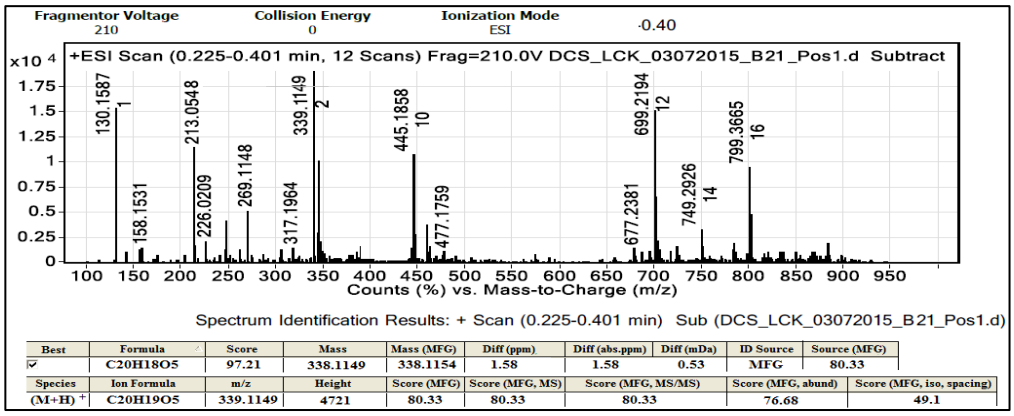



**Figure 4.21: HRESIMS spectrum of compound 26**

In addition, compound **26** has a melting point of 249-251 $^{o}$C (Lit. 244-245 $^{o}$C, Crombie, et al., 1987). In TLC analysis, a single spot with a retention factor, $R_f$ value of 0.58 was obtained using a mixture of hexane and ethyl acetate (1:3) as mobile phase. Due to its aromatic nature, it appeared as dark grey spot when visualized under short λ UV light and stained with iodine vapour to form a dark brown spot. The presence of a coumarin moiety in the chemical structure of compound **26** was deduced from the UV-Vis spectrum (Figure 4.22), in which the characteristic absorption peaks appeared at 279 and 330 nm. Absorption peaks were resulted from $\pi \rightarrow \pi^*$ transitions of benzene and pyrone moiety.

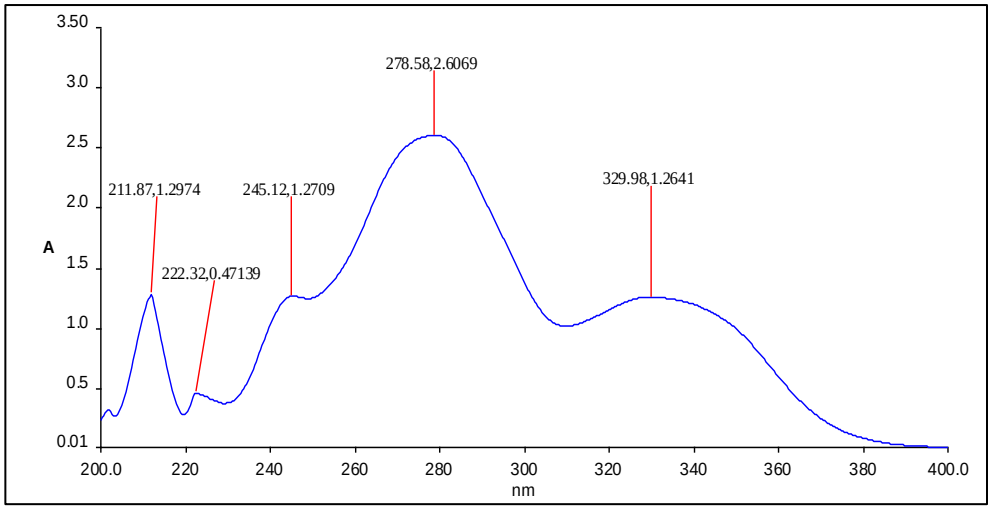



**Figure 4.22: UV-Vis spectrum of compound 26**

Comparison of the $^{1}$H-NMR spectra (Figure 4.10 and 4.25) for compound **25** and **26** showed very obvious for several proton signals in the upfield region below $\delta_H$ 4.0. In particular, a doublet signal at $\delta_H$ 0.95 integrated for six protons were assigned to the two groups of chemically equivalent methyl protons H-4" and H-5". However, the two groups of methyl protons H-4" and H-5" in compound **25** gave a triplet and doublet signals at $\delta_H$ 0.87 and 1.11, respectively. Difference was also observed in the splitting pattern and integration number for the H-2" protons, whereby in compound **26**, a doublet signal integrated for two protons was observed, but a multiplet signal integrated for one proton was found in compound **25**. At the meantime, H-3" showed a peak integrated for one proton in compound **26**, whereas in compound **25**, the integration number for H-3" is 2. The remaining proton signals appeared above $\delta_H$ 5.0 were found to be identical in the $^{1}$H-NMR spectra of these two compounds.

According to $^{13}$C-NMR spectrum (Figure 4.26), compound **26** gave almost identical spectral characteristics as compound **25** as discussed in Section 4.2. The only difference was the carbon signal at $\delta_C$ 22.1 which was assigned to carbons C-4" and C-5" as they are symmetrical carbons. However, in compound **25**, carbons C-4" and C-5" gave different carbon signals in the $^{13}$C-NMR spectrum (Figure 4.27). Since these two compounds showed the same coumarin nucleus skeleton linked to a phenyl substituent group, therefore both compounds gave almost similar carbon signals in the aromatic regions of $\delta_C$ 170-90 in their $^{13}$C-NMR spectra.

The assignment was further confirmed by 2D-NMR experiments. In HMQC spectrum (Figure 4.28), a proton signal integrated for one proton at $\delta_H$ 6.41 (H-8) is directly coupled with carbon signal at $\delta_C$ 94.9 (C-8) through $^1J$ coupling, indicating that C-8 is a methine carbon. On the other hand, in HMBC spectrum (Figure 4.29), this methine proton showed $^3J$ couplings with carbons C-4a and C-6, as well as $^2J$ couplings with carbons C-7 and C-8a, which confirmed the presence of fused benzene ring to α-pyrone ring in the coumarin nucleus. Other correlations were listed in Table 4.2. Apart from NMR data, the chemical structure of compound **26** was further verified by FTIR spectroscopic analysis. From the IR spectrum (Figure 4.23), compound **26** was confirmed to have functionalities such as hydroxyl O-H (3420 $cm^{-1}$), carbonyl C=O (1688 $cm^{-1}$), carbinol C-O (1233 $cm^{-1}$) and aromatic C=C (1615 and 1469 $cm^{-1}$). Besides stretching, C-H and C-C bending vibrations can be found below 1000 $cm^{-1}$. Based on various spectral information, compound **26** was identified as 5,7-dihydroxy-6-(3-methylbutyryl)-4-phenylcoumarin.

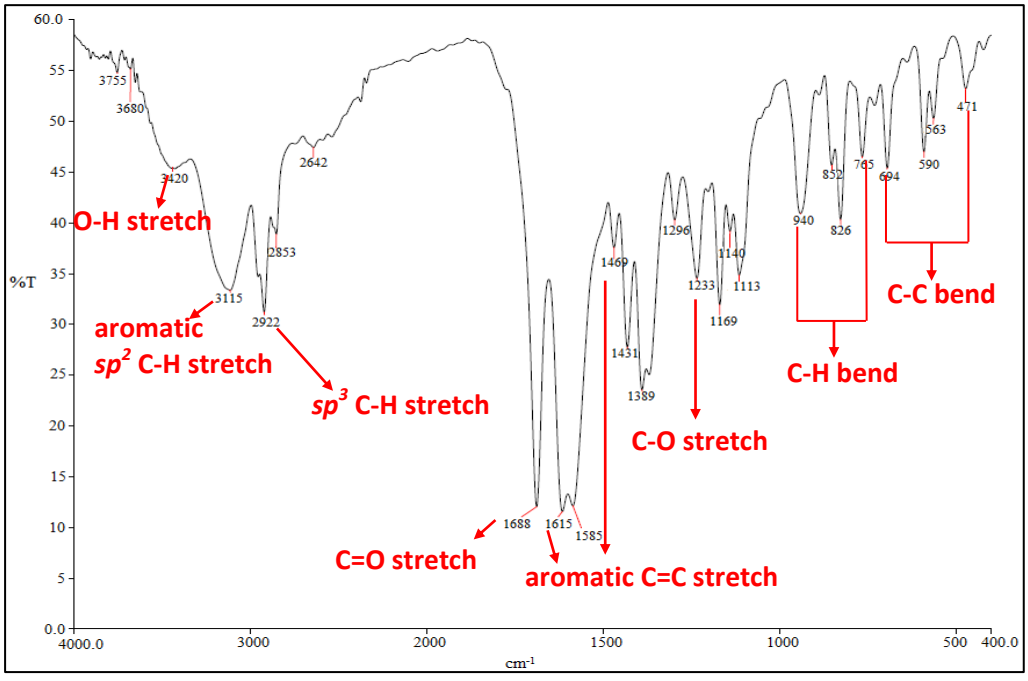



**Figure 4.23: IR spectrum of compound 26**

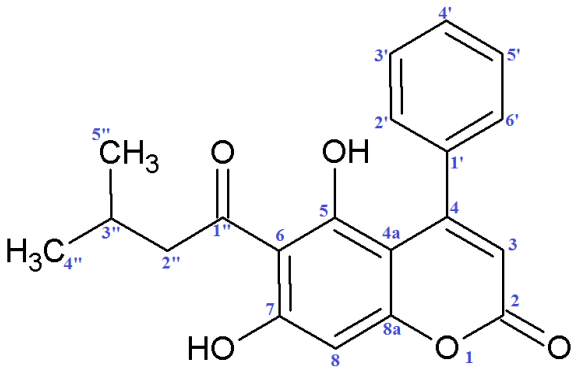
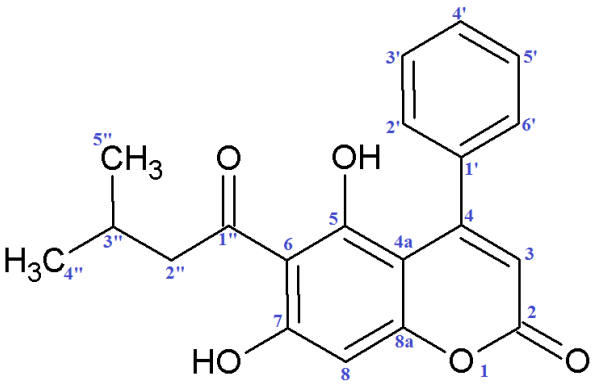



**Table 4.2: Summary of NMR data for 5,7-dihydroxy-6-(3-methylbutyryl)-4-phenylcoumarin [26]**

| Position | $\delta_H$ (ppm), Integration and Multiplicity | $\delta_C$ (ppm) | HMBC |
|---|---|---|---|
| 1 | - | - | - |
| 2 | - | 158.9 | - |
| 3 | 5.83 (1H, s) | 112.0 | C-1', 2, 4a |
| 4 | - | 156.0 | - |
| 4a | - | 101.5 | - |
| 5 | - | 165.5 | - |
| 6 | - | 106.6 | - |
| 7 | - | 163.5 | - |
| 8 | 6.41 (1H, s) | 94.9 | C-4a, 6 , 7, 8a |
| 8a | - | 160.4 | - |
| 1' | - | 139.6 | - |
| 2' & 6' | 7.37 (2H, m) | 127.3 | C-3', 4', 5' |
| 3' & 5' | 7.39 (2H, m) | 127.5 | C-2', 4', 6' |
| 4' | 7.41 (1H, m) | 128.0 | C-2', 3', 5', 6' |
| 1'' | - | 207.4 | - |
| 2'' | 3.03 (2H, d, *J* = 6.7 Hz) | 52.9 | C-1'', 3'', 4'', 5'' |
| 3'' | 2.22 (1H, m) | 24.9 | - |
| 4'' | 0.95 (6H, d, *J* = 6.7 Hz) | 22.1 | C-2'',3'', 4'',5'' |
| 5'' | | | |
| 5-OH | 14.96 (1H, s) | - | C-4a, 5, 6 |
| 7-OH | 11.24 (1H, brs) | - | - |




**5,7-dihydroxy-6-(3-methylbutyryl)-4-phenylcoumarin [26]**

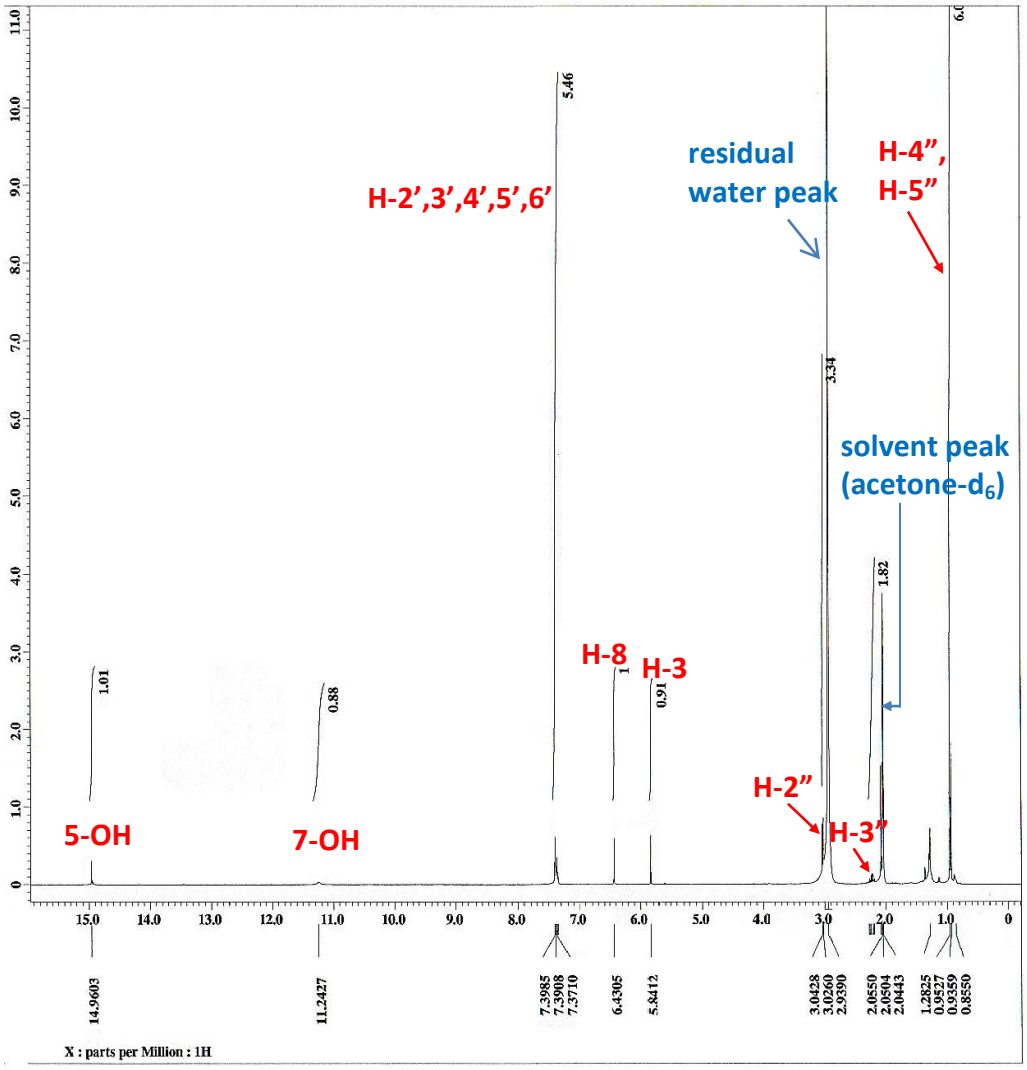
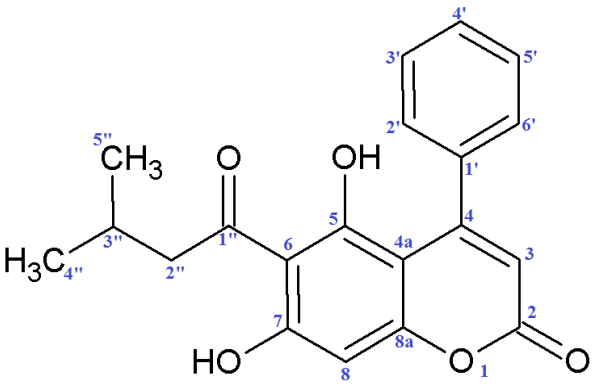



**Figure 4.24: $^{1}$H-NMR spectrum of compound 26**




**5,7-dihydroxy-6-(3-methylbutyryl)-4-phenylcoumarin [26]**

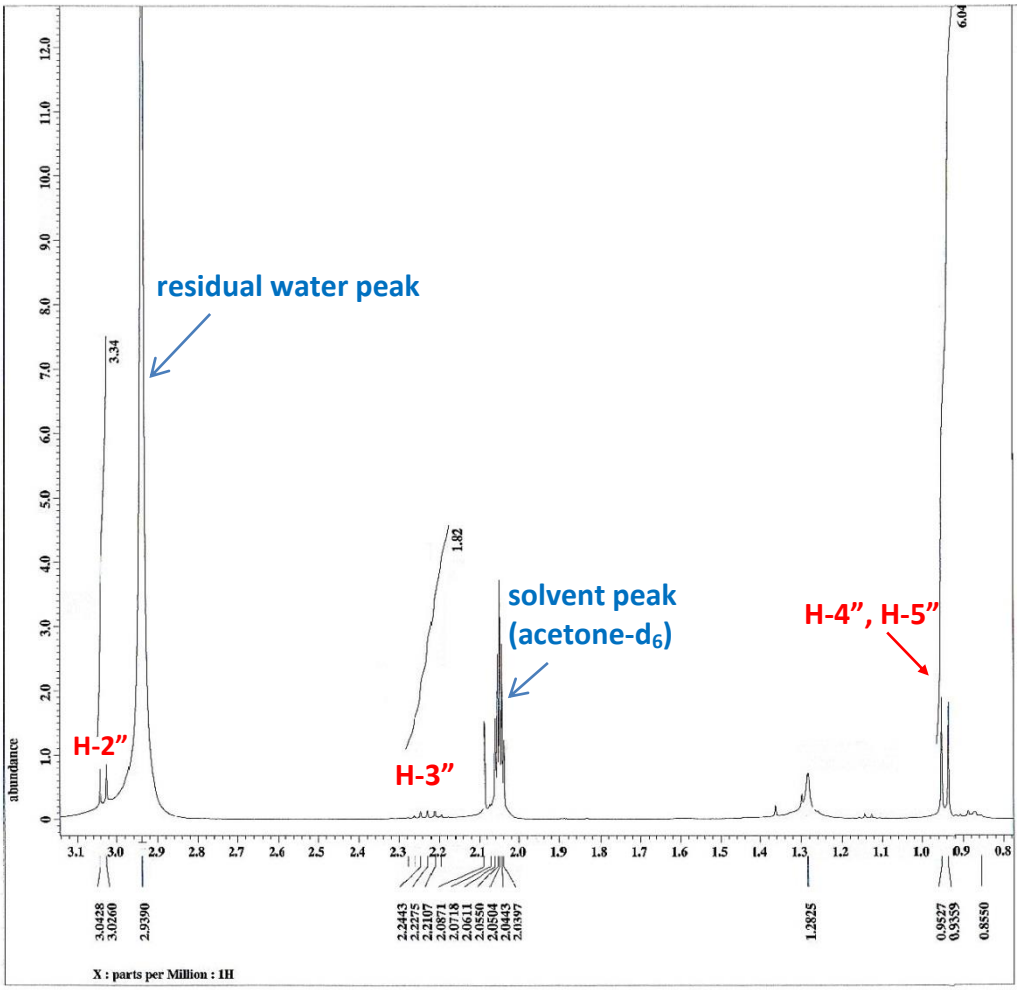
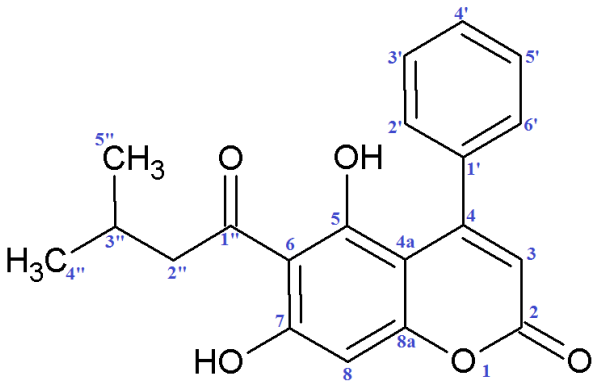



**Figure 4.25: Expanded $^{1}$H-NMR spectrum of compound 26**




**5,7-dihydroxy-6-(3-methylbutyryl)-4-phenylcoumarin [26]**

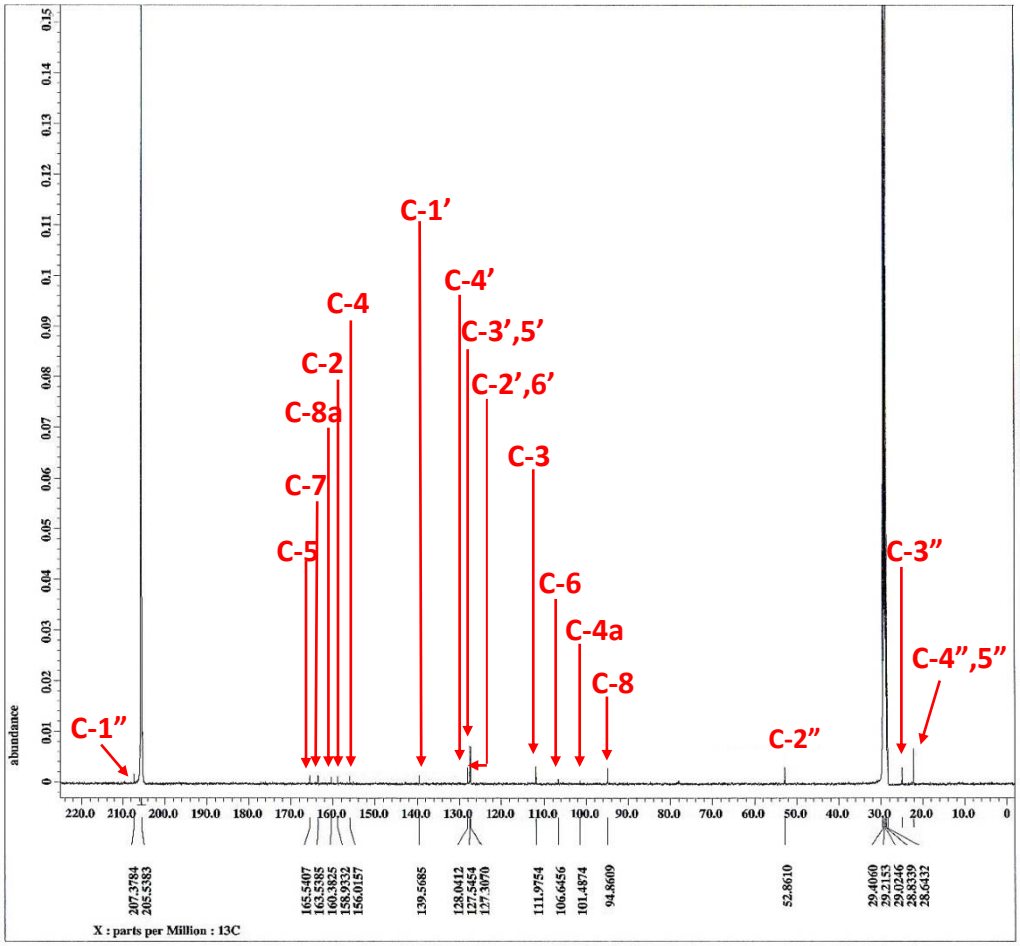
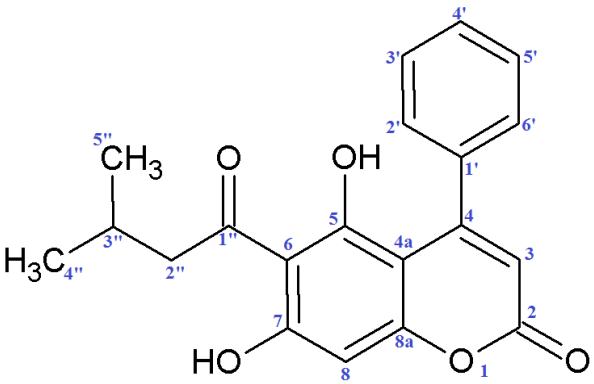



**Figure 4.26: $^{13}$C-NMR spectrum of compound 26**




**5,7-dihydroxy-6-(3-methylbutyryl)-4-phenylcoumarin [26]**

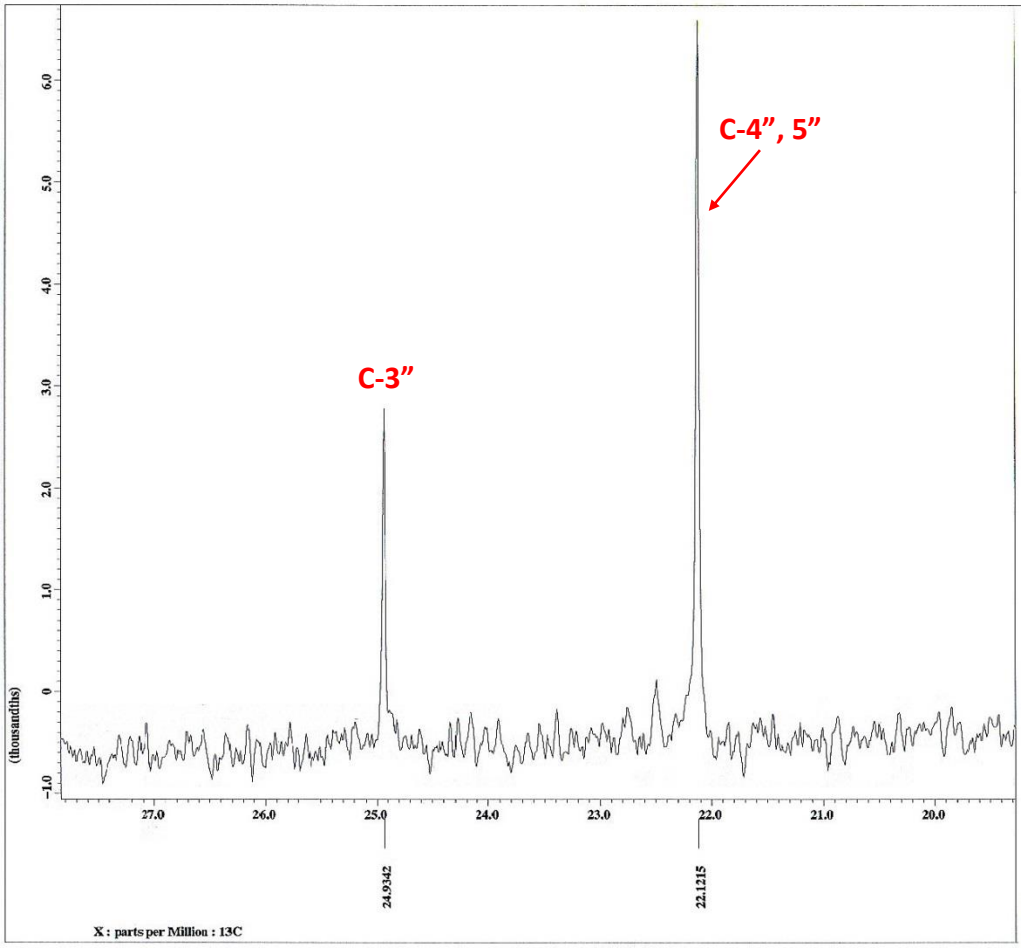
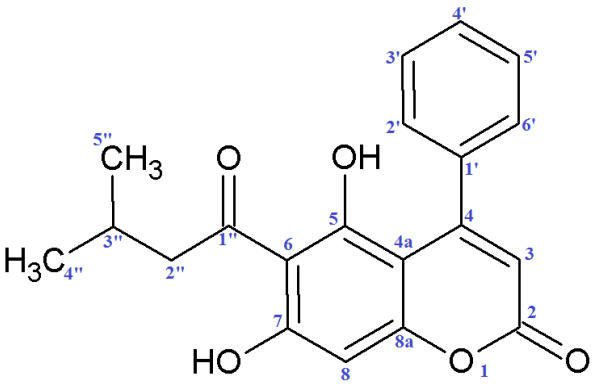



**Figure 4.27: Expanded $^{13}$C-NMR spectrum of compound 26**




**5,7-dihydroxy-6-(3-methylbutyryl)-4-phenylcoumarin [26]**

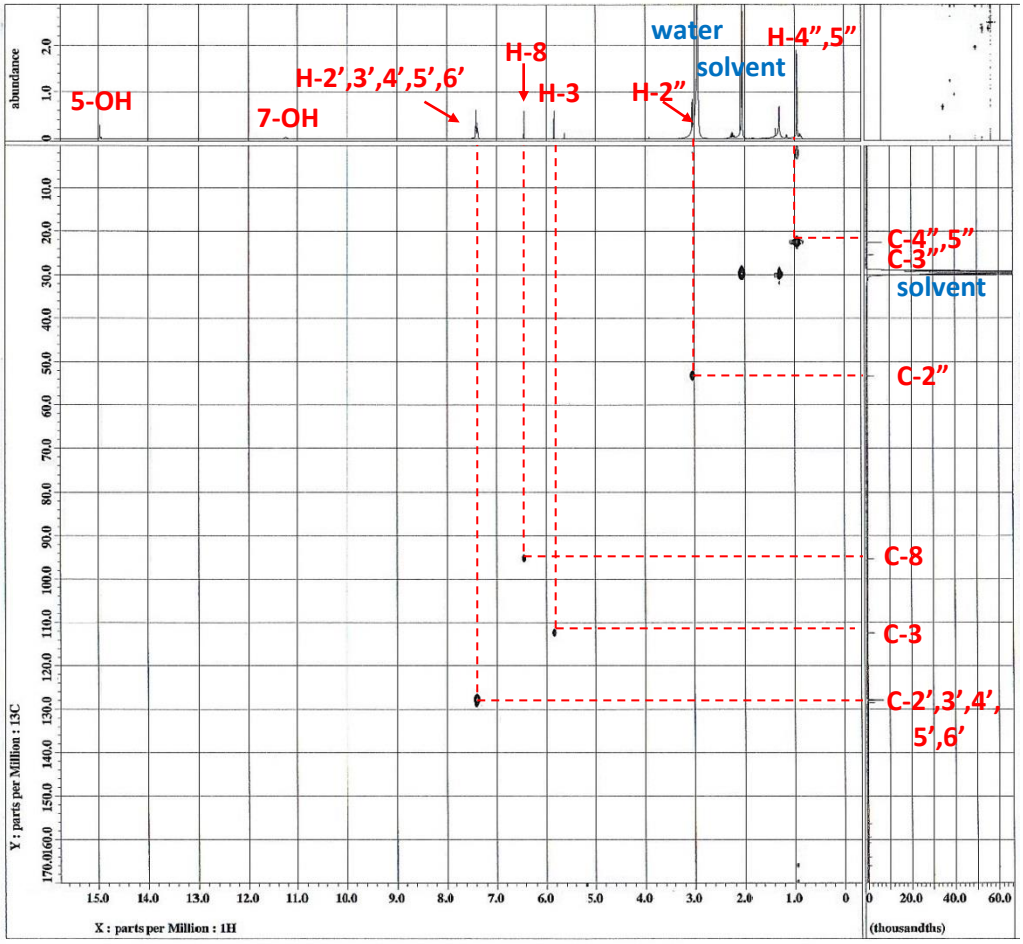
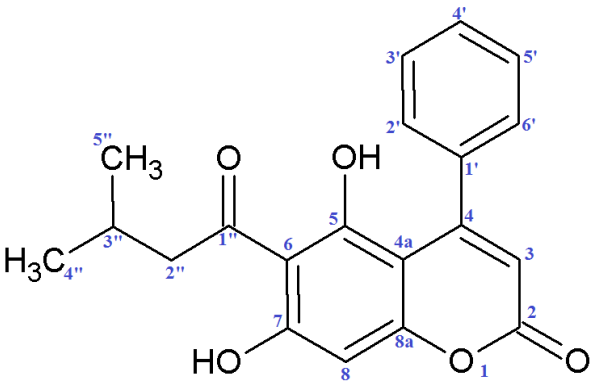



**Figure 4.28: HMQC spectrum of compound 26**




**5,7-dihydroxy-6-(3-methylbutyryl)-4-phenylcoumarin [26]**

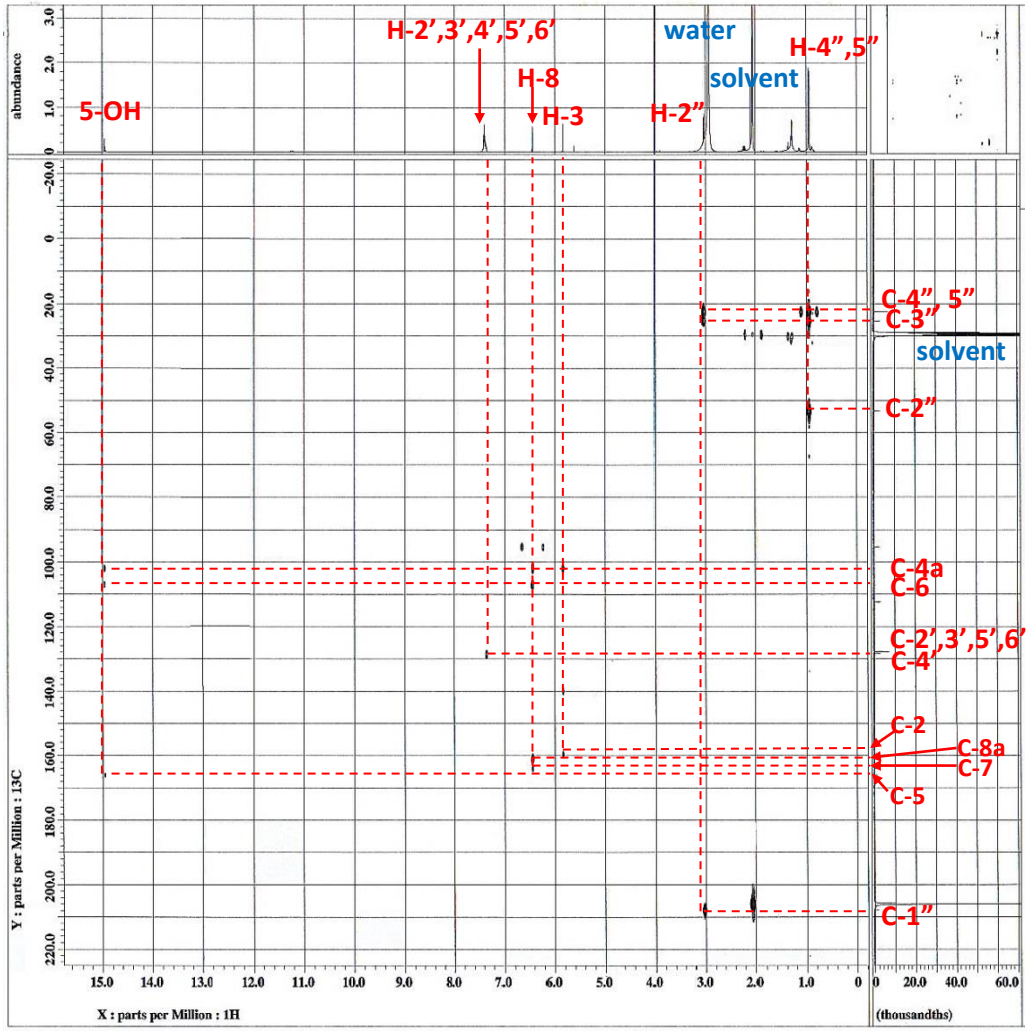
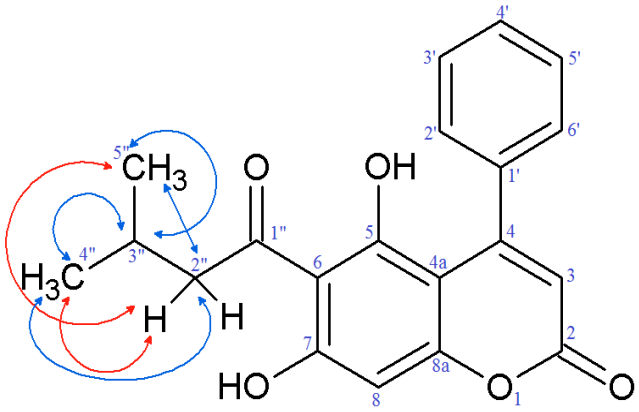



**Figure 4.29: HMBC spectrum of compound 26**




**5,7-dihydroxy-6-(3-methylbutyryl)-4-phenylcoumarin [26]**

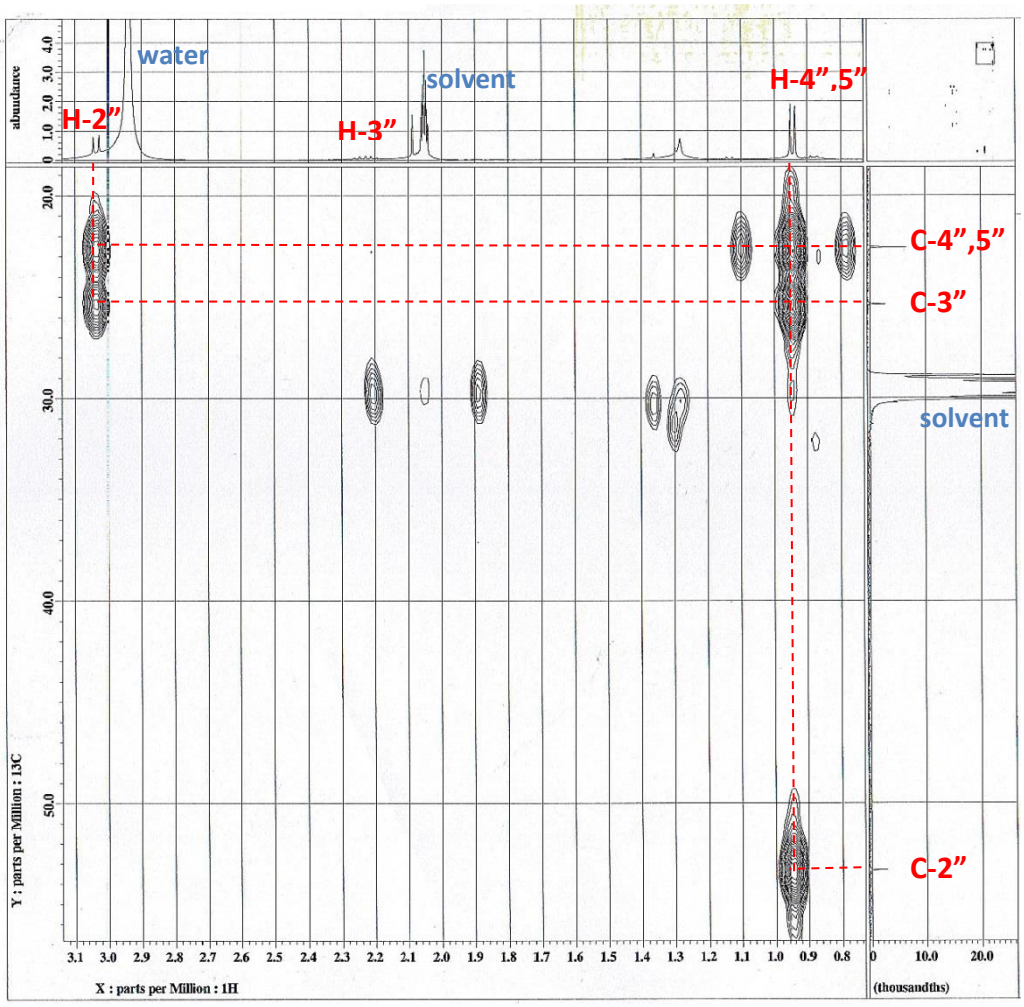



**Figure 4.30: Expanded HMBC spectrum of compound 26**

## 4.4 Chemical Identification and Structural Characterization of Isodispar B [27]

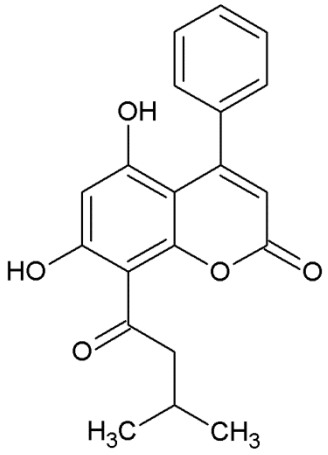



**Figure 4.31: The molecular structure of isodispar B [27]**

Compound **27** was obtained as pale yellow needle-like crystals with a melting point of 202-204 $^{o}$C (Lit. 200-202 $^{o}$C, Crombie, et al., 1987). It is a coumarin, namely isodispar B or 5,7-dihydroxy-8-(3-methylbutyryl)-4-phenylcoumarin. Similarly to compounds **25** and **26**, chemical structure of isodispar B also comprises of coumarin nucleus with 4-phenyl and 5,7-dioxygenation patterns, but a different in the position of acyl group attached. Instead of carbon C-6, the 3-methylbutyryl group is attached to carbon C-8 in isodispar B. Since isodispar B containing conjugated aromatic ring, thus it appeared as single dark grey spot under short wavelength UV light at 254 nm and a brown spot in iodine chamber. The retention factor, $R_f$ value of single spot is 0.60 via a solvent mixture of hexane and ethyl acetate (1:3) as mobile phase.

In GC-MS analysis, compound **27** gave a molecular ion peak $[M]^+$ at *m/z* 338 which is corresponding to the molecular formula of $C_{20}H_{18}O_5$. Meanwhile, a protonated molecular ion peak $[M+H]^+$ observed in HRESI mass spectrum (Figure 4.33) showed compound 27 to have an accurate mass of 338. 1157 g $mol^{-1}$, which was in good agreement with the calculated value of 338.1154 g $mol^{-1}$.

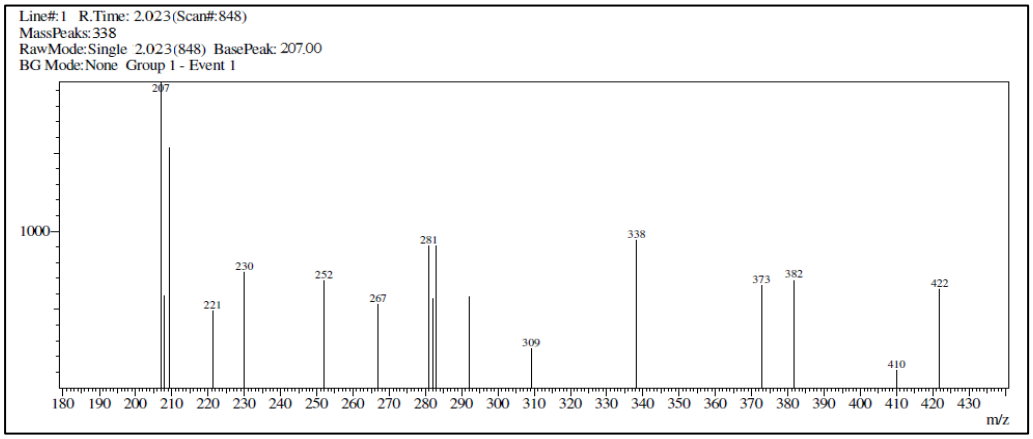
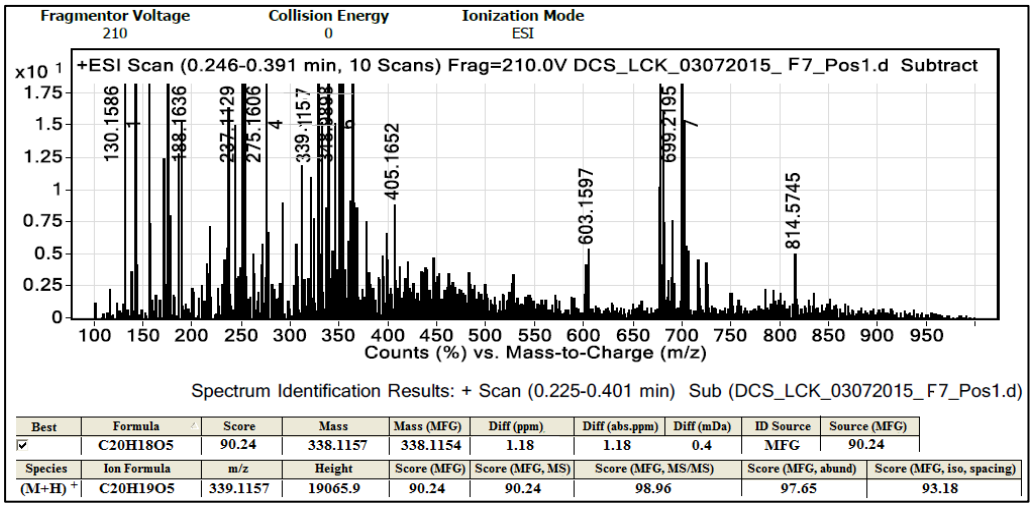



**Figure 4.32: GC-MS spectrum of isodispar B [27]**




| Best | Formula | Score | Mass | Mass (MFG) | Diff (ppm) | Diff (abs.ppm) | Diff (mDa) | ID Source | Source (MFG) |
|---|---|---|---|---|---|---|---|---|---|
| ☑ | C20H18O5 | 90.24 | 338.1157 | 338.1154 | 1.18 | 1.18 | 0.4 | MFG | 90.24 |

| Species | Ion Formula | m/z | Height | Score (MFG) | Score (MFG, MS) | Score (MFG, MS/MS) | Score (MFG, abund) | Score (MFG, iso, spacing) |
|---|---|---|---|---|---|---|---|---|
| $(M+H)^+$ | C20H19O5 | 339.1157 | 19065.9 | 90.24 | 90.24 | 98.96 | 97.65 | 93.18 |

**Figure 4.33: HRESIMS spectrum of isodispar B [27]**

The UV-Vis spectrum (Figure 4.34) shows the characteristic absorption maxima of compound **27** at 226, 257, 309 and 347 nm. The electronic

transition that responsible for the absorption peaks in the range of 270-350 nm is due to $\pi \rightarrow \pi^*$ transition. Whereas the absorption peak due to $n \rightarrow \sigma^*$ transition is within the range of 150-250 nm. These transitions were found to occur in compound **27** as it contains oxygen atoms with non-bonding electrons. Moreover, the conjugation of multiple double bonds linked to oxygen groups in compound **27** has resulted in red shift (increased in λ) of principal absorption peaks of coumarin (274 and 311 nm) to 309 and 347 nm. Apart from red shift, highly conjugated double bonds form the chromophore and gives pale yellow color to compound **27**.

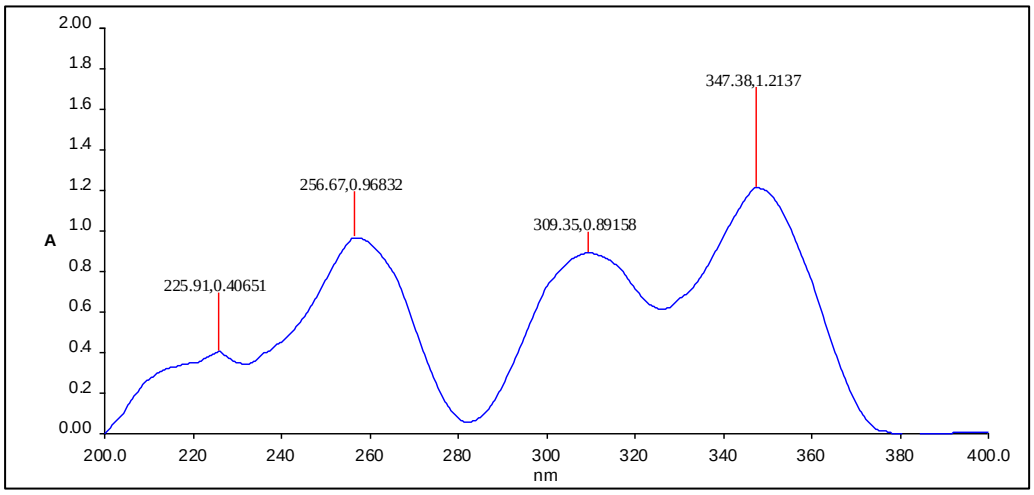



**Figure 4.34: UV-Vis spectrum of isodispar B [27]**

The molecular structure of compound **27** was characterized based on NMR data. In $^1$H-NMR spectrum (Figure 4.36), a sharp singlet signal at $\delta_H$ 14.14 was assigned to the chelated hydroxyl proton attached to carbon C-7. On the contrary, broad singlet signal at $\delta_H$ 10.25 was assigned to the free hydroxyl proton, 5-OH. Furthermore, the presence of monosubstituted benzene ring moiety in the structure gave an intense multiplet peaks at $\delta_H$ 7.3. Two relatively deshielded singlet signals observed at $\delta_H$ 6.21 and 5.92 were allocated

separately to methine protons H-6 and H-3. Those proton signals appeared in the upfield region below 4 ppm were attributed to protons which are linked to $sp^3$ hybridized carbons. Among these signals, the most shielded doublet signal at $\delta_H$ 1.02 (6H, d, $J$ = 6.7 Hz) was assigned to two groups of chemically equivalent methyl protons, H-4" and H-5".

In $^{13}$C-NMR spectrum (Figure 4.37), keto carbonyl carbon C-1" was presented as the most deshielded carbon signal at $\delta_C$ 209.8. A very intense peak at $\delta_C$ 21.1 was indicative of two magnetically equivalent carbon signals assigned to symmetrical carbons C-4" and C-5". Generally, the spectrum can be divided into three chemical shift regions, whereby signals in the range of $\delta_C$ 150 to 220 ppm were assigned to $sp^2$ hybridized carbons linked to oxygen groups. Whereas signals for those $sp^2$ hybridized carbons without linking to electronegative atoms were found to appear in a lower range of $\delta_C$ 90 to 150. Lastly, the presence of $sp^3$ hybridized carbons was observed as signals in the chemical shift region below $\delta_C$ 90 ppm.

The types of carbon were further investigated based on the HMQC analysis. According to HMQC spectrum (Figure 4.38), two symmetrical methyl carbons C-4" and C-5" were deduced as their carbon signal at $\delta_C$ 22.1 was correlated to proton signal H-4" and H-5" at $\delta_H$ 1.02, which was integrated for 6 protons. In addition, proton signal H-2" integrated for two protons at $\delta_H$ 3.14 showed correlation with carbon signal C-2" at $\delta_C$ 53.1, indicating that C-2" is a methylene carbon. Similarly, a total of 8 methine carbons was identified from

the direct coupling interactions between protons and carbons. There are 9 quaternary carbons signals which have no correlated peak in the HMQC spectrum. Meanwhile, long range heteronuclear coupling interactions can be established through HMBC analysis. For instance, in the HMBC spectrum (Figure 4.39), methine proton H-6 showed $^{2}J$ correlation with both carbons C-5 and C-7, in addition to $^{3}J$ correlation with quaternary carbons C-4a and C-8. The remaining correlations were summarized in Table 4.3.

From the IR analysis, functional groups of compound **27** can be determined. The IR spectrum (Figure 4.35) showed O-H stretch at 3286 $cm^{-1}$, C=O stretch at 1694 $cm^{-1}$, aromatic C=C stretch at 1598 $cm^{-1}$ and C-O stretch at 1269 $cm^{-1}$ corresponding to the presence of hydroxyl, carbonyl, aromatic C=C and carbinol functionalities in the structure. Moreover, C-H and C-C bending vibrations were observed below 1000 $cm^{-1}$. On the basis of the spectroscopic analyses, compound **27** was identified as isodispar B.

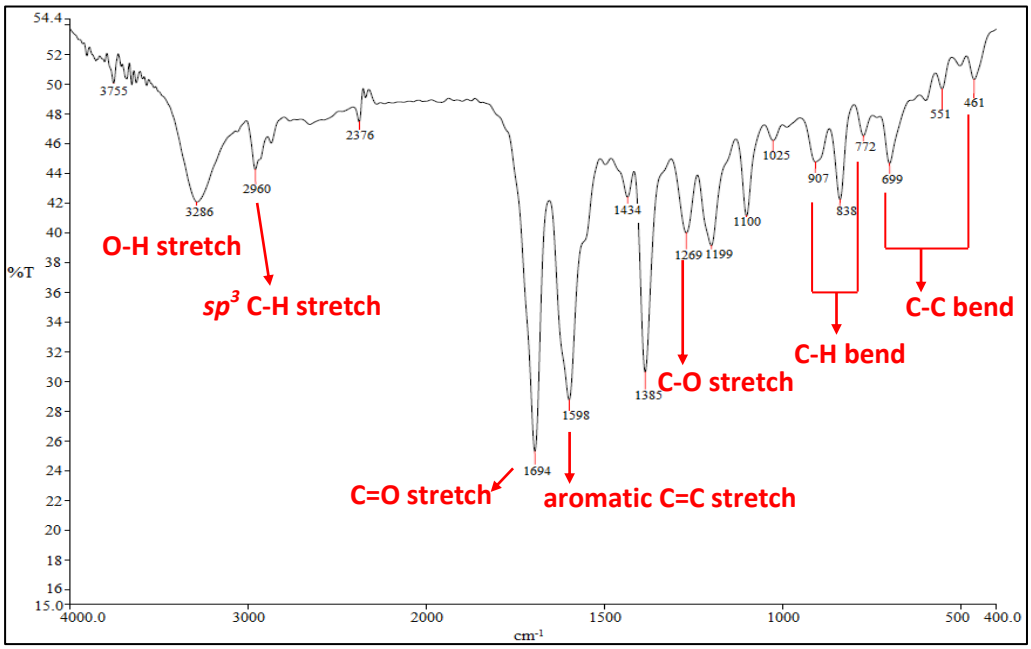



**Figure 4.35: IR spectrum of isodispar B [27]**

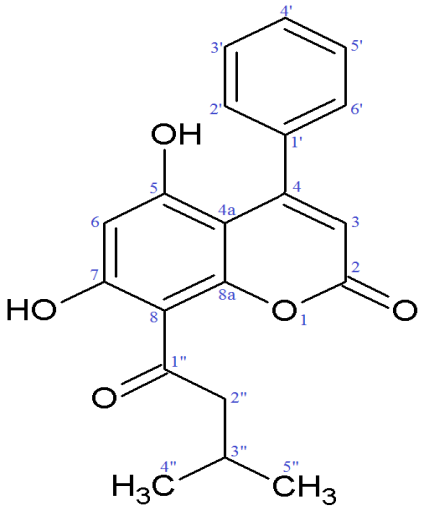
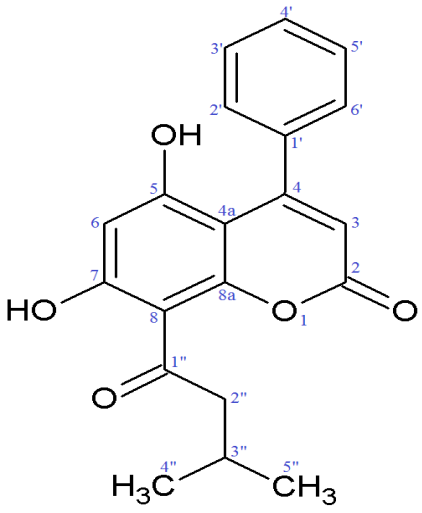



**Table 4.3: Summary of NMR data for isodispar B [27]**

| Position | $\delta_H$ (ppm), Integration and Multiplicity | $\delta_C$ (ppm) | HMBC |
|---|---|---|---|
| 1 | - | | - |
| 2 | - | 158.2 | - |
| 3 | 5.92 (1H, s) | 111.5 | C-1', 2, 4a |
| 4 | - | 156.3 | - |
| 4a | - | 101.7 | - |
| 5 | - | 161.8 | - |
| 6 | 6.21 (1H, s) | 99.5 | C-4a, 5, 7, 8 |
| 7 | - | 168.5 | - |
| 8 | - | 104.0 | - |
| 8a | - | 158.4 | - |
| 1' | - | 139.9 | - |
| 2' & 6' | 7.35 (2H, m) | 127.3 | C-1', 2', 3',4', 5', 6', 4 |
| 3' & 5' | 7.36 (2H, m) | 127.5 | |
| 4' | 7.38 (1H, m) | 128.0 | |
| 1" | - | 209.8 | - |
| 2" | 3.14 (2H, d, *J* = 6.7 Hz) | 53.1 | C-1", 3", 4", 5" |
| 3" | 2.29 (1H, m) | 25.4 | C-1", 2", 4", 5" |
| 4" | 1.02 (6H, d, *J* = 6.7 Hz) | 22.1 | C-2", 3" |
| 5" | | | |
| 5-OH | 10.25 (1H, brs) | | - |
| 7-OH | 14.14 (1H, s) | | C-6, 7, 8 |




**Isodispar B [27]**

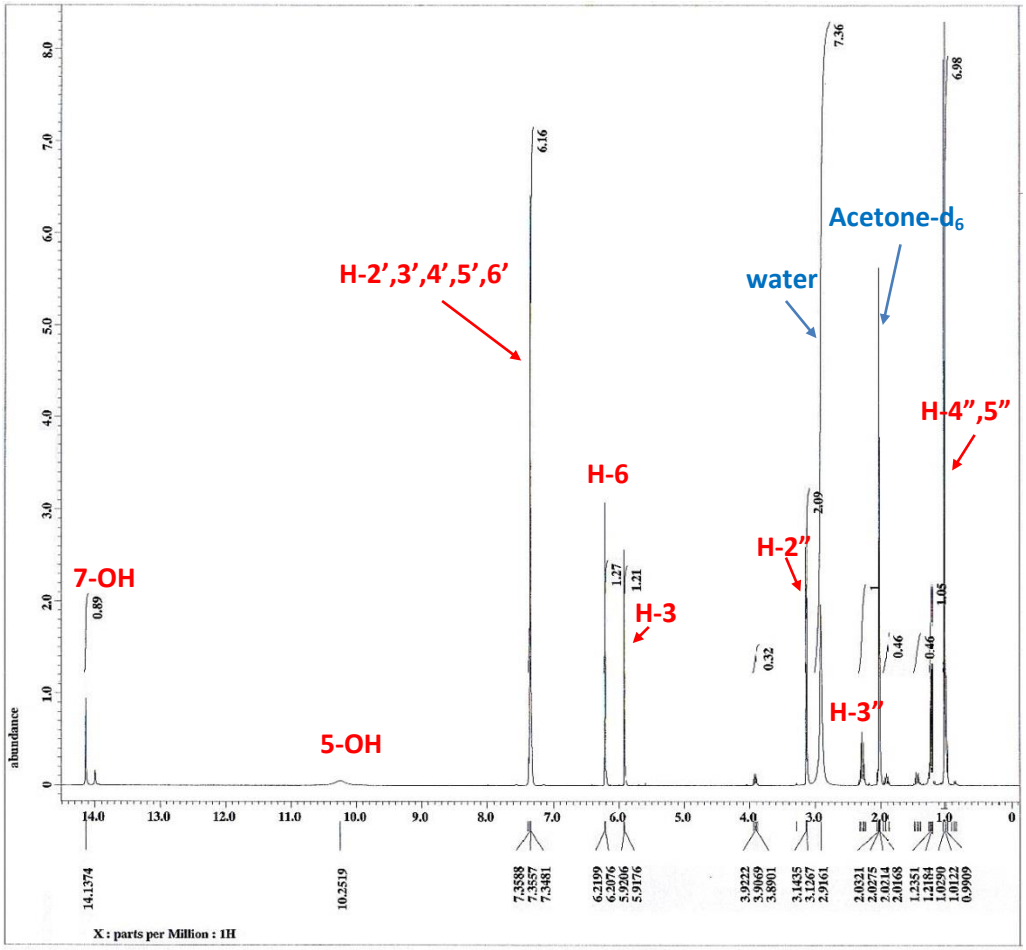
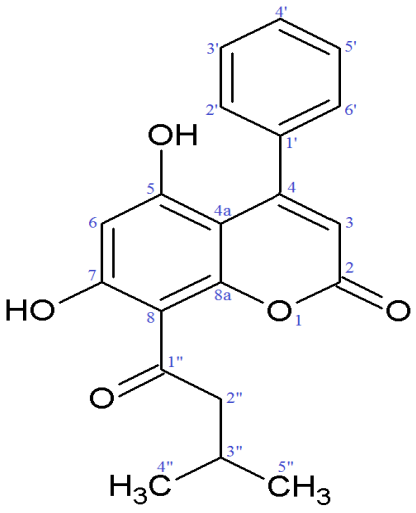



**Figure 4.36: $^{1}$H-NMR spectrum of isodispar B [27]**




**Isodispar B [27]**

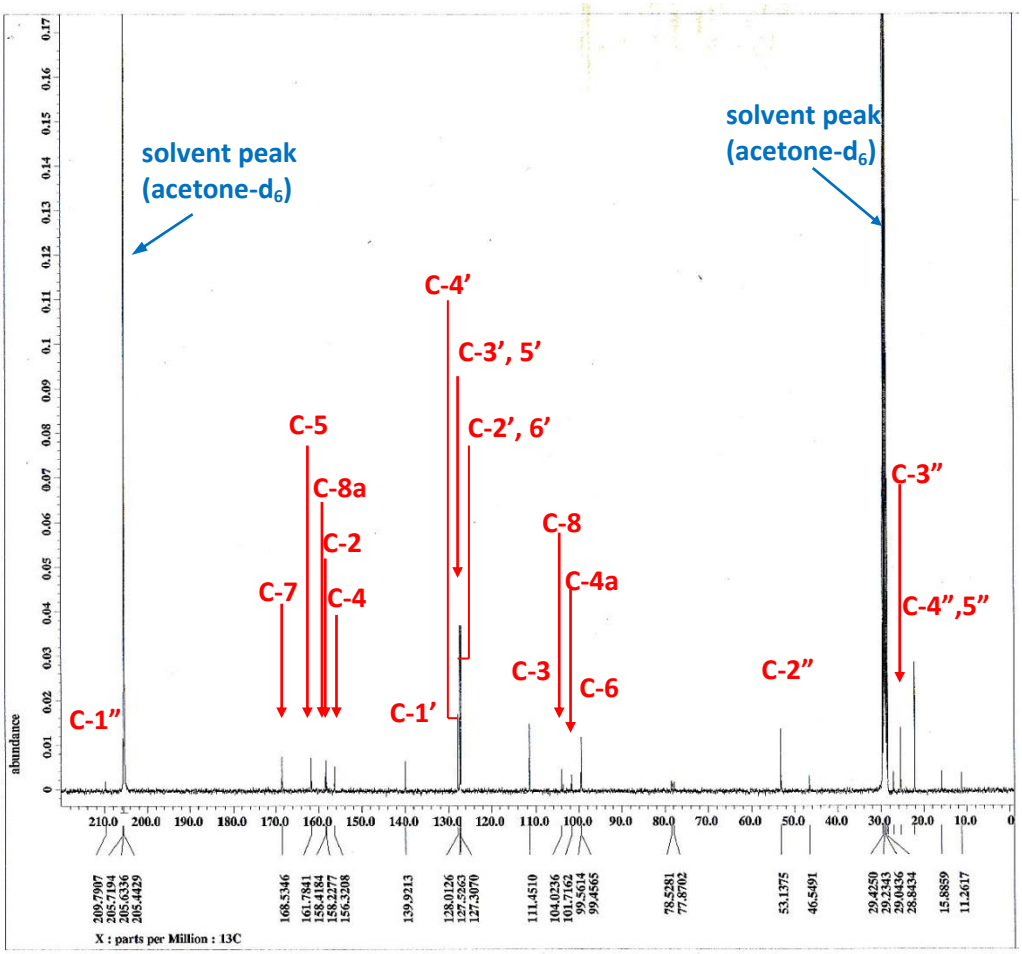
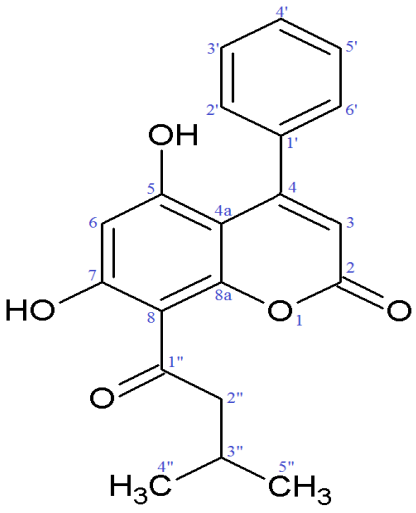



**Figure 4.37: $^{13}$C-NMR spectrum of isodispar B [27]**




**Isodispar B [27]**

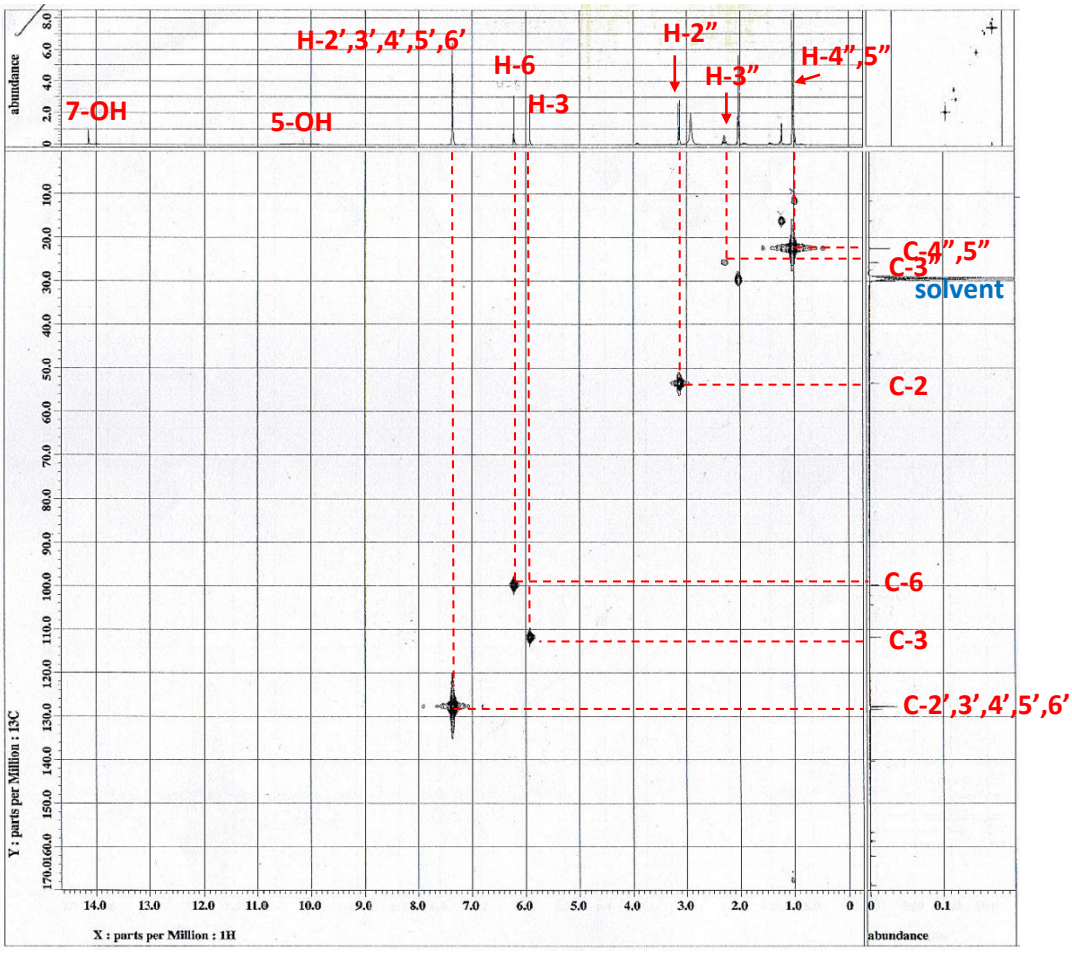
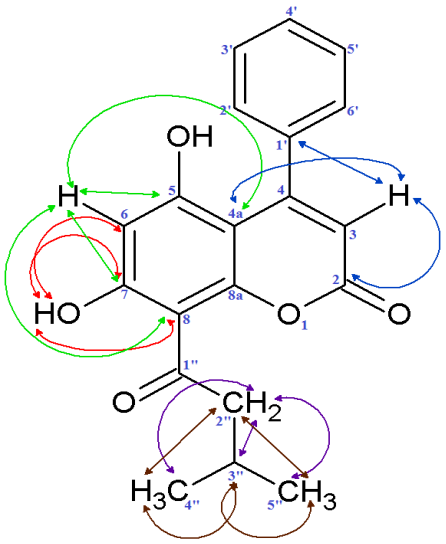



**Figure 4.38: HMQC spectrum of isodispar B [27]**




**Isodispar B [27]**

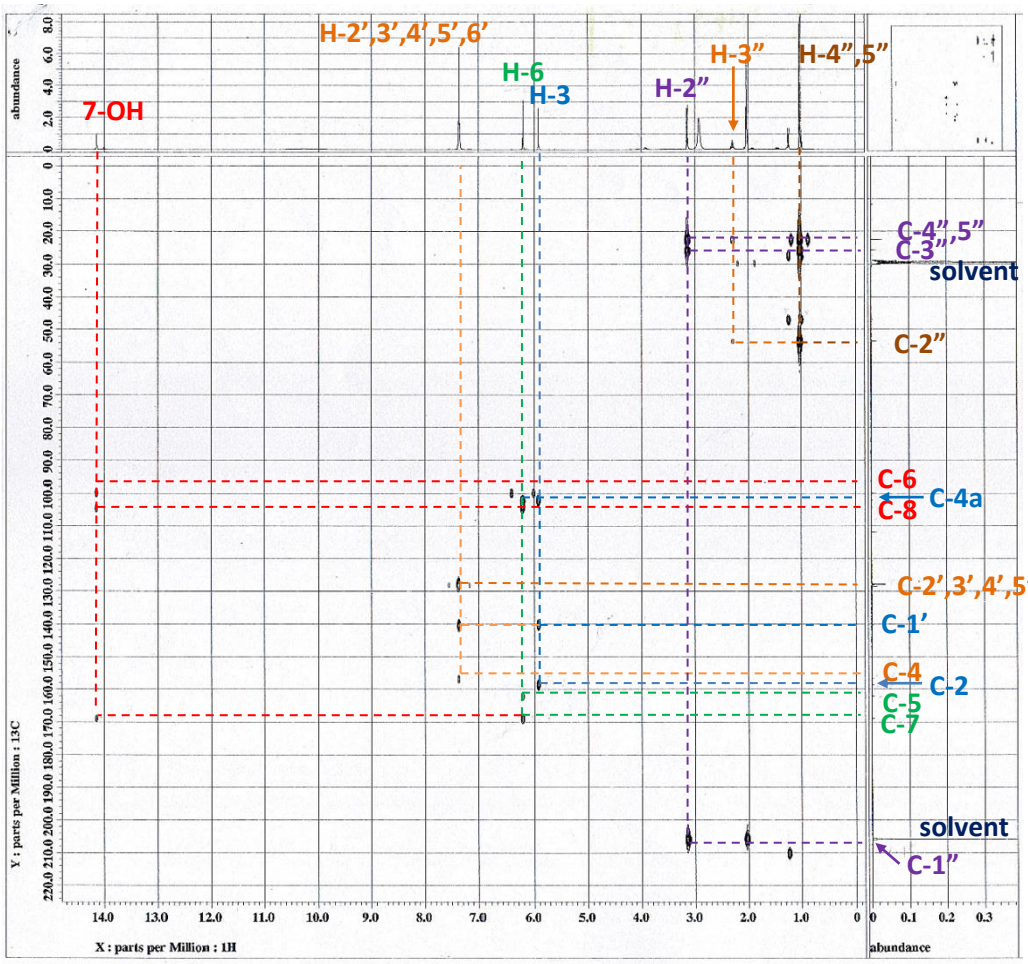



**Figure 4.39: HMBC spectrum of isodispar B [27]**

## 4.5 Chemical Identification and Structural Characterization of Friedelin [24]

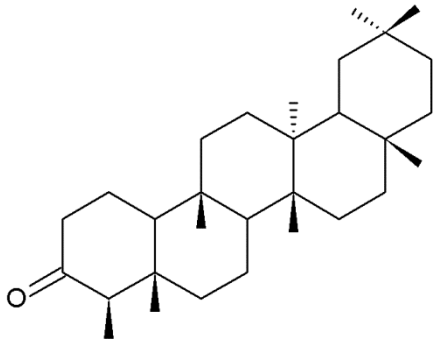


**Figure 4.40: The molecular structure of friedelin [24]**

About 98.2 mg of friedelin was obtained as white needle-like crystals with a melting point of 258-260 $^{o}$C (Lit. 259-260 $^{o}$C, Abbas, et al., 2006). The purity of compound **24** was examined by TLC analysis showing a single spot with retention factor, $R_f$ value of 0.75 eluted with a mobile phase of 90% hexane and 10% ethyl acetate. However, compound **24** do not absorb UV light but stained with iodine vapour to give a dark brown spot on TLC plate. Hence, the absence of conjugated system in the structure was deduced.

Elemental composition of compound **24** was analyzed by GC-MS resulting in a molecular ion peak $[M]^+$ at *m/z* 426 which was corresponding to molecular formula of $C_{20}H_{18}O_5$. Furthermore, compound **24** was ionized into protonated cations during LC-MS analysis. The molecular formula of $C_{20}H_{19}O_5$ was determined from the pseudo-molecular ion peak $[M+H]^+$ at *m/z* 427.3934 in the HRESIMS spectrum (Figure 4.42). The measured molecular weight of the

compound was reported to be 426.3862 g $mol^{-1}$ which showed 99.56% consistency with the calculated value, 426.3862 g $mol^{-1}$.

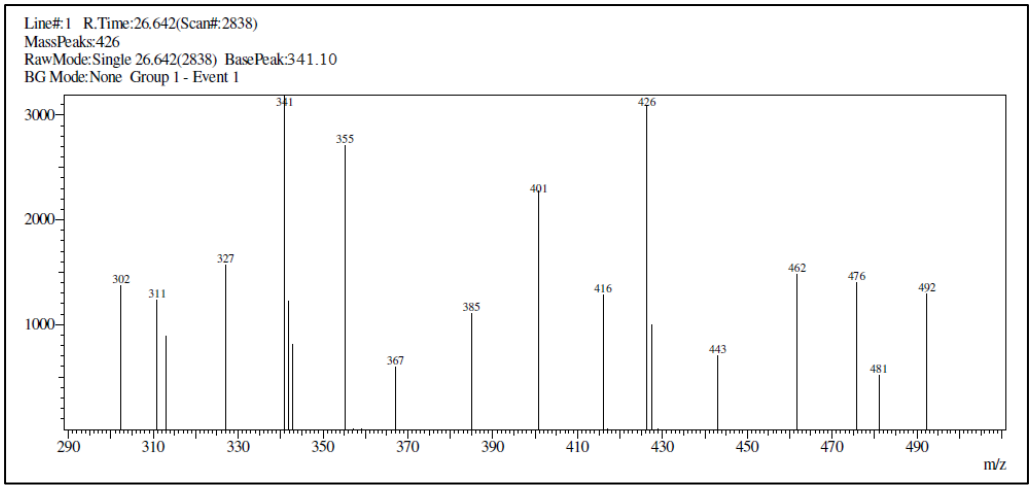
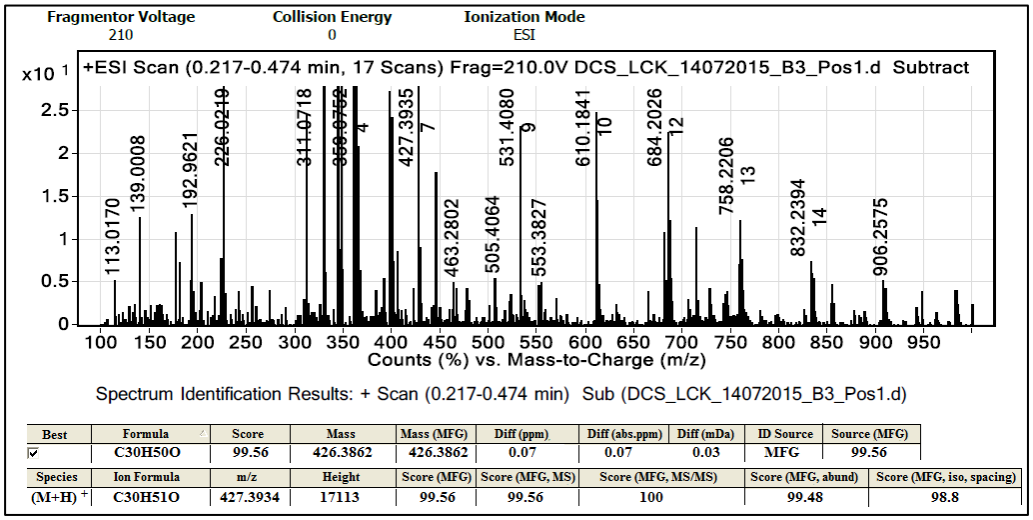



**Figure 4.41: GC-MS spectrum of friedelin [24]**




| Best | Formula | Score | Mass | Mass (MFG) | Diff (ppm) | Diff (abs.ppm) | Diff (mDa) | ID Source | Source (MFG) |
|---|---|---|---|---|---|---|---|---|---|
| ☑ | C30H50O | 99.56 | 426.3862 | 426.3862 | 0.07 | 0.07 | 0.03 | MFG | 99.56 |

| Species | Ion Formula | m/z | Height | Score (MFG) | Score (MFG, MS) | Score (MFG, MS/MS) | Score (MFG, abund) | Score (MFG, iso, spacing) |
|---|---|---|---|---|---|---|---|---|
| $(M+H)^+$ | C30H51O | 427.3934 | 17113 | 99.56 | 99.56 | 100 | 99.48 | 98.8 |

**Figure 4.42: HRESI spectrum of friedelin [24]**

Since all the protons in compound **24** were covalently bonded to $sp^3$ hybridized carbons, therefore all the proton signals were found to appear in the upfield region, below 2.5 ppm in the $^1$H-NMR spectrum (Figure 4.45). A total of seven intense singlet signals, each integrated for 3 protons were assigned to seven

groups of methyl protons. They were protons H-28 (1.16 ppm), H-27 (1.03 ppm), H-29 (0.99 ppm), H-30 (0.98 ppm), H-26 (0.93 ppm), H-25 (0.85 ppm) and H-24 (0.70 ppm). In addition, a doublet signal was assigned to methyl protons H-23 (0.86 ppm). Besides, two groups of non-equivalent methylene gave signals at $\delta_H$ 1.95 (H-$1_a$), 1.72 (H-$1_b$), 2.36 (H-$2_a$) and 2.29 (H-$2_b$). A multiplet signal of methine proton (H-4) was observed at 2.24 ppm. There was no vinylic proton signals observed, and the remaining 21 protons consisting of methylene and methine protons showed signals in the range of $\delta_H$ 1.20-1.70.

A total of 30 carbon signals were observed in $^{13}$C-NMR spectrum (Figure 4.46) as compound **24** composed of 30 carbons. The most deshielded signal at $\delta_C$ 213.0 was assigned to keto carbon, C-3. Furthermore, eight methyl carbon signals appeared in the upfield region at $\delta_C$ 6.9, 14.7, 18.0, 20.3, 18.8, 32.2, 35.1 and 31.9 were attributed to carbons C-23, C-24, C25, C-26, C-27, C-28, C-29 and C-30, respectively. The remaining assignment of carbon signals was done by comparing with the literature values reported by Abbas, et al., in year 2007.

FTIR analysis further revealed stretching vibrations such as $sp^3$ C-H stretch at 2927 $cm^{-1}$ and C=O stretch at 1713 $cm^{-1}$. On the other hand, compound **24** gave an absorption peak at 218 nm in the UV-Vis spectrum (Figure 4.44), which is resulted from n $\rightarrow$ $\sigma^*$ transition. Based on the spectral data and comparison with the literature values, it was confirmed that compound **24** is friedelin, a common triterpenoid.

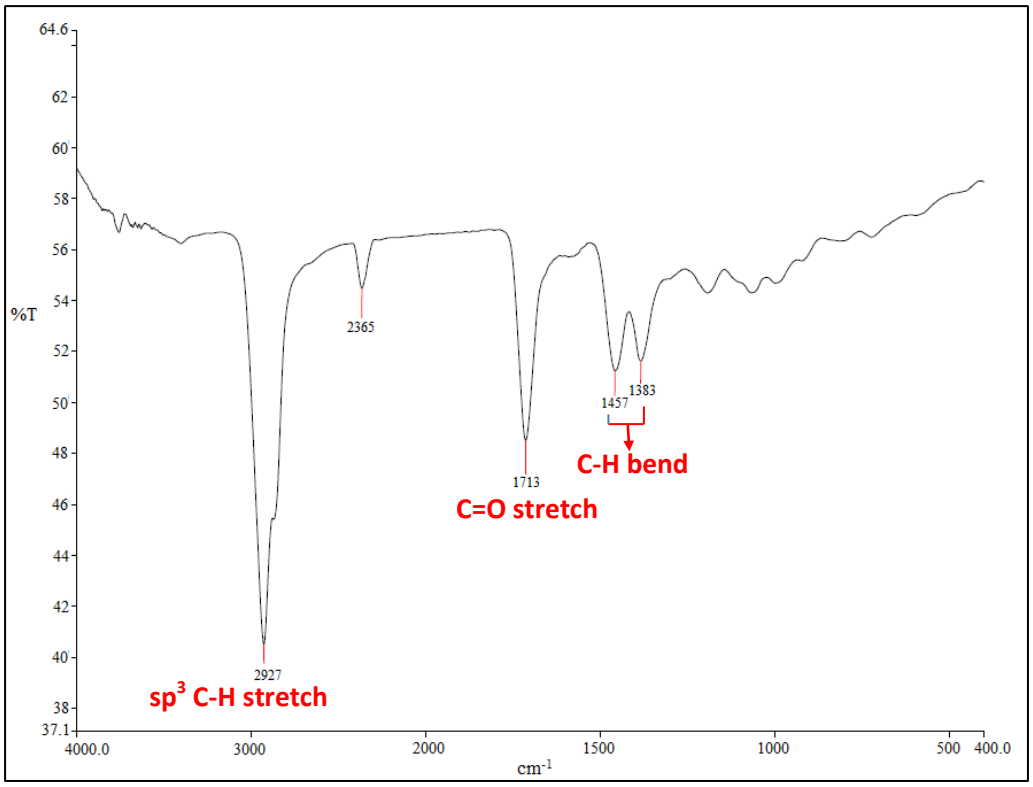



**Figure 4.43: IR spectrum of friedelin [24]**

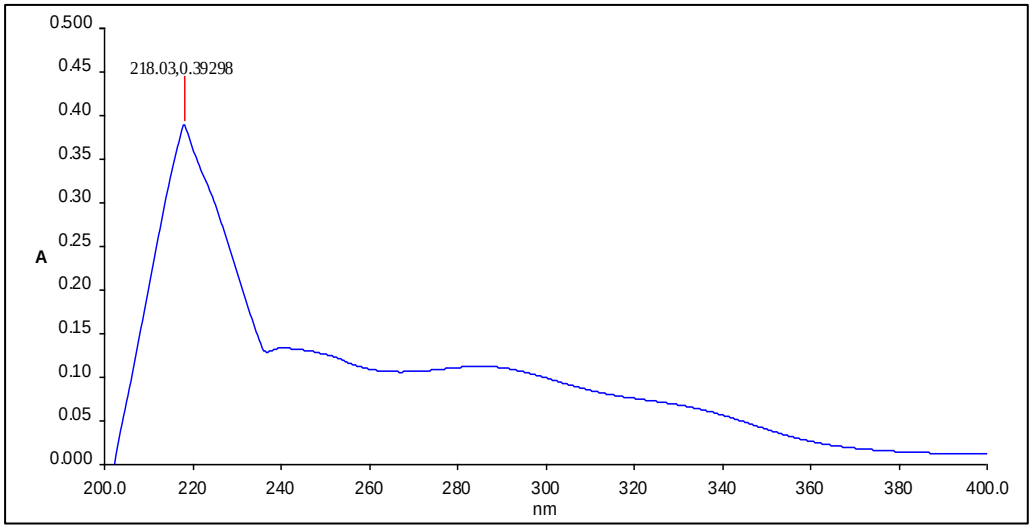
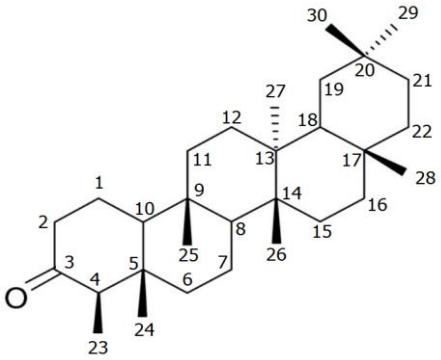



**Figure 4.44: UV-Vis spectrum of friedelin [24]**

**Table 4.4: Summary of NMR data for friedelin in comparison with literature values.**

| Position | $\delta_H$ (ppm) | *$\delta_H$ (ppm) | $\delta_C$ (ppm) | *$\delta_C$ (ppm) |
|---|---|---|---|---|
| 1 | 1.95 ($1H_a$, m)<br>1.72 ($1H_b$, m) | 1.97 (m)<br>1.71 (m) | 22.4 | 22.3 |
| 2 | 2.36 ($1H_a$, dd, *J* = 13.4 Hz, 3 Hz,)<br>2.29 ($1H_b$, m) | 2.41 (dd, *J*= 13 Hz, 3.5 Hz)<br>2.31 (m) | 41.6 | 41.5 |
| 3 | - | - | 213.4 | 213.2 |
| 4 | 2.24 (1H, m) | 2.28 (m) | 58.3 | 58.2 |
| 5 | - | - | 42.2 | 42.2 |
| 6 | - | 1.78 (m)<br>1.31 (m) | 41.3 | 41.3 |
| 7 | - | 1.51 (m)<br>1.41 (m) | 18.3 | 18.2 |
| 8 | - | 1.41 (m) | 53.2 | 53.1 |
| 9 | - | - | 37.5 | 37.4 |
| 10 | - | 1.55 (m) | 59.5 | 59.5 |
| 11 | - | 1.40 (m) | 35.7 | 35.6 |
| 12 | - | 1.30 (m)<br>1.28 (m) | 30.6 | 30.5 |
| 13 | - | - | 39.8 | 39.7 |
| 14 | - | - | 38.4 | 38.3 |
| 15 | - | 1.50 (m)<br>1.30 (m) | 32.8 | 32.8 |
| 16 | - | 1.40-1.60 (m) | 36.1 | 36.0 |
| 17 | - | - | 30.1 | 30.0 |
| 18 | - | 1.60 (m)<br>1.40 (m) | 42.8 | 42.8 |
| 19 | - | 1.60 (m) | 35.4 | 35.3 |
| 20 | - | - | 28.3 | 28.2 |
| 21 | - | 1.51 (m)<br>1.31 (m) | 32.5 | 32.5 |
| 22 | - | 1.51 (m)<br>0.97 (m) | 39.3 | 39.3 |
| 23 | 0.86 (3H, d, *J* = 6.7 Hz) | 0.92 (d, *J*= 7.0 Hz) | 6.9 | 6.8 |
| 24 | 0.70 (3H, s) | 0.75 (s) | 14.7 | 14.7 |
| 25 | 0.85 (3H, s) | 0.90 (s) | 18.0 | 18.0 |
| 26 | 0.93 (3H, s) | 1.03 (s) | 20.3 | 20.3 |
| 27 | 1.03 (3H, s) | 1.07 (s) | 18.8 | 18.7 |
| 28 | 1.16 (3H, s) | 1.20 (s) | 32.2 | 32.1 |
| 29 | 0.99 (3H, s) | 1.02 (s) | 35.1 | 35.0 |
| 30 | 0.98 (3H, s) | 0.98 (s) | 31.9 | 31.8 |

*Literature source: Abbas, et al., 2007.




**Friedelin [24]**

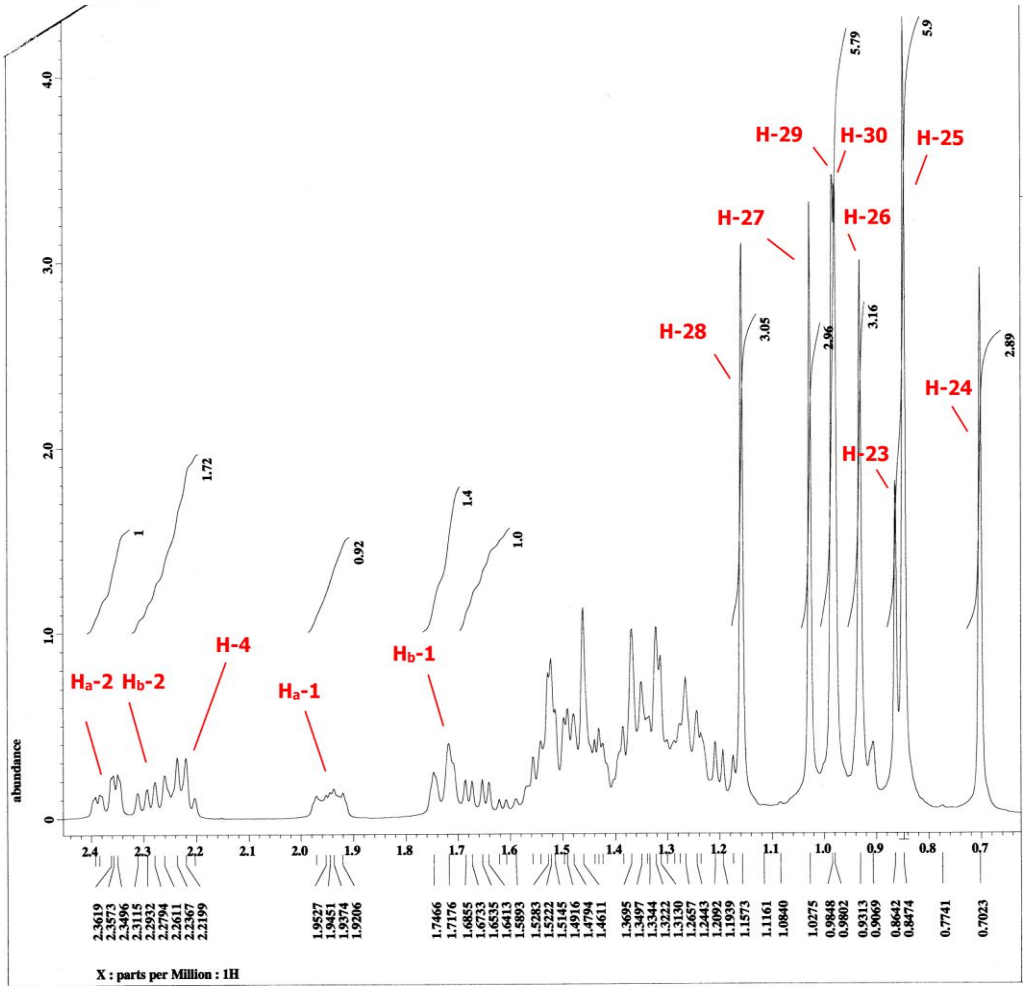
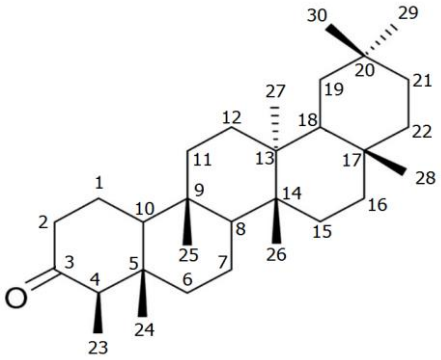



**Figure 4.45: $^{1}$H-NMR spectrum of friedelin [24]**




**Friedelin [24]**

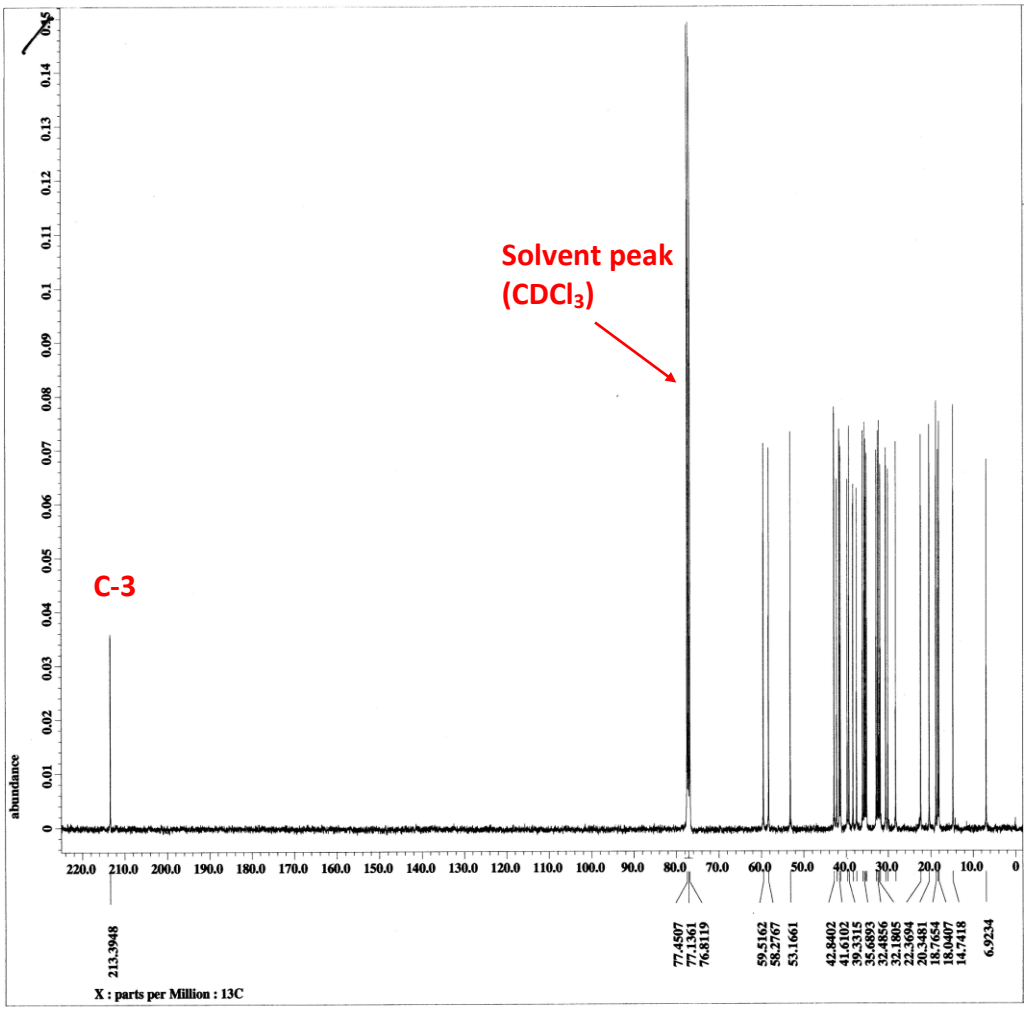
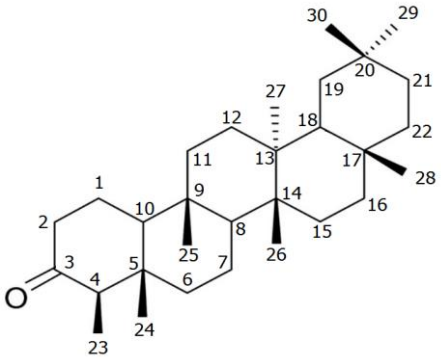



**Figure 4.46: $^{13}$C-NMR spectrum of friedelin [24]**




**Friedelin [24]**

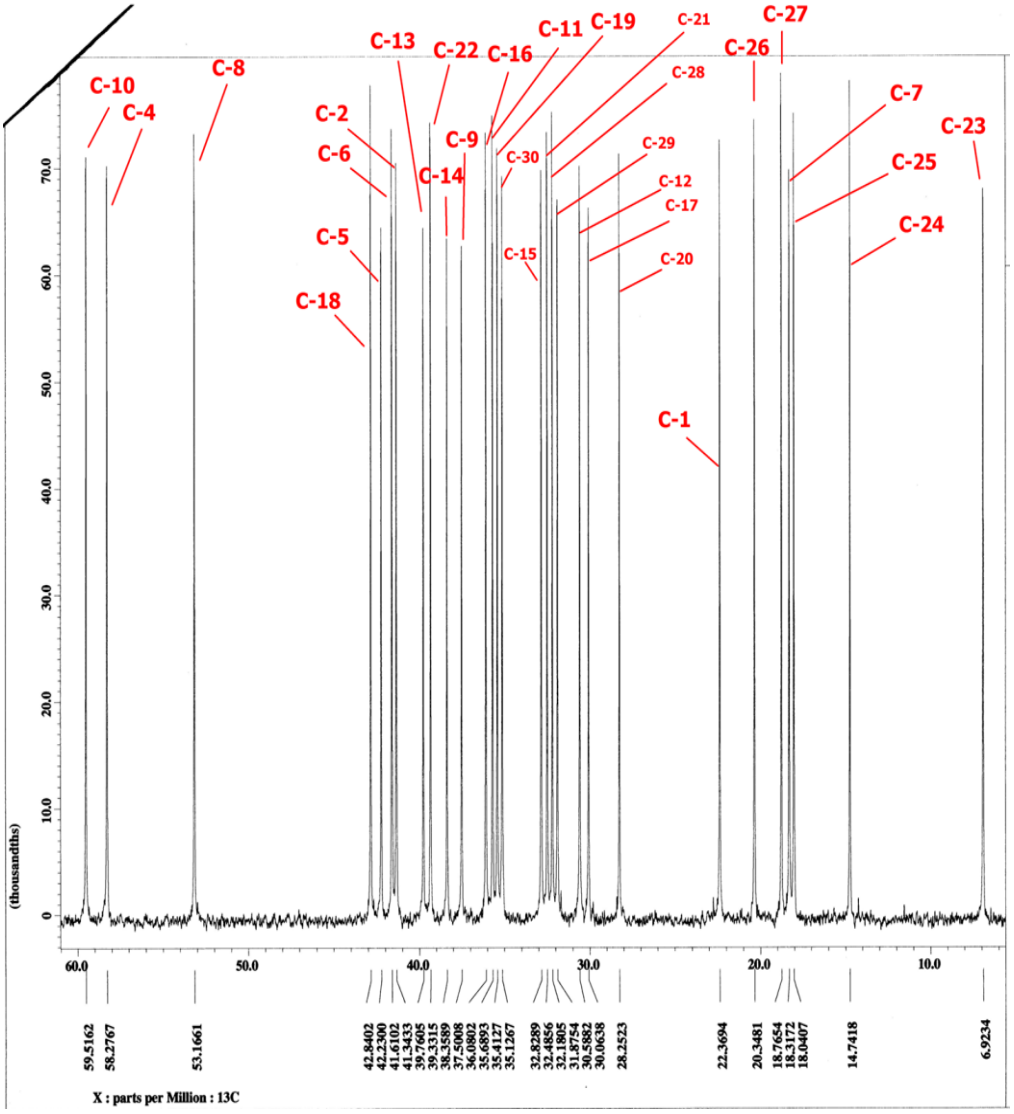



**Figure 4.47: Expanded $^{13}$C-NMR spectrum of friedelin [24]**

## 4.6 Antioxidant Test

Reactive oxygen species (ROS) such as peroxides, superoxide and hydroxyl radical are formed in living cell. They are important in cell signaling and homeostasis. However, oxidative stress can occurs if the level of ROS is too high. This may results in cell damage and hence increases the risk of cancers and aging. Generally, polyhydroxy (phenolic) compounds are known to act as antioxidant in biological system. Antioxidant functions to react with ROS to form a neutral species or a resonance-stabilized species by transferring an electron or hydrogen atom (Lin, et al., 2011). Resonance-stabilized species is also a free radical but less reactive than ROS as the lone electron can be delocalized into the aromatic ring.

In this study, the radical scavenging activity of crude extracts and isolated compounds were evaluated by means of 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, a relatively simple cell-free method to perform. DPPH is a free radial with violet colour that changes to yellow when it is scavenged. Besides, it also acts as an indicator to allow clear observation. $IC_{50}$ is defined as the concentration of sample that required to inhibit 50% of DPPH radical activity. When DPPH reacts with antioxidant, it is reduced, resulting in a decrease in absorbance at 520 nm. Therefore, the lower $IC_{50}$ value obtained, the stronger the antioxidant activity. $IC_{50}$ values were determined from the graph of percent inhibition rate against concentration of sample as shown in Figures 4.50 and 4.51.

**Table 4.5: Antioxidant results of test samples in DPPH assay**

| Test sample | $IC_{50}$ (μg/mL) |
|---|---|
| **Positive controls:** | |
| Ascorbic acid | 4.0 |
| Kaempferol | 12.5 |
| **Crude extracts:** | |
| Dichloromethane | 80.0 |
| Ethyl acetate | 19.0 |
| Methanol | 9.0 |
| **Isolated compounds:** | |
| 5,7-dihydroxy-6-(2-methylbutyryl)-4-phenylcoumarin **[25]** | 22.5 |
| 5,7-dihydroxy-6-(3-methylbutyryl)-4-phenylcoumarin **[26]** | 82.5 |
| Isodispar B **[27]** | > 240.0 |
| Friedelin **[24]** | > 240.0 |

As shown in Table 4.5, dichloromethane, ethyl acetate, and methanol crude extracts of *Calophyllum sclerophyllum* gave strong to weak antioxidant activities in DPPH radical scavenging test, with their $IC_{50}$ values of 80.0, 19.0, and 9.0 μg/mL, respectively. In comparison with positive controls ascorbic acid (4.0 μg/mL) and kaempferol (12.5 μg/mL), methanol crude extract presented a comparable antioxidant potential. Whereas ethyl acetate crude extract showed a relatively weaker antioxidant activity than the positive controls. Among all the crude extracts, dichloromethane extract was found to be weakest in scavenging the DPPH radicals. This is because dichloromethane extract contained mostly low polarity compounds such as terpenoids and hydrocarbons which may not be antioxidants.

Apart from that, isolated compound **25** and **26** showed positive results in the DPPH assay as they are phenolic compounds which contained hydroxyl groups and conjugated double bonds in their structures. The mechanism involved

donation of proton from hydroxyl group at carbon 7 (7-OH) to DPPH free radical and thereby forming a neutral DPPH species. Chelated hydroxyl group (5-OH) is unable to act as proton donor as it forms intramolecular hydrogen bond with the carbonyl group. Theoretically, both compounds should give almost similar $IC_{50}$ values as they comprised of the same coumarin nucleus with 5,7-dihydroxyl and 4-phenyl substituent groups. However, compound **25** (22.5 µg/mL) exhibited a much stronger antioxidant properties than compound **26** (82.5 µg/mL). This is probably due to the role played by the acidic α-hydrogens located next to the keto group in the radical scavenging mechanism. After α-hydrogen is being donated, compound **25** tends to form a resonance-stabilized tertiary radical which is more stable than secondary radical formed by compound **26**, and hence a stronger antioxidant than compound **26**.

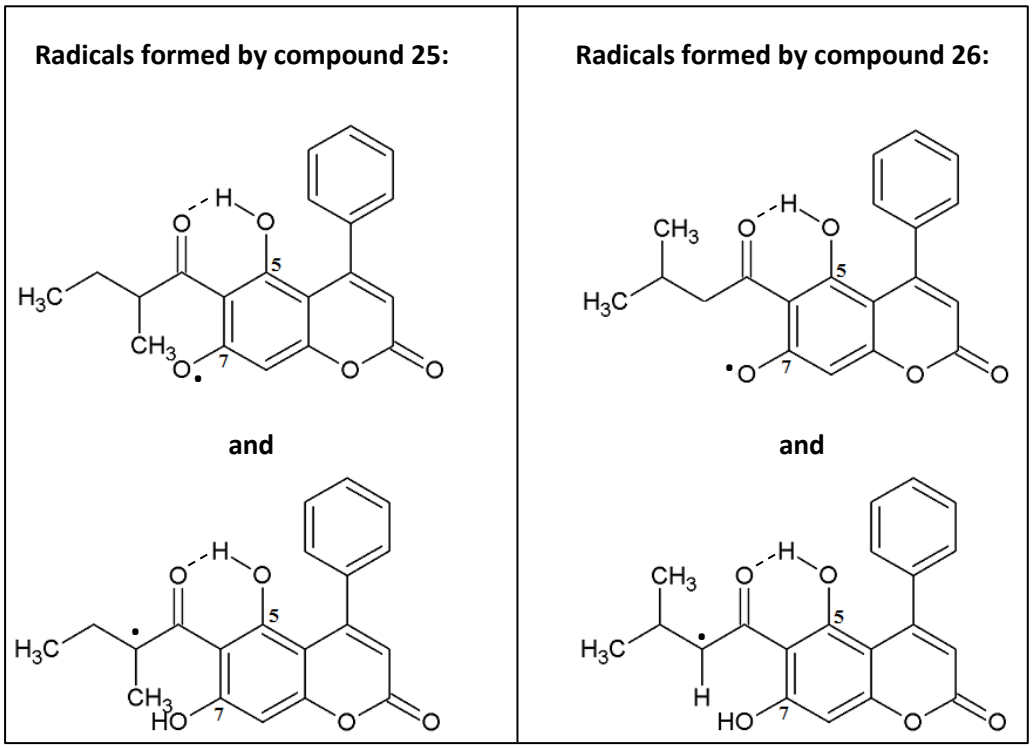



**Figure 4.48: Resonance-stabilized free radicals formed by different coumarin compounds**

On the other hand, isodispar B and friedelin were showing negative results in DPPH assay, both with $IC_{50}$ values more than 240 μg/mL. This is because lack of phenolic nature and conjugated system in the structure of friedelin. Meanwhile for isodispar B, both chelated hydroxyl group (7-OH) and α-hydrogens are unavailable as proton donors due to formation of hydrogen bonds.

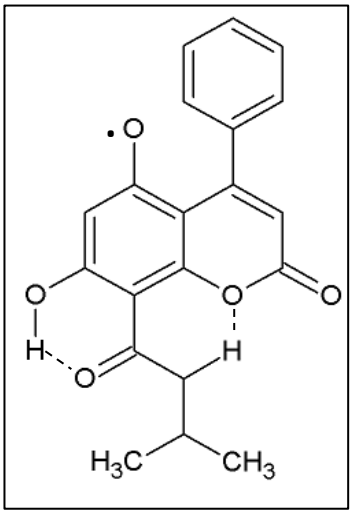



**Figure 4.49: Resonance-stabilized radical formed by isodispar B**

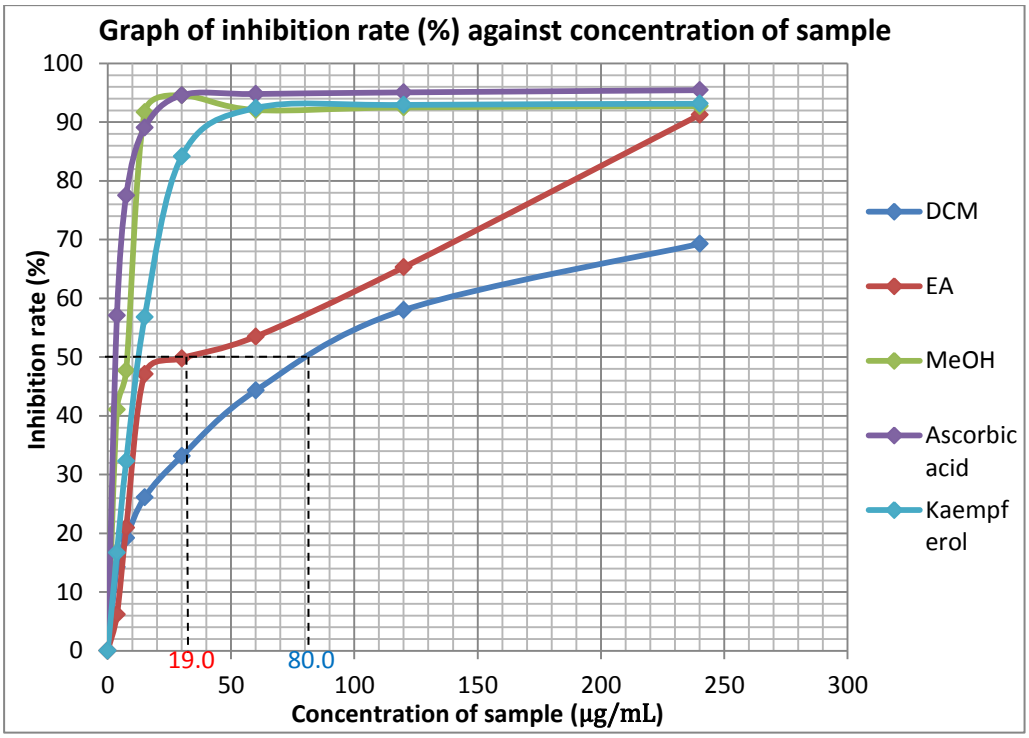



**Figure 4.50: Graph of inhibition rate (%) against concentration of crude extracts**

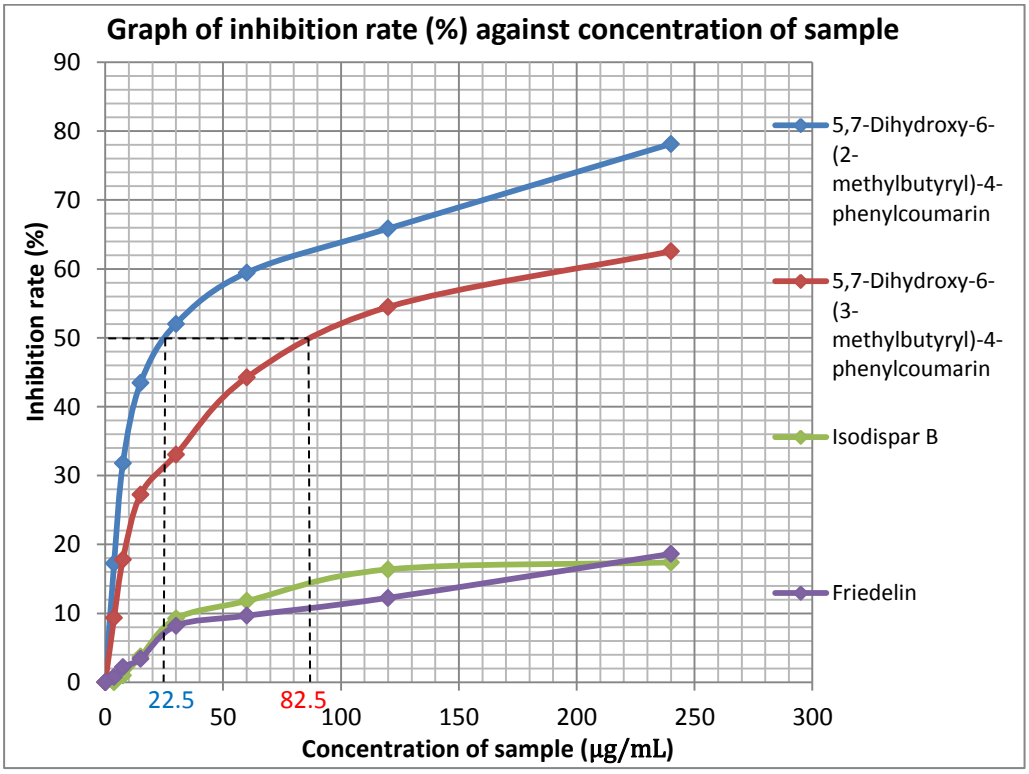



**Figure 4.51: Graph of inhibition rate (%) against concentration of isolated sample**

# CHAPTER 5

# CONCLUSION

## 5.1 Conclusion

In this study, a total of four chemical compounds have been isolated from the ethyl acetate crude extract of *Calophyllum sclerophyllum*. Their chemical structures have been elucidated via various modern spectroscopic techniques including 1D- and 2D-NMR, UV-Vis, IR and mass spectrometry. Compounds isolated were identified as friedelin **[24]**, 5,7-dihydroxy-6-(2-methybutyryl)-4-phenylcoumarin **[25]**, 5,7-dihydroxy-6-(3-methybutyryl)-4-phenylcoumarin **[26]** and isodispar B **[27]**.

All the crude extracts and isolates were investigated for their antioxidant potential via DPPH assay. Interestingly, among three coumarin compounds, compound **25** exhibited the highest antioxidant activity with $IC_{50}$ value of 22.5 μg/mL, while the compound **26** and **27** showed weak or inactive in antioxidant test. In addition, friedelin **[24]** also gave negative result in DPPH assay. At the meantime, crude extracts showed strong to weak antioxidant activities in comparison with the standards, ascorbic acid and kaempferol.

## 5.2 Future Perspectives

In this project, three coumarins and one triterpenoid had been successfully isolated. For future studies, it is suggested that these isolated compounds can be subjected for further investigation on other biological activities such as anti-fungi, antimicrobial and anti-cancer. Secondly, more advance chromatographic technique such as high performance liquid chromatography (HPLC) is recommended to be applied in order to improve the separation efficiency. Due to rapid separation and high resolution, preparative HPLC is usually an excellent technique used to isolate compounds with close similarity in structures. In addition, centrifugal chromatography and preparative TLC chromatographies are also suggested to be used in separation of compounds that exist in trace quantity.

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