INVESTIGATION OF POTENTIAL ANTIOXIDANTS

FROM THE ENDEMIC PLANT OF SARAWAK,

CALOPHYLLUM CASTANEUM

By

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ABSTRACT

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Lai Jing Yi

In this study, the stem bark of *Calophyllum castaneum* was subjected to sequential solvent extraction by using dichloromethane, ethyl acetate and methanol solvents. The crude extracts were subsequently subjected to column chromatography to give pure compounds.

A total amount of three compounds were isolated from the stem bark of *Calophyllum castaneum*, which are isoblancoic acid [66], blancoic acid [67] and β-sitosterol [68]. The pure compounds obtained were physically characterized for their physical appearance, melting point and relative polarity while their structures were elucidated by using spectroscopic methods, such as ^{1}H NMR, ^{13}C NMR, UV, IR and MS. The structural assignments were further confirmed by 2D-NMR analyses including ^{1}H-^{1}H COSY, DEPT, HMQC and HMBC.

All the crude extracts of *Calophyllum castaneum* and isolated compounds were subjected to DPPH radical scavenging assay. In this assay, methanol and ethyl acetate extracts of *Calophyllum castaneum* were found to give positive results. The methanol crude extract showed a comparable antioxidant activity

with the positive control used, kaempferol and ascorbic acid, with an IC_{50} value of 12 μg/mL, whereas ethyl acetate crude extract showed a much weaker antioxidant potential with an IC_{50} value of 84 μg/mL. Conversely, dichloromethane crude extracts and all the isolated compounds gave insignificant antioxidant activity in the DPPH assay.

ABSTRAK

Dalam kajian ini, kulit kayu batang *Calophyllum castaneum* telah diekstrak berturutan dengan menggunakan diklorometana, etilasetat dan metanol. Kemudian, ekstrak-ekstrak mentah telah disubjekkan kepada lajur kromatografi untuk menghasilkan sebatian-sebatian tulen.

Sejumlah tiga sebatian telah didapati daripada kulit pokok *Calophyllum castaneum*, iaitu asid isoblancoic **[66]**, asid isoblancoic **[67]** dan β-sitosterol **[68]**. Ciri-ciri sebatian-sebatian tulen telah ditentukan berdasarkan rupa fizikal, takat lebur dan kekutuban relatif manakala struktur mereka telah diperolehi melalui kaedah spektroskopi seperti ^{1}H NMR, ^{13}C NMR , UV, IR dan MS. Bentuk-bentuk struktur telah dipastikan dengan lebih lanjut melalui ^{1}H-^{1}H COSY, DEPT, HMQC dan HMBC.

Ekstrak-ekstrak mentah dan sebatian-sebatian tulen daripada *Calophyllum castaneum* telah dijalankan kajian DPPH. Dalam kajian ini, ekstrak-ekstrak metanol dan etilasetat daripada *Calophyllum castaneum* telah menunjukkan keputusan yang positif. Ekstrak mentah metanol telah menunjukkan aktiviti antioksidan yang lebih kurang sama berbanding dengan sebatian-sebatian kawalan positif, kaempferol dan asid askorbik dengan nilai IC_{50} iaitu 12 µg/mL, manakala ekstrak mentah eilasetat menunjukkan potensi antioksidan yang lemah dengan nilai IC_{50} iaitu 84 µg/mL. Sebaliknya, ekstrak mentah diklorometana dan semua sebatian-sebatian tidak menunjukkan aktiviti antioksidan yang nyata dalam kajian DPPH.

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Last but not least, I most deeply appreciate my family members and friends for their unlimited patience, encouragement, and mental support for my successful academic.

DECLARATION

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

_____

(LAI JING YI)

APPROVAL SHEET

This project report entitled **"INVESTIGATION OF POTENTIAL ANTIOXIDANTS FROM THE ENDEMIC PLANT OF SARAWAK, *CALOPHYLLUM CASTANEUM*"** was prepared by LAI JING YI and submitted in partial fulfillment of the requirements for the degree of Bachelor of Science (Hons.) in Chemistry at Universiti Tunku Abdul Rahman.

Approved by:

_____

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FACULTY OF SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

Date:_____

PERMISSION SHEET

It is hereby certified that **LAI JING YI** (ID No: **10ADB02881**) has completed this final year project entitled **"INVESTIGATION OF POTENTIAL ANTIOXIDANTS FROM THE ENDEMIC PLANT OF SARAWAK,** *CALOPHYLLUM CASTANEUM***"** 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 Instituional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

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(LAI JING YI)

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LIST OF ABBREVIATIONS

$[\alpha]$	Specific Rotation
α	Observed Optical Rotation
^{13}C	Carbon-13
1D-NMR	One Dimension Nuclear Magnetic Resonance
^{1}H	Proton
2D-NMR	Two Dimension Nuclear Magnetic Resonance
A_1	Absorbance of the test compound
A_o	Absorbance of the negative control (blank)
c	Concentration of sample in g/mL
C=C	Carbon=Carbon
C=O	Carbon=Oxygen
$CDCl_3$	Deuterated chloroform
C-H	Carbon-Hydrogen
cm	Centimetre
C-O	Carbon-Oxygen
COSY	Correlation Spectroscopy
d	Doublet
DCM	Dichloromethane
dd	Doublet of doublets
DEPT	Distortionless Enhancement by Polarisation Transfer
DPPH	1,1-diphenyl-2-picryhydrazyl
EtOAc	Ethyl acetate

FeCl$_3$	Ferric chloride
FTIR	Fourier-Transform Infrared Spectroscopy
g	Gram
GFC	Gel Filtration Chromatography
GPC	Gel Permeation Chromatography
HIV-1	Human Immunodeficiency Virus
HMBC	Heteronuclear Multiple Bond Coherence
HMQC	Heteronuclear Multiple Quantum Coherence
HPLC	High Performance Liquid Chromatography
HRESIMS	High Resolution Electrospray Ionisation Mass Spectroscopy
Hz	Hertz
IC$_{50}$	Half Maximal Inhibitory Concentration
IR	Infrared
J	Coupling constant in Hertz
KBr	Potassium Bromide
kg	Kilogram
l	Optical Path Length (1.0 dm)
LC-MS	Liquid Chromatography- Mass Spectroscopy
m	Multiplet
Me$_2$CO	Acetone
MeOH	Methanol
MHz	Megahertz
mL	Millilitre
mm	Millimetre

mol	Mole
MS	Mass Spectrometry
nm	Nanometre
NMR	Nuclear Magnetic Resonance
^{o}C	Degree in Celsius
O-H	Hydroxyl
ppm	Parts per million
R_f	Retention factor
s	Singlet
t	Triplet
T	Temperature
tdd	Triplet of doublet of doublets
TLC	Thin Layer Chromatography
TMS	Trimethylsilane
UV	Ultraviolet
UV-Vis	Ultraviolet-Visible
β	Beta
δ	Chemical shift
δ_C	Chemical shift of carbon
δ_H	Chemical shift of proton
λ	Wavelength
λ_{max}	Maximum wavelength
mg	Microgram
ρ	Density

CHAPTER 1

INTRODUCTION

1.1   General Introduction

Natural products, also known as secondary metabolites, are produced by living organisms such as flowers, plants, vegetables, insects and so on. Cragg and Newman (2005) stated that the oldest records of natural products were represented from Mesopotamia (2600 B.C.) which showed approximately 1000 plants and plant-derived substances. Before 19th century, human had discovered some uses and benefits of certain natural products in the form of traditional medicines, remedies and oils, but their active compounds were remained unknown. The knowledge in application of natural products is mainly based on human's trial and error in searching for available living organisms such as plants for healing and other purposes.

Throughout history, scientists had put a lot of efforts to discover new active ingredients by studying the chemistry of natural products. During the 19th century, chemistry has been used for the study and use of natural products, which was called phytochemistry (Ji, Li and Zhang, 2009). Phytochemistry involves study of plants and their secondary metabolites. Secondary metabolites are not essential in organisms' development and growth. The new evolution of analytical and structural chemistry has afforded better chromatographic and spectroscopic instruments to isolate unique compounds

from plants and to determine for their chemical structures (Ji, et al., 2009). Ji, et al. (2009) stated that the first pure natural compound, morphine was isolated from opium by Friedrich Wilhelm Sertürner, a German pharmacist in 1805. It was first to be commercialised as pain killer by Merck in 1826.

Originally, bioactive compounds isolated from flowers, plants and insects can affect their colour and odour for several functions (Dixons, 2005). For example, plant hormones as natural products to help in regulating role, and provide protection against pests. Other than that, some natural products in insects function as sex attractants (chemical messengers).

Humans grab an evolutionary benefit from natural products in the fields of industry, agriculture and medicine. Within industrial field, humans utilize natural products as ingredients of cosmetics, fragrances, flavouring agents and other applications. Besides that, scientists have discovered genetically modified plants which enhance the effects of introduced secondary metabolites in a true dietary context (Dixon, 2005). Newman (2008) predicted that around 60% of available drugs are coming from natural products in either direct or indirect way. For example, scientists synthesise anti-inflammatory agent, aspirin from the natural product, salicin. Meanwhile, quinine isolated from the bark of *Cinchona succiruba* Pav. Ex Klotsch is used as anti-malarial drug (Dias, Urban and Roessner, 2012).

Methods of natural products chemistry are divided into four steps, which are extraction, purification, isolation and elucidation. Nowadays, new and modern

chromatographic and analytical tools such as 2D-NMR, EIMS and HPLC have made phytochemical studies become much easier and faster. Although there has a turn down in interest towards phytochemistry field, but recently scientists grab interests in discovering new natural bioactive chemical compounds and then chemically synthesise them as useful drugs due to its potential benefits related to human health. Natural products chemistry has gained a growing interest because most of the world's biodiversity have not been evaluated for their potential biological activities. As a result, natural products chemistry is engaged with other science fields, such as pharmaceutical, clinical and genetic engineering to make a significant contribution for the benefit of mankind (Dias, Urban and Roessner, 2012).

1.2   Botany of Plant Species Studied

1.2.1   The Family Guttiferae

The Guttiferae family comprises of about 28 genera and 2027 species of trees or shrubs in Asia and Africa. The plants generally with opaque or coloured sap and the fruits are in capsule or berry forms. The trees have simple leaves with opposite, whorled or seldom alternate patterns, and without any stipules. Moreover, the flowers are actinomorphic and are typically unisexual function. The perianth commonly contains a calyx of 2 to 10 regular overlapped, often crossed sepals and 4 to 12 petals. The stamens are abundant and are distinct. The gynoecium consists of a single unit of pistil, a similar amount of stigmas, and a superior ovary. In addition, a single compound pistil has 3 to 5 or more

carpels, while a superior ovary has 3 to 5 or more locules, each containing one or many axile ovules (Stang, 2013).

1.2.2   The Genus *Calophyllum*

Calophyllum is one of the four genera from Guttiferae family, locally known as *"Bintangor"*, consists of around 180 - 200 species in the hot and humid tropics regions, mainly in Asia, others are distributed in East Africa, tropical America, Madagascar, the Mascarenes and Australasia (Li, Li and Stevens, 2007). According to Li, et al. (2007), the *Calophyllum* plants are trees or shrubs, with clear or milky latex. Apical buds commonly fruitful and lack of scales. The appearance of leaves is opposite, petiolate, leathery and generally glabrous. The plants normally have bisexual flowers and totally 4 to 12 decussate sepals and imbricate petals. Besides that, this plant has a locus of ovary, a large seed and drupe-like berries with thin skin.

The species of plant studied, *Calophyllum castaneum*, is a sub-canopy tree up to 30 m tall and 65 cm diameter with white to yellow latex in stem. The pattern of leaves is simple, opposite and penni-veined. Additionally, secondary veins are upright and packed closely together in an orderly arrangement. The appearance of inferior surface midrib is densely brown hairy. Flowers are white in colour with about 10 mm diameter. Fruits are approximately 21 mm diameter with fleshy green drupe appearance (Stevens, 1980). According to Stevens (1980), *Calophyllum castaneum* are grown in undisturbed mixed dipterocarp forests up to 500 m altitude, and sometimes they can be found on

hillsides and ridges. The plants are distributed in Sarawak, Brunei, West and East Kalimantan. The taxonomy of the plant studied is shown in Table 1.1 and the appearance of *Calophyllum castaneum* is shown in Figure 1.1.

Table 1.1: Taxonomy of *Calophyllum castaneum*

Kingdom	Plantae
Division	Tracheophyta
Class	Spermatopsida
Order	Malpighiales
Family	Guttiferae
Genus	*Calophyllum*
Species	*castaneum*

Figure 1.1: The tree of *Calophyllum castaneum*

Various species of *Calophyllum* have been used as traditional medicine to heal some injuries such as pain, infection, inflammation, bronchitis, gastric and ulcers (Noldin, Isaias and Filho, 2006). For example, wounds and ulcers can be healed with the gums, while inflamed eyes can be treated with the leaves soaked in water (Alkhamaiseh, 2011). Additionally, hard timber of "*bintangor*" tree is used as building materials. Xanthones, coumarins, triterpenes, bioflavonoids, benzofurans and chalcones are the common secondary metabolites present in *Calophyllum* (Ito, et al., 2002, 2003; Isaias, et al., 2004; Noldin, et al., 2006).

1.3   Objectives of Study

The purposes of carrying out this study are:

- To extract and isolate chemical constituents from the stem bark of *Calophyllum castaneum*.

- To identify and characterize the structures of pure compounds through modern spectroscopic analyses.

- To investigate the antioxidant activity of crude extracts and isolated compounds via DPPH method.

CHAPTER 2

LITERATURE REVIEW

2.1   Phytochemical Studies

Natural products are known as secondary metabolites, not as essential nutrient for human body. According to Newman and Cragg (2010), the compounds isolated from plants were chemically modified on their saccharidic-based structures for clinical uses. They are so important due to their biological properties such as antibacterial, antiviral, antifungal, anticancer and antioxidant (Newman and Cragg, 2010), which can be further studied to be potential drugs. Examples of common secondary metabolites are benzopyrans, coumarins, xanthones, triterpenes and steroids, which can be isolated from plants under phytochemical studies.

2.1.1   Benzopyrans

Benzopyrans (chromenes) are compounds with a molecular formula of C_9H_8O, mainly isolated from higher plants. These compounds are in heterocyclic ring system, due to a benzene ring fused to a pyran ring (Van Otterlo, et al., 2005). There are two isomers for this compound based on the vary orientation of fused rings with oxygen atom. 1-Benzopyrans are common compounds isolated in nature while 2-benzopyrans are rather unusual. The structures of 1-

benzopyrans have skeletons of chromane, 2*H*-chromene and 4*H*-chromene.

Figure 2.1 shows the molecular structures of 1-benzopyran skeletons.
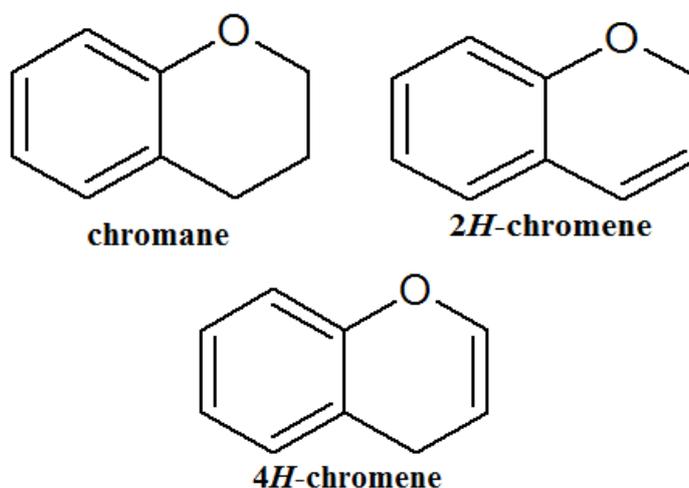
Figure 2.1: Molecular structures of 1-benzopyran skeletons

Benzopyran derivatives are formed because of different oxidation and saturation occurs in nature. Examples of benzopyran derivatives are coumarins, chromanones, chromans and chromones. The derivatives usually have been reported to show several biological activities. This is due to the presence of lipophilic nature in benzopyran derivatives helps to cross the cell membrane easily (Thomas and Zachariah, 2013). Thus, the derivatives able to interact with target cells which leading to a wide range of biological activities such as antioxidant, anti-inflammatory, antitumor, antimicrobial and antiviral. Thomas and Zachariah (2013) also mentioned that many secondary metabolites contain backbones of chromene skeleton such as alkaloids, tocopherols, anthocyanins and flavonoids.

2.1.2 Coumarins

Coumarins (*2H*-chromen-2-one) are the largest class of 1-benzopyran derivatives with molecular formula of $C_9H_6O_2$, which consist of a fusion between a benzene ring and α-pyrone ring (Lacy and Kennedy, 2004). Figure 2.2 shows the molecular structure of coumarins.
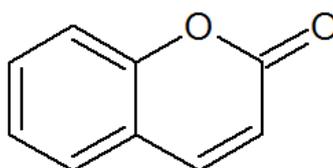
Figure 2.2: Molecular structure of coumarins

Usually these compounds prefer in odourless complex state and conjugated to sugars and acids. Under action of enzymes, acids or UV radiation, coumarins are released and produce a characteristic odour (Egan, et al., 1990). Coumarin compounds have been reported for their biological activities include anti-HIV, antitumor, antispasmodic and anti-inflammatory (Egan, et al., 1990).

The *2H*-chromen-2-one nucleus for the compounds are derived from phenylalanine phenylacrylic skeleton of cinnamic acids (Dewick, 2011). At first, *ortho*-hydroxylation of cinnamic acids leads to 2-coumaric acid formation. Side chain of the structures is subjected to configuration change from *trans* to the less stable *cis* form, and followed by lactone formation. *Trans* isomers are unfavourable in the metabolism due to fully conjugated system for the structures (Dewick, 2011). Figure 2.3 shows the synthetic pathway for coumarins metabolism.
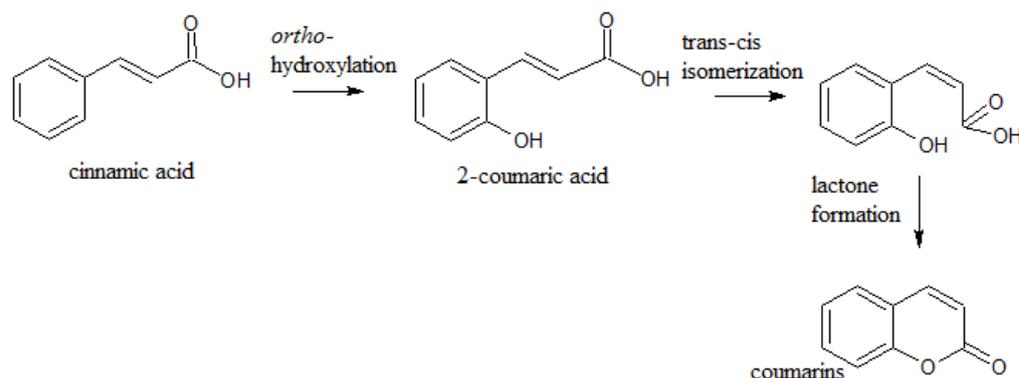
Figure 2.3: Synthetic pathway for coumarins metabolism

2.1.3 Xanthones

Garcinia mangostana, famous for its mangosteen fruit, is a rich source of xanthones, which is distributed in Southeast Asia (Kondo, et al., 2009). Xanthones, also known as 9*H*-xanthen-9-ones, are heterocyclic compounds with molecular formula $C_{12}H_8O_2$. These compounds has three-ring skeleton in dibenzo-γ-pyrone framework (Pedro, et al., 2002). Figure 2.4 shows the basic molecular structure of xanthones.
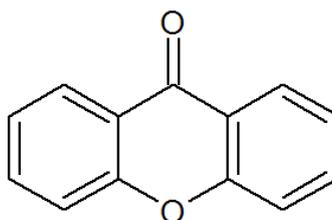
Figure 2.4: Basic molecular structure of xanthones

Akao and coworkers (2008) found that xanthones exhibited anti-inflammatory, anticancer and antibacterial activities.

2.1.4   Triterpenes

Triterpenes or triterpenoids are common compounds isolated from animals and plants, containing 30 carbons skeleton due to combination of isoprene units according to "head-to-tail" manner (Noller, 1945). Figure 2.5 shows the molecular structure of isoprene.

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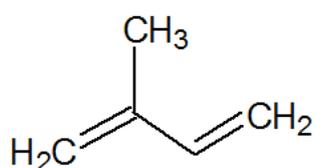
Figure 2.5: Molecular structure of isoprene

The molecular formula of isoprene is C_5H_8, hence triterpenes have molecular formula of $C_{30}H_{48}$. Triterpenoids are derivatives from triterpenes. Triterpenes are hydrocarbon compounds while triterpenoids are compounds containing extra function groups such as hydroxyl and carbonyl group. In natural product study, triterpenoids are biosynthesized from squalenes and likely present in cyclic form (Noller, 1945). Figure 2.6 shows molecular structure of squalene.
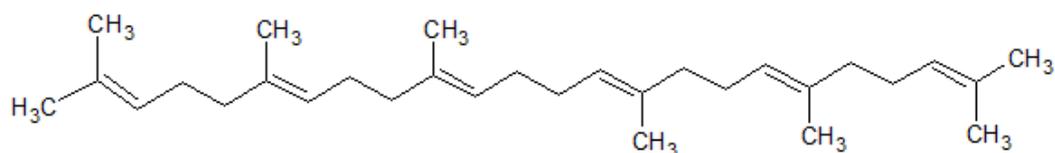
Figure 2.6: Molecular structure of squalene

2.1.5 Steroids

Steroids as hormones are biosynthesized from cholesterol in organisms. Examples of steroid hormones are sex steroids, glucocorticoids and mineralocorticoids. Steroids structurally have the simplest skeleton of four fused rings: three six-member rings and one five-member ring (Miller, Brieggemeier and Dalton, 2013). Figure 2.7 shows the basic skeleton of steroids.
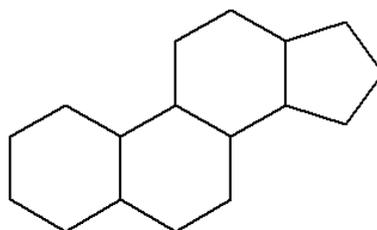
Figure 2.7: Basic skeleton of steroids

Elks (1976) reported that the chemical change in structure leads a diversity effects on biological activities. For example, some steroids act as hormone in transmitting signals, while steroids like cholesterol reduces membrane fluidity due to its phospholipid properties.

2.2   Chemistry of the Genus *Calophyllum*

The genus *Calophyllum* consists of 180-200 species, so far only 48 species from this genus have been phytochemically studied for their stem barks, flowers, fruits and leaves (McKee, et al., 1998). Some of the plant species that

have been extensively studied in this genus are *Calophyllum brasiliense*, *Calophyllum teysmannii*, *Calophyllum inophyllum*, *Calophyllum soulattri* and *Calophyllum thwaitesii*. These species share a common name as "*Bintangor*". Summary of previous studies on the genus *Calophyllum* from a total of 70 relevant publications are shown in Table 2.1.

Table 2.1: Summary of literature review on the genus *Calophylllum*

Plant species	Types of compound	Biological activities	Reference
C. apetalum	Chromanone acids, triterpenoids	Antitumor	Govindachari, Prakash and Viswanathan, 1967 Govindachari, Prakash and Viswanathan, 1968(a) Nigam and Mitra, 1969
C. blancoi	Chromanones acids, xanthones	Anticancer	Stout and Sears, 1968 Shen, et al., 2005
C. brasiliense	Xanthones, coumarins, terpenes, triterpenes, biflavonoids, chromanone acids	Anticancer, antimicrobial, antifungal, antiviral, antibacterial, antiulcerogenic, anti-HIV-1, inhibition of tumor promotion, molluscicide, analgesic	King, King and Manning, 1953 Plattner, et al., 1974 Ito, et al., 2002 Ito, et al., 2003 Abe, et al., 2004 Cottiglia, et al., 2004 Huerta-Reyes, et al., 2004 Isaias, et al., 2004 Pretto, et al., 2004 César, et al., 2011
C. bracteatum	Xanthones, triterpenoids, chromanone acids	--	Somanathan and Sultanbawa, 1972
C. calaba	Xanthones, triterpenoids	--	Somanathan and Sultanbawa, 1972
C. caledonicum	Xanthones, coumarins, chromenes,	Antiplasmodial, antimalarial, antifungal,	Morel, et al., 2000 Morel, et al., 2002 Hay, et al., 2004

	flavonoids, triterpenes	antimicrobial, anti-HIV-1	
C. canum	Xanthones	--	Carpenter, Locksley and Scheinmann, 1969
C. chapelieri	Chromanones	--	Guerreiro, Kunesch and Polonsky, 1971
C. cordato-oblongum	Xanthones, pyranocourmarins, chromanone acids, triterpenoids, biflavonoids	Antifungal	Gunasekera and Sultanbawa, 1975 Dharmaratne and Wijesinghe, 1997 Dharmaratne et al., 1998
C. cuneifolium	Xanthones, chromanone acids, triterpenes	--	Gunasekera, et al., 1977
C. decipiens	Triterpenes, chromanones, xanthones	Anti-oxidation, antibacterial	Ajithabai, et al., 2012
C. dispar	Coumarins	Cytotoxic activity	Guilet, et al., 2001
C. drybalanoides	Chromanone acids, xanthones, triterpenoids, flavonoids	--	Ha, et al., 2012
C. enervosum	Xanthones, ketones, flavonoids, benzophenones	Antimicrobial, antibacterial	Taher, et al., 2005
C. flavoramulum	Flavonoids, biflvonoids, xanthones, triterpenes, benzoic acids	Anti advanced glycation end-products (AGEs), antioxidant, anti-inflammation, antidiabetic	Ferchichi, et al., 2012
C. fragrans	Xanthones	--	Locksley and Murray, 1969
C. gracilipes	Xanthones, triterpenes	Cytotoxic activity	Nasir, et al., 2013
C. incrasaptum	Triterpenes	Antimicrobial	Abbas, et al., 2007
C. inophyllum	Biflavonoids, xanthones, chromanones,	Antibacterial, antimicrobial, anti-HIV-1,	Itiogawa, et al., 2001 Cheng, et al., 2004 Yimdjo, et al., 2004

	triterpenoids, coumarins, calophyllic acids	anticancer, antitumor, antiviral, antioxidant, antidylipidemic	Ha, et al., 2009 Li, et al., 2010 Indrakumar, et al., 2012 Prasad, et al., 2012
C. lanigerum var austroindicum	Coumarins, xanthones, chromanone acids	Anti-HIV-1	Iinuma, et al.,1996 McKee, et al., 1996 McKee, et al., 1998
C. macrocarpum	Chromanone acids, triterpenoids	--	Ampofo and Waterman, 1986
C. membrane-ceum	Xanthones, glycoside, chromanone acids, coumarins, triterpenoids	Anti-inflammatory	Zou, et al., 2005 Chen, et al., 2008
C. mucigerum	Coumarins, xanthones, triterpenes	Anti-luekemic, insecticide	Ee, et al., 2004
C. nodusum	Xanthones, terpenoids	--	Nasir, et al., 2011
C. panciflorum	Xanthones, biflavonoids	Antitumor, antiviral	Ito, et al., 1999
C. papuanum	Chromanones	--	Stout, Hickernell and Sears, 1968
C. pinetorum	Xanthone, chromanones, triterpenes, flavonoids	--	Alarcón, et al., 2008
C. polyanthum	Coumarins, benzoic acids, triterpenoids	--	Ma, et al., 2004
C. rubiginosum	Flavonol, flavonoid	Antioxidant, antimicrobial, antibacterial	Taher, et al., 2010 Alkhamaiseh, et al., 2011
C. sclerophyllum	Xanthones	--	Jackson, Locksley and Scheinmann, 1966
C. scriblitifolium	Xanthones	--	Jackson, Locksley and Scheinmann, 1967a Jackson, Locksley and Scheinmann, 1967b
C. soulattri Var moonii	Xanthones, coumarins, chromanones,	Cytotoxic activity	Gunasekera, et al., 1977 Ee, et al., 2011

	triterpenes, steroids		Mah, et al., 2011 Mah, et al., 2012
C. sundaicum	Phloroglucinol	--	Cao, et al., 2006
C. symingtonianum	Xanthones	Antifungal	Kawamura, et al., 2012
C. tetrapterum	Coumarins	Antimicrobial	Abbas, et al., 2007
C. thwaitesii	Xanthones, triterpenoids	--	Dahanayake, et al., 1974 Dharmaratne and Wanigasekera, 1996
C. thorelii	Phloroglucinol, xanthones, benzophenones	Cytotoxic activity	Nguyen, et al., 2012
C. teysmannii	Xanthones, coumarins	Anti-HIV-1	Pengsuparp, et al., 1996 McKee, et al., 1996 McKee, et al., 1998 Kijjoa et al., 2000
C. trapezifolium	Xanthones, triterpenoids	--	Somanathan and Sultanbawa, 1974
C. tomentosum	Triterpenoids	--	Govindachari, Prakash and Viswanathan, 1968(b) Nigam and Mitra, 1969
C. venulosum	Biflavonoids	--	Cao, et al., 1997
C. walker	Xanthones, triterpenes, neoflavonoids	--	Dahanayake, et al., 1974 Ampofo and Waterman, 1986
C. wightianum	Xanthones, phloroglucinols, fatty acids	--	Dean, et al., 1980 Dean, et al., 1984
C. zeylanicum	Xanthones, triterpenoids	--	Gunasekera, Sotheeswaran and Sultanbawa, 1981

2.2.1   Chemistry and Biological Activities of *Calophyllum castaneum*

Calophyllum castaneum was the plant material used in this study. By far there has been no study documented on this species. However, some other species from the genus have been studied for their phytochemistry, and many of them have been reported to be a rich source of natural products, such as xanthones, triterpenoids and coumarins (Noldin, et al., 2006).

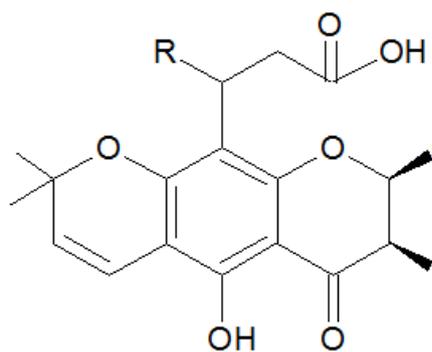**2.2.2   Chemistry of *Calophyllum brasiliense***

A phytochemical study reported that three homologous chromanone acids in *cis* isomers, apetalic acid **[1]**, compound 2, compound 3 and *trans* isomers, isoapetalic acid **[4]**, compound 5, blancoic acid **[6]** were isolated from *Calophyllum brasiliense* seed oil (Plattner, et al., 1974).

Besides chromanone acids, xanthones were also commonly isolated from the stem bark of *Calophyllum brasiliense*. Seven new xanthones were discovered in year 2002 which were brasixanthones A **[7]**, B **[8]**, C **[9]**, D **[10]**, E **[11]**, F **[12]** and G **[13]**. In addition, another 10 known xanthones were isolated from the same extract, namely latisxanthone C **[14]**, garcinone B **[15]**, pyranojacareubin **[16]**, 1,2-dimethoxyxanthone **[17]**, 4-hydroxyxanthone **[18]**, cudraxanthone   F   **[19]**,   8-desoxygartanin   **[20]**,   3,8-dihydroxy-1,2-dimethoxyxanthone **[21]**, 6-deoxyjacareubin **[22]** and toxyloxanthone A **[23]**. In biological study, compounds **[8]**, **[9]**, **[10]** and **[20]** were reported to show a

good cancer chemopreventive activity towards Epstein-Barr Virus Early Antigens (EBV-EA) (Ito, et al., 2002).

Later, Ito and coworkers (2003) further reported the isolation of three new coumarins, brasimarins A **[24]**, B **[25]** and C **[26]** from stem bark of *Calophyllum brasiliense*. By comparing spectral data with literature values, 11 known coumarins were characterised, which were calocoumarin A **[27]**, 5-methoxy-2,2-dimethyl-6-(2-methyl-1-oxo-2-butenyl)-10-propyl-2H,8H-benzo[1,2-b; 3,4-b']dipyran-8-one **[28]**, calophyllolide **[29]**, calanone **[30]**, calanolide A **[31]**, calanolide C **[32]**, inophyllum A **[33]**, inophyllum C **[34]**, inophyllum D **[35]**, inophyllum E **[36]** and mammea B/BB **[37]**.

In 2004, three anti HIV-1 dipyranocoumarins, soulattrolide, calanolides A and B **[38]** were isolated from *Calophyllum brasiliense* leaves, together with known compounds such as friedelin **[39]**, canophyllol **[40]**, amentoflavone **[41]**, apetalic acid and isoapetalic acid. The dipyranocoumarins and triterpenes were subjected to anti HIV-1 reverse transcription test, but only soulattroide, calanolides A and B were found to be active against HIV-1 RT (Huerta-Reyes, et al., 2004).
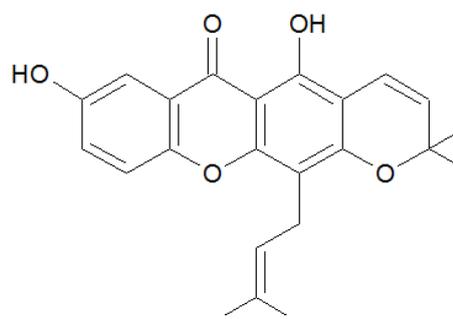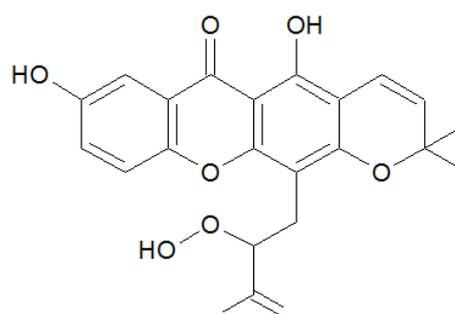
[1] R = propyl
[2] R = n-butyl
[3] R = n-pentyl
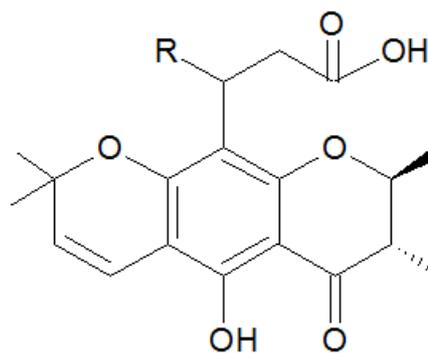
[4] R = propyl
[5] R = n-butyl
[6] R = n-pentyl
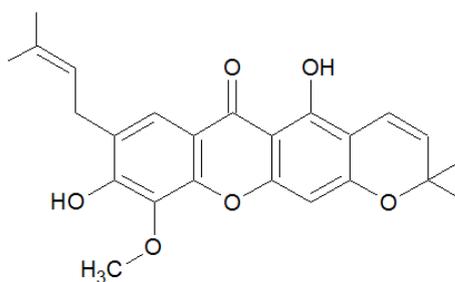
[7]

[8]

[9]

[10]

Figure 2.8: Structures of chemical compounds isolated from
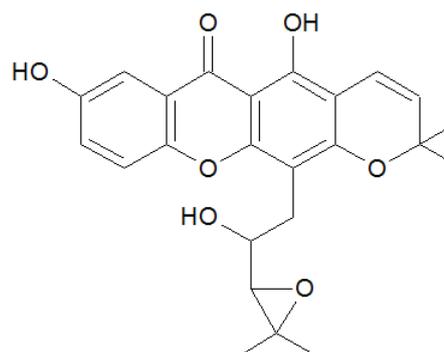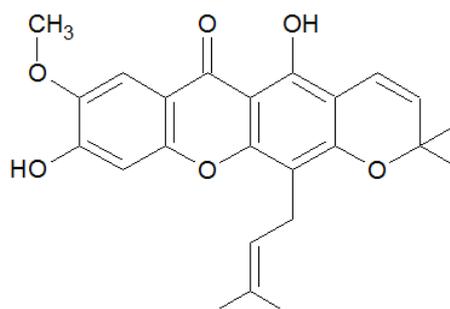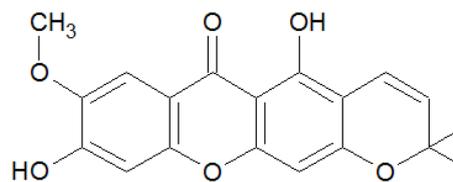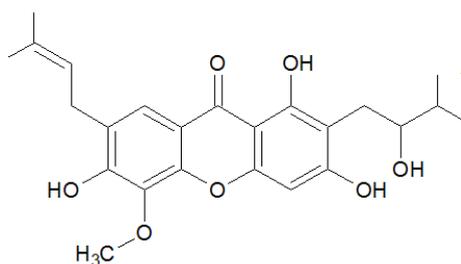*Calophyllum brasiliense*

[11]

[12]

[13]

[14]

[15]

[16]

[17]
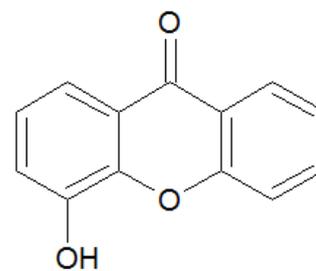
[18]

Figure 2.9: Structures of chemical compounds isolated from
Calophyllum brasiliense **(continued)**

[19]　　　　　　　　　　　　　　　**[20]**

[21]　　　　　　　　　　　　　　　**[22]**
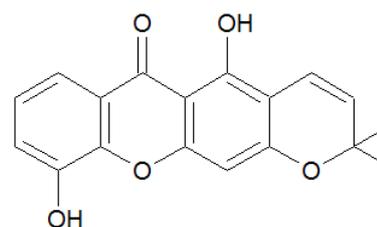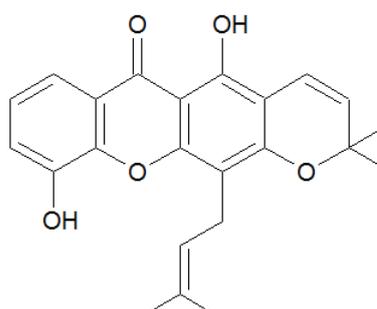
[23]　　　　　　　　　　　　　　　**[24]**
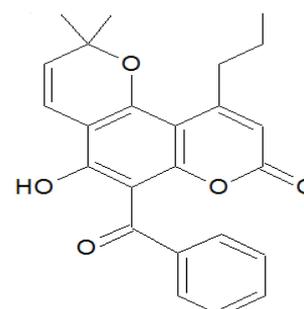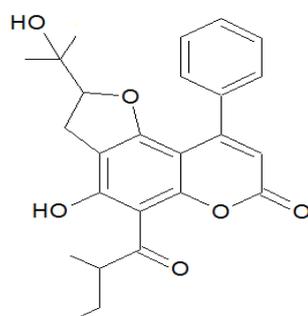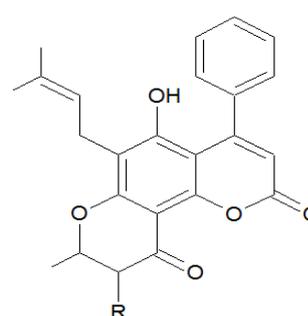
[25]　　　　　　　　　　**[26]** R = α-CH$_3$
　　　　　　　　　　　　　　　　　[27] R = β-CH$_3$

Figure 2.10: Structures of chemical compounds isolated from
Calophyllum brasiliense **(continued)**
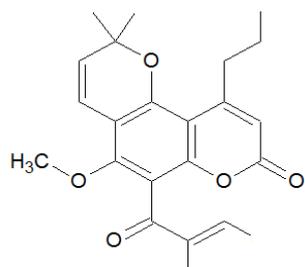
[28]
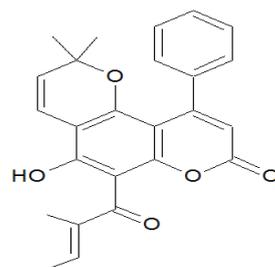
[29]
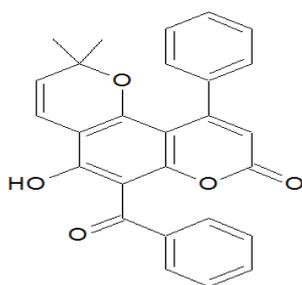
[30]
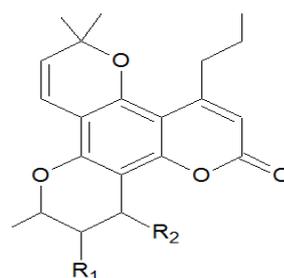
[31] $R_1 = \alpha\text{-}CH_3$; $R_2 = \beta\text{-}OH$
[32] $R_1 = \beta\text{-}CH_3$; $R_2 = \beta\text{-}OH$
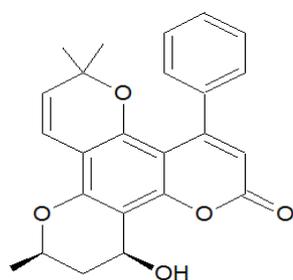
[33]
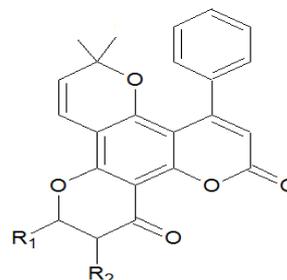
[34] $R_1 = \beta\text{-}CH_3$; $R_2 = \alpha\text{-}CH_3$
[36] $R_1 = \beta\text{-}CH_3$; $R_2 = \beta\text{-}CH_3$
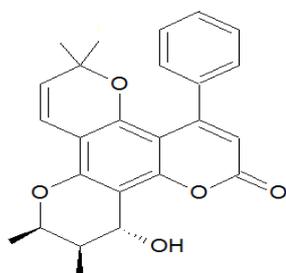
[35]
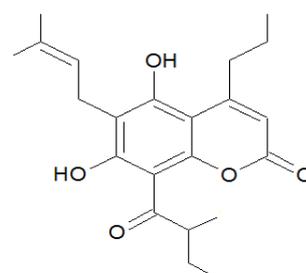
[37]

Figure 2.11: Structures of chemical compounds isolated from
Calophyllum brasiliense **(continued)**

23
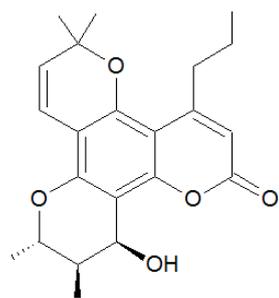
[38]
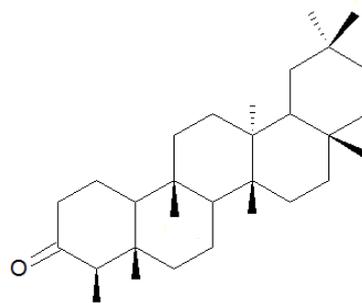
[39]
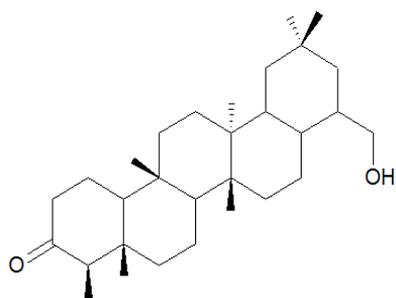
[40]
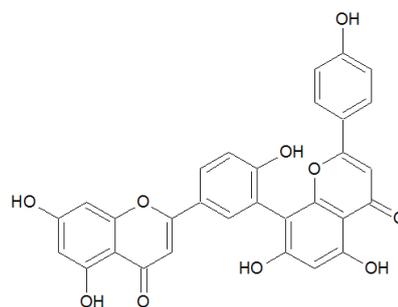
[41]

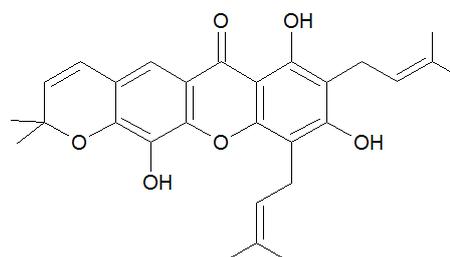**Figure 2.12: Structures of chemical compounds isolated from**
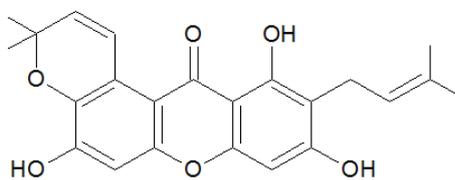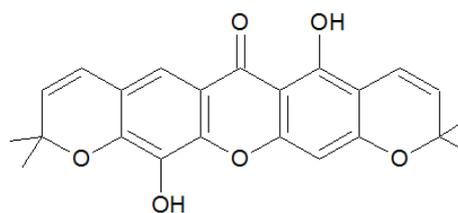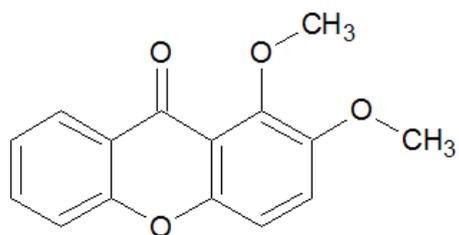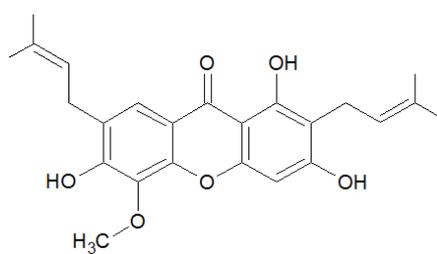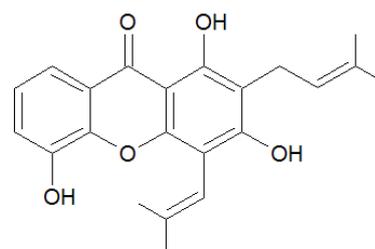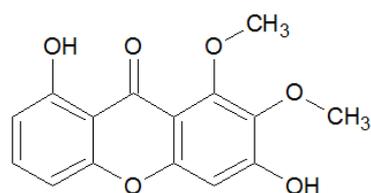*Calophyllum brasiliense* **(continued)**

2.2.3 Chemistry and Biological Activities of *Calophyllum soulattri*

In 1977, phytochemical study on the timber extract of *Calophyllum soulattri* afforded a new coumarin alcohol, soulattrolide **[42]** and three common triterpenoids, taraxerol **[43]**, taraxerone **[44]** and β-sitosterol **[45]**. Besides that, four known xanthones were isolated, which were calabaxanthone **[46]**, buchanoxanthone **[47]**, 1,7-dihydroxyxanthone **[48]** and 1-hydroxy-5-methoxyxnthone **[49]**. In addition, a new trihydroxyxanthone, 1,3,5-trihydroxy-2-(3-methylbut-2-enyl)xanthone **[50]** was also discovered from the timber extract (Gunasekere, et al., 1977).

In 2011, two phytochemical studies on stem bark of *Calophyllum soulattri* were reported. One of the studies reported isolation of a new prenylated pyranoxanthone, soulattrin **[51]**, whereas the other study reported isolation of a new pyranocoumarin, soulamarin **[52]**. Apart from that, five known xanthones, namely trapezifolixanthone **[53]**, macluraxanthone **[54]**, brasixanthone B **[8]**, caloxanthone B **[55]** and caloxanthone C **[56]**, together with two triterpenes, friedelin and stigmasterol **[57]** were also isolated (Mah, et al., 2011; Ee, et al., 2011).

A year later, Mah and coworkers (2012) discovered a new diprenylated xanthone, phylattrin **[60]** from the stem bark of the plant. All the compounds 53 to 58 were assayed for their cytotoxic activity against HeLa, NCI-H23, Raji, IMR-32, SUN-1, Hep G2, K562, LS174T and SK-MEL-28 cancer cells. All

the compounds gave a moderate activity in the assay with soulattrin **[51]** showed a broad cytotoxic activity against several types of cancer cells.
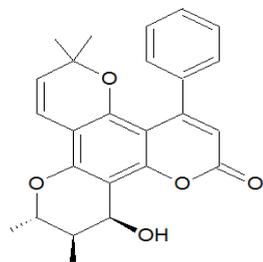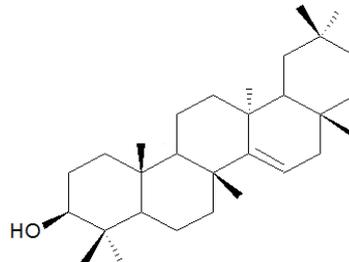
[42]   **[43]**
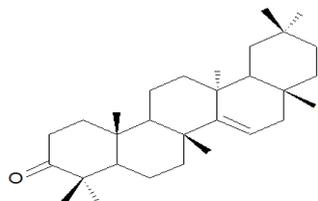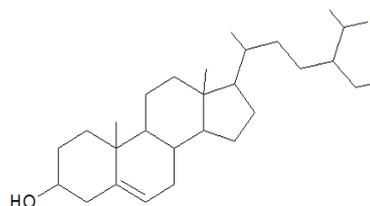
[44]   **[45]**
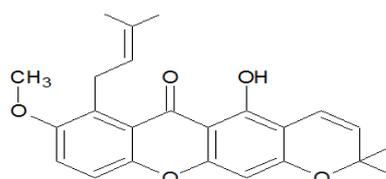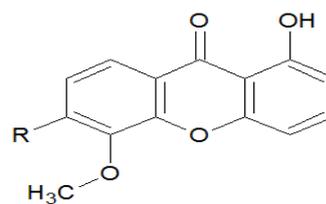
[46]   **[47]** R = OH
                            [49] R = H
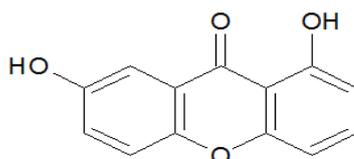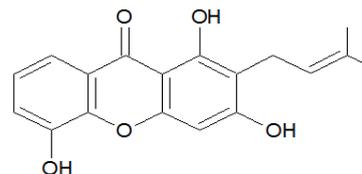
[48]   **[50]**

Figure 2.13: Structures of chemical compounds isolated from
Calophyllum soulattri
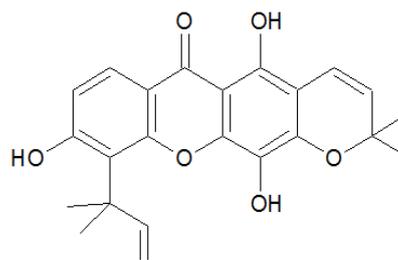
[51]
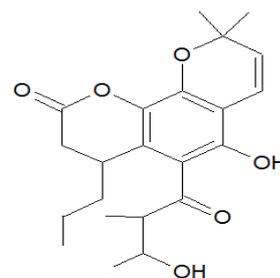
[52]
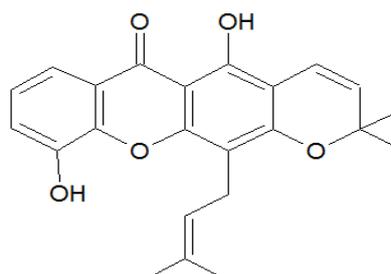
[53]
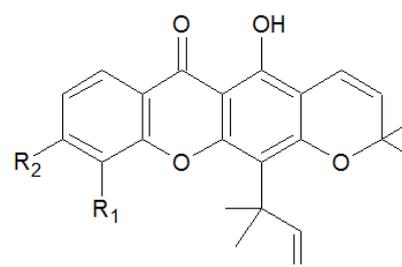
[54] R₁ = OH; R₂ = OH
[56] R₁ = OH; R₂ = H
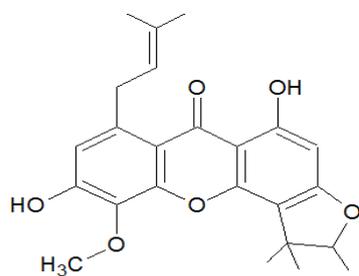
[55]
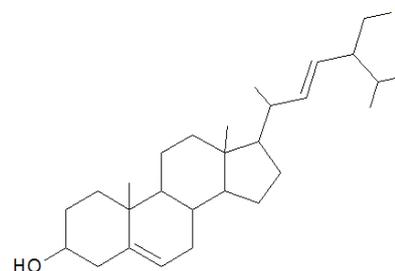
[57]
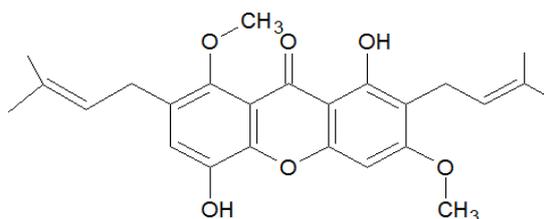
[58]

Figure 2.14: Structures of chemical compounds isolated from
Calophyllum soulattri **(continued)**

2.2.4   Chemistry of *Calophyllum venulosum*

A phytochemical study established by Cao and coworker (1997) found that *Calophyllum venulosum* is rich in biflavonoids. A total of seven biflavonoids were isolated from the leaves of *Calophyllum venulosum*. The four new isolated biflavonoids were 6"-(2-hydroxy-3-methyl-3-butenyl)amentoflavone **[59]**, 6"-(3-methyl-2-butenyl)amentoflavone **[60]**, pyranoamentoflavone 7,4'-dimethyl ether **[61]** and pyranoamentoflavone 7,4'''-dimethyl ether **[62]**. In addition, the other three known biflavonoids, 2,3-dihydroamentoflavone **[63]**, amentoflavone **[64]** and pyranoamentoflavone **[65]** were also isolated from the leave extract.

[59] R = (structure)

[60] R = (structure)

[64] R = H

[61] R$_1$ = OCH$_3$; R$_2$ = OCH$_3$; R$_3$ = OH

[62] R$_1$ = OCH$_3$; R$_2$ = OH; R$_3$ = OCH$_3$

[65] R$_1$ = OH; R$_2$ = OH; R$_3$ = OH

[64]
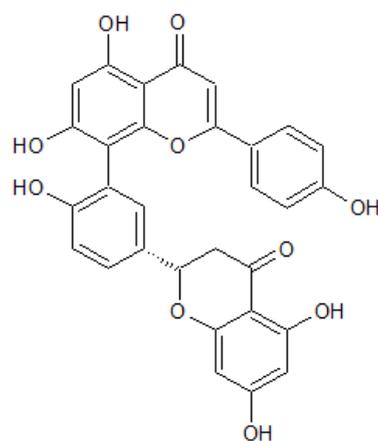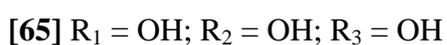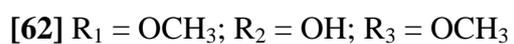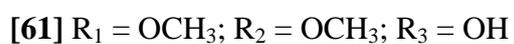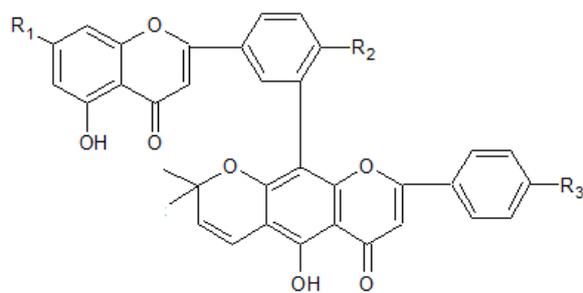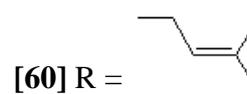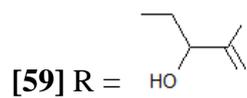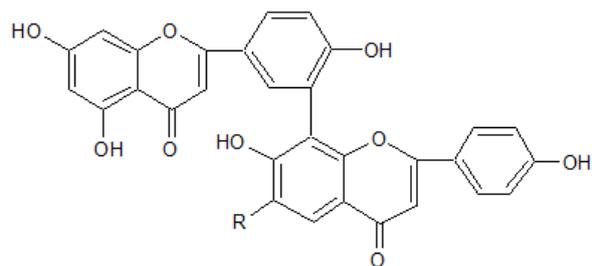
Figure 2.15: Structures of chemical compounds isolated from
Calophyllum venulosum

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1   Plant Material

The plant studied in this project was *Calophyllum castaneum*. The stem bark of this plant, approximately 2.0 kg, was collected from the jungle in Landeh, Sarawak. A voucher specimen of the plant was kept in herbarium at Universiti Teknologi MARA, Sarawak.

3.2   Chemicals

The materials and solvents used for extraction, isolation and purification of chemical compounds from *Calophyllum castaneum* are listed in Table 3.1. The materials and HPLC grade solvents used in LC-MS analysis are listed in Table 3.2. The deuterated solvents used in NMR analysis are listed in Table 3.3. All the chemical reagents and materials used in chemical analysis are listed in Table 3.4. Chemical reagents and materials used in antioxidant assay are listed in Table 3.5.

Table 3.1: Materials and solvents used for extraction, isolation and purification of chemical compounds

Materials/Solvents	Molecular formula	Density, ρ (g mL^{-1})	Source, Country
Sea sand	-	-	Merck, Germany
Sephadex® LH-20	-	-	New Jersey, USA
Silica gel (60Å) 230-400 Mesh	-	-	Merck, Germany
Sodium sulphate anhydrous	Na$_2$SO$_4$	-	John Kollin Corporation, USA
Methanol	CH$_3$OH	0.791	Mallinckrodit Chemicals, Philipsburg
Acetone	CH$_3$COCH$_3$	0.791	QREC, Malaysia
Ethyl acetate	CH$_3$COOC$_2$H$_5$	0.902	Lab- Scan, Ireland
Dichloromethane	CH$_2$Cl$_2$	1.325	Fisher Scientific, UK
n-Hexane	CH$_3$(CH$_2$)$_4$CH$_3$	0.659	Merck, Germany

Table 3.2: Materials and solvents used in LC- MS analysis

Materials/Solvents	Molecular formula	Molecular weight (g mol^{-1})	Source, Country
Nylon syringe filter	-	-	Membrane-Solution, USA
Methanol	CH$_3$OH	32.04	Fisher Scientific, UK
Acetonitrile	C$_2$H$_3$N	41.05	Fisher Scientific, UK

Table 3.3: Deuterated solvents used in NMR analysis

Deuterated solvents	Source, Country
Methanol-d_4	Acros Organics, Belgium
Acetone-d_6	Acros Organics, Belgium
Deuterated chloroform ($CDCl_3$)	Acros Organics, Belgium

Table 3.4: Materials and chemicals reagents used in chemical analysis

Solvents/Materials	Molecular formula	Source, Country
TLC silica gel 60 F_{254}	-	Merck, Germany
Ferric chloride	$FeCl_3$	Uni-chem, India
Iodine	I_2	Fisher Scientific, UK

Table 3.5: List of materials and reagents used in antioxidant assay

Materials/Reagents	Source, Country
96-well plate	Techno Plastic Products AG, Switzerland
DPPH (1,1-diphenyl-2-picrylhydrazyl)	Sigma- Aldrich, USA
Ascorbic acid	Sigma- Aldrich, USA
Kaempferol	Sigma- Aldrich, USA

3.3   Extraction, Isolation and Purification of Chemical Constituents

       from *Calophyllum castaneum*

Approximately 2.0 kg of stem bark of *Calophyllum castaneum* was collected, air dried and finely ground into powder form. The powdered stem bark was soaked in dichloromethane for two days at room temperature. The dichloromethane crude extract was filtered and concentrated via a rotary evaporator in a water bath at 40℃. The steps were repeated twice and the extracts were combined.

The stem bark material was then extracted subsequently with ethyl acetate and lastly with methanol, twice for each solvents as indicated in the first extraction. The dried masses of the dichloromethane, ethyl acetate and methanol extracts collected were 40.6336 g, 27.8285 g and 41.9768 g, respectively.

The dichloromethane crude extract of *Calophyllum castaneum* was subjected to vacuum column chromatography and separated into a series of fractions, and the chemical composition of fractions collected were analysed using TLC. Fractions that showed similar pattern of spots on TLC were combined for further purification via adsorption chromatography and size exclusion chromatography. The effectiveness of separation was monitored by TLC analyses. The procedures of isolation, purification and detection were repeated until pure compounds were successfully isolated. Compounds that gave a single spot on the developed TLC were sent for spectroscopic and spectrometric analyses such as NMR, IR, UV and MS. The crude of ethyl

acetate and methanol extracts were also subjected to column chromatography as above to obtain pure chemical compounds of interest.

3.4 Chromatography Methods

3.4.1 Column Chromatography

Column chromatography is a technique used to separate and purify chemical constituents from the crude extract. Two types of column chromatography were applied in this study, which are vacuum column chromatography and gravity column chromatography.

The size of column used in column chromatography depends on amount of sample. In this study vacuum column with 80 mm in diameter was used for first separation of each crude extracts, while gravity columns with either 40 mm or 20 mm in diameter were used in gravity column chromatography for further isolation and purification of a smaller quantity of fractions.

The sample was prepared via the dry packing method whereby the sample was firstly dissolved in an appropriate amount of solvent. Subsequently the sample solution was introduced dropwise and mixed gently with a minimum amount of dry silica gel. The wet mixture was left overnight at ambient temperature to ensure the completely dried of prepared sample. The dried sample was then introduced into the silica gel packed column. The column packing material used was Merck Kieselgel 60, 230-400 Mesh (40-60 microns). Firstly, a

vertical column was set up with a minimum hexane solvent was poured into the column, and a thin layer of sea sand or anhydrous sodium sulphate was introduced. After that, the column was half-filled with hexane solvent, and then slurry of silica gel in hexane was introduced into the column until a proper level. The slurry was allowed to settle down and the side of column was tapped using a rubber tube to make sure a compact packing to the column. After the stationary phase was densely packed, the prepared sample was followed by a thin layer of protection layer were introduced into the column. Subsequently, a series of mobile phase in increasing polarity (hexane/ dichlromethane/ ethyl acetate/ acetone/ methanol) was eluted out from the column to effect separation of compounds. The set up of column chromatographic apparatus is shown in Figure 3.1. The eluted fractions from column chromatography were analysed via TLC plates.

Figure 3.1: Set up of column chromatographic apparatus

3.4.2   Size Exclusion Chromatography (SEC)

Gel filtration chromatography (GFC) and gel permeation chromatography (GPC) are the two techniques used in size exclusion chromatography. GFC is a chromatographic method with use of aqueous solution as mobile phase, whereas GPC uses organic solvent as mobile phase. This technique is commonly used to separate large molecules on the basis of difference in their size and molecular weight. In this study, gel permeation chromatography was used to separate nearly pure compounds, which cannot be further purified by adsorption chromatography.

In this project, a highly porous rigid resins which is known as Sephadex® LH-20, was used as stationary phase, whereas a polar organic solvent mixture was used as mobile phase. Sample was dissolved in methanol and was then introduced as a thin layer of sample solution onto the top of packed column. An isocratic solvent mixture of 80% methanol and 20% dichloromethane was eluted. As the mobile phase flown down the column, large molecules will penetrate fewer pores than small molecules. Hence, large molecules will eluted out from column faster than small molecules.

3.4.3   Thin Layer Chromatography (TLC)

To determine the purity and chemical composition of compounds, TLC analysis of each fraction was carried out by using 8 cm x 4 cm dimensions of aluminium plate adhered with silica gel 60 F_{254} in Merck brand. In this

analysis, a small quantity of sample solution was transferred and dotted onto the baseline drawn on the TLC plate by using a thin capillary tube. Meanwhile, a chamber was prepared with a suitable amount of solvent mixture as mobile phase. After the developing chamber was saturated with solvent vapour, the TLC plate was put into the chamber. The solvent was allowed to move up through the stationary phase via capillary action until the solvent reached the solvent front line. The spots on developed plate were visualised under ultra-violet lamp, iodine vapour and ferric chloride solution. The polarity and identity of each spot was determined based on their retention factors, R_f values according to following equation:

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

With the aid of a picture tool, the set up of a developed TLC plate is illustrated.
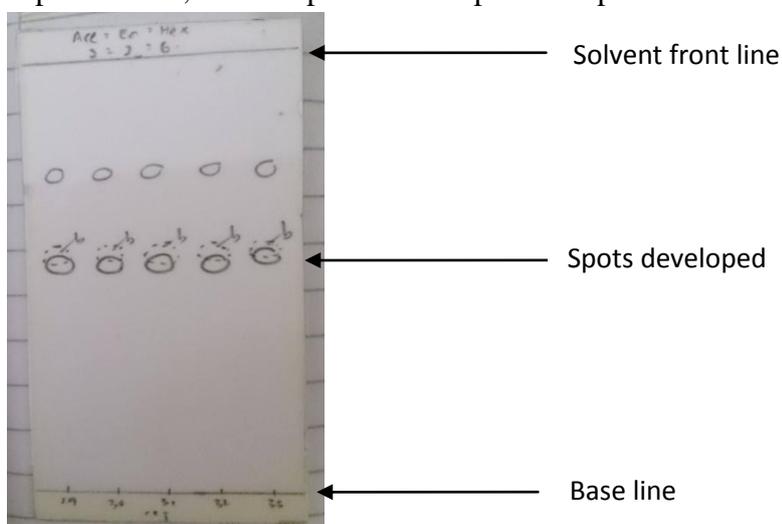
Figure 3.2: Developed TLC plate set up

3.5   TLC Detection Methods

3.5.1   Ultra-Violet (UV) Detection

All developed TLC plates were visualised with UV lamp with both short wavelength at 254 nm and long wavelength at 365 nm. Due to fluorescence quenching of compounds on the plate, chemical compounds were presented in dark spots under bright greenish blue background when irradiated with short wavelength of UV. Generally, UV lamp is used to detect conjugated or aromatic compounds.

3.5.2   Iodine Vapour Detection

The iodine vapour chamber was prepared by introducing an appropriate amount of iodine crystal into a closed chamber and a filter paper was put inside to ensure saturation of iodine vapour. The developed TLC plate was placed into the iodine vapour chamber using a pair of forceps for few minutes. The spots appeared were marked immediately as the spots might disappear over time. This detection is useful to identify non-conjugated compounds or terpenoids which give distinct yellow spots on the plate.

3.5.3 Ferric Chloride Solution

1.0 g of ferric chloride was dissolved in 100 mL of methanol to form ferric chloride solution. For confirmation of compounds, ferric chloride solution was sprayed on the developed spots of TLC. Formation of coloured complex on the spots indicates a positive result. For example, phenolic compounds give dark blue or greenish spots while hydroxamic acids give red spots.

3.6 Instruments

3.6.1 Nuclear Magnetic Resonance (NMR)

Nuclear Magnetic Resonance (NMR) spectroscopy is a technique used to structurally elucidate organic compounds by studying their protonated carbon nuclei. JEOL JNM-ECX 400 MHz spectrometer was used to carry out ^{1}H-NMR, ^{13}C-NMR, HMQC and HMBC experiments in this project. ^{1}H-NMR and ^{13}C-NMR were used to determine carbon skeleton and structure of compounds. The purpose of application of 2D-NMR was established correlation between protons and their neighbouring carbons such as HMQC was used to study direct ^1J coupling between proton and carbon, while HMBC was used to detect long range ^2J and ^3J couplings between two nuclei.

In the preparation of each NMR sample, dry sample was dissolved in a suitable of deuterated solvents such as methanol-d_4, acetone-d_6 and deuterated dichloromethane, meanwhile tetramethylsilane (TMS) was used as an internal

standard and reference. Usually, selection of solvent used was based on solubility of sample in the solvent. The dissolved samples were then filled into NMR tube to a height of around 4 cm, and the tube was capped and sealed with parafilm tightly to avoid leakage or evaporation during NMR analysis.

3.6.2 Infrared (IR) Spectroscopy

Infrared (IR) spectroscopy was applied to study the presence of different functional groups in chemical compound. Besides, IR spectrum also provides a characteristic fingerprint region that is helpful to structural determination of compounds. Perkin Elmer 2000-FTIR spectrohotometer was used to obtain IR spectrum in the range of 4000 to 400 cm^{-1} for the sample. In this study, each test sample was prepared in potassium bromide (KBr) sample pellet by grinding small portion of solid sample with KBr in a ratio of 1:10, after that the mixture was compressed under high pressure to give KBr pellet for IR analysis.

3.6.3 Ultraviolet-Visible (UV-Vis) Spectroscopy

Ultraviolet-visible (UV) spectroscopy is a technique used for analysis of conjugated compounds which absorb radiation in the range of wavelengths from 190 to 800 nm. The model of UV-Vis spectrophotometer used in this project was Perkin-Elmer Lambda (25/35/45). A 98% absolute ethanol was used as solvent blank because of its good solubility to most of the compounds.

The test compounds were dissolved and diluted with the solvent and UV absorption was measured in the range of 200 to 400 nm.

3.6.4 Liquid Chromatography-Mass Spectrometry (LC-MS)

This coupled technique is applied by coupling of liquid chromatography (LC) with mass spectrometry (MS) to analyse non-volatile compounds in high molecular weight through electrospray ionization (ESI). In this study, G6520B Q-TOF LC/MS spectrometer was used to determine the accurate mass of compound. A solution of sample in appropriate solvent was sprayed out from a fine capillary into a heated compartment, and further desolvation produce free ions for analysis. In sample preparation, the samples were dissolved in HPLC grade methanol or acetonitrile at the concentration below 1000 ppm. The HPLC grade solvent was chosen due to its high purity properties and the selection of solvent depends largely on the dissolution of samples in the solvent used. The sample solutions were filtered by using nylon syringe filter with 0.22 μm pore size to eliminate undissolved solid or impurities before the samples were introduced into the LC-MS.

3.6.5 Melting Point Apparatus

Melting point apparatus was used to study the melting point and purity of solid compound. A pure solid compound gives a narrower and sharper range of melting point than an impure substance. Barnstead Electrothermal 9100 melting point apparatus was used to measure the melting points of samples. To

carry out this test, a solid sample was placed into a haematocrit capillary tube and heated until it melted completely. The temperature range at which the compounds start and completely melt was recorded.

3.6.6 Polarimeter

Polarimeter was used to study the optical activity of a chiral molecule by measuring angle of rotation of the compound. The change in polarization orientation was caused by passing of a polarized beam (589 nm) through the optical actively compound in the instrument. Jasco Europe P-2000 digital polarimeter was used to measure observed specific rotation of samples. In this study, test solution was prepared by diluting 30 mg of sample into 10 mL of chloroform solvent. The specific rotation $[\alpha]_{\lambda}^{T}$ of sample with chiral carbon was calculated according to following equation:

$$[\alpha]_{\lambda}^{T} = \frac{\alpha}{c.l}$$

where

$[\alpha]$	=	Specific rotation
α	=	Observed optical rotation
l	=	Optical path length (1.0 dm)
c	=	concentration of sample in g/mL
T	=	Temperature (25°C)
λ	=	Wavelength (589 nm)

3.7   Antioxidant Assay

The isolated compounds and the standard compounds (Kaempferol and ascorbic acid) were separately dissolved in methanol to prepare master stocks at 2 mg/mL. The master stocks were sonicated for 5 minutes to form homogeneous solution. Then, DPPH solution with a concentration of 4 mg/mL was prepared in methanol, and was then sonicated. Master stocks for samples and DPPH solution were stored in a 4℃ chiller in dark condition to avoid decomposition chemicals in the solutions.

 Positive controls and test compounds were prepared from master stocks at concentrations of 240, 120, 60, 30, 15, 7.5 and 3.75 μg/mL in a 96-well plate by serial dilution followed by adding 10 μL of DPPH solution. Each compound was assayed in three independent replicates and the readings were averaged. The DPPH methanolic solution without the test samples was treated as negative control.

Immediately after the addition of reagents, the plate was wrapped with aluminium foil and stored in dark at room temperature for 30 minutes to avoid evaporation and exposure to light. The absorbance of the mixtures in each well was detected at 520 nm using a microplate reader (Model 670, Bio-Rad Laboratories, Hercules, Ca, USA) and results were analysed by the Microplate Manager®, Version 5.2.1 software. The wavelength at 520 nm was chosen because of the characteristic absorption at this wavelength.

3.8 **Calculation**

3.8.1 Inhibition Rate

The formula below was applied to determine inhibition rates of test compounds:

Inhibition Rate (%) = $\frac{A_0 - A_1}{A_0}$ x 100%

where

A$_0$ = Absorbance of the negative control (without plant extract)

A$_1$ = Absorbance of the test compound

The resulting data were presented as a function of inhibition rate versus concentration of the samples to obtain IC$_{50}$ value. This value is defined as the concentration of sample needed to inhibiting 50% of DPPH radical scavenging activity. The antioxidant activity was classified as strongly active (>80% inhibition), moderate active (50-80% inhibition) and inactive (<50% inhibition). Figure below shows the set up of DPPH antioxidant assay in this project.

Figure 3.3: DPPH antioxidant assay using 96-well plate

CHAPTER 4

RESULT AND DISCUSSION

4.1   Isolation of Compounds from *Calophyllum castaneum*

The *Calophyllum castaneum* stem bark material was subjected to sequential extraction by using dichloromethane, ethyl acetate and methanol. As a result, 40.6336 g of dichloromethane extract, 27.8285 g of ethyl acetate extract and 41.9768 g of methanol extract were produced. All of the methanol extract obtained was subjected to gravity column chromatography via a 100 mm diameter column packed with silica gel and eluted with mobile phase of increasing polarity hexane-DCM, DCM-Me$_2$CO and Me$_2$CO -MeOH to give 30 fractions, CCA 1-30.

Fractions 11-13 (CCA 11-13) were combined and subjected to a 40 mm diameter silica gel column eluted with solvent mixtures of hexane-EtOAc, DCM-Me$_2$CO and Me$_2$CO-MeOH, which resulted a total of 30 subfractions (CCB 1-30). The subfractions 9-10 (CCB 9-10) were fractionated via a silica gel column with 24 mm in diameter eluted with hexane-EtOAc gradient to give 37 subfractions (CCC 1-37). Subractions of 8 to 11 (CCC 8-11) with 341 mg were combined and further purified by similar column and resulted 18 subfractions (CCE 1-18). Subfractions 3 and 4 (CCE 3-4) afforded 79 mg of blancoic acid.

The subfractions of CCB 11-12 was subjected to a 40 mm diameter silica gel column with gradient elution of hexane-ethyl acetate to furnish 53 subfractions (CCD 1-53). The subfractions CCD 18-23 afforded 144 mg of isoblancoic acid compound (144 mg). On the other hand, the subfractions CCD 9-10 and 12-14 were combined and further fractionated via a silica gel packed column with 24 mm in diameter eluted with mobile phase hexane-EtOAc gradient, which yielded 32 fractions (CCG 1-32). The ninth to twelfth subfractions (CCG 9-12) were further purified to furnish 25 mg of β-sitosterol in white needle-like crystal.

Figure 4.1 illustrates the isolation pathway of blancoic acid, isoblancoic acid and β-sitosterol.
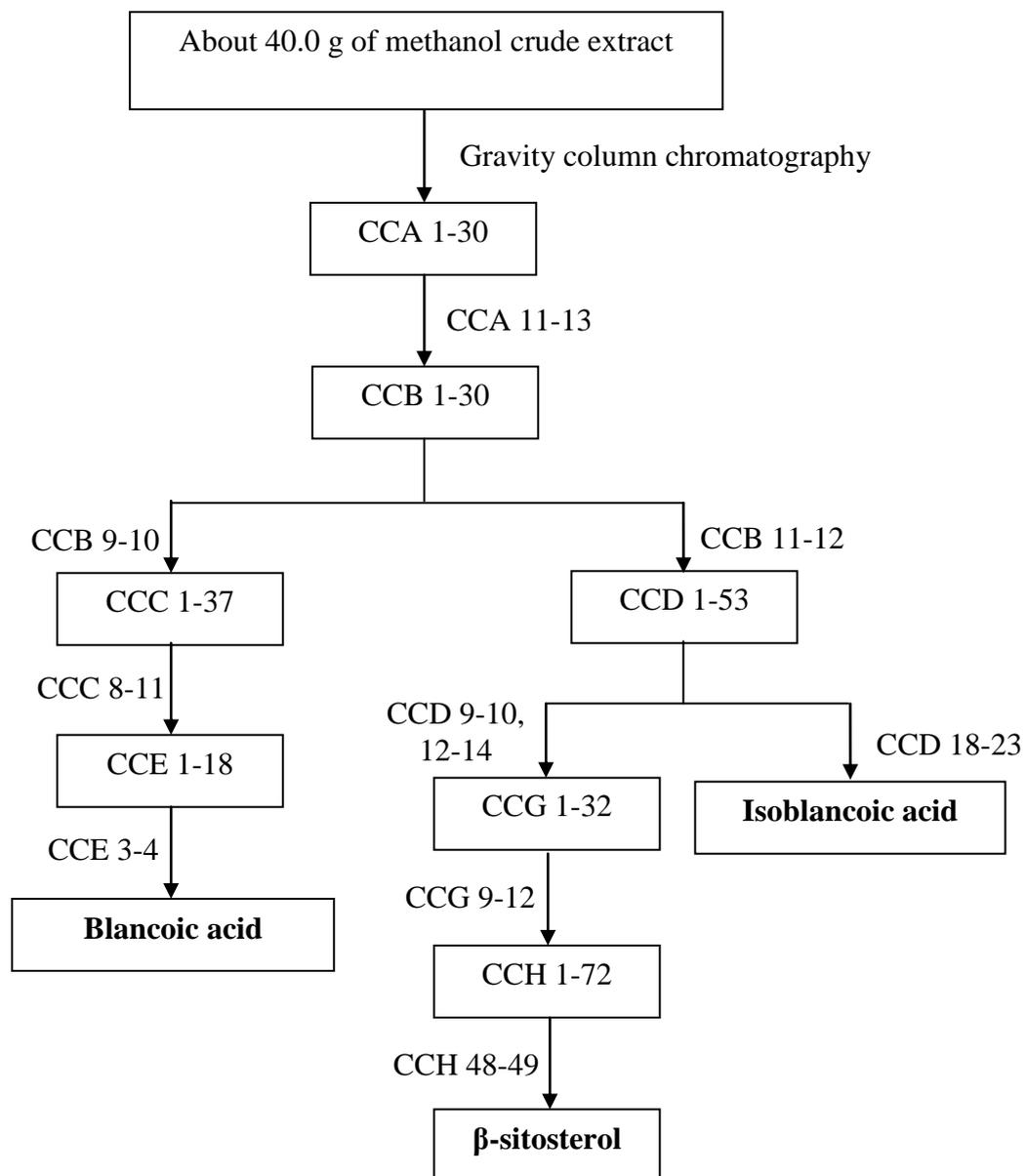
Figure 4.1: The isolation pathway of chemical compounds from
Calophyllum castaneum

4.2 Structural Elucidation and Characterisation of Isoblancoic Acid [66]
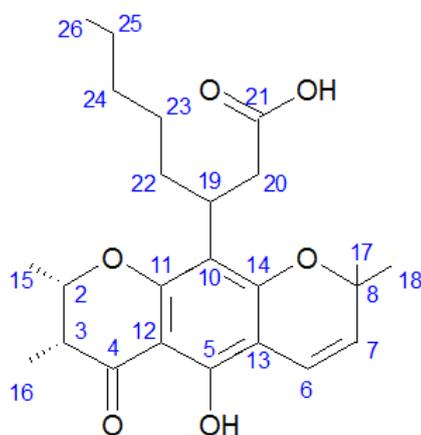
[66]

Figure 4.2: Chemical structure of isoblancoic acid [66]

A total 144 mg of yellow resin was isolated from methanol crude extract. The compound isolated was isoblancoic acid with specific rotation, $[\alpha]_D$ of -41.7°. Compound **66** was subjected to High Resolution Electrospray Ionization Mass Spectrum (HRESIMS) analysis and was confirmed to have a molecular formula of $C_{24}H_{32}O_6$ corresponding to molecular weight of 416.2198 g/mol. The HRESI mass spectrum is shown in Figure 4.3.

The compound was subjected to thin layer chromatography analysis and obtained a single spot with a retention factor, R_f value of 0.48 using a solvent mixture of 20% acetone, 20% dichloromethane and 60% hexane. It was detected as a single spot either in visualisation under UV wavelength of 254 nm or exposure to iodine vapour. Besides that, compound **66** also gave a

positive result in $FeCl_3$ test by showing a dark blue spot which revealing the phenolic nature of the compound. Figure 4.4 shows the development of TLC plate of isoblancoic acid.
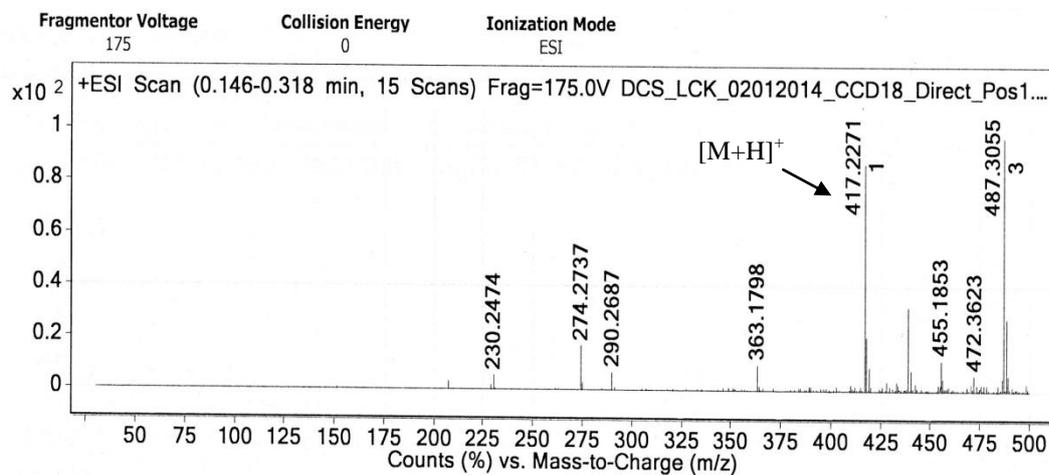
Figure 4.3: HRESIMS spectrum of isoblancoic acid [66]
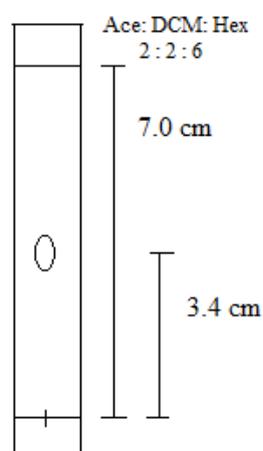
Figure 4.4: TLC development of isoblancoic acid [66]

Chemical structure of compound **66** was elucidated by using 1D- and 2D-NMR. From ^{1}H NMR spectrum (Figure 4.5), a highly deshielded signal at δ_H 12.52 was assigned to the chelated hydroxyl proton attached to carbon C-5. However, no signal was detected for hydroxyl proton attached to carbon C-21

presents due to the rapid hydroxyl proton exchange effect. In addition, a pair of doublet signals observed at δ_H 6.56 (1H, *d*, *J* = 10.0 Hz) and 5.59 (1H, *d*, *J* = 10.0 Hz) was assigned as olefinic protons, H-6 and H-7. Furthermore, five intense peaks observed at δ_H 1.45 (H-17), 1.43 (H-18), 1.38 (H-15), 1.12 (H-16) and 0.81 (H-26), were attributed to six methyl groups in the structure. The triplet signal observed at most upfield region at δ_H 0.81 (3H, *t*, *J* = 7.0 Hz) was assigned to the methyl protons, H-26 in long n-pentyl chain. Based on ^{1}H NMR spectrum (Figure 4.6), compound **66** showed some characteristic peaks similar to blancoic acid. For instance, the signals at δ_H 6.56, 5.59, 1.45 and 1.43 were characteristics proton signals for 2,2-dimethylchromene ring, while a set of proton signals at δ_H 4.60, 2.61, 1.38 and 1.12 indicated the presence of 2,3-dimethylchromanone ring in compound **66**.

The ^{13}C NMR spectrum (Figure 4.7) showed a total of 23 carbon signals assignable to 24 carbons in compound **66**. A very intense peak at δ_C 27.7 was indicative of two combined carbon signals assigned to carbons C-17 and C-18. In general, the spectrum can be divided into two chemical shift regions, whereby signals in the region below δ_C 95.0 were assigned to *sp^3* hybridised carbons while signals in the region above δ_C 95.0 were assigned to *sp^2* hybridised carbons. The most deshielded carbon signal at δ_C 201.6 was assigned to carbonyl carbon C-4. Moreover, the carbon in carboxylic acid group gave a deshielded carbon signal at δ_C 173.4 (C-21). The further assignment for the structure of compound **66** was assisted by DEPT, ^{1}H-^{1}H COSY, HMQC and HMBC spectral analyses.

In DEPT analysis (Figure 4.8), carbon signals were further differentiated into primary, secondary, tertiary and quaternary carbons. DEPT 90° showed a total of five methine (CH) carbon signals assignable to carbons C-7, C-6, C-2, C-3 and C-19. DEPT 135° is used to identify methylene and methyl carbons. Negative peaks in DEPT 135° showed the presence of five methylene (CH_2) carbon signals which were assigned to carbons C-20, C-22, C-24, C-23 and C-25. Moreover, five methyl (CH_3) carbon signals assignable to C-17, C-18, C-15, C-26 and C-16, gave positive peaks in DEPT 135° but showed no signal in DEPT 90[deg]. On the other hand, the remaining nine quaternary carbons C-4, C-5, C-8, C-10, C-11, C-12, C-13, C-14 and C-21 gave signals in DEPT 45°, but no signal was shown in DEPT 90° and 135°. Besides that, these quaternary carbons did not show a direct correlation in HMQC spectrum.

In ^1H-^1H COSY spectrum (Figure 4.9), ^3J coupling between two adjacent protons in compound **66** was studied. There were a total of four ^3J couplings observed in the spectrum. The proton signal δ_H 6.56 was ^3J coupled to proton signal δ_H 5.59 which were assigned to olefinic protons of H-6 and H-7. The correlation of these two adjacent protons was further confirmed with their similar J coupling value of 10.0 Hz. The remaining correlation signals were δ_H 4.60 (H-2) with δ_H 1.38 (H-15), δ_H 2.61 (H-3) with δ_H 1.12 (H-16), and δ_H 1.59 (H-25) with δ_H 0.81 (H-26).

The HMQC analysis helps to establish direct ^1J coupling between protons to their carbons. In the HMQC spectrum (Figure 4.10), proton signals at δ_H 6.56 (H-6), δ_H 5.59 (H-7) and δ_H 4.60 (H-2) were correlated to carbon signals at δ_C

115.3 (C-6), δ_C 126.0 (C-7), δ_C 76.3 (C-2), respectively. The remaining of assigned correlations were indicated in the expanded HMQC spectrum (Figure 4.11).

The HMBC analysis studies the long range coupling between proton signals and their neighbouring carbons. Based on the HMBC spectrum (Figure 4.12), the chelated hydroxyl proton signal (δ_H 12.52, 1H, *s*) showed ^2J coupling with carbon signal C-5 and ^3J coupling with carbon signal C-13, confirming the hydroxyl group to be attached to carbon C-5. Moreover, proton signal at δ_H 6.56 (1H, *d*, *J* = 10.0 Hz) gave cross peaks to carbon signals δ_C 160.0, 157.1, 102.3 and 78.1, indicating proton H-6 to have long range correlations with carbons C-14, C-5, C-13 and C-8, respectively. Based on 1D- and 2D-NMR analyses, compound **66** was unambigiously assigned as isoblancoic acid. Table 4.1 shows the summary of the NMR data for compound **66**.

The structure assignment for compound **66** was further supported by IR and UV-Vis analyses. The IR spectrum (Figure 4.15) shows O-H stretch at 3419 cm^{-1}, C=O stretch at 1645 cm^{-1}, aromatic sp^2 C=C stretch at 1442 cm^{-1} and C-O stretch at 1132 cm^{-1} corresponding to the presence of hydroxyl, carbonyl, aromatic C=C and carbinol functionalities in the structure. On the other hand, the UV-Vis spectrum (Figure 4.16) gives three absorption peaks at 206.27 nm, 274.69 nm and 311.89 nm indicating compound **66** to be a highly conjugated compound.

Table 4.1: Summary of NMR data for isoblancoic acid [66]

Position	δ$_H$ (ppm) & multiplicity	δ$_C$ (ppm)	DEPT	^{1}H-^{1}H COSY	HMBC
2	4.60 (1H, qd, *J*=6.7Hz, 3.0Hz)	76.3	CH	H-15	-
3	2.61 (1H, qd, *J*=7.3Hz, 3.0Hz)	43.9	CH	H-16	C4, 16
4	-	201.6	C	-	-
5	-	157.1	C	-	-
6	6.56 (1H, d, *J*=10.0Hz)	115.3	CH	H-7	C-5, 8, 13, 14
7	5.59 (1H, d, *J*=10.0Hz)	126.0	CH	H-6	C-8, 13, 17, 18
8	-	78.1	C	-	-
10	-	109.6	C	-	-
11	-	159.9	C	-	-
12	-	101.1	C	-	-
13	-	102.3	C	-	-
14	-	160.0	C	-	-
15	1.38 (3H, d, *J*=6.1Hz)	15.7	CH$_3$	H-2	C-2, 3
16	1.12 (3H, d, *J*=7.3Hz)	8.7	CH$_3$	H-3	C-2, 3, 4
17	1.45 (3H, s)	27.7	CH$_3$	-	C-7, 8, 18
18	1.43 (3H, s)	27.7	CH$_3$	-	C-7, 8, 17
19	3.73 (1H, m)	30.8	CH	-	C-10, 11, 14, 20, 22
20	2.70 (2H, dd, *J*=15.3Hz, 7.4Hz)	38.3	CH$_2$	-	C-10, 19, 21
21	-	173.4	C	-	-
22	1.59 (1H, m) 1.50 (1H, m)	33.0	CH$_2$	-	C-10
23	1.17 (2H, m)	27.4	CH$_2$	-	-
24	1.26 (2H, m)	31.7	CH$_2$	-	-
25	1.21 (2H, m)	22.5	CH$_2$	H-26	C-26
26	0.81 (3H, t, *J*=7.0Hz)	13.5	CH$_3$	H-25	C-24, 25
5-OH	12.52 (1H, s)	-	-	-	C-5, 13

Figure 4.5: ¹H NMR spectrum of isoblancoic acid [66]
(400 MHz, acetone *d₆*)
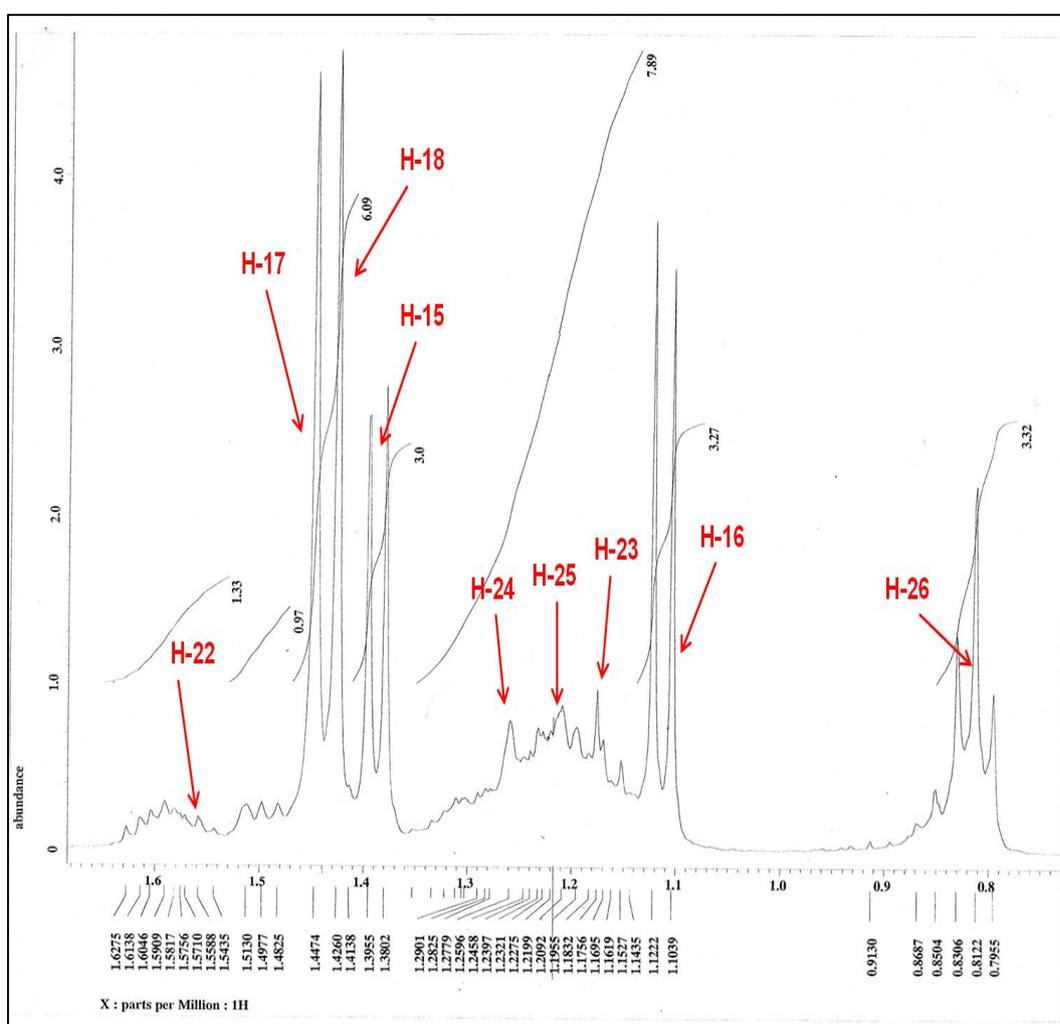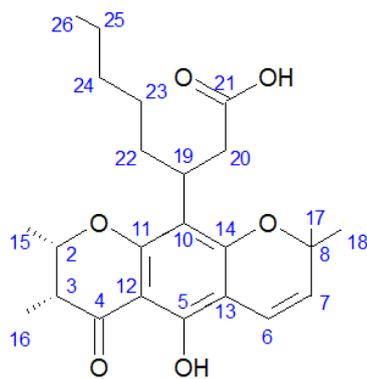
Figure 4.6: ^{1}H NMR spectrum of isoblancoic acid [66]
(400 MHz, acetone-d_6) (expanded)
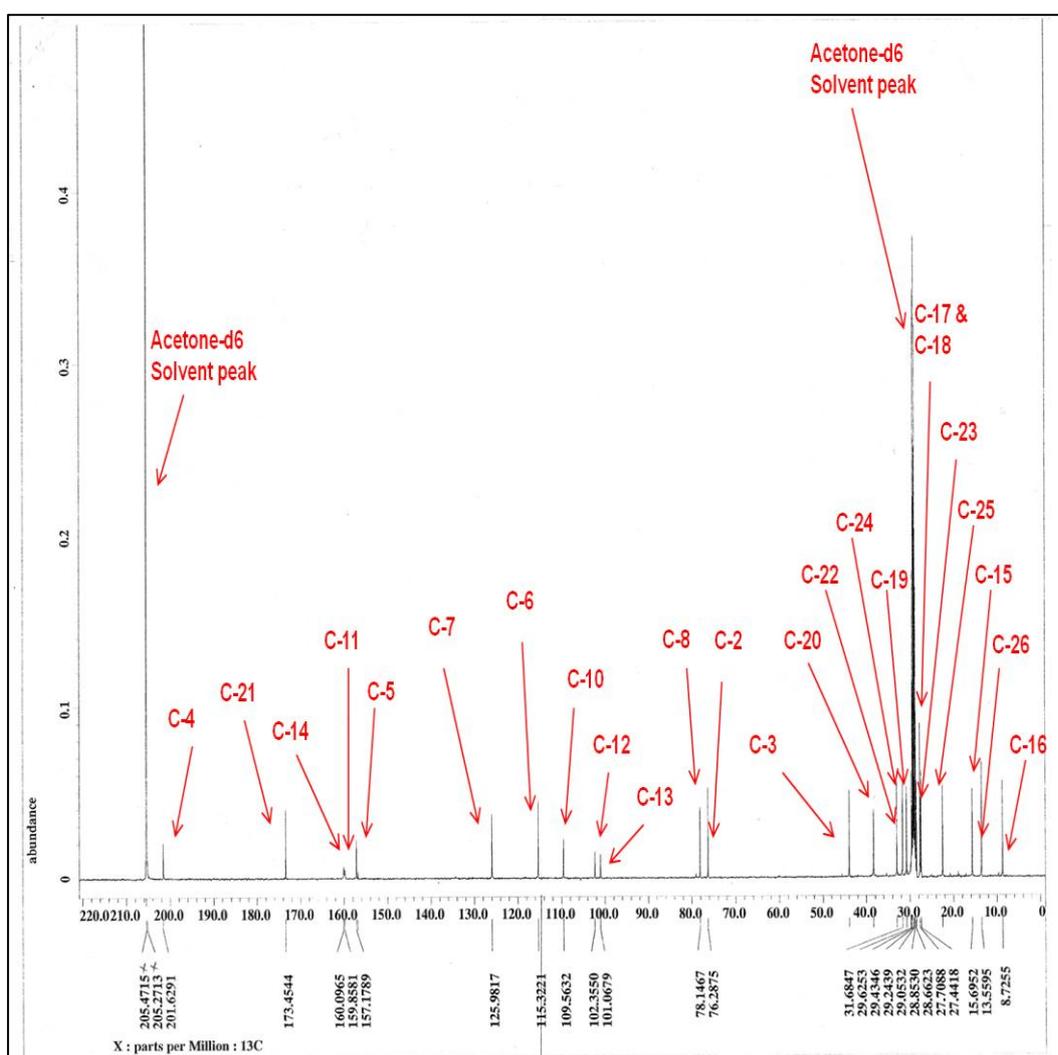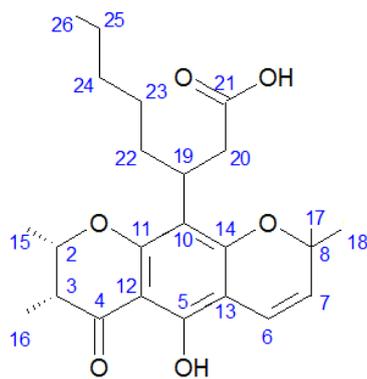
Figure 4.7: ^{13}C NMR spectrum of isoblancoic acid [66]
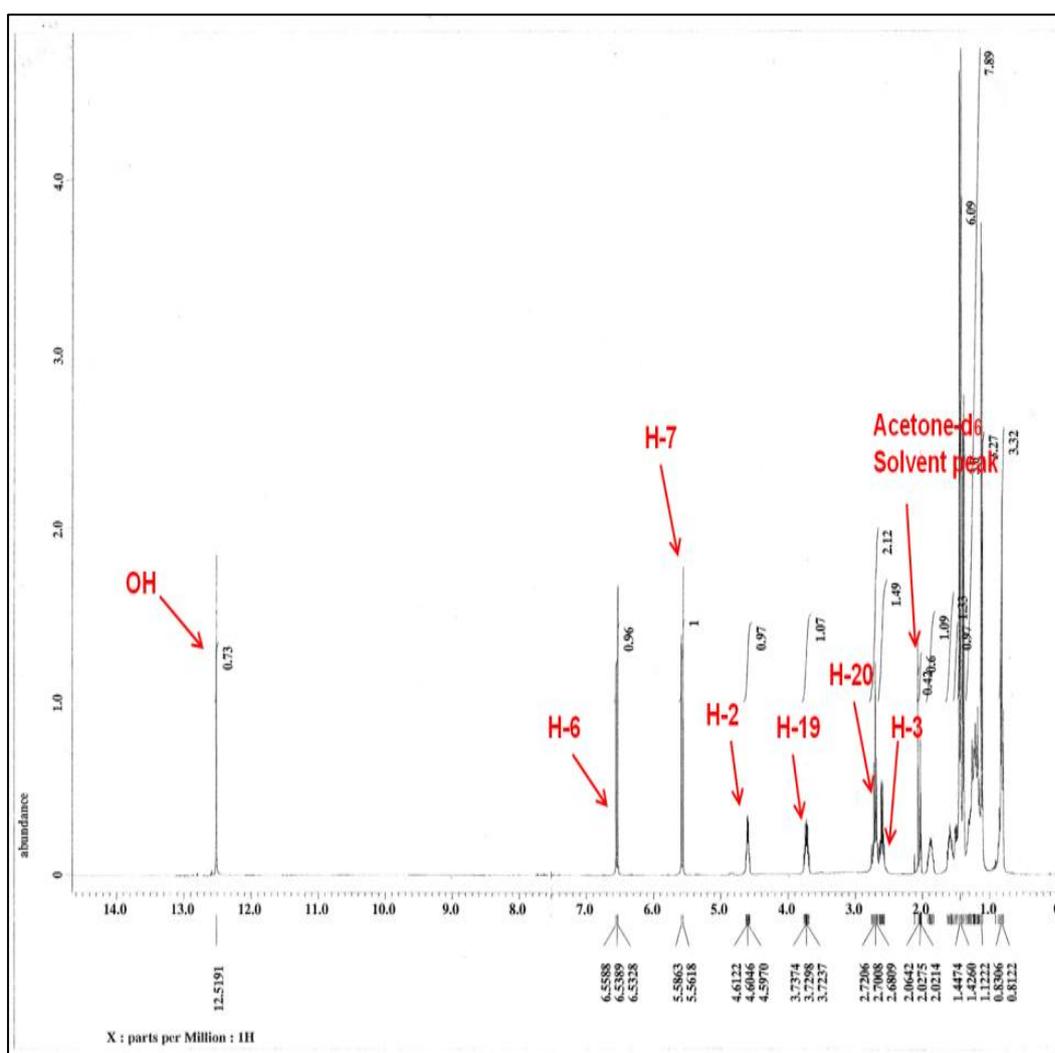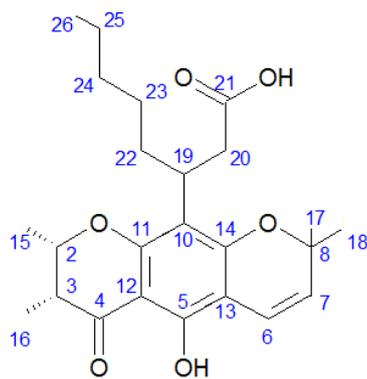(100 MHz, acetone-d_6)
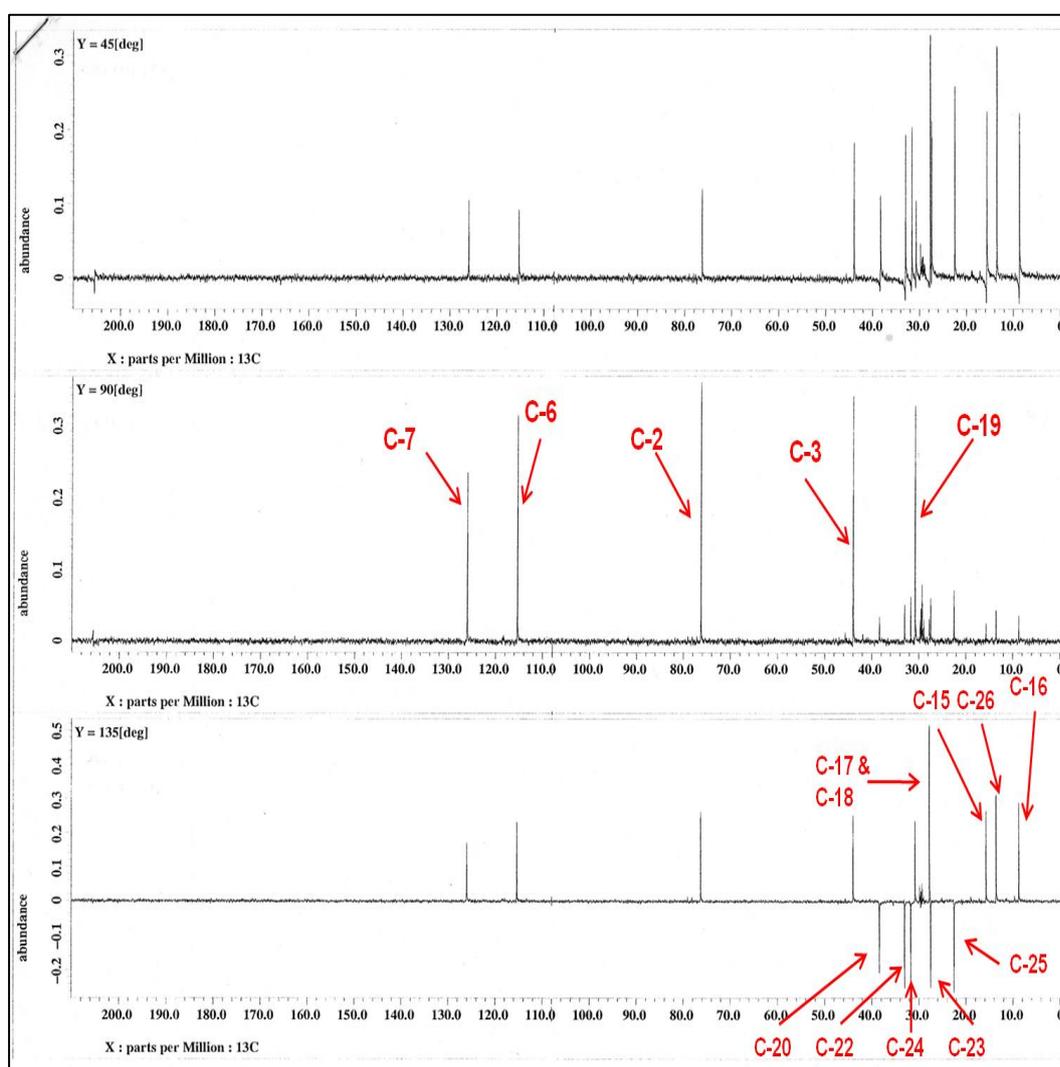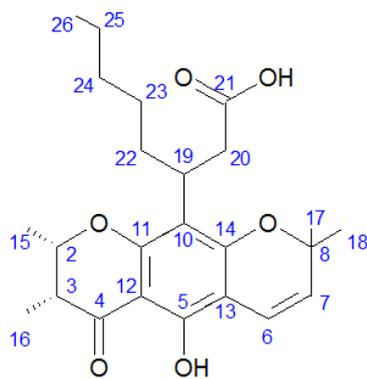
Figure 4.8: DEPT spectrum of isoblancoic acid [66] (100 MHz, acetone-d_6)
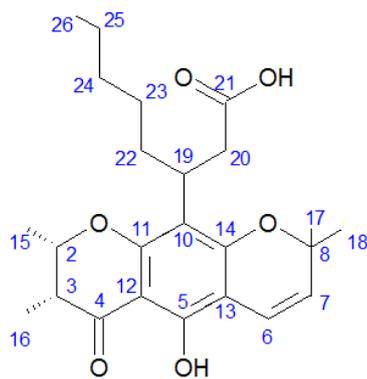
Figure 4.9: ^{1}H-^{1}H COSY spectrum of isoblancoic acid [66]
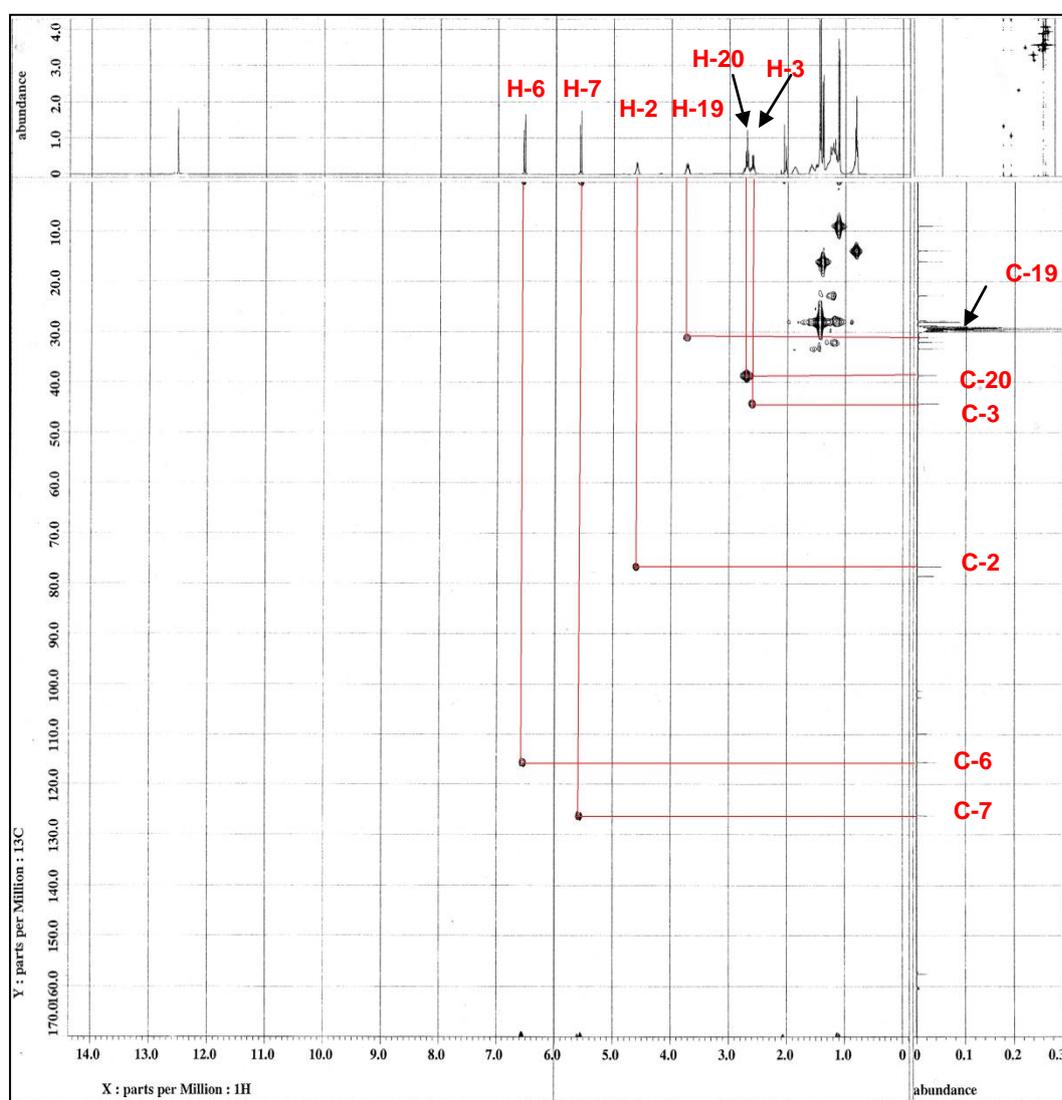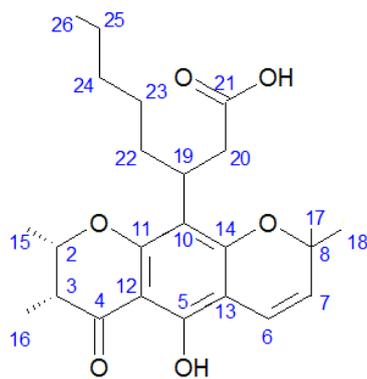
Figure 4.10: HMQC spectrum of isoblancoic acid [66]
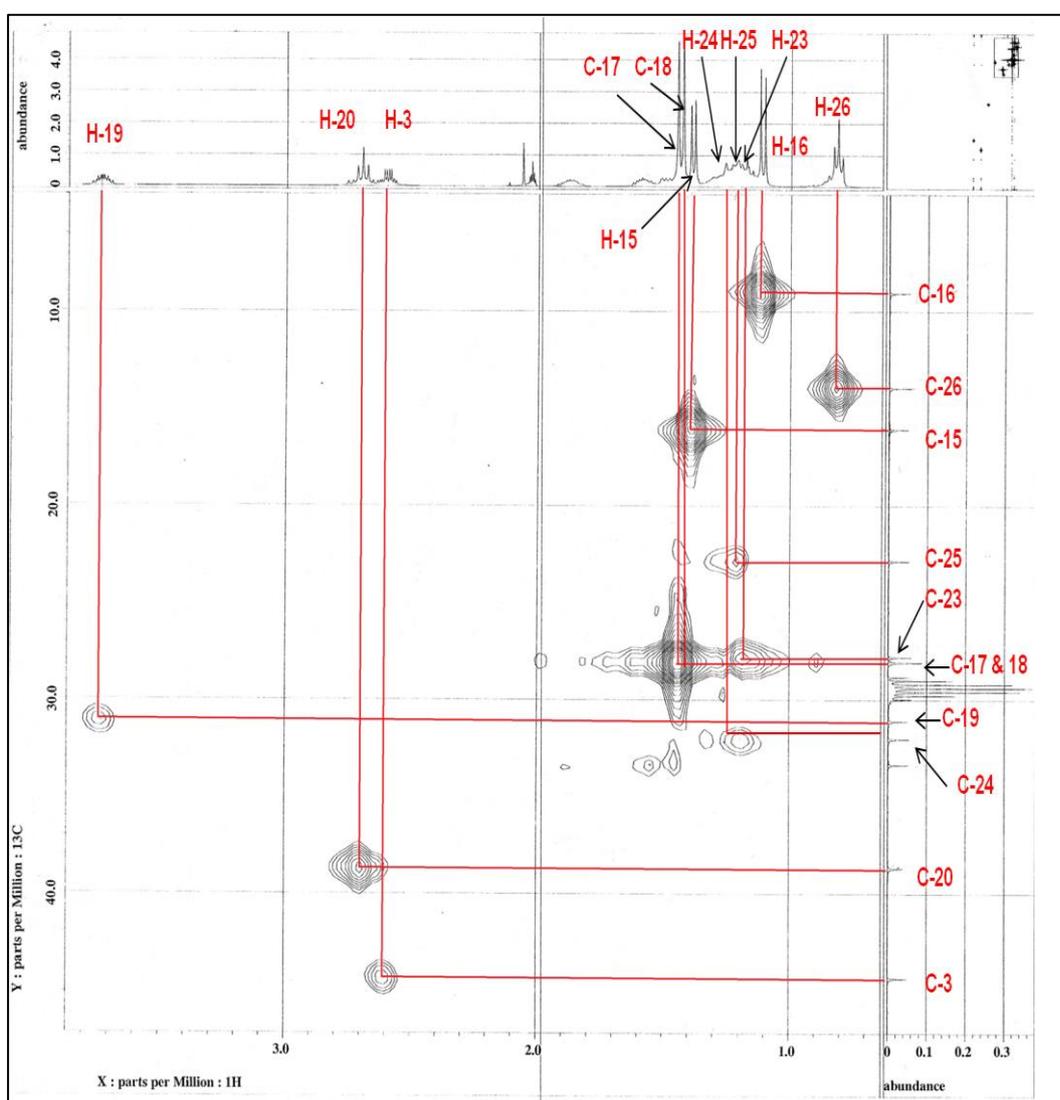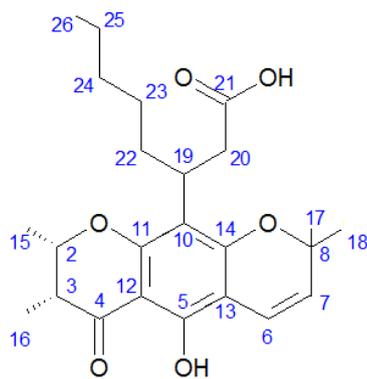
Figure 4.11: HMQC spectrum of isoblancoic acid [66] (expanded)
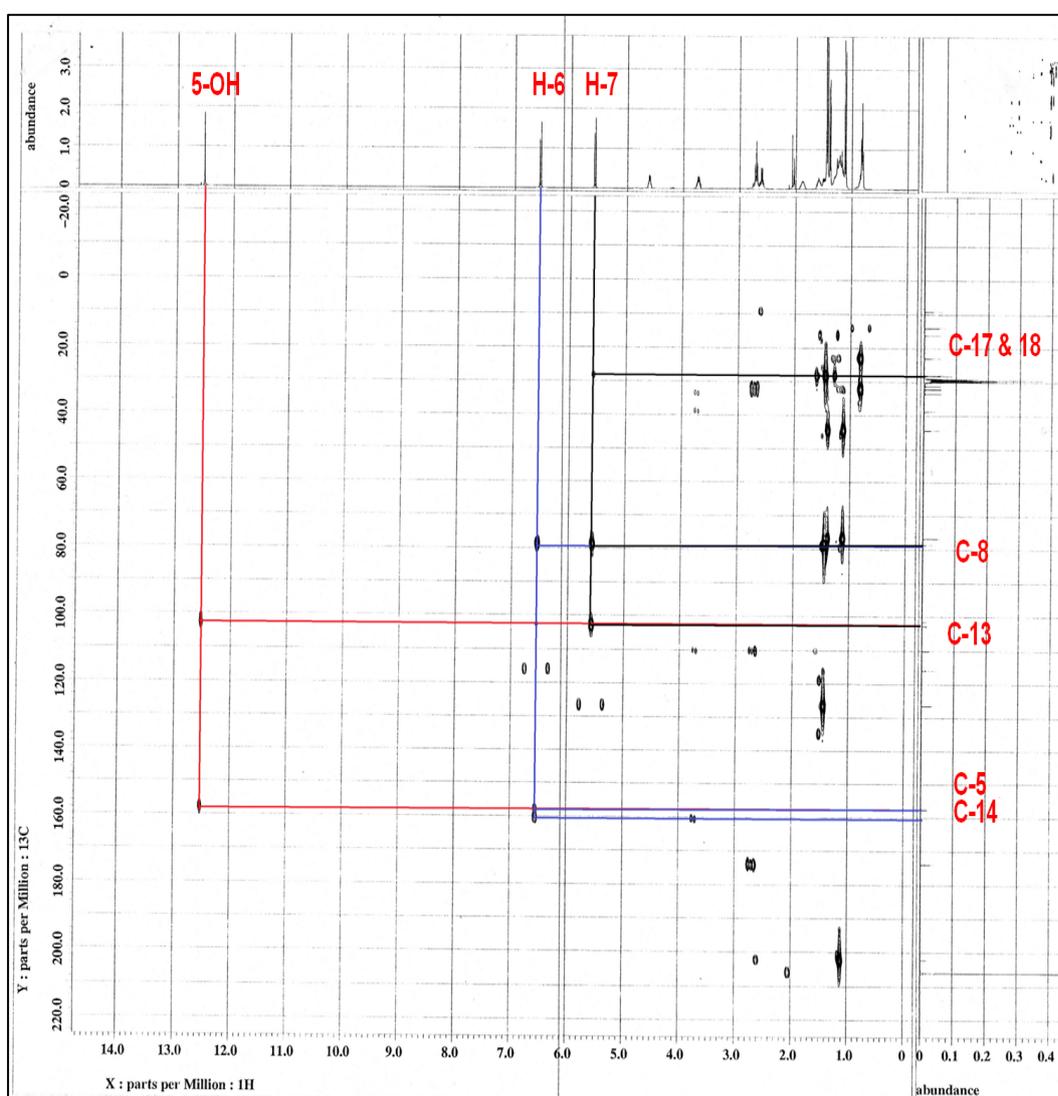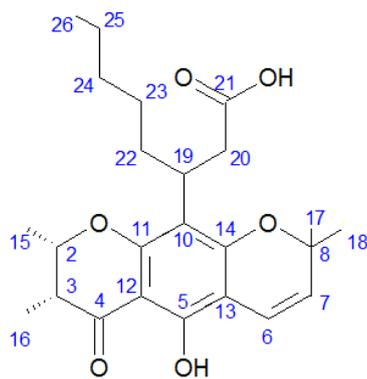
Figure 4.12: HMBC spectrum of isoblancoic acid [66]
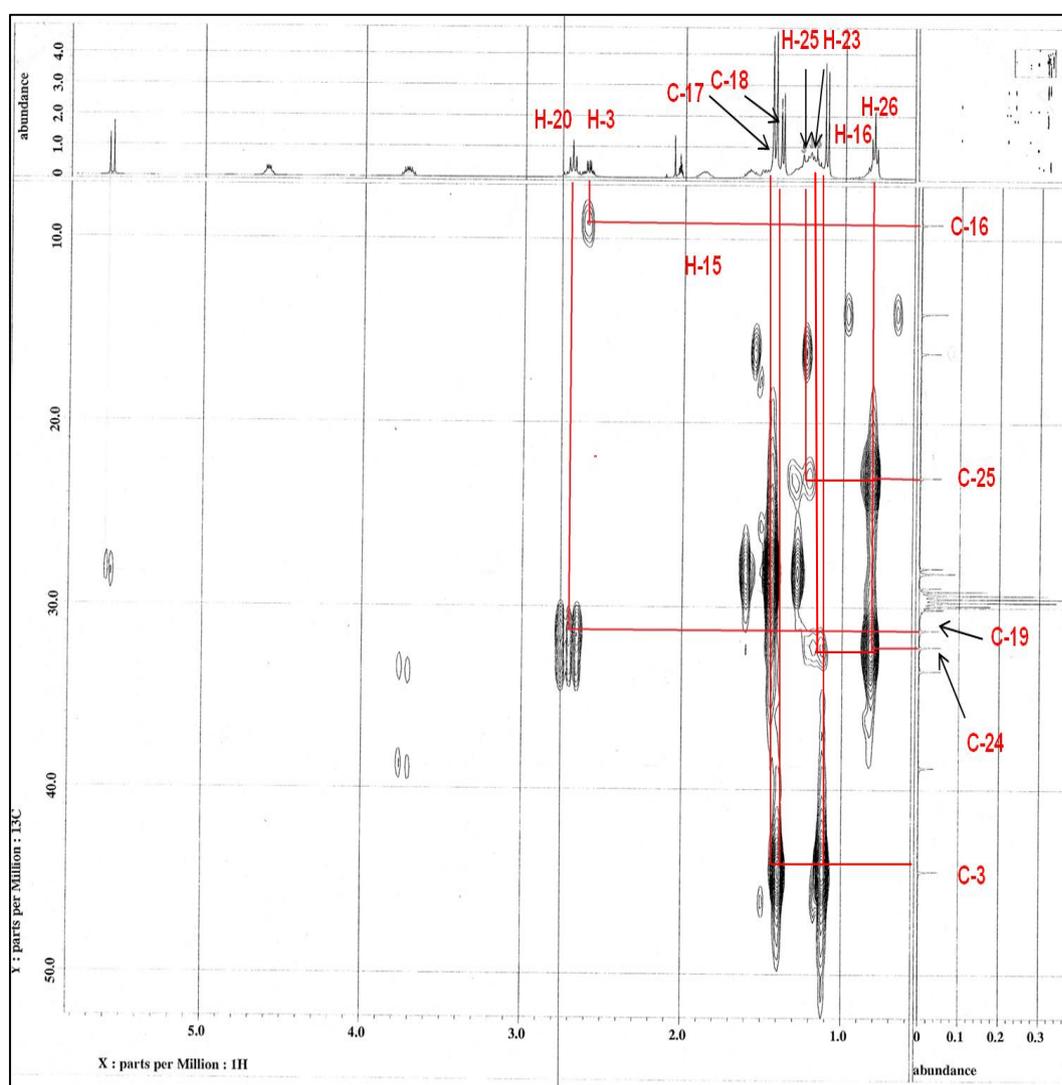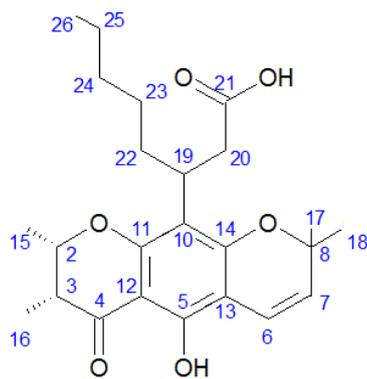
Figure 4.13: HMBC spectrum of isoblancoic acid [66] (expanded)

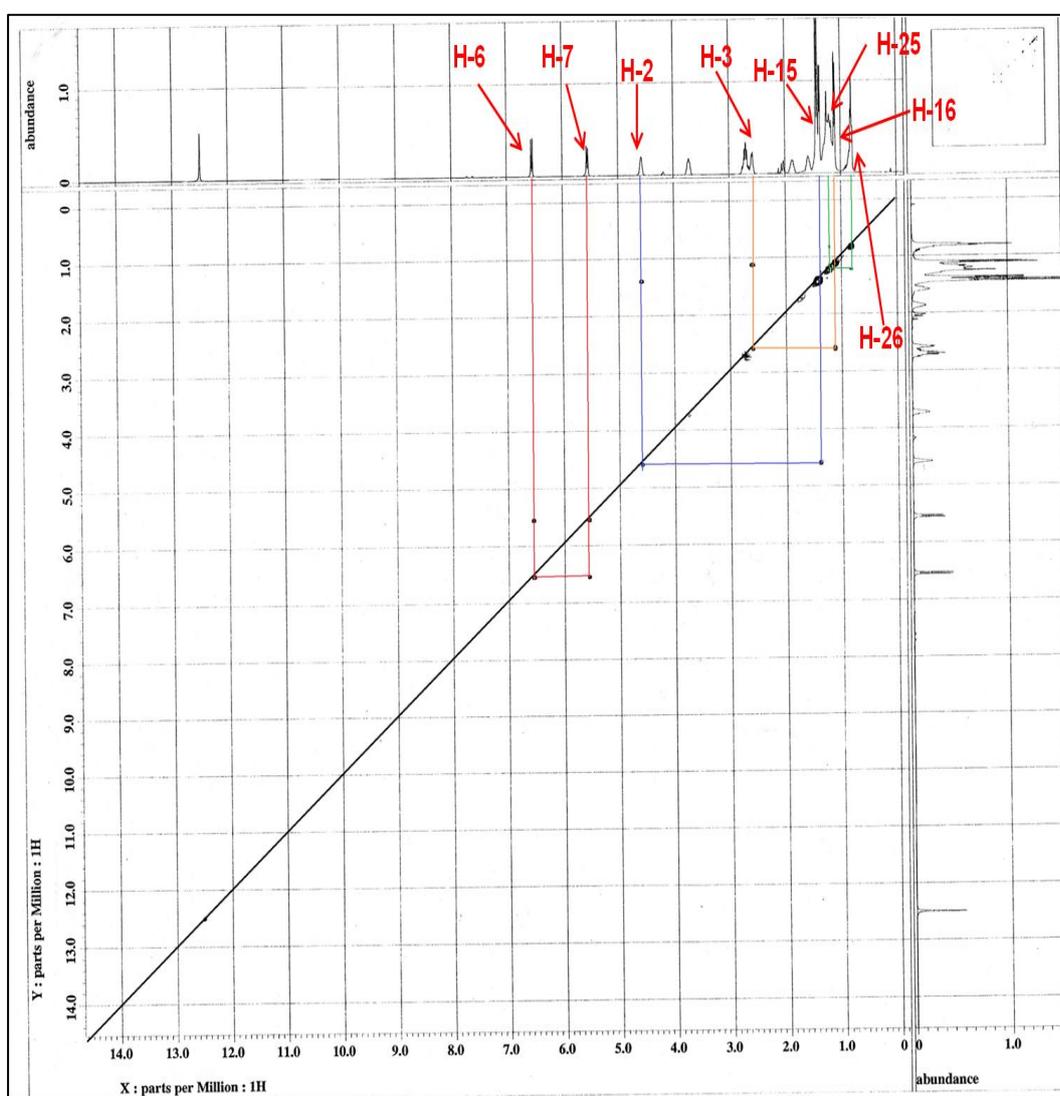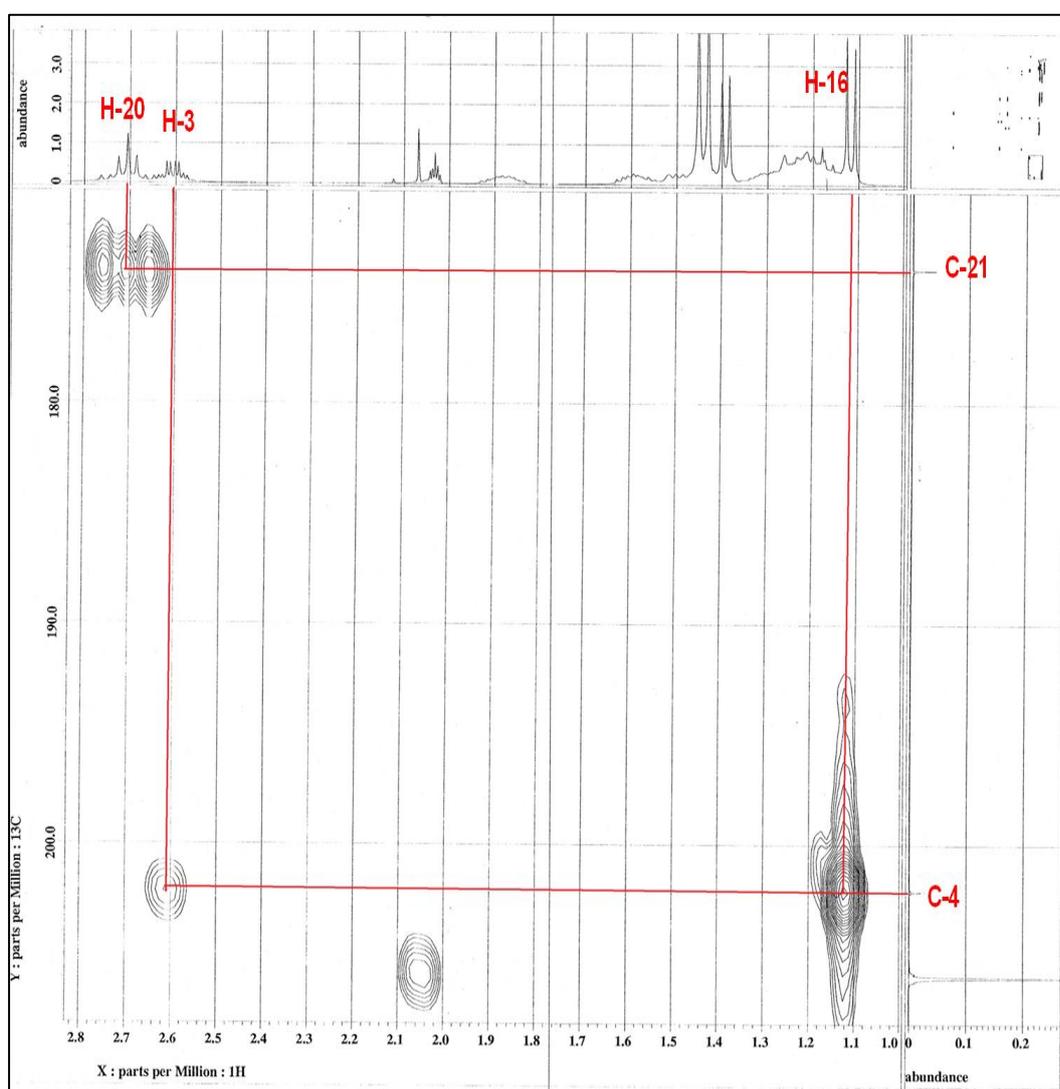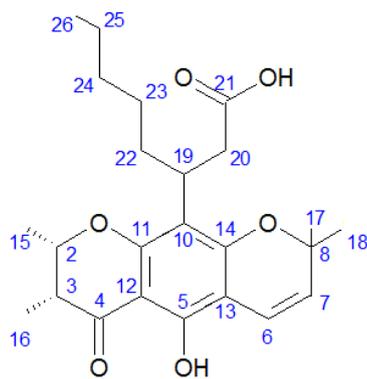**Figure 4.14: HMBC spectrum of isoblancoic acid [66] (expanded)**
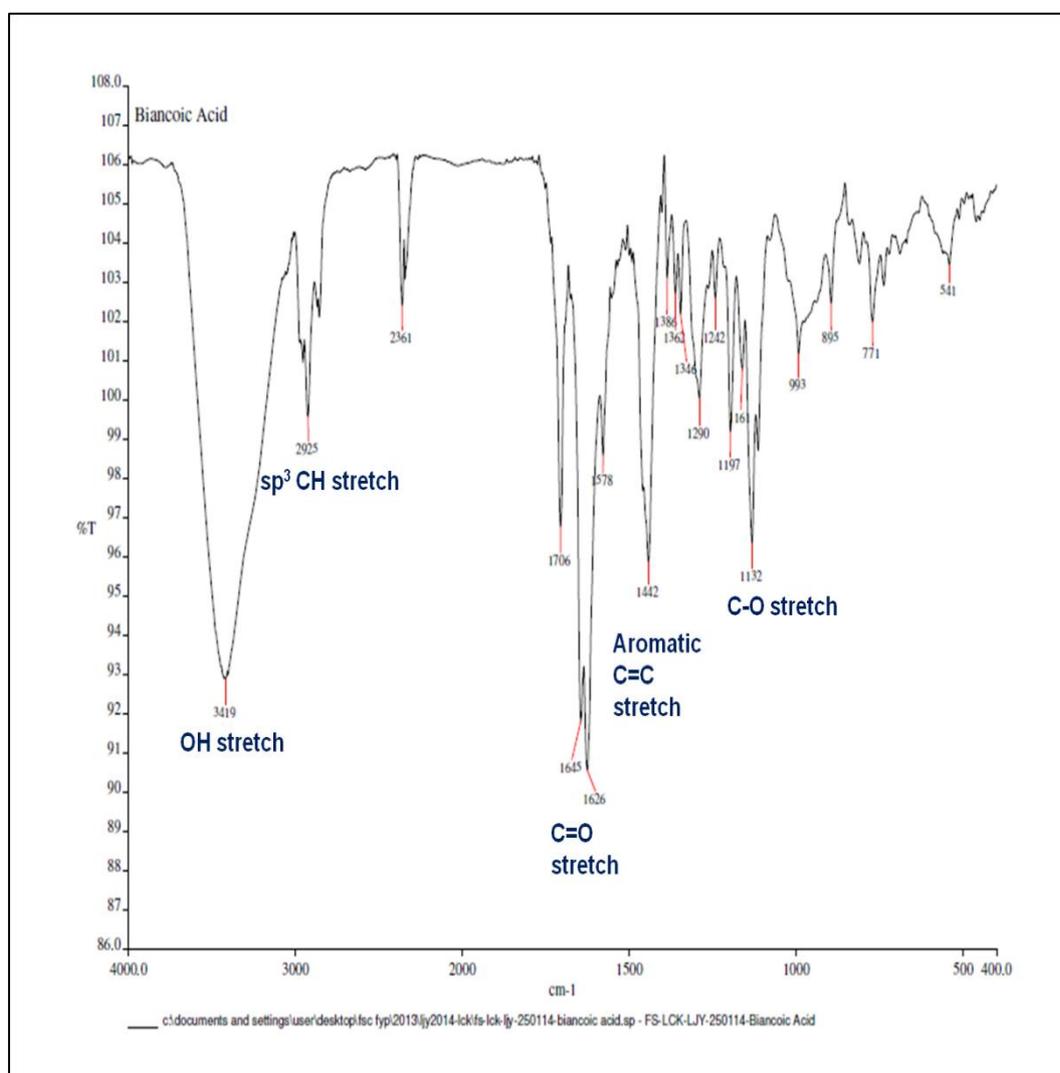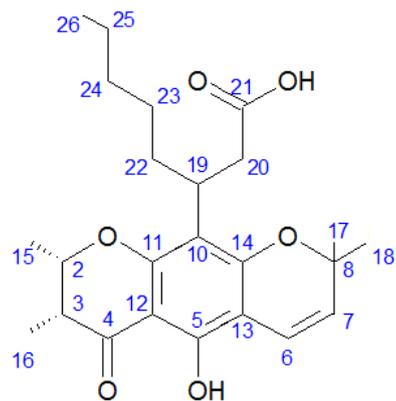
Figure 4.15: IR spectrum of isoblancoic acid [66]
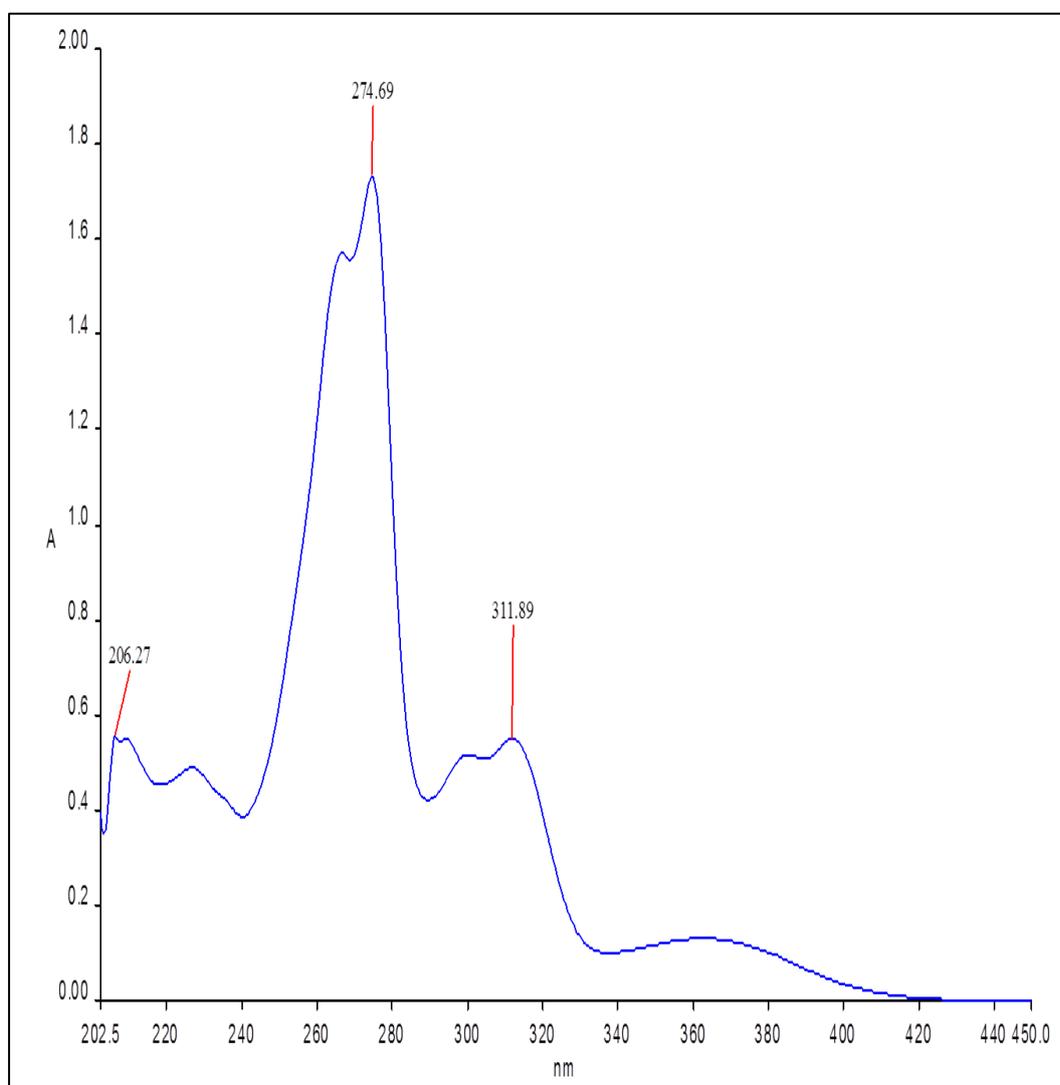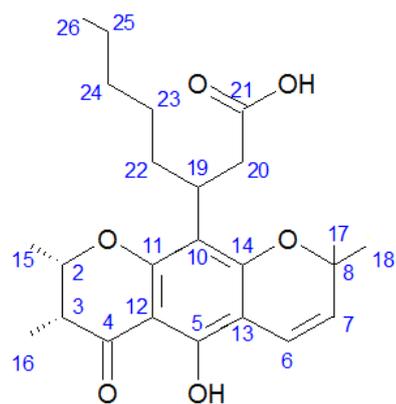
Figure 4.16: UV-Vis spectrum of isoblancoic acid [66]

4.3 Structural Elucidation and Characterisation of Blancoic Acid [67]
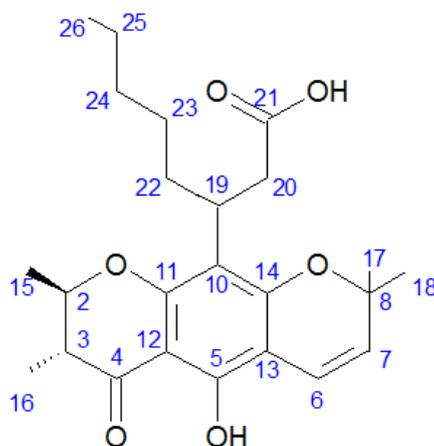
[67]

Figure 4.17: Chemical structure of blancoic acid [67]

Compound **67** was isolated as green resin with the amount of 79 mg. The compound has a molecular formula $C_{24}H_{32}O_6$ corresponding to molecular weight of 416.2204 g/mol from the HRESI mass spectrum (Figure 4.18). Since it is a stereoisomer of isoblancoic acid, thus it shares almost similar characteristic with isoblancoic acid, such as non-crystalline appearance. Compound **67** was subjected to optical activity test and obtained a specific rotation, $[\alpha]_D$ of -66.9°, being different from compound **66**.

In TLC analysis, a single spot with a retention factor, R_f value of 0.67 was obtained using a mixture of acetone: dichloromethane: hexane in 1:1:3 ratio as mobile phase. Compound **67** showed a dark spot on TLC plate when visualized under UV short wavelength (254 nm) and iodine vapour. It gave a positive result in $FeCl_3$ test indicating the compound to have phenolic nature.

The R_f value of *trans* isomer **[67]** was higher than *cis* isomer **[66]**, which revealed that *trans* isomer was less polar than *cis* isomer. Figure 4.19 shows the developed TLC plate of blancoic acid **[67]**.
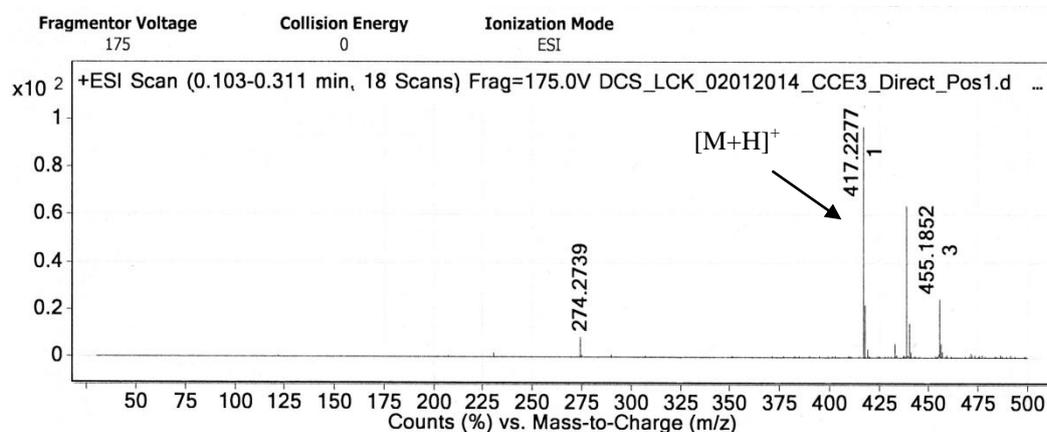
Figure 4.18: HRESIMS spectrum of blancoic acid [67]
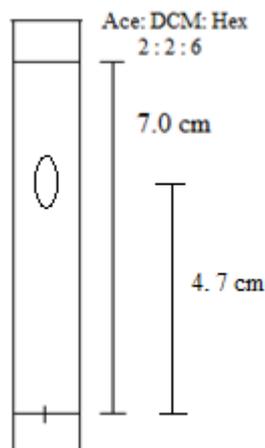
Figure 4.19: TLC development of blancoic acid [67]

According to ^{1}H and ^{13}C NMR spectra (Figures 4.20, 4.21 and 4.22), compound **67** gave almost identical spectral characteristics as isoblancoic acid **[66]** discussed in **section 4.2**. Spectral analysis revealed the presence of chelated hydroxyl proton [δ_H 12.45 (1H, s, 5-OH)] with its related chelating

carbonyl carbon [δ_C 199.5 (C-4)], a carboxylic acid group [δ_C 179.7 (C-21)], a 2,2-dimethyl chromene system [δ_H 6.59 (1H, d, J = 10.4 Hz, H-6), δ_H 5.45 (1H, d, J = 9.8 Hz, H-7), δ_H 1.41 (3H, s, H-17) and δ_H 1.37 (2H, d, J = 6.1 Hz, H-18); δ_C 125.7 (C-7), 157.1 (C-5), 78.2 (C-8), 28.4 (C-17) and 28.3 (C-18)], a 2,3-dimethyl chromanone moiety [δ_H 4.06 (1H, m, H-2), δ_H 2.50 (1H, m, H-3), δ_H 1.46 (3H, d, J = 6.7 Hz, H-15) and δ_H 1.18 (3H, d, J = 6.7 Hz, H-16); δ_C 199.5 (C-4), 79.0 (C-2), 45.8 (C-3), 19.6 (C-15) and 10.5 (C-16)]. In addition, the 1D-NMR also showed the presence of four methylene groups, one methine group [δ_H 3.65 (1H, m, H-19); δ_C 30.7 (C-19)] and one methyl group [δ_H 0.81 (3H, t, J = 6.7 Hz, H-26); δ_C 14.2 (C-26)] for the carboxypentyl side chain.

The assignment was further confirmed by HMBC analysis. According to HMBC spectrum (Figure 4.26), the methylene protons at δ_H 2.80 (H-20a) and 2.66 (H-20b) were ^2J coupled to the carboxyl carbon of COOH group (δ_C 179.7, C-21), and also ^2J coupled to a methine carbon (δ_C 30.7, C-19). Moreover, the methylene protons H-20a and 20b showed a crosspeak with a methylene carbon, C-22 at δ_C 33.2. In addition, the proton signals for H-19 and H-20 showed correlations to a substituted aromatic carbon signal at δ_C 109.1 which was assigned to carbon C-10. Apart from that, the proton signal for H-19 also furnished a crosspeak with oxygenated carbon signal (C-14) at δ_C 160.0, which indicated the carboxypentyl side chain was attached to the chromanone-chromene nucleus at carbon C-10.

As a summary of NMR data analysis, blancoic acid [67] showed a very similar assignment with reference to the stereoisomer isoblancoic acid [66]. The only structural difference between the two stereoisomers is the placement of methyl groups at carbon positions C-2 and C-3 in the dimethylchromanone ring, which can be observed for their chemical shift difference for their ^{1}H NMR data. In ^{1}H NMR spectra (Figures 4.5 and 4.20), blancoic acid gave proton signals for H-2 and H-3 at δ_H 4.09 and 2.50, respectively which revealed the two methyl group were separately attached to carbons C-2 and C-3 in *trans*-configuration in the 2,3-dimethylchromanone ring. Meanwhile, isoblancoic acid [66] showed proton signals for H-2 and H-3 at δ_H 4.60 and 2.61, respectively revealing the two methyl groups attached were in *cis*-configuration. Based on this comparison, chemical shift of proton H-2 in blancoic acid [67] was found to be shifted to more downfield region when the 2,3-dimethyl groups were changed from *trans* to *cis* configuration. Table 4.2 shows the summary of the NMR data for blancoic acid [67].

The structure of blancoic acid was further confirmed by IR and UV-Vis analyses. The presence of various functional groups in the structure can be identified from IR analysis. The IR spectrum (Figure 4.27) exhibited absorption bands at 3430 cm^{-1} (O-H stretch), 1628 cm^{-1} (C=O stretch), 1445 cm^{-1} (aromatic sp^2 C=C stretch) and 1189 cm^{-1} (C-O stretch). On the other hand, the UV-Vis spectrum (Figure 4.28) showed absorption maxima at 209.5 nm, 274.41 nm and 311.31 nm, which revealed conjugation nature of compound 67.

Table 4.2: Summary of NMR data for blancoic acid [67]

Position	δ_H (ppm) & multiplicity	δ_C (ppm)	^{1}H-^{1}H COSY	HMBC
2	4.09 (1H, m)	79.0	-	-
3	2.50 (1H, m)	45.8	H-16	C-2, 4,15, 16
4	-	199.5	-	-
5	-	157.1	-	-
6	6.59 (1H, d, J=10.0Hz)	115.7	H-7	C-5, 8, 13, 14
7	5.45 (1H, d, J=10.0Hz)	125.7	H-6	C-8, 13, 17
8	-	78.2	-	-
10	-	109.1	-	-
11	-	157.1	-	-
12	-	101.9	-	-
13	-	102.6	-	-
14	-	160.0	-	-
15	1.46 (3H, d, J=6.7Hz)	19.6	-	C-2, 3
16	1.18 (3H, d, J=6.7Hz)	10.5	H-3	C-2, 3, 4
17	1.41 (3H, s)	28.4	-	C-7, 8, 18
18	1.37 (3H, s)	28.3	-	C-7, 8, 17
19	3.65 (1H, m)	30.7	-	C-10, 14, 20, 22
20	2.80 (1H, dd, J=15.8Hz, 8.5Hz) 2.66 (1H, dd, J=15.8Hz, 6.7Hz)	38.8	-	C-10, 19, 21, 22
21	-	179.7	-	-
22	1.82 (1H, m) 1.54 (1H, m)	33.2	-	C-10, 23
23	1.20 (2H, m)	27.5	-	-
24	1.29 (2H, m)	31.8	-	-
25	1.24 (2H, m)	22.6	H-26	-
26	0.81 (3H, t, J=6.7Hz)	14.2	H-25	C-24, 25
5-OH	12.45 (1H, s)	-	-	C-5, 12, 13
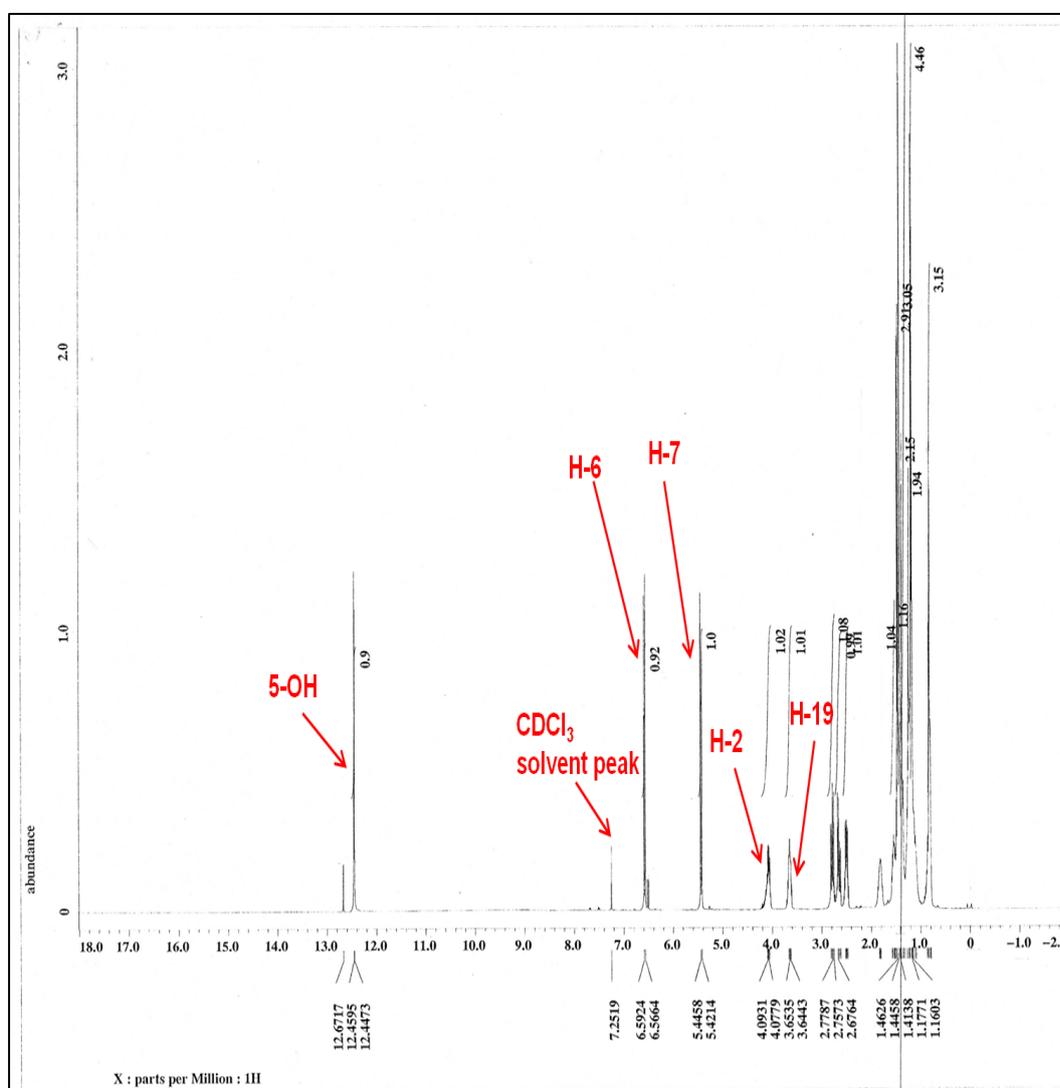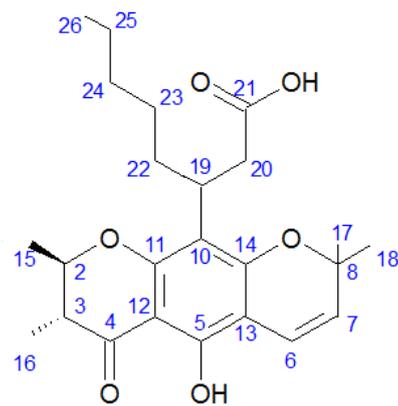
Figure 4.20: ^{1}H NMR spectrum of blancoic acid [67] (400 MHz, CDCl$_3$)
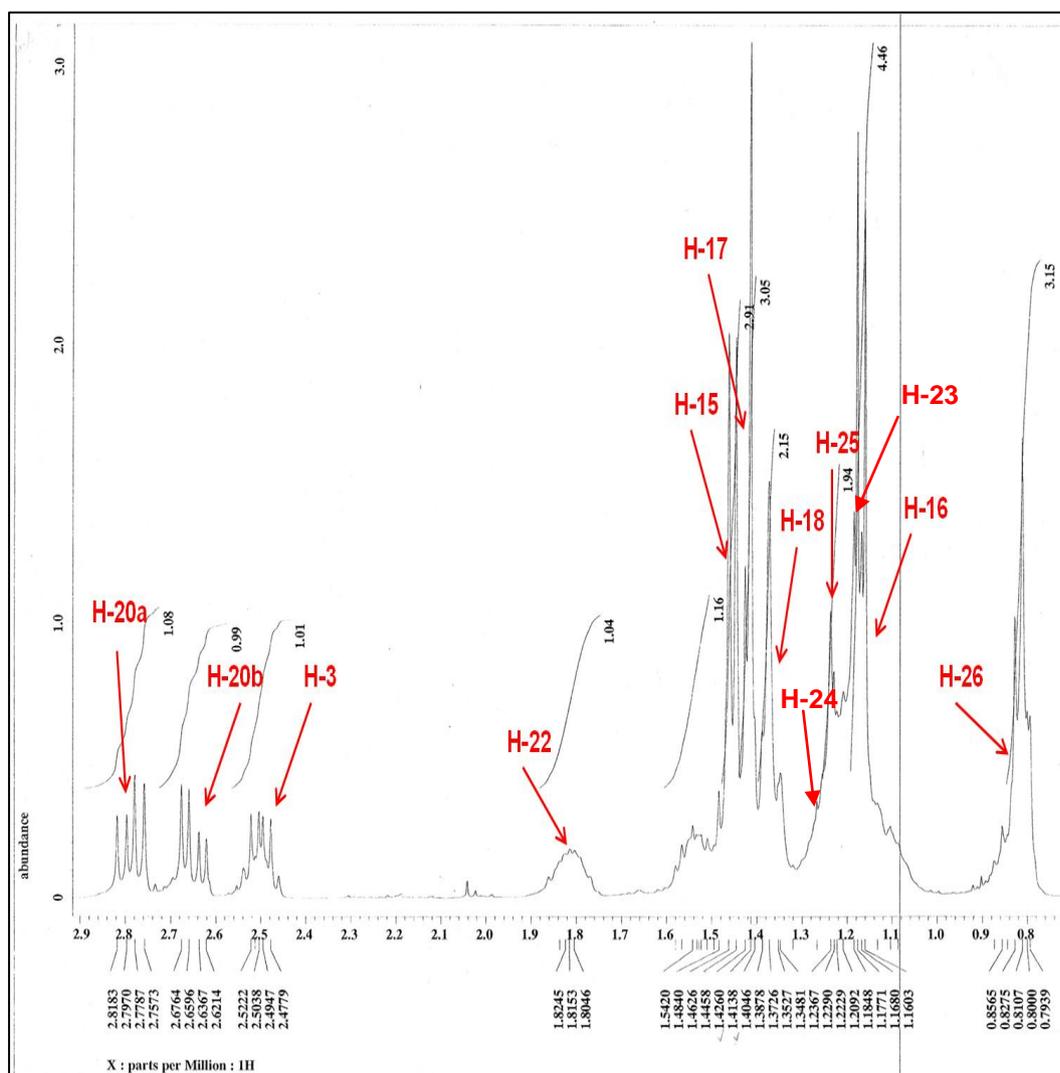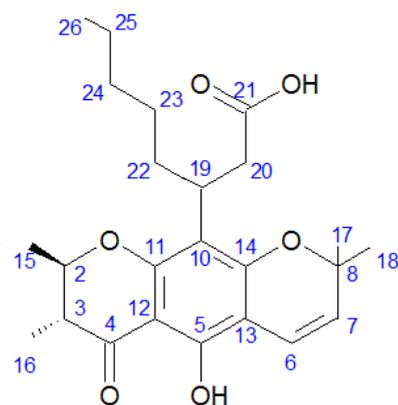
Figure 4.21: ^{1}H NMR spectrum of blancoic acid [67] (400 MHz, CDCl$_3$) (expanded)
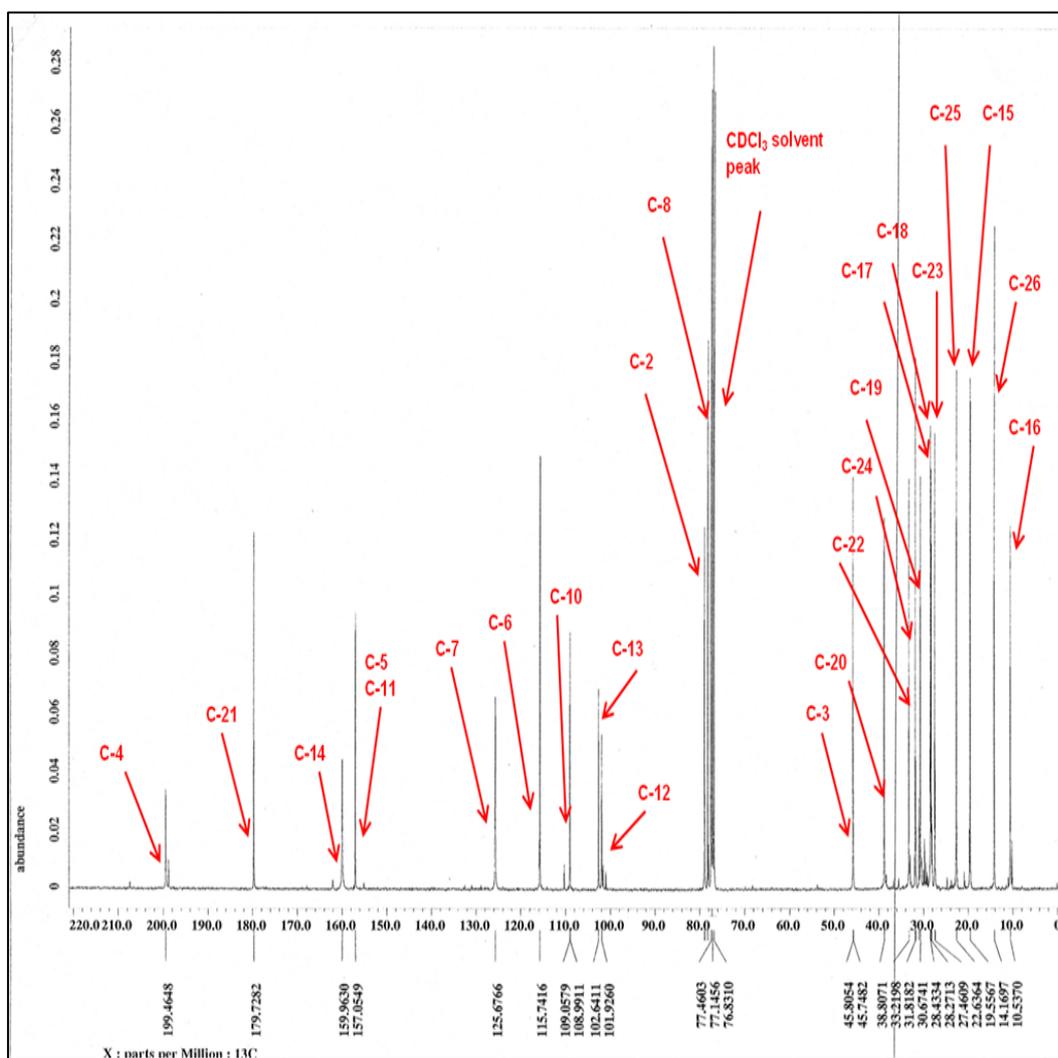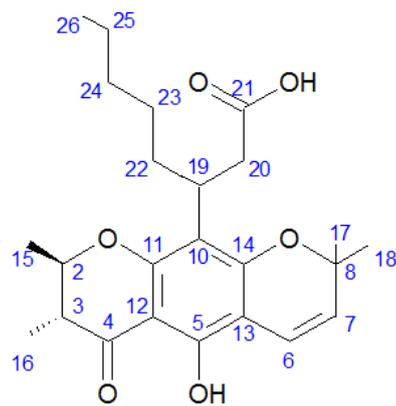
Figure 4.22: ^{13}C NMR spectrum of trans-blancoic acid [67]
(100 MHz, CDCl$_3$)
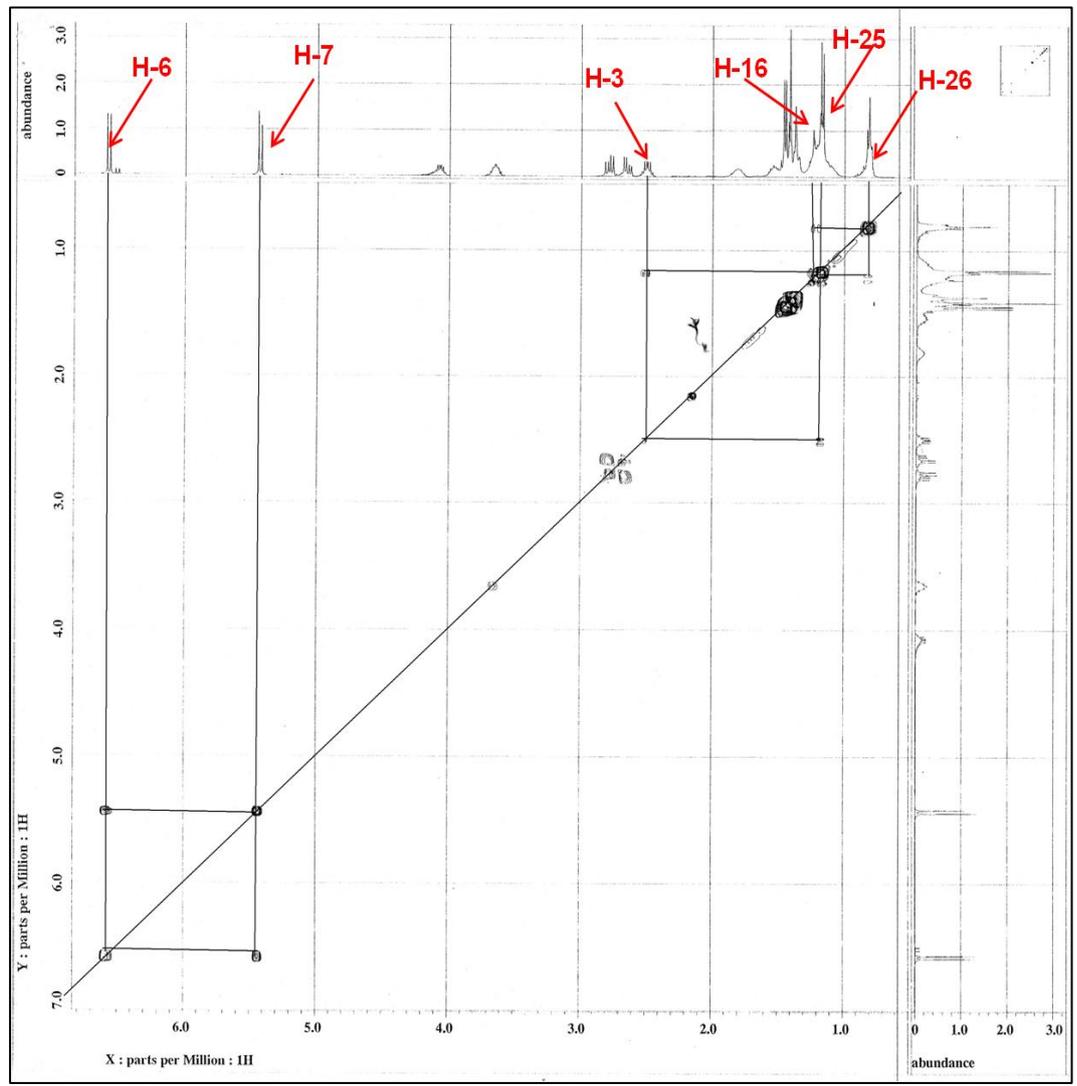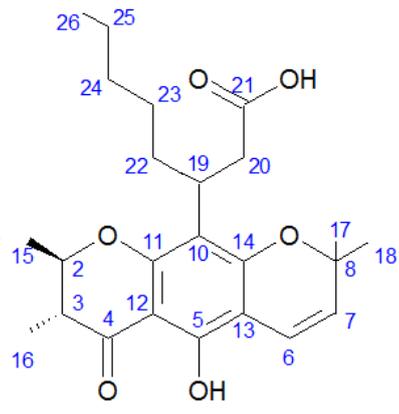
Figure 4.23: ¹H-¹H COSY spectrum of blancoic acid [67]
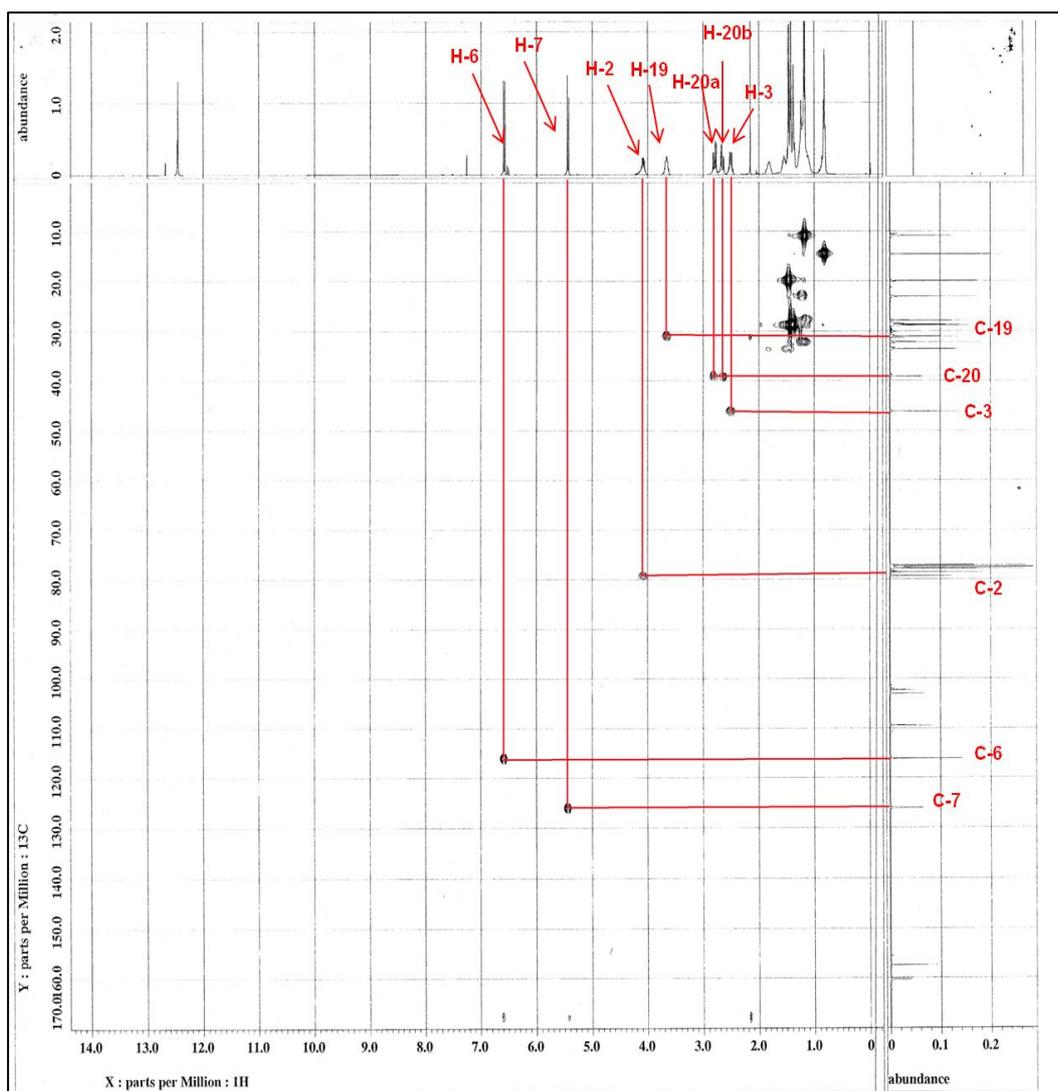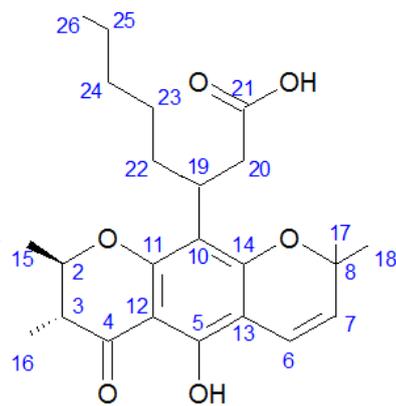
Figure 4.24: HMQC spectrum of blancoic acid [67]
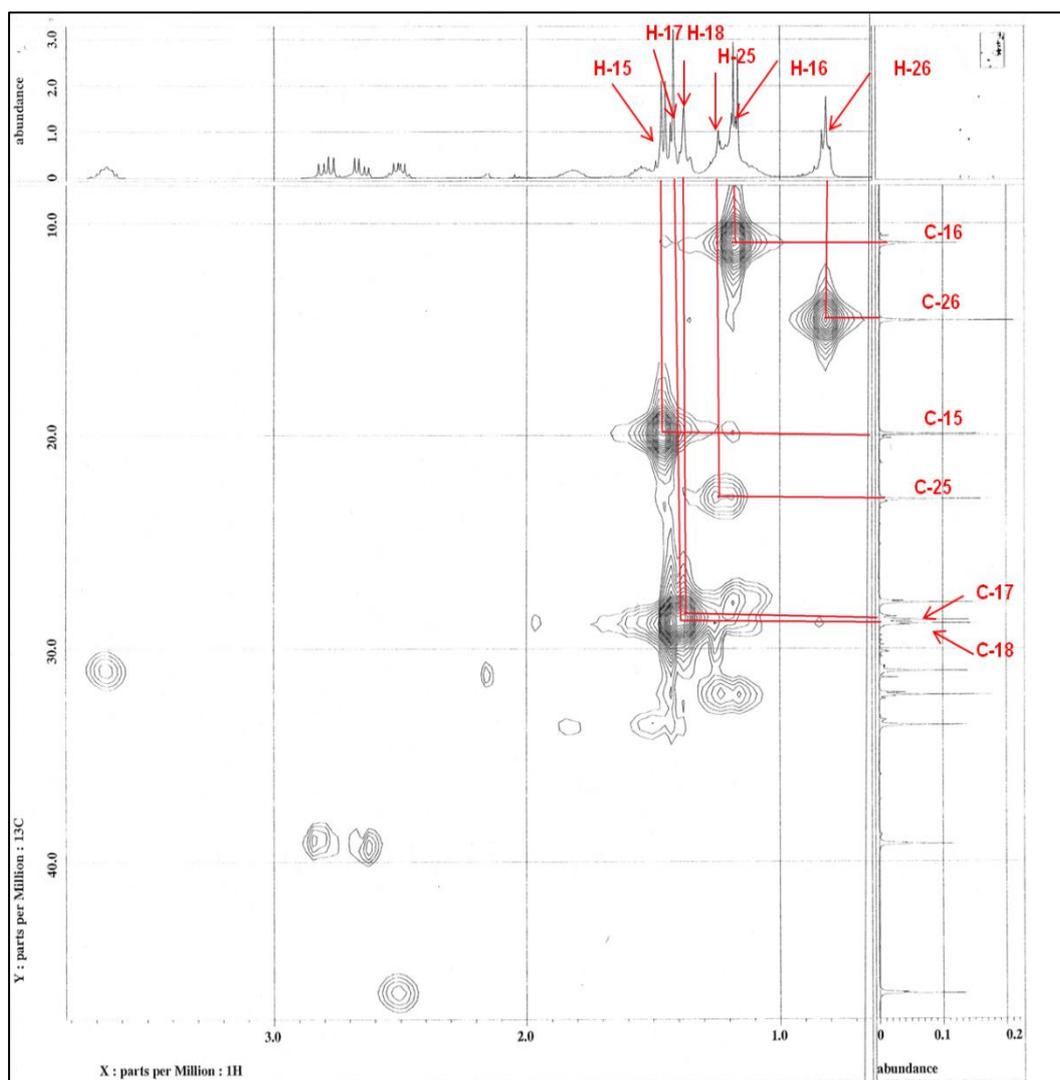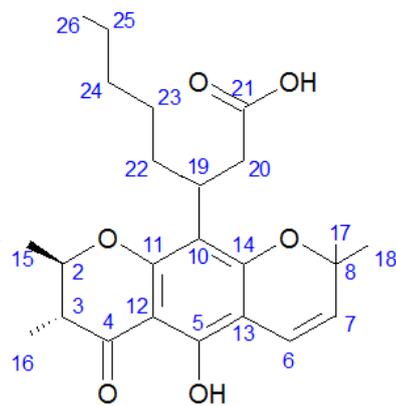
Figure 4.25: HMQC spectrum of blancoic acid [67] (expanded)
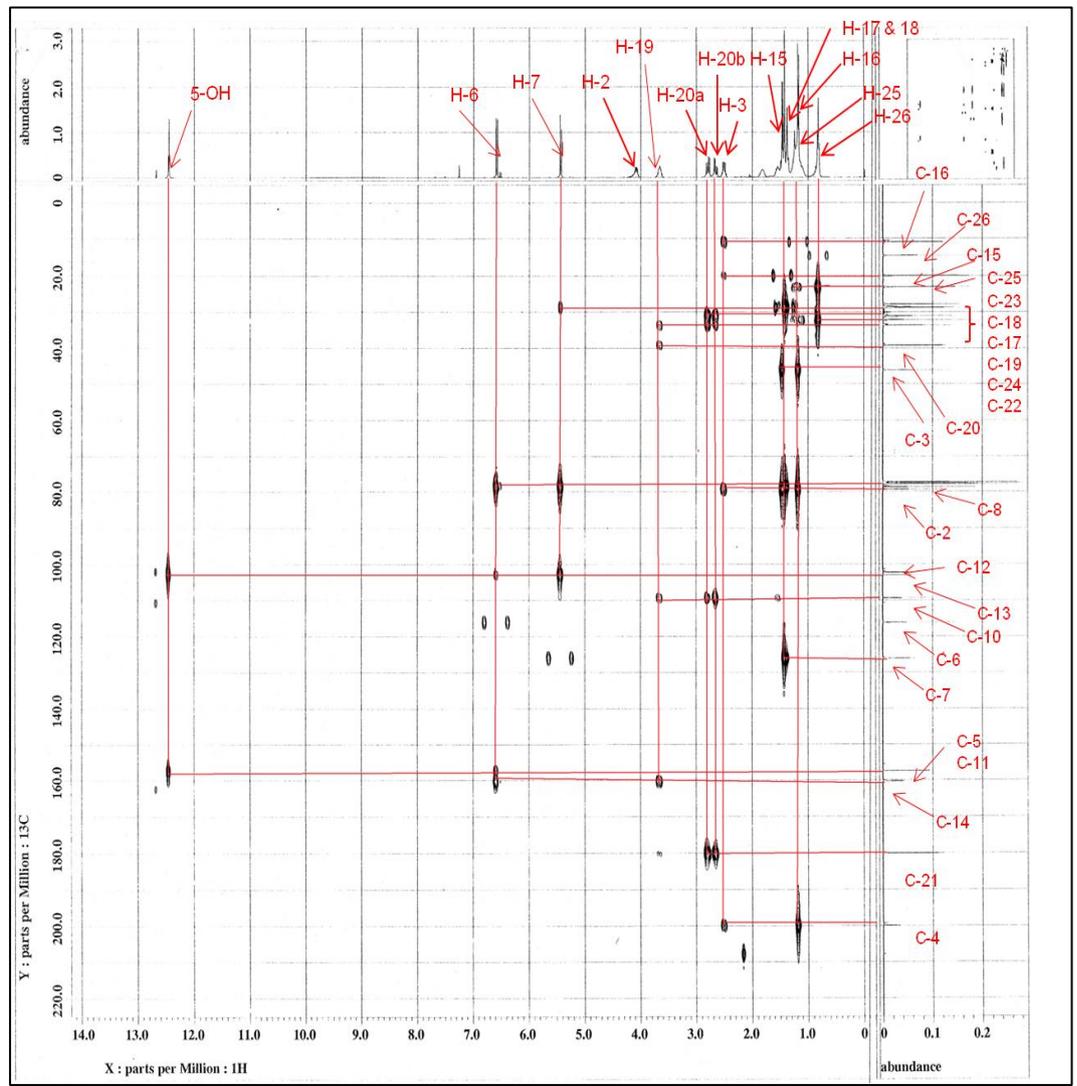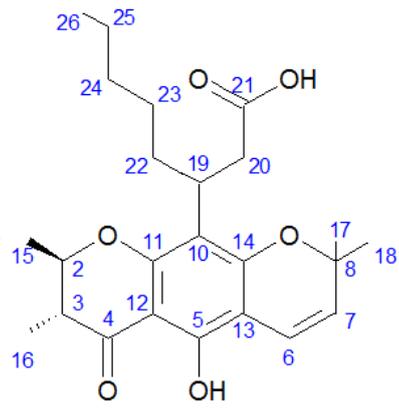
Figure 4.26: HMBC spectrum of blancoic acid [67]

Figure 4.27: IR spectrum of blancoic acid [67]
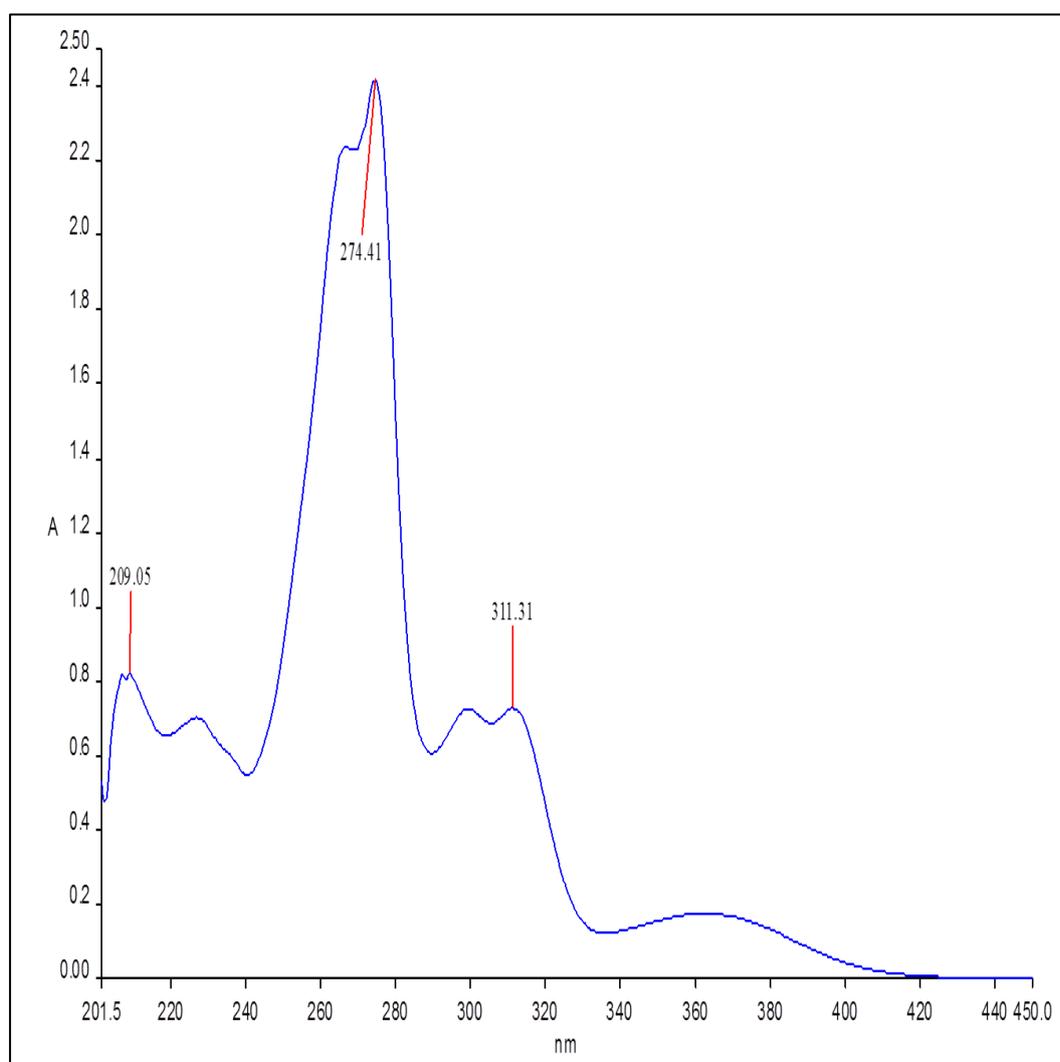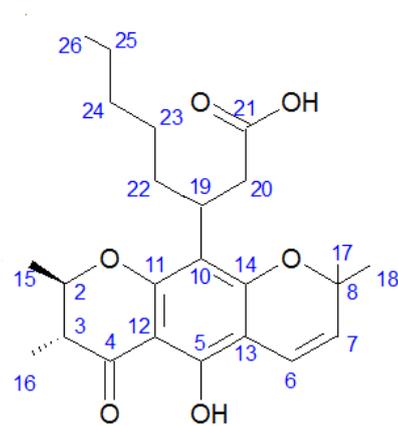
Figure 4.28: UV-Vis spectrum of blancoic acid [67]

4.4 Structural Elucidation and Characterisation of β-Sitosterol [68]
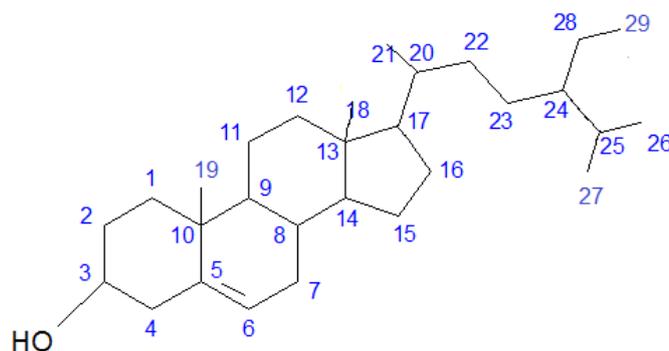
Figure 4.29: Chemical structure of β-sitosterol [68]

Compound **68** (25 mg) was isolated as white needle-like crystals with a melting range of 138-139 ^{0}C. The compound was analysed to have a molecular weight of 414.7067 g/mol in agreement with the molecular formula of $C_{29}H_{50}O$. The purity of the compound isolated was validated by TLC analysis showing a single spot at retention factor, R_f value of 0.56 eluted with mobile phase of 60% dichloromethane and 40% hexane. However, the compound also gave a negative result in $FeCl_3$ test, revealing the compound to be non-phenolic. Figure 4.30 shows the developed TLC plate of β-sitosterol **[68]**.
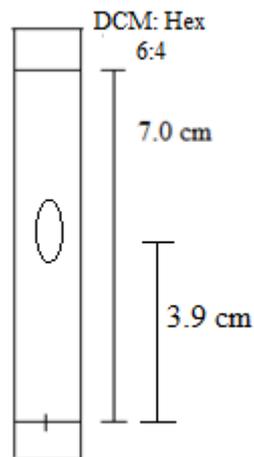
Figure 4.30: TLC development of β-sitosterol [68]

In ^{1}H NMR spectrum (Figure 4.31), the most deshielded proton signal at δ_H 5.35 (1H, d, J = 9.2 Hz) was assigned to olefinic proton H-6, while the oxymethine proton H-3 gave a mutiplet signal at δ_H 3.52. In the expanded spectrum (Figure 4.32), most signals appeared at upfield region below δ_H 2.50 which are characteristic signals for a terpenoid compound. The spectrum further revealed one intense triplet signal at δ_H 0.85, three intense doublet signals at δ_H 0.94, 0.83 and 0.82 and two intense singlet signals at δ_H 1.02 and 0.68 corresponding to the methyl protons of H-29, H-21, H-27, H-26, H-19 and H-18, respectively.

The ^{13}C NMR spectrum (Figure 4.33) showed 29 carbon signals with the two most deshielded signals at δ_C 140.8 and 121.8, were assigned to a pair of olefinic carbons C-5 and C-6, respectively. The assignment of structure of compound **68** was further confirmed by comparison of ^{1}H and ^{13}C NMR data with the reported literature values of β-sitosterol. Table 4.3 shows the

comparison of NMR data of compound **68** with literature values reported by Patra (2010).

With the aids of IR spectroscopic analysis, structure of compound **68** was further confirmed to have O-H, C=C, C-O functionalities. In the IR spectrum (Figure 4.35), alkene C=C (1600 cm^{-1}), hydroxyl O-H (3144 cm^{-1}) and carbinol C-O (964 cm^{-1}) stretchings were observed. Additionally, the UV-Vis spectrum (Figure 4.36) revealed that compound **68** to be a non-conjugated compound showing one absorption band at 206.90 nm.

Table 4.3: Comparison of NMR data of compound 68 with literature values for β-sitosterol

Position	δ_H (ppm) & multiplicity	δ_C (ppm)	δ_H* (ppm)	δ_C* (ppm)
1	1.47 (m)	37.3	1.47	37.3
2	1.55 (m)	31.7	1.56	31.7
3	3.52 (1H, m)	71.9	3.52 (tdd, J=4.2, 1.1Hz)	71.8
4	2.26 (1H, m)	42.4	2.28	42.3
5	-	140.8	-	140.7
6	5.35 (1H, d, J=9.16Hz)	121.8	5.36	121.7
7	2.00 (m)	32.0	2.03	31.7
8	1.65 (m)	32.0	1.67	31.9
9	1.48 (m)	50.2	1.48	50.2
10	-	36.6	-	36.5
11	1.52 (m)	21.2	1.52	21.1
12	1.58 (m)	39.8	1.49	39.8
13	-	42.4	-	42.3
14	1.50 (m)	56.8	1.50	56.8

Position	δ_H (ppm) & multiplicity	δ_C (ppm)	δ_H* (ppm)	δ_C* (ppm)
15	1.60 (m)	24.4	1.60	24.4
16	1.85 (m)	28.3	1.84	28.3
17	1.48 (m)	56.1	1.49	56.1
18	0.68 (3H, s)	11.9	0.68	11.9
19	1.02 (3H, s)	19.5	1.02	19.4
20	1.65 (m)	36.2	1.64	36.5
21	0.94 (3H, d, J=6.7Hz)	19.1	0.94	18.8
22	0.85 (2H, m)	34.0	0.88	34.0
23	1.02 (m)	26.1	1.04	26.1
24	1.51 (m)	45.9	1.50	45.9
25	1.65 (m)	29.2	1.65	28.9
26	0.82 (3H, d, J=6.7Hz)	19.9	0.83	19.8
27	0.83 (3H, d, J=6.7Hz)	18.9	0.85	18.8
28	1.02 (m)	23.1	1.04	23.1
29	0.85 (3H, t, J=8.0Hz)	12.1	0.88	12.0

* Patra, et al., 2010
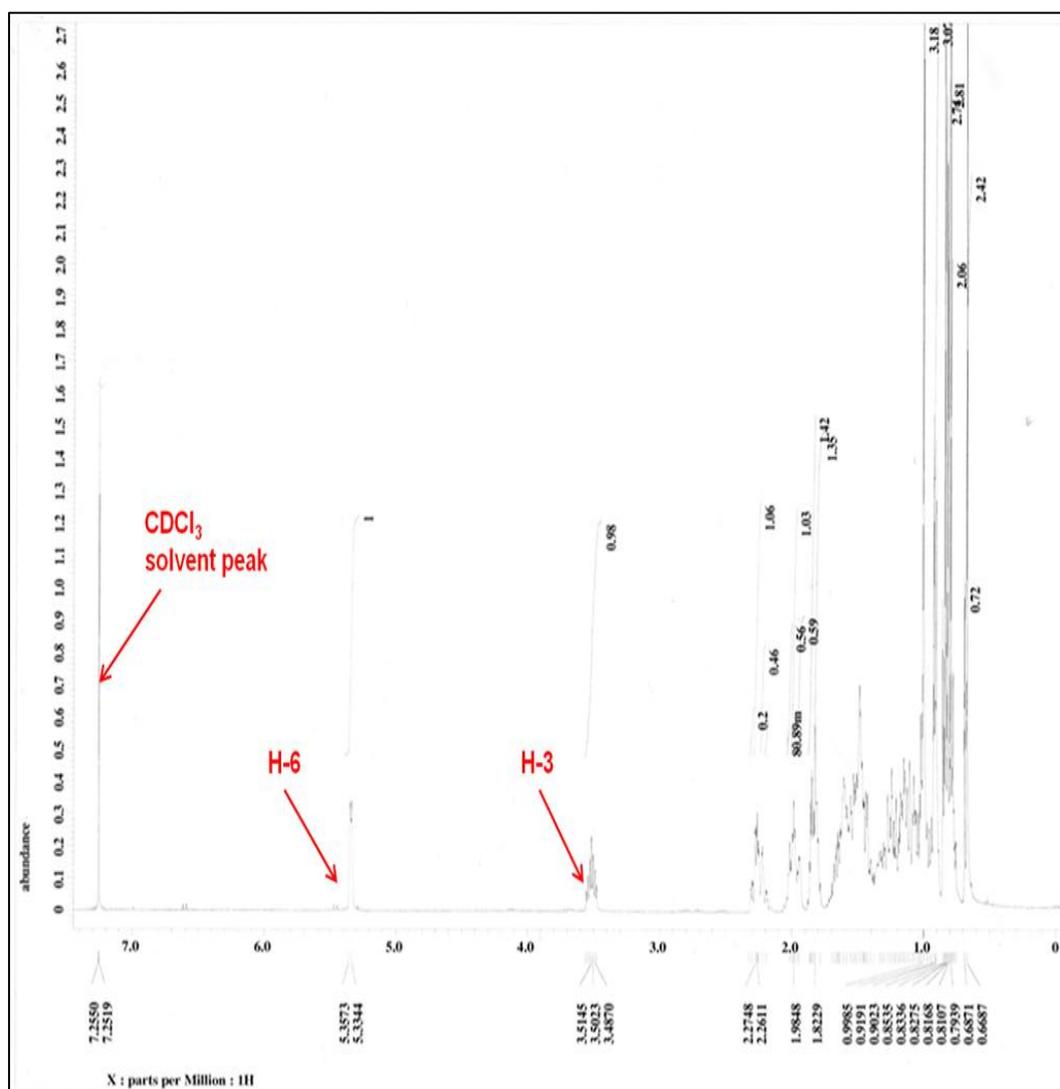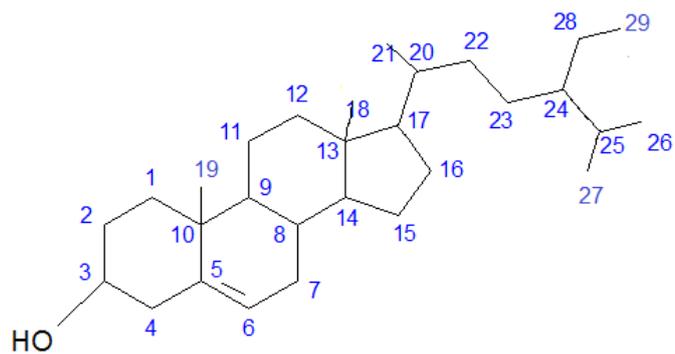
Figure 4.31: ^{1}H NMR spectrum of β-sitosterol [68] (400 MHz, CDCl$_3$)
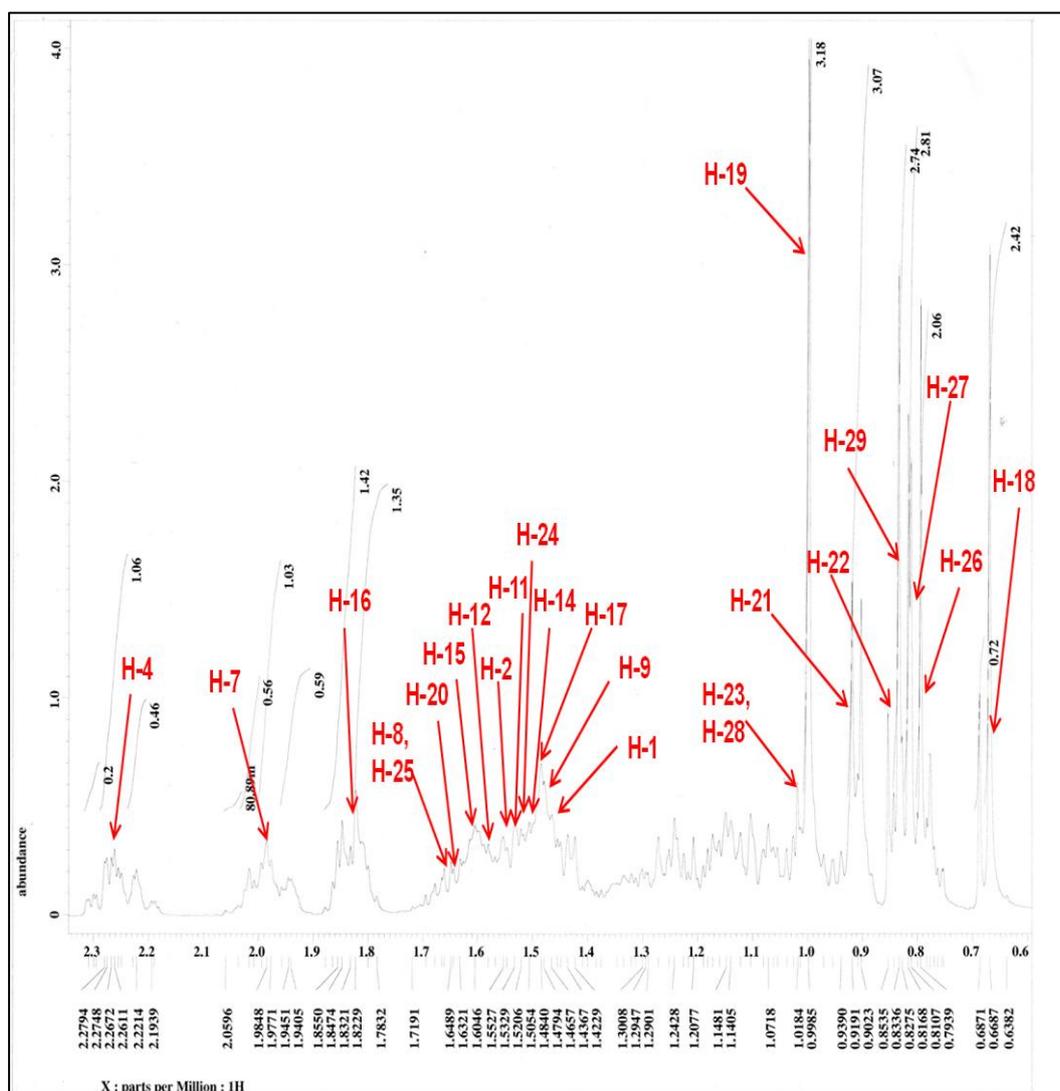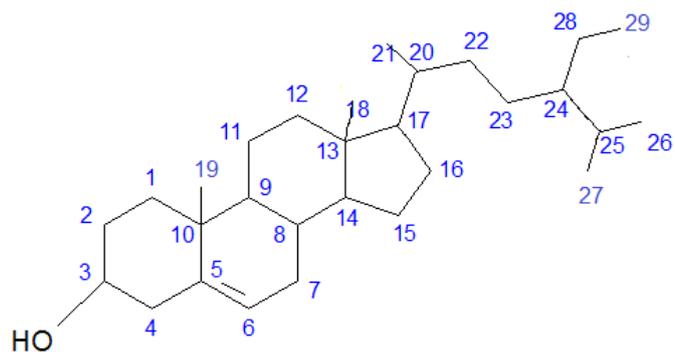
Figure 4.32: ^{1}H NMR spectrum of β-sitosterol [68] (400 MHz, CDCl$_3$) (expanded)
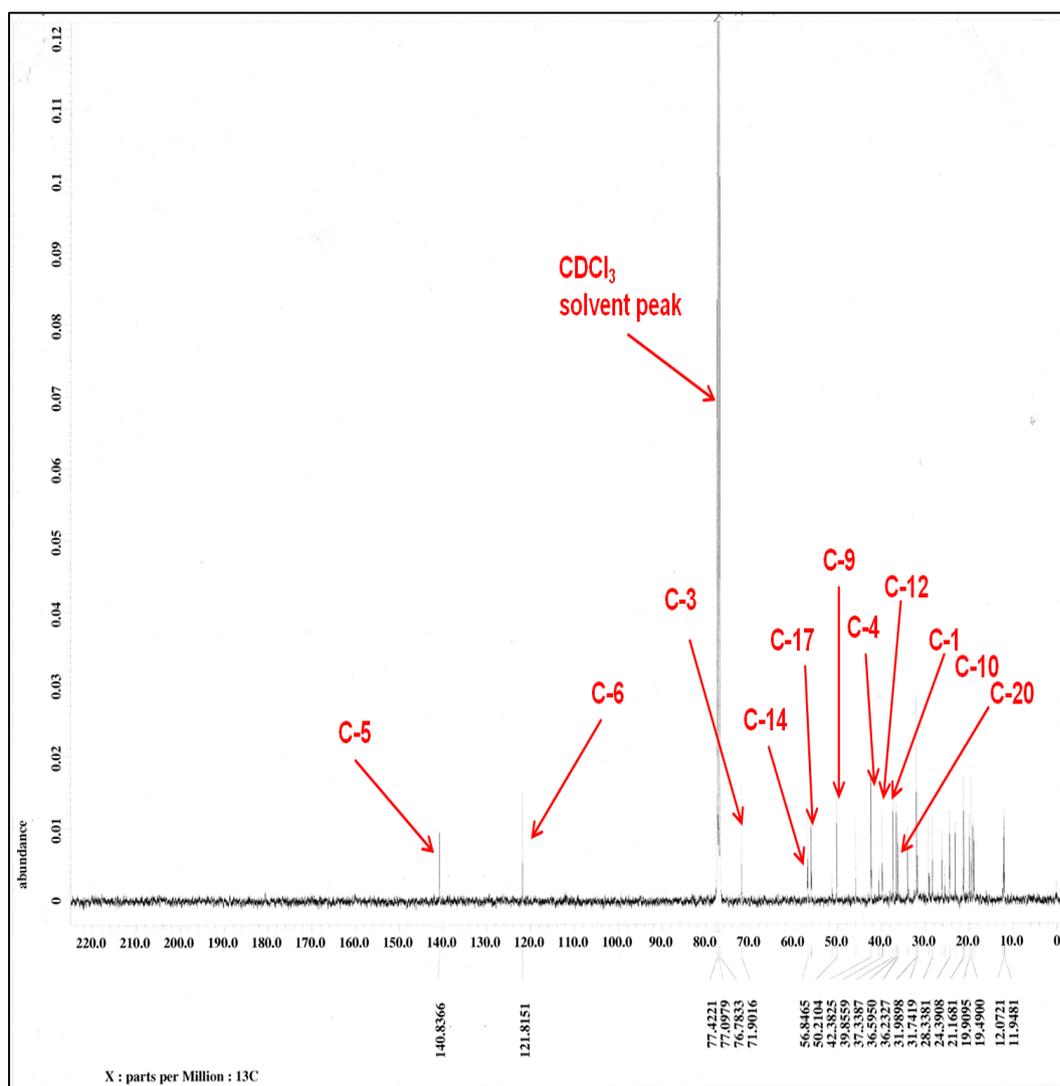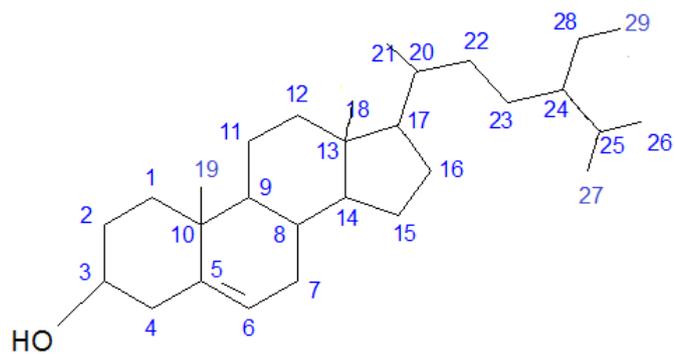
Figure 4.33: ^{13}C NMR spectrum of β-sitosterol [68] (100 MHz, CDCl$_3$)
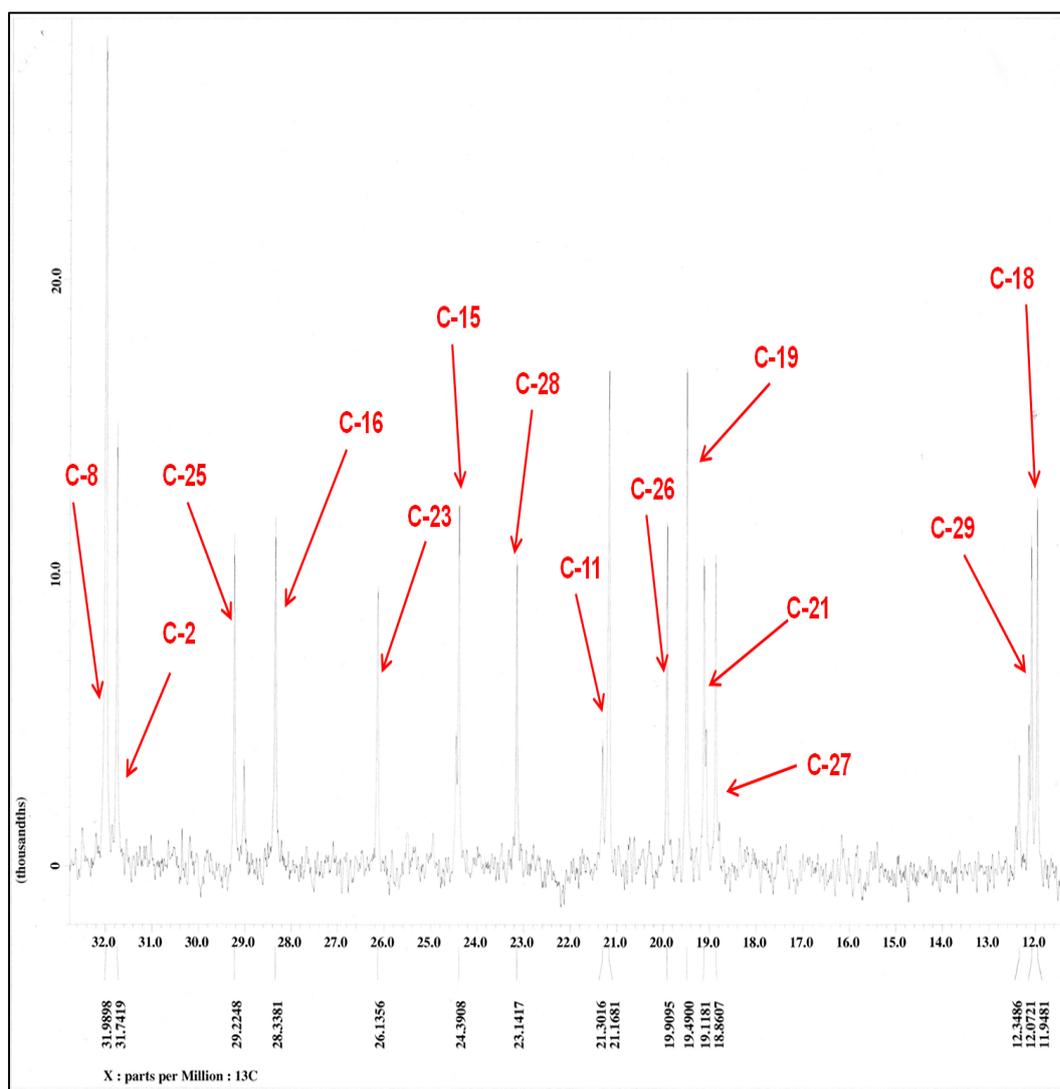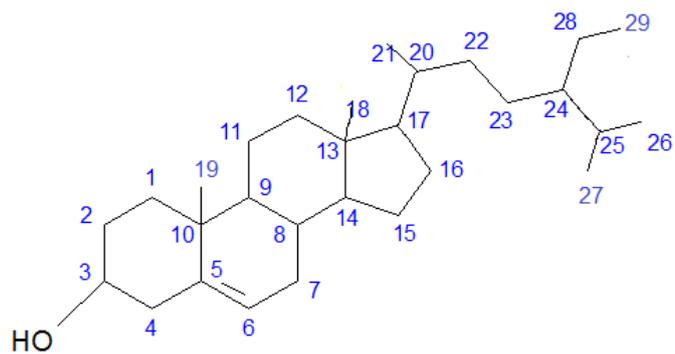
Figure 4.34: ^{13}C NMR spectrum of β-sitosterol [68] (100 MHz, CDCl$_3$) (expanded)
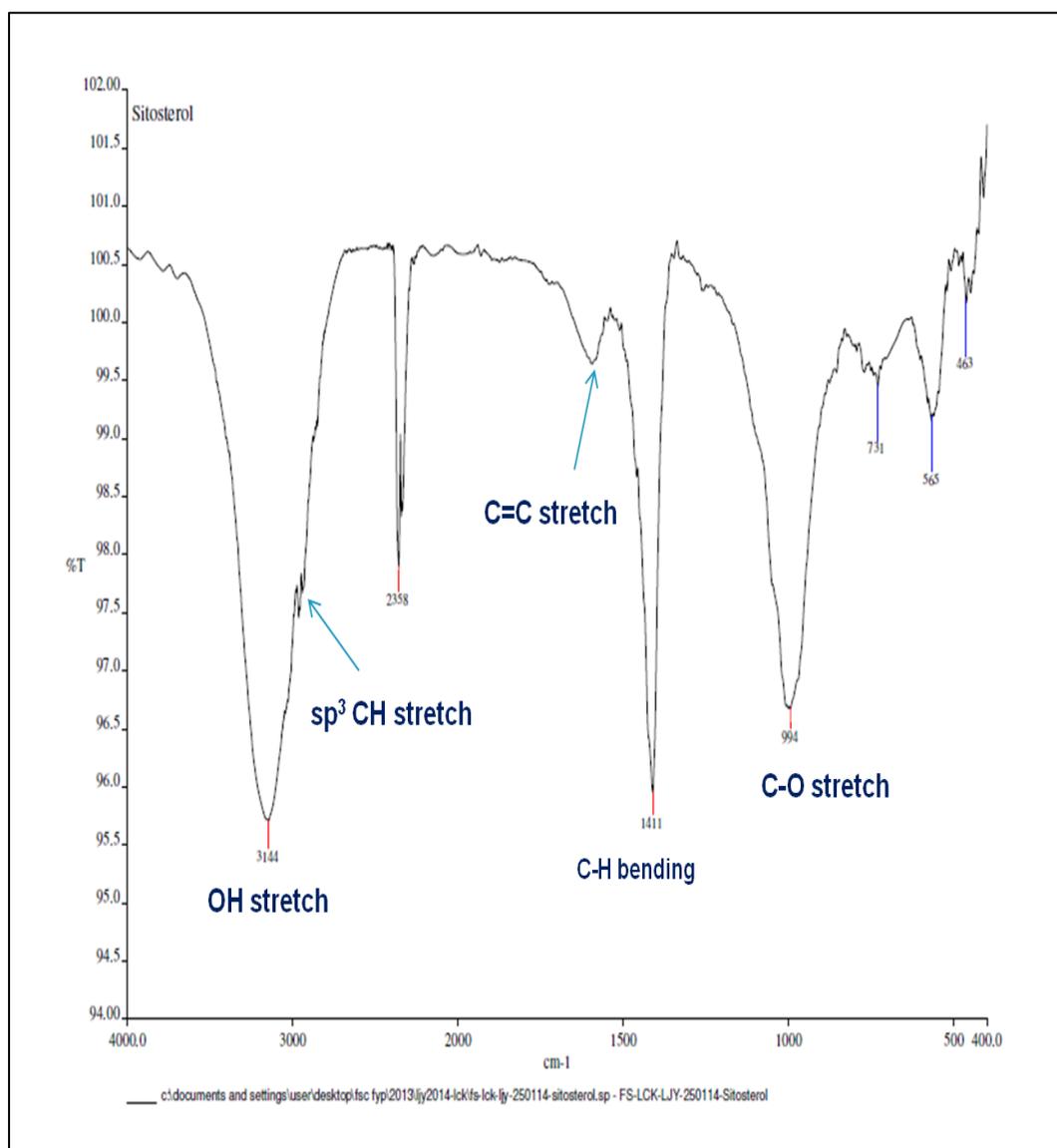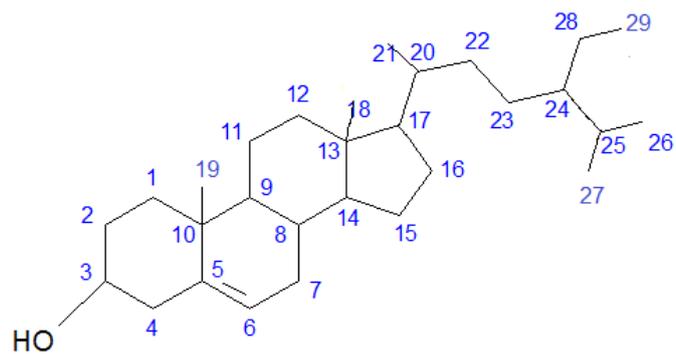
Figure 4.35: IR spectrum of β-sitosterol [68]
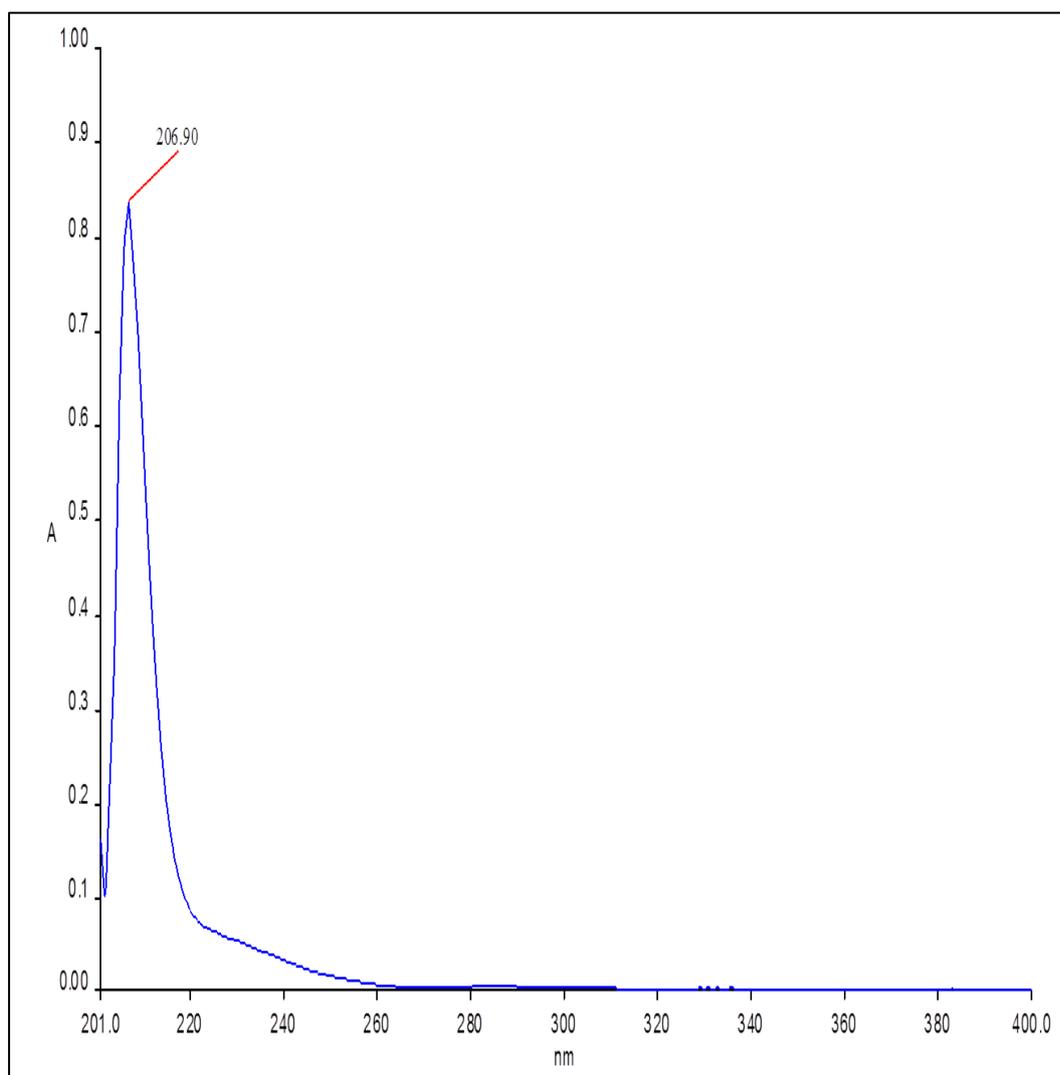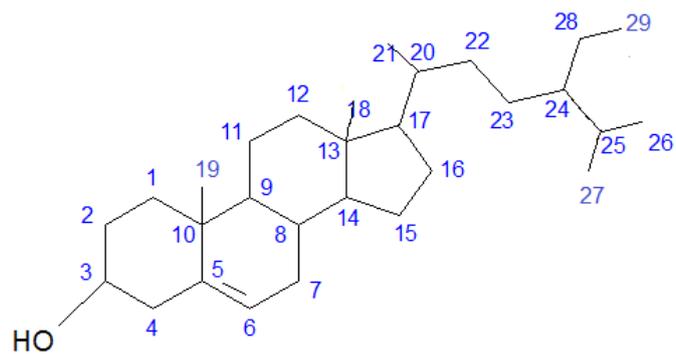
Figure 4.36: UV-Vis spectrum of β-sitosterol [68]

4.5   Antioxidant Assay

Antioxidants are compounds that capable to provide protection against free radical by minimizing cell damage caused by reactive oxygen species, ROS (Bergendi, et al., 1999). Most of the flavonoids and phenolics compounds, especially isolated from natural sources have a great capability in free radical scavenging activity due to their phenolic nature in stabilizing the free radicals (Chanda and Dave, 2009). Hence, there is a growing interest in the study of natural antioxidants either in the form of crude extracts or pure compounds.

The dichloromethane, ethyl acetate and methanol crude extracts, as well as the three isolated compounds, namely isoblancoic acid **[66]**, blancoic acid **[67]** and β-sitosterol **[68]** were subjected to DPPH assay to evaluate for their antioxidant potentials. The concentration to inhibit 50% DPPH free radical activity (IC_{50} values) was determined based on the plot of graph of inhibition rate versus concentrations as shown in Figures 4.37, 4.38, 4.39 and 4.40.

Methanol and ethyl acetate crude extracts of *Calophyllum castaneum* gave a positive result in the DPPH free radical scavenging test, with their IC_{50} values of 12 μg/mL and 84 μg/mL, respectively. In comparison with positive controls used kaempferol (IC_{50} = 16 μg/mL) and ascorbic acid (IC_{50} = 10 μg/mL), methanol crude extract showed a comparable antioxidant potential. Meanwhile, the ethyl acetate crude extract showed a much weaker antioxidant activity than the positive controls. However, dichloromethane extract was found to be

inactive in the DPPH test, which was revealed that active compounds are less soluble in non-polar solvent than polar solvent.

Apart from that, none of the isolated compounds showed positive results in the DPPH assay, albeit isoblancoic acid **[66]** and blancoic acid **[67]** are phenolic compounds. This was due to the hydroxyl proton at carbon C-5 is found to form hydrogen bonding to carbonyl carbon C-4 and thus making it to be unavailable as proton donor in the assay.

Table 4.4: Antioxidant results of test compounds in the DPPH assay

Test compound/ extract	IC_{50} (μg/mL)
Methanol crude extract	12
Ethyl acetate crude extract	84
Dichloromethane crude extract	> 200
Isoblancoic acid	> 200
Blancoic acid	> 200
β-sitosterol	> 200
Kaempferol	16
Ascorbic acid	10
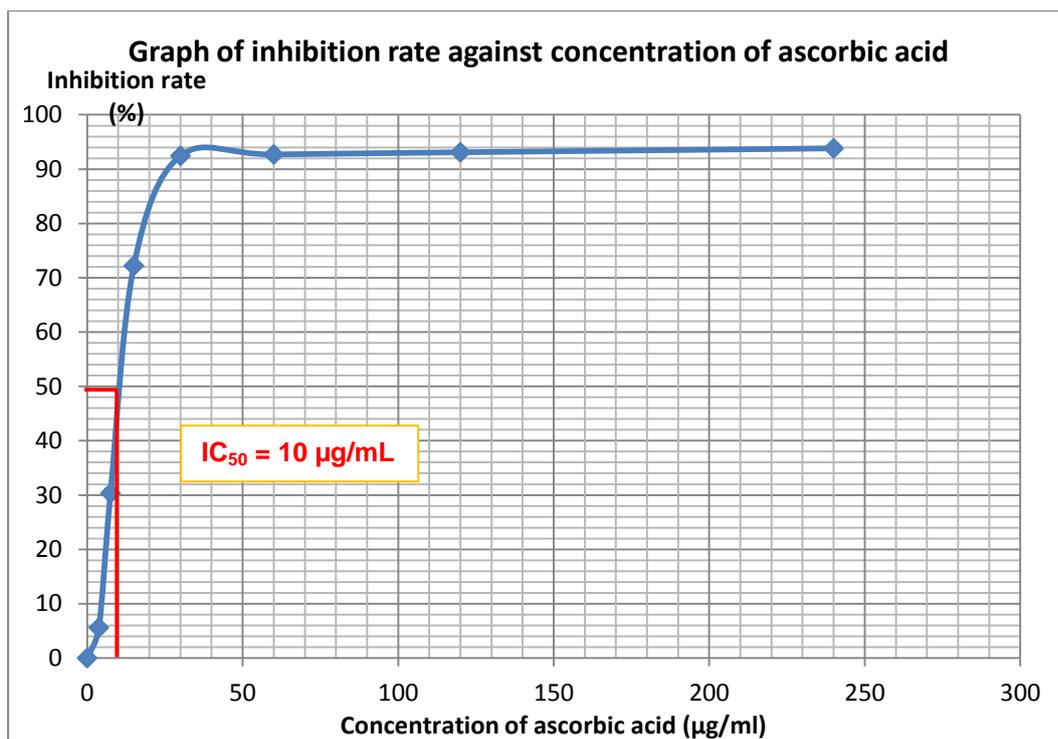
Figure 4.37: Graph of inhibition rate against concentration for ascorbic acid
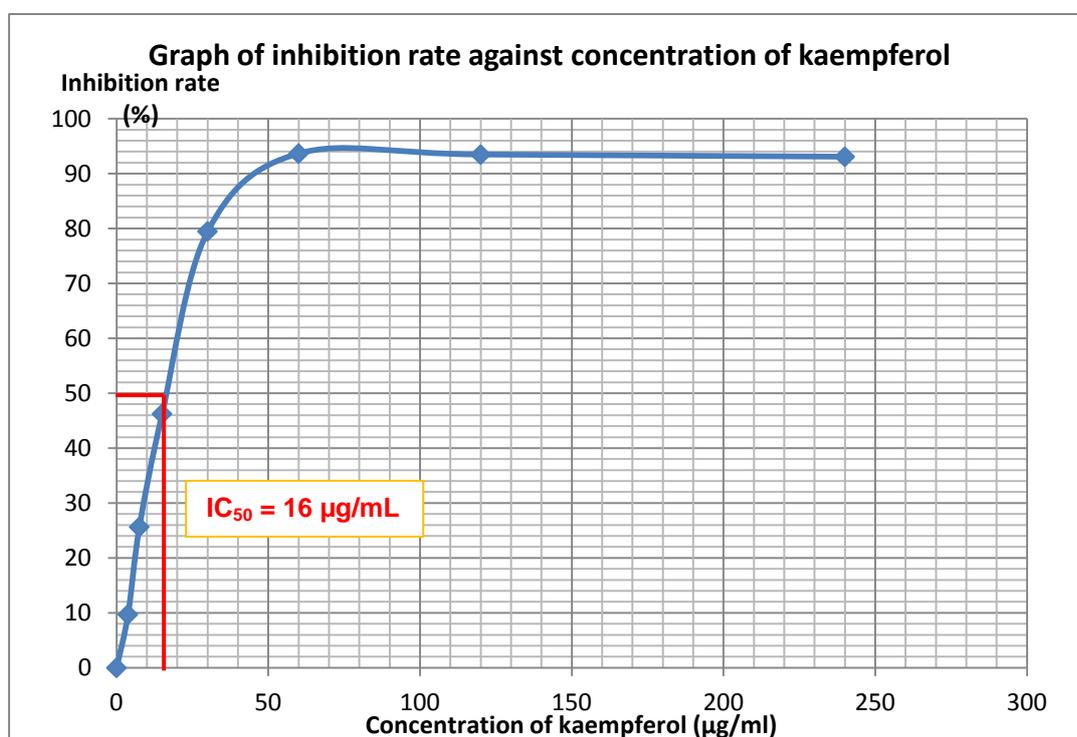
Figure 4.38: Graph of inhibition rate against concentration for kaempferol
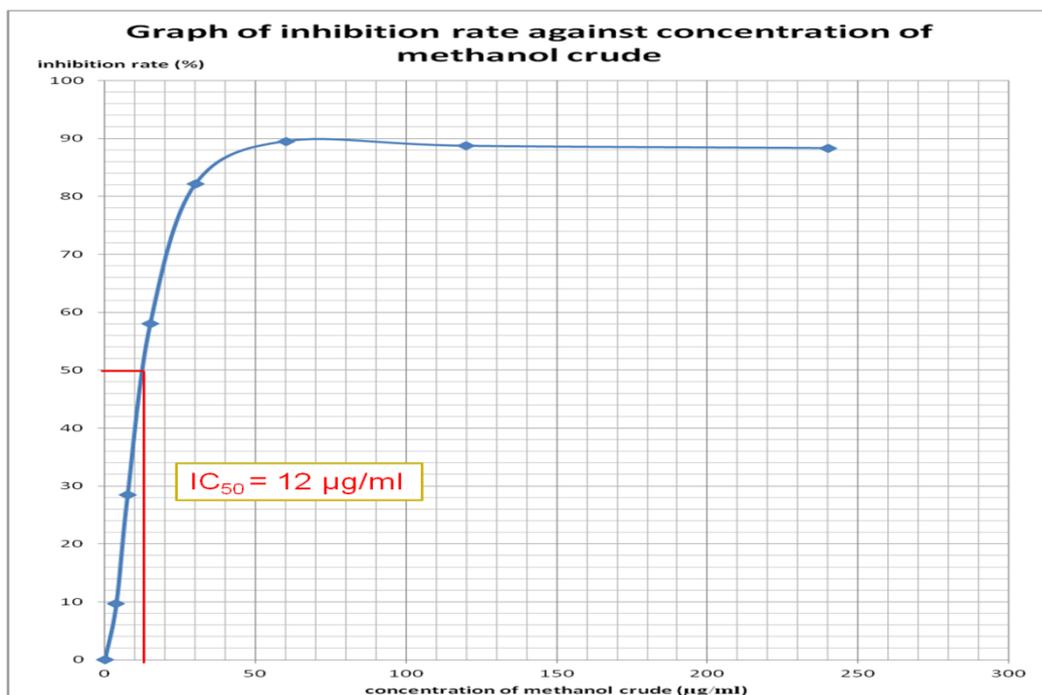
Figure 4.39: Graph of inhibition rate against concentration for methanol crude extract
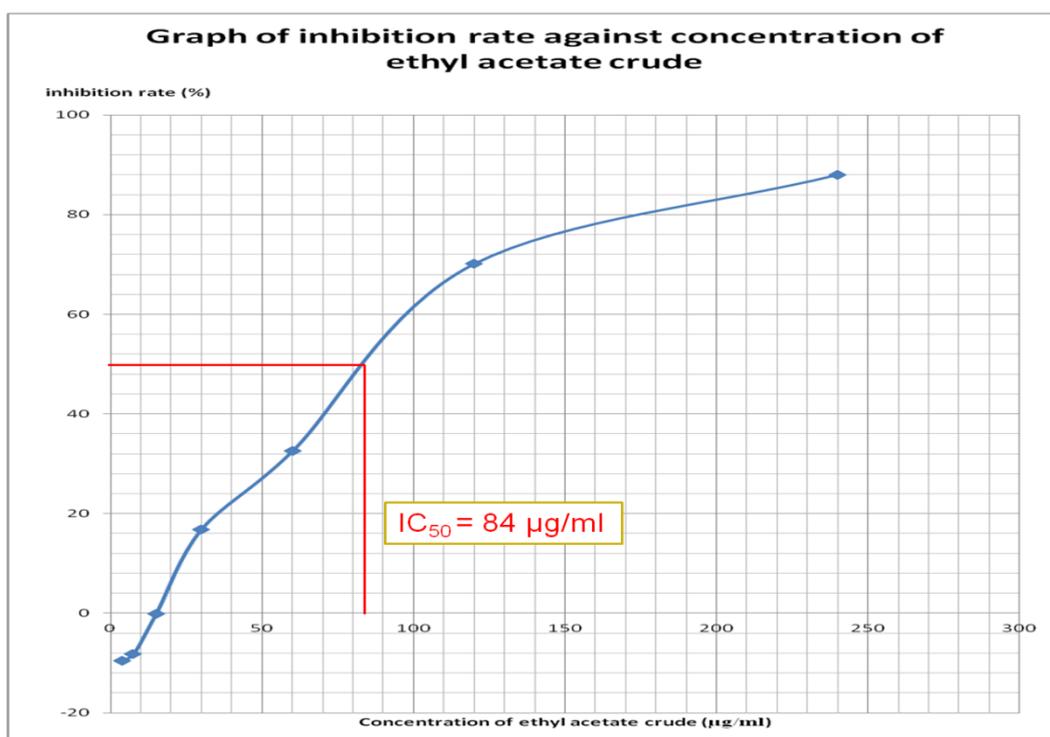
Figure 4.40: Graph of inhibition rate against concentration for ethyl acetate crude extract

4.6   Future Studies

In DPPH assay, methanol extract of *Calophyllum castaneum* was reported to show a strong antioxidant activity but the isolated compounds from the crude extract were found to be inactive in the test, meaning that active constituents which could be the minor compounds were still remained inseparable from the crude extract. To isolate minor active components from the plant extract, the use of more advance separation techniques such as high performance liquid chromatography, flash column chromatography and centrifugal chromatography are recommended.

In addition, the crude extracts and isolated pure chemical constituents are subjected to be further evaluated for their other biological activities such as anti-bacterial, anti-fungal and cytotoxic properties in future study.

CHAPTER 5

CONCLUSION

In this study, a total three natural compounds were isolated from the stem bark of *Calophyllum castaneum*, namely isoblancoic acid **[66]**, blancoic acid **[67]** and *β*-sitosterol **[68]**. The pure compounds obtained were characterized from their physical appearance, melting point and relative polarity while their structures were elucidated by using spectroscopic methods, such as ^{1}H NMR, ^{13}C NMR, UV, IR and MS. The structural assignments of the compounds **66**-**68** were further confirmed by ^{1}H-^{1}H COSY, DEPT, HMQC and HMBC analyses.

The crude extracts of *Calophyllum castaneum* and isolated compounds were subjected to DPPH free radical scavenging test. As a result, methanol and ethyl acetate extracts exhibited positive results with their IC_{50} values of 12 µg/mL and 84 µg/mL, respectively. Methanol crude extract displayed a comparable antioxidant activity with the standard compounds, kaempferol (IC_{50} = 16 µg/mL) and ascorbic acid (IC_{50} = 10 µg/mL). Conversely, dichloromethane crude extracts and all the isolated compounds were found to be inactive in the DPPH assay.

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APPENDICES

APPENDIX A

The table below shows the data on inhibition rates at different concentrations for ascorbic acid from the DPPH assay.

Concentration	Absorbance				Inhibition
(µg/mL)	1	2	3	Mean	rate[1] (%)
240.0	0.055	0.085	0.057	0.066	93.83
120.0	0.058	0.068	0.064	0.063	93.10
60.0	0.059	0.075	0.067	0.067	92.70
30.0	0.074	0.064	0.070	0.069	92.45
15.0	0.361	0.278	0.126	0.255	72.22
7.50	0.676	0.535	0.707	0.639	30.36
3.75	0.859	0.791	0.950	0.867	5.59

[1] Each value was obtained by dividing with value of average based negative control, $A_0 = 0.918$

The table below shows the data on inhibition rates at different concentrations for kaempferol from the DPPH assay.

Concentration ($\mu g/mL$)	Absorbance				Inhibition rate[1] (%)
	1	2	3	Mean	
240.0	0.065	0.078	0.083	0.075	93.09
120.0	0.081	0.064	0.068	0.071	93.49
60.0	0.081	0.060	0.066	0.069	93.67
30.0	0.403	0.160	0.108	0.224	79.50
15.0	0.726	0.547	0.486	0.586	46.26
7.50	0.72	0.865	0.848	0.811	25.66
3.75	0.929	1.006	1.020	0.985	9.72

[1] Each value was obtained by dividing with value of average based negative control, $A_0 = 1.091$

APPENDIX C

The table below shows the data on inhibition rates at different concentrations for methanol crude of *Calophyllum castaneum* extract from the DPPH assay.

Concentration	Absorbance				Inhibition
(µg/mL)	1	2	3	Mean	rate[1] (%)
240.0	0.084	0.122	0.116	0.107	88.31
120.0	0.103	0.097	0.111	0.104	88.71
60.0	0.087	0.111	0.091	0.096	89.51
30.0	0.088	0.098	0.306	0.164	82.13
15.0	0.256	0.334	0.567	0.386	57.99
7.50	0.468	0.613	0.888	0.656	28.50
3.75	0.666	0.843	0.979	0.829	9.66

[1] Each value was obtained by dividing with value of average based negative control, $A_0 = 0.918$

APPENDIX D

The table below shows the data on inhibition rates at different concentrations for ethyl acetate crude of *Calophyllum castaneum* extract from the DPPH assay.

Concentration	Absorbance				Inhibition
(µg/mL)	1	2	3	Mean	rate[1] (%)
240.0	0.130	0.097	0.103	0.110	88.02
120.0	0.269	0.270	0.284	0.274	70.12
60.0	0.573	0.620	0.665	0.619	32.53
30.0	0.744	0.757	0.789	0.763	16.85
15.0	0.881	0.909	0.967	0.919	-0.11
7.50	1.018	0.985	0.978	0.994	-8.24
3.75	1.010	0.980	1.026	1.005	-9.51

[1] Each value was obtained by dividing with value of average based negative control, $A_0 = 0.918$