KISANTINI A/P MURUGESU B.Sc. (Hons) Chemistry 2014

INVESTIGATION OF PHYTOCHEMICALS FROM *CALOPHYLLUM CASTANEUM* FOR THEIR ANTIOXIDANT PROPERTIES

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INVESTIGATION OF PHYTOCHEMICALS FROM *Calophyllum castaneum* FOR THEIR ANTIOXIDANT PROPERTIES

By

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ABSTRACT

INVESTIGATION OF PHYTOCHEMICALS FROM *Calophyllum castaneum* FOR THEIR ANTIOXIDANT PROPERTIES

Kisantini A/P Murugesu

Since ancient times, plants have been used as traditional medicines for various kinds of diseases. Now, after millions of years, advancement in technology and research methods have successfully brought phytochemicals and biological studies of plants to a new level for drug discovery purposes. *Calophyllum* is one of the genera among 40 genera belonging to Guttiferae family. Various plant species of this genus have been investigated for their phytochemicals and bioactivities. However, so far there is no research has been reported on the plant studied, *Calophyllum castaneum*. In this study, the trunk bark material of *C.castaneum* was extracted using methanol, ethyl acetate and dichloromethane. Methanol crude extract afforded two triterpenes namely, friedelinol **[34]** and friedelin **[35]** while dichloromethane crude extract yielded a chromanone acid, isoblancoic acid **[36]**. The structures of isolated compounds were elucidated and characterized using advanced spectroscopic techniques such as 1D-NMR (^{1}H and ^{13}C), HMBC, HMQC, mass spectrometry, UV-Vis and IR. All the pure

compounds and crude extracts of *C.castaneum* were investigated for their free radical scavenging activity via DPPH assay. The methanol and ethyl acetate crude extracts were found to have showed significant antioxidant activities with IC_{50} values of 12 μg/ml and 37 μg/ml, respectively. However, DCM crude extract and all the compounds **34**, **35**, and **36** were found to be inactive when tested for their antioxidant potentials in the assay.

ABSTRAK

KAJIAN FITOKIMIA DARI

Calophyllum castaneum UNTUK AKTIVITI ANTIOKSIDAN

Kisantini A/P Murugesu

Sejak zaman dahulu lagi, fitokimia dari tumbuh-tumbuhan telah digunakan dalam pelbagai jenis rawatan penyakit. Kini, selepas berjuta-juta tahun, kemajuan dalam bidang teknologi dan kaedah penyelidikan telah berjaya membawa kajian fitokimia dan bioaktiviti tumbuh-tumbuhan ke tahap yang baru untuk tujuan penemuan ubat. *Calophyllum* adalah salah satu genus antara 40 genera dibawah keluarga Guttiferae. Pelbagai spesis daripada genus *Calophyllum* telah dikaji untuk fitokimia dan bioaktiviti, tetapi setakat ini tiada kajian telah dilaporkan ke atas *C.castaneum*. Dalam kajian ini, kulit batang pokok *C.castaneum* telah diekstrak menggunakan pelarut organik metanol, etil asetat and diklorometana. Ekstrak mentah metanol telah menghasilkan dua triterpene iaitu friedelinol **[34]** dan friedelin **[35]** manakala ekstrak mentah diklorometana menghasilkan asid kromanon, asid isoblancoic **[36]**. Struktur-struktur kompaun tulen telah dianalisis dengan menggunakan teknik-teknik spektroskopi yang maju seperti 1D-NMR (^{1}H

dan ^{13}C), HMBC, HMQC, spektrometri jisim, UV-Vis dan IR. Kompaun-kompaun tulen dan ekstrak mentah telah disiasat untuk aktiviti antioksidan dengan menggunakan kaedah DPPH. Ekstrak mentah metanol dan etil acetat didapati menunjukkan ciri-ciri antioksidan yang nyata dengan nilai IC_{50} iaitu 12 μg/ml dan 37 μg/ml. Walaubagaimanapun, ekstrak mentah diklorometana dan sebatian- sebatian **34**, **35** dan **36** didapati tidak aktif dalam ujian DPPH.

ACKNOWLEDGEMENTS

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Without you all, I wouldn't have achieved this position in my life. I dedicate this thesis for you, Mum and Dad. I love you all.

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.

_____

(KISANTINI A/P MURUGESU)

APPROVAL SHEET

This project report entitled "INVESTIGATION OF PHYTOCHEMICALS FROM *Calophyllum castaneum* FOR THEIR ANTIOXIDANT PROPERTIES**"** was prepared by KISANTINI A/P MURUGESU and submitted as partial fulfillment of the requirements for the degree of Bachelor of Science (Hons) Chemistry at Universiti Tunku Abdul Rahman.

Approved by:

_____

(Dr. Lim Chan Kiang) Date:

Supervisor

Department of Chemical Science

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UNIVERSITI TUNKU ABDUL RAHMAN

Date: _____

PERMISSION SHEET

It is hereby certified that **KISANTINI A/P MURUGESU** (ID No: **11ADB05924**) has completed this final year project entitled "INVESTIGATION OF PHYTOCHEMICALS FROM *Calophyllum castaneum* FOR THEIR ANTIOXIDANT PROPERTIES" under the supervision of Dr. Lim Chan Kiang (Supervisor) from the Department of Chemical Science, Faculty of Science.

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

Yours truly,

_____

(KISANTINI A/P MURUGESU)

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LIST OF ABBREVIATIONS

β	Beta
π	Pi
π*	Pi star
σ*	Sigma star
σ	Sigma
ε	Molar absorptivity
µg	microgram
µM	Micro Molar
µL	Microliter
A.G	Analytical Grade
^{0}C	Degree Celcius
^{13}C	Carbon-13
cm^{-1}	Unit for wavenumber
1D	1-Dimensional
2D	2-Dimensional
d	doublet
dd	doublet of doublets
dt	doublet of triplets
dbh	Diameter at breast height
DPPH	2,2-diphenyl-1-picrylhydrazyl
DNA	Deoxyribonucleic acid

EI-MS	Electron ionized mass spectrometry
EC_{50}	Concentration that causes 50% of maximum effect
FRAP	Ferric Reducing Ability of Plasma
GI_{50}	Concentration that causes 50% growth inhibition
HIV	Human immunodeficiency virus
HREIMS	High resolution electron impact mass spectrometry
HRFABMS	High resolution fast atom bombardment mass-spectrometry
HMQC	Heteronuclear Multiple Quantum Correlation
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Performance Liquid Chromatography
^{1}H	Proton
Hz	Hertz
IC_{50}	Concentration that causes 50% inhibition activity
I.G	Industrial Grade
IR	Infra red
J	Coupling constant in Hertz
^{2}J coupling	Coupling over two bonds
^{3}J coupling	Coupling over three bonds
KBr	Potassium Bromide
KB cells	KERATIN-forming tumor cell
LCMS	Liquid Chromatography Mass Spectrometry
m/z	Mass-to-charge ratio
mg	milligram

m	multiplet
MS	Mass spectroscopy
nm	nanometer
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser effect
ORAC	Oxygen radical absorbance capacity
ppm	Parts per million
RNA	Ribonucleic acid
R_f	Retention Factor
R	Right-handed configuration
s	singlet
S	Left-handed configuration
t	triplet
TRAP	Total Radical Trapping Antioxidant Parameter
TPA	Tissue plasminogen activator
TLC	Thin layer chromatography
UV-Vis	Ultraviolet-visible

CHAPTER 1

INTRODUCTION

1.1 General Introduction

In today's world, natural products are generally referred to traditional Chinese medicine, herbal concoctions and dietary supplements (Spainhour, 2005). A natural product is a chemical compound extracted from a living organism that exhibits biological activity to be used in pharmacological or medicinal field (Lahlou, 2013). But, within organic chemistry context, the definition is often restricted to secondary metabolites which are the molecules that are not essential for growth and development but provides them evolutionary advantage in plant defense (Bart, 2011).

Plant derived secondary metabolites have been studied and used by humans in the treatment of illnesses for thousands of years. Thousands of years ago, therapeutic uses of plants have been well documented. The earliest documentation on plant based natural products started in Mesopotamia (2600 B.C.) which recorded the

use of two plant species, and they are still being used till to date as colds, coughs and inflammation medicines (Dias, Urban and Roessner, 2012). Then, many preserved knowledge about storage and uses of medicinal herbs were collected from Egypt, China, Greek, England, Ireland, France, Germany and Arab in the form of documented collections which were written by their great physicians, pharmacists, philosophers and scientists (Dias, Urban and Roessner, 2012). All the way through the history, information regarding plant derived traditional medicine has been inherited from generation to generation in different part of the world and it has contributed for today's medicinal system development (Brahmachari, 2011).

Now after millions of years, the natural products have evolved to produce novel, unique and structurally diverse secondary metabolites to become drug candidates. This has led to the isolation of many, novel natural products that have become very famous in pharmaceuticals and chemical industries. Some examples of biological activities possessed by therapeutic compounds are antifungal, anti-HIV, anti-microbial, anti-malarial, immunomodulators, anti-oxidant, antineoplastics, neurotransmission modulators and others (Spainhour, 2005). Researchers have estimated that there are about 250,000 different plant species around the globe but only 5-15% of them have been explored for biologically active compounds for drug discovery (Williams, 2002).

The World Health Organization states that 80% of the world's population or more accurately 3.5 to 4 billion people mainly in the developing countries still depend on herbal medicines extracted from plants for their primary healthcare (Farnsworth, 1988). The interest in plant based medicines among us was due to the minor side effects and good efficiency of them in curing human illnesses (Goyal, 2012).

In the period of 1970-1980, advancement in science and technology has caused investigation of natural products in drug discovery to reach its peak (Koehn and Carter, 2005). However, introduction of high-throughput synthesis and combinatorial chemistry to obtain non-synthetic molecules has caused a hold up for the classical natural products chemistry (Koehn and Carter, 2005). The declining interest with natural products is due to several problems associated with effort, time and cost to find a novel natural product. Furthermore, long product development cycle is also one of the reasons for the declining interest in the natural products (Koehn and Carter, 2005). In addition, there were also some major difficulties with the supply of authenticated plant resources (McChesney, Venkataraman and Henri, 2007). Research on natural products also requires determination and patience as search for novel compounds involves tough and lengthy methodologies such as extraction, compounds isolation, pure compounds purification and structural elucidation (Dias, Urban and Roessner, 2012).

However, natural product research process started to show pick up after year 2000 when technology advancement brought in sensitive instruments to assist in the identification and structure elucidation of potentially active compounds (Dias, Urban and Roessner, 2012). Usage of high-tech instruments with advance capabilities allows the research process to be carried out in a very short period of time. With the most recent high-resolution spectroscopy technologies such as HPLC-MS and HPLC-NMR, rapid and straight forward compound separation and identification have become achievable (Dias, Urban and Roessner, 2012). Clear-cut structure and pure compound determination have become even easier by carrying out spectrometric and spectroscopic analyses using IR, UV-Vis, NMR and MS (Dias, Urban and Roessner, 2012).

1.2 Botany of Plant Species Studied

1.2.1 The Family

Clusiacae, which is also known as Guttiferae Juss is one of the families of floras with 40 genera and 1200 species. This family is economically essential as the products of the plants from this family can be very useful in many fields. Some examples of products that can be obtained from the plants of this family are edible

fruits, valuable resins, hard wood, oily seeds and potentially active compounds that turn into drug candidates (Taher, et al., 2010).

Researchers have proven that this family shows a large amount of variation in plant morphology among its genera and species. They are normally herbs, shrubs, lianas or trees which sometimes bear essential oil in schizogenous spaces or canals. Some Clusiacae genera may have red or black coloured glands that contain pseudohypericin or hypericin (Li, et al., 1990; Watson and Dallwitz, 1992). The leaves of this plant family can be plain, gland fringed entirely or rarely, whorled or exstipulated at times. Fruits of Clussicae can be fleshy or non-fleshy and are usually in drupe, berry or capsule form with seeds range from one to several. Fruits in capsule form can be septicidal or septifragal but rarely loculicidal (Li, et al., 1990; Watson and Dallwitz, 1992). They may exist with or without endosperms in the seeds. The flowers of this family are either unisexual or bisexual and often are actinomorphic. Bracteoles of the flower are often placed underneath calyx and normally cannot be easily noticeable from sepals. The sepals are combined in bud wholly, decussated or imbricated. The flowers might have 4 to 12 petals that are imbricate, aggregated in 'inflorescences', free or twisted in bud. When the petals are in aggregated form, they are either, in cymes, in umbels, or in panicles. The gynoecium of the flower consists of big ovary with single or compound pistils, 1 or more ovules on every placenta and 1 to 12 styles and stigmas. They have numerous stamens which exist as united or separated (Li,

et al., 1990; Watson and Dallwitz, 1992). The plants of this family are usually with colored or milky sap and most of them can be found mainly in tropical regions such as in Eastern Africa, Latin America, Australasia, Pacific islands, Madagascar, West Indies, South and Southeast Asia (Taher, et al., 2010).

1.2.2 The Genus

The genus *Calophyllum*, from the Clusiacae family is a tropical flowering plant that encompasses approximately 200 diverse species (Morel, et al., 2000). The full name of this genus is *Calophyllum Linnaeus,* and the word '*Calophyllum*' was originated from Greek which means 'beautiful leaf' (Ong, et al., 2011). This genus can be found in many parts of the world, but is mainly distributed in tropical regions such as Australasia, Australia, Eastern Africa, Madagascar, the Mascarenes, China, tropical America, Southern coastal India, Southeast Asia, West Indies, Latin America, and the Pacific islands (Ong, et al., 2011). The genus normally develops as shrubs or trees and grows up in various habitats such as alongside coastal areas, flat forests, coral cays and edge of mountains (Ong, et al., 2011). The genus has many local names according to its origins. To name a few, it is known as *damanu* in Fiji, *canoe tree* in Andaman island, *galaba* in West Indies, *kathing* in Thailand and *poon* in Myanmar (Department of Agriculture, Fisheries

and Forestry, 2010). In Malaysia, the plant is commonly recognized as *bintagor* tree (Taher, et al., 2010).

The plants of this genus are very well known for their medicinal values. The parts of this genus are used to treat diseases such as inflammation, gastric ulcers, rheumatism, varicose veins, hemorrhoids, tumors, infections and many more (Filho, Silva and Niero, 2009). Studies have also reported that plant species of *Calophyllum* are very rich in secondary metabolites such as flavonoids, triterpenes, xanthones and coumarins (Kashman, et al., 1992). The presence of these compounds in this genus allows it to possess biological activities such as anti-fungal, anti-HIV, anti-microbial, anti-malarial, molluscicidal, cytotoxic and others (Spainhour, 2005).

Other than biological values, the parts of this genus also have high economical values. The plant species of this genus are famous for their woods which are used to make plywood, boats, flooring and furniture (Department of Agriculture, Fisheries and Forestry, 2010). Other than this, *Calophyllum* can also be source of other valuable products such as scented oil, drugs, weapons and medicines.

1.2.3 *Calophyllum castaneum*

Calophyllum castaneum which means ‘chestnut brown’ in Latin is one of the *Callophyllum* species that has never been phytochemically studied before. It is a sub-canopy tree that can grow up to 30 m tall and 65 cm dbh. The stem of this tree produces latex which is white or yellow in color. The leaves of this species normally are simple, oppositely arranged and penni-veined. The midrib at lower surface of the leaves is brown in color and hairy. Many secondary veins are present on the leaves and they are positioned very near to each other. The tree produces white flowers of which the diameter is approximately 10 mm. The fruits produced by this species are drupes in which the single seed is placed in hardened endocarp which is surrounded by fleshy mesocarp part and thin exocarp part. They are approximately 21 mm in diameter and are green in color. *Calophyllum castaneum* normally prefers habitats such as mixed dipterocarp forests with altitude up to 500 m that are free from disturbance, ridges and hillsides of mountain forests. This species is a native plant of Borneo (Sarawak, Brunei, West- and East-Kalimantan) (Stevens and Arbor, 1980).

Table 1.1: Profile of *Calophyllum castaneum*.

Domain	Eukaryota
Kingdom	Plantae
Subkingdom	Viridaeplantae
Phylum	Tracheophyta
Subphylum	Euphyllophytina
Infraphylum	Angiospermae
Class	Magnoliopsida
Subclass	Rosidae
Super order	Theanae
Order	Theales
Family	Guttiferae / Clusiaceae
Subfamily	Kielmeyeroideae
Tribe	Calophylleae
Genus	*Calophyllum*
Specific species	*castaneum*
Scientific name	*Calophyllum castaneum*

* BayScience Foundation, Inc., 1986; Arctos: Multi-Institution, Multi-Collection Museum Database, 2011

Figure 1.1: The tree of *Calophyllum castaneum.*

1.3 Phytochemical Studies

Phytochemical studies are related to the study of plant chemicals. The term 'Phyto' means 'plant' in Greek language (American Institute for Cancer Research, 2014). Phytochemicals are any chemical compounds that are found in plants which can have positive effects on human health (American Cancer Society, 2014). These chemicals which are also known as secondary metabolites are responsible for the flavor, odor and color of the plant (American Institute for Cancer Research, 2014). Their function in plant body is as defense mechanism and to attract pollinating agents (Kennedy and Wightman, 2011). They don't possess any nutritive values and hence are not responsible for the growth and development of the plants. But, scientists have found that these phytochemicals

can be used by humans to protect themselves from certain diseases (American Cancer Society, 2014). Each photochemical from different plant species or genera have different function on human body. The presence of these phytochemicals in plants allows them to acquire certain biological activities such as antioxidant, antibacterial, mollucidal and others. Some examples of phytochemicals that can be isolated from plants are flavanoids, terpenes, xanthones and polyphenols (American Cancer Society, 2014).

1.3.1 Xanthones

Xanthones are one of the secondary metabolites found in plants that fall in the category of polyphenolic compounds (Sahelian, 2014). Xanthones, whose structure is similar to flavanoids have different names such as *9H*-xanthen-9-one and 9-oxo-xanthene. Xanthones normally can be found in lichen, fungi, higher plant families, and seldom found in fruits and vegetables (Negi, et al., 2013). There are documentations stating that this compound occurs in the families of Polygalaceae, Gentianaceae, Moraceae, and Guttiferae (Negi, et al., 2013). But its occurrence in nature is very limited as the majority of them are only found in Gentianaceae and Guttiferae family. To date, approximately 200 different types of xanthones have been discovered by scientists and 43 types are solely from mangosteen fruit (Xango, 2014).

Scientists have found that, apart from antioxidant activity, xanthones possess other valuable biological activities too such as anti-allergic, anti-convulsion, anti-cancer activities, antithrombotic, anti-bacterial, anti-inflammatory and platelet aggregation inhibitory (Negi, et al., 2013). These biological properties allow them to give positive effects to human health when they are being consumed. They can be used in the treatments of diseases such as meningitis, leukemia, cancer, infections, septimia, inflammation, skin disorders, allergies, pneumonia, tuberculosis, cholesterol levels, fatigue and gastro-intestinal disorders (SidoMuncul Herbal, 2014).

1.3.2 Terpenoids

Terpenoids or isoprenoids are the largest class of phytochemicals produced and there are approximately 40,000 of them for both primary and secondary metabolisms (Bishayee, Ahmed and Perloff, 2011). Majority of the terpenoids are produced by plants but only a small fraction has been studied for their biological properties. They exist as colorless, volatile and aromatic liquid most of the time (Bishayee, Ahmed and Perloff, 2011). This phytochemical is produced from the combination of two or more isoprene units. Isoprene units are five carbon units and its chemical name is 2-methyl-1,3-butadiene. General formula for terpenoid hydrocarbon is $(C_5H_8)n$ (Szakiel, Paczkowski and Bertsch, 2012).

Terpenoids are normally found in the body parts of conifers, mosses, citrus, liverworts, eucalyptus, algae, lichens and higher plants. They possess numerous biological activities such as anti-spasmodic, antiseptic, anti-carcinogenic, anti-bacterial, anti-depressant and anti-malarial to name a few (Szakiel, Paczkowski and Bertsch, 2012). They are used in the treatment of cancer, acne, skin fungus, cold sores, malaria, inflammation, viral and bacterial infections, arthritis, headache, toothache and menstrual pain relief (Szakiel, Paczkowski and Bertsch, 2012).

1.4 Antioxidant Assay

Antioxidants are compounds that inhibit oxidation process caused by scavenging radicals while radicals are actually unstable and very reactive species with unpaired electrons (Apak, et al., 2013). Radicals are the byproducts produced during body metabolism and are responsible for ageing process, cell mutation, cancer, stroke and others. Antioxidants protect our cells from being damaged by free radicals and oxidizing species. These antioxidants are naturally produced in our body and also can be found in many foods such as fruits and vegetables (Anwar, et al., 2006). Antioxidant activity refers to the ability to inhibit oxidation process caused by scavenging radicals. Some examples of phytochemicals that

have radical scavenging ability are flavonoids, coumarins and anthocynins (Khalaf, et al., 2008).

A few methods have been developed by scientists to determine antioxidant activity of plants and food products. Some extensively used methods are: DPPH, TRAP, ORAC and FRAP to name a few (Institute of Food Technologists, 2008). The method that was used in this study was DPPH assay in which the antioxidant and radical scavenging activity of a plant metabolites is measured based on the ability of antioxidants present to reduce 2,2- diphenylpicrylhydrazyl (DPPH), a stable free radical (Kedare and Singh, 2011). This assay method is the most common used to determine antioxidant activity in labs as it is considered easy, economical, the results are accurate and highly reproducible (Kedare and Singh, 2011).

1.5 Objectives of study

The objectives of this study are:

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

2. To elucidate and identify the structures of isolated compounds using modern instrumental methods such as UV-Vis, IR, MS and NMR.

3. To evaluate the radical scavenging activities of the crude extracts as well as pure compounds via DPPH assay.

CHAPTER 2

LITERATURE REVIEW

2.1 Chemistry of *Calophyllum* Species

About hundreds of journal papers have reported on the phytochemical and biological studies of the genus *Calophyllum*. This genus comprises of approximately 200 different plant species. However, only 25 to 30 species have been investigated. Among the species that have been studied are *C. inophyllum*, *C. brasiliense*, *C. soulattri*, *C. lanigerum*, *C. venulosum*, *C. pinetorum*, *C. membranaceum*, *C. sundaicum*, *C. dispar*, *C. antillanum*, *C. rubiginosum*, *C. enervosum*, *C. thorelii*, *C. caledonicum*, *C. blancoi*, *C. dryobalanoides*, *C. polyanthum*, *C. teysmannii*, *C. panciflorum*, *C. rivulare* and others.

2.1.1 *Calophyllum polyanthum*

In 2004, Ma et al. isolated two novel diastereoisomer pyranocoumarins namely

calopolyanolides D **[1]** and calopolyanolides C **[2]** with 12 other established compounds from the seed of this plant species. The two novel compounds isolated which were in the form of pale yellow needles were tested for antiherpatic activity, but both compounds gave negative results against the Herpex virus by giving GI_{50} value of more than 250 μg/ml. The 12 known compounds were 5,7,3',4'-tetrahydroxyisoflavone, *β*-daucosterol, ursolic acid, calanolide E2, 3,4-dihydroxybenzoic acid, 3,5-dihydroxy-4-methoxybenzoic acid, 2- hydroxy-4-methoxybenzoic acid, calopolyanolide A, 3,5-dimethoxybenzoic acid, 3,4,5-trihydroxybenzoic acid, calopolyanolide B and *β*-sitosterol (Ma, et al., 2004).

2.1.2 *Calophyllum blancoi*

Pyranoxanthones were isolated from the acetone extract of the roots of this plant species in the year 2005 by Shen and coworkers. The acetone extract of this plant afforded three novel and two known pyranoxanthones. The new compounds in yellow powdered form were blancoxanthone **[3]**, acetyl blancoxanthone **[4]** and 3-hydroxyblancoxanthone **[5]** while the known compounds were pyranojacaeubin **[6]** and caloxanthone **[7]**. The structures of new compounds were elucidated using EI-MS and NMR data while the structures of known compounds were confirmed by comparison with literature data. The isolated compounds were subjected to anti-coronavirus test and it was concluded that compounds **3** and **6** possess anti-

coronavirus activity with EC_{50} value of 3 and 15 ug/ml, respectively. Compound **3** is believed to be a good candidate to cure corona virus disease (Shen, et al., 2005).

2.1.3 *Calophyllum teysmannii*

In 1996, Pengsuparp et al. isolated a coumarin named soulattrolide **[8]** from the latex of this plant. In the previous study of the same plant species, costatolide **[9]**, a coumarin which is the major constituent has been isolated and it showed positive result for anti-HIV activity. Since, the structures of both compounds **8** and **9** have high similarity and extraction of **9** gave a significant quantity of **8**, the **8** was subjected to Reverse Transcriptases (RT), DNA polymerase and RNA polymerase assays. From the test, it was observed that compound **8** successfully inhibited HIV-1 RT which allows it to function as HIV-1 non-nucleoside reverse transcriptase inhibitor. Compound **8** also managed to inhibit the activities of RNA-dependent and DNA-dependent polymerase of HIV-1 RT with IC_{50} values of 0.34 μM and 0.73 μM, respectively (Pengsuparp, et al., 1996).

In the same year, McKee and coworkers isolated two new pyranocoumarins, calanolide F **[10]** and simple prenylated coumarin precursor **[11]** from the leaves

& twigs and latex of the plant species respectively. Compound **10** was determined using HREIMS which gives the molecular formula of $C_{22}H_{26}O_5$ and the configuration was confirmed as 10*S*, 11*R*, 12*S*. Compound **11** was established using HRFABMS which revealed its molecular formula, $C_{19}H_{22}O_5$. Both compounds **10** and **11** were screened for anti-HIV property and only compound **10** showed positive result in the test (McKee, et al., 1996).

2.1.4 *Calophyllum venulosum*

In 1997, Chao and coworkers isolated four new and three known biflavonoids from the leaves of this plant species collected from Sabah, Malaysia. The new compounds isolated were in the form of yellow powder namely pyranoamentoflavone7,4'-dimethyl ether **[12]**, pyranoamentoflavone 7,4'''-dimethyl ether **[13]**, 6''-(2-hydroxy-3-methyl-3-butenyl)amentoflavone **[14]**, 6''-(3-methyl-2-butenyl)amentoflavone **[15]**, and their structures were elucidated using advanced spectroscopic techniques. While the known compounds isolated were pyranoamentoflavone **[16]** and amentoflavone **[17]**. Compound **17** is a biflavonoid that can be found frequently and the structure was confirmed by comparison with literature values. Compound **6** is a rare biflavonoid with prenyl groups and its structure was confirmed using 1D and 2D NMR data (Chao, Sim and Goh, 1997).

2.1.5 *Calophyllum inophyllum*

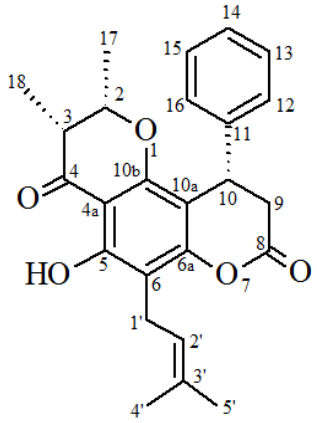
In 2004, Yimdjo and coworkers reported the phytochemical and biological studies on this plant. The extraction of the root bark of this species using CH_2Cl_2-MeOH solvent yielded a new compound inoxanthone, **[18]**, yellow colored needles with melting point of 217^0C and the test with $FeCl_3$ and Gibbs reagents indicated the presence of phenolic group in its structure. This extract also afforded eight other established compounds namely, caloxanthones B, calophynic acid, friedelan-3-one, caloxanthones A, 1,5-dihydroxyxanthone**,** inophylloidic acid, macluraxanthone and brasiliensic acid. The CH_2Cl_2-MeOH extract of nuts of the plant yielded four known pure compounds after repeated column chromatography: inophyllums C, calaustralin, inophyllums E and calophyllolide. All the isolated compounds were subjected to cytotoxicity test against KB cell line and antimicrobial test against three types of microorganisms. Among all, only compound calophyllolide with its IC_{50} value of 3.5 μg/ml gave significant result against KB cells. For the antimicrobial test, compounds, calophynic acid, 1,5-dihydroxyxanthone, inophylloidic acid, inophyllums C, calaustralin, inophyllums E and calophyllolide gave positive results in inhibiting the bacteria, *Staphylococcus aureus* (Yimdjo, et al., 2004).

In 2010, Li and coworkers isolated a novel triterpenoid with seven other known triterpenoids from the ethanolic extract of stem and leaf parts of this species. The structure of the new compound, 3β,23-epoxy-friedelan-28-oic acid **[19]** was elucidated using HREIMS which suggested that it is a fridelane-type triterpenoid. The structures of seven other known compounds canophyllol **[20]**, 3-oxo-friedelan-28-oic acid **[21]**, friedelin **[22]**, canophyllal **[23]**, canophyllic acid **[24]**, epifriedelanol **[25]** and oleanolic acid **[26]** were confirmed by comparison with established compounds. Selected compounds **19**, **21**, **24** and **26** were tested against leukemia cells to study their inhibitory activity and it was found that compound **21** inhibited leukemia cells in human body efficiently with IG_{50} value of 2.67 μM (Li, et al., 2010).

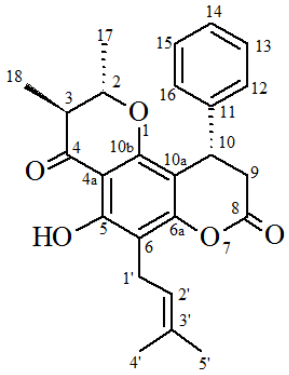
2.1.6 *Calophyllum caledonicum*

In 2004, Hay et al. isolated seven xanthones from the hexane extract of root bark of the plant species. The xanthones isolated were caloxanthone C **[27]**, calozeyloxanthone **[28]**, demethylcalabaxanthone **[29]**, dombakinaxanthone **[30]**, 6-deoxy-g-mangostin **[31]**, macluraxanthone **[32]** and calothwaitesixanthone **[33]**. The isolated xanthones were studied to understand the relationship between the structures of xanthones and their biological activity against malaria disease. Due to the ineffectiveness of chloroquinone antimalarial drug on the *Plasmodium*

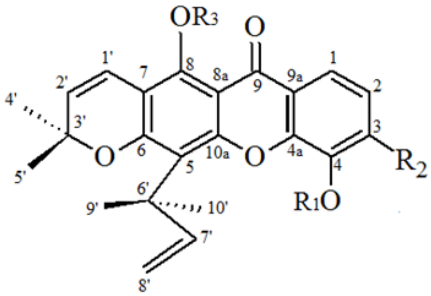
falciparum, one of the deadliest parasites that cause malaria, the isolated compounds were tested in order to develop new antimalarial drugs. From the study it was found that, compounds **29**, **31** and **33** showed potent antimalarial activity against *Plasmodium falciparum* with their IC_{50} values of 0.9, 0.8 and 1.0 μg/ml, respectively. Similarities between these three xanthones include, oxygenated at positions 1,3,7 and isoprenylated at positions 2 and 8 (Hay, et al., 2004).



[1]



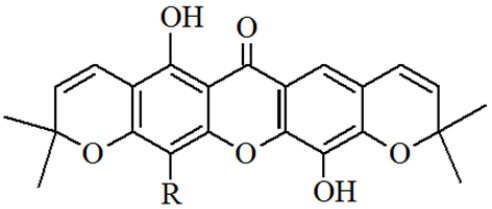
[2]



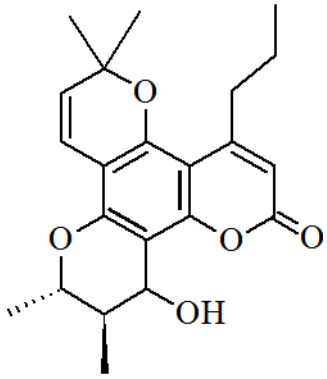
[3] R_1= Ac, R_2=H, R_3=H

[4] R_1= H, R_2=H, R_3=H

[5] R_1= H, R_2=OH, R_3=H



[6] R= H [7] R= Prenyl



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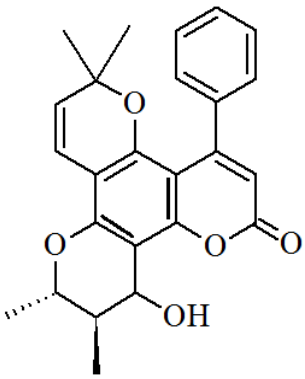
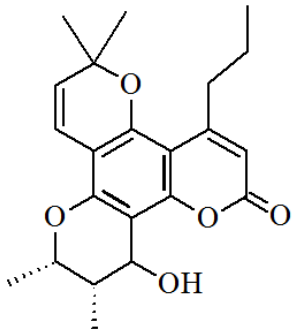
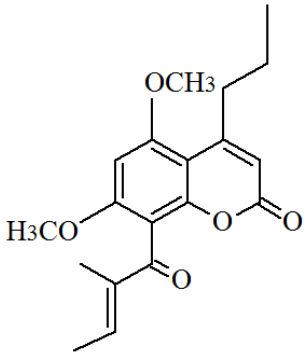


[9]

Figure 2.1: Chemical structures of natural products.



[10]



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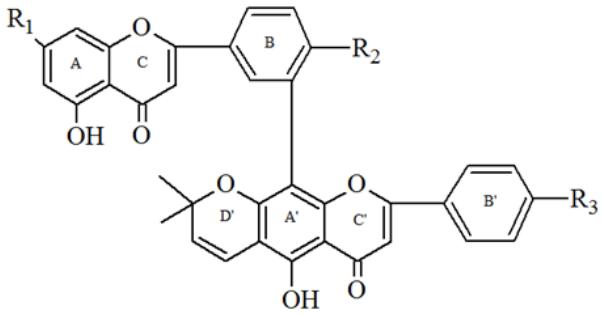
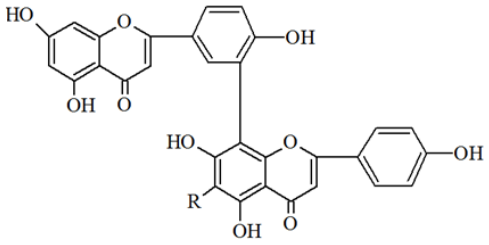


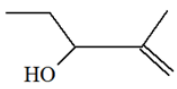
[12] R_1= OMe, R_2=OMe, R_3=OH

[13] R_1= OMe, R_2=OH, R_3= OMe

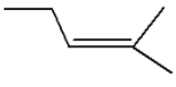
[16] R_1= OH, R_2=OH, R_3=OH



[14] R=

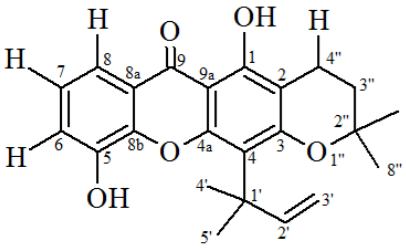


[15] R=



[17] R= H

Figure 2.2: Chemical structures of natural products (continued).



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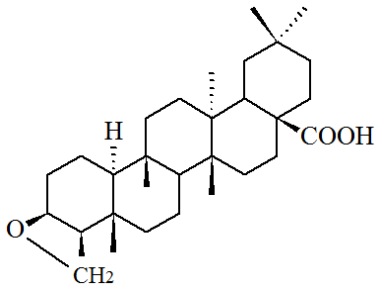
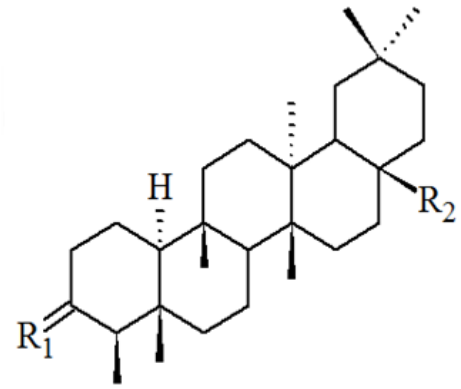


[19]



[20] R_1= O R_2= CH_2OH

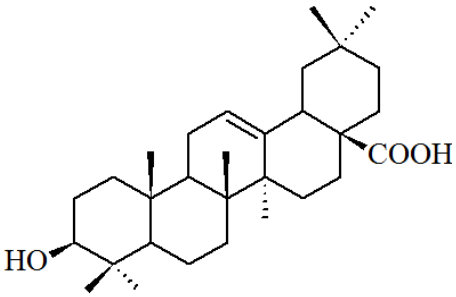
[21] R_1= O R_2= COOH

[22] R_1= O R_2= CH_3

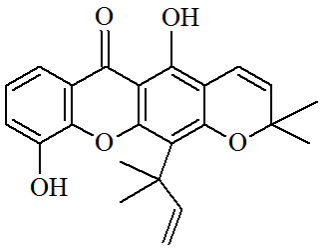
[23] R_1= O R_2= CHO

[24] R_1= βOH R_2= COOH

[25] R_1= βOH R_2= CH_3

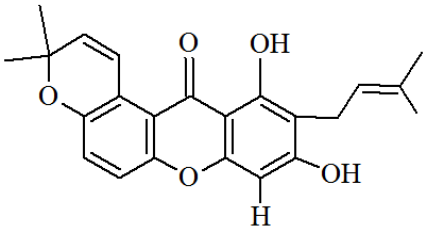


[26]

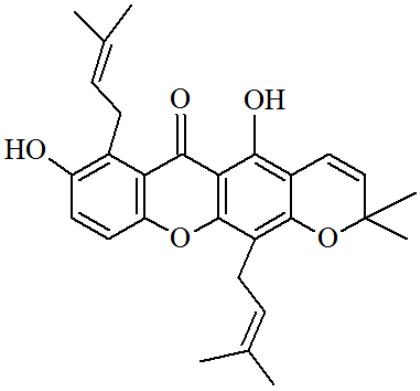


[27]

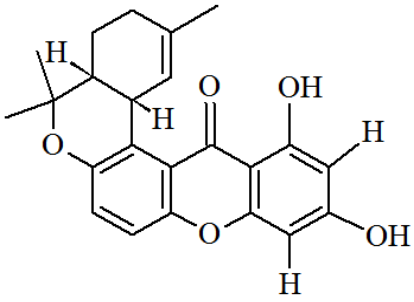
Figure 2.3: Chemical structures of natural products (continued).



[28]



[29]



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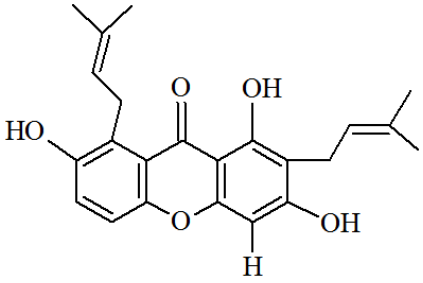
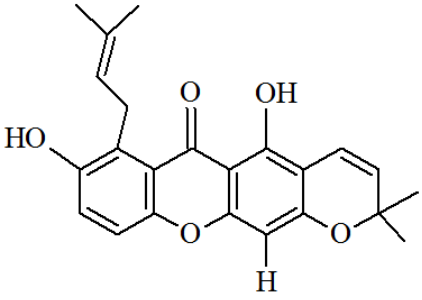
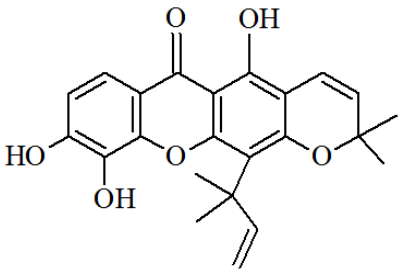


[31]



[32]



[33]

Figure 2.4: Chemical structures of natural products (continued).

Table 2.1: Summary of Chemistry and Biological Activities of *Calophyllum* Species.

Plant name	Compounds isolated	Biological details	Literature reference
C. brasilience	- Chromanone acids - Xanthones - Phenolic acids - Triterpenoids - Biflavonoid - Steroids - Terpenes - Coumarins	- Antibacterial activity - Anticancer agents - Anti ulcerogenic - Antifungal activity - Analgesic activity - Anti-HIV - Leishmanicidal effects - Antiviral activity - Molluscicidal activity - Anti-microbial	- Leonti, et al.,2001 - Sartori, et al.,1999 - Souza, et al., 2009 - Ito, et al., 2002 - Chilpa, Estrada and Muniz, 1997 - Plattner, et al.,1974
C. inophyllum	- Xanthones - Triterpene - Coumarin - Benzopyrano derivatives - Calophyllic acid - Flavonoids	- Cytotoxic activity - Antioxidant activity - Anticancer - Anti HIV - Antimicrobial activity	- Bruneton, 1993 - Breck and Stout,1969 - Kawazu, Ohigashi and Mitsui, 1968 - Prasad, et al., 2012 - Polonsky, 1957

Table 2.1 continued: Summary of Chemistry and Biological Activities of *Calophyllum* Species.

			- Ling, Kian and Hoon, 2009 - Dweck and Meadowsy, 2002 - Bhat, Kane and Sreenivasan, 1954 - Khan, et al., 1996
C. venulosum	- Biflavonoids	- Nil	- Chao, Sim and Goh, 1997
C. pinetorum	- Flavonoids - Triterpenes - Xanthones - chromanone acid	- Nil	- Roig,1988 - Alarcon, et al., 2008 - Piccinelli, et al.,2013
C. membranaceum	- Xanthone	- Inhibitory activity against cyclooxygenase -2	- Jiangsu New Medical College,1986 - Zou, et al., 2005
C. sundaicum	- Polyprenylated acylphloroglucinols	- Antidepressant - Anticancer activity - Anti HIV - Anti-inflammatory	- Taher, et al., 2005 - Velioglu, et al., 1998

Table 2.1 continued: Summary of Chemistry and Biological Activities of *Calophyllum* Species.

C. dispar	- Coumarin	- Molluscicidal - Piscicidal - Anti-HIV - Cytotoxic activity	- Kashman, et al., 1992 - Spino, Marco and Subramaniam, 1998 - Guilet, et al., 2001
C. enervosum	- Xanthone -Polyisoprenylated ketone	- Antimicrobial activity	- Taher, et al., 2005
C. rubiginosum	- Xanthone - Coumarin	- Antioxidant activity	- Taher, et al., 2010
C. thorelii	- Xanthones - Phenolic compounds	- Cytotoxic activity	- Nguyen, et al., 2012
C. caledonicum	- Xanthones - Chromanone acid	- Antimalarial - Antifungal - Antiplasmodial activity	- Morel, et al., 2000 - Hay, et al., 2003 - Hay, et al., 2004 - Morel, et al., 2002
C. blancoi	- Xanthones	- Anti-corona virus activity	- Shen, et al.,2005
C. soulattri	- Coumarin	- Anti-HIV	- Sartori, et al.,1999

Table 2.1 continued: Summary of Chemistry and Biological Activities of *Calophyllum* Species.

C. dryobalanoides	- Xanthones - Flavonoid - Triterpenoid - Chromanone acids	- Nil	- Vo, 1997 - Ha, et al., 2012
C. polyanthum	- Pyranocoumarin	- Anti-HIV	- Ma, et al., 2004 - Kashman, et al.,1992
C. teysmannii	- Coumarin - Xanthones	- Anti-HIV - Therapeutical agent	- Pengsuparp, et al., 1996 - McKee, et al., 1996 - Maia, et al., 2005 - Gonzalez, et al., 1999 - Kijjoa, et al., 2000
C. lanigerum	- Coumarin	- Anti-HIV	- McKee, et al., 1996 - Pengsuparp, et al., 1996 - Kashman, et al., 1992
C. panciflorum	- Biflavonoid - Xanthones	- Anti tumor activity	- Ito, et al., 1999

Table 2.1 continued: Summary of Chemistry and Biological Activities of *Calophyllum* Species.

C. antillanum	- Chromanone acid	- Nil	- Roig, 1974 - Piccinelli, et al., 2013
C. rivulare	- Chromanone acid	- Nil	- Roig, 1974 - Piccinelli, et al., 2013
C. cordato-oblongum	- Xanthones - Pyranocoumarins - Chromene acid - Triterpenoids	- Anti-HIV	- Dharmaratne, et al., 1999
C. papuanum	- Chromanone acid	- Nil	- Stout, Hickernell and Sears, 1968
C. apetalum	- Coumarin - Triterpenoid - Chromanone acid	- Nil	- Govindachari, Prakash and Viswanathan, 1967 - Nigam and Mitra, 1969
C. tomentosum	- Coumarin	- Nil	- Nigam and Mitra, 1969

Table 2.1 continued: Summary of Chemistry and Biological Activities of *Calophyllum* Species.

C. chapelieri	- Chromanone acid	- Nil	- Guerreiro, Kunesch and Polonsky, 1971
C. austroindicum	- Xanthone - Chromanone acid	- Nil	- Linuma, et al., 1996
C. macrocarpum	- Flavonoid - Chromanone acid	- Nil	- Ampofo and Waterman, 1986
C. walkeri	- Flavonoid - Chromanone acid - Xanthone	- Nil	- Ampofo and Waterman, 1986

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials and Instruments

3.1.1 Collection of Plant Material

The plant studied in this project was *Calophyllum castaneum* and the stem bark material of this plant was collected from the jungle in Landeh, Sarawak. The voucher specimen detailing the collection of plant was deposited at the herbarium of Universiti Teknologi MARA, Sarawak. The dry weight of the collected plant was 2.0689 kg.

3.1.2 Chemicals

Solvents and materials used for isolation and purification of chemical constituents

are listed in the Table 3.1. Staining chemicals used for chromatographic visualization techniques are listed in the Table 3.2. Solvents and materials used for chemical analysis (NMR and TLC) are listed in the Table 3.3. Chemicals used for the preparation of antioxidant assay are listed in the Table 3.4.

Table 3.1: Solvents and materials used for isolation and purification of chemical constituents.

Solvents/Materials	**Molecular Formula**	**Density, $\rho(gml^{-1})$**	**Source, Country**
Hexane (I.G)	$CH_3(CH_2)_4CH_3$	0.659	Merck (Germany)
Dichloromethane (I.G)	CH_2Cl_2	1.325	Fisher Scientific (UK)
Ethyl Acetate (I.G)	$CH_3COOC_2H_5$	0.902	Lab-Scan (Ireland)
Acetone (I.G)	CH_3COCH_3	0.791	QReC (Malaysia)
Methanol (I.G)	CH_3OH	0.791	Mallinckrodit Chemicals (Phillipsburg)
Anhydrous Sodium Sulphate	Na_2SO_4	-	John Kollin Coperation (USA)
Sea Sand	-	-	Merck (Germany)
Silica gel 60 (230-400 mesh)	-	-	Carl Roth (Germany)
Sephadex LH-20			

*(I.G) refers to industrial grade

Table 3.2: Staining chemicals used for chromatographic visualization techniques.

Chemicals	Molecular Formula	Source, Country
Ferric Chloride	$FeCl_3$	UNI-CHEM
Iodine crystals	I_2	Fisher Scientific (UK)

Table 3.3: Solvents and materials used for chemical analysis (NMR and TLC).

Solvents / Materials	Molecular formula	Source, Country
Acetone-d_6	CD_3COCD_3	ACROS Organics (Belgium)
Chloroform-d	$CDCl_3$	ACROS Organics (Belgium)
Methanol-d_4	CD_3OH	ACROS Organics (Belgium)
Hexane(A.G)	$CH_3(CH_2)_4CH_3$	QReC (Malaysia)
Dichloromethane(A.G)	CH_2Cl_2	QReC (Malaysia)
Ethyl Acetate(A.G)	$CH_3COOC_2H_5$	R&M Chemicals (UK)
Acetone(A.G)	CH_3COCH_3	QReC (Malaysia)
Methanol(A.G)	CH_3OH	QReC (Malaysia)
TLC silica gel 60 F_{254}	-	Merck(Germany)
Nylon Syringe Filter		Membrane-Solution (USA)

*(A.G) refers to analytical grade

Table 3.4: Chemicals used for the preparation of antioxidant assay.

Chemicals/Materials	Source, Country
DPPH	Sigma Aldrich (USA)
Vitamin C	Sigma Aldrich (USA)
Kaempferol	Sigma Aldrich (USA)
96-well plate	Techno Plastic Product AG (Switzerland)

3.2 Instruments

3.2.1 Nuclear Magnetic Resonance (NMR)

NMR is a non-destructive spectroscopic technique that uses magnetic property of atom called nucleus spin to elucidate structure by determining the composition of organic compounds. The NMR spectra of the samples were obtained from JEOL JNMECX 400 MHz spectrometer using trimethylsilane, TMS as internal standard. The samples were prepared by dissolving in deuterated solvents such as acetone-d_6 (CD_3COCD_3), chloroform-*d* ($CDCl_3$), and methanol-d_4 (CD_3OD) in a uniform 5 mm internal diameter NMR glass tube. Solvent choice depends on the degree of solubility of compound in the solvent. A reasonable amount of sample was diluted in deuterated solvent and was transferred into NMR tube up to height of 4 cm.

The labeled tube was capped and sealed tightly using parafilm to prevent solvent evaporation. Chemical shifts were reported in the units of parts per million (ppm).

3.2.2 Infrared Spectrometer (IR)

Infrared spectrometry is a technique used to determine the functional groups present in organic compounds. IR spectra of samples were obtained from Perkin Elmer Lambda 25 Spectrum RX1 using KBr pellet in the range of 4000 cm^{-1} to 400 cm^{-1}. The sample was prepared by mixing with KBr at 1:10 ratio, which was then compressed under high pressure to produce thin KBr disc. The prepared sample was placed in IR instrument for measurement.

3.2.3 Ultraviolet-visible Spectrometer

UV-Vis method measures absorbance of light after the light passes through the analyte in solution form. Measurement is done at specific wavelength or a range of wavelength. UV-Vis spectra were recorded on Perkin Elmer Lambda 35 in the region from 200 nm (near UV) to 800 nm (near infra-red). The analysis was done

by dissolving 3 mg of samples in 2 ml analytical grade ethanol and the solutions were transferred into quartz cuvette to be placed in UV-Vis spectrometer.

3.2.4 Gas Chromatography – Mass Spectrometry (GC-MS)

GC-MS is a common analytical instrument used in the isolation and identification of thermally stable and volatile organic compounds. The samples were prepared by dissolving 1 mg test compounds in analytical grade solvents. Choice of solvent depends on the extent of solubility of compounds. The mixture was sonicated for thorough mixing and filtered into sample vials using MS® Nylon Syringe Filter of 0.45 μm pore size and 25 mm diameter. The instrument used to record CGMS spectra was Shimadzu GCMS-QP 2010 Plus model.

3.2.5 Polarimeter

Polarimeter is used to measure the optical rotation of optical isomers which are able to rotate the plane polarized light either clockwise or anti-clockwise. 30 mg of sample was weighed and dissolved in 10 ml chloroform. The diluted sample was then transferred into polarimeter sample tube and placed in the polarimeter

for the measurement. The optical rotation of the sample was measured using JASCO, P-2000 Polarimeter.

3.2.6 Melting Point Instrument

Melting point determination is done to know the purity of solid compounds obtained. Melting points of pure compounds were determined using Stuart Melting point SMP 10 apparatus and were uncorrected. A very small amount of sample was inserted into capillary tube and the test tube was then inserted into the melting point instrument. The measurement was taken from the temperature where solid started to melt until it completely becomes liquid.

3.2.7 Microplate Reader

Microplate reader is a colorimetric method used to determine the biological activities of sample being analyzed. The microplate format used is 96-well (8 by 12 matrix) with a typical reaction volume of (100 and 200 µL) per well. The detection mode used is absorption detection.

The wells of microplate were filled with test compounds, methanol and DPPH solution of specific volumes and kept in the dark for 30 minutes. After 30 minutes, the well plate was sent for the measurement of absorbance using the microplate reader. The absorbances of the mixtures in the well plate were measured using Model 680, Bio-Rad Laboratories, Hercules, CA, USA microplate reader at 520 nm and the results were interpreted by the Microplate Manager®, Version 5.2.1 software.

3.3 Extraction, Isolation and Purification of Chemical Constituents from *Calophyllum castaneum*

About 2 kg of dried stem bark of *Calophyllum castaneum* collected was finely ground and transferred into three 5 L conical flasks using filter funnel. The conical flasks were then filled with 5 L industrial grade dichloromethane solvent and allowed to soak with irregular shaking for two days under room temperature and pressure. After two days, the material was filtered and the extract was subjected to rotary evaporation under reduced pressure to remove the solvent. The solvent was recovered to be further used in soaking to minimize wastage. The concentrated extracts were transferred into 500 ml beaker for air drying.

The plant material was soaked again with recovered dichloromethane and left for two days. The extraction process was repeated twice using recovered solvent to ensure maximum extraction of plant chemicals. The concentrated dichloromethane extracts were combined and left open for air drying. The above procedures were repeated using ethyl acetate followed by methanol. The recorded masses for dichloromethane, ethyl acetate and methanol extracts were 40.6336 g, 27.8285 g and 41.9765 g respectively. About two grams of each crude extract were kept for DPPH assay.

The remaining of crude extracts were then separately subjected to silica gel column chromatography using gradient elution in which the crude extracts were separated into a number of fractions. The collected fractions were concentrated using rotary evaporator under reduced pressure at 40^{o}C. The concentrated fractions were then transferred into sample vials and continued with TLC analysis. The fractions that show similar TLC separation patterns were combined together and the fractions of interest were continued with further purification using column chromatography method. The process of purification and TLC analysis were repeated until pure compounds were obtained. The partially pure compounds which were inseparable by silica gel column were subjected to gel filtration chromatography using Sephadex LH-20 as stationary phase eluted with methanol to give pure compounds.

Isolated pure compounds were analyzed using various instrumental techniques such as mass spectrometry, NMR, UV-Vis, and IR spectroscopies to elucidate the structure of the isolated compounds. Melting point determination was done on pure compounds to confirm their purity.

3.4 Chromatographic Method

3.4.1 Column Chromatography

Column chromatography was used to purify a mixture of compounds. The stationary phase used in column chromatography was silica gel while mobile phase was pure solvent or mixture of solvents. ROTH Kieselgel 60 (230-400 mesh) was used for column and sample packings. Type of column chromatography used was gravity column chromatography. The sizes of columns used for separations were 40 mm, 30 mm or 20 mm in diameter depending on the amount of sample used. Initially, the crude sample was subjected to column separation and the resulting sample fractions were subjected to further column separations until desired outcome is achieved.

Dry packing method was used to pack the samples before they were introduced as a sample layer onto the packed column. Samples to be purified were dissolved in solvent and blended in minimal amount of silica gel in a fume hood. They were ground to powder form and allowed to dry.

The column packing was prepared using sintered glass column. A minimum amount of hexane was poured into the column and sea sand or anhydrous salt was added into the column to cover the sintered disc. This was done to prevent silica gel from running down the column due to the larger porosity of sintered disc and to absorb moisture from eluted samples. The column was tapped using rubber tubing to ensure the sand is distributed evenly. Silica slurry was prepared by mixing silica gel with hexane. The slurry then introduced into the column and was left to settle down in the column. The column was tapped again to condense the packing to prevent cracking during separation. Next, the sample was introduced into the column. Then, a layer of sand or anhydrous salt was added above sample layer to prevent the sample packing from being disturbed and to absorb moisture in mobile phase.

Subsequently, mobile phases were added into the column to elute out the compounds by gravity force. The mobile phases used for elution were hexane, dichloromethane, ethyl acetate, acetone and methanol. Gradient elution was used

in all column chromatography separation so that compounds of different polarity can be separated out from the sample or crude. Often series of increasing polar solvent system is used starting from less polar solvent which elutes out less polar compound followed by polar solvent which elutes polar compounds. Separations can be achieved because, the components of mixture have different affinity for stationary and mobile phases and thus they will be eluted out from the column with different retention times.

The eluents were collected according to separated color bands or according to volumes. The solvents from collected fractions were removed using rotary evaporator. They were then analyzed using TLC to determine quantitatively the chemical composition of each fraction collected. Fractions that show similar chemical composition on TLC plate were combined together to be subjected for further separation.

Figure 3.1: Gravity column set up.

3.4.2 Thin Layer Chromatography (TLC)

Coated aluminium sheets of silica gel (Merck TLC Silica gel 60 F_{254}) were used for TLC analysis. TLC plates used were obtained as 20 cm × 20 cm sheets. Each sheet was cut into smaller plates sized 8 cm×4 cm. The plates were then measured 0.5 cm from bottom and above to draw two horizontal lines across the plate with a pencil. The bottom line drawn is called baseline while the upper line on the plate is called solvent front line. Five sample spots were marked on the baseline using pencil.

The samples were prepared in analytical grade solvents. The solvents used must be able to completely dissolve the samples. Micro capillary tube was used to spot the sample solutions on the TLC plate. Developing chamber was prepared by placing folded filter paper inside a beaker with watch glass covered on top. About 10 ml solvent was added into the developing chamber, swirled gently and the plate was then placed in. The beaker was covered with watch glass to prevent solvents from evaporated away and filter paper was used to give saturated vapor atmosphere in the chamber. The chamber was left undisturbed for the TLC to develop. The plate was removed from the chamber when the solvent reached the solvent front line. The plate was allowed to dry and spot visualization was carried

out using UV lamp (short and long wavelengths), iodine vapor and ferric chloride solution.

Retention factors, R_f were calculated for pure compounds using the formula below:

R_f = (Distance travelled by the compound) / (Distance travelled by the solvent front)

The purpose of calculating R_f value for pure compounds was to determine and confirm the identity of the isolated compound with the R_f value of reference compound.

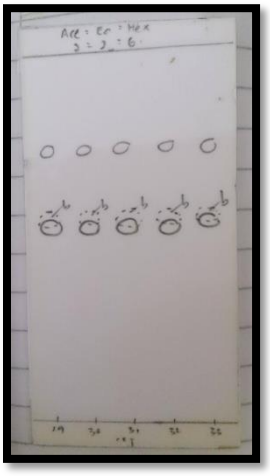


Figure 3.2: TLC plate.

3.4.3 Gel Filtration Chromatography

Gel filtration chromatography was used to purify partially impure compound mixture which showed two or three spots on TLC plate. The stationary phase used was polymer gel, Sephadex LH-20 while mobile phase used was a mixture of solvents. The size of column used for separation of compounds was 20 mm diameter column.

The slurry of stationary phase was prepared by mixing the sephadex gel in a solvent where it was mixed thoroughly and sonicated to eliminate air bubbles. The column was then filled with approximately 50 ml solvent. Next, the stirred slurry was poured into the column using a filter funnel and the sephadex packing was allowed to settle down the column. The packing was continued by adding more slurry into the column until it reached the level, 3 cm from the top part of column. The samples were dissolved in solvent in which the solvent choice is dependent on the extent of sample dissolution. The dissolved samples were filtered to eliminate particulate matters. Pasteur pipette was used to load sample gently onto the packed column.

The column was run by adding mobile phase using Pasteur pipette. Isocratic elution was used in this chromatography. Compounds in the column were eluted out according to the differences in their molecular weights. Larger molecules will be eluted first from the column followed by the smaller molecules. The stationary phase was composed of porous gel beads. The elution time of smaller size molecule was longer because they took a longer travelling route down the column by diffusing into the gel beads. Meanwhile larger molecules were too big to get diffused into the pores.

The fractions were collected in small volumes of 3 or 4 ml per fraction. The collected fractions were allowed to air dry and TLC analyses were carried out. Pure compounds isolated were sent for NMR analysis for structural elucidation.

3.5 TLC Detection Methods

Once the TLC plate was developed, the spots separated were visualized using various visualization and staining techniques. These techniques allow us to identify spots present on the TLC plate, and to have a general idea about the type of compounds and functional groups present on the studied compound. The spots observed on the TLC were marked down using a pencil.

3.5.1 Natural Colors

The compounds separated can be seen easily on the silica plate right after the development using naked eyes if they appear as colored compounds. No visualization technique was used if the compound is naturally colored and the color is visible in daylight. Some examples of compounds that possess natural colors are blancoic acid and chlorophyll. However, most of the time the organic compounds appear to be colorless on the TLC plate. So, methods below were used to reveal the spots on the plate.

3.5.2 UV Detection

The developed TLC plates were observed under UV light of both short (254 nm) and long (365 nm) wavelengths using UV lamp SASTEC ST-UVL7. The components appear as fluorescent or dark spots under UV light were marked down using a pencil. The TLC plates are normally impregnated with fluorophor which exhibits fluorescence under UV light. The area of compound separated usually appears as a dark spot with bright background under UV short wavelength or as fluorescence spot with dark background under UV long wavelength. Dark spot is due to the compound containing chromophore which can quench the

fluorescence by absorbing UV light in that particular area, while fluorescence spot is because of the excitation by UV light. This method allows the detection of UV-active compounds containing conjugated systems and aromatic groups.

3.5.3 Iodine Vapor Test

Iodine chamber was prepared by introducing appropriate amount of iodine crystals into a closed bottle. The developed TLC plate was then placed in iodine chamber saturated with iodine vapor for a few minutes before taking it out. The staining effect on the TLC plate was temporary as it will disappear upon evaporation against time. Iodine forms dark-brown complex with colorless compounds and were marked down using a pencil.

3.5.4 Ferric Chloride Solution

The solution was prepared by dissolving 1.5 g ferric chloride in 100 ml methanol. The separated components on the developed plates were sprayed with staining reagent to determine the presence of phenols or hydroxamic acids as indicated by

formation of dark blue and red color spots respectively after the treatment with the reagent.

3.6 Antioxidant Assay

The isolated compounds and crude extracts were tested for their antioxidant activity via DPPH free radical scavenging assay. The protocol of antioxidant assay carried out was similar to that described by Susanti et al (2007) with minor alteration.

The master stocks of isolated pure compounds and standards were dissolved in analytical grade methanol solvent to prepare master stocks at concentration of 1 mg/ml. The prepared master stocks were sonicated for 5 minutes to ensure complete dissolution of samples before they were kept in refrigerated condition at 4^{o}C in the dark. Next, DPPH solution of 2 mg/ml was prepared by dissolving the DPPH powder in methanol. The solution prepared was then sonicated for thorough dissolution and kept in dark condition in a chiller at 4^{o}C.

In a 96-well plate, different concentrations of isolated and standard compounds at concentration of 240, 120, 60, 30, 15, 7.5, and 3.75 µg/mL were prepared in triplicates by diluting them in methanol through serial dilutions. Then, 10 µl of freshly prepared DPPH solution was added into each well followed by the addition of methanol. Table 3.5 shows the volumes of test compounds, methanol and DPPH solution added into each well.

The plate was wrapped with aluminium foil immediately after the addition of reagents and solvent to prevent evaporation. The plate was then stored in the dark condition at room temperature for 30 minutes. After 30 minutes, the absorbances of the mixtures in the plate were measured using microplate reader at 520 nm and the results were interpreted by the Microplate Manager®, Version 5.2.1 software.

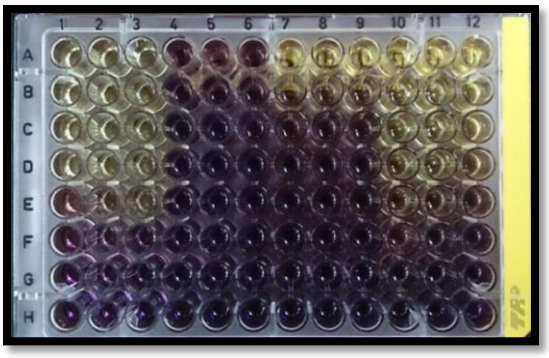


Figure 3.3: 96 well plate filled with test compounds.

Table 3.5: Volumes of test compounds, methanol and DPPH solution added into each well.

Row	Final Concentrations of Test compounds (µg/ml)	Test compound (µl)	DPPH solution (µl)	Methanol (µl)	Final Volume (µl)
A	240.00	100 (96 stock + 104 MeOH)	10	90	200
B	120.00	100 (100 from A + 100 MeOH)	10	90	200
C	60.00	100(100 from B + 100 MeOH)	10	90	200
D	30.00	100(100 from C + 100 MeOH)	10	90	200
E	15.00	100(100 from D + 100 MeOH)	10	90	200
F	7.50	100(100 from E + 100 MeOH)	10	90	200
G	3.75	100(100 from F + 100 MeOH)	10	90	200
H	-	0	10	190	200

(Row H serves as negative control)

The inhibition rates of isolated and standard compounds were calculated using formula given below:

Inhibition rate (%) = $\frac{A_O - A_1}{A_0} \times 100\%$

Where A_o = Absorbance of the negative control (blank)

A_1 = Absorbance of the test compound

Graphs of inhibition rate against concentration of samples were plotted for each test compound and IC_{50} values were obtained from the plots.

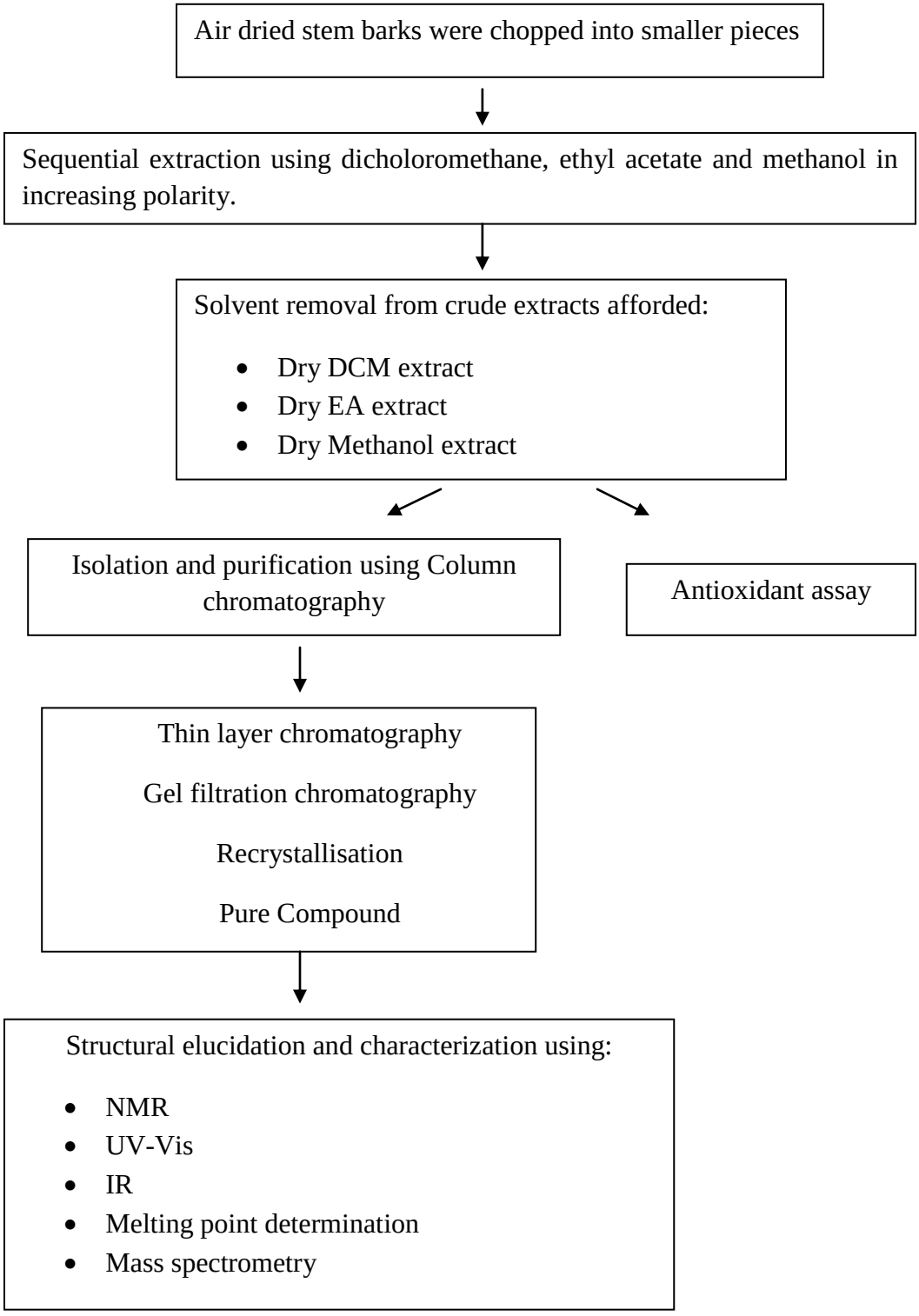


Figure 3.4: Summary of extraction and isolation of chemical constituents from *C.castaneum*.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation of Friedelinol [34]

Friedelinol was isolated from the methanol crude extract of *C.castaneum*. The dry methanol crude extract (41.9768 g) was subjected to gravity column chromatography over Carl Roth silica gel 60 of 230-400 Mesh packed in hexane. Gradient elution was used to elute the column. Mobile phase used for the elution were mixtures of hexane: dichloromethane, dichloromethane: acetone and acetone: methanol in increasing polarity. The separation afforded a total of 30 fractions and they were labeled as CCA. The fractions were then subjected to TLC analysis and grouped according to similar TLC patterns. Combined fractions CCA 8-10 were chromatographed over silica gel using 30 mm diameter column and eluted with hexane: ethyl acetate, dichloromethane: acetone and acetone: methanol mixtures in increasing polarity. A total of 37 sub-fractions were collected according to separated bands and volumes which were labeled as CCA 1.1-4.7. The fractions were then combined on the basis of TLC analysis. Sub-fraction CCA 1.6 was recrystallized and washed using methanol to give white

flakes (75 mg). The isolated pure compound was physically characterized and the structure was elucidated using various spectroscopic techniques. The compound was identified as friedelinol, **[34]** according to spectroscopic data obtained and was further confirmed by comparison with literature data.

4.1.1 Characterization of Friedelinol [34]

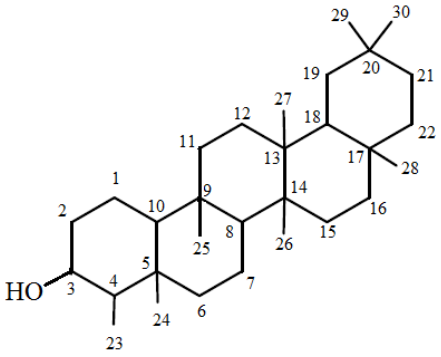


Figure 4.1: Structure of friedelinol [34].

Isolated compound was subjected to TLC analysis. Compound **34** gave a single spot on the TLC plate when the plate was developed using dichloromethane: hexane (5:5) showing an R_f value of 0.28. The compound gave no spot under UV light detection and treatment with $FeCl_3$ reagent. Negative result under UV light of both long and short wavelength indicated that the compound has no conjugated system or aromatic group in its structure. Negative result for $FeCl_3$ test proved the

compound to be non-phenolic. However, staining with iodine vapor gave a single brown spot.

The compound gave a molecular ion peak at m/z 428 in the mass spectrum (Figure 4.2) revealing the compound to have molecular formulae of $C_{30}H_{52}O$. The structure of the compound was elucidated using NMR technique. Compound **34** was characterized as a triterpenoid based on 1D NMR data. Chloroform-*d* was used to prepare the NMR samples giving a solvent peak at 7.2 ppm in the ^{1}H NMR spectrum (Figure 4.3). The spectrum showed most of the proton signals appeared at high field region below 2 ppm due to the presence of hydrogen bonded to sp^3 hybridized carbons in the structure. A relatively deshielded singlet signal at 3.7 ppm was assigned to the methine proton H-3 which is attached to carbon holding hydroxyl group. Multiplets in the region of 1.5-1.9 ppm were assigned to methylene and methine protons. Signals appeared at shielded region between 1.15-0.8 ppm were assigned to methyl protons at positions H-23, H-24, H-25, H-26, H-27, H-28, H-29 and H-30. Remaining proton signals belonging to the rest of methylene and methine protons appeared clumped together in the region between 1.2 ppm and 1.5 ppm.

^{13}C NMR spectrum (Figures 4.5- 4.6) of compound **34** showed a total number of 30 carbon peaks indicated a total of 30 carbon atoms in the structure. Solvent

peak of chloroform-*d* appeared around 77.6 ppm in the spectrum. The most deshielded signal at 73.3 ppm in the spectrum was assigned to carbon C-3 bonded to a hydroxyl group. The deshielded signal is due to the deshielding effect of electronegative atom, oxygen bonded to carbon C-3. The eight carbon signals belonging to methyl groups were assigned in the following positions: C-23 (12.2 ppm), C-24 (16.9 ppm), C-25 (18.8 ppm), C-26 (19.2 ppm), C-27 (20.7 ppm), C-28 (32.9 ppm), C-29 (35.6 ppm) and C-30 (32.4 ppm). The positions of six quaternary carbons at C-5, C-9, C-13, C-14, C-17 and C-20 gave signals at 37.7 ppm, 38.4 ppm, 39.0 ppm, 39.9 ppm, 30.6 ppm and 28.7 ppm respectively. The remaining carbon signals of compound **34** belong to eleven methylene and five methine carbons. ^{13}C NMR spectrum proved that the isolated compound has 30 carbons and that it is a triterpenoid. The assignments of both carbon and protons matched well with the literature values.

Melting point of compound **34** was found to be in the range of 282-283^{o}C (Lit m.p, 282-294^{o}C, Kumar, Raj and Khare, 2009). The UV-Vis spectrum (Figure 4.7) of the compound showed intense absorption at 206.33 nm (ε = 32.86 Lmol^{-1}cm^{-1}). The IR spectrum (Figure 4.8) of compound **34** showed absorption peak at 3429 cm^{-1} indicating the presence of hydroxyl group in the structure. Absorption band at 2924 cm^{-1} was due to sp^3 C-H stretching while, absorption band at 1000 cm^{-1} was because of the C-O stretching. Peaks at 1446 cm^{-1} and 772 cm^{-1} were attributed to the bending vibrations of methyl and methylene groups, respectively.

Table 4.1: Comparison of ^{1}H NMR data of compound 34 with the literature values of friedelinol.

Position	δ_H	δ_H*
2	a- 1.89 (1H,dt, *J*= 10.4Hz, 2.44Hz) b- 1.58 (1H,m)	a- 1.90 (1H,dt, *J*=10.1Hz, 3.0Hz) b- 1.57 (1H,m)
3	3.73 (1H, s)	3.73 (1H, s)
6	a- 1.73 (1H,dt, *J*= 12.2Hz, 3.3Hz)	a- 1.73 (1H,dt, *J*=12.8Hz, 3.0Hz)
23	0.93 (3H,d, *J*=6.7Hz)	0.94 (3H,d, *J*= 7.0Hz)
24	0.93 (3H,s)	0.93 (3H,s)
25	0.84 (3H,s)	0.84 (3H,s)
26	0.98 (3H,s)	0.98 (3H,s)
27	0.98 (3H,s)	0.98 (3H,s)
28	1.16 (3H,s)	1.16 (3H,s)
29	0.99 (3H,s)	0.99 (3H,s)
30	0.95 (3H,s)	0.95 (3H,s)

*Utami, et al., 2013

Table 4.2: Comparison of ^{13}C NMR data of compound 34 with the literature values of friedelinol.

Position	δ_C	δ_C*	Position	δ_C	δ_C*
1	16.3	15.8	16	36.1	36.1
2	35.9	35.3	17	30.6	30.0
3	73.3	72.8	18	43.3	42.1
4	49.7	49.2	19	36.7	35.6
5	37.7	37.1	20	28.7	28.2
6	42.3	41.7	21	32.7	32.1
7	18.1	17.5	22	40.3	39.3
8	53.8	53.2	23	12.2	11.6
9	38.4	37.8	24	16.9	16.4
10	61.9	61.3	25	18.8	18.6
11	35.7	35.2	26	19.2	18.6
12	31.2	30.6	27	20.7	20.1
13	39.0	38.4	28	32.9	32.3
14	39.9	39.3	29	35.6	35.0
15	33.4	32.8	30	32.4	31.8

*Utami, et al., 2013

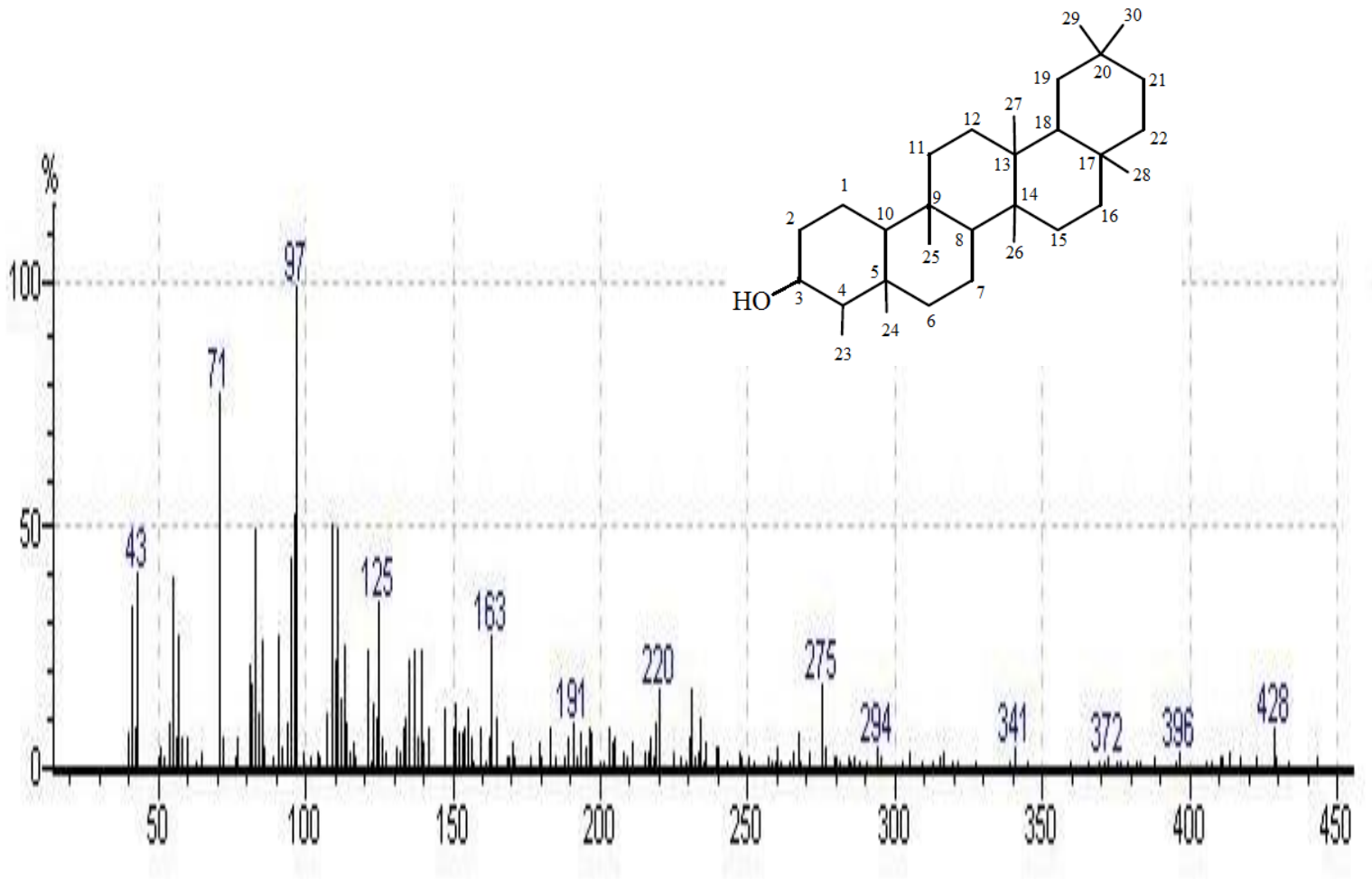


Figure 4.2: Mass Spectrum of friedelinol [34].

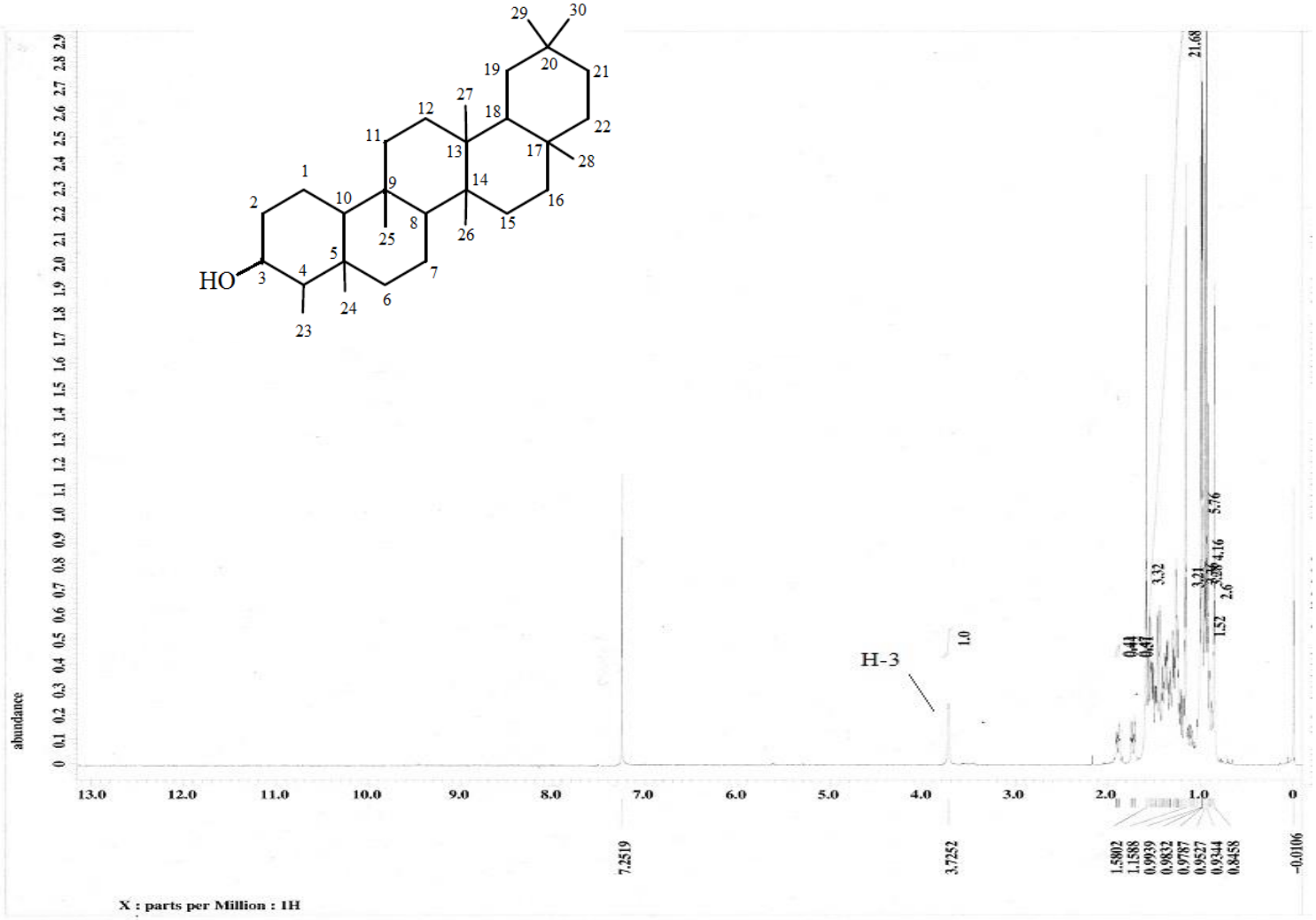


Figure 4.3: ^{1}H NMR spectrum of friedelinol [34] (400MHz, $CDCl_3$).

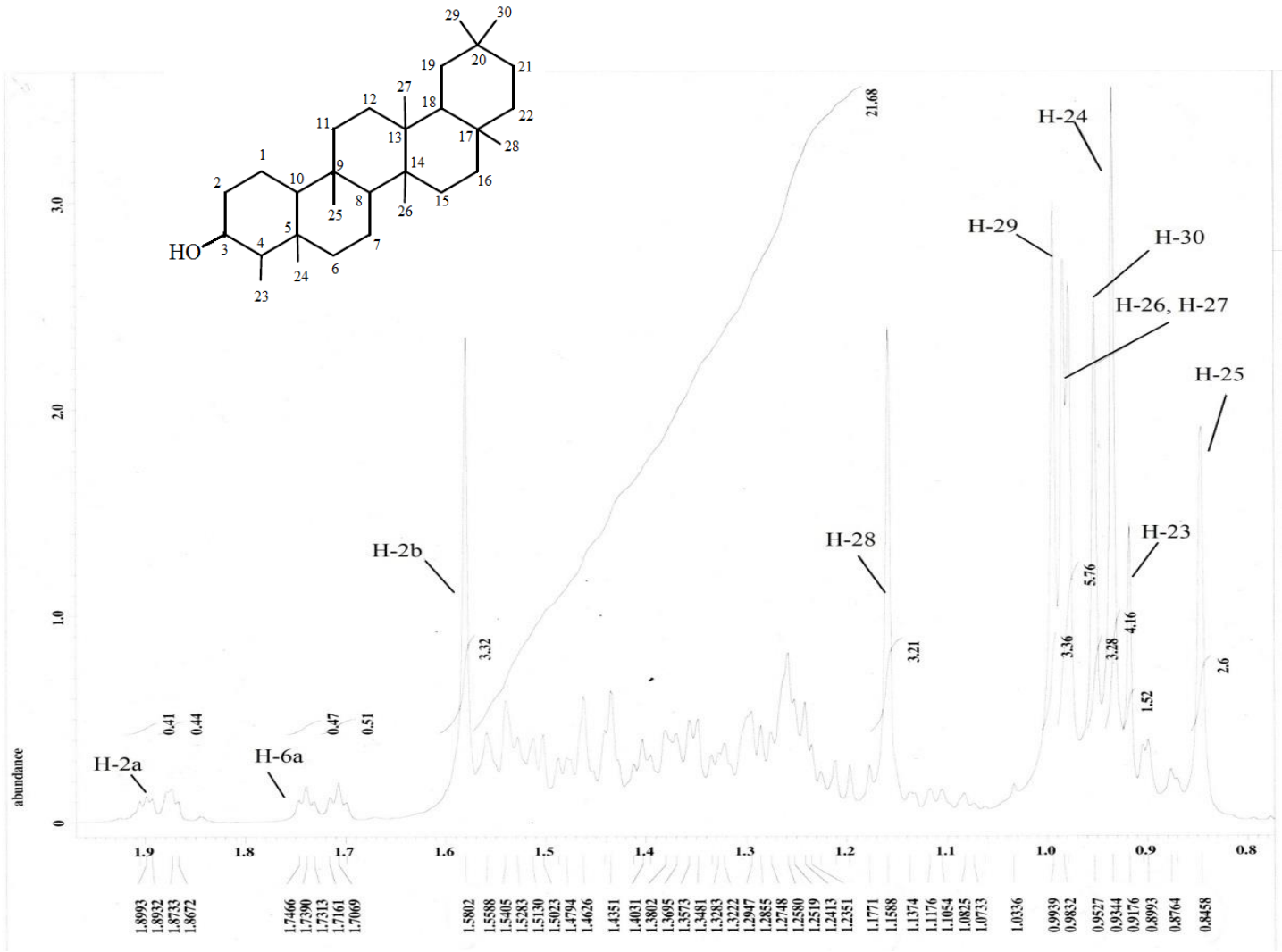


Figure 4.4: ^{1}H NMR spectrum of friedelinol [34] (400MHz, $CDCl_3$) (Expanded).

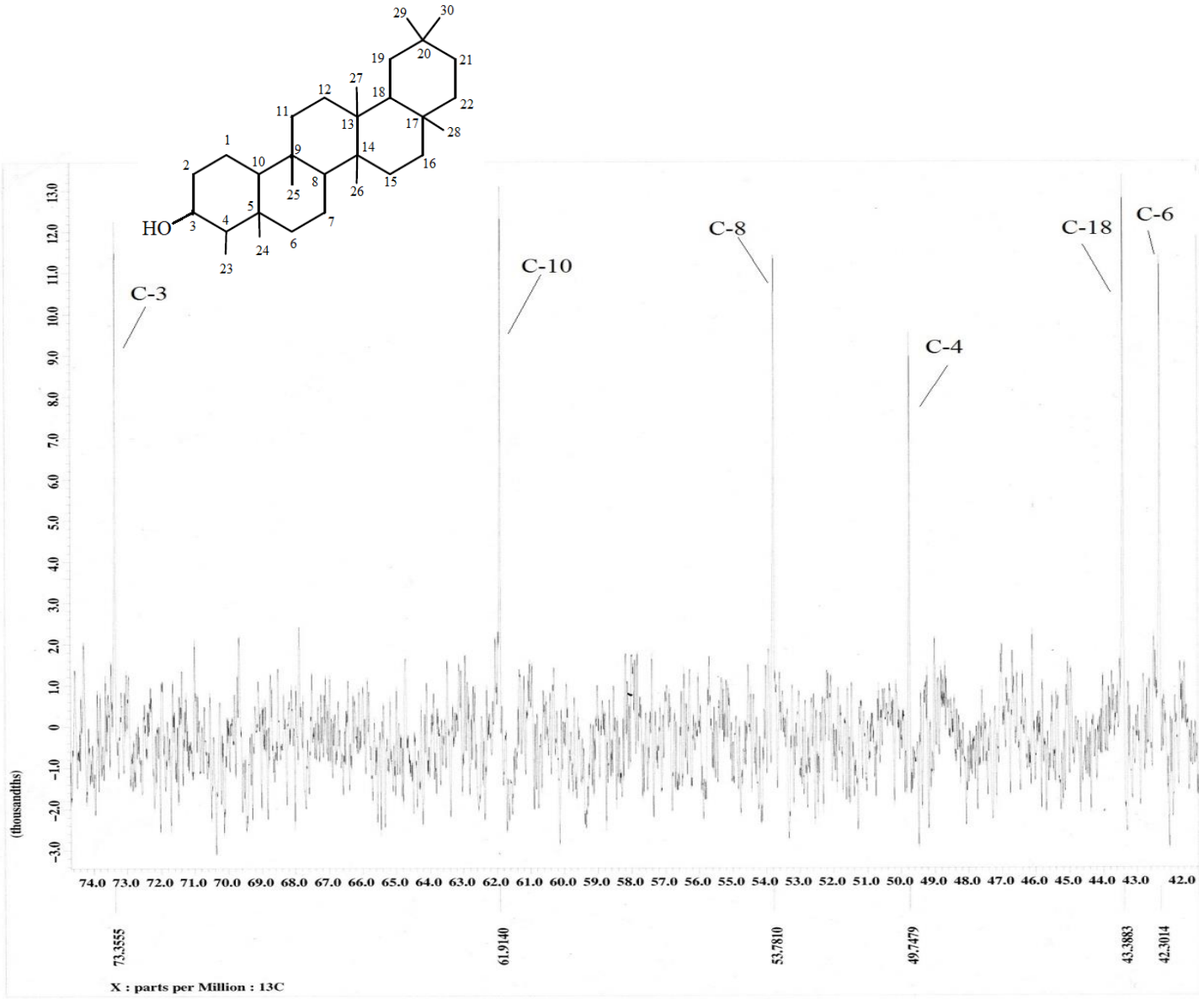


Figure 4.5: ^{13}C NMR spectrum of friedelinol [34] (100MHz, $CDCl_3$) (Expanded).

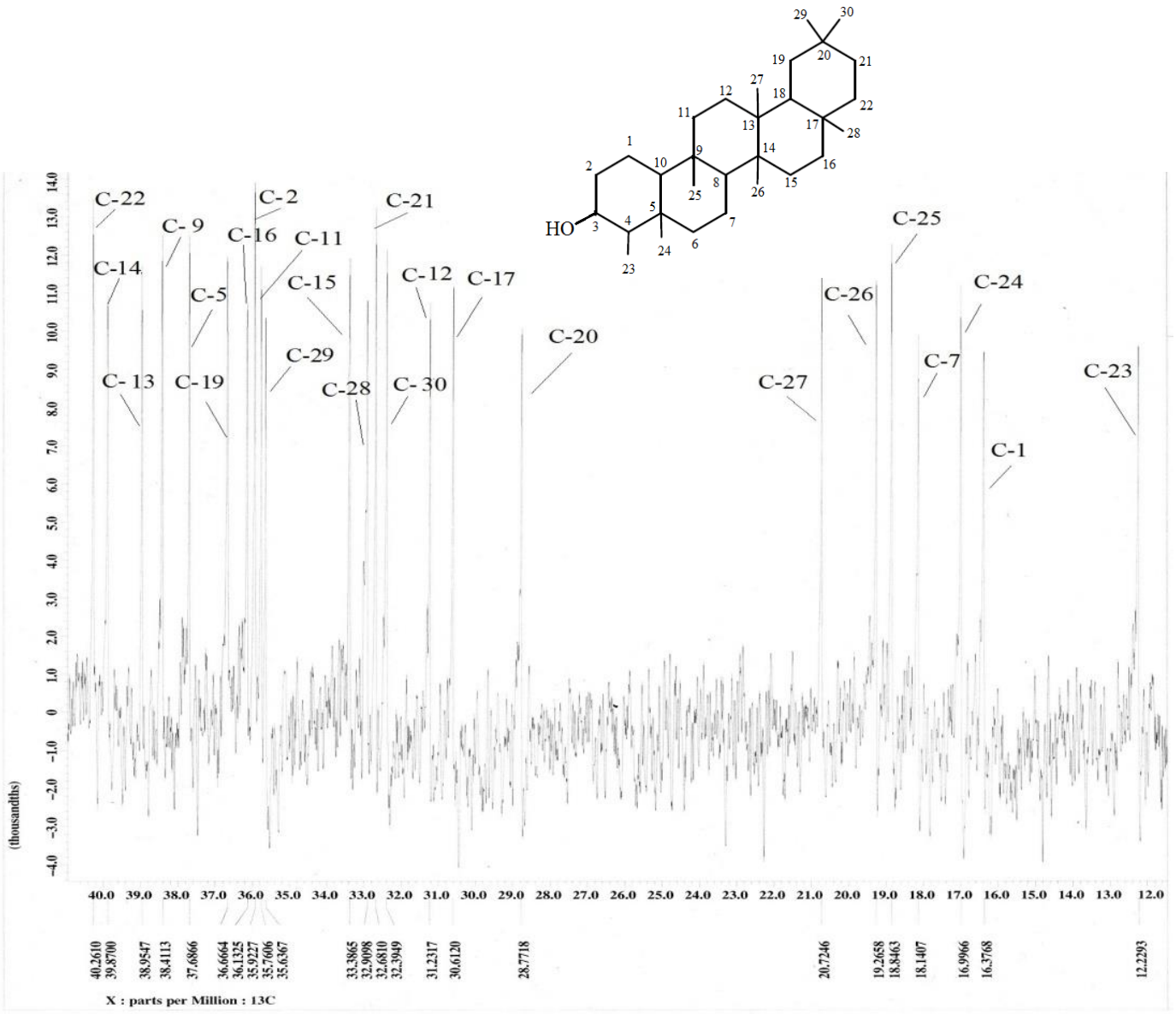


Figure 4.6: ^{13}C NMR spectrum of friedelinol [34] (100MHz, $CDCl_3$) (Expanded).

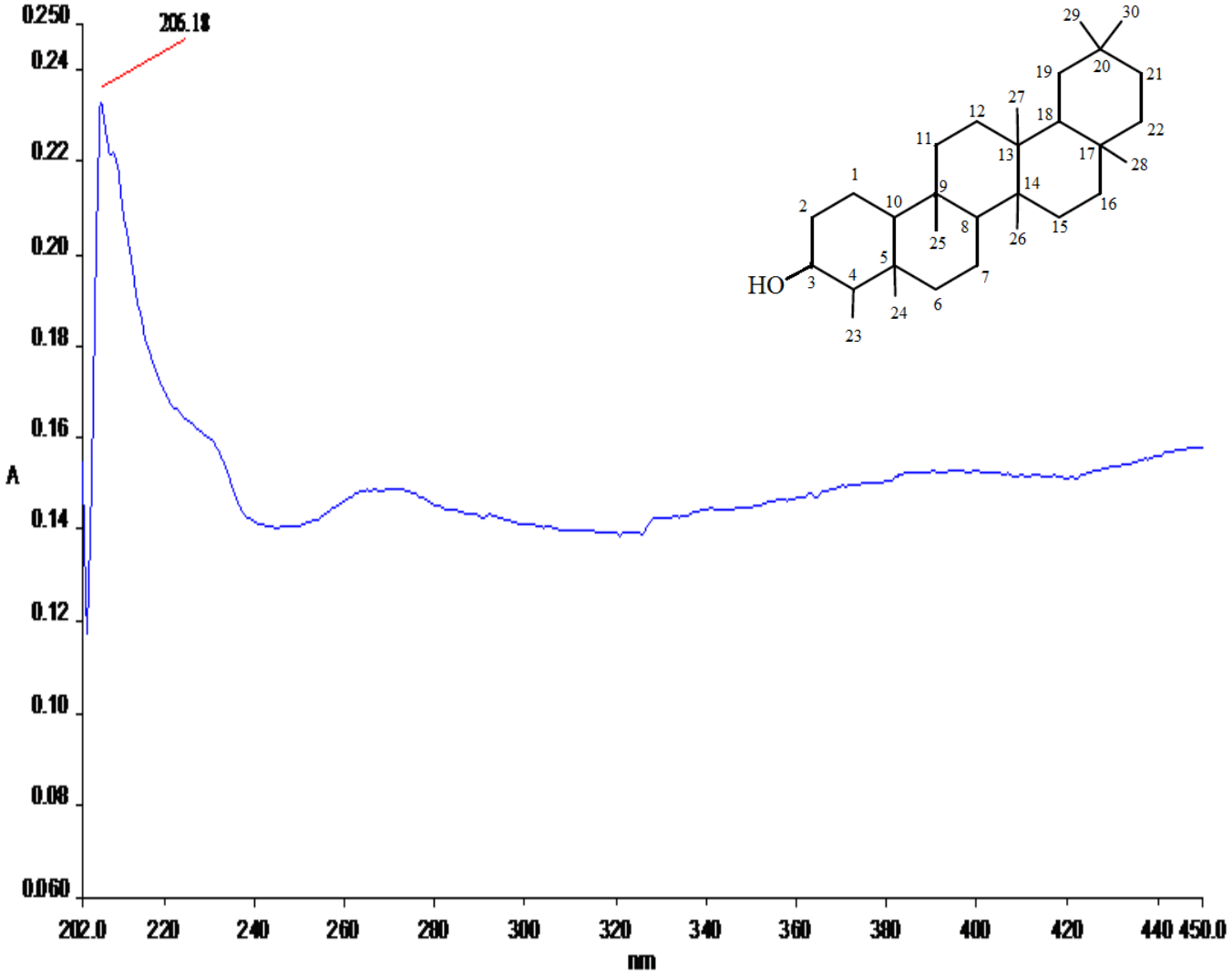


Figure 4.7: UV Vis spectrum of friedelinol [34].

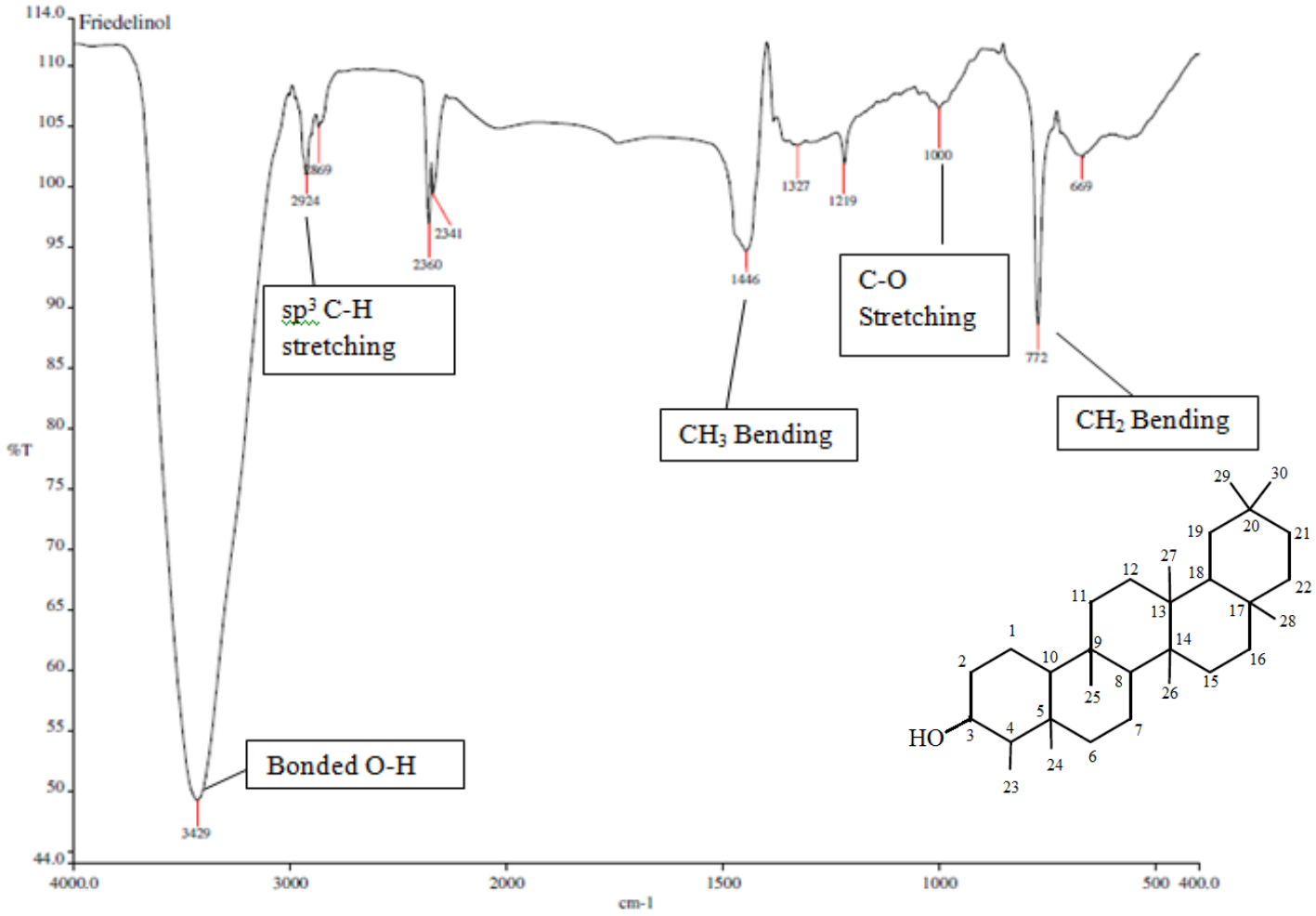


Figure 4.8: IR spectrum of friedelinol [34].

4.2 Isolation of Friedelin [35]

Another compound extracted from the methanol crude extract of *C. castaneum* apart from compound **34** was friedelin **[35]**. Subfraction CCA1.5 from CCA 1.1-4.7 series was subjected to column chromatography over silica gel 60 of 230-400 Mesh (Carl Roth) and eluted using gradient elution of solvent mixture (acetone: hexane) in increasing polarity. The separation yielded 8 sub-fractions and they were labeled as CCC. The sub-fractions were further subjected to TLC analysis where they were grouped according to the TLC patterns. Fractions CCC 4 and 5 were combined and chromatographed over silica gel using dichloromethane: hexane solvent system to give another 54 sub-fractions (CCD). Compound **35** appeared as white needle-like crystals was obtained from sub-fraction CCD 45-49 after repetitive recrystallization using methanol.

4.2.1 Characterization of Friedelin [35]

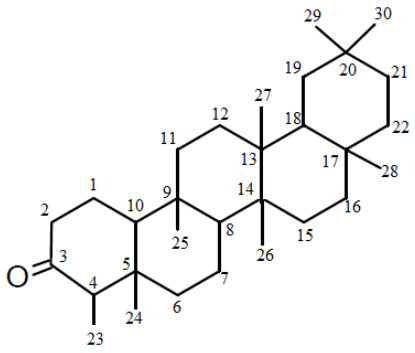


Figure 4.9: Structure of friedelin [35].

Compound **35** was subjected to TLC analysis in which the analysis resulted in single spot using ethyl acetate: hexane (1:9) as mobile phase. The R_f value of compound **35** which gave positive result when stained with iodine vapor was 0.75. This compound gave negative result when visualized under UV light which indicated the absence of conjugated system in the structure. Detection method using $FeCl_3$ gave no color change proving the absence of phenolic groups in the compound.

The compound is found to have molecular weight of 426 g/mol corresponding to molecular formula, $C_{30}H_{50}O$. Since compound **35** is a known compound, the structure was elucidated using 1D-NMR technique and positions of both carbons and protons were assigned by comparison with literature data. Chloroform-*d* was used to prepare the sample for NMR analysis and thus solvent peak appeared at 7.2 ppm in the ^{1}H NMR spectrum (Figure 4.10) of the compound. All the signals in ^{1}H NMR spectrum of compound **35** appeared in the upfield region, below 2.5 ppm due to the presence of hydrogens bonded to sp^3 hybridized carbons. No aromatic or vinylic signals were observed in the spectrum. The proton NMR spectrum showed significant signals for methyl protons which were found to confine in the upfield region between 0.7 ppm and 1.17 ppm. The seven singlet signals of methyl protons were assigned to hydrogens H-24 (0.71 ppm), H-25 (0.86 ppm), H-26 (1.04 ppm), H-27 (0.99 ppm), H-28 (1.17 ppm), H-29 (0.99 ppm) and H-30 (0.94 ppm), while doublet methyl signal at 0.87 ppm was assigned

to hydrogen H-23. Multiplet at 2.27 ppm was assigned to methine proton H-4, and multiplets at 1.95 ppm, 1.74 ppm, 2.37 ppm and 2.27 ppm were assigned to methylene protons H-1a, H-1b, H-2a and H-2b, respectively. Protons at carbon positions 1,2 and 4 appeared at a relatively more downfield region due to the deshielding effect caused by electronegative atom attached to carbon C-3. Remaining signals belonging to methylene and methine protons resided in the region between 1.2 ppm and 1.5 ppm. The signals displayed on the proton spectrum were characteristic signals of common triterpenoids and the readings matched well with the literature values reported by Abbas et al (2007).

The ^{13}C NMR spectrum (Figures 4.13- 4.15) of compound **35** showed a total of 30 signals which were attributed to 8 methyl carbons, 11 methylene carbons, 4 methine carbons, 6 quaternary carbons and a keto carbon in the structure. The keto carbon appeared at the most deshielded region of the spectrum at 213ppm was assigned to carbonyl carbon C-3. The spectrum contains two different groups of low intensity and high intensity signals. The low intensity signals of 6 quaternary carbons were due to the lack of NOE enhancement and they were assigned to the carbons C-5 (42.2 ppm), C-9 (37.5 ppm), C-13 (39.8 ppm), C-14 (38.4 ppm), C-17 (30.1 ppm) and C-20 (28.3 ppm). High intensity signals of 8 methyl group carbons displayed on the spectrum were assigned to carbons C-23, C-24, C-25, C-26, C-27, C-28, C-29 and C-30 at 6.9 ppm, 14.7 ppm, 18.0 ppm, 20.3 ppm, 18.7 ppm, 32.1 ppm, 31.9 ppm, and 35.1 ppm, respectively. Remaining

signals in the spectrum were attributed to methylene and methine carbon signals. Solvent peak of chloroform-*d* appeared around 77.1 ppm in the spectrum. The spectroscopic data of compound **35** was identical with the literature values reported by Abbas et al (2007) for friedelin. The compound is confirmed as triterpenoid by referring to the total number of carbon and proton signals that appear at shielded region. These two facts are characteristic criteria for triterpenoids.

The melting point value obtained for compound **35** was in the range of 250-252^{0}C (Lit m.p 251-254^{0}C, Sousa, et al., 2012). The UV-Vis spectrum (Figure 4.16) showed strong absorption at 206.18 nm which is the characteristic absorption maxima for triterpenes. The hypsochromic shift of high energy and low intensity was due to the non-conjugated structure of compound **35**. The possible electronic transition responsible for the absorption is $\sigma \rightarrow \sigma^*$ transition (ε=32.67 Lmol^{-1}cm^{-1}) which is due to sigma bonds. The IR spectrum (Figure 4.17) showed intense absorption peak at 1638 cm^{-1} which indicated the presence of carbonyl group in the structure. Absorption bands at 2900 cm^{-1} and 1443 cm^{-1} were due to C-H stretching of sp^3 hybridized carbons and C-H bending of methyl groups, respectively. Additional peaks at 3434 cm^{-1} and 2359 cm^{-1} were caused by absorption of moisture and carbon dioxide by KBr pellet.

Table 4.3: Comparison of ^{1}H NMR data of compound 35 with the literature values of friedelin.

Position	δ_H	δ_H*
1	1.95 (H_a, m)	1.96
	1.74 (H_b, m)	1.68
2	2.37	2.38
	(H_a,dd, *J*= 2.7Hz, 5.4Hz)	2.28
	2.27 (H_b, m)	
4	2.27 (1H, m)	2.25
23	0.87 (3H, d,	0.87
	J = 6.7Hz)	
24	0.71 (3H, s)	0.71
25	0.86 (3H, s)	0.86
26	1.04 (3H, s)	1.00
27	0.99 (3H, s)	1.05
28	1.17 (3H, s)	1.17
29	0.99 (3H, s)	1.00
30	0.94 (3H, s)	0.95

*Abbas, et al., 2007

Table 4.4: Comparison of ^{13}C NMR data of compound 35 with the literature values of friedelin.

Position	δ_C	δ_C*	Position	δ_C	δ_C*
1	22.4	22.3	16	36.1	36.0
2	41.6	41.5	17	30.1	30.0
3	213.3	213.2	18	42.8	42.8
4	58.3	58.2	19	35.4	35.3
5	42.2	42.1	20	28.3	28.2
6	41.3	41.3	21	32.5	32.4
7	18.3	18.2	22	39.3	39.2
8	53.2	53.1	23	6.9	6.8
9	37.5	37.4	24	14.7	14.7
10	59.5	59.5	25	18.0	17.9
11	35.7	35.6	26	20.3	20.3
12	30.6	30.5	27	18.7	18.7
13	39.8	39.7	28	32.1	32.1
14	38.4	38.3	29	31.9	31.8
15	32.8	32.8	30	35.1	35.0

*Abbas, et al., 2007

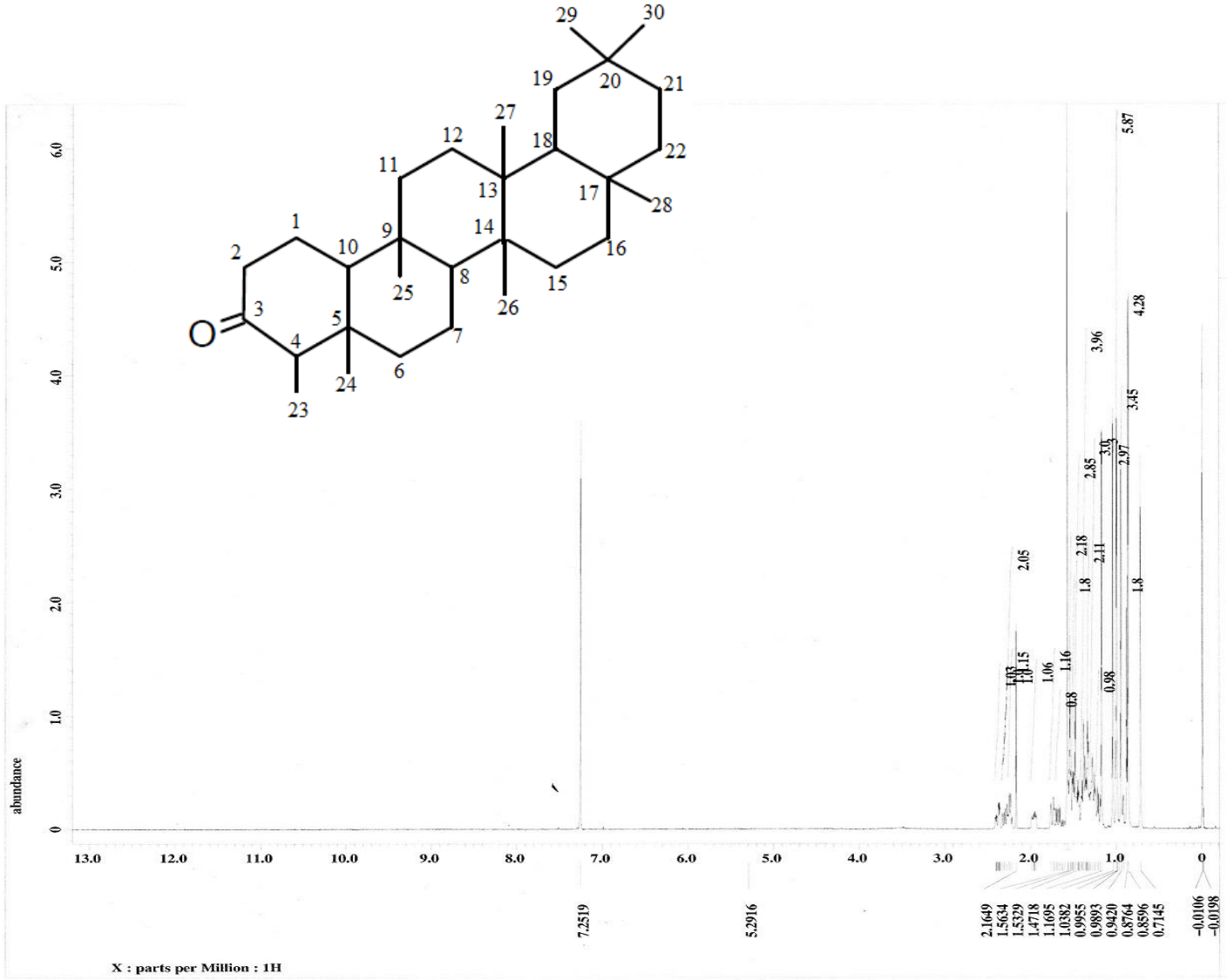


Figure 4.10: ^{1}H NMR spectrum of friedelin [35] (400MHz, $CDCl_3$).

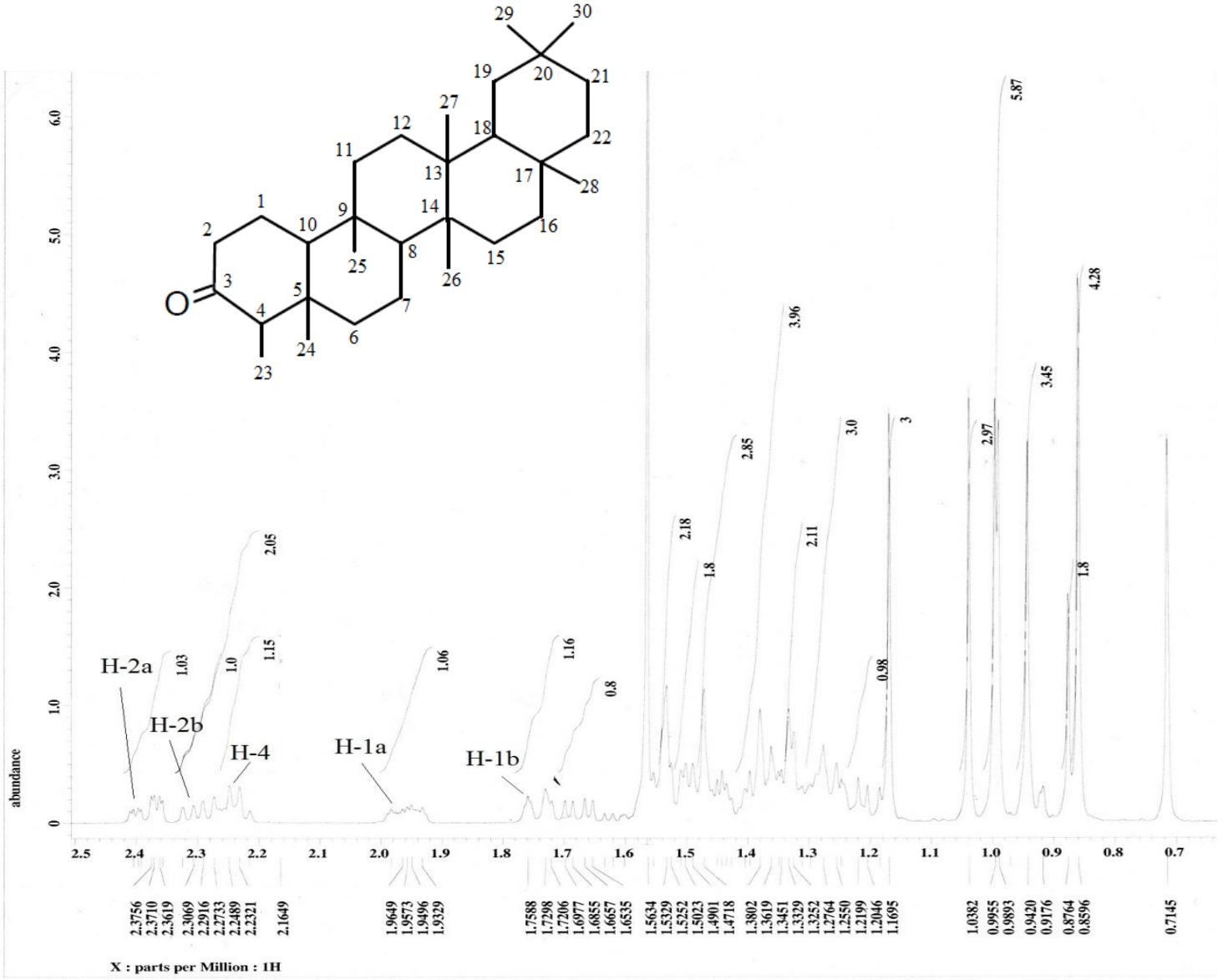


Figure 4.11: ^{1}H NMR spectrum of friedelin [35] (400MHz, $CDCl_3$) (Expanded).

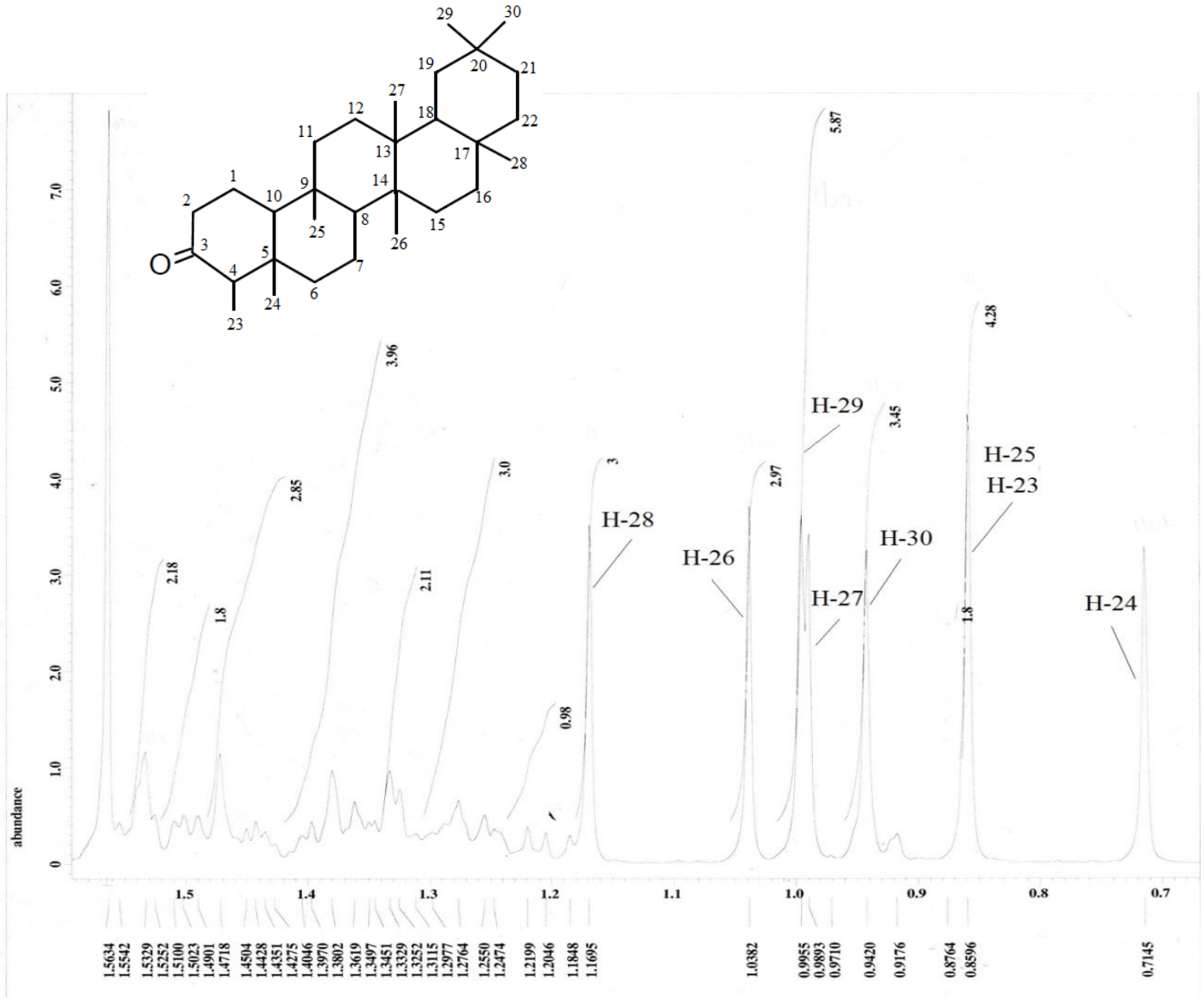


Figure 4.12: ^{1}H NMR spectrum of friedelin [35] (400MHz, $CDCl_3$) (Expanded).

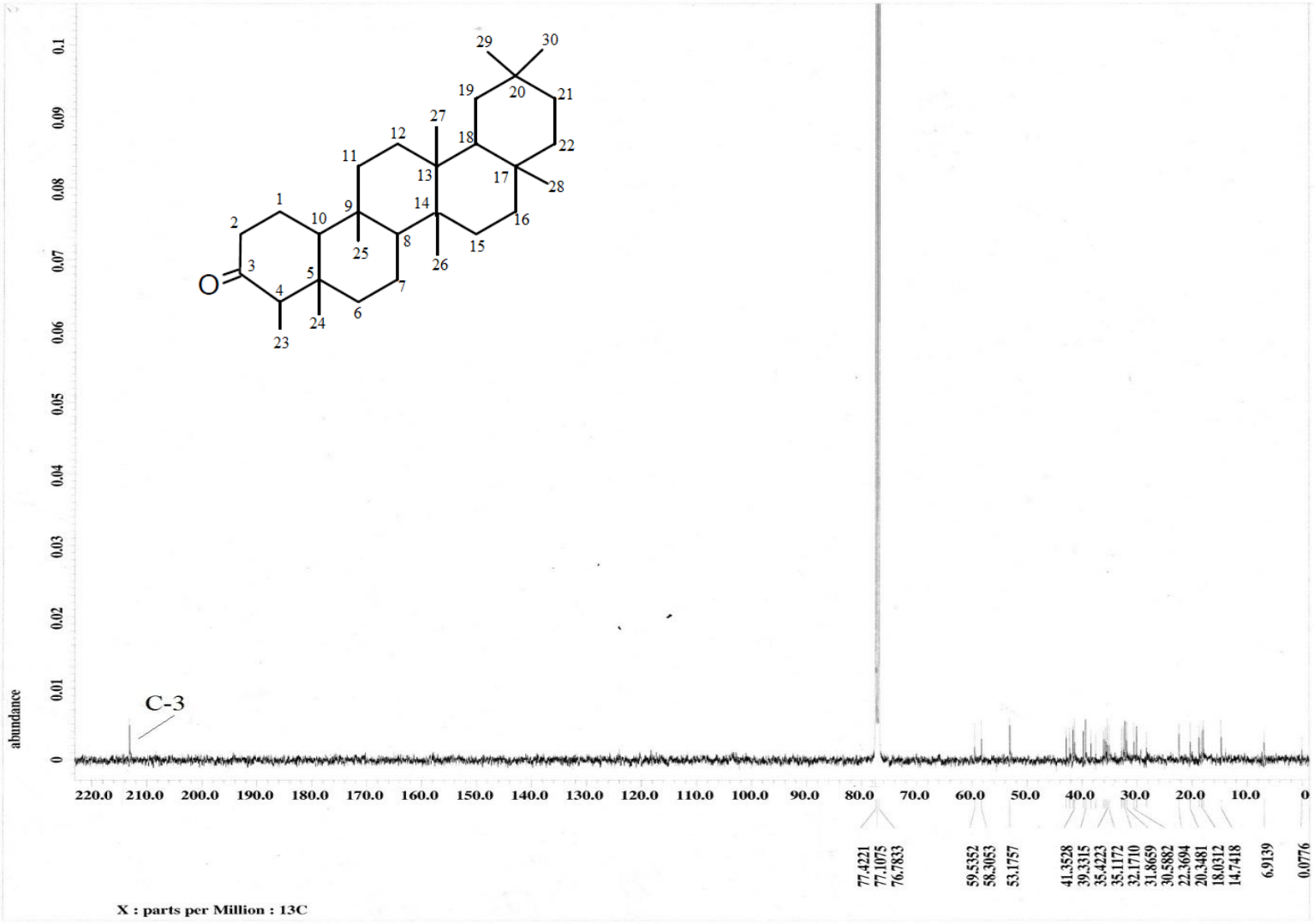


Figure 4.13: ^{13}C NMR spectrum of friedelin [35] (100MHz, $CDCl_3$).

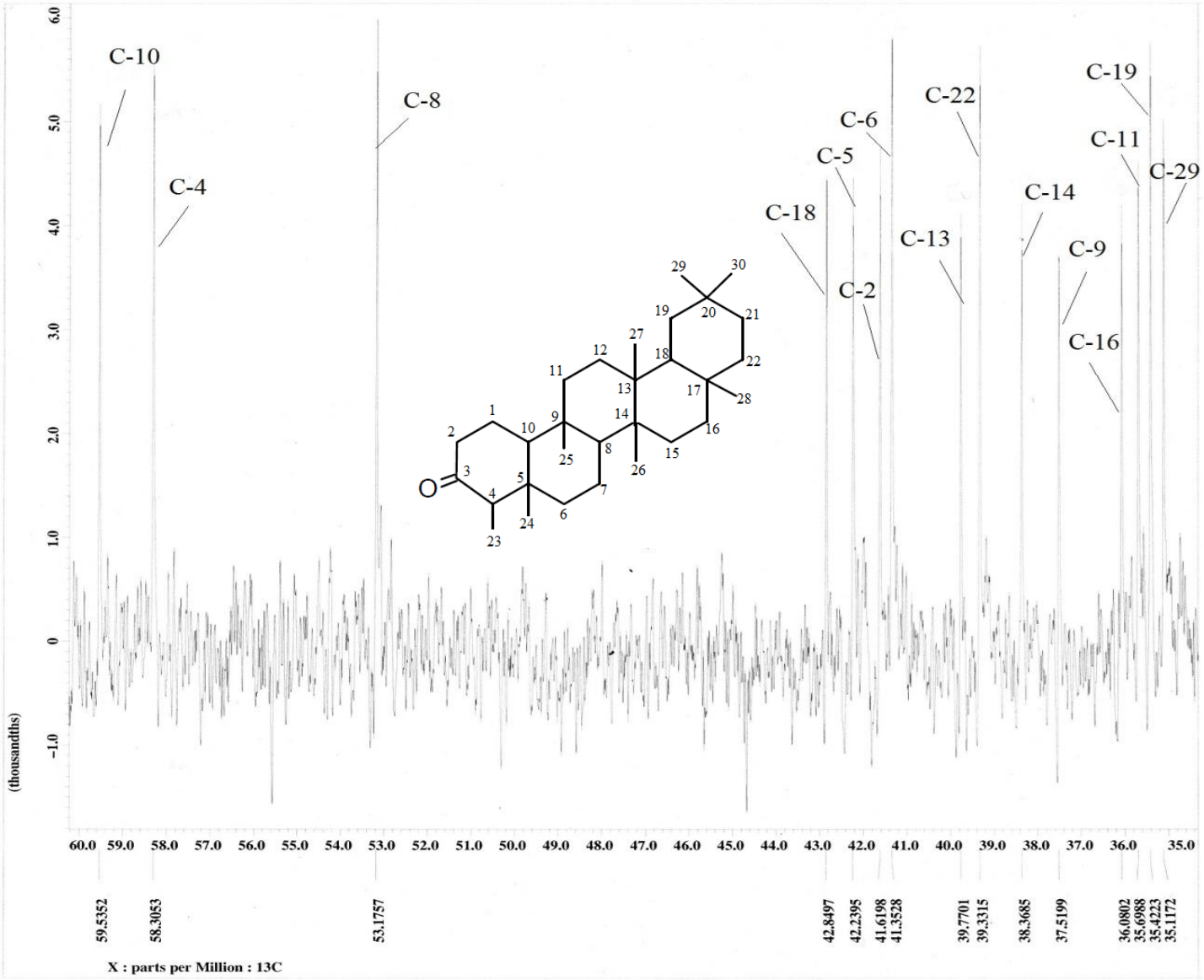


Figure 4.14: ^{13}C NMR spectrum of friedelin [35] (100MHz, $CDCl_3$) (Expanded).

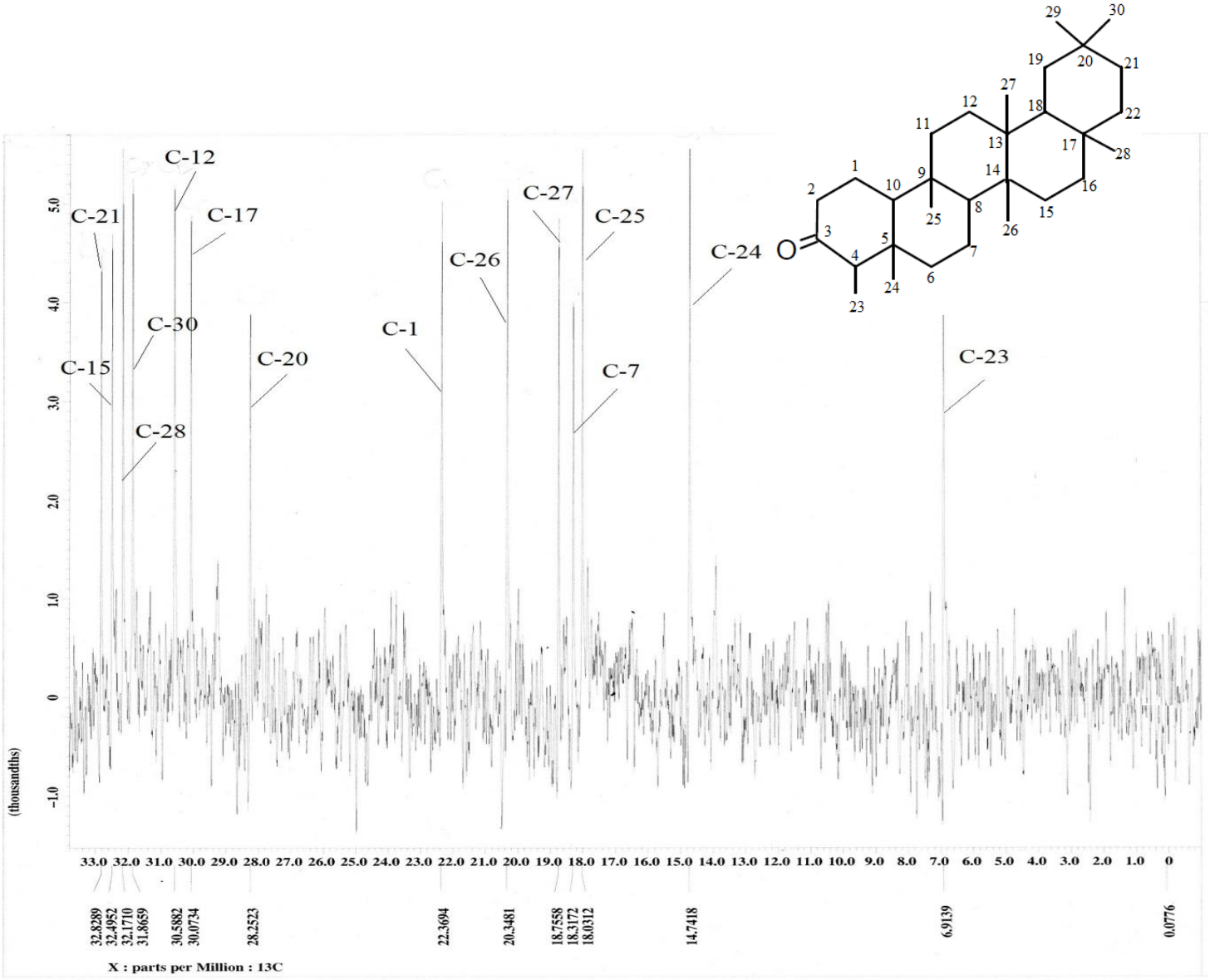


Figure 4.15: ^{13}C NMR spectrum of friedelin [35] (100MHz, $CDCl_3$) (Expanded).

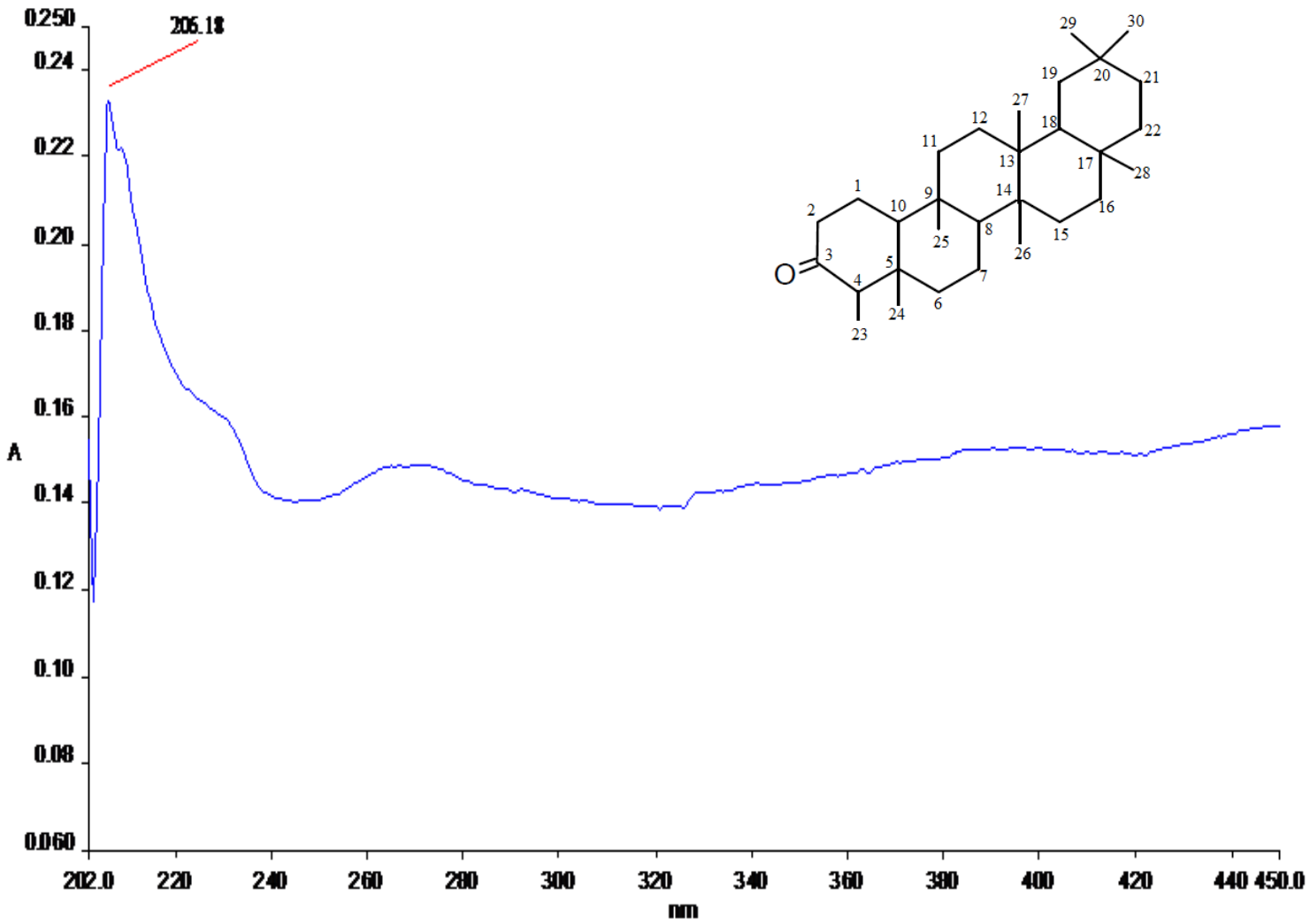


Figure 4.16: UV Vis spectrum of friedelin [35].

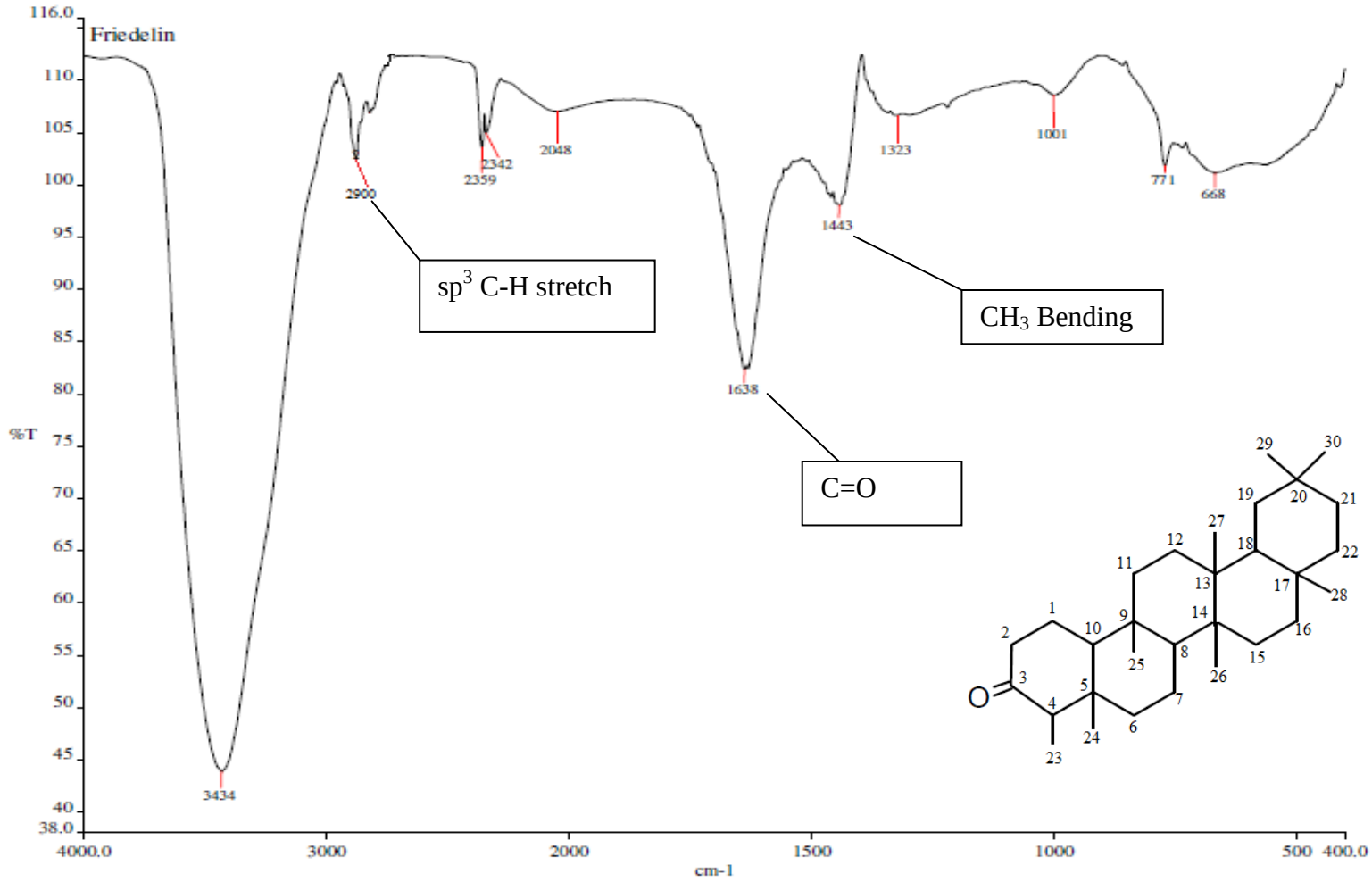


Figure 4.17: IR spectrum of friedelin [35].

4.3 Isolation of Isoblancoic Acid [36]

Isoblancoic acid was obtained from dichloromethane crude extract of *C. castaneum* plant. The dry crude extract which weighed 40.64 g was introduced onto a silica gel 60, 230-400 Mesh packed column. Gradient elution in increasing polarity was used to elute the compounds from the column. A total number of 40 fractions (F1-F40) were collected using solvent mixtures of hexane: ethyl acetate, ethyl acetate: acetone and dichloromethane: methanol. The collected fractions were then subjected for TLC analysis where fractions with similar chemical composition were grouped together. Fraction 19 was subjected to further separation via a silica gel packed gravity column (30 mm in diameter) eluted with solvent mixtures of hexane: ethyl acetate, ethyl acetate: acetone and dichloromethane: methanol in increasing polarity. The separation afforded a total of 43 sub-fractions and they were labeled as CCE. Sub-fraction CCE 13 was found to be partially pure when analyzed by TLC, thus it was subjected to gel filtration chromatography packed with Sephadex LH-20 eluted with solvent mixture dichloromethane: methanol (1:9). The separation afforded 47 sub-fractions (S1-S47) and they were combined on the basis of TLC analysis. Sub-fractions S 23-25 were found to give a single spot on TLC plate and thus further analyzed using NMR technique. The yellow-gummy resin was identified as isoblancoic acid **[36]** according to NMR result obtained and was further confirmed by other spectroscopic analyses such as UV and IR.

4.3.1 Characterization of Isoblancoic Acid [36]

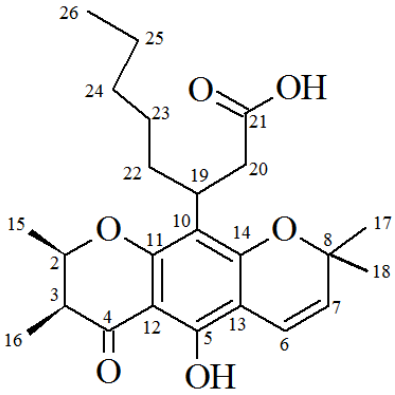


Figure 4.18: Structure of isoblancoic acid [36].

The isolated pure compound was found to give a single yellow spot on TLC plate. It gave a R_f value of 0.45 when eluted with a solvent mixture of acetone: ethyl acetate: hexane (2:2:6). The compound gave positive results for all the TLC detection methods. Detection using UV light of both short and long wavelengths gave dark spot which indicated the presence of conjugated system in the compound structure. The $FeCl_3$ test gave a color change from brown to blue indicating the existence of phenolic group in the structure while staining with iodine vapor resulted in a single brown spot.

The compound is found to have molecular weight of 416 g/mol corresponding to molecular formula, $C_{24}H_{32}O_6$. The structure of compound **36** was elucidated using

1D and 2D NMR techniques. The ^{1}H NMR spectrum (Figure 4.19) showed the most deshielded signal at 12.3 ppm which suggested the presence of a chelated hydroxyl group. Doublet signals at 6.5 ppm and 5.4 ppm were assigned to olefinic protons H-6 and H-7 respectively. Broad singlet signal at 4.42 ppm was assigned to deshielded proton H-2 which is located next to oxygen atom. Multiplets at 3.6, 2.8, 2.6 and 2.4 ppm were assigned to protons H-19, H-20a, H-20b and H-3, respectively. These proton signals were found to appear at a relatively deshielded region of the spectrum due to the anisotropic effect from the neighboring carbonyl groups. The remaining signals in the upfield region suggested the presence of methyl, methylene and methine groups in the compound **36**. Signals at 1.34, 1.14, 1.35, 1.42 and 0.81 ppm were allocated to methyl protons H-15, H-16, H-17, H-18 and H-26, respectively. Signals at 1.53, 1.19, 1.15 and 1.24 ppm were assigned to methylene protons H-22, H-23, H-24 and H-25, respectively while methine proton H-19 gave a multiplet signal at 3.66 ppm. The solvent used to prepare the sample was chloroform-*d* and the solvent peak was found at 7.2 ppm in the spectrum.

The ^{13}C NMR spectrum (Figure 4.22) of compound **36** showed two most deshielded carbon signals at 201.3 and 179.1 ppm which suggested the presence of two carbonyl carbons in the compound, were assigned to carbons C-4 and C-21, respectively. Other carbon signals in the aromatic region from 159 to 101 ppm were assigned to aromatic carbon atoms C-5 (157.3 ppm), C-7 (125.7 ppm), C-11

(157.3 ppm), C-12 (101.2 ppm), C-13 (102.6 ppm) and C-14 (159.9 ppm) in the structure. Signals at 125.7 and 115.6 ppm were attributed to olefinic carbons C-7 and C-6, respectively. The remaining 14 carbon signals lying in the shielded region below 90 ppm were assigned to 14 sp^3 hybridized carbons in the compound. Solvent peak of chloroform-*d* appeared around 77.1 ppm in the spectrum.

2D NMR (HMBC and HMQC) were used to assist the assignment of signals for compound **36**. HMQC spectrum (Figures 4.24 and 4.25) indicates the direct attachment of proton to carbon through ^1J coupling. From the spectrum, it was found that protonated carbons showed direct ^1J correlation to their respective protons. However, quaternary carbons C-4, C-5, C-8, C-10, C-11, C-12, C-13, C-14 and C-21 did not show any correlation in the HMQC spectrum.

HMBC spectrum (Figure 4.26) reveals long range connectivity between proton and their neighboring carbons via ^2J and ^3J couplings. From the spectrum, it was found that hydrogen belongs to chelated hydroxyl group showed ^2J correlation to carbon C-5 and ^3J correlation to carbon C-13. In addition, proton signal H-16 was found to correlate with carbon C-3 through ^2J coupling, C-2 and C-4 through ^3J coupling. Remaining ^2J and ^3J coupling correlations are shown in Table 4.7. The

optical rotation of the compound was found to be, $[\alpha]_D$ -41.7^o (c 0.003, $CHCl_3$) (Lit optical rotation -66.7°, Stout, Hickernell and Sears, 1968).

Intense absorption peaks were observed in the UV-Vis spectrum (Figure 4.27) of compound **36** at 311.31, 274.41 and 209.05 nm. The wavelength of absorption maxima of the compound were observed to be shifted to longer wavelength region (bathochromic shift) compared to previous compounds, **34** and **35** which is due to the presence of conjugated structure. Electronic transition that is responsible for the absorption at 209.05 nm might be due to $\pi \rightarrow \pi^*$ (ε=116.50 $Lmol^{-1}cm^{-1}$) transition caused by conjugated double bonds. Remaining absorptions might be due to $n \rightarrow \pi^*$ (ε=332.87 $Lmol^{-1}cm^{-1}$) and $\pi \rightarrow \pi^*$ (ε=97.09 $Lmol^{-1}cm^{-1}$) transitions of carbonyl groups. Absorption band at 311.31 nm belongs to $\pi \rightarrow \pi^*$ forbidden transition which causes it to appear at low intensity. IR spectrum (Figure 4.28) of compound **36** showed several significant peaks at 3156, 1705 and 1624 cm^{-1} which indicated the presence of hydroxyl group, carboxylic acid C=O group and keto C=O group, respectively. Absorption bands at 2976 and 2928 cm^{-1} were due to stretching by aromatic C-H and sp^3 hybridized C-H, respectively. Absorptions due to aromatic double bonds are shown by band at 1401 cm^{-1} while band at 1132 cm^{-1} was due to C-O stretching. Absorption bands at 1439, 729, 1388 and 895 cm^{-1} were due to CH_2 stretching, CH_2 bending, CH_3 bending and aromatic C-H bending, respectively.

Table 4.5: Comparison of ^{1}H NMR data of compound 36 with the literature values of isoblancoic acid.

Position	δ_H	δ_H*	Position	δ_H	δ_H*
2	4.42 (1H, broad)	4.57 (qd, *J*=6.5Hz, 3.3Hz)	19	3.66 (1H, m)	3.70 (m)
3	2.51 (1H, m)	2.59 (qd, *J*=7.0, 3.3Hz)	20	2.81 (1H, dd, *J*=15.3, 6.7Hz) 2.64 (1H, dd, *J*=15.3, 8.6Hz)	2.76 (dd, *J*=17.8, 7.5Hz) 2.71 (dd, *J*=17.8, 7.5Hz)
6	6.58 (1H, d, *J*=9.8Hz)	6.61 (d, *J*=10.0Hz)	22	1.53(2H, m)	-
7	5.44 (1H, d, *J*=9.8Hz)	5.49 (d, *J*=10.0Hz)	23	1.19(2H, m)	-
15	1.34 (3H, d, *J*=6.1Hz)	1.39 (d, *J*=6.5Hz)	24	1.15 (2H, d, *J*=6.1Hz)	1.18 (d, *J*=7.0Hz)
16	1.14 (3H, d, *J*=7.3Hz)	1.16 (d, *J*=7.0Hz)	25	1.24 (2H, s)	1.29 (d, *J*=7.0Hz)
17	1.35 (3H, s)	1.40 (s)	26	0.81 (3H, t, *J*=6.7Hz)	0.85 (t, *J*=6.5Hz)
18	1.42 (3H, s)	1.45 (s)	5-OH	12.38 (1H,s)	-

(Stout, Hickernell and Sears, 1968)

Table 4.6: Comparison of ^{13}C NMR data of compound 36 with the literature values isoblancoic acid.

Position	δ_C	δ_C*	Position	δ_C	δ_C*
2	76.1	76.5	15	16.4	16.3
3	44.2	44.2	16	9.4	9.4
4	201.4	200.8	17	28.4	28.3
5	157.3	159.2	18	28.2	28.2
6	115.6	115.5	19	30.8	25.7
7	125.7	126.0	20	38.7	35.1
8	78.3	78.9	21	179.2	172.7
10	108.8	109.3	22	33.2	34.1
11	157.3	158.8	23	27.5	22.6
12	101.2	102.9	24	31.8	34.5
13	102.6	101.4	25	22.7	22.6
14	159.9	157.3	26	14.1	13.7

*Stout, Hickernell and Sears, 1968

Table 4.7: Summary of HMQC and HMBC data for compound 36.

Position of proton, δ_H	HMQC	HMBC
0.81 (H26)	14.1(C26)	22.7(C25), 31.7(C24)
1.14 (H16)	9.4(C16)	201.3(C4),76.1(C2), 44.2(C3)
1.15 (H24)	31.7(C24)	22.7(C25)
1.24 (H25)	22.7(C25)	31.7(C24)
1.33 (H15)	16.41(C15)	76.1(C2), 44.2(C3)
1.35 (H17)	28.4(C17)	125.7(C7), 78.3(C8), 28.2(C18)
1.42(H18)	28.2(C18)	125.7(C7), 78.3(C8)
1.53(H22)	33.2 (C22)	108.8 (C10)
2.51 (H3)	44.2(C3)	201.3(C4), 9.4(C16)
2.64 (H20b)	38.7(C20)	108.8(C10), 179.2(C21), 30.8(C19), 33.2(C22)
2.81(H20a)	38.7(C20)	108.8(C10), 179.2(C21), 30.8(C19), 33.2(C22)
3.66 (H19)	30.8 (C19)	33.2 (C22), 38.7 (C20), 108.8 (C10), 159.9 (C14)
5.44 (H7)	125.8(C7)	102.6(C13), 78.3(C8), 28.2(C18)
6.5 (H6)	115.7(C6)	102.6(C13), 157.3(C5), 159.9(C14), 78.3(C8)
12.38 (5-OH)	-	102.6(C13), 157.3(C5)

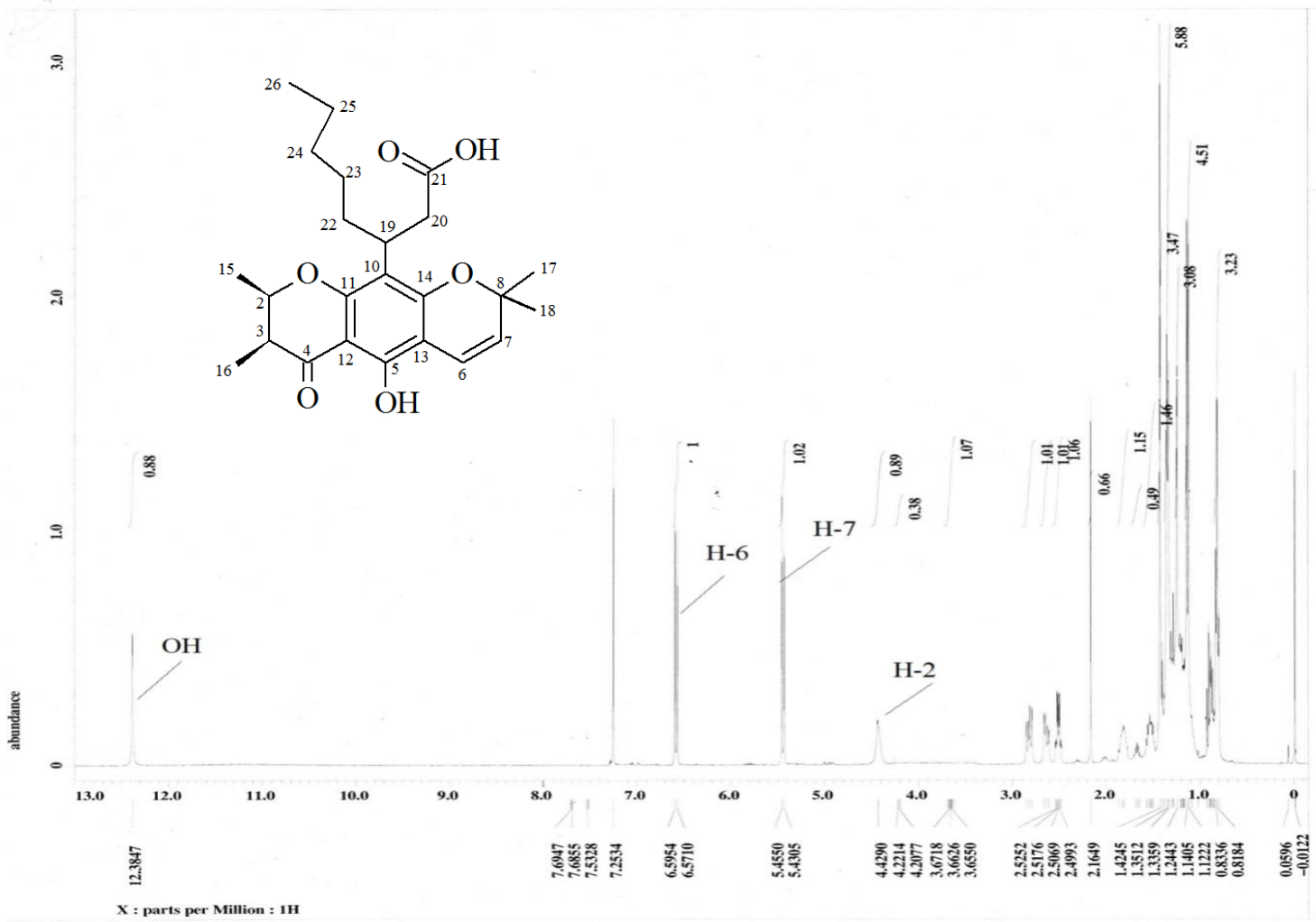


Figure 4.19: ^{1}H NMR spectrum of isoblancoic acid [36] (400MHz, $CDCl_3$).

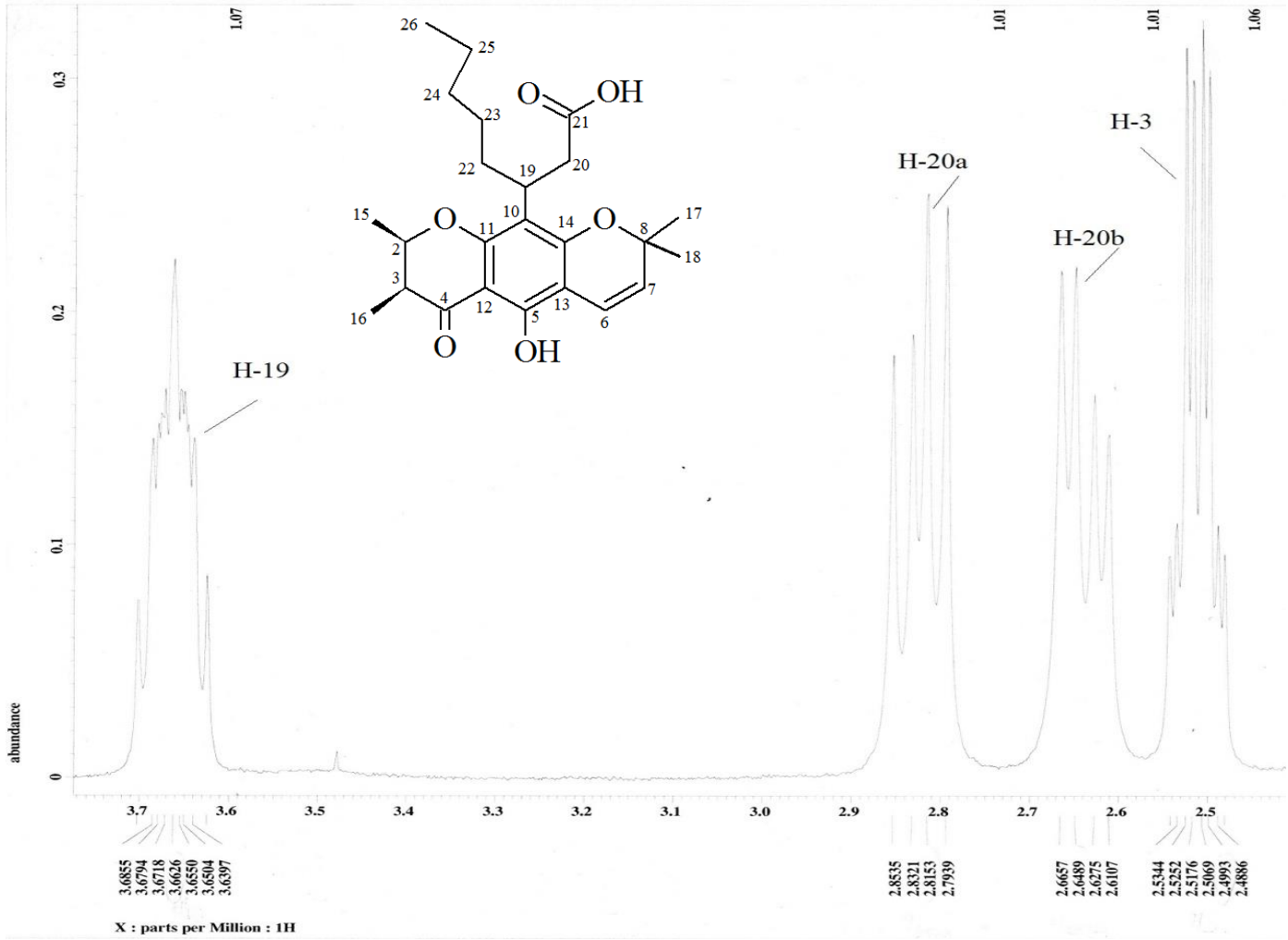


Figure 4.20: ^{1}H NMR spectrum of isoblancoic acid [36] (400MHz, $CDCl_3$) (Expanded).

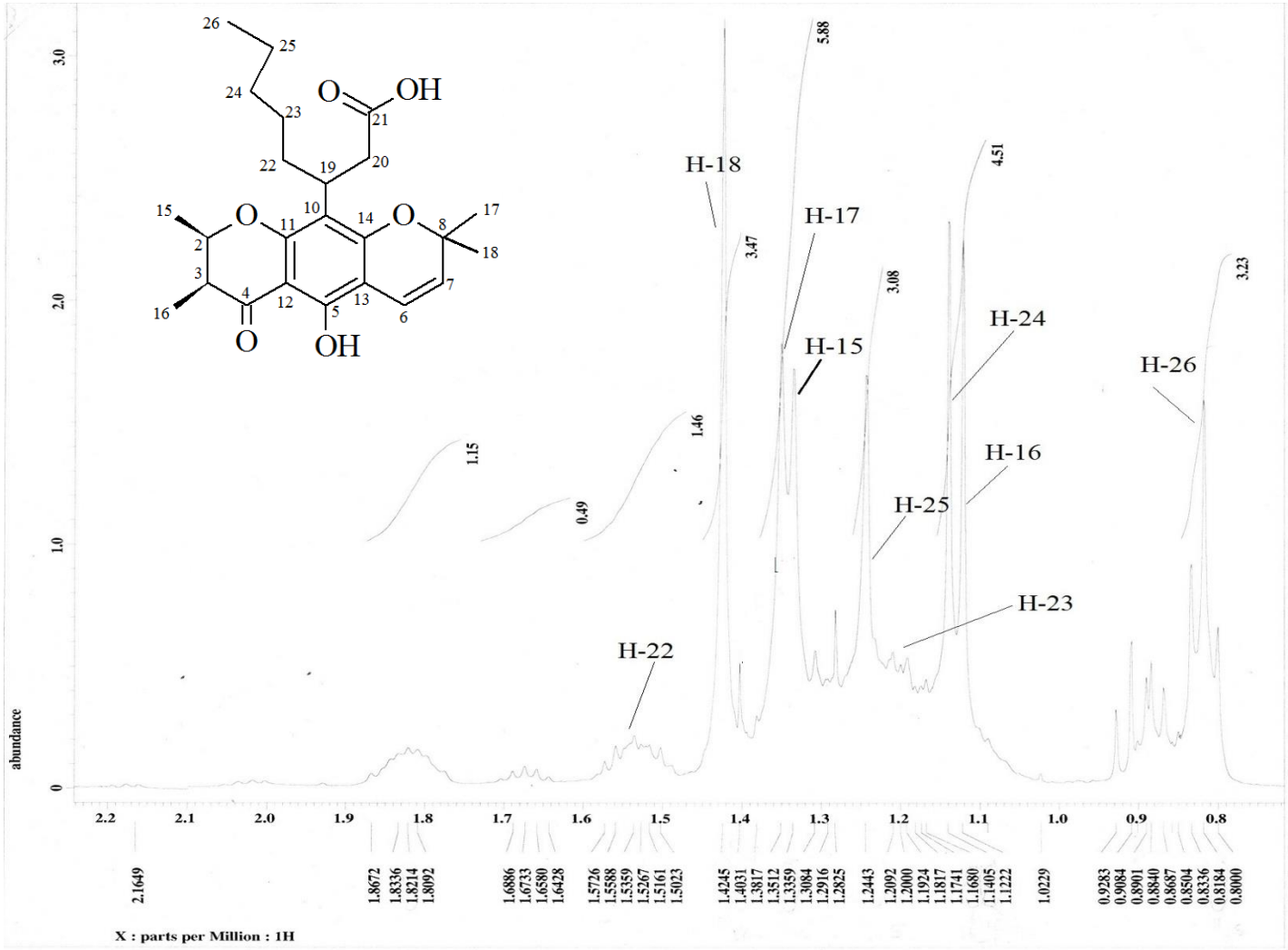


Figure 4.21: ^{1}H NMR spectrum of isoblancoic acid [36] (400MHz, $CDCl_3$) (Expanded).

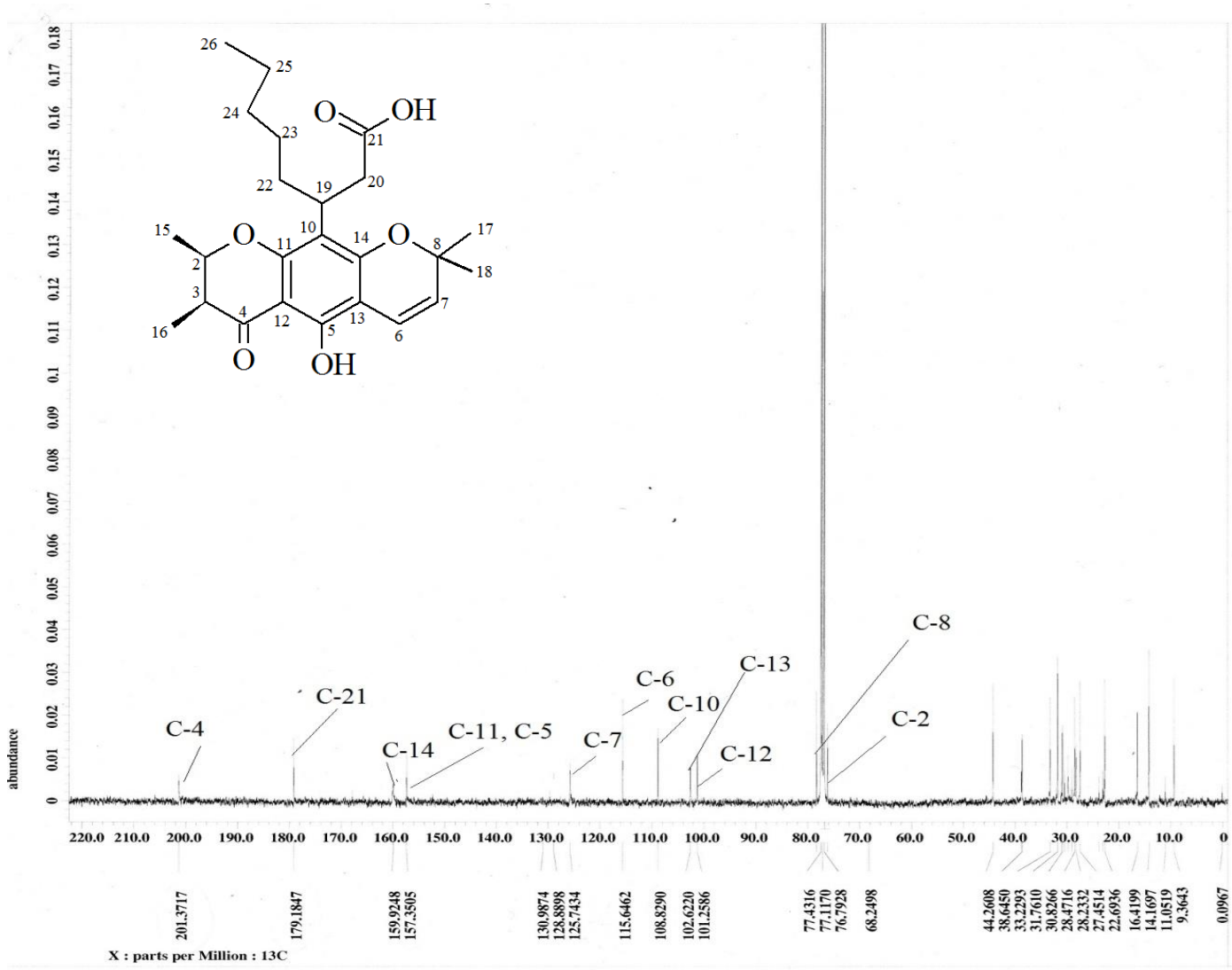


Figure 4.22: ^{13}C NMR spectrum of isoblancoic acid [36] (100MHz, $CDCl_3$).

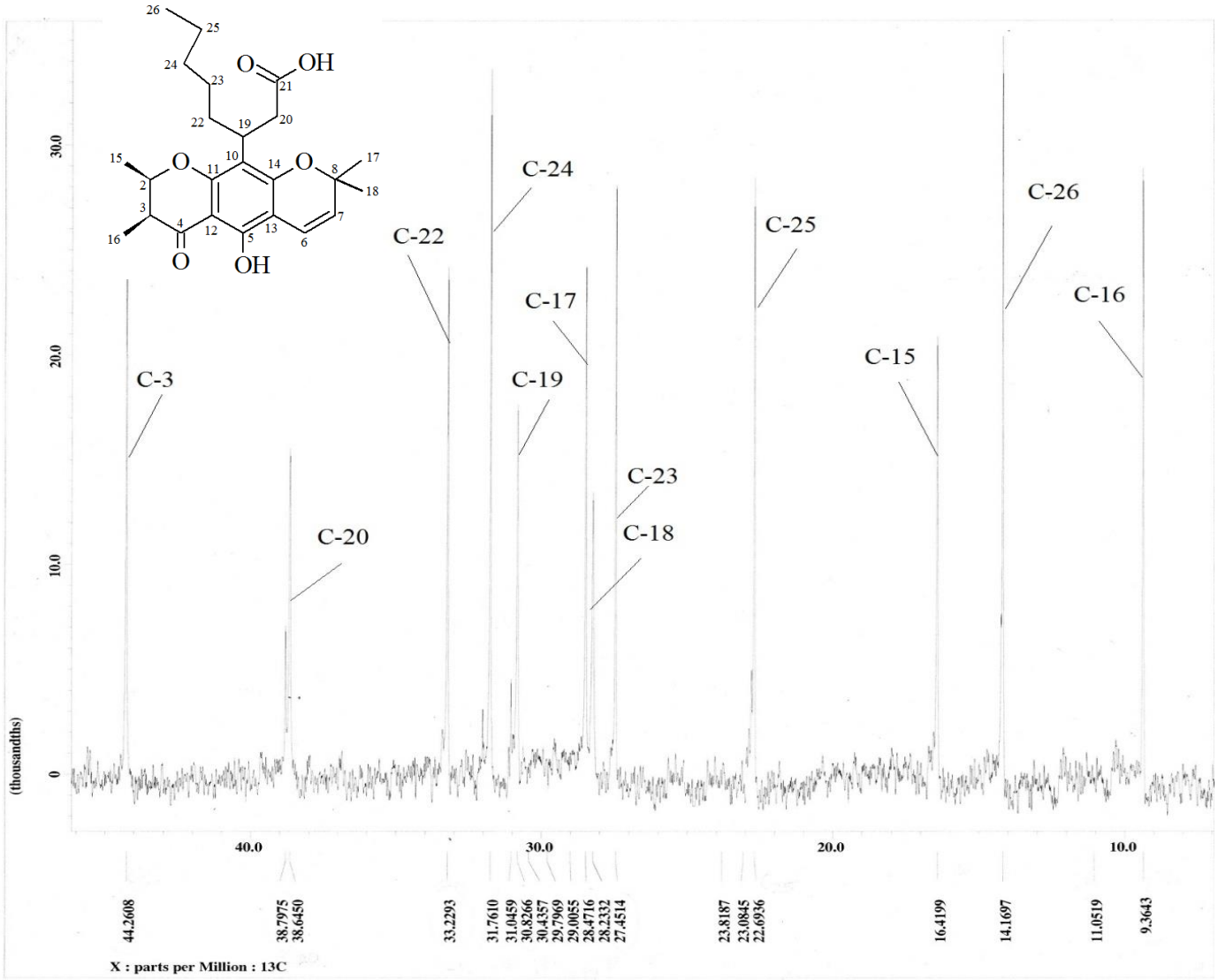


Figure 4.23: ^{13}C NMR spectrum of isoblancoic acid [36] (100MHz, $CDCl_3$) (Expanded).

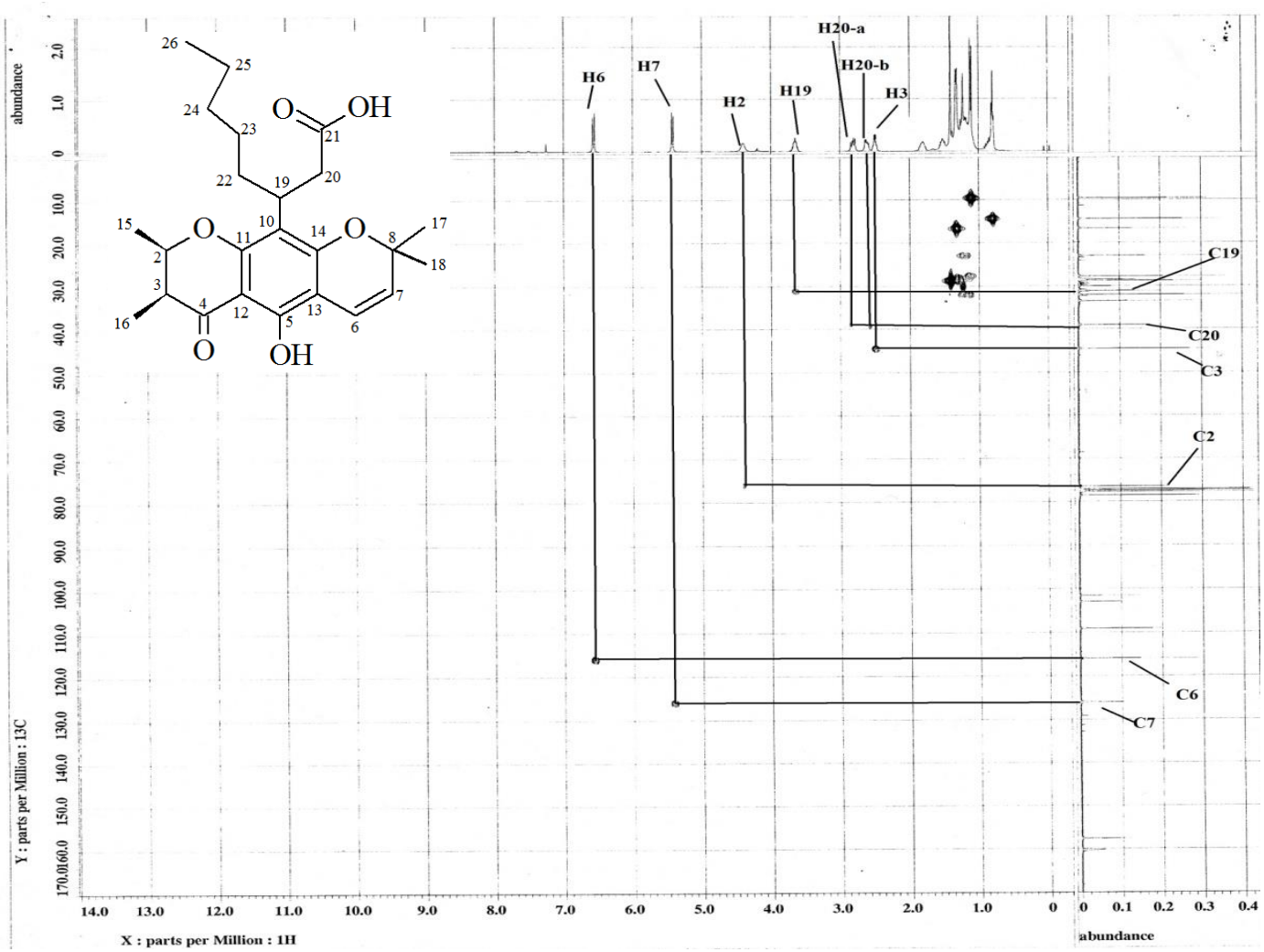


Figure 4.24: HMQC spectrum of isoblancoic acid [36].

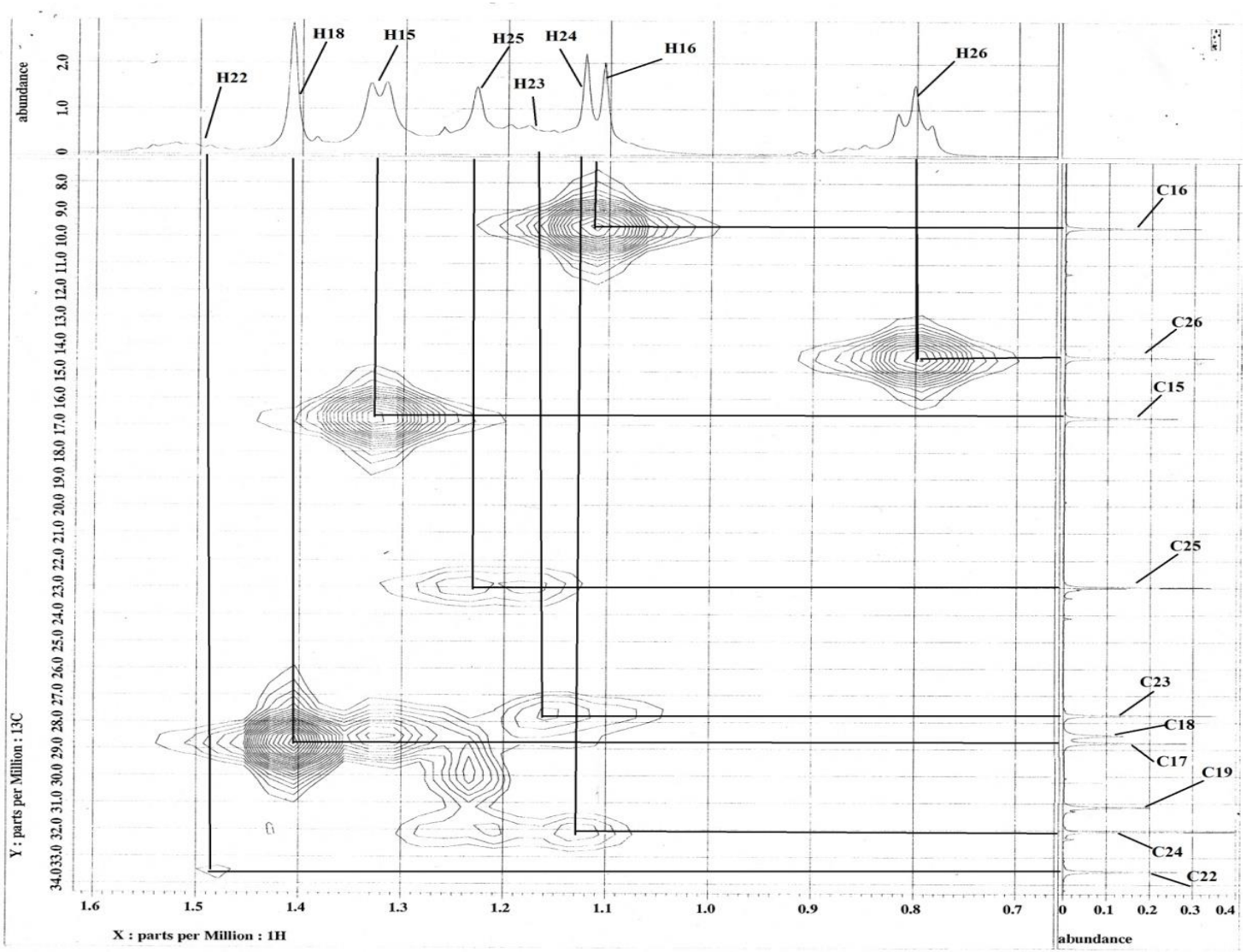


Figure 4.25: HMQC spectrum of isoblancoic acid [36] (Expanded).

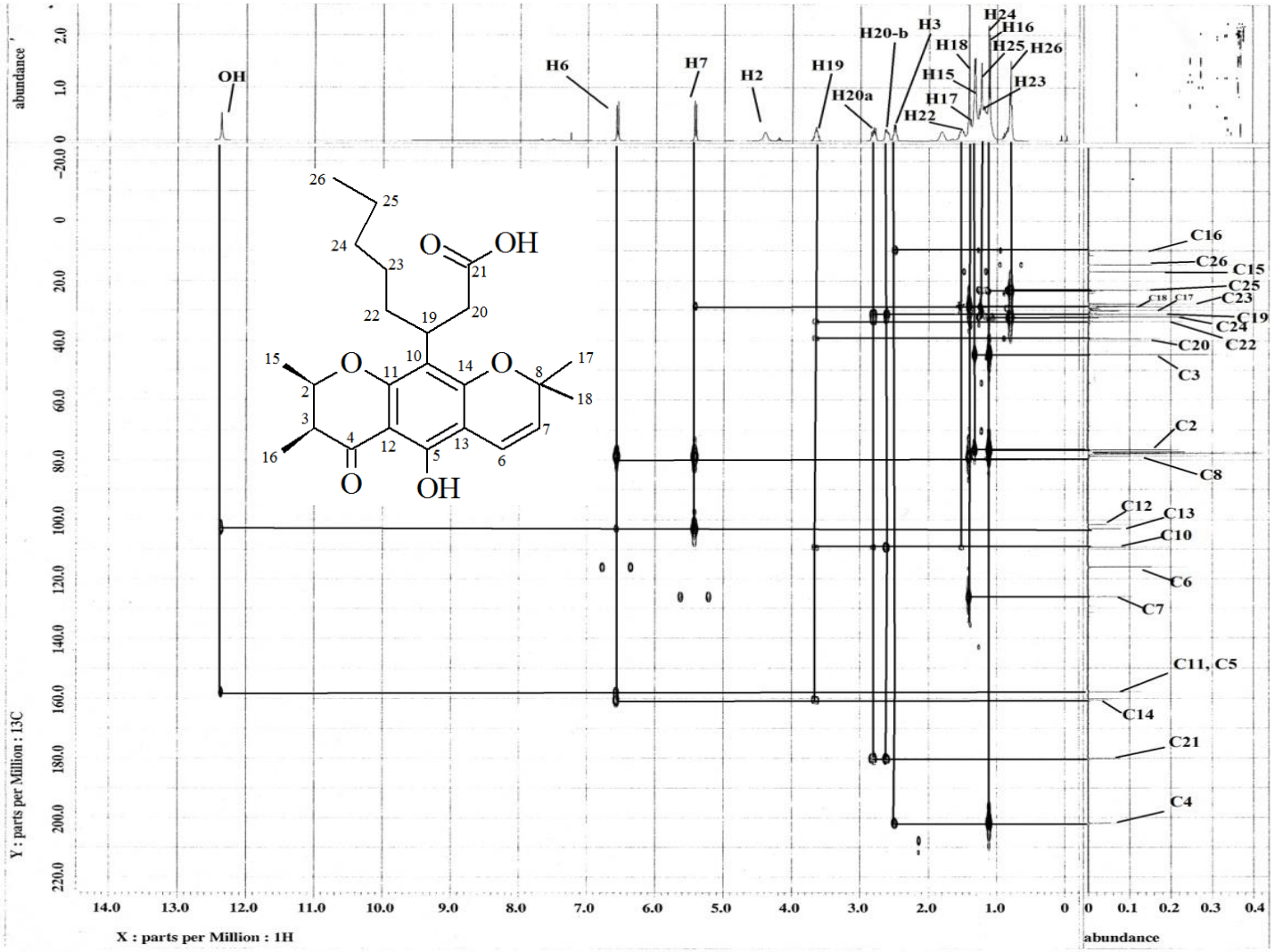


Figure 4.26: HMBC spectrum of isoblancoic acid [36].

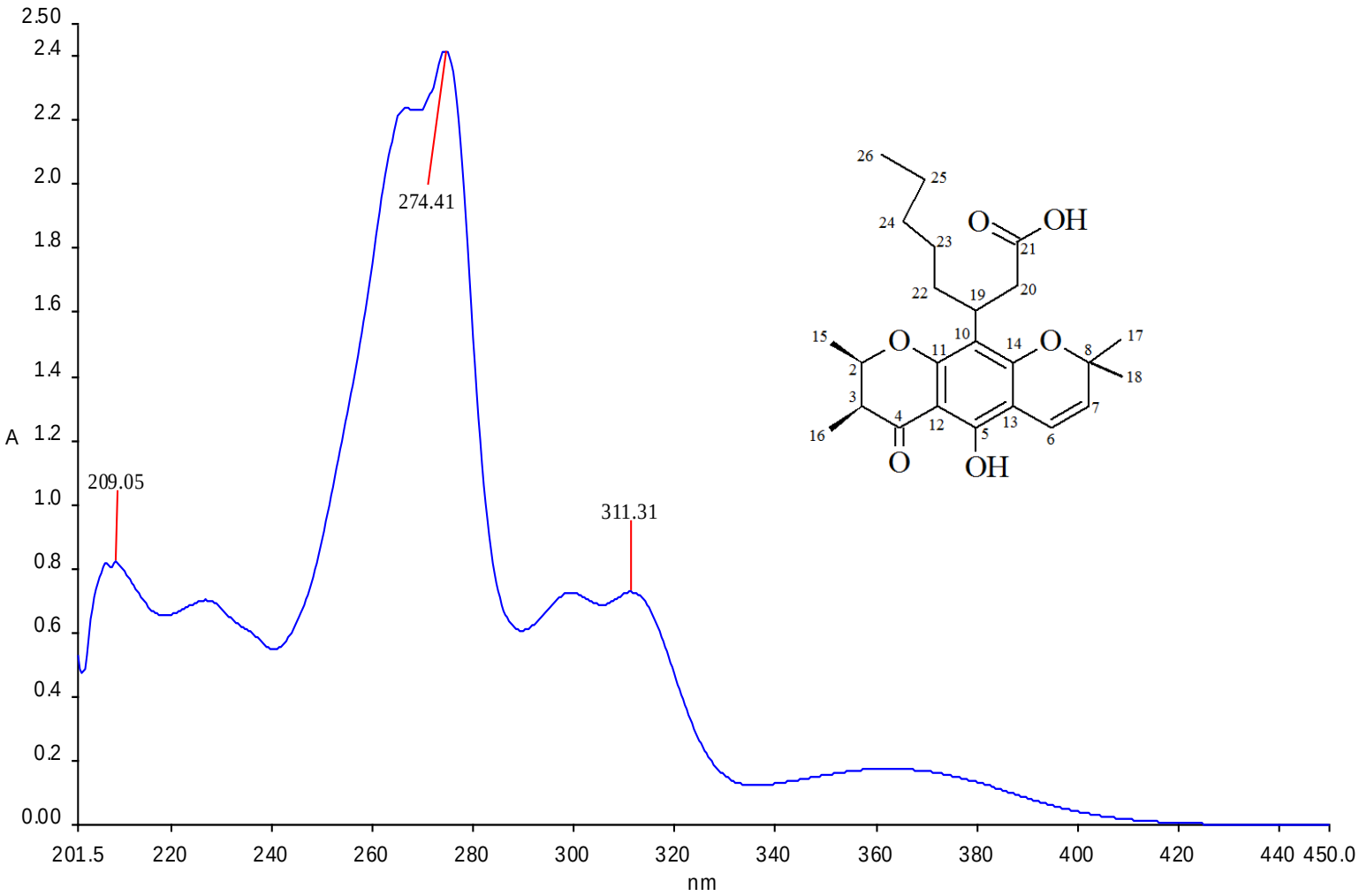


Figure 4.27: UV Vis spectrum of isoblancoic acid [36].

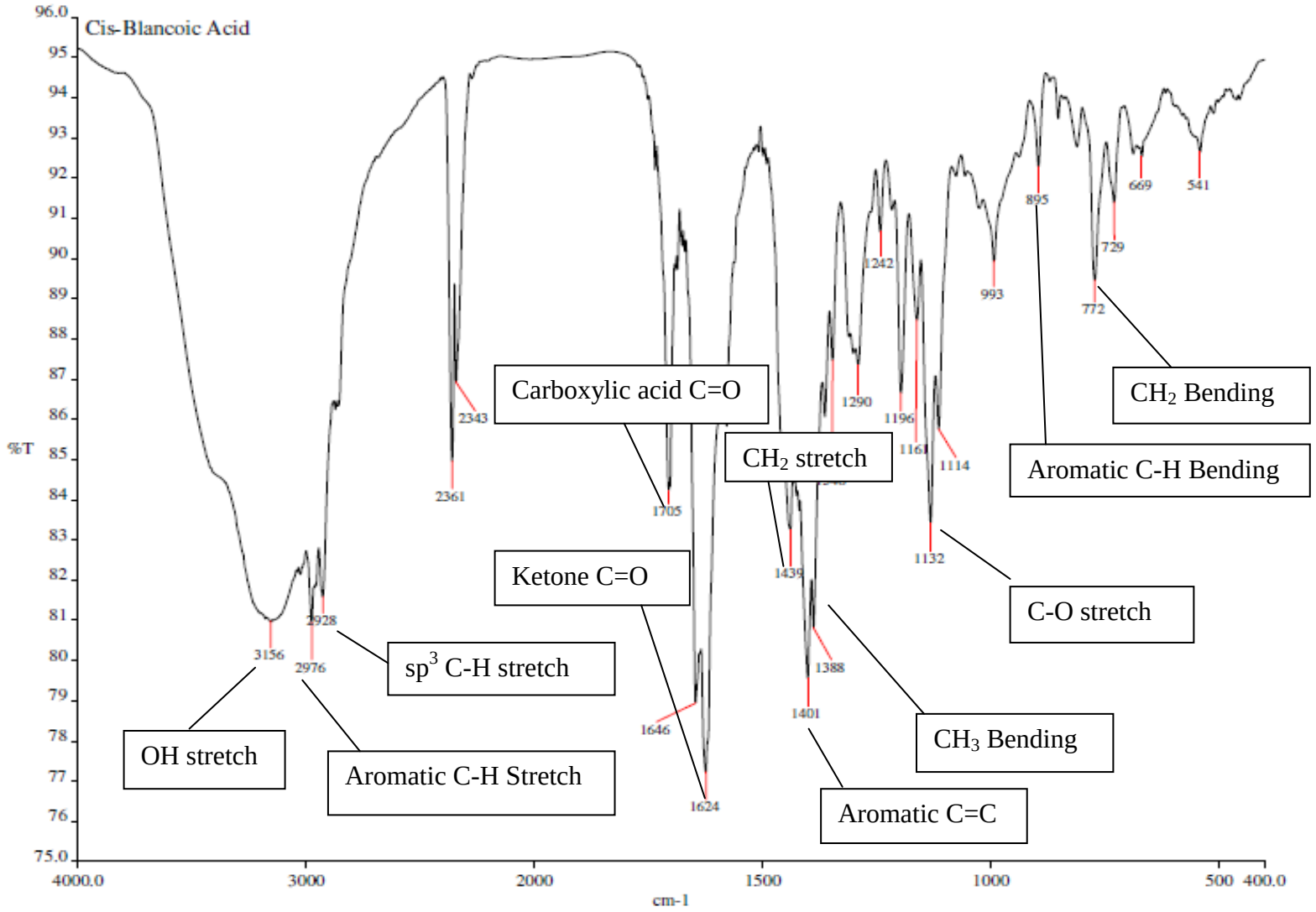


Figure 4.28: IR spectrum of isoblancoic acid [36].

4.4 Antioxidant Assay

The antioxidant activity of isolated pure compounds and crude extracts were tested using DPPH method described by Susanti et al (2007) with slight modifications. Antioxidant activity of a compound measures the ability of the compound to inhibit the oxidation activity of free radicals. In our study, methanol and ethyl acetate crude extracts were found to show significant scavenging activity against DPPH radicals with their IC_{50} values of 12 and 37 μg/ml, respectively. However, DCM crude extract and other isolated compounds did not give positive results in the assay.

DPPH• is a stable radical which functions to oxidize other compounds. In solution form, it exists as purple color and absorbs light in the UV-Vis region of 520 nm. The purple color of the radical is due to its conjugated structure and it bears a nitrogen atom with odd number electron. When ethyl acetate and methanol crude extracts (antioxidant) were introduced into the DPPH solution, it gets itself reduced by accepting a hydrogen atom. Upon reduction the radical changed to its stable form, 2,2-diphenylpicrylhydrazyl and became yellow. The ability of an antioxidant to reduce DPPH• is measured by the extent of discoloration. The higher the antioxidant activity, the more intense the yellow color forms. IC_{50} value is the amount of antioxidant needed to inhibit the radical activity by 50%.

The lesser the amount of antioxidant used to reduce radical, the better the antioxidant activity. Graphs of inhibition rate (%) against concentration of samples were plotted to indicate the strength of antioxidant activity of the samples. From the graphs (Figures 4.28 and 4.29), it was found that both methanol and ethyl acetate crude extracts showed maximum inhibition of more than 80%.

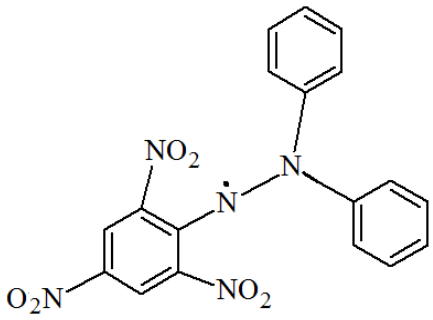


Figure 4.29: Structure of DPPH radical.

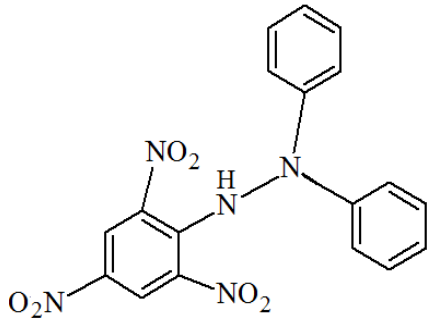


Figure 4.30: Structure of stable DPPH molecule.

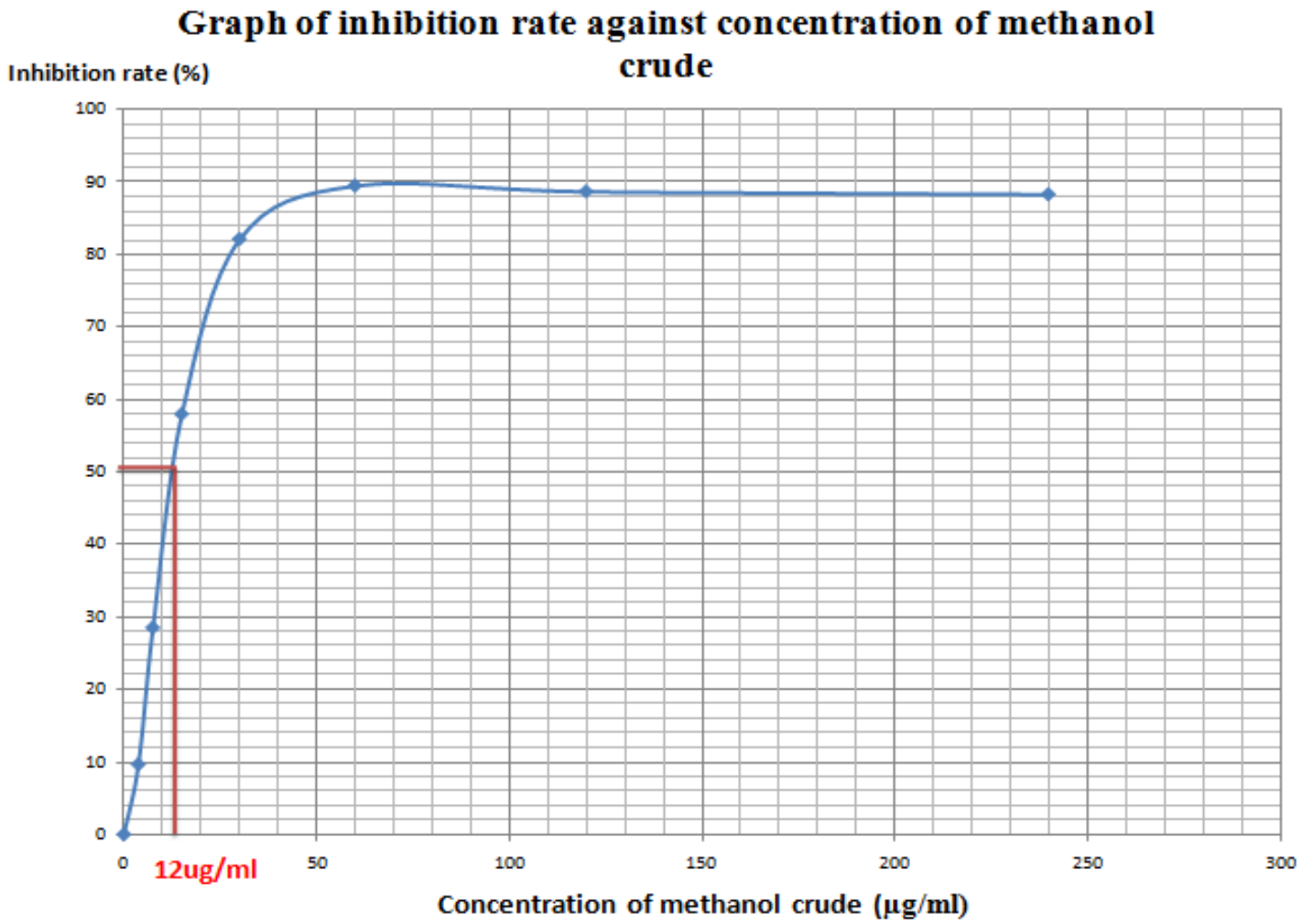


Figure 4.31: Antioxidant activity graph for methanol crude.

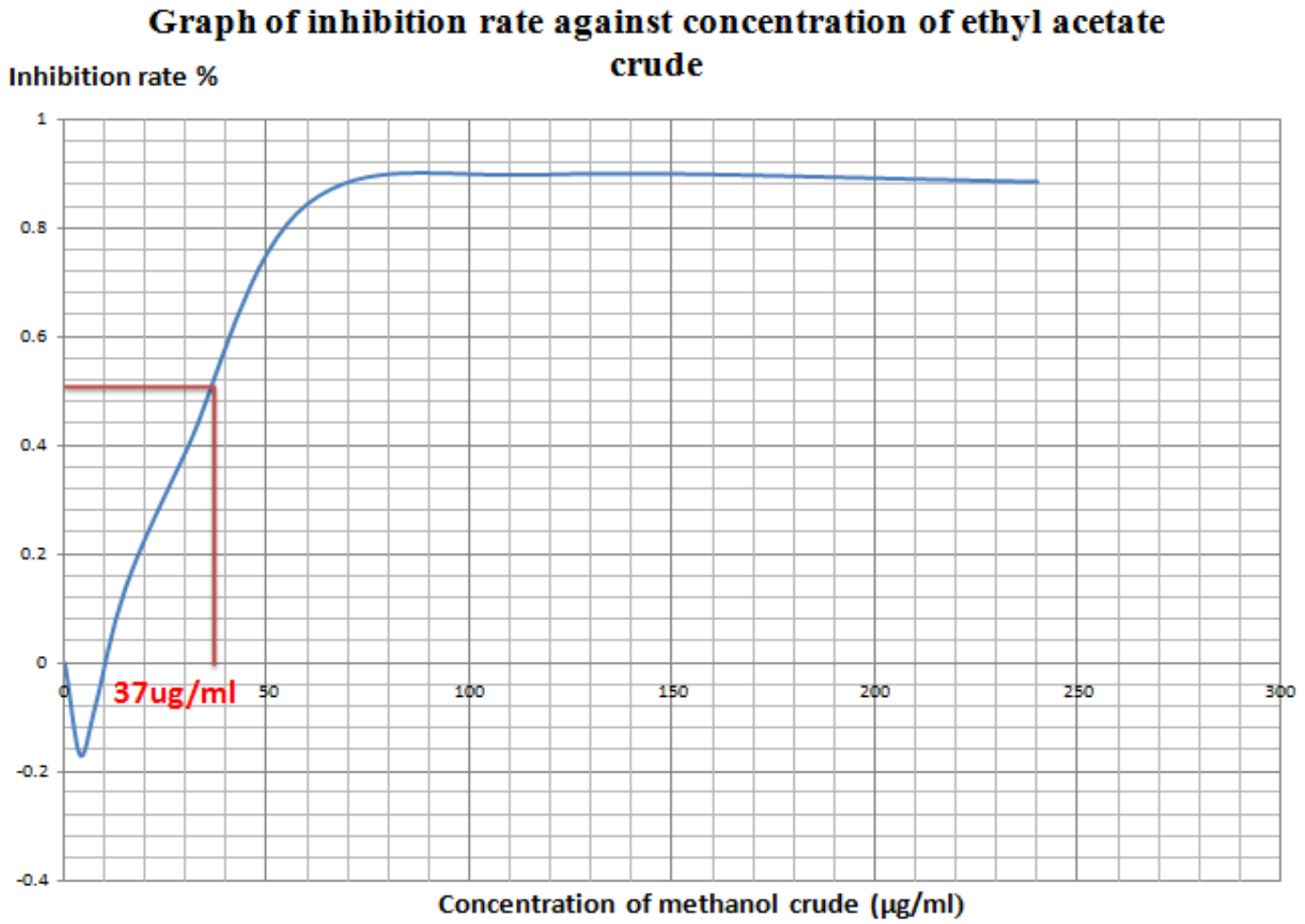


Figure 4.32: Antioxidant activity graph for ethyl acetate crude.

CHAPTER 5

CONCLUSIONS

Three secondary metabolites were isolated from the stem bark of endemic plant species of Sarawak, *Calophyllum castaneum*. The isolated compounds were friedelin, friedelinol and isoblancoic acid. Friedelin and friedelinol are categorized as triterpenoids and they were isolated from methanol crude extract, while isoblancoic acid which is a chromanone acid, was isolated from the dichloromethane crude extract of the plant material. The structures of isolated pure compounds were elucidated and characterized using modern spectroscopic analyses.

The crude extracts and isolated pure compounds were investigated for their antioxidant potential via DPPH assay. From the antioxidant assay, the crude extracts of methanol and ethyl acetate were found to give IC_{50} values of 12 and 37 μg/ml, respectively. The antioxidant activity of ethyl acetate and methanol crude were found to be strongly active with inhibition more than 80%. However, dichloromethane crude extract and other isolated compounds showed negative results. In future studies, isolation of minor compounds from the same plant

species using more advance separation techniques such as HPLC should be considered. Furthermore, the crude extracts and isolated pure compounds should also be subjected to further investigations for their other biological activities such as anti-microbial, anticancer, anti-malarial and others.

REFERENCES

Abbas, F.A., Massarany, S.M.A., Khan, S., Howiriny, T.A.A., Mossa, J.S. and Abourashed, E.A., 2007. Phytochemical and biological studies on Saudi *Commiphora opobalsamum*. *Natural Product Research*, 21(5), pp. 383–391.

Alarcon, A.B., Rubio, O.C., Pe´rez, J.C., Piccinelli, A.L. and Rastrelli, L., 2008. Constituents of the Cuban Endemic Species *Calophyllum pinetorum*, *Journal of Natural Products*, 71, pp. 1283–1286.

American Cancer Society, Inc., 2014. *Phytochemicals*. [online] Available at: <http://www.cancer.org/treatment/treatmentsandsideeffects/complementaryandalternativemedicine/herbsvitaminsandminerals/phytochemicals> [Accessed 15 February 2014].

American Institute for Cancer Research, 2014. *Phytochemicals: The Cancer Fighters in the Foods We Eat.* [online] Available at: < http://www.aicr.org/reduce-your-cancer risk/diet/elements_phytochemicals.html> [Accessed 17 February 2014].

Ampofo, S.A. and Waterman, P.G., 1986. Xanthones and Neoflavonoids from two Asian species of *Calophyllum. Phytochemistry*, 25, pp. 2617-2620.

Anwar, F., Jamil, A., Iqbal, S. and Sheikh, M.A., 2006. Antioxidant activity of various plant extracts under ambient and accelerated storage of sunflower oil. *Journal of Food Science,* 57 (2), pp. 189-197.

Apak, R., Gorinstein, S., Böhm, V., Schaich, K.M., Özyürek, M. and Güçlü, K., 2013. Methods of measurement and evaluation of natural antioxidant capacity/activity. *Pure and Applied Chemistry,* 85(5), pp. 957–998.

Arctos: Multi-Institution, Multi-Collection Museum Database, 2011. *Taxonomy Details for Calophyllum castaneum*. [online] Available at: <http://arctos.database.museum/name/Calophyllum%20castaneum> [Accessed 21 March 2014].

Bart, H.J., 2011. Extraction of Natural Products from Plants – An Introduction. In: H.J.Bart and S.Pilz, eds. *Industrial Scale Natural Products Extraction.* Wiley-VCH Verlag GmbH & Co. KGaA. pp. 1-23.

BayScience Foundation, Inc., 1986. *Taxonomy of Calophyllum castaneum*. [online] Available at: <http://zipcodezoo.com/Plants/C/Calophyllum_castaneum/Default.asp> [Accessed 19 March 2014].

Bhat, S.G., Kane, J.G. and Sreenivasan, A., 1954. The in vitro evaluation of the antibacterial activity of undi oil (*Calophyllum inophyllum Linn*.). *Journal of the American Pharmaceutical Association,* 43, pp. 543–546.

Bishayee, A., Ahmed, S. and Perloff, M., 2011.Triterpenoids as potential agents for the chemoprevention and therapy of breast cancer. *Frontiers in Bioscience.* [online] Available at: < http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3057757/> [Accessed 15 July 2013].

Brahmachari, G., 2011. Natural Products in Drug Discovery: Impacts a Opportunities — An Assessment. In: G.Brahmachari, ed. *Bioactive Natural Products: Opportunities and Challenges in Medicinal Chemistry.* World Scientific Pub Co Inc. pp. 1-114.

Breck, G.D. and Stout, G.H., 1969. *Calophyllum* products: A new 4-phenylcoumarin from *C. australianum*. *Journal of Organic Chemistry*, 34, pp. 4203–4204.

Bruneton, J., 1993. *Pharmacognosie-Phytochimie: Plantes Medicinales*. Paris: Technique & Documents Lavoisier.

Chao, S.G., Sim, K.Y. and Goh, S.H., 1997. Biflavonoids of *Calophyllum venulosum*. *Journal of Natural Products*, 60, pp. 1245-1250.

Chilpa, R.R., Estrada, M.J. and Muniz, E.E., 1997. Antifungal xanthones from *Calophyllum brasiliensis* heartwood. *Journal of Chemical Ecology*, 23 (7), pp. 1901–1911.

Department of Agriculture, Fisheries and Forestry, 2010. *Calophyllum*. [online] Available at: < http://www.daff.qld.gov.au/forestry/using-wood-and-its-benefits/wood-properties-of-timber-trees/calophyllum> [Accessed 21 March 2014].

Dharmaratne, H.R.W., Perera, D.S.C., Marasinghe, G.P.K. and Jamie, J., 1999. A chromene acid from *Calophyllum cordato-oblongum*. *Phytochemistry*, 51, pp. 111-113.

Dias, D.A., Urban, S. and Roessner, U., 2012. A Historical Overview of Natural Products in Drug Discovery. *Journal of Metabolites*, 2, pp. 303-336.

Dweck, A.C. and Meadowsy, T., 2002. Tamanu (*Calophyllum inophyllum*) – the African, Asian, Polynesian and Pacific Panacea. *International Journal of Cosmetic Science*, 24, pp. 1–8.

Farnsworth, N. R., 1988. *Biodiversity*. [e-book] Washington DC: National Academy Press. Available at: <http://www.nap.edu/openbook.php?record_id=989&page=R1 > [Accessed 19 February 2014].

Filho, C., Silva, C.M. and Niero, R., 2009. Chemical and pharmacological aspects of the genus *Calophyllum*. *Chemistry and Biodiversity*, 6(3), pp. 313-327.

Guerreiro, E., Kunesch, G. and Polonsky, J., 1971. Les Constituants Des Graines De *C.chapelieri* (Guttiferae). *Phytochemistry*, 10, pp. 2139 - 2145.

Guilet, D., He´lesbeux, J.J., Se´raphin, D., Se´venet, T., Richomme, P. and Bruneton, J., 2001. Novel Cytotoxic 4-Phenylfuranocoumarins from *Calophyllum dispar*. *Journal of Natural Products,* 64, pp. 563-568.

Gonzalez, M.J., Nascimento, M.S.J., Cidade, H.M., Pinto, M.M., Kijjoa, A. and Anantachoke, C., 1999. Immunomodulatory activity of xanthones from *Callophylum teysmannii var*. *inuphylloide*. *Planta*, 65, pp. 368–371.

Govindachari, T.R., Prakash, D. and Viswanathan, N., 1967. Chemical Constituents of *Calophyllum apetalum. Tetrahedron Letters*, 42, pp. 4177-4181.

Goyal, P.K., 2012. Cancer Chemoprevention by Natural Products: Current & Future Prospects. *Journal of Integrative Oncology*, 1, pp. 1-2.

Ha, L.D., Hansen, P.E., Duus, F., Pham, H.D. and Nguyen, L.H.D., 2012. A new chromanone acid from the bark of *Calophyllum dryobalanoides*. *Phytochemistry Letters,* 5, pp. 287–291.

Hay, A.E., Guilet, D., Morel, C., Larcher, G., Macherel, D., Ray, A.M.L., Litaudon, M. and Richomme, P., 2003. Antifungal chromans inhibiting the mitochondrial respiratory chain of pea seeds and new xanthones from *Calophyllum caledonicum*. *Planta Medica*, 69, pp. 1130–1135.

Hay, A.E., Helesbeux, J.J., Duval, O., Labaied, M., Grellier, P. and Richomme, P., 2004. Antimalarial xanthones from *Calophyllum caledonicum* and *Garcinia vieillardii*. *Life Sciences*, 75, pp. 3077–3085.

Institute of Food Technologists, 2008. *Determining Antioxidant Activity*. [online] Available at: < http://www.ift.org/ > [Accessed 21 February 2014].

Ito, C., Itoigawa, M., Mishina, Y., Filho, V.C., Mukainaka, T., Tokuda, H., Nishino, H. and Furukawa, H., 2002. Chemical constituents of *Calophyllum brasiliensis*: structure elucidation of seven new xanthones and their cancer chemopreventive activity. *Journal of Natural Products*, 65 (3), pp. 267–272.

Ito, C., Itoigawa, M., Miyamoto, Y., Rao, K.S., Takayasu, J., Okuda, Y., Mukainaka, T., Tokuda, H., Nishino, H. and Furukawa, H., 1999. A New Biflavonoid from *Calophyllum panciflorum* with Antitumor-Promoting Activity. *Journal of Natural Products*, 62, pp. 1668-1671.

Jiangsu New Medical College, 1986. *Dictionary of Chinese Herb Medicines*, Shanghai: Shanghai Scientific and Technologic Press.

Kashman, Y., Gustafson, K., Fuller, R., Cardellina, J., McMahon, J., Currens, M., Buckeit, R., Hughes, S., Cragg, G. and Boyd, M., 1992. The calanolides, a novel HIV Inhibitory class of coumarin derivatives from the tropical rainforest tree, *Calophyllum lanigerum. Journal of Medical Chemistry*, 35, pp. 2735–2743.

Kawazu, K., Ohigashi, H. and Mitsui, T., 1968. The piscicidal constituents of *Calophyllum inophyllum*. *Tetrahedron Letters*, 19, pp. 2383–2385.

Kedare, S.B. and Singh, R.P., 2011. Genesis and development of DPPH method of antioxidant assay. *Journal of Food Science and Technology*, 48(4), pp. 412-422.

Kennedy, D.O. and Wightman, E.L., 2011. Herbal Extracts and Phytochemicals: Plant Secondary Metabolites and the Enhancement of Human Brain Function. *Advances in Nutrition.* [online] Available at: <http://advances.nutrition.org/content/2/1/32.full> [Accessed 15 July 2013].

Khalaf, N.A., Shakya, A.K., Othman, A.A., Agbar, Z.E. and Farah, H., 2008. Antioxidant Activity of Some Common Plants. *Turkish Journal of Biology*, 32, pp. 51-55.

Khan, N.U.D., Perveen, N., Singh, M.P., Singh, R., Achari, B. and Partha, P.G., 1996. Two isomeric benzodipyranone derivatives from *Calophyllum inophyllum. Phytochemistry*, 42, pp. 1181–1183.

Kijjoa, A., Gonzalez, M.J., Pinto, M.M., Silva, A.M., Anantachoke, C. and Herz, W., 2000. Xanthones from *Calophyllum teysmannii var. inophylloide*. *Phytochemistry*, 55, pp. 833–836.

Koehn, F.E. and Carter, G.T., 2005. The Evolving Role of Natural Products in Drug Discovery. *Journal of Drug Discovery*, 4, pp. 206-220.

Kumar, S., Raj, K. and Khare, P., 2009. Flavones and acridones from *Atalantia wightii*. *Indian Journal of Chemistry*, 48, pp. 291-294.

Lahlou, M., 2013. The Success of Natural Products in Drug Discovery. *Journal of Pharmacology & Pharmacy*, 4, pp. 17-31.

Leonti, M., Vibrans, H., Sticher, O. and Heinrich, M., 2001. *Proceedings of the 42nd Annual Meeting of the American Society of Pharmacognosy*. Mexico: Oaxaca.

Li, X.W., Li, J., Robson, N.K.B. and Stevens, P., 1990. Clusiaceae (GUTTIFERAE). *Flora of China*, [e-journal] 13, pp. 1. Available at: < http://www.efloras.org/florataxon.aspx?flora_id=2&taxon_id=10203> [Accessed 25 January 2014].

Li, Y.Z., Li, Z.L., Yin, S.L., Shi, G., Liu, M.S., Jing, Y.K. and Hua, H.M., 2010. Triterpenoids from *Calophyllum inophyllum* and their growth inhibitory effects on human leukemia HL-60 cells. *Fitoterapia*, 81, pp. 586–589.

Ling, K.H., Kian, C.T. and Hoon, T.C., 2009. *A guide to medicinal plants.* Singapore: World Scientific.

Linuma, M., Tosa, H., Toriyama, N., Tanaka, T. and Ito, T., 1996. Six xanthones from *Calophyllum austroindicum. Phytochemistry,* 43, pp. 681–685.

Ma, C.H., Chen, B., Qi, H.Y., Li, B.G. and Zhang, G.L., 2004. Two pyranocoumarins from the seeds of *Calophyllum polyanthum*. *Journal of Natural Products*, 67, pp. 1598–1600.

Maia, F., Almeida, M.R., Gales, L., Kijjoa, K., Pinto, M.M., Saraiva, M.J. and Damas. A.M., 2005. The binding of xanthone derivatives to transthyretin. *Biochemical Pharmacology,* 70, pp. 1861–1869.

McChesney, J.D., Venkataraman, S.K. and Henri, J.T., 2007. Plant natural products: Back to the future or into extinction?. *Journal of Phytochemistry,* 68, pp. 2015–2022.

McKee, T.C., Fuller, R.W., Covington, C.D., Cardellina, J.H., Gulakowski, R.J., Krepps, B.L., McMahon, J.B. and Boyd, M.R., 1996. New Pyranocoumarins Isolated from *Calophyllum lanigerum* and *Calophyllum teysmannii*. *Journal of Natural Products*, 59, pp. 754-758.

Morel, C., Se´raphin, D., Oger, J.M., Litaudon, M., Se´venet, T. and Richomme, P., 2000. New xanthones from *Calophyllum caledonicum*. *Journal of Natural Products*, 63, pp. 1471–1474.

Morel, C., Se´raphin, D., Teyrouz, A., Larcher, G., Bouchara, J.P., Litaudon, M., Se´venet, T., Richomme, P. and Bruneton, J., 2002. New and antifungal xanthones from *Calophyllum caledonicum*. *Planta Medica*, 68, pp. 41–44.

Negi, J. S., Bisht, V. K., Singh, P., Rawat, M. S. M. and Joshi G. P., 2013. Naturally Occurring Xanthones: Chemistry and Biology. *Journal of Applied Chemistry.* [online] Available at: <http://www.hindawi.com/journals/jac/2013/621459/> [Accessed 15 July 2013].

Nguyen, L.T.Y., Nguyen, H.T., Barbic, M., Brunner, G., Heilmann, J., Pham, H.D., Nguyen, D.M. and Nguyen, L.H.D., 2012. Polyisoprenylated acylphloroglucinols and a polyisoprenylated tetracyclic xanthone from the bark of *Calophyllum thorelii*. *Tetrahedron Letters*, 53, pp. 4487–4493.

Nigam, S.K. and Mitra, C.R., 1969. Constituents of *C.apetalum* and *C.tomentosum* trunk bark. *Phytochemistry*, 8, pp. 323 – 324.

Ong, H.C., Mahliaa, T.M.I., Masjukia, H.H. and Norhasyima, R.S., 2011. Comparison of palm oil, *Jatropha curcas* and *Calophyllum inophyllum* for biodiesel. *Journal of Renewable and Sustainable Energy Reviews*, 15, pp. 3501–3515.

Pengsuparp, T., Serit, M., Hughes, S.H., Soejarto, D.D. and Pezzuto, J.M., 1996. Specific Inhibition of Human Immunodeficiency Virus Type 1 Reverse Transcriptase Mediated by Soulattrolide, a Coumarin Isolated from the Latex of *Calophyllum teysmannii*. *Journal of Natural Products,* 59, pp. 839-842.

Piccinelli, A.L., Kabani, A.O., Lotti, C., Alarcon, A.B., Rubio, O.C. and Rastrelli, L., 2013. A fast and efficient HPLC-PDA–MS method for detection and identification of pyranochromanone acids in *Calophyllum* species. *Journal of Pharmaceutical and Biomedical Analysis*, 76, pp. 157– 163.

Plattner, R.D., Spencer, G.F., Weisleder, D. and Kleiman, R., 1974. Chromanone carboxylic acids in *Calophyllum brasiliense* seed oil. *Phytochemistry*, 13(11), pp. 2597–2602.

Polonsky, J., 1957. Structure chimique du calophyllolide, del_inophyllolide et de l_acide calophyllique, constituants des noix de *Calophyllum inophyllum*. *Bulletin de la Société Chimique de France*, pp. 1079–1087.

Prasad, J., Shrivastava, A., Khanna, A.K., Bhatia, G., Awasthi, S.K. and Narender, T., 2012. Antidyslipidemic and antioxidant activity of the constituents isolated from the leaves of *Calophyllum inophyllum. Phytomedicine*, 19, pp. 1245– 1249.

Roig, J.T., 1974. *Plantas Medicinales, Aromaticas o Venenosas de Cuba*. La Habana: Editorial Ciencia y Tecnica.

Roig, J. T., 1988. *Plantas Medicinales, Aroma´ticas o Venenosas de Cuba.* Havana: Editorial Cientı´fico-Te´cnica.

Sahelian, R., 2014. *Xanthones in mangosteen fruit juice, antioxidant and cancer benefits, any adverse effects?*.[online] Available at: <http://www.raysahelian.com/xanthones.html> [Accessed 15 March 2014].

Sartori, N.T., Canapelle, D., Júnior, P.T.S. and Martins, D.T.O., 1999. Gastroprotective effect from *Calophyllum brasiliense Camb*. bark on experimental gastric lesions in rats and mice. *Journal of Ethnopharmacology*, 67, pp. 149–156.

Shen, Y.C., Wang, L.T., Khalil, A.T., Chiang, L.T. and Cheng, P.W., 2005. Bioactive Pyranoxanthones from the Roots of *Calophyllum blancoi*. *Chemical and Pharmaceutical Bulletin*, 53(2), pp. 244- 247.

SidoMuncul Herbal, 2014. *Xanthones-Sources and Benefits*. [online] Available at: < http://www.sidomunculherbal.com/en/content/10-xanthones-sources-and-benefits> [Accessed 21 January 2014].

Sousa, G.F., Duarte, L.P., Alcântara, A.F.C., Silva, G.D.F., Filho, S.A.V., Silva, R.R., Oliveira, M.D. and Takahashi, J.A., 2012. New Triterpenes from *Maytenus robusta*: Structural Elucidation Based on NMR Experimental Data and Theoretical Calculations. *Molecules*, 17, pp. 13439-13456.

Souza, M.C., Beserra, M.A.S., Martins, D.C., Real, V.V., Santos, R.A.N., Rao, V.S., Silva, R.M. and Martins, D.T.O., 2009. In vitro and in vivo anti-Helicobacter pylori activity of *Calophyllum brasiliense Camb*. *Journal of Ethnopharmacology*, 123, pp. 452–458.

Spainhour, C.B., 2005. Natural Products. In: S.C.Gad, ed. *Drug Discovery Handbook.* John Wiley & Sons, Inc. pp. 12-63.

Spino, C., Marco, D. and Subramaniam, S., 1998. Anti-HIV coumarins from *Calophyllum* seed oil. *Bioorganic and Medicinal Chemistry*, 24, pp. 3475–3478.

Stevens, P.F. and Arbor, J.A., 1980. *Calophyllum castaneum*. [online] Available at: <http://www.asianplant.net/Calophyllaceae/Calophyllum_castaneum.htm> [Accessed 24 January 2014].

Stout, G.H., Hickernell, G.K. and Sears, K.D., 1968. *Calophyllum* products: Papuanic and Isopapuanic acids. *The Journal of Organic Chemistry*, 33(11), pp. 4191-4200

Susanti, D., Sirat, F.H., Ahmad, M., Ali R.M. and Aimi, N., 2007. Antioxidant and cytotoxic flavonoids from the flowers of *Melastoma malabathricum*. *L. Food Chemistry,* 103, pp. 710-716.

Szakiel, A., Pączkowski, C. and Bertsch, C., 2012. Fruit cuticular waxes as a source of biologically active triterpenoids. *Phytochemistry Reviews.* [online]
Available at: < http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3601259/> [Accessed 15 July 2013].

Taher, M., Attoumani, N., Susanti, D., Ichwan, S.J.A. and Ahmad, F., 2010. Antioxidant Activity of Leaves of *Calophyllum rubiginosum. American Journal of Applied Sciences*, 7 (10), pp. 1305-1309.

Taher, M., Idris, M.S., Ahmad, F. and Arbain, D., 2005. A polyisoprenylated ketone from *Calophyllum enervosum*. *Phytochemistry,* 66, pp. 723–726.

Utami, R., Khalid, N., Sukari, M.A., Rahmani, M., Abdul, A.B. and Dachriyanus., 2013. Phenolic contents, antioxidant and cytotoxic activities of *Elaeocarpus floribundus Blume. Pakistan Journal of Pharmaceutical Sciences,* 26 (2), pp. 245-250.

Velioglu, Y.S., Mazza, G., Gao, L. and Oomah, B.D., 1998. Antioxidant activity and total phenolics in selected fruits, vegetables and grain products. *Journal of Agricultural and Food Chemistry,* 46, pp. 4113-4117.

Vo, V.C., 1997. *Dictionary of Medicinal Plants in Vietnam*. Ho Chi Minh City: Medical Publishing House.

Watson, L. and Dallwitz, M.J., 1992. *The families of flowering plants: descriptions, illustrations, identification, and information retrieval*. [online]
Available at: < http://delta-intkey.com>
Accessed 15 March 2014]

Williams, R.B., 2002. Medicinal Natural Products. *Medicinal Natural Products*. [online]
Available at: <http://scholar.lib.vt.edu/theses/available/etd-11182002-> [Accesed 12 March 2014].

Xango, LLC, 2014. *Xanthones*. [online]
Available at: < http://www.xango.com/about/science/xanthones> [Accessed 21 January 2014].

Yimdjo, M.C., Azebaze, A.G., Nkengfack, A.E., Meyer, A.M., Bodo, B. and Fomum, Z.T., 2004. Antimicrobial and cytotoxic agents from *Calophyllum inophyllum. Phytochemistry,* 65, pp. 2789–2795.

Zou, J., Jin, D., Chen, W., Wang, J., Liu, Q., Zhu, X. and Zhao, W., 2005. Selective Cyclooxygenase-2 Inhibitors from *Calophyllum membranaceum*. *Journal of Natural Products,* 68, pp. 1514-1518.