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PHYTOCHEMICALS AND BIOLOGICAL ACTIVITIES OF *Aquilaria subintegra* Ding Hou

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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (CHEMISTRY)

FACULTY OF SCIENCE AND MATHEMATHICS
SULTAN IDRIS EDUCATION UNIVERSITY
2020

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ACKNOWLEDGEMENT

"In The Name of Allah, Most Gracious, Most Merciful"

First and foremost, I show my gratitude to The Almighty God for giving me the strength to complete this thesis. Alhamdulillah with His permission, I managed to settled PhD work eventually. I am deeply grateful to everyone who has helped me in completing this work.

Million thanks go to my supervisors, Assoc. Prof. Dr Saripah Salbiah Syed Abdul Azziz and Assoc. Prof. Dr Wong Chee Fah for their enthusiasm, guidance, and unrelenting support throughout this work. I want to thank the Department of Chemistry, Faculty of Science and Mathematics UPSI for the access of UV, IR, and NMR instruments.

Sincerely thanks to all lab assistants, especially to Mr Ibrahim, Mrs Johana, Mr Muhammad Hazim, and Mr Zurin for their help throughout these four years. Special thanks to my lab mates and friends; Munirah, Nor Izzati, Kan Shieh Ting, Syafawati, and Nur Hidayat for their moral support, advice, and encouragement to make the lab work meaningful.

I am grateful to the MyPhD scholarship by the Ministry of Higher Education for my doctoral fellowship. My thanks go to FRGS Grant from Ministry of Higher Education (MOHE) under vote 2014-0030-101-02 and GPU grant from Universiti Pendidikan Sultan Idris under vote 2018-0140-103-01 for the support throughout the entire research.

Special thanks to my husband, Mohd Zaid, my daughter, Aileen Medina who cheer me up all the time, my mother Nor Hamidah, brothers, and family in law for their love, spirits, and encouragement in every single thing I do. Last but not least, this PhD specially dedicated to my late grandmother, Badariah Mahidin, who always loves me and gives full of support in everything I do. All of you made everything possible for me. Thank you.

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ABSTRACT

This study aims to investigate the phytochemicals and biological activities from the bark and leaf of *Aquilaria subintegra* Ding Hou. The samples were air-dried, ground, and gradient extracted by cold extraction using *n*-hexane, dichloromethane, and methanol successively. The bark and leaf crude extracts were purified *via* chromatographic techniques to produce pure compounds. The chemical structure of compounds were determined using spectroscopic methods such as nuclear magnetic resonance, ultraviolet, infrared, mass spectrometry and comparison from the literature review. The crude extracts and isolated compounds were tested for their anti-lipase, antioxidant and anticancer activities through colorimetric test, DPPH free radical scavenging and cytotoxicity assay, respectively. This study has successfully afforded 22 compounds, including three new compounds, namely aquilene A, aquilene B, and subintegranol. Other compounds were 5-hydroxy-7,4′-dimethoxyflavone, luteolin-7,3′,4′-trimethyl ether, 5,3′-dihydroxy-7,4′-dimethoxyflavone, 7,3′-dimethoxyluteolin, 5,7-dihydroxy-4′-methoxyflavone, *β*-sitosterol, *β*-sitostenone, stigmasterol, friedelin, epifriedelanol, phytol, and hentriacontane. Anti-lipase activity of *n*-hexane extracts of leaf and methanol extracts of bark showed the highest inhibition, 46% and 52% compared with the control, respectively. Meanwhile, through computational study of 5-hydroxy-7,4′-dimethoxyflavone exhibited the highest inhibition and predicted as a competitive lipase inhibitor. Besides, the leaf of methanolic extract showed the strongest DPPH radical scavenger with IC_{50} value of 29.56 ± 6.37 µg/mL. However, the tested isolated compounds showed less ability in antioxidant activity. In cytotoxic activity, the leaf and bark of methanolic extracts exhibited highest anticancer activity with IC_{50} values of 24.30 ± 0.07 and 17.46 ± 0.08 µg/mL, respectively. Besides, 5-hydroxy-7,4′-dimethoxyflavone showed the highest cytotoxicity activity with IC_{50} value of 38.48 ± 0.06 µg/mL. In conclusion, this study successfully revealed the presence of various phytochemicals in *A. subintegra* and their potential as anti-lipase, antioxidant and anticancer agents. In implication, this study enhances knowledge on the diversity of chemical compounds and their potential in modern medicine.

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FITOKIMIA DAN AKTIVITI BIOLOGI DARIPADA *Aquilaria subintegra* Ding Hou

ABSTRAK

Kajian bertujuan untuk mengenal pasti fitokimia dan aktiviti biologi daripada kulit batang dan daun *Aquilaria subintegra* Ding Hou. Sampel dikering, dikisar dan diekstrak secara pengekstrakan dingin menggunakan pelarut *n*-heksana, diklorometana dan metanol secara berturutan. Ekstrak mentah kulit batang dan daun dipisahkan melalui teknik kromatografi untuk mendapatkan sebatian tulen. Struktur kimia sebatian tulen dikenalpasti menggunakan kaedah spektroskopi seperti resonans magnet nukleus, ultra lembayung, infra merah dan spektrometri jisim dan perbandingan daripada kajian lepas. Ekstrak mentah dan sebatian tulen diuji terhadap aktiviti anti-lipase, antioksidan dan antikanser masing-masingnya menerusi ujian warna, DPPH radikal bebas dan assai kesitotoksikan. Kajian ini berjaya mengasingkan 22 sebatian termasuk tiga sebatian baharu iaitu aquilen A, aquilen B dan subintegranol. Sebatian lain adalah 5-hidroksi-7,4′-dimetoksiflavon, luteolin-7,3′,4′-trimetil eter, 5,3′-dihidroksi-7,4′-dimetoksiflavon, 7,3′-dimetoksi luteolin, 5,7-dihidroksi-4′-metoksiflavon, *β*-sitosterol, *β*-sitostenon, stigmasterol, fridelin, epifridelanol, phytol dan hentriakontan. Aktiviti anti-lipase ke atas ekstrak *n*-heksana daun dan ekstrak metanol kulit batang masing-masingnya menunjukkan perencatan tertinggi, 46% dan 52% berbanding kawalan. Manakala, kajian simulasi berkomputer terhadap sebatian 5-hidroksi-7,4′-dimetoksiflavon mempamerkan perencatan tertnggi dan diramalkan sebagai perencat lipase yang kompetitif. Selain itu, ekstrak daun mentah metanol menunjukkan aktiviti perencatan radikal DPPH yang kuat dengan nilai IC_{50} 29.56 ± 6.37 μg/mL. Namun, sebatian tulen yang diuji mempamerkan kebolehan yang rendah bagi aktiviti antioksidan. Bagi aktiviti sitotoksik, ekstrak metanol daun dan kulit batang masing-masingnya menunjukkan aktiviti antikanser yang kuat dengan nilai IC_{50}, 24.30 ± 0.07 dan 17.46 ± 0.08 μg/mL. Selain itu, 5- hidroksi-7,4′-dimetoksiflavon menunjukkan kesan sitotoksik yang kuat dengan nilai IC_{50} 38.48 ± 0.06 μg/mL. Kesimpulannya, kajian ini berjaya mendedahkan kepelbagaian fitokimia daripada *A. subintegra* dan potensinya sebagai agen anti-lipase, antioksidan dan antikanser. Implikasinya, kajian dapat meningkatkan pengetahuan mengenai kepelbagaian sebatian kimia dalam tumbuhan serta potensinya dalam perubatan moden.

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LIST OF ABBREVIATIONS

α	-	Alpha
AA	-	Ascorbic acid
Abs	-	Absorbance
ASBH	-	*n*-hexane crude of bark *A. subintegra*
ASBD	-	Dichloromethane crude of bark *A. subintegra*
ASBM	-	Methanol crude of bark *A. subintegra*
ASLH	-	*n*-hexane crude of leaf *A. subintegra*
ASLD	-	Dichloromethane crude of leaf *A. subintegra*
ASLM	-	Methanol crude of leaf *A. subintegra*
β	-	Beta
br	-	broad
δ	-	Chemical Shift
CC	-	Column Chromatography
^{13}C-NMR	-	Carbon NMR
$CDCl_3$	-	Chloroform-D
CD_3OD	-	Methanol-D
CH_2Cl_2	-	Dichloromethane
COSY	-	Correlation spectroscopy
1D	-	1-dimensional
2D	-	2-dimensional
d	-	Doublet
dd	-	Doublet of doublet
DEPT	-	Distortionless Enhancement by Polarization Transfer
DMSO	-	Dimethyl Sulfoxide
DL	-	Dried Leaves
DPPH	-	2,2-diphenyl-1-picrylhy-drazyl
EIMS	-	Electron Ionization Mass Spectrometry

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ESI-MS	-	Electron Spray Ionization Mass Spectrometry
EtOAc	-	Ethyl acetate
n-Hex	-	Hexane
HCl	-	Hydrochloric acid
H_2SO_4	-	Sulfuric acid
Hz	-	Hertz
^{1}H-NMR	-	Proton NMR
HMBC	-	Heteronuclear Multiple Bond Correlation
HMQC	-	Heteronuclear Multiple Quantum Coherence
IR	-	Infrared
J	-	Coupling constant
M	-	Multiplet
MeOH	-	Methanol
m/z	-	Mass to charge ion
MS	-	Mass Spectrometry
mg	-	miligram
mL	-	mililiter
min	-	minute
NMR	-	Nuclear Magnetic Resonance
nm	-	nanometer
PPL	-	Porcine Pancreatic Lipase
s	-	Singlet
sec	-	second
SD	-	Standard deviation
SiO_2	-	Silica gel
t	-	triplet
TLC	-	Thin Layer Chromatography
UV	-	Ultra Violet
µL	-	microliter
µmole	-	micromole
PHE	-	Phenylalanine
ILE	-	Isoleucine

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ASP	-	Aspartate
TYR	-	Tyrosine
HIS	-	Histidine
SER	-	Serine
LEU	-	Leucine
ALA	-	Alanine
PRO	-	Proline
ILE	-	Isoleucine
PHE	-	Phenylalanine
TRP	-	Tryptophan
ARG	-	Arginine
VAL	-	Valine
GLU	-	Glutamate
THR	-	Threonine

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G Conference Attendance

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CHAPTER 1

INTRODUCTION

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1.1 General Introduction

Natural products (NPs) give a massive impact on science because of the discovery of plentiful medicinal drugs precursor (Crane & Gademann, 2016). NPs universally refer to substances which are isolated from living organism, and it has usually formed in secondary metabolites. NPs have always been a keystone in finding new lead molecules for drug candidates (Orhan, 2016). They are representing a vast family of diverse chemical constituents with their valuable biological activities, particularly in human, veterinary medicine, and agriculture, as well as significant agents in pharmaceuticals, herbicides, and insecticides (Katz & Baltz, 2016). NPs have brought significant effects of economic due to their role in pharmaceuticals, and fragrances (Anulika, Ignatius, Raymond, Osasere, & Abiola, 2016).

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Historically, since 60,000 years ago, NPs such as plants, animals, microorganisms and marine organisms have been used as a medicinal treatment. However it faced a massive challenge in the early stage to the humans because of the consumption risks such as vomiting, diarrhoea, coma or other toxic reactions and perhaps led to the death; still, early humans knowledgeable about edible materials and natural medicines (Yuan, Ma, Ye, & Piao, 2016).

The NPs such as bacterial, fungal, and the plant has served as front line therapeutics to treat diseases. For example, antibiotics, chemotherapeutics, immunosuppressants, cholesterol-lowering agents and anaesthetics. These molecules derive from large groups of chemicals including polyketides, nonribosomal peptides, saccharides, alkaloids, terpenoids, and comprise a staggering diversity of chemical scaffolds (Medema & Fischbach, 2015; Tang, Zou, Watanabe, Walsh, & Tang, 2017).

Today, natural medicines have been used principally in medical treatment, especially in developing countries (Greenwell & Rahman, 2015). Herbal plants, either one part (flowers, leaves, branches or roots) or entire parts of it might use in the treatment of acute and chronic diseases. Also, in the development of dietary products due to continuous advantages, showed minimal side effect contrasting to synthetic medicine which contributes a severe impact for the long term uses (Farzaneh & Carvalho, 2015). Therefore, in the 21st century, medicinal herbs gained attention as an alternative way to replace the usage of synthetic drugs.

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The statistical data from the World Health Organization (WHO), showed about 80% of people in the world depend on traditional herbal medicine for their health care needs. Traditional Chinese Medicine, Indian Ayurvedic Medicine, and Unani Medicine of Arab cultures were globally used as references for herbal medicine (Rehman, Choe, & Yoo, 2016).

Most researchers attract Malaysian herbs due to the massive areas of rainforest and tropical weather. In Malaysia, approximated 15,000 known plant species, in which 2,000 species have medicinal values, and the rest species are not exploited and cultivated (Saad et al., 2015). Some examples of medicinal plants reported in Malaysia shown in Table 1.1 (Madaleno, 2015).

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Table 1.1

Medicinal plants reported in Malaysia

Plant Species	**Part**	**Medicinal action**
Strobilanthes crispus (L.) Bremek (Pecah Kaca)	Leaf	Anticancer, antioxidant, antidiabetic
Cananga odorata (Lam.) Hook. F. & Thomson (Ylang-ylang)	Flower	As a relaxing body oil
Cuminum cyminum L. (Cumin)	Seed	Herbal supplement for fatigue
Cocos nucifera L. (Chamomile)	Fruit	As a healing oil
Plectranthus barbatus Andrews (Long Pepper)	Whole plant	Healing body oils

(*continued*)

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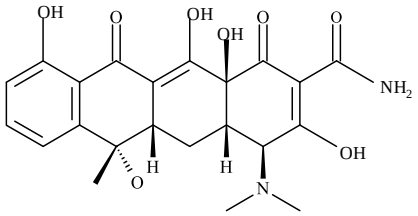
Table 1.1 *(continued)*

Plant Species	**Part**	**Medicinal action**
Pinellia nti-in (Thunb.) Ten. Ex Breitenb. (Musli)	Rhizome	Chinese remedy for cough and cold
Mentha arvensis L. (Jamun)	Leaf	Chinese remedy for pimples, skin rashes and bruises
Cinnamomum camphora (L.) J. Presl (PolygalaRhubarb)	Bark, leaf	As nti-inflammatory cream for itching and burns

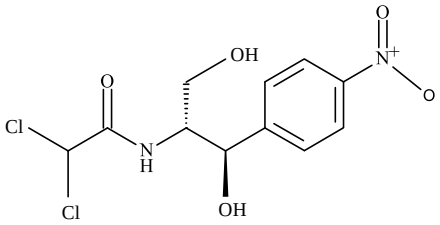
The US Food and Drug Administration approved 1453 new NPs in the year 2013, and prominently used in medicinal treatment. For example, tetracycline (**1**) and chloramphenicol (**2**) as antibacterial, amphotericin B (**3**) (antifungal and antiparasitic), mitomycin C (**4**) and daunorubicin (**5**) as antitumor agents (Katz & Baltz, 2016). Thus, the significance of NPs are undoubtedly enormous and contribute massive benefits. Therefore, the scientist worldwide has a vital responsibility to identify medicinal plants which valuable to commercialize as herbal medicines.

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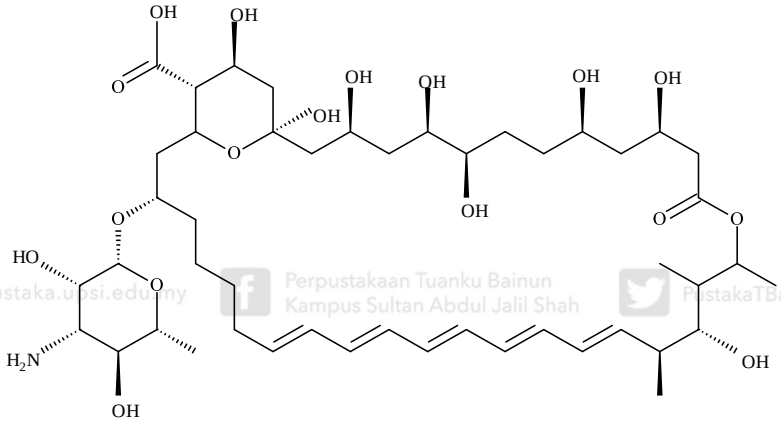
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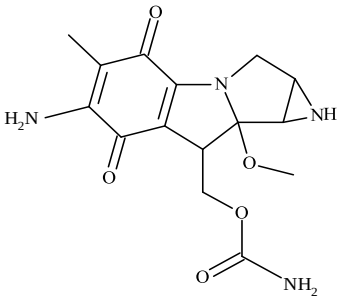
(1)



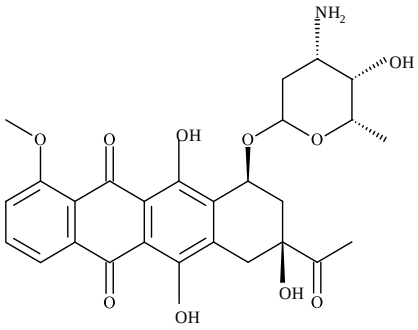
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(3)



(4)



(5)

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1.2 Problem Statement

Aquilaria sp. is a fragrant tree which widely distributed in Asia including, Malaysia, Thailand, and Indonesia. This species was valuable due to the production of scented and aromatic wood which known as gaharu or agarwood. Besides that, agarwood tea was produced from *Aquilaria* leaves which mainly manufactured in China, Malaysia, and Indonesia. Yet, there is no official report commenced date of agarwood tea became as a commercial product, however, in 2007, there was first reported (Wu et al., 2007) toward toxicological safety of agarwood tea in the local market Hainan, China (Adam, Lee, & Mohamed, 2017).

However, there is a deficiency information specific population of *A. subintegra*. *A. subintegra* has found in Thailand, Narathiwat province, with an altitude range between 300 -500 m asl (Lee & Mohamed, 2016).

Furthermore, there is a limited number of phytochemical and pharmacological studies of *A. subintegra*. To the best of our knowledge, there is a tiny reference for isolation of *A. subintegra* in the phytochemical study. Besides, an inadequate study on the pharmacological study of the isolated compound from *A. subintegra* triggered us to reveal the chemical constituents and expected to obtained new bioactive potential compounds from this plant.

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1.3 Significance of Study

This study revealed the chemical constituents obtained for the first time and examined their biological activities. Current results would be able to enhance knowledge, especially on species of study, the diversity of its chemical compounds derived from this plant, and its potential as a precursor to modern medicine.

1.4 Objectives of Study

The main objectives of the current study are:

i- To extract, isolate, and purify chemical constituents from the crude extracts of *A. subintegra* bark and leaf using chromatographic techniques.

ii- To identify and elucidate the chemical structures of the isolated compounds using several spectroscopic analyses such as nuclear magnetic resonances (NMR), ultraviolet-visible spectroscopy (UV), Fourier transform infrared spectroscopy (FTIR) and mass spectrometry (MS).

iii- To investigate biological properties including antioxidant, anti-lipase, and anticancer toward crude extracts and isolated compounds. Antioxidant activity was determined using DPPH free radical scavenging assay. The anti-lipase activity was measured by colorimetric assay; meanwhile,

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anticancer activity was examined using Cell Counting Kit-8 (CCK-8)/ WST-8 toward breast cancer cell, MCF-7.

iv- To determine the interaction of isolated compounds with receptor using computational study.

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CHAPTER 2

LITERATURE REVIEW

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2.1 Thymelaeaceae Family

Thymelaeaceae is one medium-size family tree, comprises about 50 genera and 900 species, and widely distributed in latitudes south of 40^{o}N and can found in Asia, Africa, and Australia (Elias, Ibrahim, & Mahamod, 2017; Herber, 2002). The name of the Thymelaeaceae family given by Michel Adanson, who is a French botanist in 1763, and this family is divided into subfamilies Octolepidoidae and Thymelaeoideae (Farah et al., 2018). In contrast, the earlier study indicated that this family has four small families, including Gonystyloideae, Aquilarioideae, Synandrodaphnoideae, and Thymelaeoideae (Herber, 2002). There have some arguments in the ordering of subfamilies of Thymelaeaceae. The changes in taxonomy affinities of this family started in 1836 by taxonomist Lidney. Lidney (1836) proposed *Aquilaria* under the order Aquilariaceae, Alliance Daphnales, and while Herber (2002) proposed *Aquilaria*

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under two prominent families, Octolepidoideae; Thymelaeoideae, and Lee and Mohamed (2016) proposed *Aquilaria* and *Gyrinops* were under Thymelaeoideae subfamily and tribe Aquilarieae. The summary of the changes in taxonomy affinities shown in Table 2.1.

Thymelaeaceae family said to be exclusive due to most of the genus such as *Aquilaria*, *Gyrinops,* and *Wikstroemia* that can produce fragrant, aromatic, and scented wood which is known as gaharu or agarwood (Elias et al., 2017; Lok, 2016). Two genera from tribe Aquilarieae, namely, *Aquilaria* Lam. And *Gyrinops* Gaertn. Renowned as an agarwood producing tree which widely distributed from northeast India, through southeast Asia and south of China to Papua New Guinea (Farah et al., 2018).

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Table 2.1

The chronology of changes of taxonomy affinities in family Thymelaeaceae and genus Aquilaria

Taxonomist	**Year proposed**	**Change made**	
Lidney	1836	Alliance	Daphnales
		Order	Aquilariaceae; Elaeagnaceae;
		Genera	*Hernandiaceae*; *Thymelaeaceae*; *Aquilaria*; *Gyrinops*; *Ophiospermum*
Bentham and Hooker	1880	Genera	*Aquilaria* was removed

(*continued*)

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Table 2.1 *(continued)*

Taxonomist	**Year proposed**	**Change made**	
Gilg	1894	Subfamily	Aquilarioideae; Phalerioideae;
		Tribe	Thymelaeoideae; Drapetoideae
		Genera	*Aquilarieae*
			Aquilaria; *Gyrinopsis*; *Gyrinops*
Gilg	1921	Subfamily	Aquilarioideae; Phalerioideae;
			Thymelaeoideae; Drapetoideae;
			Microsemmatoideae; Octolepidoideae;
			Synandrodaphnoideae
Domke	1934	Subfamily	Aquilarioideae; Gilgiodaphnoideae;
		General	Gonystyloideae; Thymelaeoideae
		Tribe	Aquilariidae
		Genera	*Aquilaria*; *Gyrinops*
Hutchinson	1967	Order	Thymelaeales
		Family	Aquilariaceae; Thymelaeaceae
		Genera	*Aquilaria*; *Deltaria*; *Gyrinops*; *Lethedon* ;
			Octolepis; *Solmsia*
The Angiosperm Phylogeny Group	1998	Order	Malvales
Herber	2002	Subfamily	Octolepidoideae; Thymelaeoideae
		Tribe	Aquilarieae; Daphneae; Synandrodaphneae
		Genera	*Aquilaria*; *Gyrinops*

Adapted from Lee & Mohamed, 2016

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2.2 Genera *Aquilaria*

Aquilaria is one of the precious trees in Asia due to its highest value of resin-impregnated heartwood and including in the family Thymelaeaceae and belongs to the subfamily Thymelaeideae (formerly Aquilariodeae) (Lee & Mohamed, 2016). The *Aquilaria* genus slowly grows up to 40 m height and 60 cm diameter of the tree and usually found in tropical forests (Hashim et al., 2016). The tree produces bitter fruit and has a small white flower (Elias et al., 2017; Hashim et al., 2016). *Aquilaria* species distributed between altitudes of 0-850 m, in locations with typical temperatures of 20-22°C (Khalil, Rahim, Taha, & Abdallah, 2013). This plant widespread in Asia and Southeast Asia, for instance, India, Bhutan, Bangladesh, Myanmar, Philippines, Malaysia and Thailand (Chen et al., 2012; Faridah-Hanum et al., 2009; Lok, 2016; Monggoot, Popluechai, Gentekaki, & Pripdeevech, 2017).

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The resin derived from the wounded tree of *Aquilaria* genera makes it highly valuable trees is known as agarwood. There are also known by several names which are aloeswood, eaglewood, oud, clambake, Chen Xiang, and jinkoh (Ahmaed & Kulkarni, 2017). There are 21 accepted species of *Aquilaria* genus (The Plant List, 2013). A total of nine species of *Aquilaria* had reported producing agarwood including *Aquilaria beccariana* Tiegh., *Aquilaria crassna* Pierre ex Lecomte, *Aquilaria piculat* (Oken) Merr., *Aquilaria hirta* Ridl., *Aquilaria khasiana* Hallier f., *Aquilaria malaccensis* Lamk., *Aquilaria microcarpa* Baill., *Aquilaria piculat* Ridl., and *Aquilaria sinensis* (Lour.) Spreng (Hashim et al., 2016). Meanwhile, another study stated that thirteen species able to produce scented, aromatic agarwood (Elias et al., 2017; Lee & Mohamed, 2016). In Thailand, there have four common species of agarwood, including

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A. malaccensis, A. subintegra, A. picul, and A. crassna (Kamonwannasit, Nantapong, Kumkrai, Luecha, & Kupittayanant, 2013). All species are now listed under Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (UNEP-WCMC 2015) (Pern, Lee, Ludin, & Mohamed, 2018). Table 2.2 below shows the distribution of 21 *Aquilaria* species (Hashim et al., 2016; Lee & Mohamed, 2016).

Table 2.2

Distribution of Aquilaria species

Species	**Distribution**	**Year reported**
Aquilaria piculate	Bukidnon	1922
Aquilaria baillonii	Cambodia	1915
Aquilaria banaensis	Vietnam	1986
Aquilaria beccariana	East Malaysia, Brunei, Kalimantan (Indonesia)	1983
Aquilaria brachyantha	Cagayan (Philippines)	1922
Aquilaria citrinicarpa	Mindanao (Philippines)	1922
Aquilaria crassna	Cambodia, Laos, Thailand, Vietnam	1915
Aquilaria cumingiana	Mindanao (Philippines)	1901
Aquilaria decemcostata	Laguna (Philippines)	1922
Aquilaria filaria	Indonesia, Philippines	1950
Aquilaria hirta	South Thailand, Peninsular Malaysia, Singapore	1901
Aquilaria khasiana	Khasi, Meghalaya (India)	1922

(continued)

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Table 2.2 *(continued)*

Species	Distribution	Year reported
Aquilaria malaccensis	Bangladesh, Bhutan, Indoesia, Malaysia, Iran, Myanmar, Philippines, Thailand, Singapore	1783
Aquilaria microcarpa	Peninsular Malaysia, Singapore, Indonesia	1875
Aquilaria parvifolia	Camarines (Philippines)	1960
Aquilaria rostrata	Peninsular Malaysia	1924
Aquilaria rugosa	Vietnam, north Thailand	2005
Aquilaria sinensis	China, Taiwan, Hong Kong, Hainan Island	1825
Aquilaria subintegra	South Thailand	1964
Aquilaria urdanetensis	Mindanao (Philippines)	1922
Aquilaria yunnanensis	Yunnan (China)	1985

Also, Figure 2.1 showed the map of the distribution of *Aquilaria* sp in Indomalesia. **(A)** the most extensive parts dominated of a population of *A. crassna*, *A. malaccensis,* and *A. sinensis* in the northwest end including Malaysia, Thailand, Cambodia, and China, meanwhile, **(B)** the Philippines, the only country have six endermic species including *A. brachyantha*, *A. decemcostata*, *A. parvifolia*, *A. apiculata*, *A. citrinicarpa* and *A. urdanetensis* (Lee & Mohamed, 2016).

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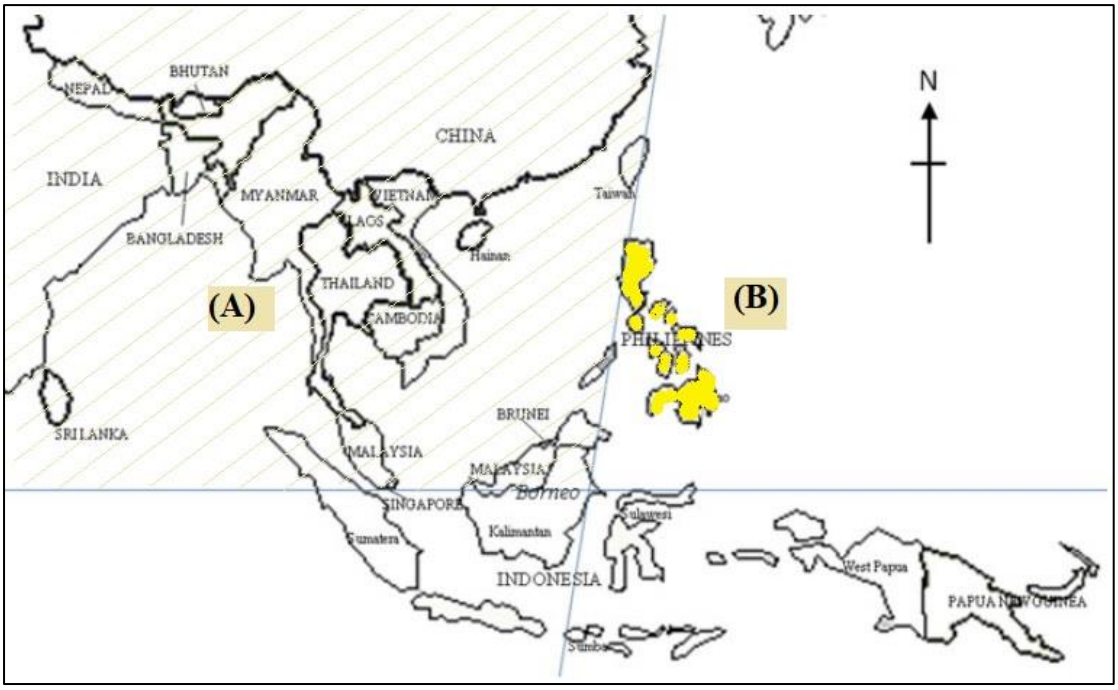


Figure 2.1. Distribution of Aquilaria species in Indomalesia region. Adapted from Lee & Mohamed, 2016.

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Aquilaria or agarwood has widely used in many cultures for a long time ago. It plays an essential role in traditional medicine, cultural and religious practices in Buddhist, Hindu, Muslim, Jewish, and Christian (Lim & Noorainie, 2010). In Buddhist religious ritual, agarwood play as a central between human and God, whereby it burned to purify the space surrounding the Buddha statue (Compton & Ishihara, 2004). Agarwood incense also applies in Islamic culture where it used in mosques, shops, and houses during ceremony or ritual (Karimi et al., 2012). Besides that, the Egyptians believe that they have been used agarwood incense since 3000 years ago in death rituals (Gerard, 2007).

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In addition, *Aquilaria* sp also plays an essential role in medicine, including the preparation of Chyawanprash, as an adaptogen and antiaging cure in Ayurvedic medicine (Parle & Bansal, 2011). Moreover, in the pharmaceutical industry, it is employed as an antiemetic, tranquillizer, and digestive in oriental medical treatment (Okudera & Ito, 2009).

Nowadays, the range of agarwood products and their uses is expanding and continually. The essential oil and perfume are top-rated and high demand especially in the Arab world meanwhile, beads as well as bracelets of agarwood pieces, are valuable 'natural art' in Japan, Korea, and Taiwan (Gerard, 2007).

Accessible literature and information in Table 2.3 show that several *Aquilaria* species have traditionally used as medicine in different parts of the world. This genus has economic importance as forest products for use as timber and herbal medicines. The important ethnomedicinal properties possessed by this genus can be the basis for further research in the future.

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Table 2.3

Medicinal uses of various Aquilaria species

Species	Part	Uses	Reference
A. sinensis	Leaf	Treat constipation problem	(Kakino et al., 2010)
A. crassna	Several parts of plant	Treatment for diarrhoea, dysentery and skin diseases	(Kamonwannasit et al., 2013)
A. sinensis	Resinous wood	treatment for abdominal pain, vomiting, circulatory disorders, and dyspnea	(Wang et al., 2018)
A. *agallocha*	Resinous bark	For rheumatism treatment	(Rana, Sohel, Akhter, & Islam, 2010)

2.2.1 *Aquilaria subintegra*

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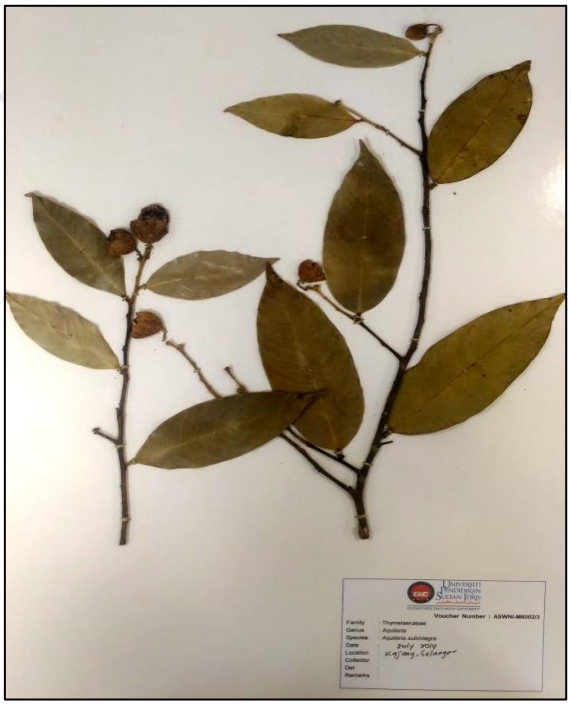
Aquilaria subintegra (Figure 2.2) was known locally in Malaysia as gaharu or karas (Hashim et al., 2016). This species is the main cultivation species and principally found in Thailand. The tree is a shrub, has an elliptic-oblong or slightly obovate-oblong leaf shape and leaf is papery, and the surface slightly smooth (Lee & Mohamed, 2016). The fruit of *A. subintegra* has a diamond shape, and its size approximately 4.5 cm × 1.5 cm and the surface appearance is wrinkle, covered with fine hair; each year the species will crop flower in April and May then fruiting during July and August (Pern et al., 2018). Also, this plant might be considered as fast-growing species (Farah et al., 2018).

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(a) (b)



(c)

Figure 2.2.(a) Bark, (b) leaf and (c) herbarium specimen of *A. subintegra*

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2.3 Phytochemical Studies of *Aquilaria s*pecies

Aquilaria species have known for a long time as rich sources of bioactive secondary metabolites. A review of the literature discovered that the phytochemical studies of *Aquilaria* species before the present study. The research findings have reported the presence of a few classes of organic compounds, including flavonoids, phenolic acids, terpenoids, steroids, fatty acid, and alkanes. Thus, phytochemical studies are discussed in this chapter.

2.3.1 Phenolic Compounds

Phenolic compounds are secondary metabolites of plants and contain various compounds depending on the type of the target of plants (Farzaneh & Carvalho, 2015; Manach, Scalbert, Morand, Rémésy, & Jime' nez, 2004). Phenolic compounds found as one of the important secondary metabolites due to having health superiority, such as antioxidant (Ganesan et al., 2018; Wojdyło, Oszmiański, & Czemerys, 2007), anti-lipase agents in obesity treatment (Buchholz & Melzig, 2015; Sergent, Vanderstraeten, Winand, Beguin, & Schneider, 2012), antiinflammatory (Zhang & Tsao, 2016) and antibacterial (Karou, Dicko, Simpore, & Traore, 2005). Phenolic compounds were considered as significant bioactive components owing to several allied health benefits with repute to lifestyle diseases and oxidative stress. There are several factors for the shifting trend to herbal medicines including the rising proportion of aged people in Japan and Europe, as well as augmented health awareness and the onset of various metabolic disorders because of ageing (Ameer, Shahbaz, & Kwon, 2017).

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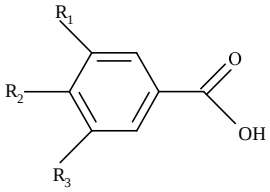
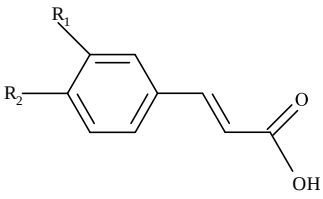
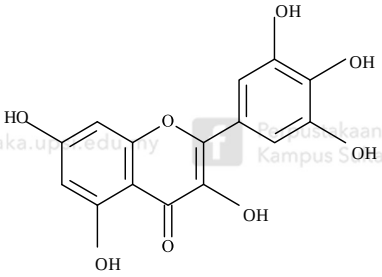
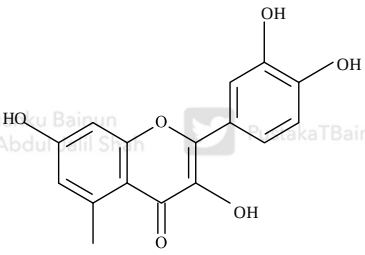
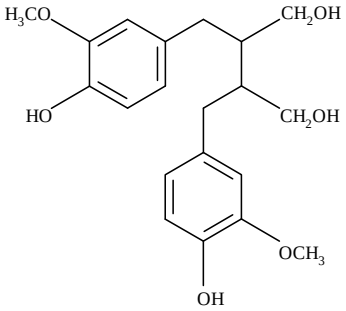
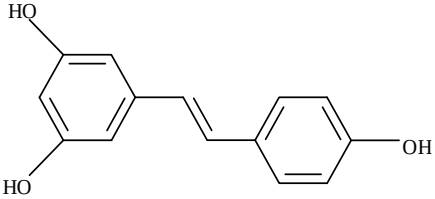
The phenolic compounds can be distinguished by three main classes, namely, phenolic acid, flavonoid, and non-flavonoid (Zhang & Tsao, 2016). These classes can be differentiated by their number of phenol rings that contain and the structural elements that attach these rings (Manach et al., 2004). Phenolic acid richly discovered in food and grouped into two classes, namely derivatives of benzoic acid and derivatives of cinnamic acid. Flavonoid involves a large group of phenolic which contains more than 4,000 identified compounds. Non-flavonoid consists of stilbenes which comprise two phenyl moieties connected by a two-carbon methylene bridge. Lignan contains a 2,3-dibenzylbutane structure formed from the dimerization of two cinnamic acid residues (Kanti & Syed, 2009). Tannins (hydrolyzable tannins) are also in the polyphenolic group, consist of gallotannins, and ellagitannins. They mostly exist in fruit such as raspberry, cranberries, strawberries, and grapes (Marín, Elisa M. Miguélez, J Villar, & Lombó, 2015). Another study found that tannins are a part of the plant's defence system as antibacterials and antiviruses (Butnariu, 2014). Table 2.4 showed the distinction of classes of phenolic compounds together with their examples.

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Table 2.4

Classes of Polyphenolic Compounds

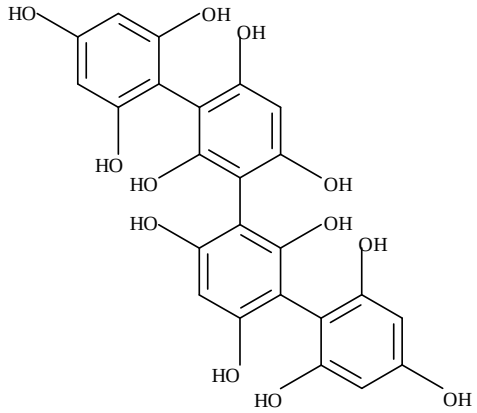
Classes	**Examples**		**Reference**
Phenolic acid		R_1= R_2= OH, R_3 = H: protacatechuic acid (**6**) R_1= R_2= R_3 = OH: gallic acid (**7**)	(Działo et al., 2016; Manach et al., 2004)
		R_1= OH: coumaric acid (**8**) R_1= R_2= OH: caffiec acid (**9**) R_1=OCH_3, R_2= OH: ferulic acid (**10**)	
Flavonoids			(Działo et al., 2016; Manach et al., 2004)
	Myricetin (**11**)	Quercetin (**12**)	
Lignans			(Działo et al., 2016; Manach et al., 2004)
	Secoisolariciresinol (**13**)		
Stilbenes			(Działo et al., 2016; Manach et al., 2004)
	Resveratrol (**14**)		

(continued)

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Table 2.4 *(continued)*

Classes	Examples	Reference
Tannins	 Tetrafucol A (**15**)	(Działo et al., 2016)

2.3.1.1 Flavonoids

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Flavonoid is one of a large group of polyphenolic compounds present in plants that consist fifteen-carbon skeleton comprising of two aromatic rings connected through heterocyclic pyrane ring, and the basic structure of flavonoid was shown in Figure 2.3. Flavonoids are habitually existing in plant tissues in the form of diverse derivatives, such as sugar *O*-conjugates at position C2 (chalcones), at C3 (flavonols, anthocyanidins, and flavan-3-ols), or C7 (flavanones, flavones, and isoflavones) (Gentile et al., 2018; Kumar & Pandey, 2013; Manach et al., 2004; Marín et al., 2015). Flavonoids also commonly attached to several types of sugars such as glucose, galactose, rhamnose, xylose, rutinose, arabinopyranose, and arabinofuranose (Marín et al., 2015).

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Over the past decade, most research in the natural product has emphasized the use of flavonoids in various epidemiological studies due to its versatile health benefits (Kumar & Pandey, 2013). Numerous studies had reviewed the bioavailability of dietary flavonoids. For example, as an antiinflammatory (Feng et al., 2012; Zhang et al., 2006), antiobesity (Hossain et al., 2016), antioxidant (Djeradi, Rahmouni, & Cheriti, 2014), allergy preventive (Ogawa, Oku, Iwaoka, Iinuma, & Ishiguro, 2007), antidiabetic (Babu, Liu, & Gilbert, 2013), and α- amylase inhibitor (Dat, Dang, & Thanh, 2016). Table 2.5 is shown the example of constituents for each sub-group of flavonoid.

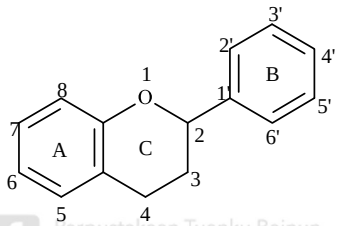


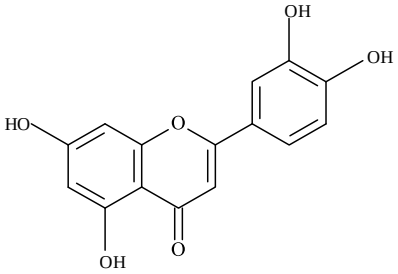
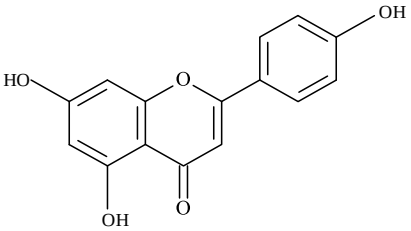
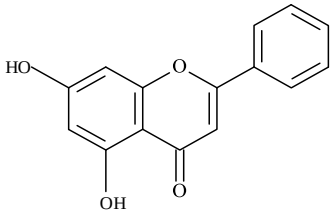
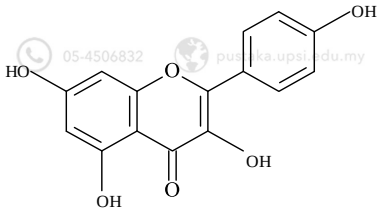
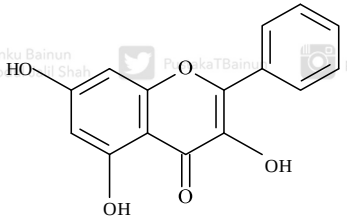
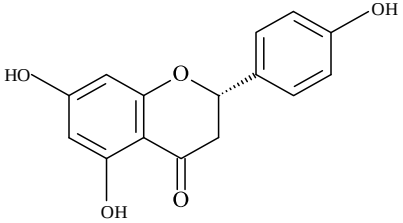
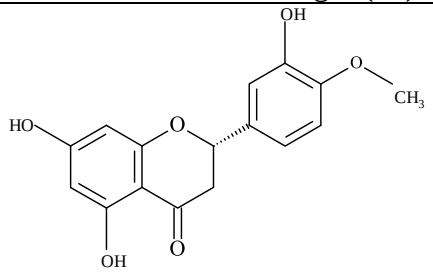
Figure 2.3. Basic skeleton of flavonoid

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Table 2.5

Chemical structures of flavonoid groups

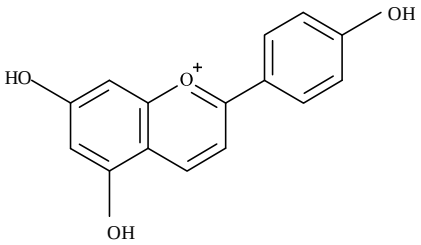
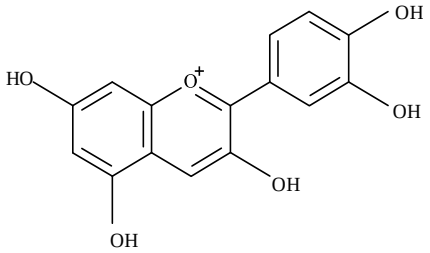
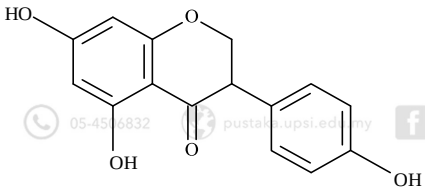
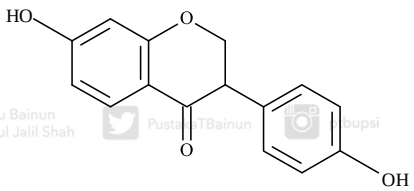
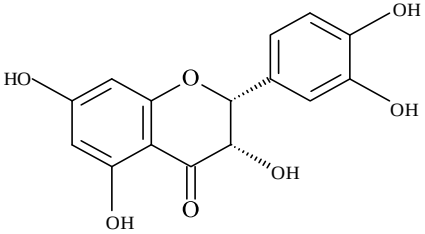
Group of Flavonoid	Example		
Flavones			
	Luteolin (**16**)	Apigenin (**17**)	Chrysin (**18**)
Flavonols			
	Kaempferol (**19**)	Galangin (**20**)	
Flavanones			
	Hesperetin (**21**)	Naringenin (**22**)	

(continued)

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Table 2.5 *(continued)*

Group of Flavonoid	Example
Anthocyanidin	 Apigenidin (**23**)  Cyanidin (**24**)
Isoflavones	 Genistein (**25**)  Daidzein (**26**)
Flavan-3-ols	 Epicatechin (**27**)

Adapted from Gentile et al., 2018, Kumar & Pandey, 2013, Manach et al., 2004, and Marín Fernández et al., 2015

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2.3.1.2 Reported Flavonoids in *Aquilaria* Species

There are numerous phytochemical studies have been done on *Aquilaria* species in the universe. For instance, researcher Alfinda Novi Kristanti and colleagues (2017) had reported flavonoid compounds, namely 5,3′,4′-trihydroxy-7-methoxyflavon or 3′-hydroxy genkwanin (**28**), which isolated from the stem bark of *A. microcarpa*. On the other hand, twelve flavonoids were successfully isolated from the stem of *A. sinensis* and identified as aquilarinoside A_1 (**29**), lethedioside A (**30**), 7,4′-dimethylapigenin-5-*O*-xylosylglucoside (**31**), lethedoside A (**32**), 7-hydroxyl-4′-methyl-5-*O*-glucosideflavonoid (**33**), 7,3′-dimethyl-4′-hydroxyl-5-*O*-glucosideflavonoide (**34**), 7, 4′-dimethyl-5-*O*-glucosideflavonoide (**35**), 5-hydroxyl-7, 4′-dimethoxyflavone (**36**), luteolin-7, 3′, 4′-trimethyl (**37**), hydroxylgenkwanin (**38**), 5, 7-dihydroxyl-4′-methoxyflavone (**39**), and formononetin (**40**) (Dong, Dan, Yue-lin, & Peng-fei, 2012). In addition, leaf of *A. sinensis* also contained the flavonoid compounds, for instance, 5-hydroxy-7,4′-dimethoxyflavonoid (**36**), 5,3′-dihydroxy-7,4′-dimethoxyflavone (**41**) and luteolin-7,3′,4′-trimethyl ether (**37**) (Kang et al., 2014). Compound (**41**) or known as pilloin, also has been found in the stem bark of *A. sinensis* and appeared as yellowish needles (Tsai et al., 2018).

Another study also identified the flavone compounds, comprising compounds (**40**), (**36**), (**37**), compound 5, 7, 4′-trihydroxyflavone or apigenin (**17**) and 3′, 4′, 5, 7-tetrahydroxyflavone or luteolin (**16**) from the stem of *A. sinensis* and branches of *A. agallocha* (Liu, Perng, & Chen, 2018). Isolation from leaf extract of *A. sinensis* also has been studied by Yang and friends (2012) and revealed a new flavonoid glycoside named aquisiflavoside (**42**). *A*nother finding of *A. sinensis* showed the presence of

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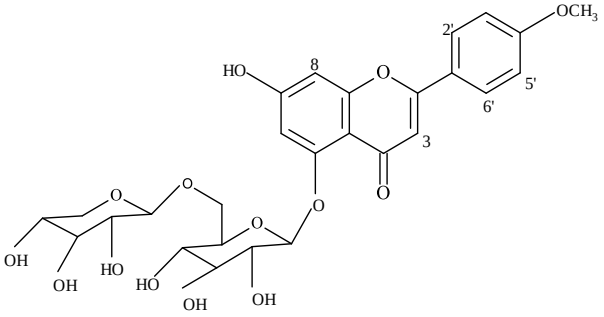
major bioactive compounds, such as genkwanin 5- *O* –*β*-primeveroside (**43**) and genkwanin (**44**) (Ito et al., 2012).

Phytochemical study of leaves of *A. sinensis* from China revealed the existence of flavonoid compounds including hypolaetin-5-*O*-*β*-D-glucuronopyranoside **(45)**, aquilarisin **(46)**, 7,4′-di-*O*-methylapigenin-5-*O*-xylosylglucoside **(31)**, hydroxygenkwanin **(33)**, genkwanin **(44)**, 7,4′-di-*O*-methylluteolin or compound **(41)** 7,3′,4′-tri-*O*-methylluteolin or compound **(37)**, 7,4′-di-*O*-methylapigenin or compound **(36)** (Yu et al., 2013).

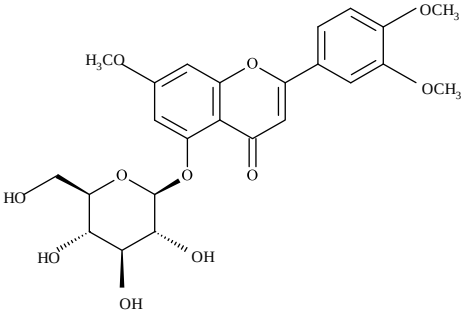
Besides, Chu and colleagues (2016) reported that six flavonoid compounds, namely, apigenin (**17**), luteolin (**16**), kaempferol (**19**), quercetin (**12**), kaempferol 3-*O*-*α*-L-rhamnopyranoside or afzelin (**47**), and quercetin 3-*O*-*α*-L-rhamnopyranoside or quercitrin (**48**), were obtained for the first time from the flower of *A. sinensis*. Another prior study obtained another flavonoid constituent, namely, 7-*β*-D-glucoside of 5-*O*-methylapigenin (**49**) which appeared as a pale yellow amorphous, from the leaves of *A. sinensis* (Qi, Lu, Liu, & Yu, 2009).

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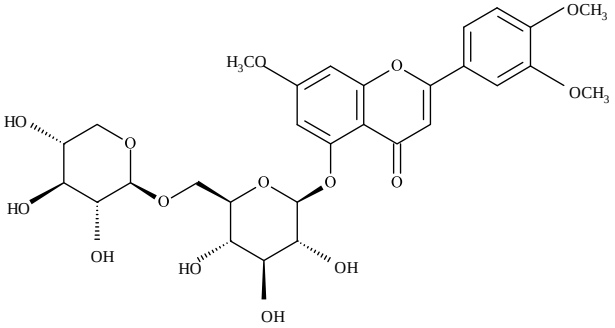
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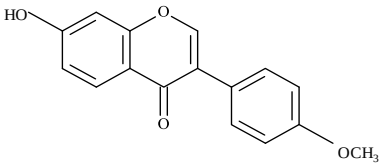
(**29**)



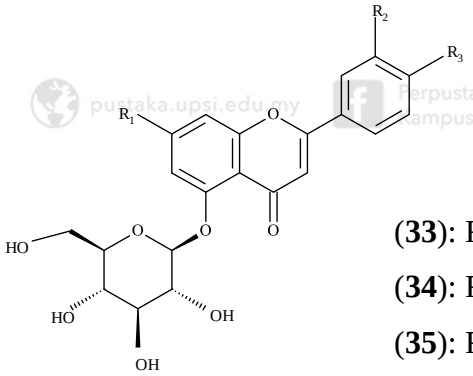
(**32**)



(**30**)



(**40**)



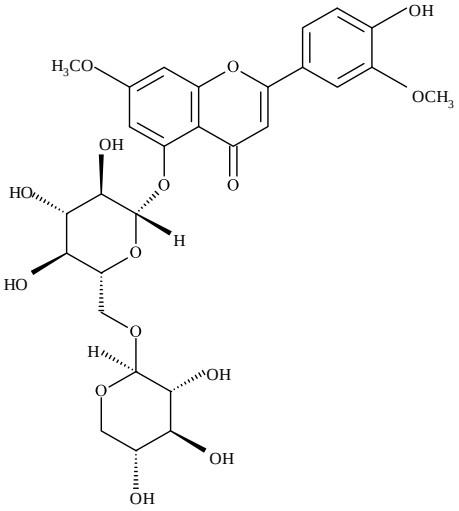
(**33**): R_1= OH, R_2= H, R_3= OCH_3

(**34**): R_1= OCH_3, R_2= OCH_3, R_3= OH

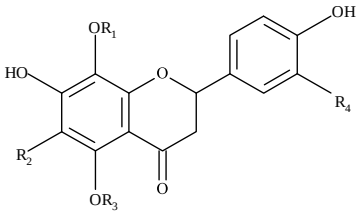
(**35**): R_1= OCH_3, R_2= H, R_3= OCH_3

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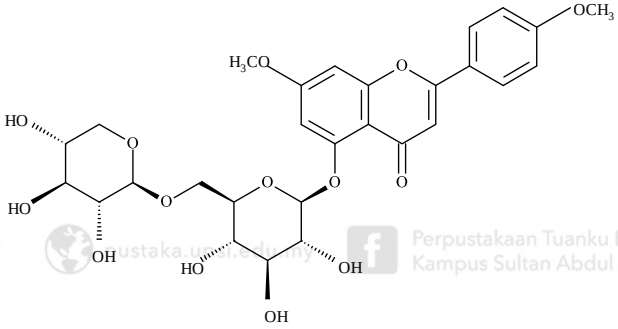


(**42**)

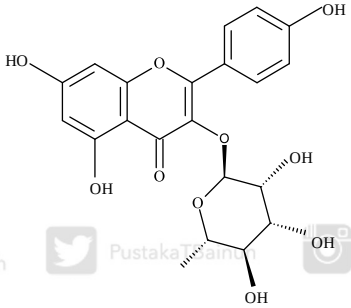


(**45**): R_1= R_2= H, R_3= GluA, R_4= OH ;

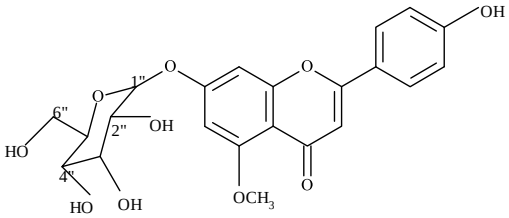
(**46**): R_1= GluA, R_2= O, R_3= CH_3, R_4= H



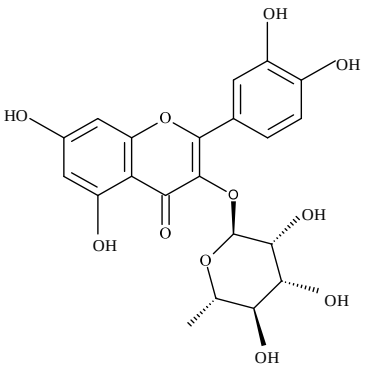
(**31**)



(**47**)



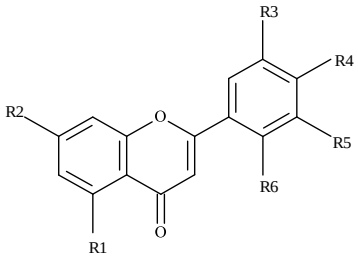
(**49**)



(**48**)

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Compound	R1	R2	R3	R4	R5	R6
28	OH	OCH_3	OH	OH	H	H
36	OH	OCH_3	H	OCH_3	H	H
37	OH	OCH_3	OCH_3	OCH_3	H	H
38	OH	OCH_3	H	OH	OH	H
39	OH	OH	H	OCH_3	H	H
41	OH	OCH_3	OH	OCH_3	H	H
43	O-β-primeveroside	OCH_3	H	OH	H	H
44	OH	OCH_3	H	OH	H	H

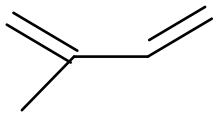
2.3.2 Terpenes

Terpenes are one of the largest groups of secondary metabolites and mostly are insoluble in water. It's derived from mevalonic acid and consists of five carbons bonded with each other and has the branched carbon skeleton of isopentane and the basic structure of terpenes is called isoprene or 2-methylbutadiene (**50**) (Anulika et al., 2016; Wang et al., 2018). Terpene group consists of more than 40,000 structures reported mainly from various plants and a small amount from fungi and marine invertebrates (Cho et al., 2017; Pan et al., 2017). Terpene exists in three provinces of life and

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acknowledged to fulfil a diversity of functions, such as membrane constituents, chemical attractants, or feeding deterrents (Pan et al., 2017).



(**50**)

There are several categories of terpene, including monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterpenes (C_{25}) and triterpenes (C_{30}). In-plant, terpenes derived from two pathways (Figure 2.4); the cytosolic mevalonate (MVA) pathway which comprises enzymatic reaction from acetyl coenzymeA condensation through mevalonate iphosphate decarboxylation and providing precursors for the synthesis of the sesquiterpene (C_{15}) and triterpene (C_{30}), meanwhile plastidial methylerythritol phosphate (MEP) pathway began with the reaction between pyruvate and glyceraldehyde-3-phosphate and offering precursors for monoterpenes, diterpenes and tetraterpenes (Mendoza-Poudereux et al., 2017). Table 2.6 shows the summary classification of terpene groups.

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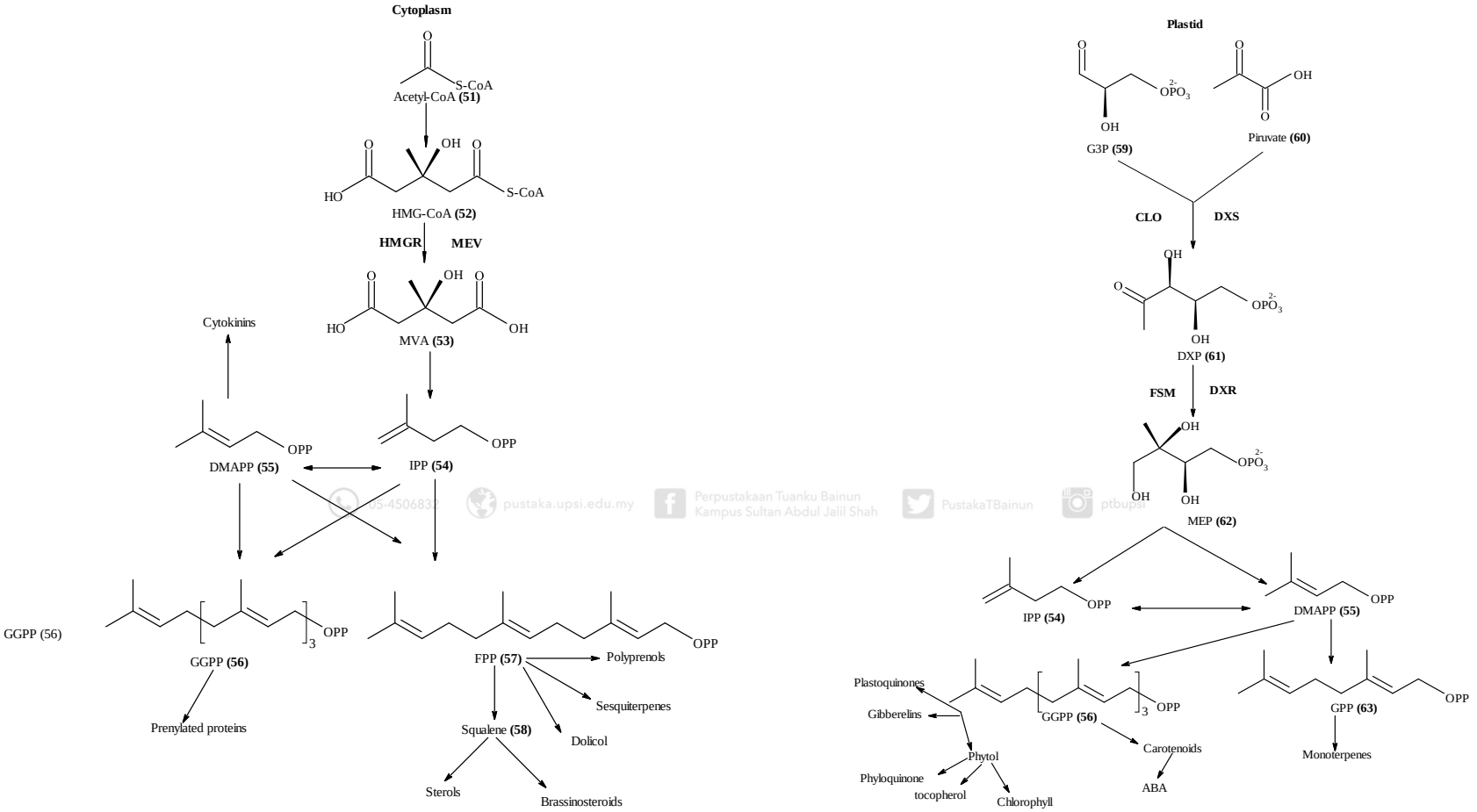


Figure 2.4. Biosynthesis of terpenes and their inhibitors. ABA: abscisic acid. CLO: clomazone. DMAPP: isomer dimethylallyl diphosphte. DXP: 1-deoxy-D-xylulose 5-phosphate. DXR: DXP reductoisomerase. DXS: DXP synthase. FPP: farnesyl diphosphate. FSM: fosmidomycin. GGPP: geranylgeranyl diphosphate. G3P: D-glyceraldehyde-3 phosphate. HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzyme A. HMGR: HMG-CoA reductase. IPP: isopentenyl diphosphate. MEP: methyl-D-erytritol-4-phosphate. MEV: mevinoline. MVA: mevalonate. Adapted from Mendoza-Poudereux et al., 2017.

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Table 2.6

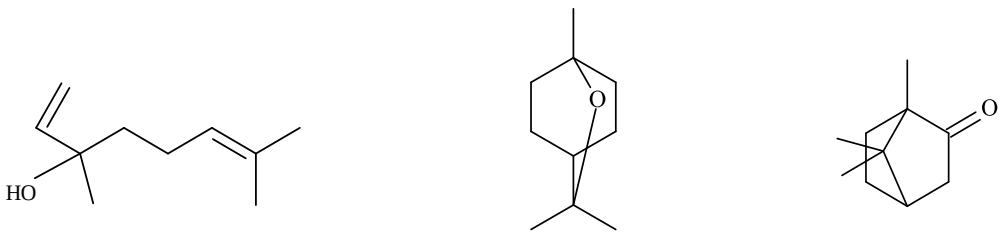
The classification of terpenes based on the number of isoprene units in their structure

Group	**Number of Carbon**	**Isoprene C_5 unit**
Hemiterpene	5	1
Monoterpene	10	2
Sesquiterpene	15	3
Diterpene	20	4
Sesterterpenes	25	5
Triterpene	30	6
Tetraterpene	40	8
Polyterpene	> 40	> 8

2.3.2.1 Monoterpene

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Monoterpene is C_{10} consists of 2-isoprene units. Monoterpenes are derived from geranyl diphosphate molecule (GPP) and mainly found in essential oils such as linalool (**64**), cineole (**65**), and camphor (**66**) (Mendoza-Poudereux et al., 2017). The chemical structures of these compounds were displayed in Figure 2.5.



(64) **(65)** **(66)**

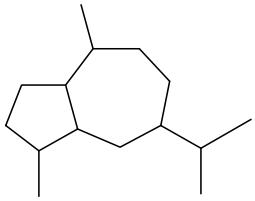
Figure 2.5. Examples of monoterpene compounds

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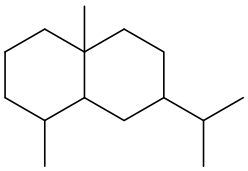
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2.3.2.2 Sesquiterpenes

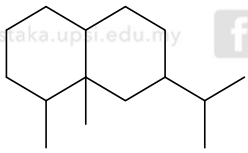
Sesquiterpenes, C_{15}, comprises of 3-isoprene units and usually found in essential oils. The examples of sesquiterpene are guaianes (**67**), eudesmanes (**68**), eremophilanes (**69**), agarofurans (**70**), vetispiranes (**71**), and prezizanes (**72**) and most of them are oxygenated (Kristanti, Tanjung, & Aminah, 2018; Yang et al., 2014). Figure 2.6 showed the skeleton of sesquiterpenes.



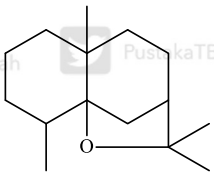
guaiane (**67**)



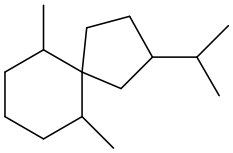
eudesmane (**68**)



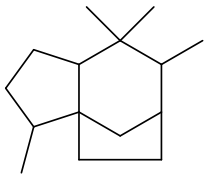
eremophilane (**69**)



agarofuran (**70**)



vetispirane (**71**)



prezizane (**72**)

Figure 2.6. Skeleton of sesquiterpenes

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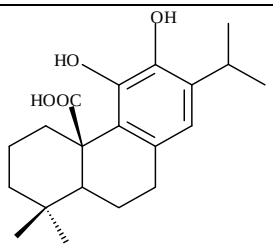
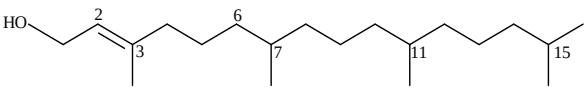
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2.3.2.3 Diterpenes

Diterpenes group, C_{20} are consists of twenty carbons, and mainly present in plant secondary metabolites. Diterpenes are a vital source for pharmaceuticals, food additive, and fragrance, yet their low natural abundance and high structural complexity limit their wide-range industrial consumption (Andersen-ranberg et al., 2016). Diterpenes are believed to have few biological properties such as antiinflammatory, redox-protective pharmacological effects (Silva et al., 2014), cancer chemotherapy (Chen, Cho, Toyomasu, Cane, & Christianson, 2017) and pancreatic lipase (PL) inhibitor (Birari & Bhutani, 2007). The examples of diterpene constituents are displayed in Table 2.7.

Table 2.7

Examples of diterpenes in various species

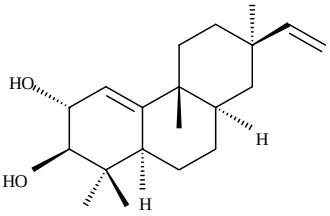
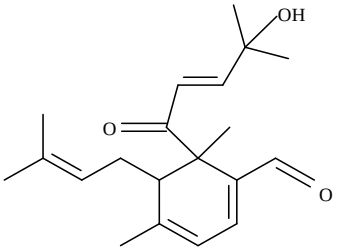
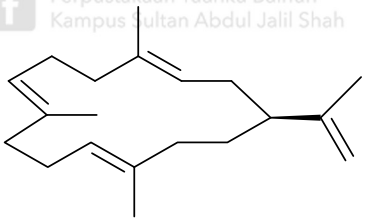
Plant/ Species	Compound	Reference
Salvia officinali	 Carnosic acid **(73)**	(Birari & Bhutani, 2007)
Not stated	 Phytol **(74)**	(Silva et al., 2014)

(continued)

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Table 2.7 *(continued)*

Plant/ Species	Compound	Reference
Euphorbia ebracteolata	 Yuexiandajisu F **(75)**	(Shi et al., 2005)
Litsea cubeba	 Cubelin **(76)**	(Trisonthi, Sato, Nishiwaki, & Tamura, 2014)
Sinularia flexibilis	 Cembrene A **(77)**	(Palaniveloo, Ramachandram , & Vairappan, 2014)

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2.3.2.4 Triterpenes and Steroids

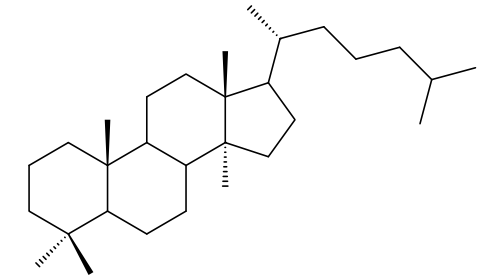
Triterpene group, C_{30}, consist of thirty carbons, usually present in all organism, especially in plants and demonstrated significant pharmacology effects such as antiinflammatory, antimicrobial, antiviral, cytotoxic and cardiovascular (Silva et al., 2012). Most of their skeletons are tetracycles which consist of three six-membered and one five-membered rings, and pentacycles, consist of either four six-membered and one five-membered rings or five six-membered rings and also acyclic, mono-, di-, tri- and hexacyclic scaffolds have been isolated from nature (Sandjo & Kuete, 2013). The classes of triterpenes are including lanostane (**78**), dammarane (**79**), tetranortriterpenoids (**80**), fridelane (**81**), lupine (**82**), oleanane (**83**), ursane (**84**) and hopane (**85**) groups (Hill & Connolly, 2018; Sandjo & Kuete, 2013). Figure 2.7 was shown the skeletons of triterpenes.

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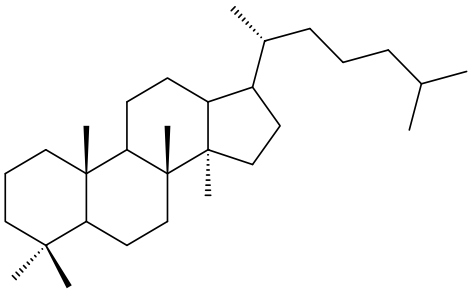
On the other hand, steroids occupy fully or partly condensed cyclopenta[a]phenanthrene scaffold, sometimes bearing methyl groups at C-10 and C-13, as shown in Figure 2.8. Meanwhile in Figure 2.9 displayed the different skeleton of steroids especially at the backbone of the side chain at C-17, its length, and the stereochemistry of some of its chiral centres (Sandjo & Kuete, 2013). Sterols are believed to have significant structural components of membranes and functions in signalling as steroidal hormones (Thimmappa, Geisler, Louveau, O'Maille, & Osbourn, 2014).

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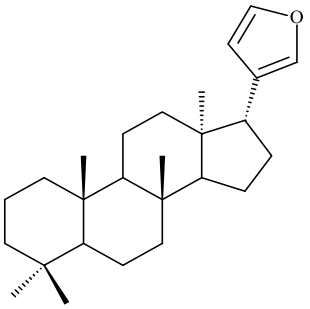
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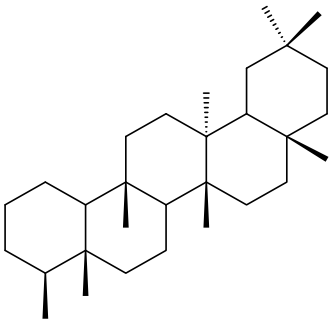
(**78**)



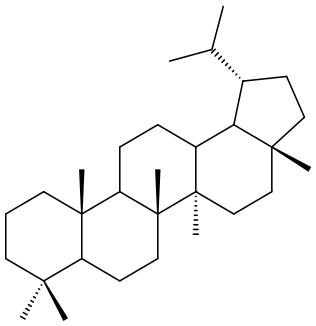
(**79**)



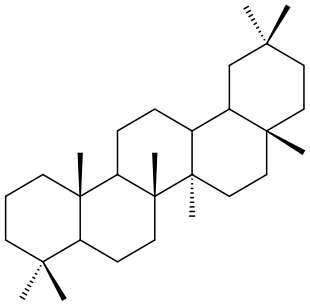
(**80**)



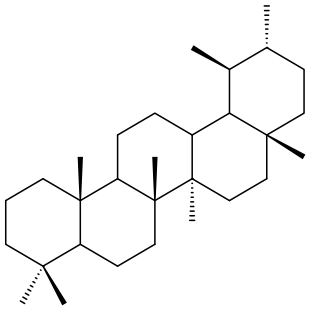
(**81**)



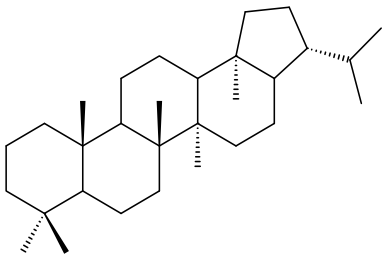
(**82**)



(**83**)



(**84**)



(**85**)

Figure 2.7. Skeleton of triterpenes: lanostane (**78**), dammarane (**79**), tetranortriterpenoid (**80**), fridelane (**81**), lupine (**82)**, oleanane (**83**), ursane (**84**), hopane (**85**)

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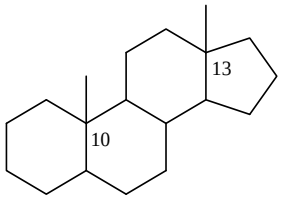
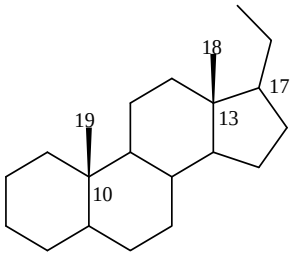
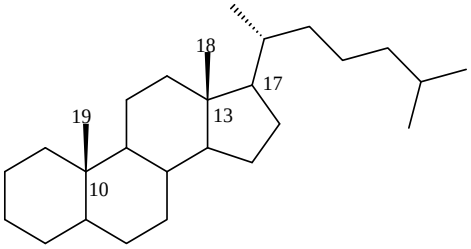


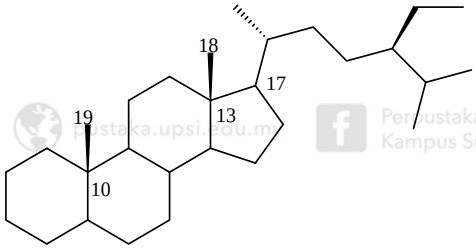
Figure 2.8. Backbone skeleton of steroid



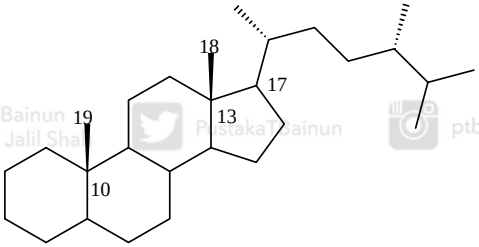
(**86**)



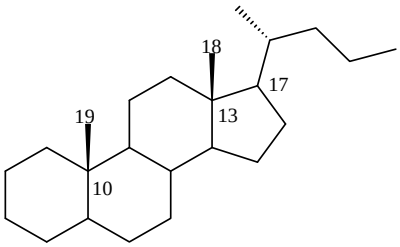
(**87**)



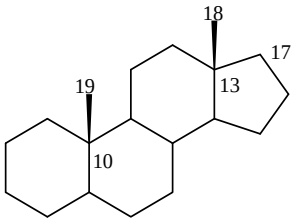
(**88**)



(**89**)



(**90**)



(**91**)

Figure 2.9. Different skeleton of steroids; pregnane (**86)**, cholestane (**87**), stigmastane (**88**), ergostane (**89**), cholane (**90**) and androstane (**91**)

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The biosynthesis of triterpenes and sterols (Figure 2.10) done through the mevalonate pathway, involving isoprenoids, isopentenyl diphosphate (IPP) (**54)**, and dimethylallyl diphosphate (**55**) forming the precursor, squalene (**58**); sterols synthesized via cyclization of 2,3-oxidosqualene (**92**) to the sterol lanosterol (**93**) or cycloartenol **(94**) via chair-boat-chair (CBC) conformation, and triterpenes synthesized thru chair-chair-chair (CCC) conformation prior to cyclization of 2,3-oxidosqualene (**92**) into a massive range of triterpenes several skeletons (Sandjo & Kuete, 2013; Thimmappa et al., 2014).

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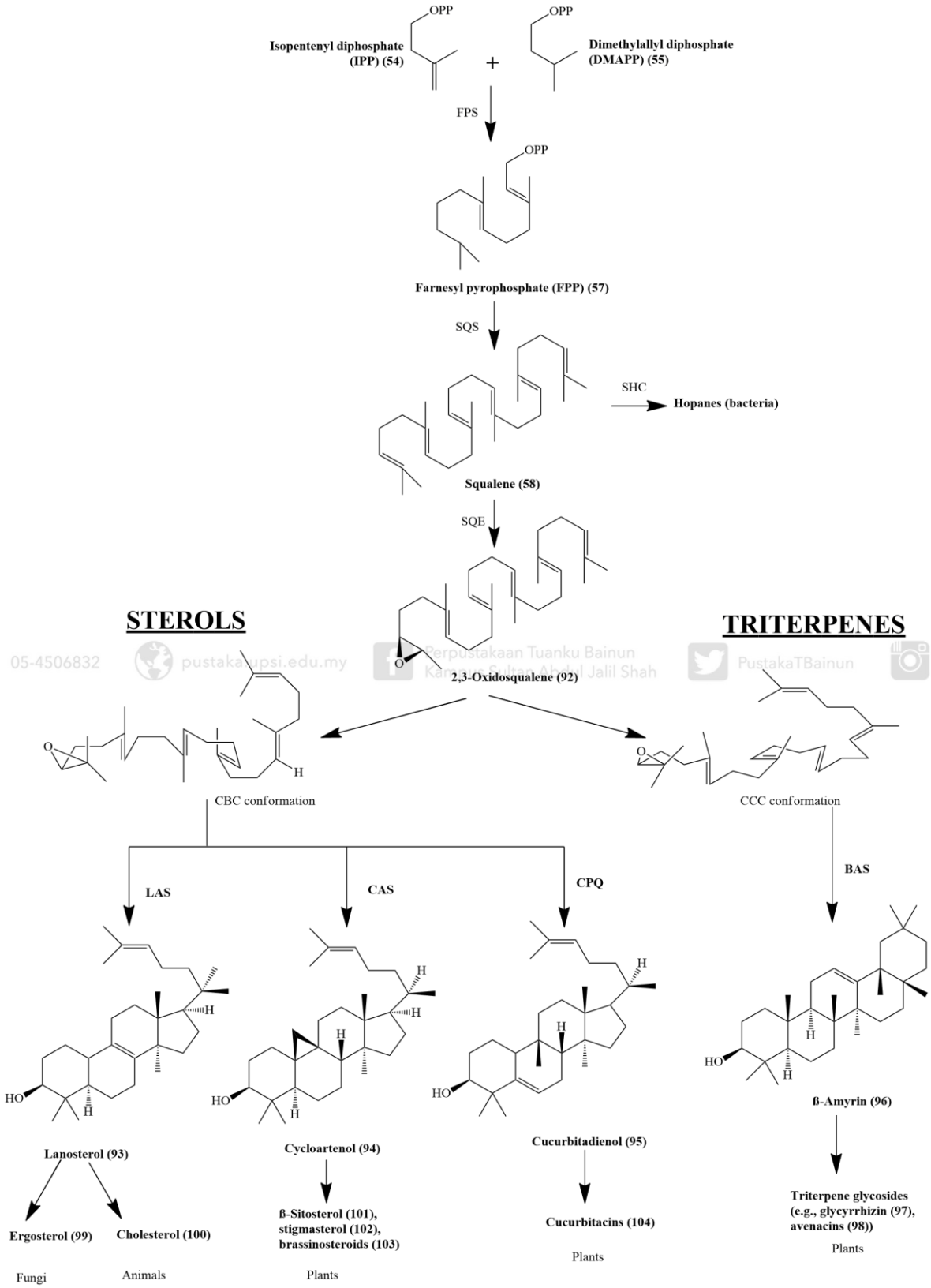


Figure 2.10. The biosynthesis of sterols and triterpenes. Adapted from Thimmappa et al., 2014.

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2.3.2.5 Terpenes in *Aquilaria* Species

Sesquiterpene has been discovered to be the primary class of the essential oil of *Aquilaria* sp. In 2010, Tajuddin and Yusoff discovered the presence of sesquiterpenes in essential oils from *A. malaccensis* spp such as *β*-agarofuran (**105**), agarospirol (**106**), jinkoh-eremol (**107**), kusunol (**108**), selina-3,11-dien-9-one (**109**), guaia-1(10),11-dien-9-one (**110**) and karanone (**111**).

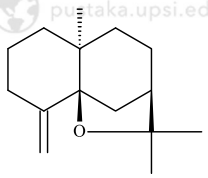
Another study, Yang et al., (2014) discovered three new sesquiterpenes from agarwood *A. sinensis*, namely 4-hydroxyl- baimuxinol (**112**), 7*β*-*H*-9(10)-ene-11,12-epoxy-8-oxoeremophilane (**113**), 7*α*-*H*- 9(10)-ene-11,12-epoxy-8-oxoeremophilane (**114**) and a known compound, neopetasane (**115**). A recent study had revealed four new sesquiterpenes from agarwood *A. malaccensis*, namely aquilanol A (**116**), aquilanol B (**117**), daphnauranol D (**118**) and chamaejasmone E (**119**) as well as five known compounds including daphnauranol B (**120**), daphnauranol C (**121**), chamaejasmone D (**122**), auranticanol A (**123**) and 12-hydroxyhumula- *2Z,6E,9E*-triene (**124**) (Ma et al., 2017).

On the other hands, the leaves of *A. sinensis* revealed two new tirucallane triterpenoids, aquilacallanes A (**125**) and aquilacallanes B (**126**) from ethanolic extract together with known compounds, namely 24-methylene-25-methyltirucall -7-en-3-one (**127**), 11-oxo-*β*-amyrin (**128**), hederagenin (**129**), 3*β*-acetoxyfriedelane (**130**) and ursolic acid (**131**) (Cheng et al., 2013). In the following year, two megastigmane glycosides, *viz*, (9S) megastigma-4,7-diene-2,3,9-triol 9-*O*-*β*-D-glucopyranoside (**132**) and (9S) megastigma-4(13),7-diene-3,6,9-triol 9-*O*-*β*-D-glucopyranoside (**133**) and

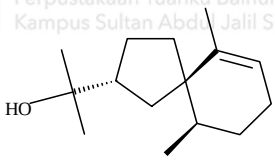
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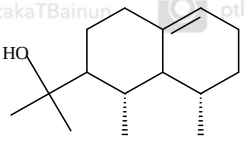
two cucurbitacin glycosides namely 2-*O*-*β*-D-glucopyranosyl cucurbitacin I (**134**), bryoamaride (**135**) were obtained from leaves of *A. sinensis* (Sun et al., 2015). More study from the leaves and petioles of *A. sinensis* has been carried and discovered the presences of triterpene compounds, for instance, aquilacallane B (**126**), aquilacallane A (**125**), cucurbitacin L (**136**), bryoamaride (**135**), 2-*O*-*β*-D-glucopyranosyl cucurbitacin I (**134**), 2-*O*-*β*-D-glucopyranosylcucurbitacin J (**137**), and 2-*O*-*β*-D-glucopyranosylcucurbitacin K (**138**) (Wang, Wang, & Yue, 2015). Furthermore, the prior study also exhibited steroid compounds from *A. sinensis* leaves, named as *β*-sitosterol (**101**), stigmasterol (**102**), *β*-sitostenone (**139**) and stigmasta-4,22-dien-3-one (**140**) (Kang et al., 2014). These triterpenes constituents also have been summarized by Kristanti and friends (2018) in their mini-review article.



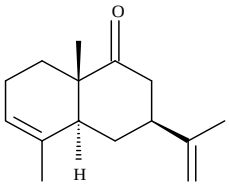
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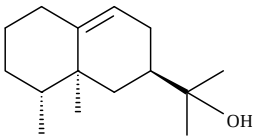
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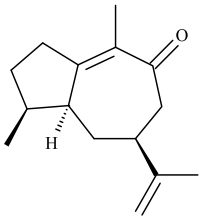
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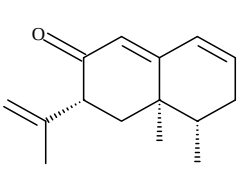
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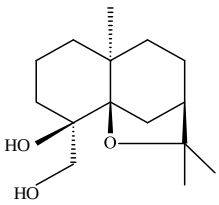
(107)



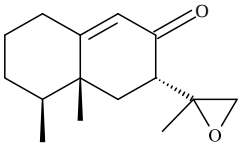
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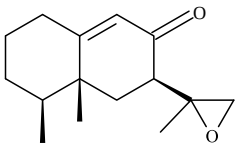
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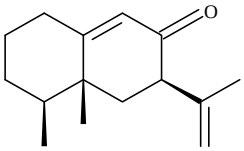
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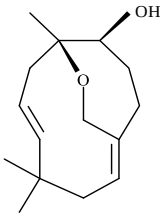
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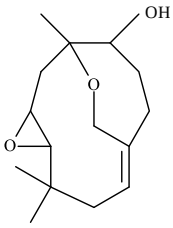
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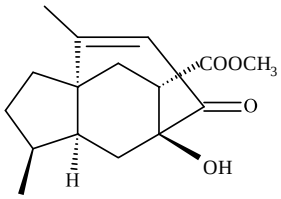
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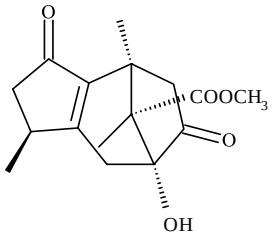
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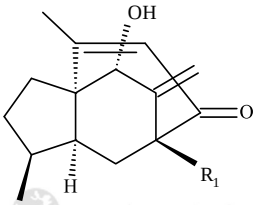
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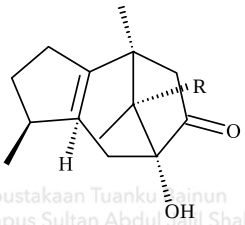
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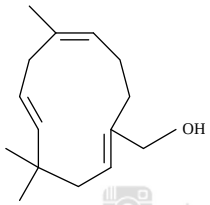
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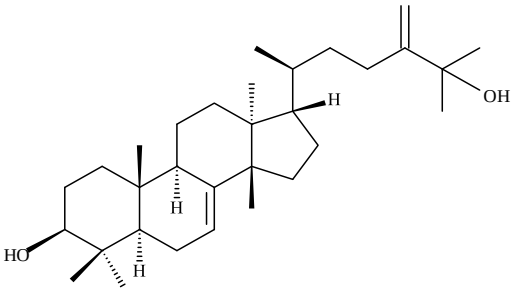
(120): R_1= OH;
(121): R_1= H



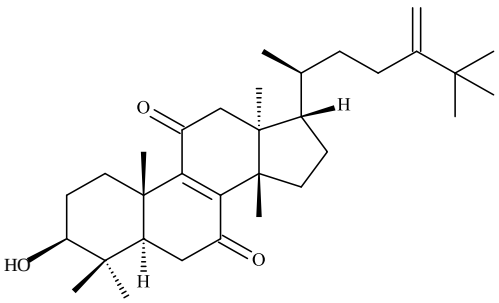
(122): R= CH_2OH;
(123): R= CHO



(124)



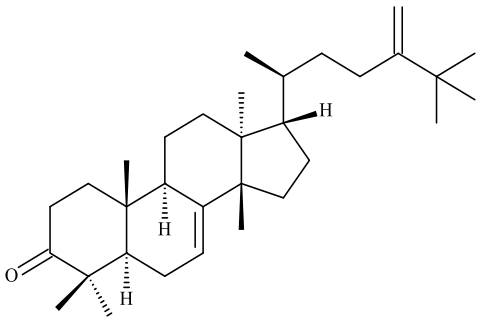
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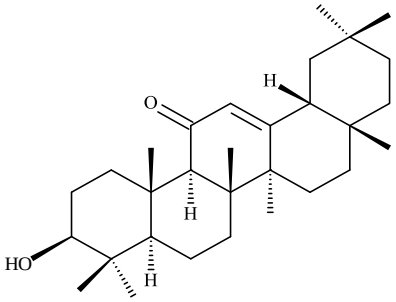
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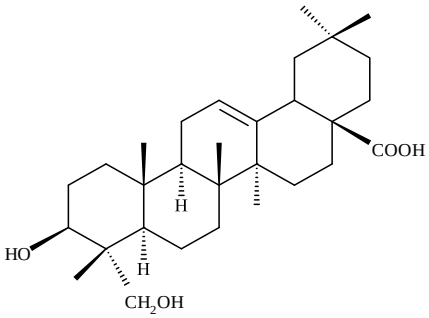
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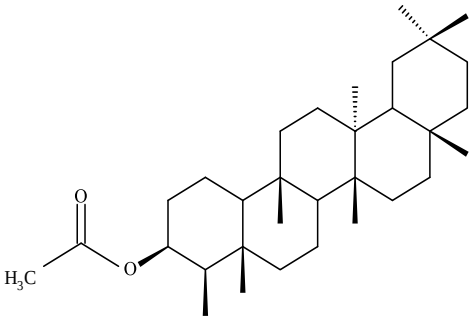
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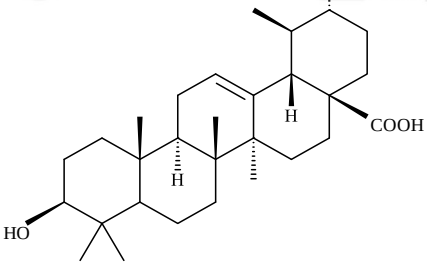


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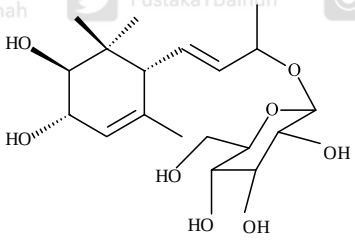


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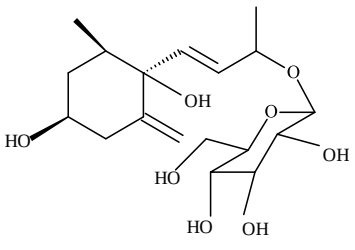
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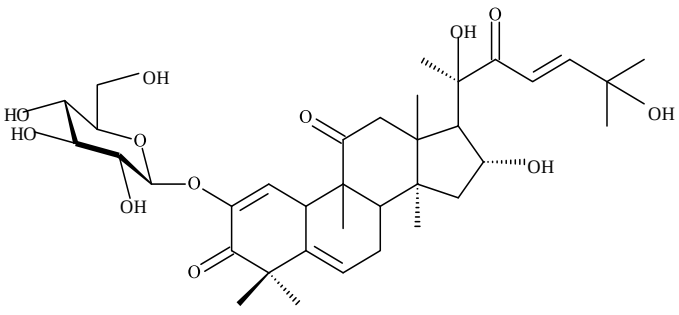
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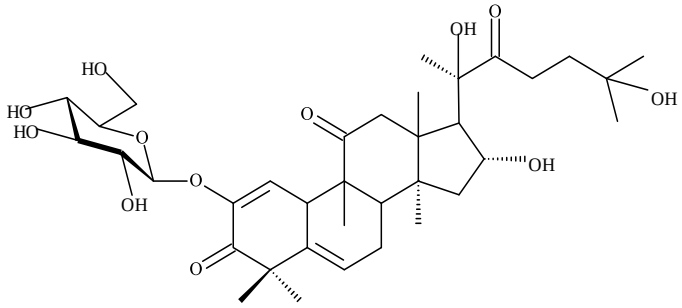
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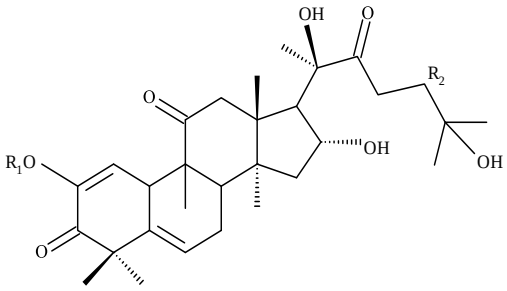
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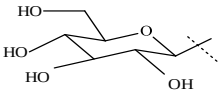
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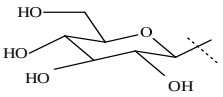


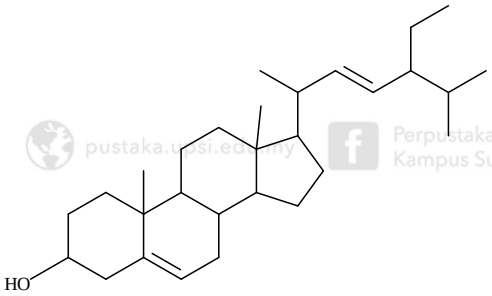
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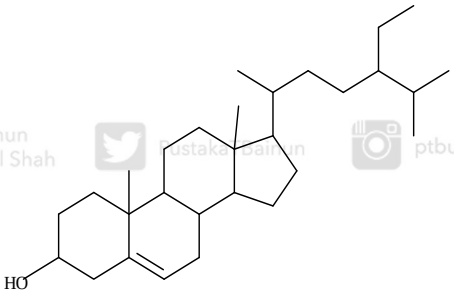
(136): R_1= R_2= H

(137): R_1=  R_2= (R)OH

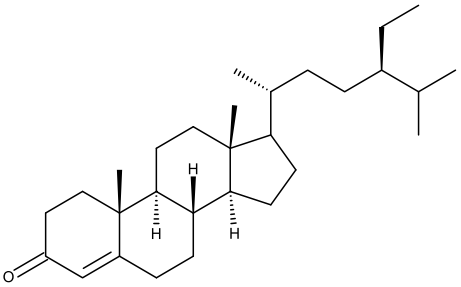
(138): R_1=  R_2= (S)OH



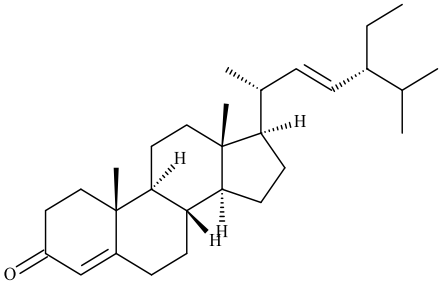
(102)



(101)



(139)



(140)

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2.3.3 Miscellaneous Phytochemicals Reported in *Aquilaria* Species

Other secondary metabolites from *Aquilaria* species were included xanthones, benzophenones, phenolic acids, alkane, and 2-(2-phenylethyl) chromones. These compounds were tabulated in Table 2.8. This table also showed there is no phytochemical studies have been reported in *A. subintegra*.

Table 2.8

Other phytochemicals isolated from Aquilaria species

Compound	**Species**	**Part**	**References**
9H-Xanthen-9-one,1,2,3,6,7-pentahydroxy (**141**)	*A. sinensis*	Leaves	(Yu et al., 2013)
Neomangiferin (**142**)	*A. sinensis*	Leaves	(Yu et al., 2013)
Iriflophenone-3,5-*C*-*β*-diglucoside (**143**)	*A. sinensis*	Leaves	(Yu et al., 2013)
Iriflophenone 3-*C*-*β*-glucoside (**144**)	*A. sinensis*	Leaves	(Yu et al., 2013)
Aquilarixanthone (**145**)	*A. sinensis*	Leaves	(Yu et al., 2013)
Mangiferin (**146**)	*A. sinensis*	Leaves	(Yu et al., 2013)
Isomangiferin (**147**)	*A. sinensis*	Leaves	(Yu et al., 2013)
Iriflophenone- 2-O-α-L-rhamnopyranoside (**148**)	*A. sinensis*	Leaves	(Yu et al., 2013)
Homomangiferin (**149**)	*A. sinensis*	Leaves	(Yu et al., 2013)
Iriflophenone (**150**)	*A. sinensis*	Leaves	(Yu et al., 2013)
Aquilarinensides A–E (**151-155**)	*A. sinensis*	Leaves	(Sun et al., 2014)

(continued)

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Table 2.8 *(continued)*

Compound	Species	Part	References
Aquilarin A (**156**)	*A. sinensis*	Stem	(Wang, Peng, Tan, & Dai, 2010)
Balanophonin (**157**)	*A. sinensis*	Stem	(Wang et al., 2010)
(+)- Lariciresinol (**158**)	*A. sinensis*	Stem	(Wang et al., 2010)
(+)-Aquilaroside A (**159**)	*A. sinensis*	Stem	(Wu et al., 2014)
Longifloroside A (**160**)	*A. sinensis*	Stem	(Wu et al., 2014)
(-)-Simulanol (**161**)	*A. sinensis*	Stem	(Wu et al., 2014)
(-)-Syringaresinol (**162**)	*A. sinensis*	Stem	(Wu et al., 2014)
Methylparaben (**163**)	*A. sinensis*	Leaves	(Kang et al., 2014)
Vanillic acid (**164**)	*A. sinensis*	Leaves	(Kang et al., 2014)
Isovanillic acid (**165**)	*A. sinensis*	Leaves	(Kang et al., 2014)
p-Hydroxybenzoic acid (**166**)	*A. sinensis*	Leaves	(Kang et al., 2014)
6-Hydroxy-2-(2-phenylethyl) chromone (**167**)	*A. microcarpa*	Stem bark	(Kristanti, Tanjung, Rahayu, & Herdiana, 2017)
7-Methoxy-2-[2-(4′-hydroxy-phenyl)ethyl]chromone (**168**)	*A. sinensis*	resinous wood	(Wang et al., 2018)
7-Hydroxy-2-[2-(4′-methoxyphenyl)ethyl]chromone (**169**)	*A. sinensis*	resinous wood	(Wang et al., 2018)
5,6-Dihydroxy- 2-[2-(3′-hydroxy-4′-methoxyphenyl)ethyl] chromone (**170**)	*A. sinensis*	resinous wood	(Wang et al., 2018)
6-Hydroxy-5-methoxy-2-(2-phenyl- ethyl) chromone (**171**)	*A. sinensis*	resinous wood	(Wang et al., 2018)
4′-Hydroxyacetanilide **(172)**	*A. malaccensis*	leaf	(Afiffudden, Alwi, & Hamid, 2015)

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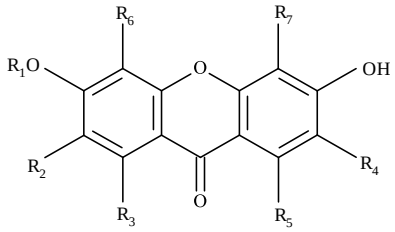
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Table 2.8 *(continued)*

Compound	Species	Part	References
Hexadecanoic acid **(173)**	*A. malaccensis*	leaf	(Adam et al., 2017)
Cucurbitacin **(174)**	*A. malaccensis* *A. crassna*	-	(Adam et al., 2017)
Friedelan-3-one **(175)**	*A. sinensis*	leaf	(Adam et al., 2017)
β-Caryophyllene **(176)**	*A. crassna*	Essential oil	(Dahham et al., 2015)
4′,7-Dimethyoxy-6-hydroxychromone **(177)**	*A. filaria*	Resinous wood	(Suzuki et al., 2017)
6-Hydroxy-2-(2-phenylethyl)chromone **(178)**	*A. filaria*	Resinous wood	(Suzuki et al., 2017)
(−)-Aquisinenone F **(179)**	*A. sinensis*	Resinous wood	(Huo et al., 2018)
(−)-Aquisinenone G **(180)**	*A. sinensis*	Resinous wood	(Huo et al., 2018)
(+)-4′-Methoxyaquisinenone G **(181)**	*A. sinensis*	Resinous wood	(Huo et al., 2018)
6,7-Dimethoxy-2-(2-phenylethyl) chromone **(182)**	*A. sinensis*	Resinous wood	(Wang et al., 2018)

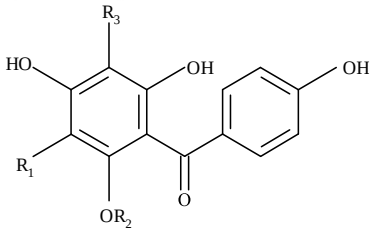
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Compound	R_1	R_2	R_3	R_4	R_5	R_6	R_7
141	H	OH	H	OH	OH	H	H
142	H	Glc	OH	OGlc	H	H	H
145	H	Xyl	OH	OH	H	OH	H
146	H	Glc	OH	OH	H	H	H
147	H	H	OH	OH	H	Glc	H
149	CH_3	Glc	OH	OH	H	H	H

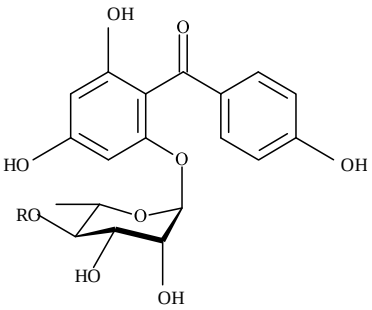
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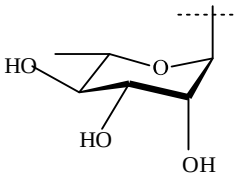


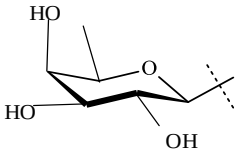
Compound	R_1	R_2	R_3
143	Glc	H	Glc
144	Glc	H	H
148	H	Rha	H
150	H	H	H

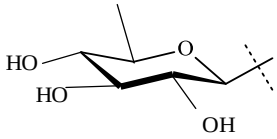
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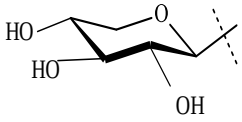
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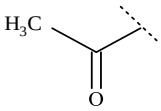


(151): R= 

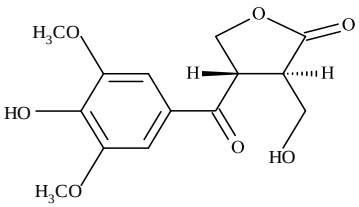
(152): R= 

(153): R= 

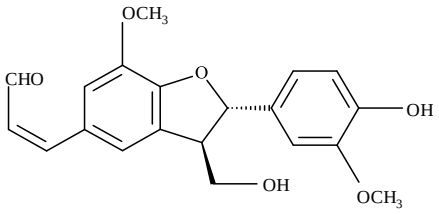
(154): R= 

(155): R= 

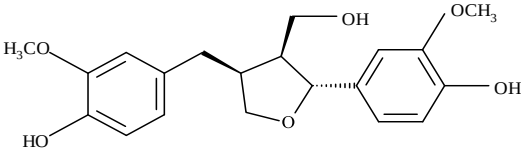
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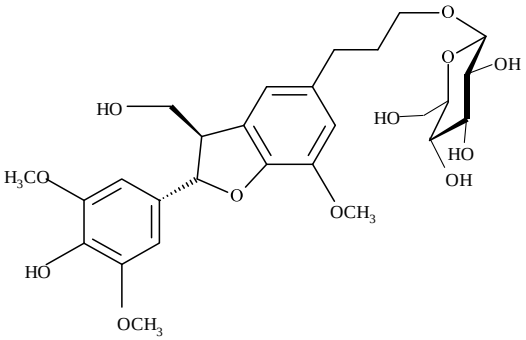
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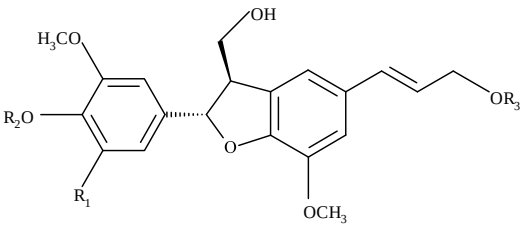
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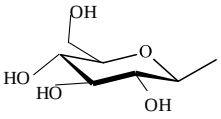


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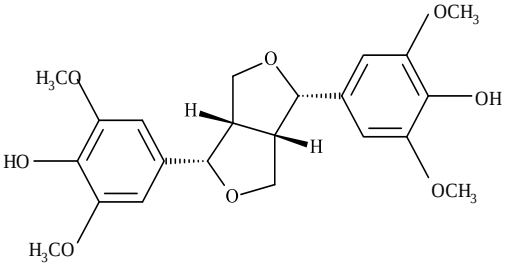
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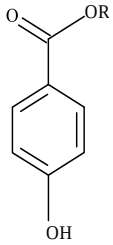


(160): R_1= H, R_2= CH_3, R_3= 

(161): R_1= OCH_3, R_2= H, R_3= H

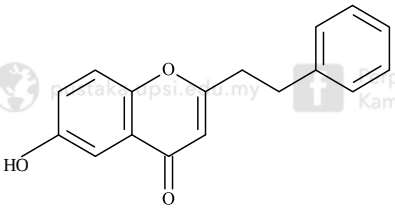


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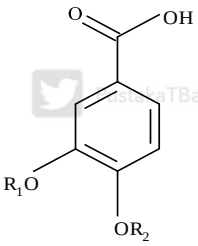


(163): R= CH_3

(166): R= H



(167)

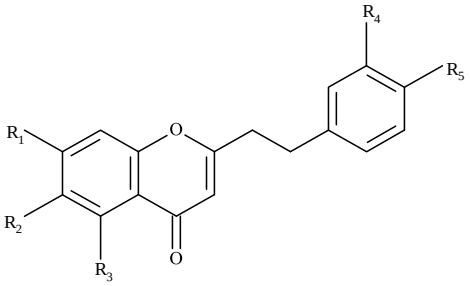


(164): R_1= CH_3, R_2= H

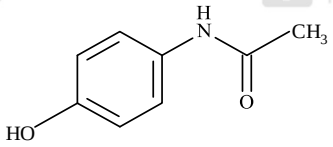
(165): R_1= H, R_2= CH_3

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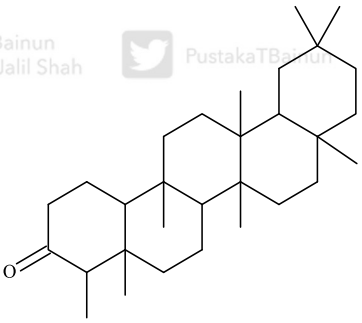
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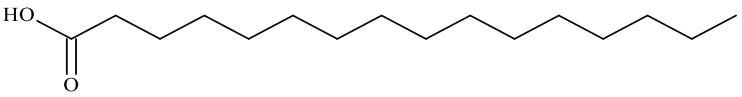
Compound	R_1	R_2	R_3	R_4	R_5
168	OCH_3	H	H	H	OH
169	OH	H	H	H	OCH_3
170	H	OH	OH	OH	OCH_3
171	H	OH	OCH_3	H	H



(172)



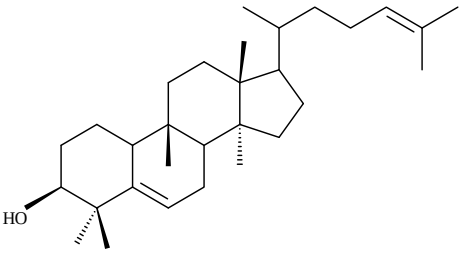
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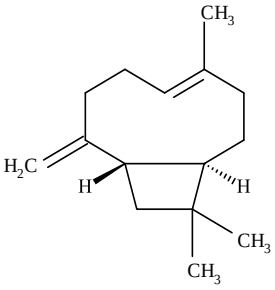
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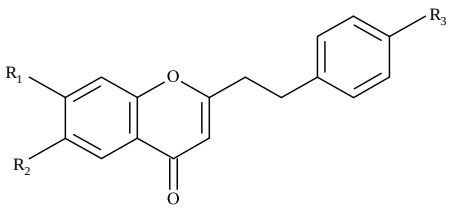
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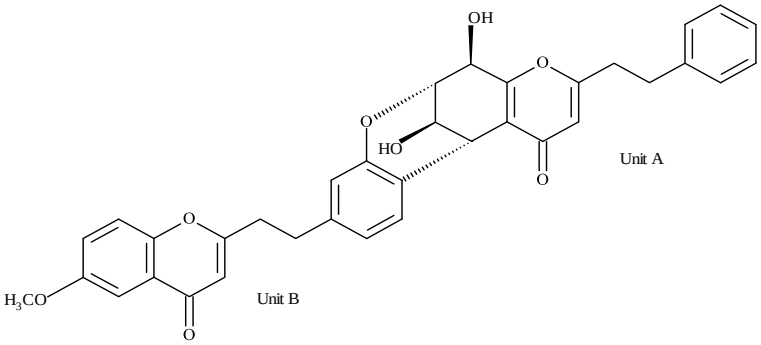


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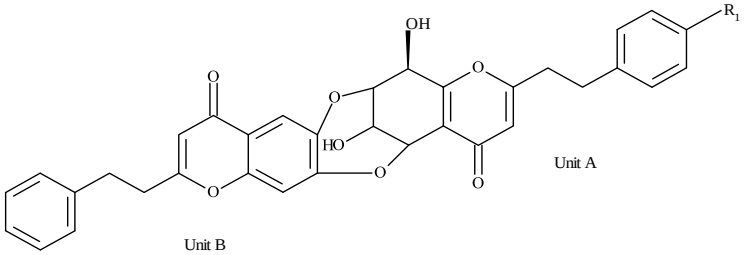


Compound	**R_1**	**R_2**	**R_3**
177	OCH_3	OH	OCH_3
178	H	OH	H
182	OCH_3	OCH_3	H

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(179)



(180): R_1= H,
(181): R_1= OCH_3

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2.4 Pharmacological Studies of Chronic Disease

On a global scale, few chronic diseases have gained attention to countries such as obesity, cancer, and associated with oxidative stress. One of the reasons makes this situation become triggered due to poor quality of lifestyles such as smoking, drinking alcohol, increase physical inactivity, and increasing intake of energy-dense food that rich fat, including processing food.

2.4.1 Obesity

Obesity is one of the significant contributors to chronic disease and associated with several pathological disorders such as diabetes, cardiovascular disease, cancer (Wu et al., 2014), hyperlipidemia, and hypertension (Kazmi et al., 2013). Obesity is occasioned from inequity energy intake and energy expenditure and characterized by enlarged fat mass and elevated lipid concentration in blood (Ado, Abas, Mohammed, & Ghazali, 2013; Chidrawar, Patel, Sheth, Shiromwar, & Trivedi, 2011; Rains, Agarwal, & Maki, 2011). World Health Organization (WHO) has reported the statistic of obesity population was about over 650 million adults, and 1.9 billion were overweight in the year 2016 (WHO, 2018).

Obesity treatment is available in a few ways, such as controlling food consumption, stimulation of energy outlay, and inhibition of lipase activity (Wu et al., 2014). Inhibition of absorption and digestion of dietary fat is one of the potential treatment of obesity (Yun, 2010). it is due to the dietary fat promotes well in storing

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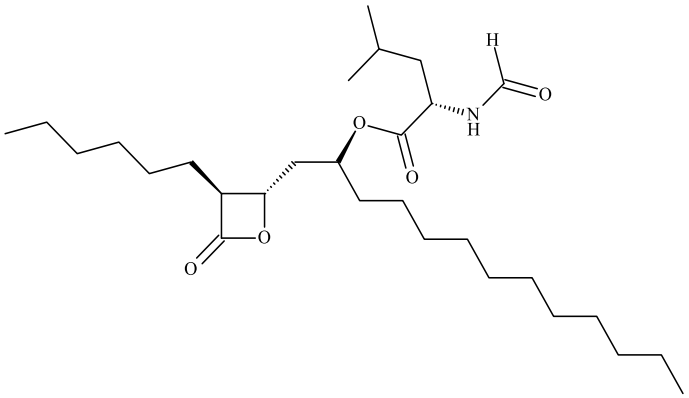
body fat compare to dietary carbohydrates; dietary fat could not be absorbed through the small intestine without subjected to the pancreatic lipase action (Kazmi et al., 2013; Yun, 2010). Pancreatic lipase (triacylglycerol hydrolase E.C. 3.1.1.3) is an essential enzyme in lipid absorption which hydrolysis about 70% of dietary fats and leads to obesity problems (Ahmed, Ashfaq, & Mirza, 2018; Ibrahim et al., 2018; Wu et al., 2014).

In the current market, there are two types of synthetic drugs, orlistat (**183**) and sibutramine (**184**) broadly consumed in obesity treatment, as a pancreatic lipase inhibitor and as an appetite suppressant, respectively however give a harmful side effect for a long term usage (Ibrahim et al., 2018; Lim, Goh, Kuan, & Loh, 2014; Yun, 2010). A previous study revealed that consumption of orlistat results in unpleasant gastrointestinal reactions such as oily stools, oily spotting, and flatulence (Buchholz & Melzig, 2015). Besides, sibutramine compromised adverse health effects like heart attack and stroke in high-risk cardiac patients and led to severe psychiatric disorders; therefore it was drawn from the recent market (Ado et al., 2013).

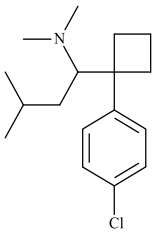
Therefore, extensive studies urge to discover a potential source from the natural product for the treatment of obesity. In earlier study had discovered the few groups of compounds such as flavonoids, sterols, tannins, and saponins which demonstrated promising effects to treat obesity problems in various mechanisms (Chidrawar et al., 2011).

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2.4.2 Oxidant or Oxidative Stress

Another current health issue is oxidative stress or oxidant, which generally signifies to a chemical reaction where the substance donates the proton or hydrogen to an oxidizing agent (Mahmod, Amella, Abdul Hamid, Mat Rashid, & Ali, 2017). Oxidant or free radical is formed from triplet oxygen, water, and unsaturated lipid molecules (Huda, Munira, Fitrya, & Salmah, 2009). Oxidative stress resulted from the inequity production of free radical and antioxidant defences (Liu et al., 2018).

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Increasing free radicals in the body might cause oxidative stress or damage in the cell by stealing electrons from cell molecules such as nucleic acids, proteins, and lipids, and its led to the development of degenerative diseases including cancer, diabetic, atherosclerosis and gastric ulcer (Ganesan et al., 2018; Huda et al., 2009; Mahmod et al., 2017).

Thus, a large and growing body of literature has investigated the natural antioxidant supplement to avoid conflict oxidative damage. Antioxidant was defined as a hydrogen or electron donor, or in the food industry, as a substance which able to prevent or delay oxidation of oxidizable biomolecules in low concentration (Morales & Paredes, 2014; Saad et al., 2015). The mechanisms involving in the antioxidant procedures are; direct hydrogen atom transfer process or an electron transfer process (Djeradi et al., 2014).

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$$FIO^{-}H + RO^{\cdot} \rightarrow F1^{-}O^{\cdot} + ROH$$

The rapid and stable procedure is essential to determine antioxidant activity due to the time-consuming aspects; therefore few methods have been developed to assay free radical scavenging activity from plant extracts (Cai, Luo, Sun, & Corke, 2004). Thus, the $ABTS^{\cdot+}$ and $DPPH^{\cdot}$ were the most commonly used in antioxidant activities due to their excellent reproducibility under certain conditions. The $ABTS^{\cdot+}$ method required enzyme or chemical reaction and may be dissolved in aqueous and organic media, while $DPPH^{\cdot}$ assay does not require any preparation, however, it only can be dissolved in organic media such as ethanol (Wojdyło et al., 2007).

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The synthetic antioxidant frequently used in human health. However, it promotes the adverse side effect; therefore, the researcher urges to find natural and safe sources of food antioxidant (Naidu, Ismail, Yeng, Sasidharan, & Kumar, 2012). Hence, there were several studies have been investigated alternative antioxidants from natural sources. Few studies indicated that secondary metabolites from medicinal plants possess antioxidant effect including phenolic group such as flavonoids, coumarins, anthocyanins, and phenolic acids (Li, Lee, Jang, Ma, & Kim, 2017; Morales & Paredes, 2014) as well as alkaloids, amines, betalains, vitamins and terpenoids (Cai et al., 2004).

2.4.3 Cancer

On the other hand, cancer usually associated with a few chronic diseases such as obesity, diabetes, hypertension, and also oxidative stress. Cancer is described by the repetitively multiplying cells with the failure to be controlled or stopped in the human body and thus forming tumours of malignant cells with the probable to be metastatic (Greenwell & Rahman, 2015). In other words, cancer results in an over-proliferation of irregular cells with a high capacity for imitation and invasion (Rai, Jogee, Agarkar, & Santos, 2016).

According to statistic data from WHO, 2018, cancer is the top two cause of death in the world and approximated 9.6 million death in the year 2018. The most common cancers reported in Malaysia and developed countries are including breast, ovarian and colorectal cancers and the five years' survival rate was 68% for cancer diagnosed in 2004 until 2010 (Nurhanan et al., 2017).

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Breast cancer is one of the common types of cancer, and the next leading cause of cancer deaths among women reported in United State (Graidist, Martla, & Sukpondma, 2015; Lu et al., 2015). Chemotherapy generally used to treat breast cancer, however, causes poor side effects and development of multidrug resistance; thus, the discovery of natural products may offer an alternative treatment of cancer (Graidist et al., 2015).

An earlier study has revealed that breast cancer stem cells (BCSCs) and their marker CD44+ and CD24- were identified as an attractive target for potentials breast cancer therapeutics due to they contained high tumorigenicity and multidrug resistance (Lu et al., 2015).

Natives from developing countries such as in Asia and Africa used to consume traditional medicines from medicinal plants for their health benefits and therapeutic purposes (Greenwell & Rahman, 2015). In addition, medicinal mushroom (Inonotus obliquus (Fr.) or chaga) has been increasingly used as traditional medicines by Estonian folks as anticancer therapy (Sak, Jürisoo, & Raal, 2014).

Furthermore, the previous report indicated that antibiotics, namely salinomycin (Sal), were efficient as anticancer agents inhibit the proliferation of numerous types of cancer cells, such as breast cancer, pancreatic cancer, ovarian, and prostate cancer (Lu et al., 2015). Also, this study showed that salinomycin 100 times more effectively than paclitaxel in the killing of breast cancer stem cells in mice.

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On the other hand, Farooqui et al., (2016) indicated that about 80% population of African and Asian including Malaysia were used Complementary and Alternative Medicine (CAM) as a principal source as an alternative to treat cancer. Besides that, Chinese Herbal Medicine (CHM) has attracted cancer patients worldwide, which showing beneficial chemo-preventives and antioxidant properties from CHM herbs (Mi, Hui, Zakaria, & Yusof, 2017). The main reason for them remains to dedicate these practices due to a lack of consciousness and a firm belief in its effectiveness (Mi et al., 2017; Othman & Farooqui, 2015). Another study discovered that ginseng, well-known Chinese herbs, showing enhance antitumor effect when combined with some anticancer drugs via in vitro and in vivo studies (Chen et al., 2014).

By engendering awareness of discovering natural product properties and benefits of natural herbs, healthcare professionals will act as a knowledge resource to the community (Prakash, Kumar, Kumar, & Ajeet, 2013). Yet, the scientist and natural product researchers are the urge to find alternative treatment and therapies, to replace current treatment such as chemotherapy, radiotherapy, and usage of synthetic drugs which led the cancer patients under strain and damage their health (Greenwell & Rahman, 2015).

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2.5 Pharmacological Properties of *Aquilaria* species

Aquilaria plants have been used for medicinal purposes for thousands of years and continue to be used in Ayurvedic, Tibetan, and traditional East Asian medicine. Extensive reports on the pharmacological properties of the *Aquilaria* plant showed that it has several biological properties.

2.5.1 Antioxidant Property

One of pharmacological study toward *Aquilaria* species is antioxidant properties. An earlier study showed that *Aquilaria* sp. (*A. malaccensis*) possesses significant antioxidant activities due to the presence of several groups of bioactive compounds (Wil et al., 2014). A few methods have been used by this study, including Total antioxidant activity (TAC), Cupric reducing antioxidant capacity (CUPRAC) and 2,2-diphenyl-1-picrylhydrazy (DPPH) radical scavenging assays to evaluate the antioxidant activity. This verdict also has proven by Huda et al. (2009), where *A. malaccensis* leaf showed positive results toward antioxidant activity. Liu et al., (2018) has revealed the flavonoid compound, 5-hydroxy-7, 3', 4'- trimethoxyflavone (**37**) was an effective DPPH scavenger. In addition, another study by Ray, Leelamanit, Sithisarn, and Jiratchariyakul (2014) indicated that polyphenolic compound mangiferin (**146**) from ethanolic extract of *A. crassna* leaf was potent as an antioxidant agent.

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2.5.2 Anti-Obesity Property

Another interesting pharmacological effect of *Aquilaria* species is in lowering bodyweight treatment. Previously, two polyphenolic compounds, mangiferin (**146**) and genkwanin 5-O-β-primeveroside (**43**) in ethanolic *A.sinensis* leaves have been tested towards mice (in vivo) and showed the positive result of laxative property, where the weight is significantly decreased (Ito et al., 2012). Another report supported this finding, Kakino et al., (2010) signified the ethanolic *A.sinensis* leaves have a laxative effect that increases contractions of the intestine to accelerate food in the intestinal zone without causing diarrhoea. Also, the laxative activity of the compound (**43**) in acetone extract had strongly shown in reducing of constipation problem without side effects such as diarrhoea (Hara et al., 2008).

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Besides that, another study showed the potential of crude extract from leaves and barks of *A. subintegra* and *A. malaccensis* to inhibit the activity of porcine pancreatic lipase from producing the fatty acid (Ibrahim et al., 2018). This finding also supported by another study where the phenolic content in leaves and hydrosol extract of *A. malaccensis* were expected to inhibit the activity of pancreatic lipase and the potential to be as antiobesity agent (Jamahseri et al., 2014). Another study revealed the presence of several compounds such as phenol, flavonoid, gallic acid, and quercetin (**12**) in *A. subintegra* leaves and possible to become as a pancreatic lipase inhibitor (Zainal, Musa, Idris, & Ku Hamid, 2018).

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2.5.3 Anticancer Property

A. subintegra species also have been tested its potential towards *in vitr*o anticancer ovarian, breast, and colorectal activities. The results indicated that the ethanolic unripe fruit extract of *A. subintegra* showed higher anticancer activity compared to methanolic and *n*-hexane fruit extract (Nurhanan et al., 2017). Moreover, further investigation on the ethanolic extract of *A. subintegra* branch against MCF-7 breast cancer was done. It showed its ability to reduce the cell growth rate from 0.0421 h^{-1} to 0.0158 h^{-1} and the potential to control cytostatic and cytotoxic effect (Abbas, Hashim, & Amid, 2017).

2.5.4 Other Biological Properties

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A potential α-glucosidase inhibitor for diabetes treatment has been studied, where eight compounds from *A.sinensis* leaves including four new compounds were identified; the primary compound, mangiferin (**146**) was shown as a first inhibitor (Feng, Yang, & Wang, 2011). Also, researchers from Thailand were believed that leaves of *A.crassna* have a few possibilities of bioactive compounds. Sattayasai et al., (2012) indicated that the leaves of *A. crassna* were played as antipyretic, analgesic, antioxidant agents and recommended as a food supplement and metabolic disorder therapy such as a diabetes treatment.

Kamonwannasit et al., (2013) found that nonexistence of acute oral toxicity in mice and proposed *A. crassna* leaves extract as an antibacterial against *Staphylococcus epidermidis*. *A.malaccensis* and *A.subintegra* were shown to have antidiabetic

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properties to inhibit the activity of α-amylase (Yunus, Zaki, & Ku Hamid, 2015). Numerous studies have attempted to explain the promising Alzheimer therapy demonstrated from stem and leaf of *A. subintegra* as well as exhibited their potential as acetylcholinesterase (AChE) inhibitor (Adam et al., 2017; Bahrani, Mohamad, Paydar, & Rothan, 2014; Jok, Che Radzi, & Ku Hamid, 2015).

Another interesting active compound is 4′-hydroxyacetanilide (**172**) or known as acetaminophen, that found in *A. malaccensis* leaf extract (Afiffudden et al., 2015). This compound had been used in the synthesis of non-steroid anti-inflammatory drugs (NSAIDs). In addition, a known flavonoid, pilloin (**41**) is believed to have an anti-inflammatory property. This study investigated its anti-inflammatory effect in bacterial lipopolysaccharide-induced RAW 264.7 macrophages and septic mice. This compound was successfully inhibited NF-κB activation and reduced the phosphorylation of IκB in LPS-stimulated macrophages (Tsai et al., 2018).

On the other hands, Michal Korinek et al., (2016) had revealed antiallergic effect from *A. malaccensis* seed ethanolic extract and an active phorbol ester, namely aquimavitalin which showed potent inhibition against A23187- and antigen-induced degranulation assay with IC_{50} values of 1.7 nM and 11 nM, respectively. Another study was showed the hepatoprotective potential of ethanolic extract from *A. agallocha* leaves towards paracetamol (PCM) induced hepatotoxicity in Sprague-Dawley (SD) rats validated by a significant decrease in the assessment of liver function test including Serum alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), bilirubin and cholesterol (Alam et al., 2017). Some previously

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reported work on isolated from *Aquilaria* species showed their pharmacological properties. These reports are tabulated in Table 2.9.

Table 2.9

Pharmacological activities of several phytochemicals from Aquilaria species

Plant Species	**Compounds**	**Description**
A. sinensis	Aquisiflavoside **(42)**	Nitric oxide production inhibitor, (Yang, Feng, Yang, Zhao, & Liu, 2012).
A. malaccensis	Hexadecanoic acid **(173)**	Showed antibacterial affect (Adam et al., 2017; Khalil et al., 2013).
A. malaccensis *A. crassna*	Cucurbitacin **(174)**	Showed antibacterial, antifungal, anticancer and anti tumor properties (Adam et al., 2017).
A. sinensis	Friedelan-3-one **(175)**	Possess anti-inflammatory effect (Adam et al., 2017).
A. crassna	β-Caryophyllene **(176)**	Exhibited anticancer, antioxidant, antimicrobial, anticlonogenic activites and as a promising chemotherapeutic agent against colorectal malignancies (Dahham et al., 2015).
A. sinensis	Iriflophenone 3-*C*-*β*-glucoside (IPG) **(144)**	Active in anti-hyperglycemic activity, lowering fasting blood glucose compared to insulin as a positive control (Pranakhon, Aromdee, & Pannangpetch, 2015).

(continued)

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Table 2.9 *(continued)*

Plant Species	Compounds	Description
A. filaria	4′,7-dimethyoxy-6-hydroxychromone **(177)**	Exhibited wide range antiproliferative activity against all tumor cell lines tested with IC_{50} values of 25– 38 µM (Suzuki et al., 2017).
A. filaria	6-hydroxy-2-(2-phenylethyl)chromone **(178)**	revealed the persuasive effect with an IC_{50} value of 319 mol ratio relative to 12-*O*- tetradecanoyl phorbol-13-acetate (Suzuki et al., 2017).
A. sinensis	(−)-Aquisinenone F **(179)** (−)-Aquisinenone G **(180)** (+)-4′-Methoxyaquisinenone G **(181)**	Possess noteworthy inhibition of nitric oxide production in lipopolysaccharide-stimulated RAW264.7 cells with IC_{50} values in the range 7.0−12.0 µM (Huo et al., 2018)
A. sinensis	6,7-dimethoxy-2-(2-phenylethyl) chromone **(182)**	Showed as a potential bioactive compounds toward inflammatory treatment or prevention (Wang et al., 2018).

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CHAPTER 3

METHODOLOGY

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3.1 General Procedures

Cold extraction technique was applied to extract the phytochemicals from the dried bark and leaf of *A. subintegra*. Several organic solvents, *n*-Hexane, dichloromethane (CH_2Cl_2), and methanol (MeOH), were used in this procedure.

Gravity column chromatography was applied for fractionation and isolation. Column chromatography was equipped with silica gel 60F 230-400 mesh ASTM (Merck 1.09385.1000) as a stationary phase. The normal silica gel was made into a slurry with hexane before packed into the column and equilibrates for few hours before use. Thin layer chromatography (TLC) was used to monitor various chemical entities present in the crude extracts, fractions that obtained from column and microcolumn chromatography, and also isolated pure compounds. TLC sheets commercially pre-

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coated with aluminium plates, silica gel $60F_{254}$ (1.05554.0001) from Merck with 0.25 mm thickness. The spots on the TLC plate were detected by ultraviolet (UV) illumination at 254 nm and 365 nm, before being sprayed with a *p*-anisaldehyde reagent.

Analysis of purified compounds was demonstrated by using Nuclear Magnetic Resonance spectra (NMR), 1D (^{1}H, ^{13}C, DEPT) and 2D (^{1}H-^{1}H COSY, HMQC, HMBC, and TOCSY) analysis from JEOL ECX (500 MHz). The NMR solvent used was deuterated chloroform ($CDCl_3$). Chemical shift (δ) was reported in ppm and coupling constants (*J*) in Hz and internally referred to the solvent signal in $CDCl_3$ [^{1}H: δ 7.26 (s); ^{13}C: δ 77.1].

The UV spectra were demonstrated by using a Perkin Elmer UV-visible spectrophotometer and obtained in CH_3OH, and the wavelength of the spectrum was determined in the range of 200 to 400 nm. The infrared spectra (IR) were determined using Nicolet 6700 FTIR spectrophotometer at FRIM, Selangor. Mass of compounds was determined using Gas Chromatography/Mass Spectrometry Agilent Technologies GCMS-5975C VL MSD spectrometer (Santa Clara, California, USA) at UPSI Tanjung Malim and liquid chromatography LTQ Orbitrap mass spectrometer (Thermo Scientific, San Jose, USA) at FRIM, Selangor.

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3.1.1 Chemical Reagents

For the extraction methods, the industrial-grade of solvents has been used, including *n*-hexane (*n*-Hex), dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and methanol (MeOH). These solvents were distilled before used in the chromatographic separation of compounds.

Other chemicals also used were sulphuric acid (H_2SO_4), hydrochloric acid (HCl), glacial acetic acid ($C_2H_4O_2$), and *p*-anisaldehyde, in analytical grade purchased from Merck (Darmstadt, Germany).

The spray reagent used for the detection and visualization of the separated constituents on the thin layer chromatography (TLC) as below:

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***p*-Anisaldehyde/sulphuric acid reagent**

Preparation of *p*-anisaldehyde/sulphuric acid:
85 mL of methanol was added with 10 mL of glacial acetic acid, followed by 0.5 mL *p*-anisaldehyde solution and concentrated sulphuric acid (5 mL).

p-Anisaldehyde/sulphuric acid test:
The *p*-anisaldehyde stain was used to detect several groups of compound since it is sensitive to most functional group, such as terpene, steroid, and flavonoid group. It tends to stain the TLC plate itself, upon mild heating (100-105ºC); the colour will

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change into light pink, purple, yellow fluorescence, coloration respect to functional group.

3.2 Plant Materials

Two fresh parts of the plant (bark and leaf) of *A. subintegra* were collected from Kajang, Selangor, Malaysia in July 2014. The species was identified by the Malaysian Timber Industry Board (MTIB). The voucher specimen was deposited at the herbarium of Faculty of Science and Mathematics, Universiti Pendidikan Sultan Idris (Voucher No: AMWNI-M6002/1).

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3.3 Extraction of *Aquilaria subintegra*

The fresh samples of the plant were dried at room temperature. They were then cut into small slices and powdered using a dry grinder (Kuao Fung, Taiwan). The dried powdered bark and leaf of the plant were extracted via maceration technique, with *n*-hexane, CH_2Cl_2, and MeOH subsequentially to obtain its phytochemicals. The extraction was conducted for 72 hours and repeated for three times. The crude extracts were then concentrated under reduced pressure using a rotary evaporator. The details extraction procedures were displayed in Scheme 3.1 and Scheme 3.2. The percentage yield and physical property of crude extracts were presented in Table 3.1.

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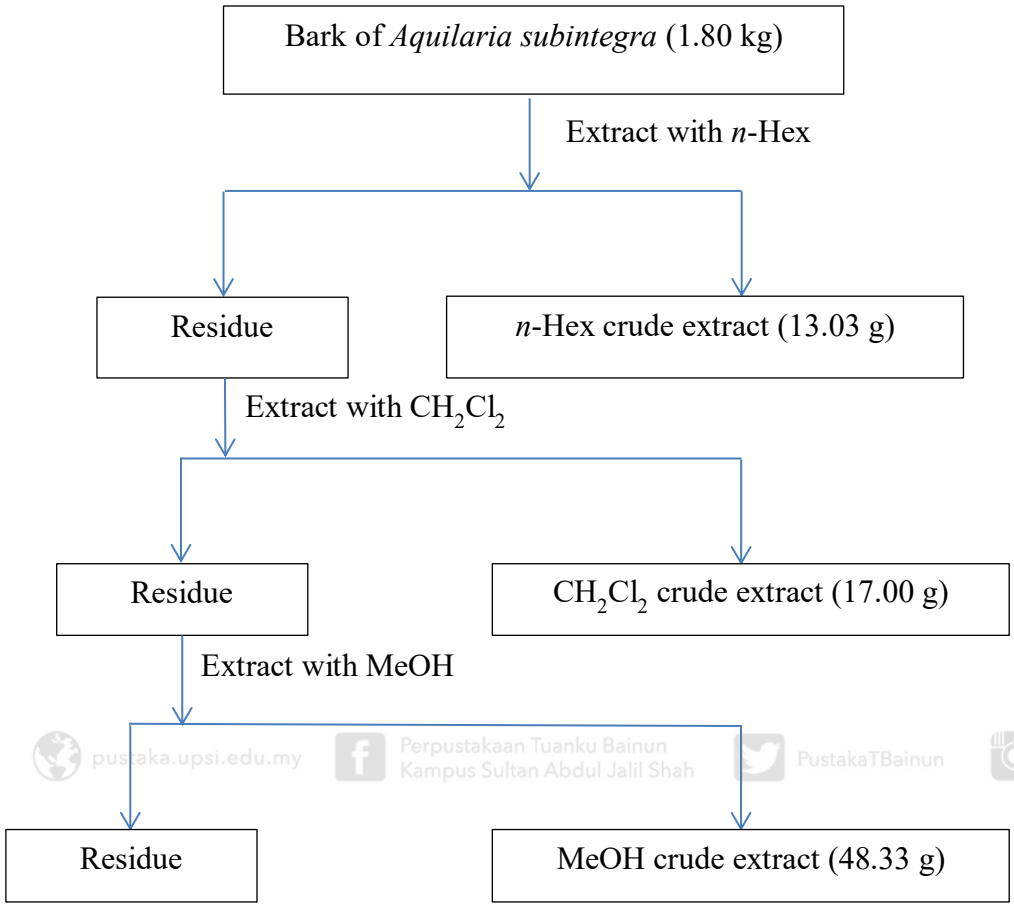
Table 3.1

Percentage yield and physical property of the crude extracts of A. subintegra

Parts	**Weight**	**Solvent**	**Yield**	**Property**
Bark	1.80 kg	*n*-Hexane (ASBH)	13.03 g (0.72%)	Green
		CH_2Cl_2 (ASBD)	17.00 g (0.94%)	Dark green
		MeOH (ASBM)	48.33 g (2.69%)	Brown gum
Leaf	3.30 kg	*n*-Hexane (ASLH)	38.06 g (1.15%)	Dark green
		CH_2Cl_2 (ASLD)	43.34 g (1.32%)	Dark green
		MeOH (ASLM)	78.29 g (2.37%)	Brown gum

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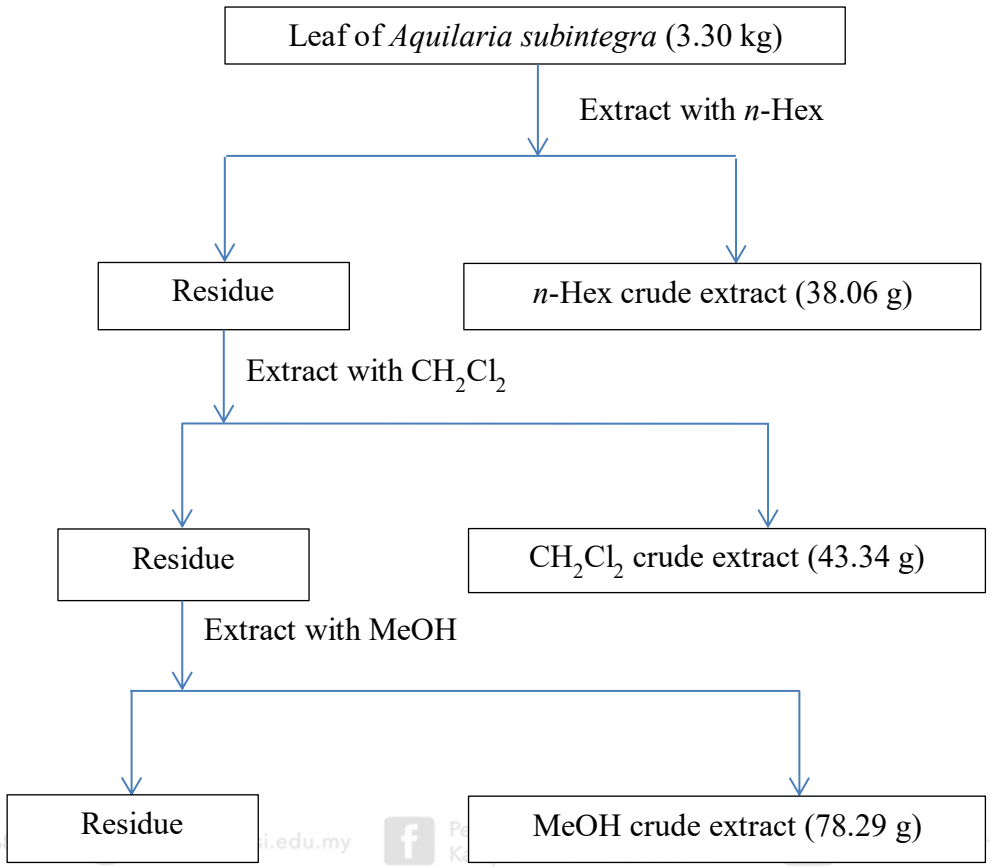
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Scheme 3.1. Extraction procedure from the bark of *Aquilaria subintega*

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Scheme 3.2. Extraction procedure from the leaf of *Aquilaria subintegra*

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3.4 Isolation and Purification

At the beginning of this stage, *A. subintegra* crude extracts (bark and leaf) were loaded into column chromatography (CC) and monitored by thin layer chromatography (TLC). Column chromatography was carried out using silica gel 60, 230-400 mesh ASTM (Merck 1.09385.1000), and TLC was executed on Kieselgel 60 F_{254} 20 X 20 cm (Merck 1.05554.0001) aluminium TLC sheets.

The crude extract of CH_2Cl_2 was subjected to isolation and purification. The crude extract was separated by using column chromatography over silica gel-based on the gradient elution method. Further purification was continued using exhaustive CC and TLC to obtain pure compounds. It is necessary for eluted fractions analysed using TLC for their chemical composition during the purification process.

The UV light model UVGL-58 was applied to observe spots on the TLC plates, visualized under ultraviolet light of 254 and 365 nm. Each of the developed TLC plates was then introduced under ultraviolet light and sprayed with the *p*-anisaldehyde reagent. The fractions, which showed the same retention factor (R_f) value on the TLC plate, were combined. The potential fractions were treated by extensive column chromatography by using normal silica or using other techniques, such as washing and recrystallization for further purification. The purified compounds were determined by a single spot on the TLC plate, visualized under UV light, and sprayed with *p*-anisaldehyde reagent subsequently.

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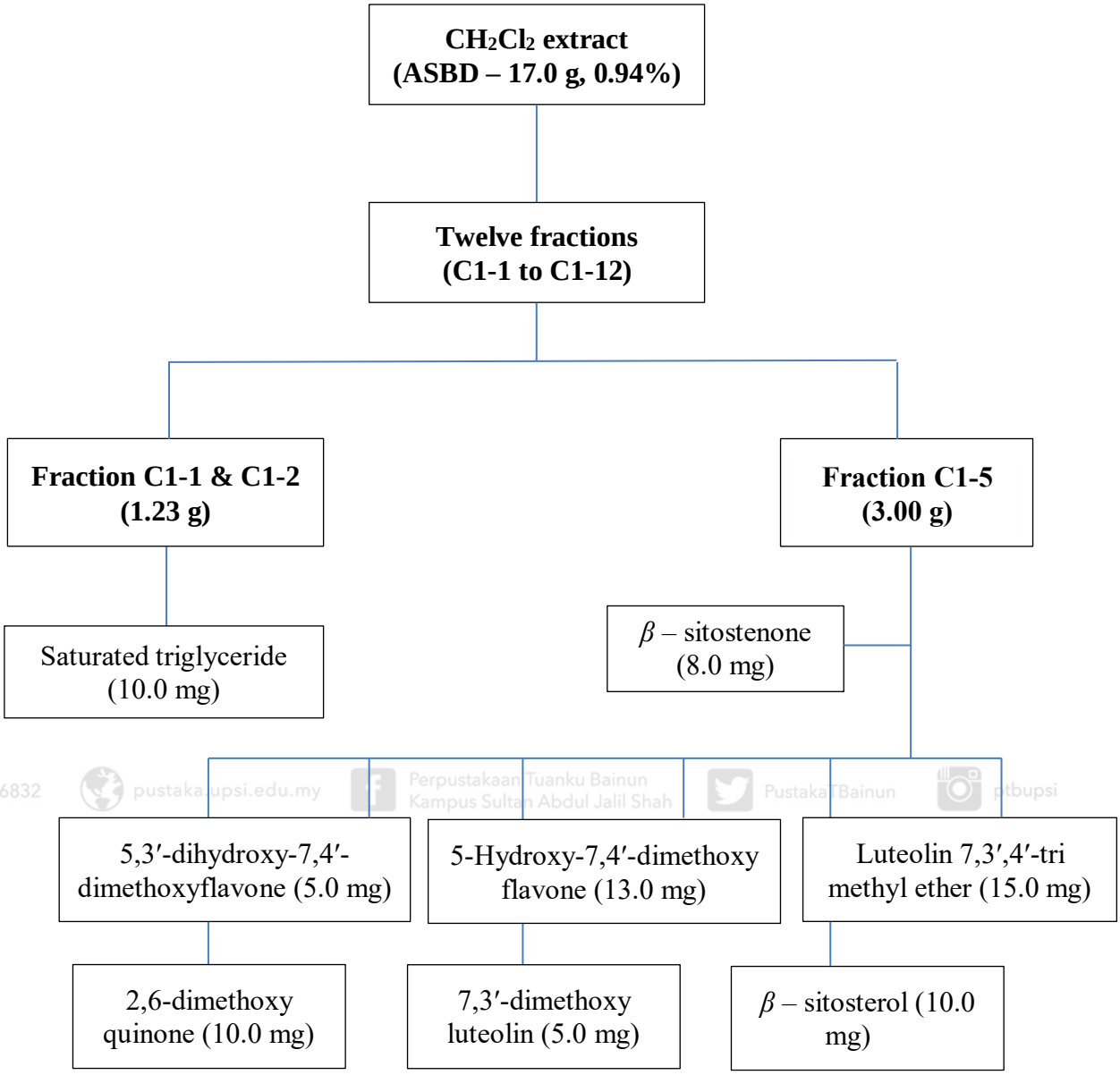
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3.5 Chemical Constituents from Bark of *A. subintegra*

Purification of CH_2Cl_2 bark extract (ASBD, 17.0 g) by gravity column chromatography eluted with gradient solvent, CH_2Cl_2 (100%), mixture of CH_2Cl_2/MeOH (99:1, 98:2, 96:4, 94:6, 92:8, 90:10, 70:30, 50:50) and MeOH (100%). From the first CC, twelve fractions were yielded, labelled as C1-1 to C1-12. Fraction C1-5 (3.00 g) was subjected to multiple CC for purification and afforded 5-hydroxy-7,4′-dimethoxyflavone (**36**), luteolin-7,3′,4′-trimethyl ether (**37**), 5,3′-dihydroxy-7,4′-dimethoxyflavone (**41**), 7,3′-dimethoxy luteolin (**185**), *β*-sitosterol (**101**), *β*-sitostenone (**139**) and 2,6-dimethoxyquinone (**187**). Meanwhile, combination fractions C1-1 and C1-2 (1.23 g) gave one compound, saturated triglyceride fatty acid (**186**) after purified by multiple CC. The purification from the bark of *A. subintegra* was summarized in Scheme 3.3.

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Scheme 3.3. Flow chart for the purification process of *A. subintegra* bark extracts.

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3.5.1 Physical and Spectral Data of Isolated Chemical Constituents from Bark

3.5.1.1 ASBD1: 5-Hydroxy-7,4′-dimethoxyflavone (36)

Sub-fraction C2-6 (90 mg) from fraction C1-5 was purified by CC on silica gel and eluted with CH_2Cl_2: EtOAc (90:10 v/v) as well as washing technique to yield 5-hydroxy-7,4′-dimethoxyflavone (**36**) (13.0 mg, 0.43%) as a yellow needles; UV (MeOH) λ_{max} nm: 270 and 321; FTIR (KBr) υ_{max} cm^{-1}: 3688, 3086, 2915, 1737, 1606; ^{1}H-NMR (500 MHz, $CDCl_3$), δ ppm: 3.88 (3H, *s*, OCH_3-7), 3.89 (3H, *s*, OCH_3-4′), 6.37 (1H, *d*, *J* = 2.3 Hz, H-6), 6.48 (1H, *d*, *J* = 2.3 Hz, H-8), 6.58 (1H, *s*, H-3), 7.02 (2H, *d*, *J* = 9.2 Hz, H-3′, 5′), 7.85 (2H, *d*, *J* = 9.2 Hz, H-2′, 6′), 12.82 (1H, br.*s*, OH-5); ^{13}C-NMR (125 MHz, $CDCl_3$), δ ppm: 164.1 (C-2), 104.4 (C-3), 182.5 (C-4), 162.2 (C-5), 98.1 (C-6), 165.5 (C-7), 92.7 (C-8), 157.8 (C-9), 105.6 (C-10), 123.6 (C-1′), 128.1 (C-2′), 114.6 (C-3′), 162.6 (C-4′), 114.6 (C-5′), 128.1 (C-6′), 55.9 (OCH_3-7), 55.6 (OCH_3-4′); ESI-MS: *m/z* 299.08871 $[M+H]^+$, $C_{17}H_{14}O$

3.5.1.2 ASBD2: Luteolin 7,3′,4′-trimethyl ether (37)

Sub-fraction C4-12 (39.6 mg) from C1-5 was subjected to CC on SiO_2 and eluted with CH_2Cl_2: EtOAc (90:10 v/v) and followed with recrystallization technique, gave a yellow needle flavonoid, known as luteolin 7,3′,4′-trimethyl ether (**37**) (15.0 mg, 0.5%); UV (MeOH) λ_{max} nm: 270 and 340; FTIR (KBr) υ_{max} cm^{-1}: 3690, 3092, 2926, 1737, 1617; ^{1}H NMR (500 MHz, $CDCl_3$), δ ppm: 3.89 (3H, *s*, OCH_3-7), 3.97 (3H, *s*, OCH_3-3′), 3.99 (3H, *s*, OCH_3-4′), 6.59 (1H, *s*, H-3), 6.38 (1H, *d*, *J* = 2.3 Hz, H-6), 6.50 (1H,

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d, *J* = 2.3 Hz, H-8), δ 6.99 (1H, *d*, *J* = 8.6 Hz, H-5′), 7.34 (1H, *d*, *J* = 1.7 Hz, H-2′), 7.53 (1H, *dd*, *J* = 8.6, 2.3 Hz, H-6′) and 12.80 (1H, br.*s*, OH-5); ^{13}C-NMR (125 MHz, $CDCl_3$), δ ppm: 165.5 (C-2), 104.7 (C-3), 182.5 (C-4), 162.2 (C-5), 98.1 (C-6), 165.5 (C-7), 92.6 (C-8), 157.8 (C-9), 105.6 (C-10), 123.8 (C-1′), 108.8 (C-2′), 149.3 (C-3′), 152.3 (C-4′), 111.2 (C-5′), 120.2 (C-6′), 56.1 (OCH_3-7), 56.1 (OCH_3-3′), 55.6 (OCH_3-4′); ESI-MS: *m/z* 329.09903 $[M+H]^+$, $C_{18}H_{16}O_6$.

3.5.1.3 ASBD4: 5,3′-Dihydroxy-7,4′-dimethoxyflavone (41)

Fraction C1-5 (3.0 g) was fractionated using CC and eluted with CH_2Cl_2: MeOH (96:4 v/v) and produced 113 fractions (C2-1 to C2-113). Fractions C2-11 and C2-12 (230 mg) were combined and subjected to several CC to yield 5,3′-dihydroxy-7,4′-dimethoxyflavone (**41**) (5.0 mg, 0.17%) as a yellow amorphous; UV (MeOH) λ_{max} nm: 270 and 330; FTIR (KBr) υ_{max} cm^{-1}: 3700, 1594; ^{1}H-NMR (500 MHz, $CDCl_3$), δ ppm: 3.89 (3H, *s*, OCH_3-7), 4.01 (3H, *s*, OCH_3-4′), 6.38 (1H, *d*, *J* =2.3 Hz, H-6), 6.49 (1H, *d*, *J* = 2.3Hz, H-8), 6.57 (1H, *s*, H-3), 7.03 (1H, *d*, *J* = 8.5 Hz, H-5′), 7.33 (1H, *d*, *J* = 2.3 Hz, H-2′), 7.48 (1H, *dd*, *J* = 8.5 Hz, 1.8 Hz, H-6′), 12.80 (1H, *s*, OH-5) and 6.06 (1H, *s*, OH-3′); ^{13}C-NMR (125 MHz, $CDCl_3$), δ ppm: 164.1 (C-2), 104.5 (C-3), 182.5 (C-4), 162.5 (C-5), 98.1 (C-6), 165.5 (C-7), 92.7 (C-8), 157.7 (C-9), 108.3 (C-10), 123.4 (C-1′), 115.0 (C-2′), 146.9 (C-3′), 149.2 (C-4′), 115.0 (C-5′), 120.8 (C-6′), 55.9 (OCH_3-7), 56.2 (OCH_3-4′); ESI-MS: *m/z* 313.07132 $[M-H]^-$, $C_{18}H_{16}O_6$.

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3.5.1.4 ASBD4: 7,3′-Dimethoxy luteolin (185)

Fraction C1-5 (3.0 g) was rechromatographic using multiple CC and eluted with *n*-hex (100%), *n*-hex/ CH_2Cl_2 (50:50, 20:80), CH_2Cl_2 (100%) solvent system and afforded pale yellow amorphous, 7,3′-dimethoxy luteolin (**185**) (5.0 mg, 0.17%); UV (MeOH) λ_{max} nm:270 and 334; FTIR (KBr) υ_{max} cm^{-1}: 3748, 2917, 1739, 1608; ^{1}H-NMR (500 MHz, $CDCl_3$), δ ppm: 3.88 (3H, *s*, OCH_3 -7), 3.99 (3H, *s*, OCH_3-3′), 6.37 (1H, *d*, *J* =2.3 Hz, H-6), 6.49 (1H, *d*, *J* = 2.3 Hz, H-8), 6.58 (1H, *s*, H-3), 6.95 (1H, *d*, *J* = 8.6 Hz, H-5′), 7.43 (1H, *dd*, *J* = 2.3 Hz, 8.0 Hz, H-6′), 7.47 (1H, *d*, *J* = 2.3 Hz, H-2′) and 12.79 (1H, *s*, OH-5, 4′); ^{13}C-NMR (125 MHz, $CDCl_3$), δ ppm: 162.2 (C-2), 104.8 (C-3), 182.6 (C-4), 161.9 (C-5), 98.2 (C-6), 163.9 (C-7), 92.7 (C-8), 157.8 (C-9), 110.4 (C-10), 122.2 (C-1′), 112.4 (C-2′), 149.6 (C-3′), 146.0 (C-4′), 119.2 (C-5′), 122.2 (C-6′), 55.9 (OCH_3-7), 56.2 (OCH_3-3′); ESI-MS: *m/z* 315.0866 $[M+H]^+$, $C_{18}H_{16}O_6$.

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3.5.1.5 ASBD5: *β* – sitosterol (101)

Purification of fraction C1-5 (3.0 g) by multiple CC with solvent system *n*-hex: CH_2Cl_2, (50:50 v/v), followed with washing and recrystallization techniques afforded β – sitosterol (**101**) (10.0 mg, 0.33%) as a white amorphous; FTIR (KBr) υ_{max} cm^{-1}: 2931, 2861, 1667, 1465, 1050; ^{1}H-NMR (500 MHz, $CDCl_3$), δ ppm: 5.34 (1H, *t*, *J* =5.2 Hz, H-6), 3.51 (1H, *tdd*, *J* = 4.6, 5.2, 6.3 Hz, H-3), 1.0 (3H, *s*, H-29), 0.92 (3H, *d*, *J* =6.5 Hz, H-19), 0.84 (3H, *t*, *J* = 7.5 Hz, H-24), 0.82 (3H, *d*, *J* = 4.6 Hz, H-26), 0.80 (3H, *d*, *J* = 6.3 Hz, H-27), 0.67 (3H, *s*, H-28); ^{13}C NMR (125 MHz, $CDCl_3$), δ ppm: 37.3 (C-1), 31.7 (C-2), 72.1 (C-3), 42.4 (C-4), 140.8 (C-5), 121.8 (C-6), 31.9 (C-7, C-8), 50.2

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(C-9), 36.6 (C-10), 21.2 (C-11), 39.8 (C-12), 42.3 (C-13), 56.8 (C-14), 26.1 (C-15, C-21), 28.3 (C-16), 56.1 (C-17), 36.2 (C-18), 19.1 (C-19), 34.0 (C-20), 45.9 (C-22), 23.1 (C-23), 12.1 (C-24), 29.2 (C-25), 19.9 (C-26), 19.5 (C-27), 18.9 (C-28), 11.9 (C-29); ESI-MS: *m/z* 415.30276 $[M+H]^+$, $C_{29}H_{50}O$.

3.5.1.6 ASBD6: *β* – sitostenone (139)

Isolation and purification of fraction C1-5 (3.0 g) using several times of CC and eluted with 100% *n*-hex, *n*-hex/ EtOAc (70:30, 50:50, 30:70) and EtOAc (100%) solvent system, gradiently, afforded a white amorphous, β – sitostenone (**139**) (8.0 mg, 0.27%). FTIR (KBr) υ_{max} cm^{-1}: 2955, 1679, 1465; ^{1}H-NMR (500 MHz, $CDCl_3$), δ ppm: 5.74 (1H, *s*, H-4), 1.18 (3H, *s*, H-19),), 0.91 (3H, *d*, *J* = 6.9 Hz, H-21), 0.82 (3H, *d*, *J* = 4.6 Hz, H-26), 0.80 (3H, *d*, *J* = 8.0 Hz, H-27), 0.83 (3H, *t*, *J* = 7.5 Hz, H-29), 0.70 (3H, *s*, H-18); ^{13}C-NMR (125 MHz, $CDCl_3$), δ ppm: 36.2 (C-1), 34.2 (C-2), 199.9 (C-3), 123.8 (C-4), 171.9 (C-5), 33.9 (C-6), 33.0 (C-7), 35.7 (C-8), 53.9 (C-9), 39.7 (C-10), 21.2 (C-11), 38.7 (C-12), 42.5 (C-13), 55.9 (C-14), 24.3 (C-15), 28.3 (C-16), 56.0 (C-17), 12.2 (C-18), 18.8 (C-19), 35.8 (C-20), 19.1(C-21), 35.8 (C-22), 26.1 (C-23), 45.9 (C-24), 29.7 (C-25), 21.1 (C-26), 19.9 (C-27), 23.1 (C-28), 11.4 (C-29); ESI-MS: *m/z* 413.26709 $[M+H]^+$, $C_{29}H_{50}O$.

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3.5.1.7 ASBD7: Saturated Triglyceride Fatty Acid (186)

Combination fraction C1-1 and C1-2 (1.23 g) was isolated and purified using numerous of CC and eluted with 100% *n*-hex, *n*-hex/ EtOAc (70:30, 50:50, 30:70) and EtOAc (100%) solvent system, gradiently, afforded a white amorphous, saturated triglyceride fatty acid (**186**) (10.0 mg, 0.81%). FTIR (KBr) υ_{max} cm^{-1}: 2916, 1737, 1422, 1179; ^{1}H-NMR (500 MHz, $CDCl_3$), δ ppm: 5.26 (1H, *m*, H-1a′), 4.27 (2H, *dd*, *J* = 4.1, 4.0 Hz, H-1a), 4.14 (2H, *dd*, *J* = 5.8, 5.7 Hz, H-1a″), 2.31 (6H, *m*, H-2, 2′, 2″), 1.59 (6H, *m*, H-3, 3′, 3″), 1.27 (60H, br. *s*, H-4, 4′, 4″ to H-13, 13′,13″), 0.87 (9H, *t*, H-14, 14′, 14′); ^{13}C-NMR (125 MHz, $CDCl_3$), δ ppm: 173.4 (C-1, 1″), 173.0 (C-1′), 34.1 (C-2, 2′, 2″), 24.9 (C-3, 3′, 3″), 29.2 – 29.8 (C-4, 4′, 4″ to C-11, 11′, 11″), 32.0 (C-12, 12′, 12″), 22.8 (C-13, 13′, 13″), 62.2 (C-1a, 1a′), 14.2 (C-14, 14′, 14″); ESI-MS: *m/z* 723.46283 $[M+H]^+$, $C_{45}H_{86}O_6$.

3.5.1.8 ASBD8: 2,6-Dimethoxyquinone (187)

Fraction C1-5 (3.0 g) was purified by multiple CC on silica gel and eluted with CH_2Cl_2: EtOAc (85:15 v/v) to afforded 2,6-dimethoxyquinone (**187**) (10.0 mg, 0.33%) as a orange-yellow needles; UV (MeOH) λ_{max} nm: 285; FTIR (KBr) υ_{max} cm^{-1}: 1695, 1592, 1447; ^{1}H-NMR (500 MHz, $CDCl_3$), δ ppm: 3.82 (6H, *s*, OCH_3-2, OCH_3-6), 5.86 (2H, *s*, H-3, H-5); ^{13}C-NMR (125 MHz, $CDCl_3$), δ ppm: 176.8 (C-1), 157.4 (C-2, C-6), 107.5 (C-3, C-5), 186.9 (C-4), 56.6 (OCH_3- 2, 6); ESI-MS: *m/z* 169.04961 $[M+H]^+$, $C_8H_8O_6$.

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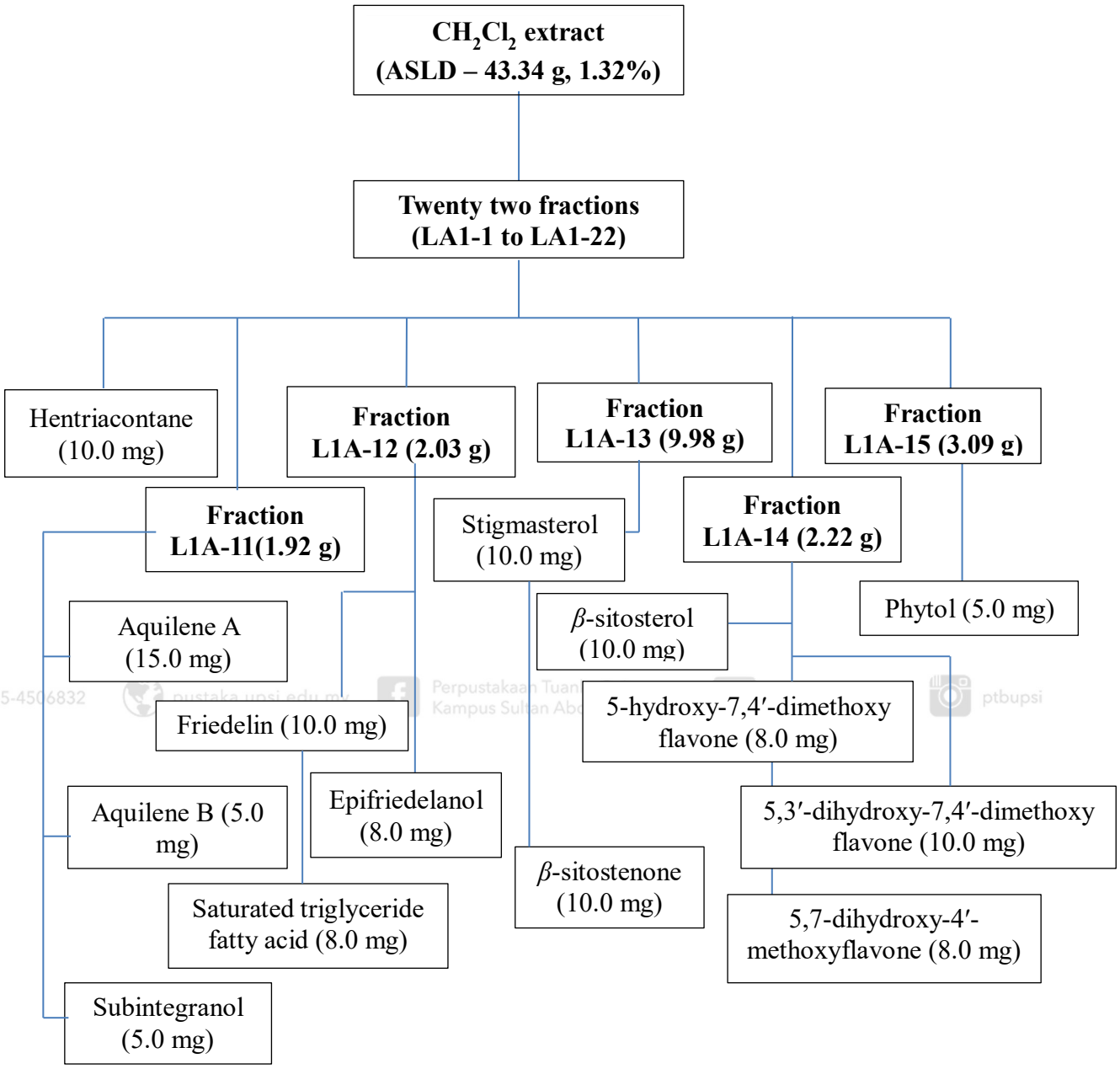
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3.6 Chemical Constituents from Leaf of *A. subintegra*

The CH_2Cl_2 crude extract of leaf (ASLD, 43.34 g) was introduced onto first CC and separated gradiently with 100% CH_2Cl_2, continue with mixture of CH_2Cl_2/MeOH (99:1, 98:2, 96:4, 94:6, 92:8, 90:10, 70:30, 50:50) and MeOH (100%) to yield 22 fractions (L1A-1 to L1A-22). The first column was afforded hentriacontane (**190**). Fraction L1A-12 (2.03 g) was purified using CC and afforded friedelin (**188**), epifriedelanol (**189**), and saturated triglyceride fatty acid (**186**). Fraction L1A-14 (2.22 g) subjected to CC for purification and gave *β*-sitosterol (**101**), 5-hydroxy-7,4′-dimethoxyflavone (**36**), 5,7-dihydroxy-4′-methoxyflavone (**39**) and 5,3′-dihydroxy-7,4′-dimethoxyflavone (**41**). Other fraction, L1A-11 (1.92 g) yielded three compounds, aquilene A (**191**), aquilene B (**192**), and subintegranol (**193**). While fraction L1A-15 (3.09 g) was afforded one diterpene known as 3,7,11,15-tetramethyl-2-hexadecen-1-ol (phytol) (**74**). Fraction L1A-13 (9.98 g) was isolated via CC and obtained compound stigmasterol (**102**) and *β*-sitostenone (**139**). The isolation and purification of the leaf of *A. subintegra* were shown in Scheme 3.4.

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Scheme 3.4. Flow chart for the purification process of *A. subintegra* leaf extracts

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3.6.1 Physical and Spectral Data of Isolated Chemical Constituents from Leaf

3.6.1.1 ASLD1: 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (Phytol) (74)

Fraction L1A-15 (3.09 g) was subjected to multiple CC, eluted with *n*-hex: EtOAc (95:5 v/v) to afford 3,7,11,15-tetramethyl-2-hexadecen-1-ol (phytol) (**74**) (5.0 mg, 0.16%) as a colourless oil; FTIR (KBr) υ_{max} cm^{-1}: 3736, 2927; ^{1}H-NMR (500 MHz, $CDCl_3$), δ ppm: 4.14 (2H, *d*, *J* = 7.6 Hz, H-1), 5.39 (1H, *t*, *J* = 6.8 Hz, H-2), 1.97 (2H, *br*. *t*, *J* = 7.6 Hz, H-4), 0.99-1.44 (18H, *m*, H-5, H-6,H-7, H-8, H-9, H-10, H-11, H-12, H-13, H-14), 1.50 (1H, hept like), 0.85 (6H, *d*, *J* = 6.9 Hz, H-16, H-17), 0.83 (6H, *d*, *J* = 6.9 Hz, H-18, H-19) and 1.67 (3H, *s*); ^{13}C-NMR (125 MHz, $CDCl_3$), δ ppm: 59.5 (C-1), 123.1 (C-2), 140.4 (C-3), 39.9 (C-4), 25.2 (C-5), 36.7 (C-6), 32.9 (C-7), 37.5 (C-8), 24.5 (C-9), 37.4 (C-10), 32.8 (C-11), 37.3 (C-12), 24.9 (C-13), 39.4 (C-14), 28.1 (C-15), 22.7 (C-16), 22.8 (C-17), 19.8 (C-18, C-19), 16.3 (C-20). ESI-MS: *m/z* 297.13068 $[M+H]^+$, $C_{20}H_{40}O$.

3.6.1.2 ASLD2: Friedelin (188)

Purification of L1A-12 (2.03 g) by several times of CC, eluted with *n*-hex: EtOAc (92:8 v/v), gave a colourless needles compound, friedelin (**188**) (10.0 mg, 0.49%); UV (MeOH) λ_{max} nm: 275; FTIR (KBr) υ_{max} cm^{-1}: 2928, 1716, 1388; ^{1}H-NMR (500 MHz, $CDCl_3$), δ ppm: 2.37 (2H, *m*, H-2), 2.24 (1H, *q*, *J* = 6.9 Hz, H-4), 0.72 (3H, *s*, H-24), 0.86 (3H, *s*, H-25), 0.88 (3H, *d*, *J* = 7.4 Hz, H-23), 0.99 (3H, *s*, H-26), 1.04 (3H, *s*, H-27), 1.17 (3H, *s*, H-28), 0.95 (3H, *s*, H-29), and 1.00 (3H, *s*, H-30); ^{13}C-NMR (125

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MHz, $CDCl_3$), δ ppm: 22.4 (C-1), 41.6 (C-2), 213.6 (C-3), 58.3 (C-4), 42.2 (C-5), 41.3 (C-6), 18.3 (C-7), 53.1 (C-8), 37.5 (C-9), 59.5 (C-10), 35.7 (C-11), 30.6 (C-12), 39.7 (C-13), 38.3 (C-14), 32.5 (C-15), 36.1 (C-16), 30.1 (C-17), 42.8 (C-18), 35.4 (C-19), 28.2 (C-20), 32.8 (C-21), 39.3 (C-22), 6.9 (C-23), 14.7 (C-24), 18.0 (C-25), 20.3 (C-26), 18.8 (C-27), 32.2 (C-28), 35.1 (C-29), 31.9 (C-30); ESI-MS: *m/z* 428.30719 $[M+H]^+$, $C_{30}H_{50}O$.

3.6.1.3 ASLD3: Epifriedelanol (189)

Purification of L1A-12 (2.03 g) by several time of CC, eluted with *n*-hex: EtOAc (92:8 v/v), also found another colourless needles compound, epifriedelanol (**189**) (8.0 mg, 0.39%); FTIR (KBr) υ_{max} cm^{-1}: 3692, 2932, 1385; ^{1}H-NMR (500 MHz, $CDCl_3$), δ ppm: 3.73 (1H, *br. d*, H-3), 0.95 (3H, *s*, H-24), 0.85 (3H, *s*, H-25), 1.00 (3H, *s*, H-26), 0.98 (3H, *s*, H-27), 0.99 (3H, *s*, H-28), 0.94 (3H, *s*, H-29), 1.16 (3H, *s*, H-30) and 1.20 - 1.58 (*m*, unresolved peak); ^{13}C-NMR (125 MHz, $CDCl_3$), δ ppm: 15.8 (C-1), 35.4 (C-2), 72.8 (C-3), 49.2 (C-4), 37.1 (C-5), 41.8 (C-6), 17.6 (C-7), 53.2 (C-8), 37.9 (C-9), 61.4 (C-10), 35.2 (C-11), 30.7 (C-12), 38.4 (C-13), 39.7 (C-14), 32.8 (C-15), 36.1 (C-16), 29.8 (C-17), 42.8 (C-18), 35.6 (C-19), 28.3 (C-20), 32.4 (C-21), 39.3 (C-22), 11.7 (C-23), 16.5 (C-24), 18.3 (C-25), 18.7 (C-26), 20.2 (C-27), 32.2 (C-28), 35.1 (C-29), 31.9 (C-30); ESI-MS: *m/z* 430.22055 $[M+H]^+$, $C_{30}H_{52}O$.

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3.6.1.4 ASLD4: Stigmasterol (102)

Fraction L1A-13 (9.98 g) was subjected to multiple CC using *n*-hex: EtOAc (90:10 v/v) as an eluent system, afforded stigmasterol (**102**) (10.0 mg, 0.10%) as white needles; FTIR (KBr) υ_{max} cm^{-1}: 3435, 2960, 2937, 1641, 1464, 1053; ^{1}H-NMR (500 MHz, $CDCl_3$), δ ppm: 3.52 (1H, *m*, H-3), 5.34 (1H, *t*, *J* = 5.2 Hz, H-6), 0.91 (3H, *d*, *J* = 6.5 Hz, H-19), 4.98 (1H, *m*, H-21), 5.12 (1H, *m*, H-22), 0.83 (3H, *t*, *J*= 7.5 Hz, H-23), 0.82 (3H, *d*, *J* = 4.6 Hz, H-26), 0.80 (3H, *d*, *J* = 6.3 Hz, H-27), 0.69 (3H, *s*, H-28), 1.02 (3H, *s*, H-29); ^{13}C-NMR (125 MHz, $CDCl_3$), δ ppm: 37.3 (C-1), 32.0 (C-2), 71.9 (C-3), 42.3 (C-4), 140.8 (C-5), 121.8 (C-6),31.7 (C-7), 31.7 (C-8), 50.2 (C-9), 36.2 (C-10), 21.1 (C-11), 39.7 (C-12), 42.2 (C-13), 56.8 (C-14), 24.4 (C-15), 29.0 (C-16), 56.0 (C-17), 40.6 (C-18), 21.2 (C-19), 138.4 (C-20), 129.3 (c-21), 45.9 (C-22), 25.5 (C-23), 12.0 (C-24), 29.2 (C-25), 19.9 (C-26), 19.4 (C-27), 18.8 (C-28), 12.3 (C-28); ESI-MS: *m/z* 413.26266 $[M+H]^+$, $C_{29}H_{48}O$.

3.6.1.5 ASLD5: Hentriacontane (190)

Isolation of ASLD (43.34 g) into CC, eluted with *n*-hex: CH_2Cl_2 (10:90 v/v), afforded a long chain alkane, hentriacontane (**190**) (10.mg, 0.02%) as a white amorphous; FTIR (KBr) υ_{max} cm^{-1}: 2955, 2915, 2847, 1461; ^{1}H-NMR (500 MHz,$CDCl_3$), δ ppm: 0.88 (6H, *s*, H-1, H-31), 1.25 (58H, *m*, H-2 to H-30); ^{13}C-NMR (125 MHz, $CDCl_3$), δ ppm: 14.2 (C-1, C-31), 22.8 (C-2, C-30), 32.0 (C-3, C-29), 29.5 (C-4, C-28), 29.8 (C-5 to C-27); ESI-MS: *m/z* 437.21381 $[M+H]^+$, $C_{31}H_{64}$.

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3.6.1.6 ASLD6: 5,7-Dihydroxy-4′-methoxyflavone (39)

Fraction L1A-14 (2.22 g) was subjected to multiple CC, eluted with CH_2Cl_2: MeOH (90:10 v/v) solvent system, afforded 5,7-dihydroxy-4′-methoxyflavone (**39**) (8.0 mg, 0.36%), as a yellow amorphous. UV (MeOH) λ_{max} nm: 270, 314; FTIR (KBr) υ_{max} cm^{-1}: 3266, 2923, 1605; ^{1}H-NMR (500 MHz, $CDCl_3$), δ ppm: 3.88 (3H, *s*, OCH_3 -4′), 6.56 (1H, *s*, H-3), 6.37 (1H, *d*, *J* = 2.3 Hz, H-6), 6.48 (1H, *d*, *J* = 1.7 Hz, H-8), 6.94 (2H, *d*, *J* = 8.1 Hz, H-3′, 5′), 7.78 (2H, *d*, *J* = 8.6 Hz, H-2′, 6′), 12.81 (1H, *s*, OH- 5); ESI-MS: *m/z* 283.06128 $[M-H]^-$, $C_{16}H_{12}O_5$.

3.6.1.7 ASLD7: Aquilene A (191)

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Fraction L1A-11 (1.92 g) was purifired using multiple CC eluted with solvent system *n*-hex:EtOAc (70:30 v/v), gradiently, afforded compound aquilene A **(191)** as a white needle (15.0 mg, 0.78%). FTIR (KBr) υ_{max} cm^{-1}: 2916, 1703, 1386 ; ^{1}H-NMR (500 MHz, $CDCl_3$), δ ppm: 5.31 (1H, *d*, *J* = 3.5, H-7); 4.66 (1H, *s*, H-30a), 4.83 (1H, *s*, H-30b), 1.04 (3H, *s*, H-23), 1.11 (3H, *s*, H-24), 1.00 (3H, *s*, H-25), 0.81 (3H, *s*, H-26), 1.01 (3H, *s*, H-27), 0.91 (3H, *d*, *J* = 6.3 Hz, H-28) and 1.06 (3H, *s*, H-29); ^{13}C-NMR (125 MHz, $CDCl_3$), δ ppm: 38.6 (C-1), 35.0 (C-2), 217.2 (C-3), 47.9 (C-4), 52.4 (C-5), 24.6 (C-6), 117.9 (C-7), 146.1 (C-8), 48.6 (C-9), 36.2 (C-10), 34.1 (C-11), 33.7 (C-12),51.3 (C-13), 43.6 (C-14), 29.7 (C-15), 18.6 (C-16), 36.6 (C-17), 53.1 (C-18), 28.3 (C-19), 159.1 (C-20), 35.1 (C-21), 36.4 (C-22), 24.4 (C-23), 21.7 (C-24), 12.8 (C-25), 22.1 (C-26), 27.5 (C-27), 18.3 (C-28), 29.4 (C-29), 105.8 (C-30); ESI-MS: *m/z* 423.19754 $[M+H]^+$, $C_{30}H_{46}O$.

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3.6.1.8 ASLD8: Aquilene B (192)

Fraction L1A-11 (1.92 g) was purified using multiple CC eluted with solvent system *n*-hex:EtOAc (70:30 v/v), gradiently, afforded compound aquilene B **(192)** as a white amorphous (5.0 mg, 0.25%). FTIR (KBr) υ_{max} cm^{-1}: 2957, 1704, 1630, 1369; ^{1}H-NMR (500 MHz, $CDCl_3$), δ ppm: 5.30 (1H, *d*, *J* = 3.5, H-7), 4.66 (1H, *s*, H-30a), 4.83 (1H, *s*, H-30b), 3.48 (1H, *d*, *J* = 5.15 Hz, H-13), 1.04 (3H, *s*, H-23), 1.11 (3H, *s*, H-24), 1.00 (3H, *s*, H-25), 1.01 (3H, *s*, H-26), 0.81 (3H, *s*, H-27), 0.91 (3H, *d*, *J* = 6.9 Hz, H-28) and 1.05 (3H, *s*, H-29); ^{13}C-NMR (125 MHz, $CDCl_3$), δ ppm: 38.6 (C-1), 35.1 (C-2), 217.2 (C-3), 47.9 (C-4), 52.4 (C-5), 24.6 (C-6), 117.9 (C-7), 148.7 (C-8), 48.5 (C-9), 36.2 (C-10), 34.1 (C-11), 217.0 (C-12), 51.3 (C-13), 43.6 (C-14), 29.4 (C-15), 18.6 (C-16), 36.6 (C-17), 53.1 (C-18), 28.3 (C-19), 158.0 (C-20), 35.0 (C-21), 36.5 (C-22), 24.4 (C-23), 21.6 (C-24), 12.8 (C-25), 27.5 (C-26), 22.0 (C-27), 18.3 (C-28), 29.3 (C-29), 105.8 (C-30); ESI-MS: *m/z* 437.23420 $[M+H]^+$, $C_{30}H_{44}O_2$.

3.6.1.9 ASLD9: Subintegranol (193)

Fraction L1A-11 (1.92 g) was purified using multiple CC eluted with solvent system *n*-hex: EtOAc (60:40 v/v), gradiently, afforded compound **(193)** as a white amorphous (5.0 mg, 0.25%). FTIR (KBr) υ_{max} cm^{-1}: 3726, 2927, 1736, 1636; ^{1}H-NMR (500 MHz, $CDCl_3$), δ ppm: 1.25 (3H, *s*, CH_3-2), 1.83 (2H, *ddd*, *J* = 6.9, 7.4, 6.3 Hz, H-3), 3.02 (2H, *t*, H-4), 10.18 (1H, *s*, H-5a), 12.10 (1H, *s*, OH-6), 2.16 (3H, *s*, CH_3-9), 7.28 (1H, *d*, *J* = 7.4 Hz, H-10), 7.21 (1H, *d*, *J* = 6.9 Hz, H-11), 2.15 (3H, *s*, CH_3-12), 1.25 (2H, *m*, H-1′), 0.98 (2H, *m*, H-2′), 1.40 (1H, *m*, H-3′), 1.26 (1H, *m*, H-4′), 1.53 (2H, *m*, H-

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5′), 1.14 (2H, *m*, H-6′), 1.36 (1H, *m*, H-7′), 1.26 (1H, *m*, H-8′), 1.25 (2H, *m*, H-9′), 1.25 (2H, *m*, H-10′), 1.05 (2H, *m*, H-11′), 1.29 (1H, *m*, H-12′), 0.85 (3H, *d*, H-13′), 0.86 (3H, *d*, H-14′), 0.85 (3H, *d*, H-15′), 0.84 (3H, *d*, H-16′), 0.86 (3H, *d*, H-17′), 1.64 (1H, *m*, H-18′), 2.30 (2H, *m*, H-19′), 4.08 (2H, *t*, H-22′), 1.61 (1H, *m*, H-23′), 2.03 (2H, *m*, H-24′), 5.34 (1H, *m*, H-25′), 5.34 (2H, *m*, H-26′); ^{13}C-NMR (125 MHz, $CDCl_3$), δ ppm: 75.2 (C-2), 23.7 (CH_3-2), 30.8 (C-3), 18.5 (C-4), 117.6 (C-4a), 114.5 (C-5), 194.1 (C-5a), 155.9 (C-6), 124.2 (C-7), 138.6 (C-8), 144.1 (C-8a), 126.9 (C-9), 13.3 (CH_3-9), 128.5 (C-10), 129.0 (C-11), 125.6 (C-12), 11.1 (CH_3-12), 39.6 (C-1′), 37.3 (C-2′), 32.8 (C-3′), 29.7 (C-4′), 28.7 (C-5′), 37.5 (C-6′), 29.7 (C-7′), 26.2 (C-8′), 23.7 (C-9′), 24.8 (C-10′), 37.4 (C-11′), 28.3 (C-12′), 14.2 (C-13′), 22.7 (C-14′), 22.8 (C-15′), 19.7 (C-16′), 19.8 (C-17′), 25.1 (C-18′), 34.6 (C-19′), 174.2 (C-20′), 64.4 (C-22′), 28.8 (C-23′), 26.2 (C-24′), 130.1 (C-25′), 130.1 (C-26′); ESI-MS: *m/z* 647.38440 $[M+H]^+$, $C_{42}H_{62}O_5$.

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3.6.1.10 ASLD10: Saturated Triglyceride Fatty Acid (186)

Fraction L1A-12 (2.03 g) was subjected to multiple CC, with eluent system *n*-hex: CH_2Cl_2 (60:40 v/v), afforded saturated triglyceride fatty acid (**186**) (8.0 mg, 0.39%), as a white amorphous. Its physical properties and spectroscopy data were alike with saturated triglyceride, which previously successful isolated from bark of *A. subintegra*.

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3.6.1.11 ASLD11: *β*-sitosterol (101)

Fraction L1A-14 (2.22 g) was subjected to CC, with eluent system *n*-hex: CH_2Cl_2 (50:50 v/v) and washed with cold MeOH, afforded *β*-sitosterol (**101**) (10.0 mg, 0.45%), as a white needles. Its physical properties and spectroscopy data were alike with *β*-sitosterol, which previously successful isolated from bark of *A. subintegra*.

3.6.1.12 ASLD12: *β*-sitostenone (139)

Purification of L1A-13 (9.98 g) by several CC, eluted with *n*-hex: EtOAc (70:30 v/v) yielded a white amorphous compound, *β*-sitostenone (**139**) (10.0 mg, 0.10%). The physical properties and its spectroscopic data analysis were identical with *β*-sitostenone, which had been determined in bark of *A. subintegra*.

3.6.1.13 ASLD13: 5-Hydroxy-7,4′-dimethoxyflavone (36)

Fraction L1A-14 (2.22 g) was submitted to CC, with eluent system *n*-hex: CH_2Cl_2 (5:95 v/v), have afforded 5-hydroxy-7,4′-dimethoxyflavone (**36**) (10.0 mg, 0.45%) as a yellow amorphous. The physical properties and its spectroscopic data analysis were identical with 5-hydroxy-7,4′-dimethoxyflavone, which had been determined in bark of *A. subintegra*.

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3.6.1.14 ASLD14: 5,3′-Dihydroxy-7,4′-dimethoxyflavone (41)

Purification of L1A-14 (2.22 g) by multiple CC, eluted with *n*-hex: EtOAc (30:70 v/v), afforded 5,3′-dihydroxy-7,4′-dimethoxyflavone (**41**) (10.0 mg, 0.45%), a yellow amorphous. The physical properties and its spectroscopic analysis data of this compound were matching with 5,3′-dihydroxy-7,4′-dimethoxyflavone which have been successfully isolated from bark of *A. subintegra*.

3.7 Bioactivity Studies

This section will discuss intensively the biological activities of crude extracts and isolated compounds of *A. subintegra*. These biological activities are antioxidant, anti-lipase, and anticancer.

3.7.1 Antioxidant Activity

Antioxidant activity was tested by using DPPH (2, 2-diphenyl-1-picrylhy-drazyl) Free Radical Scavenging method described by Bobo-Gracia et al. (2015). DPPH assay was carried out to determine the antioxidative activity of crude extracts from barks and leaves (hexane, dichloromethane, and methanol) as well as isolated compounds; 5-hydroxy-7,4′-dimethoxyflavone (**36**), luteolin-7,3′,4′-trimethyl ether (**37**), *β*-sitosterol (**101**), friedelin (**188**) and aquilene A (**191**) from *A. subintegra.*

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3.7.1.1 Instruments and Materials

The instrument used was Microplate reader (Dynatech MR5000 TECAN, Tecan Group Ltd., Switzerland). For the disposable materials, these are including flat-bottomed 96-well plate, aluminium foil, micropipette tips, and multichannel solution basin.

3.7.1.2 Chemicals

Chemicals used in this activity were DPPH (2, 2-diphenyl-1-picrylhy-drazyl), ascorbic acid (Sigma-Aldrich Chemical Co. St. Louis, USA), methanol and DMSO (Merck, Germany) and deionized water.

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3.7.1.3 DPPH radical scavenging activity

DPPH (2, 2-diphenyl-1-picrylhy-drazyl) free-radical scavenging assay was based on the 96-well plate. DPPH is organic nitrogen radical with purple colour. It is reduced by antioxidants to form pale yellow hydrazine. Ascorbic acid was used as a standard at 0.39 – 50 μg/mL to generate a calibration curve. DPPH and ascorbic acid were from Sigma-Aldrich Chemical Co. St. Louis, USA.

A total of diluted sample was added to 180 μL of DPPH solution (150 μmol/L) in methanol: water (80:20, v/v) and shaken for 60 s in a 96-well microplate. Then, after 40 min in the dark at room temperature, the absorbance was measured at 515 nm in the

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microplate reader. The percentage of DPPH quenched was calculated using the following equation:

$$\% \text{ Inhibition of DPPH radicals} = (1 - [A/B]) \times 100\%$$

Where,

A = (Absorbance at 515 nm of 20 µL of the sample with 180 µL DPPH solution after 40 min) - (Absorbance at 515 nm of 20 µL of the sample with 180 µL methanol: water (80:20, v/v) after 40 min).

B = (Absorbance at 515 nm of 20 µL of DMSO with 180 µL DPPH solution) - (Absorbance absorbance at 515 nm of 20 µL of DMSO with 180 µL methanol: water (80:20, v/v).

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The amount of extracts necessary to decrease the initial DPPH absorbance by 50 % (IC_{50}) was graphically determined by plotting the DPPH scavenging percentage as a function of extract concentration.

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3.7.2 Anti-lipase Activity

Anti-lipase activity test was assessed using a colorimetric assay and standard curves of free fatty acid methods (Kwon & Rhee, 1986; Leow et. al., 2004). Crude extracts from barks and leaves (*n*-hexane, dichloromethane and methanol) as well as isolated compounds (5-hydroxy-7,4′-dimethoxyflavone (**36**), luteolin-7,3′,4′-trimethyl ether (**37**), 5,3′-dihydroxy-7,4′-dimethoxyflavone (**41**), β-sitosterol (**101**), aquilene A (**191**) and subintegranol (**193**) from *A. subintegra* were screened of their anti-lipase *via* colorimetric assay.

3.7.2.1 Instruments and Apparatus

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Instruments that have been used including electronic balance (Shimadzu, Japan), ultrasonic cleaner (Wise Cleane, Germany), vortex mixer (VELP scientifica), water bath shaker, UV-Visible spectrophotometer (BIOMATE 3S, Madison, USA) and pH meter.

Apparatus used were Schott Duran bottle 500mL (Mainz, Germany), beaker 100 mL (Pyrex, Tewksbury, USA), scintillation vials 20 mL, micropipette 2 - 20 µL and 100 -1000 µL (Eppendorf, Hamburg, Germany), pipette tips (GEB, Zhejiang, China), cuvettes 1.5 mL.

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3.7.2.2 Chemical Reagents

Chemicals used in this study were sodium dihydrogen phosphate monohydrate (1.06346.100), di-sodium hydrogen phosphate (1.06586.0500) from Merck (Darmstadt, Germany), Copper (II) acetate AR Grade (BENDOSEN, Malaysia), Oleic Acid from Sigma-Aldrich (St. Louis, USA). Other chemicals were 2,2,4-trimethylpentane AR grade (QREC), lipase from porcine pancreas Type II from Sigma-Aldrich (St. Louis, USA), and pyridine (Merck, Germany).

3.7.2.3 Standard Curves of Free Fatty Acid

Sample oleic acid (2.0 – 25.0 µmole) was dissolved with 5 mL of isooctane in a test tube. Then 1.0 ml of copper acetate pyridine reagent was added and two phases formed. The mixture was mixed using a vortex mixer for 90 sec and stands still around 20 sec until the aqueous phase was sediment clearly from the solution of isooctane and fatty acid. The standard curves of free fatty acids against absorbance were determined by the absorbance of isooctane solution at 715 nm against the control (no free fatty acid) (Appendix A).

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3.7.2.4 Assay for inhibitory pancreatic lipase activity

In vitro pancreatic lipase activity was determined by measuring the release rate of oleic acid (free fatty acid) from the standard curve of free fatty acid. This method was performed colorimetrically with slight modification from Kwon and Rhee's method (1986). 1.0 mL of culture filtrate was mixed with 2.5 mL of natural substrate (olive oil mixed with buffer, 1:1,v/v) and 20 µl of 20 mM $CaCl_2$. The mixture was shaken 30 min in the water bath shaker at an agitation rate of 200 rpm. Then 1 ml of 6 N HCl was added to terminate the reaction of the emulsion system and followed by adding 5 mL of isooctane, thus mixing for 30 sec using a vortex mixer. The upper isooctane layer containing the fatty acid was drawn off to a test tube, and 1mL of copper reagent was added before analysis. Lipase activity was determined by measuring the amount of free fatty acids released from the standard curves of free fatty acid; one unit of lipase activity was defined as one µmole of fatty acid released by an enzyme in one minute (Leow, et al., 2004; Kwon & Rhee, 1986). Figure 3.1 shows the flow process of the colorimetric assay.

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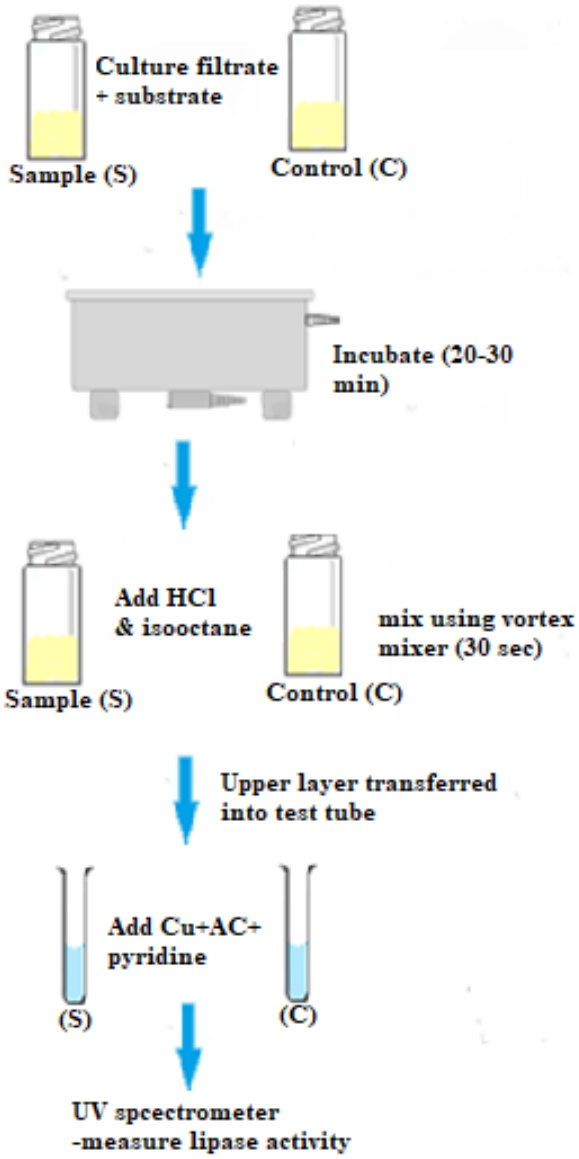


Figure 3.1. Flow process of colorimetric assay

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3.7.3 Anticancer Activity

3.7.3.1 Materials and Apparatus

The materials and apparatus used were MCF-7 breast cancer cells HTB-22™ (ATTC), CCK-8 solution, WST-8, plate reader (450 nm filter), 96-well plate, multi-channel pippettes, DMSO and carbon dioxide (CO_2) incubator.

3.7.3.2 Cytotoxic Assay

The cytotoxic assay was conducted on MCF-7 breast cancer cells using Cell Counting Kit-8 (CCK-8) (Nakagawa et al., 2016; Bao et al., 2014, Takahashi et al., 2014). At first, cancer cells were cultured to the active stage. Once the cells reached the active stage, they were transferred into a 96-well tissue culture plate at 1 x 10^4 cells/well with 100 µl of cell suspension for each well. The culture was pre-incubated for 24 hours in a humidified carbon dioxide (CO_2) incubator.

Then, the culture was observed after 24 hours, under the inverted-light microscope to check the morphology and density of cell growth. After the cell attachment and growth were confirmed, samples (crude extracts and isolated compounds), and negative control were added to each designated well at 10 µl. The culture was incubated for another 48 hours to allow the effect of samples towards MCF-7 cells to take place. Then, 10 µl of Cell Count Reagent SF was added to each well, and the culture was subjected to 2 hours of incubation followed by the absorbance measurement at 450 nm using the microplate reader.

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Upon completion, the culture plate was discarded, and the absorbance data were analysed using Equation 1 to generate a decay curve for the 50 % inhibition concentration (IC_{50}) value estimation.

Equation 1:

$$Percentage\ of\ Controlled\ Cell\ Growth = \left(\frac{Abs_{Sample} - Abs_{Blank}}{Abs_{Negative\ Control} - Abs_{Blank}}\right) \times 100\ \%$$

3.8 Mechanism of inhibition of pancreatic lipase activity

This section was purposed for exploring the mechanism of inhibition of pancreatic lipase (PL) by bioactive compounds from *A. subintegra*. This mechanism was operated using computational chemistry to view the interaction between the compounds (inhibitor) 5-hydroxy-7,4′-dimethoxyflavone (**36**), luteolin-7,3′,4′-trimethyl ether (**37**), 5,3′-dihydroxy-7,4′-dimethoxyflavone (**41**) and *β*-sitosterol (**101**) toward PL.

3.8.1 Molecular Docking

The Molecular Docking application was done using Yet Another Scientific Artifical Reality Application (YASARA) Structure Package; an easy-to-use, reliable, universal package for molecular graphics, molecular modeling, and molecular dynamics (Krieger et al., 2002) (http://www.yasara.com).

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The docking was conducted to predict the potential inhibition site of RA (inhibitor) toward enzyme (porcine pancreatic lipase, PPL) using *in silico* experimental approach. The structure of RA was obtained from the National Center for Biotechnology Information (NCBI) PubChem with the entry no CID 79370. While the structure of PPL was gained using the Protein Data Bank (PDB) crystal structure of the PPL-CLP-TGME (PDB-ID: 1ETH) as shown in Figure 3.2.

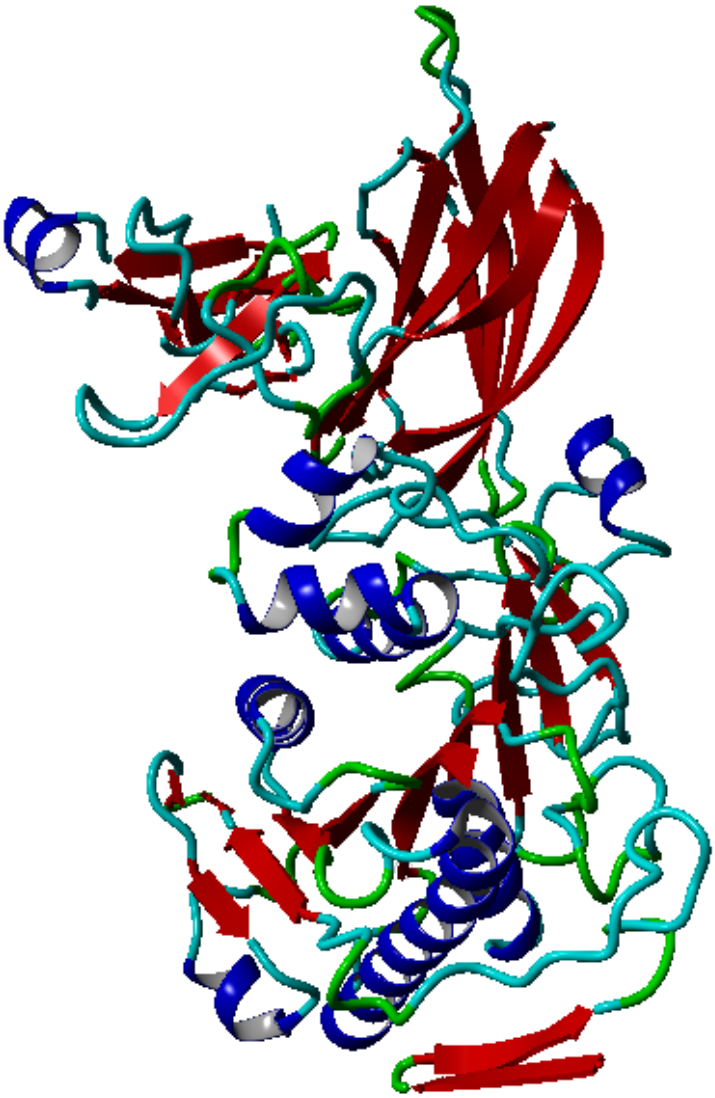


Figure 3.2. The structure of PPL-CLP-TGME (PDB-ID: 1ETH) obtained from PDB

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YASARA software has been used to examine blind docking between PPL as a target for the inhibitor, thus detecting possible voids that might serve as a binding site. The calculation was done using YASARA macro (dock_run.mcr).There have few parameters including: 25 docking runs; 25,000,000 energy evaluations; 150 population sizes; 27,000 number of generation.

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CHAPTER 4

RESULTS AND DISCUSSION

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4.1 Phytochemical Studies

In this study, *A. subintegra* was selected as a plant sample. The fresh parts of the plant were collected from Kajang, Selangor, Malaysia. Barks and leaves of the sample were air-dried to eliminate moisture content, ground into small pieces to make the extraction effective.

The extraction started from non-polar solvent (*n*-hex) and followed with a more polar solvent (CH_2Cl_2 and MeOH). This is following the principle “like dissolve like” (Ncube et al., 2008). The maceration technique has been used for the extraction due to some compounds were sensitive to the high temperature and to avoid degradation of chemical compounds. The sample was soaked into solvents for three days and repeated for thrice. Then, the crude extracts were concentrated under reduced pressure using a

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rotary evaporator. The crude extracts were then kept in the fridge at 4ºC for further analysis.

The purification process of CH_2Cl_2 crude extract from bark and leaf of *A. subintegra* was involving the chromatographic practices such as column chromatography, TLC as well as recrystallization and washing techniques. Structural elucidation of the isolated compounds was done using spectroscopic analysis such as 1D and 2D NMR, FTIR, UV, and LC-MS.

A total of 22 phytochemicals have been successfully isolated from bark and leaf of *A. subintegra*. The isolation and purification processes have afforded several classes of chemical constituents identified as flavonoids, triterpenes, diterpenes, steroids, and long-chain alkane. The phytochemicals have been summarized in Table 4.1.

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Table 4.1

Phytochemicals isolated from bark and leaf of A. subintegra

Plant part	Extracts	Chemical constituents
Bark	CH_2Cl_2	**ASBD1:** 5-Hydroxy-7,4′-dimethoxyflavone (**36**)
		ASBD2: Luteolin-7,3′,4′-trimethyl ether (**37**)
		ASBD3: 5,3′-Dihydroxy-7,4′-dimethoxyflavone (**41**)
		ASBD4: 7,3’-Dimethoxyluteolin (**185**)
		ASBD5: *β*-Sitosterol (**101**)
		ASBD6: *β*-Sitostenone (**139**)
		ASBD7: Saturated triglyceride fatty acid (**186**)
		ASBD8: 2,6-dimethoxyquinone (**187**)
Leaf	CH_2Cl_2	**ASLD1:** 3,7,11,15 – Tetramethyl – 2 – hexadecen – 1 – ol (phytol) (**74**)
		ASLD2: Friedelin (**188**)
		ASLD3: Epifriedelanol (**189**)
		ASLD4: Stigmasterol (**102**)
		ASLD5: Hentriacontane (**190**)
		ASLD6: 5,7-dihydroxy-4′-methoxyflavone (**39**)
		ASLD7: Aquilene A (**191**)
		ASLD8: Aquilene B (**192**)
		ASLD9: Subintegranol (**193**)
		ASLD10: Saturated triglyceride fatty acid (**186**)
		ASLD11: *β*-Sitosterol (**101**)
		ASLD12: *β*-Sitostenone (**139**)
		ASLD13: 5-Hydroxy-7,4′-dimethoxyflavone (**36**)
		ASLD14: 5,3′-Dihydroxy-7,4′-dimethoxyflavone (**41**)

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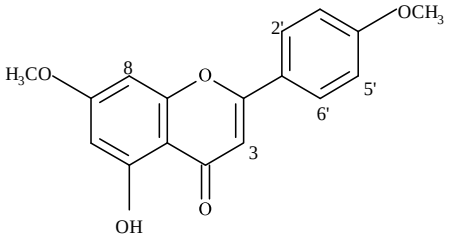
4.2 Chemical Constituents Isolated from bark of *A. subintegra*

Isolation and purification of bark extract of *A. subintegr*a were carried out using column chromatography (CC), thin layer chromatography (TLC), and several purification techniques including washing and recrystallization technique to afford pure phytochemicals.

The purification of dichloromethane (CH_2Cl_2) extracts of the bark plant successfully isolated eight compounds. These compounds were including several classes such as four flavonoids (**ASBD1**, **ASBD2**, **ASBD3**, **ASBD4**), two steroids (**ASBD5**, **ASBD6**)**,** a triglyceride (**ASBD7**) and quinone (**ASBD8**).

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4.2.1 ASBD1: 5-Hydroxy-7,4′-dimethoxyflavone (36)



Purification of fraction C1-5 afforded compound **ASBD1**. Compound **ASBD1** was obtained as a yellowish amorphous powder (13.0 mg, 0.43%). On the TLC analysis, the compound was observed as a dark spot under short wave UV (254 nm) and turned to yellow fluorescence after sprayed with *p*-anisaldehyde. The LC-MS spectrum revealed

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a significant molecular ion peak at *m/z* 299.08871 $[M+H]^+$ and corresponding with the molecular formula of $C_{17}H_{14}O_5$ (Figure 4.1).

The IR spectrum of **ASBD1** (Figure 4.2) exhibited the absorptions for OH group at 3688 cm^{-1}, sp^3 C-H stretching at 2915 cm^{-1}, and sp^2 C-H band at 3053 cm^{-1}. In addition, the carbonyl group was observed at 1740 cm^{-1}, while the C=C aromatic ring was exhibited at 1606 cm^{-1}. The UV spectrum (Figure 4.3) displayed absorption bands at 270 nm and 321 nm suggesting a flavone derivative skeleton (Meng, Krzysiak, Durako, Kunzelman, & Wright, 2008).

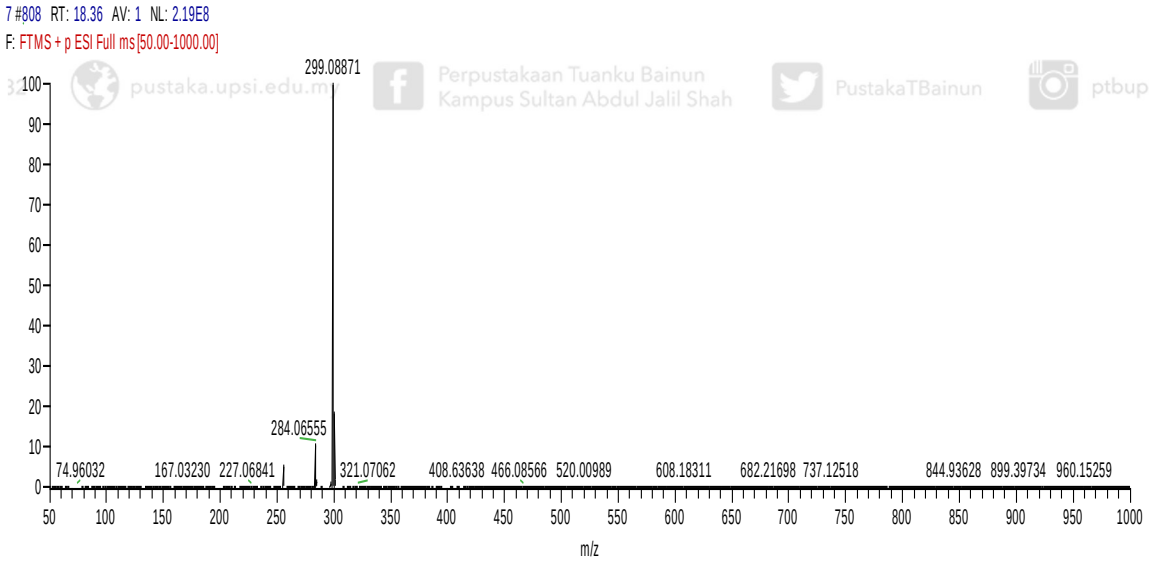


Figure 4.1. LC-MS spectrum of compound **ASBD1**

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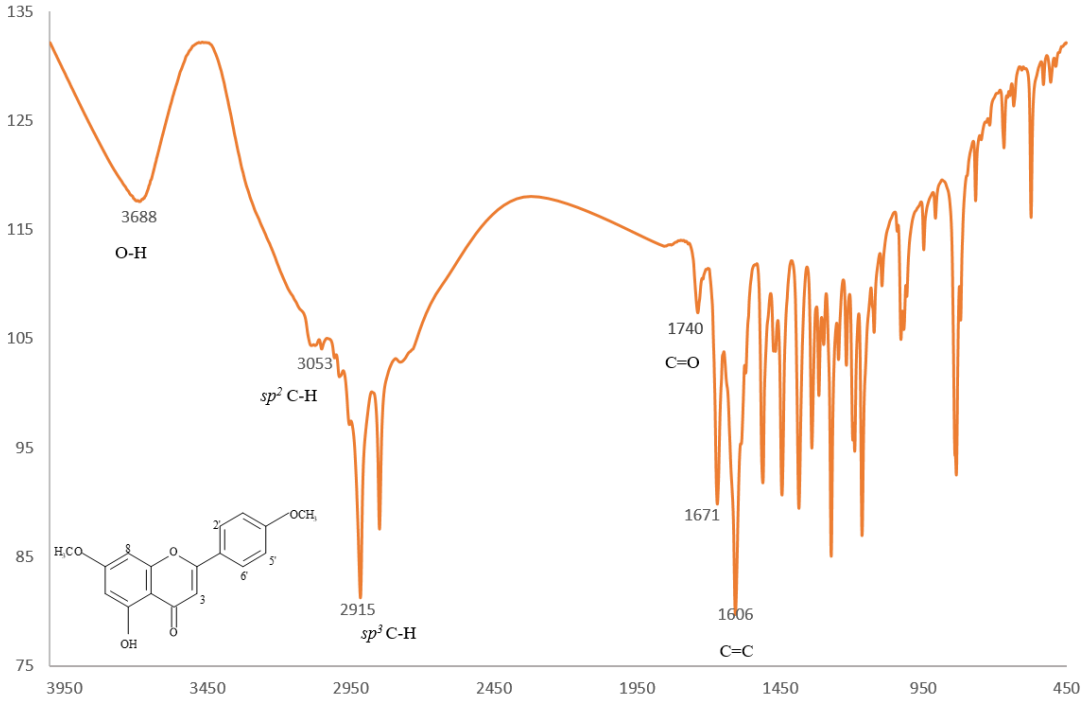


Figure 4.2. IR spectrum of compound **ASBD1**

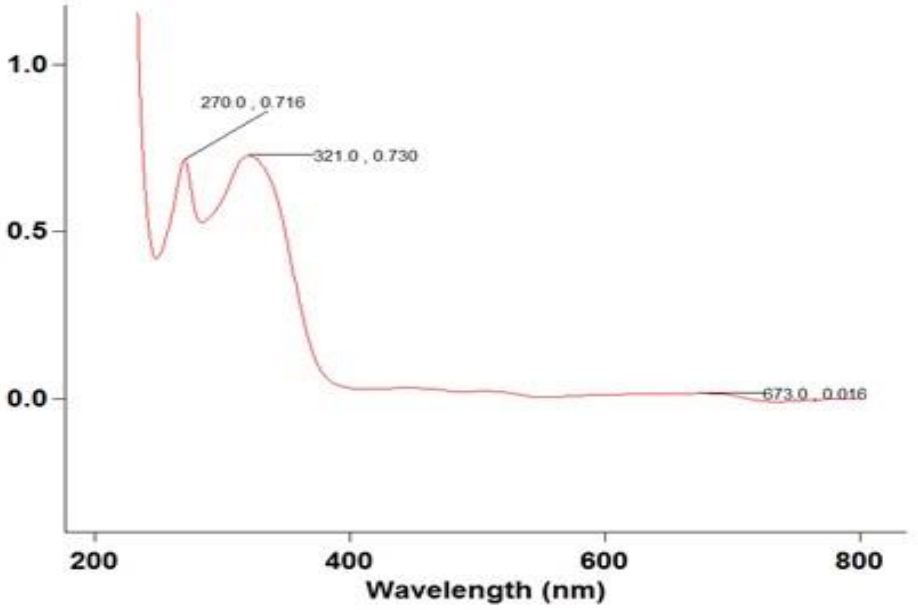


Figure 4.3. UV spectrum of compound **ASBD1**

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The ^{1}H-NMR spectrum of **ASBD1** in Figure 4.4 revealed two signal of methoxy groups that appeared at δ3.89 and 3.88, placed at C-7 and C-4′. The singlet signal at δ12.82 corresponded to a hydroxyl group at C-5. The aromatic region of the ^{1}H NMR spectrum showed the characteristic features appropriate for the flavonoid skeleton. One singlet signal appeared at δ 6.58, attributed to H-3. In addition, the spectrum displayed two doublets which characteristic of *ortho*-proton; signals at δ 7.01 (2H, *J* = 9.2 Hz) assigned to H-3′ and H-5′ as well as δ 7.85 (2H, *J* = 9.2 Hz) attributed to H-2′ and H-6′.

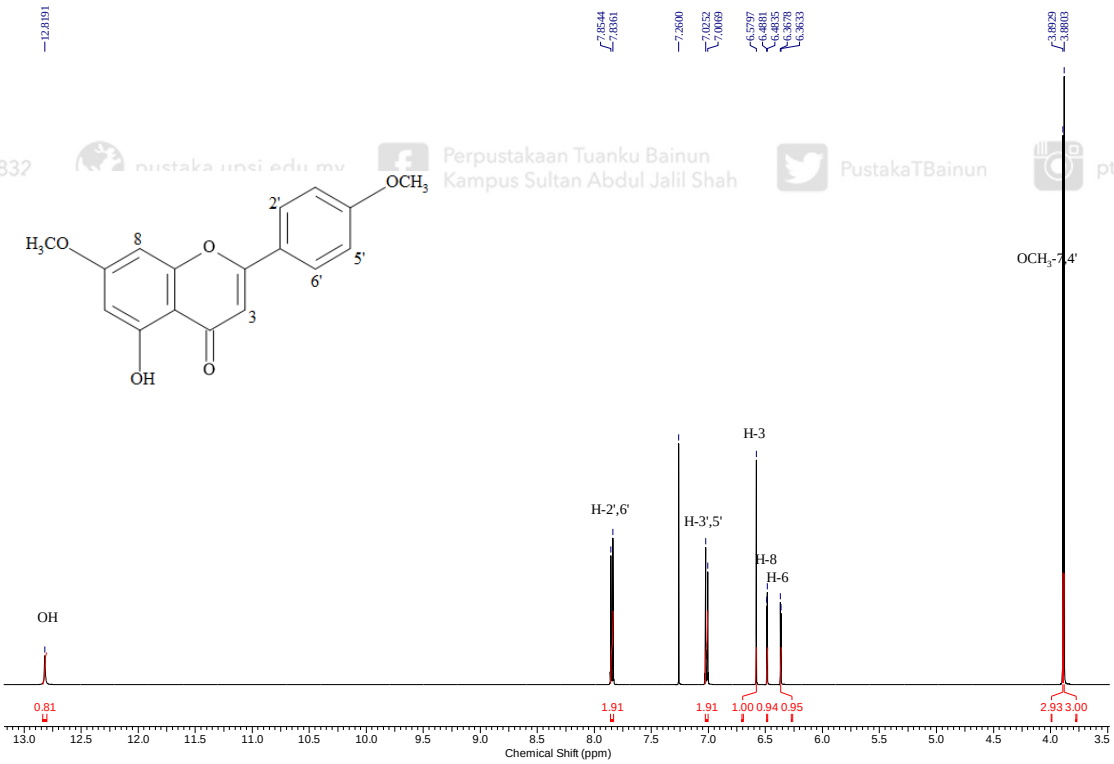


Figure 4.4. ^{1}H-NMR spectrum of compound **ASBD1**

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The ^{13}C-NMR spectrum of **ASBD1** (Figure 4.5) showed seventeen signals consisting of two methoxyl carbons at δ 55.6 and 55.9. One carbonyl carbon observed at δ 182.5 was assigned to C-4. This spectrum data was in good agreement with 5-hydroxy-7,4′-dimethoxyflavone (**36**) (Kang et al., 2014; Mangoyi, Midiwo, & Mukanganyama, 2015). The assignment of ^{1}H and ^{13}C NMR data of compound **ASBD1** was compared with the data from literature and tabulated in Table 4.2.

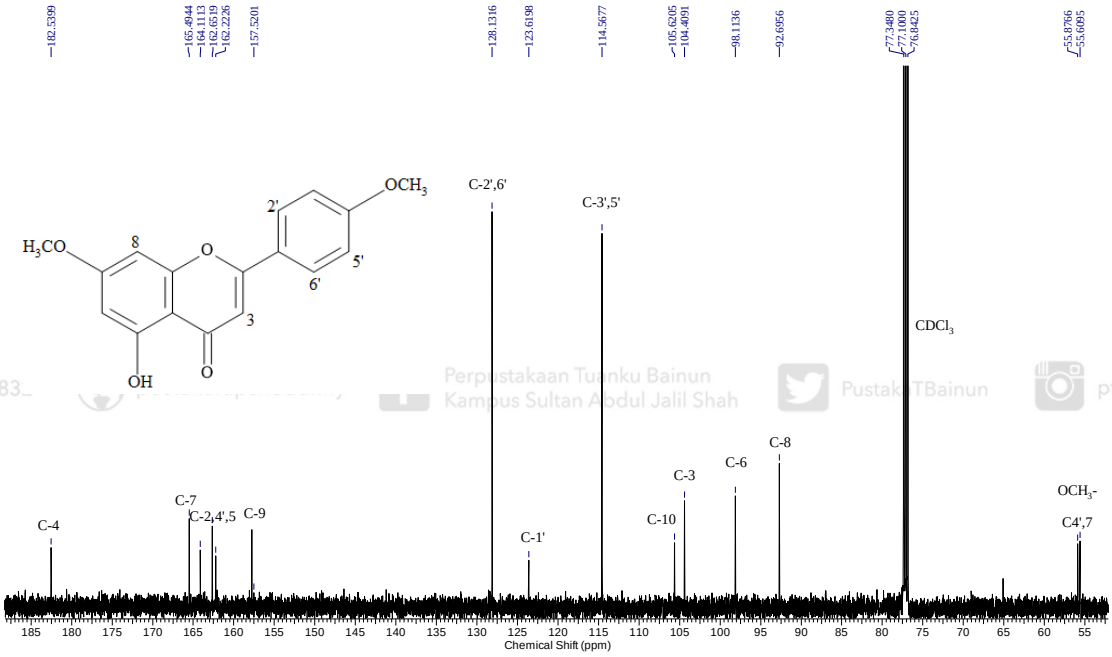


Figure 4.5. ^{1}3C-NMR of compound **ASBD1**

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Table 4.2

*^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C (125 MHz, $CDCl_3$) spectral data of **ASBD1** and 5- hydroxy-7,4′-dimethoxyflavone (**36**)*

	Compound ASBD1		**5-hydroxy-7,4′-dimethoxyflavone (36)**	
No	**δ^{1}H (ppm)**	**δ^{13}C (ppm)**	***δ^{1}H (ppm)**	****δ^{13}C (ppm)**
1				
2		164.1		163.9
3	6.58 (1H, *s*)	104.4	6.58 (*s*)	104.2
4		182.5		182.4
5				
6	6.36 (1H, *d*, *J*=2.3 Hz)	98.1	6.37 (*d*, *J*=2.5 Hz)	98.0
7		165.5		165.3
8	6.48 (1H, *d*, *J*=2.3 Hz)	92.7	6.48 (*d*, *J*=2.5 Hz)	92.5
9		157.8		157.6
10		105.6		105.4
1′		123.6		123.4
2′	7.85 (2H, *d*, *J*=9.2 Hz)	128.1	7.84 (*d*, *J*=8.5 Hz)	127.9
3′	7.01(2H, *d*, *J*=9.2 Hz)	114.6	7.01 (*d*, *J*=8.5 Hz)	114.4
4′		162.6		162.5
5′	7.01(2H, *d*, *J*=9.2 Hz)	114.6	7.01 (*d*, *J*=8.5 Hz)	114.4
6′	7.85 (2H, *d*, *J*=9.2 Hz)	128.1	7.84 (*d*, *J*=8.5 Hz)	127.9
OH-5	12.82 (1H, *s*)	162.2	12.81 (br. *s*)	162.0
OCH_3-7	3.88 (3H, *s*)	55.9	3.88 (*s*)	55.5
OCH_3-4′	3.89 (3H, *s*)	55.6	3.90 (*s*)	55.8

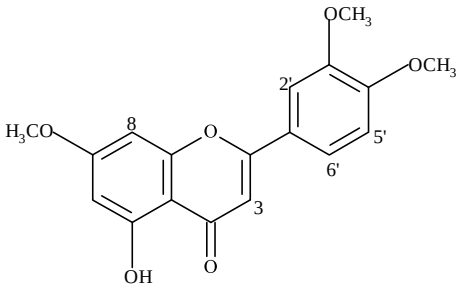
Note: * Measured at 400 MHz (^{1}H) in $CDCl_3$ (Kang et al., 2014)

** Measured at 100 MHz (^{13}C) in $CDCl_3$ (Mangoyi, Midiwo, & Mukanganyama, 2015)

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4.2.2 ASBD2: Luteolin-7,3′,4′-trimethyl ether (37)



Purification of fraction C1A-5 using multiple CC and recrystallization technique afforded 15.0 mg of compound **ASBD2** as a yellow amorphous. The compound was dissolved in CH_2Cl_2 and appeared as a yellow fluorescence spot on TLC after sprayed with the *p*-anisaldehyde reagent. The UV spectrum (Figure 4.6) for this compound showed the maximum absorption at 270 and 340 nm. The FTIR spectrum of **ASBD2** (Figure 4.7) showed the absorptions at 3074 and 2928 cm^{-1} indicating the presence of *sp2* and *sp3* C-H stretching, respectively. Absorption at 3689 cm^{-1} was attributed to alcohol (O-H) group, while the band at 1739 and 1618 cm^{-1} revealed the presence of carbonyl group and C=C, accordingly. The molecular formula of **ASBD2** was $C_{18}H_{16}O_6$ as established from the LC-MS spectrum in accordance with the appearance of a significant peak at *m/z* 329.09903 $[M+H]^+$ (Figure 4.8).

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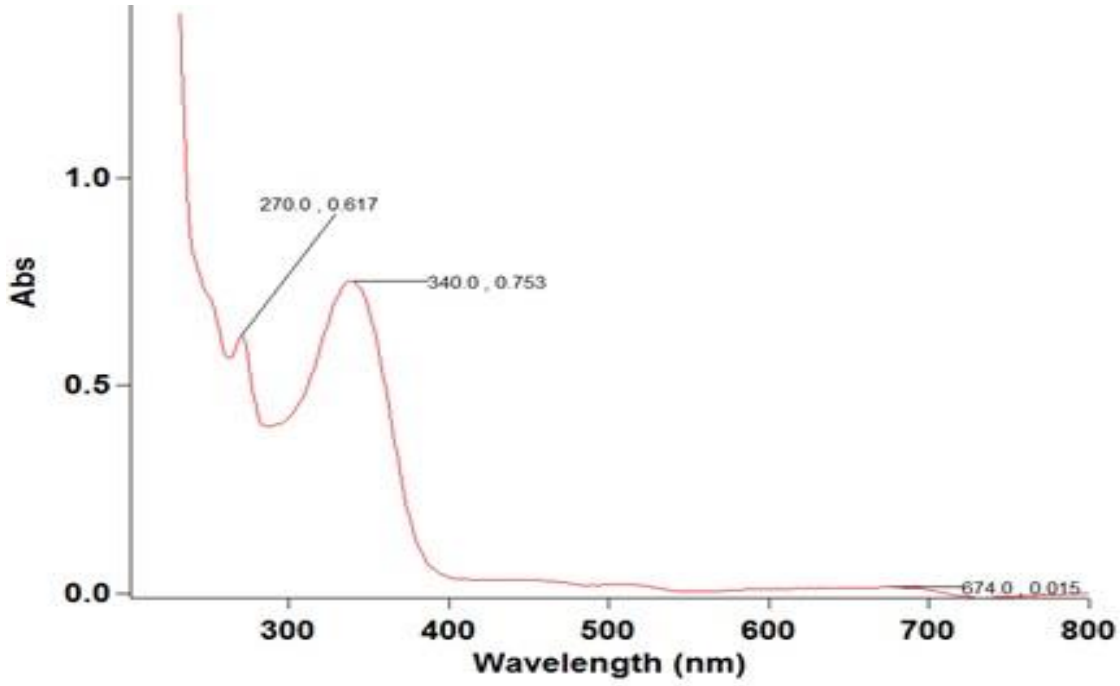


Figure 4.6. UV spectrum of compound **ASBD2**

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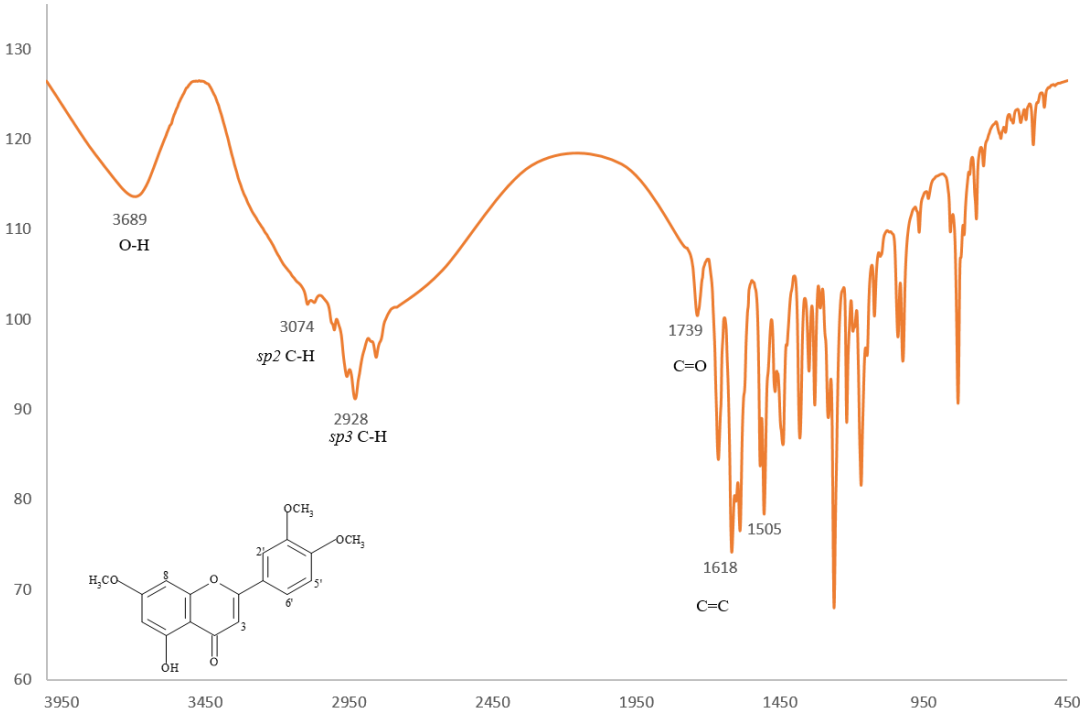


Figure 4.7. IR spectrum of compound **ASBD2**

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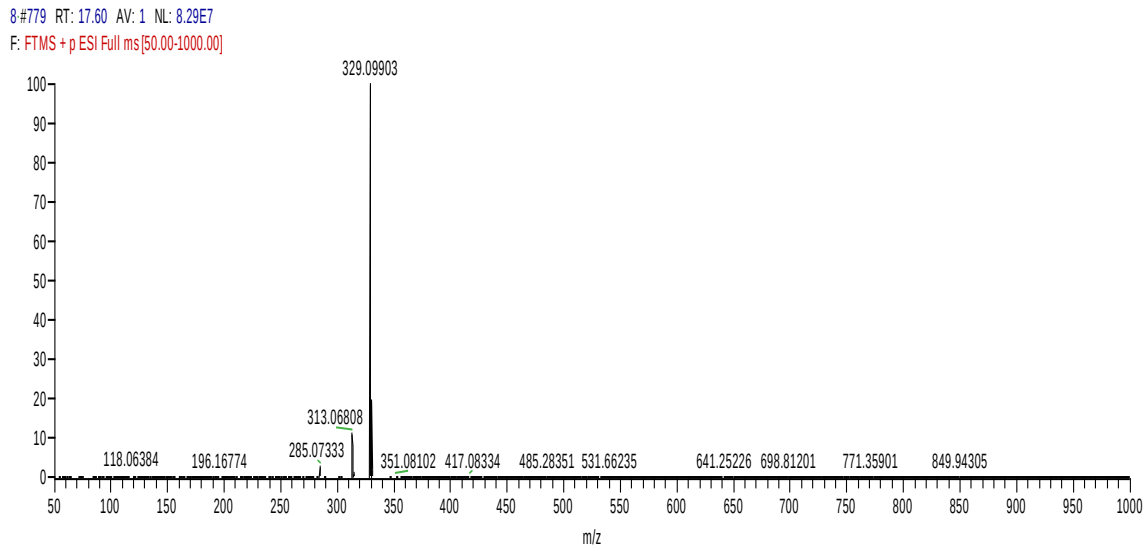


Figure 4.8. LC-MS spectrum of compound **ASBD2**

The ^{1}H-NMR spectrum of **ASBD2** ($CDCl_3$, 500 MHz) in Figure 4.9 displayed resonances of three singlets of three methoxyl groups at δ 3.89, 3.97 and 3.98, and assigned to OCH_3-7, OCH_3-3′, and OCH_3-4′, respectively. A singlet of aromatic proton H-3 was observed at δ 6.59. In addition, four doublets of aromatic proton were exhibited at δ 6.38 (H-6, *J*= 2.3 Hz), δ 6.50 (H-8, *J* = 2.3 Hz), δ 6.99 (H-5′, *J* = 8.6 Hz) and δ 7.34 (H-2′, *J* = 1.7 Hz). Another doublet of doublet (*J* = 8.6, 2.3 Hz) of H-6′ was identified at δ 7.53. A broad singlet corresponded to the hydroxyl group was observed at δ 12.80.

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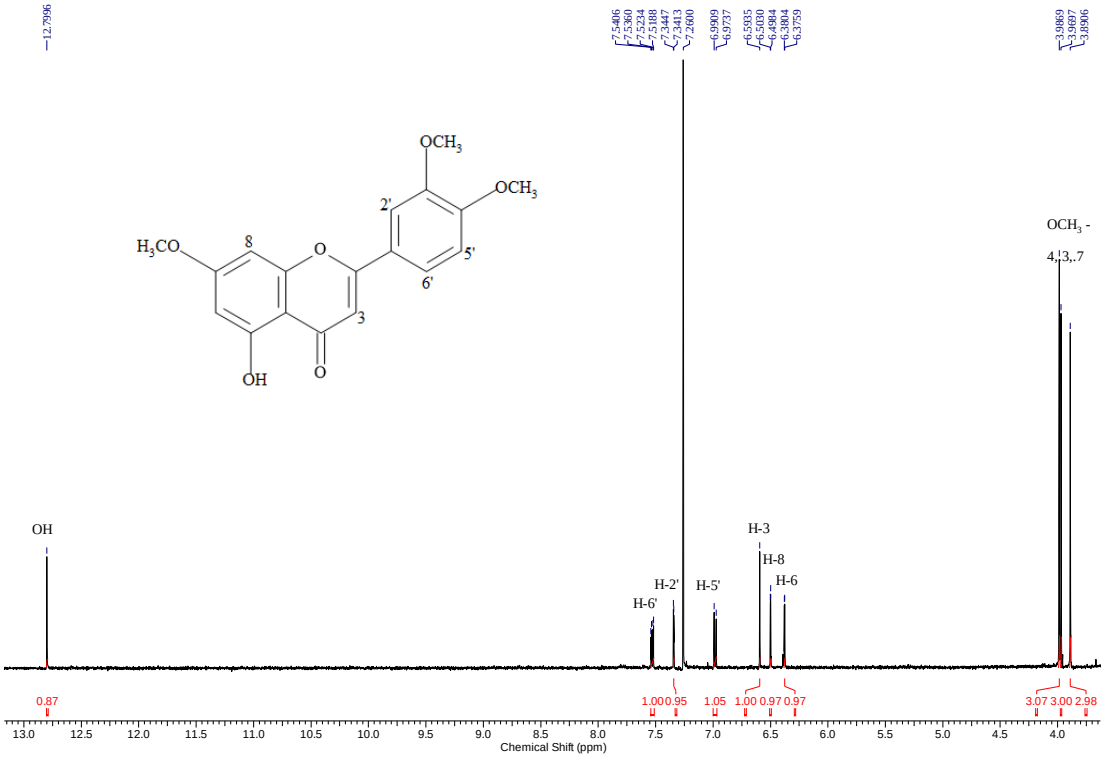


Figure 4.9. ^{1}H-NMR spectrum of compound **ASBD2**

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The ^{13}C-NMR spectrum of **ASBD2** in Figure 4.10 showed the presence of carbon C-4 (C=O) at δ 182.5 and three methoxy groups (C-7, C-3′, and C-4′) at δ 55.9 and 56.2, respectively. A comparison of the spectroscopic data from compound **ASBD2** with the literature data has led to the identification of luteolin-7,3′,4′-trimethyl ether **(37)**, which previously isolated from the leaf (Kang et al., 2014) and flower of *A. sinensis* (Chu et al., 2016).

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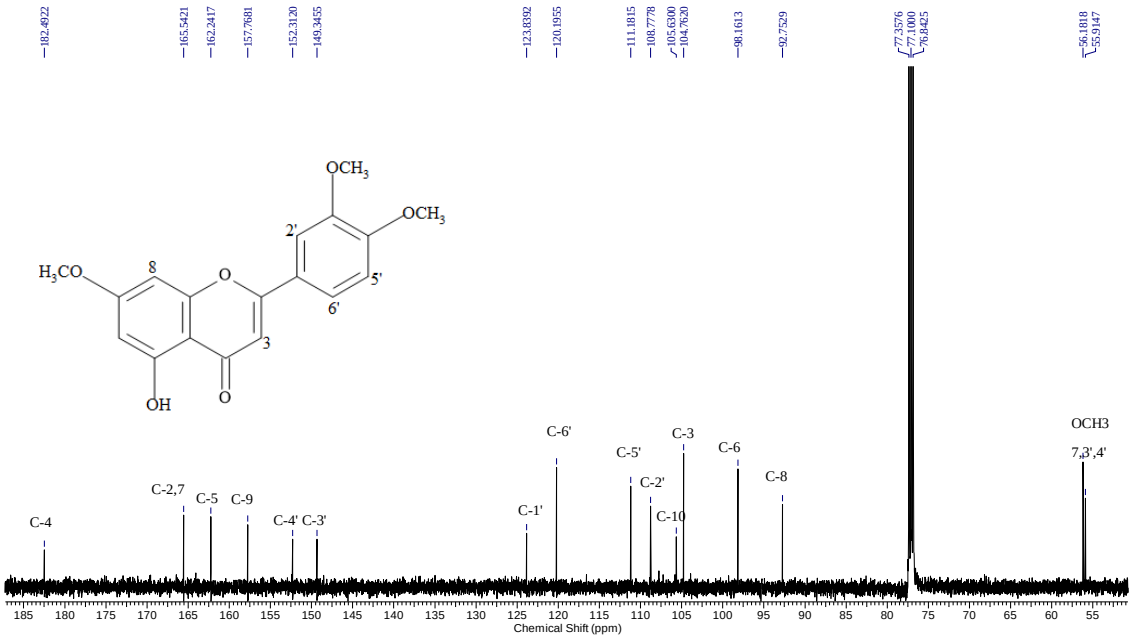


Figure 4.10. ^{13}C-NMR spectrum of compound **ASBD2**

Table 4.3

*^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C (125 MHz, $CDCl_3$) spectral data of compound **ASBD2** and luteolin-7,3′,4′-trimethyl ether (**37**)*

	Compound ASBD2		**Luteolin-7,3′,4′-trimethyl ether (37)**	
No	**δ^{1}H (ppm)**	**δ^{13}C (ppm)**	***δ^{1}H (ppm)**	****δ^{13}C (ppm)**
1				
2		165.5		163.8
3	6.59 (1H, *s*)	104.7	6.59 (*s*)	103.1
4		182.5		179.1
5		162.2		163.3
6	6.38 (1H, *d*, *J*=2.3 Hz)	98.1	6.38 (*d*, *J*=2.5 Hz)	98.2
7		165.5		167.6
8	6.50 (1H, *d*, *J*=2.3 Hz)	92.6	6.50 (*d*, *J*=2.5 Hz)	92.9
9		157.8		156.6
10		105.6		106.0
1′		123.8		122.0
2′	7.34 (1H, *d*, *J*=1.7 Hz)	108.8	7.35 (*d*, *J*=2.0 Hz)	114.9
3′		149.3		147.5

(continued)

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Table 4.3 *(continued)*

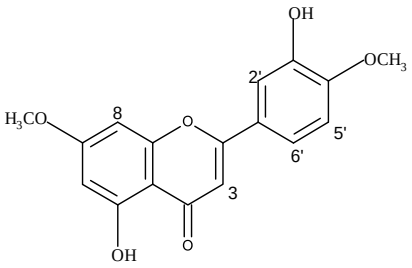
	Compound ASBD2		**Luteolin-7,3′,4′-trimethyl ether (37)**	
No	**δ^1H (ppm)**	**δ^{13}C (ppm)**	***δ^1H (ppm)**	****δ^{13}C (ppm)**
4′		152.3		149.0
5′	6.97 (1H, *d, J*=8.6 Hz)	111.2	6.98 (*d, J*=8.5 Hz)	114.9
6′	7.53(1H, *dd, J*= 8.6, 2.3 Hz)	120.2	7.53 (*dd, J*=8.5, 2.0 Hz)	121.0
OH-5	12.80 (1H, *s*)		12.80 *(s*)	
OCH_3-7	3.89 (3H, *s*)	56.1	3.89 (*s*)	55.9
OCH_3-3′	3.97 (3H, *s)*	56.1	3.97 (s)	56.2
OCH_3-4′	3.99 (3H, *s*)	55.9	3.99 (*s*)	56.5

Note: * Measured at 400 MHz (^{1}H) in $CDCl_3$ (Kang et al., 2014)

** Measured at 75 MHz (^{13}C) in $CDCl_3$ (Morales & Paredes, 2014)

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4.2.3 ASBD3: 5,3′-Dihydroxy-7,4′-dimethoxyflavone (41)



Compound **ASBD3** (5.0 mg, 0.17%) was isolated as yellow amorphous from the C1A-5 fraction using multiple CC and dissolved in CH_2Cl_2. This compound appeared as a dark spot under short wave UV (254 nm), and remained as a pale yellow spot and turned to yellow fluorescence after derivatisation with *p*-anisaldehyde's reagent. The UV spectrum (Figure 4.11) for this compound showed maximum absorptions at 270 and 333 nm identifying a flavone skeleton (Meng et al., 2008). The IR spectrum of **ASBD3**

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showed strong absorptions at 3701 cm^{-1} and 1595 cm^{-1} attributed to O-H and C=C functional groups, respectively (Figure 4.12**)**. Its molecular formula of $C_{17}H_{14}O_6$ was supported by its molecular ion peak at *m/z* 313.07132 $[M-H]^+$ analysed using ESI-MS technique (Figure 4.13).

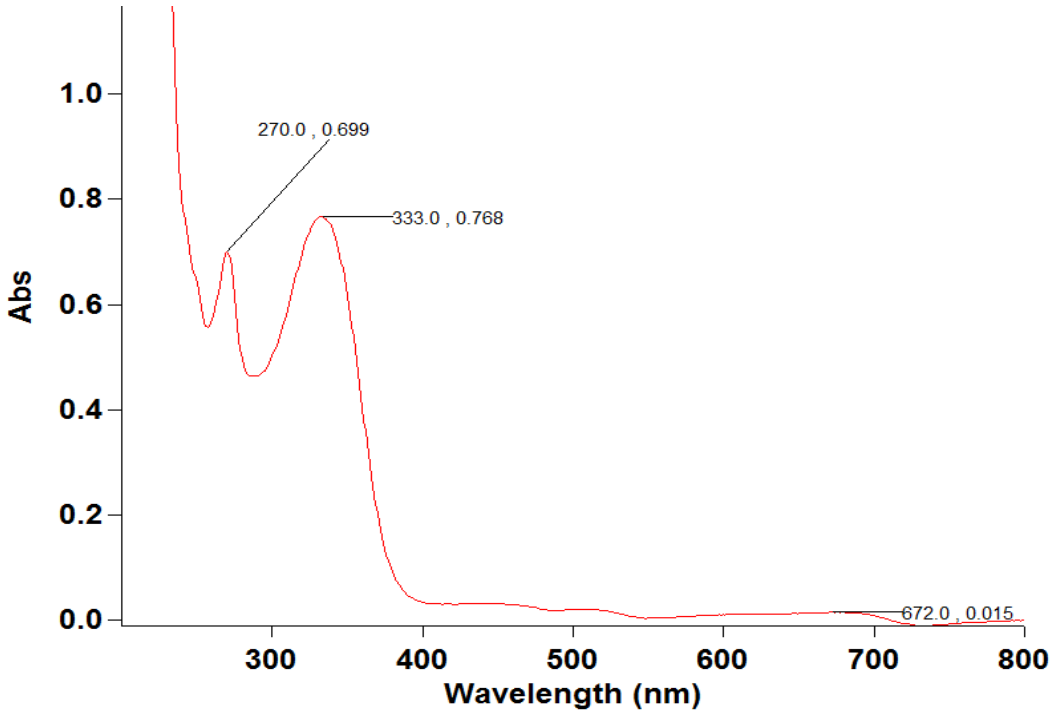


Figure 4.11. UV spectrum of compound **ASBD3**

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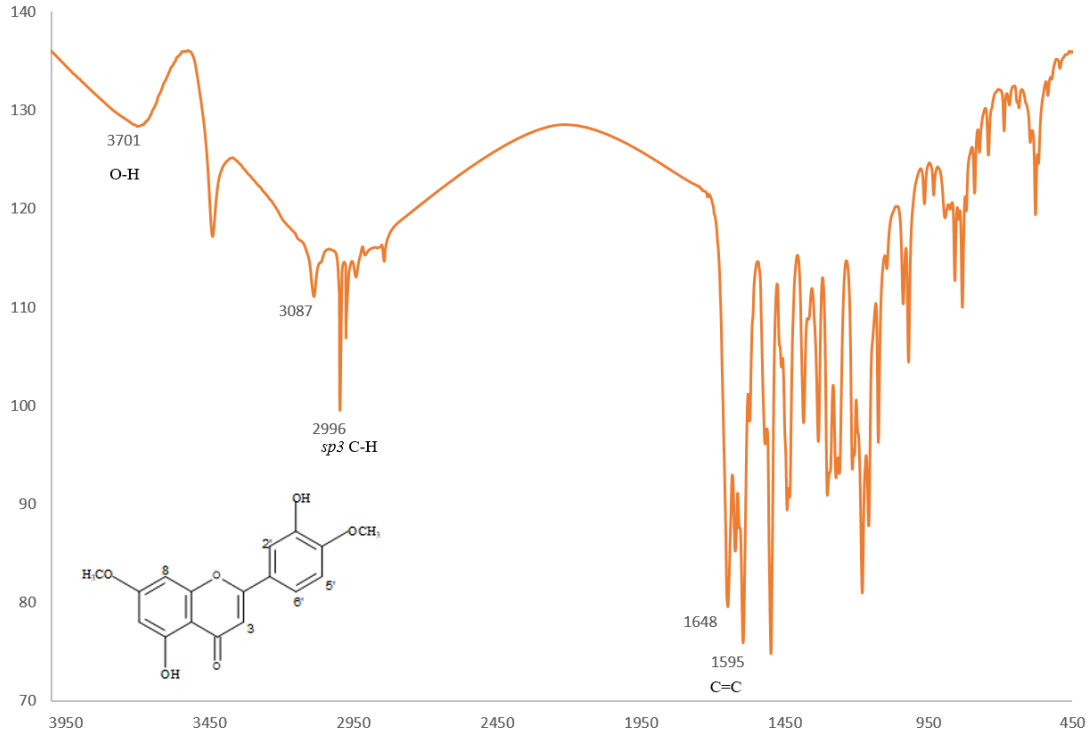


Figure 4.12. IR spectrum of compound **ASBD3**

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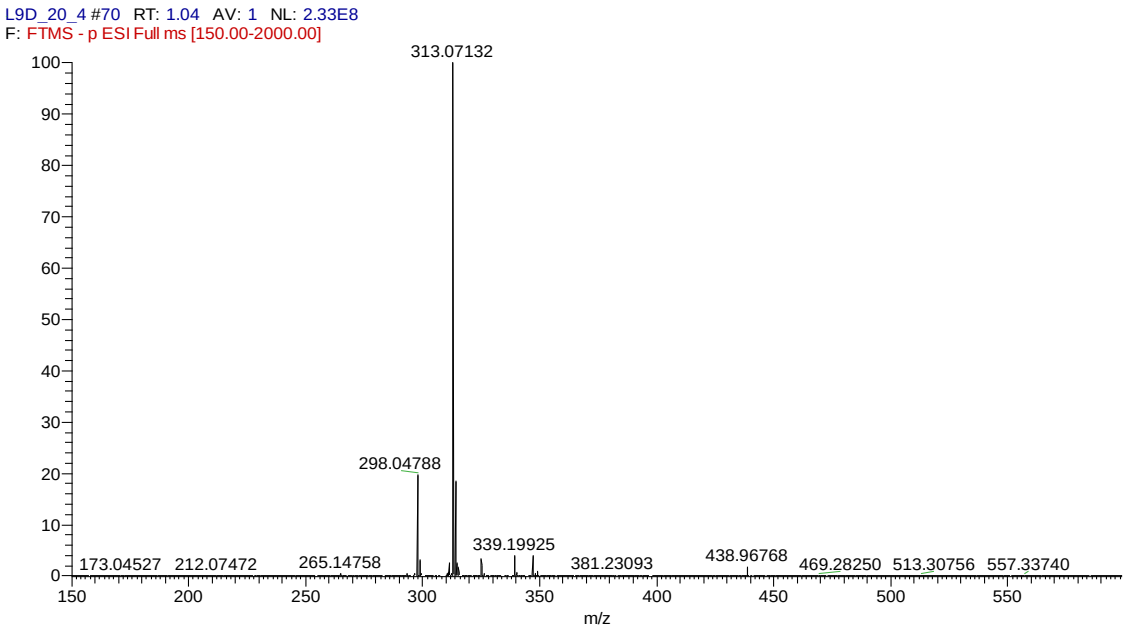


Figure 4.13. LC-MS spectrum of compound **ASBD3**

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In the ^{1}H-NMR spectrum of **ASBD3** (Figure 4.14) showed a resonance of two singlets of methoxyl at δ 4.00 (OCH_3-4′) and δ 3.88 (OCH_3-7). Besides, six aromatics have displayed; one singlet at δ 6.57 attributed to H-3 and two doublets at δ 6.37 (*J* = 2.3 Hz) and 6.49 (*J* = 2.3 Hz) characteristic of *meta*-protons assigned to H-6 and H-8, respectively. Another *meta*-proton was displayed at δ 7.33 (*J* = 1.7 Hz) allocated to H-2′. Meanwhile, one doublet with *ortho* position was shown at δ7.04 (*J* = 8.6 Hz) belonged to H-5′. Another proton δ 7.50 (H-6′) showed a signal doublet of the doublet with coupling constant, *J* = 8.6, and 2.3 Hz. Two broad singlets of the hydroxyl group at δ 12.80 and 6.06 were attributed to OH-5 and OH-3′, accordingly.

The ^{13}C-NMR spectrum of **ASBD3** in Figure 4.15 revealed 17 carbon signals. Eight methine carbons displayed at δ 164.1, 162.2, 165.5, 157.7, 108.3, 123.4, 146.9 and 149.2 were assigned to C-2, C-5, C-7, C-9, C-10, C-1′, C-3′, and C-4′, respectively. The presence of a carbonyl (δ 182.5) and two methoxyl carbons (δ 55.9 and 56.2) was assigned to C-4, OCH_3-7 and OCH_3-4′, respectively.

Based on the analysed spectral data and comparison with the literature, the structure of compound **ASBD3** was elucidated as 5,3′-dihydroxy-7,4′-dimethoxyflavone (**41**) or known as illion, which was formerly isolated from the leaf of *A. sinensis* (Kang et al., 2014).

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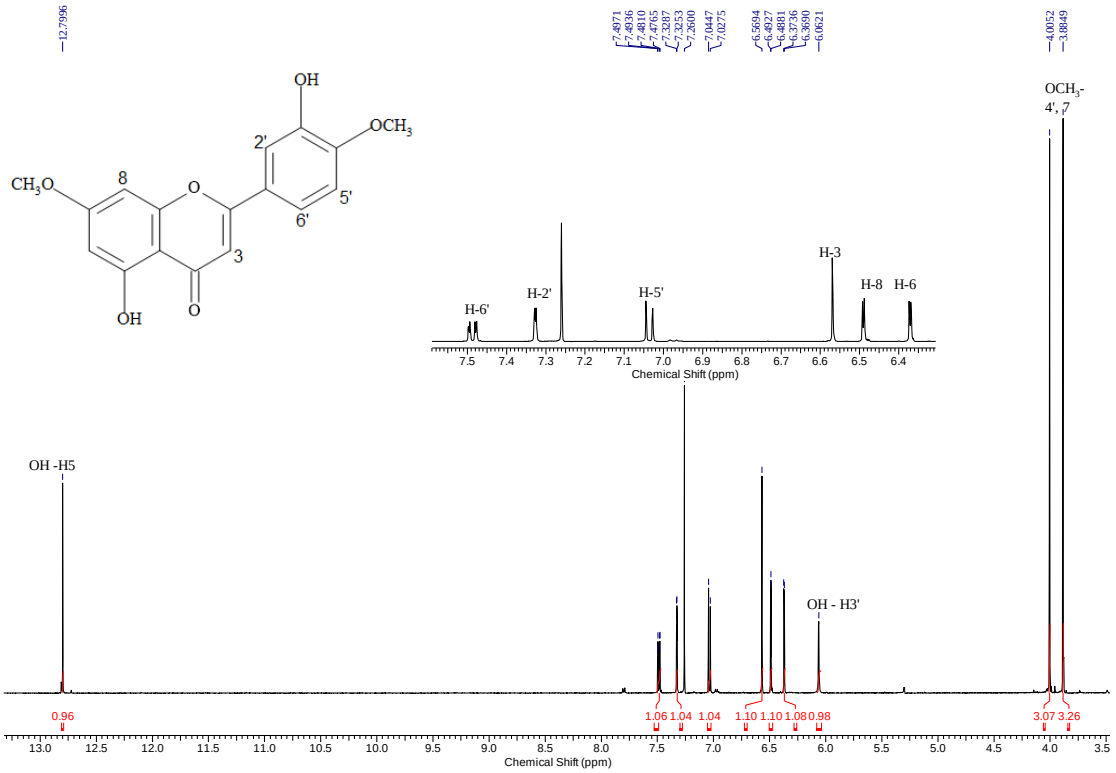


Figure 4.14. ^{1}H-NMR spectrum of compound **ASBD3**

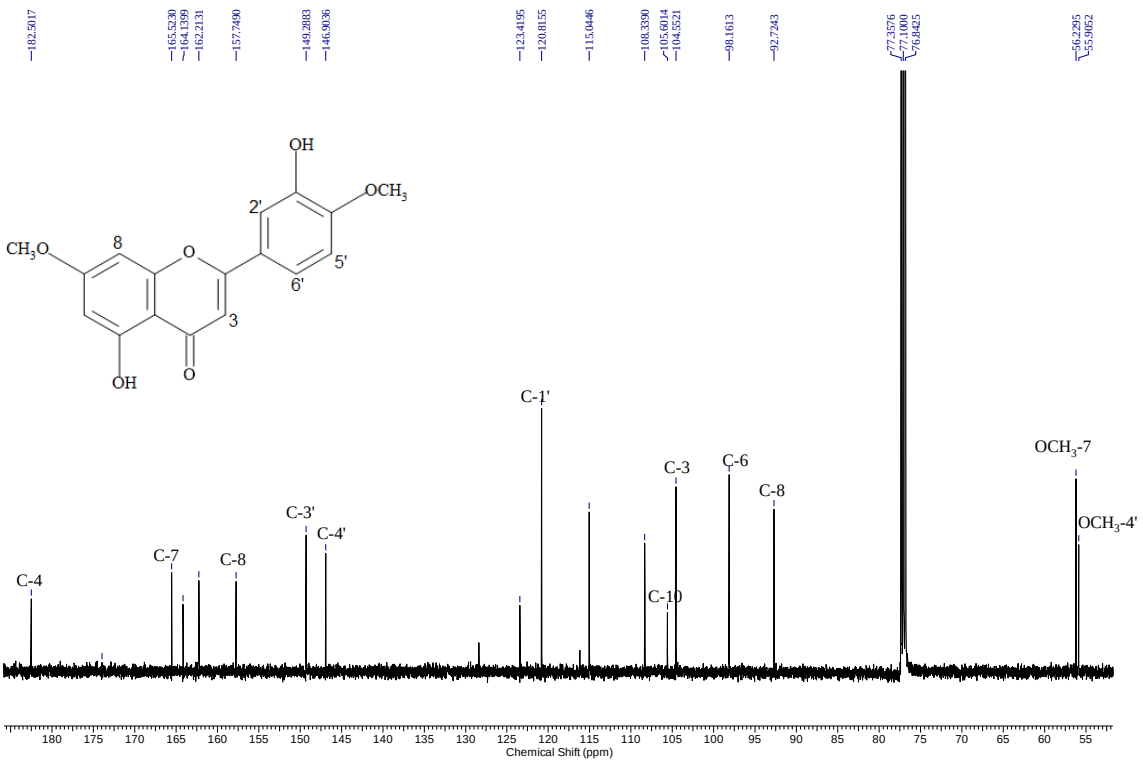


Figure 4.15. ^{13}C-NMR spectrum of compound **ASBD3**

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Table 4.4

^{1}H-NMR (500 MHz, $CDCl_3$) and ^{13}C (125 MHz, $CDCl_3$) spectral data of compound ***ASBD3*** *and 5,3′-dihydroxy-7,4′-dimethoxyflavone (**41**)*

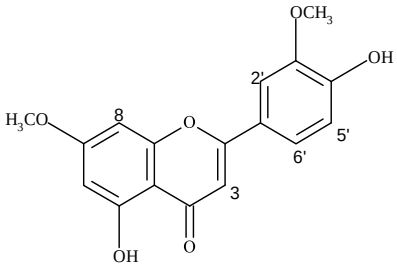
	Compound ASBD3		**5,3′-dihydroxy-7,4′-dimethoxyflavone (41)**
No	**δ^{1}H (ppm)**	**δ^{13}C (ppm)**	***δ^{1}H (ppm)**
1			
2		164.1	
3	6.57 (1H, *s*)	104.5	6.57 (*s*)
4		182.5	
5		162.2	
6	6.37 (1H, *d*, *J*=2.3 Hz)	98.1	6.37 (*d*, *J*=2.4 Hz)
7		165.5	
8	6.49 (1H, *d*, *J*=2.3 Hz)	92.7	6.49 (*d*, *J*=2.4 Hz)
9		157.7	
10		108.3	
1′		123.4	
2′	7.33 (1H, *d*, *J*=1.7 Hz)	115.0	7.35 (*d*, *J*=2.4 Hz)
3′		146.9	
4′		149.2	
5′	7.04 (1H, *d*, *J*=8.6 Hz)	115.0	7.04 (*d*, *J*=8.4 Hz)
6′	7.50 (1H, *dd*, *J*=2.3, 8.0 Hz)	120.8	7.49 (*dd*, *J*=8.4, 2.4 Hz)
OH-5	12.80 (1H, *s*)		12.80 (*s*)
OH-3′	6.06 (1H, s)		
OCH_3-7	3.88 (3H, *s*)	55.9	3.89 (*s*)
OCH_3-4′	4.01 (3H, *s*)	56.2	4.01 (s)

Note: * Measured at 400 MHz (^{1}H) in $CDCl_3$ (Kang et al., 2014)

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4.2.4 ASBD4: 7,3′-Dimethoxyluteolin (185)



Compound **ASBD4** (5.0 mg, 0.17%) was obtained as a yellow amorphous from the CH_2Cl_2 extract (fraction C1A-5). After sprayed with *p*-anisaldehyde reagent, the TLC spot of this compound displayed a yellow fluorescence, indicating the presence of a flavonoid compound. The LC-MS analysis revealed a very significant molecular ion peak at *m/z* 315.0866 $[M+H]^+$ and supported by its molecular formula, $C_{17}H_{14}O_6$ (Figure 4.16).

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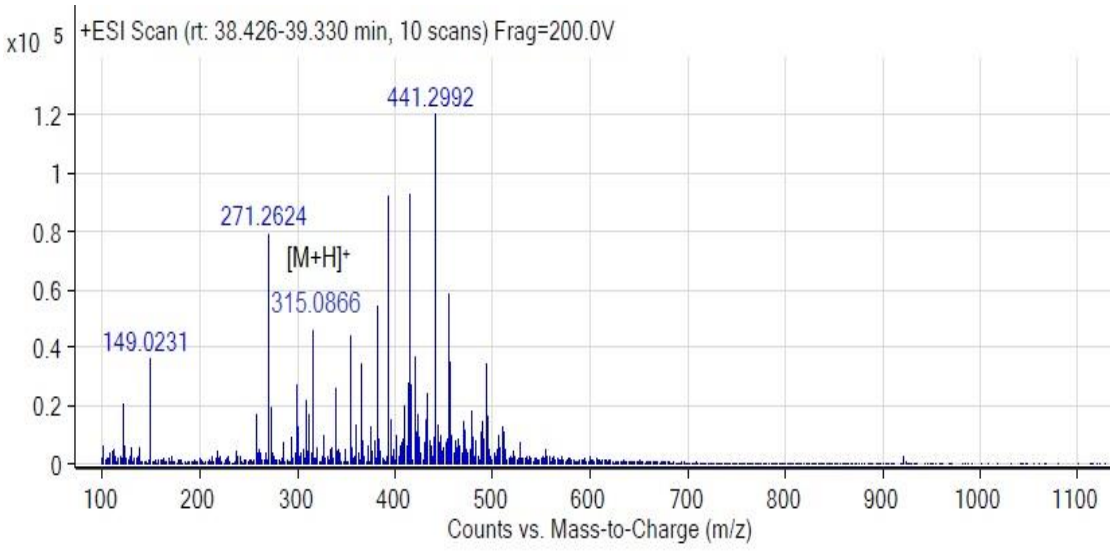


Figure 4.16. LC-MS spectrum of compound **ASBD4**

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The IR spectrum of **ASBD4** (Figure 4.17) exhibited the presence of the hydroxyl group at 3748 cm^{-1} and also showed the presence of C-H stretching at 2917 cm^{-1}. The absorption peak at 1739 and 1608 cm^{-1} displayed the existence of C=O and C=C group, respectively. Moreover, The UV spectrum of **ASBD4** (Figure 4.18) displayed absorption at 270 and 334 nm in accordance with a flavone skeleton (Meng et al., 2008).

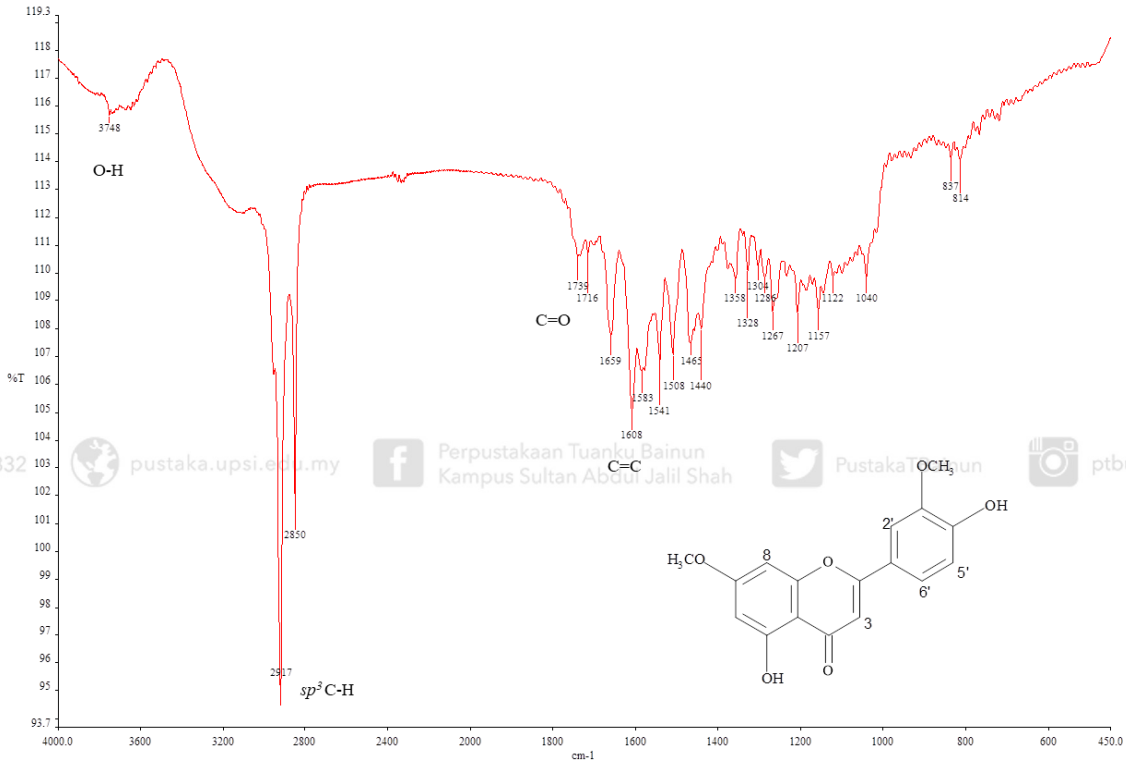


Figure 4.17. IR spectrum of compound **ASBD4**

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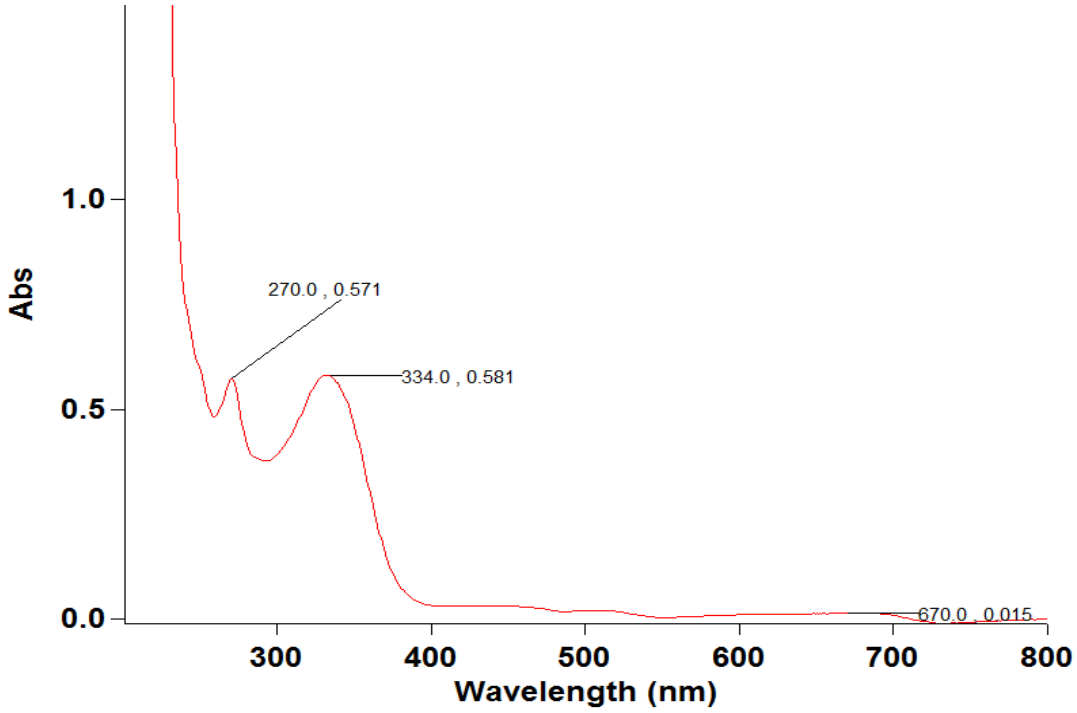


Figure 4.18. UV spectrum of compound **ASBD4**

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The ^{1}H-NMR spectrum of **ASBD4** in Figure 4.19 showed resonances for two methoxyl groups, at δ 3.88 (OCH_3-7) and δ 3.99 (OCH_3-3′). Three doublets of aromatic protons were exhibited at δ 6.37 (*J* = 2.3 Hz), 6.49 (*J* = 2.3 Hz), and 7.47 (*J* = 1.7 Hz) with *meta* position, corresponded to H-8, H-6, and H-2′, respectively. Another doublet was appeared at δ 6.95 (*J* = 8.5 Hz), characteristic of *para*-proton, attributed to H-5′. Also, a doublet of doublet aromatic proton was displayed at δ 7.43 (*J* = 8.0, 2.3 Hz, H-6′) and a sharp one-proton resonance for the one aromatic proton at δ 6.58 was also observed correspond to H-3. A broad singlet of the hydroxyl group at δ 12.79 (OH-5 and OH-4′) was also identified in this spectrum.

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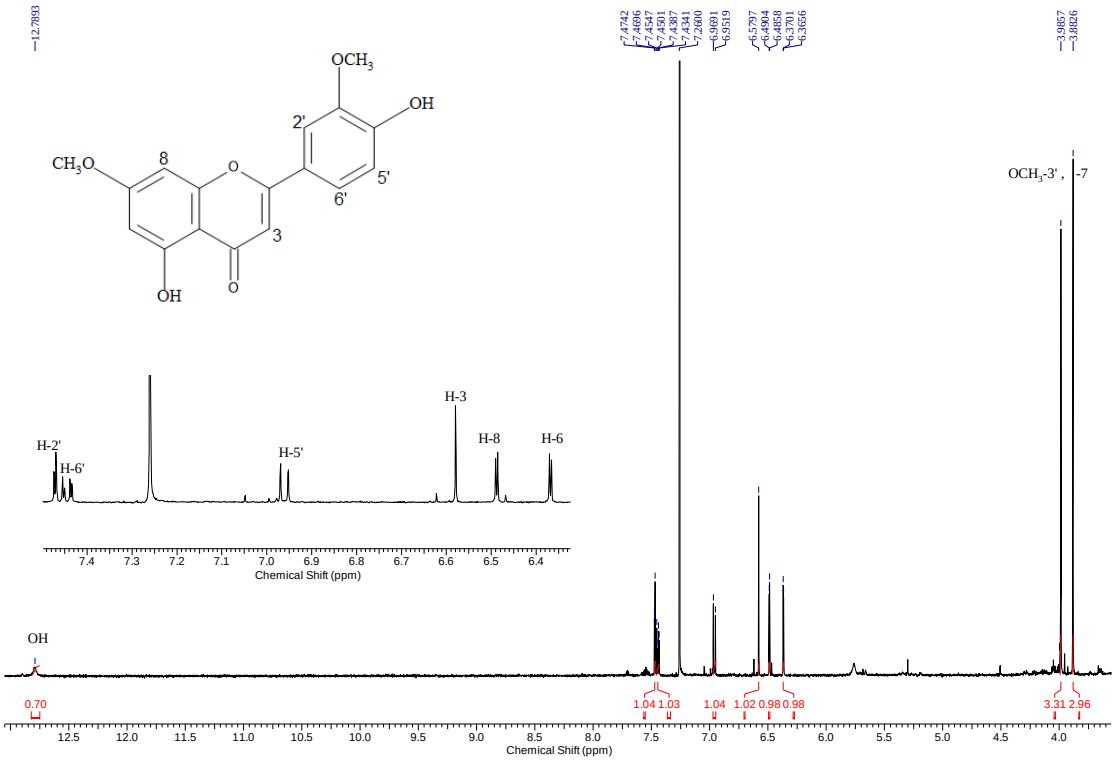


Figure 4.19. ^{1}H-NMR spectrum of compound **ASBD4**

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The ^{13}C-NMR spectrum of **ASBD4** in Figure 4.20 exhibited 17 signals of carbons. The spectrum showed the presence of a carbonyl (δ 182.5) and two methoxyl carbons (δ 55.9 and 56.2) which assigned to C-4, OCH_3-7, and OCH_3-3′, respectively.

Based on the analysis of its physical properties and comparison with spectroscopic data with literature reviews, compound **ASBD4** was determined to be 7,3′-dimethoxyluteolin (**185**) or known as 5,4′-dihydroxy-7,3′-dimethoxyflavone (Morales & Paredes, 2014) which had been isolated previously from the methanolic crude extract of *A. chinensis* (Sharmin et al., 2014).

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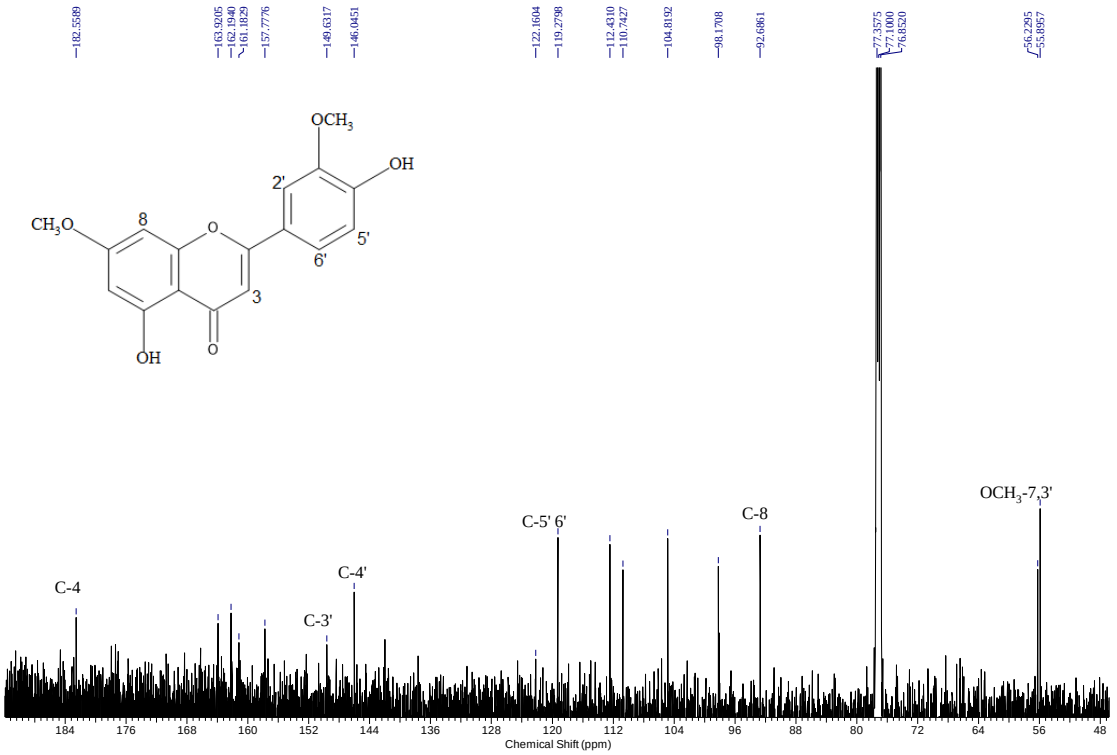


Figure 4.20. ^{13}C-NMR spectrum of compound **ASBD4**

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Table 4.5

*^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C (125 MHz, $CDCl_3$) spectral data of compound **ASBD4** and 7,3'-dimethoxyluteolin (**185**)*

	Compound ASBD4		**7,3′-dimethoxyluteolin (185)**	
No	**δ^{1}H (ppm)**	**δ^{13}C (ppm)**	***δ^{1}H (ppm)**	****δ^{13}C (ppm)**
1				
2		162.2		163.9
3	6.58 (1H, *s*)	104.8	6.56 (*s*)	103.2
4		182.6		180.6
5		161.9		161.1
6	6.37 (1H, *d*, *J*=2.3 Hz)	98.2	6.37 (*d*, *J*=2.3 Hz)	96.3
7		163.9		165.2
8	6.49 (1H, *d*, *J*=2.3 Hz)	92.7	6.48 (*d*, *J*=2.3 Hz)	92.7
9		157.8		155.9
10		110.4		106.5

(continued)

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Table 4.5 *(continued)*

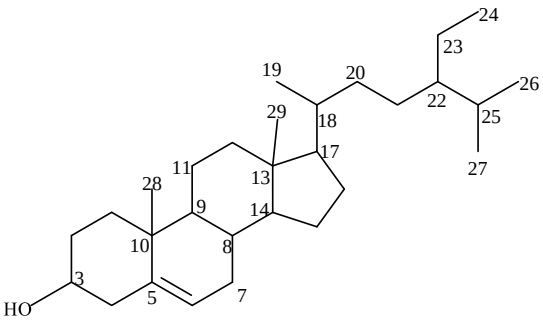
	Compound (37)		**7,3′-dimethoxyluteolin (185)**	
No	**δ^1H (ppm)**	**δ^{13}C (ppm)**	***δ^1H (ppm)**	****δ^{13}C (ppm)**
1′		122.2		123.6
2′	7.47 (1H, *d, J*=1.7)	112.4	7.47 (*d, J*=2.3)	114.9
3′		149.6		147.5
4′		146.0		145.1
5′	6.95 (1H, *d, J*=8.5)	119.2	7.03 (*d, J*=8.4)	119.0
6′	7.43 (1H, *dd, J*=8.0, 2.3 Hz)	122.2	7.46 (*dd, J*=8.4, 2.3 Hz)	121.6
OH-5	12.78 (1H, *s*)		12.96 (*s*)	
OCH_3-7	3.89 (3H, *s*)	55.9		55.9
OCH_3-3′	3.98 (3H, *s*)	56.2		56.2

Note: * Measured at 500 MHz (^{1}H) in $CDCl_3$ (Sharmin et al., 2014);

** Measured at 75 MHz (^{13}C) in $CDCl_3$ (Morales & Paredes, 2014)

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4.2.5 ASBD5: *β*-sitosterol (101)



Compound **ASBD5** was isolated from fraction C1A-5 using CC and purified by washing with cold MeOH. This white needles compound was non-active under short (254 nm) and longwave UV (365 nm), but displayed as violet spot after derivatisation

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with the *p*-anisaldehyde reagent. The LC-MS spectrum (Figure 4.21) showed an appearance of a significant peak at *m/z* 415.30276 $[M+H]^+$ which attributed to molecular formula $C_{29}H_{50}O$. Compound **ASBD5** presented a strong band of hydroxyl (O-H) group at 3403 cm^{-1}. Absorptions at 2931 and 2861 cm^{-1} showed the stretching of aliphatic alkane (C-H), while the presence of alkene group (C=C) was exhibited at band 1667 cm^{-1}. Furthermore, band at 1465 and 1050 cm^{-1} were assigned to bending vibrations of CH_2 and cycloalkane, respectively. This IR spectrum is shown in Figure 4.22.

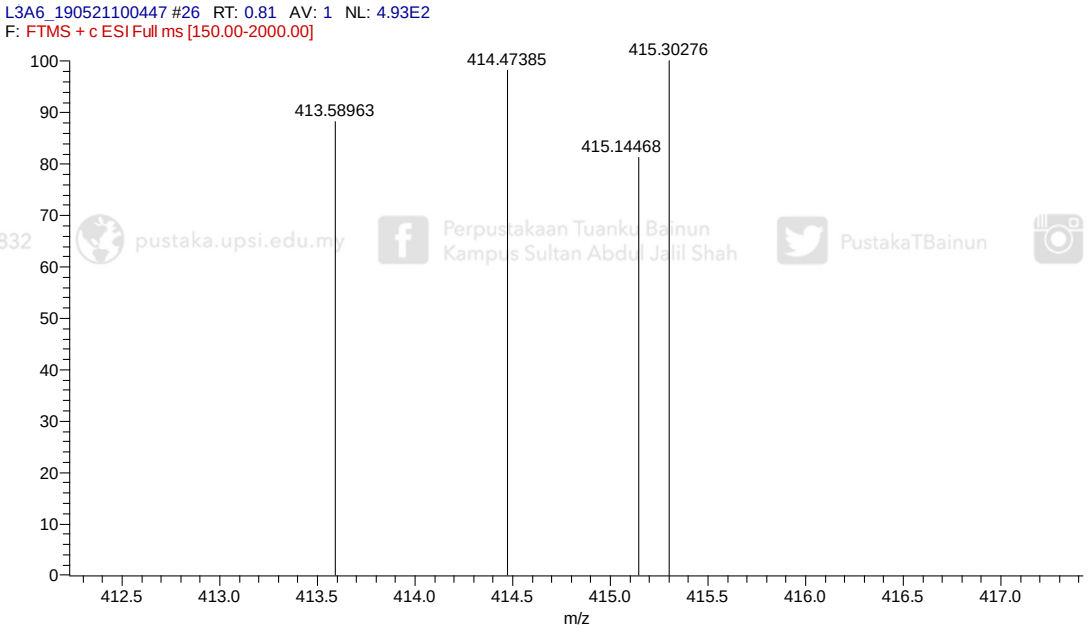


Figure 4.21. LC-MS spectrum of compound **ASBD5**

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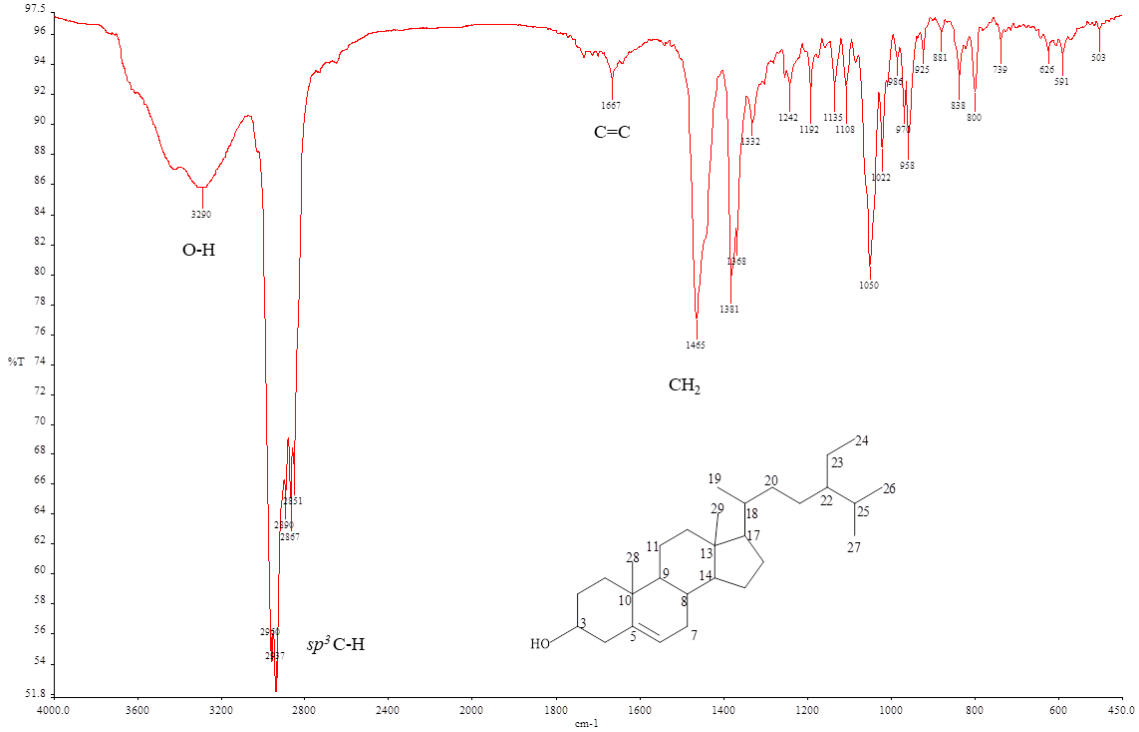


Figure 4.22. IR spectrum of compound **ASBD5**

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The ^{1}H-NMR spectrum of compound **ASBD5** stated in Figure 4.23. This spectrum showed 50 protons with the presence of two methyl singlets at δ 1.0 and 0.67 correspondings to H-29 and H-28, respectively. In addition, three methyl doublets that appeared at δ 0.92, 0.82 and 0.80, attributable to H-19, H-26, and H-27, correspondingly. A methyl triplet at δ 0.84 was assigned as H-24. A triplet with a coupling constant of 5.2 Hz resonated at δ 5.34 was attributed to an olefinic proton, H-6.

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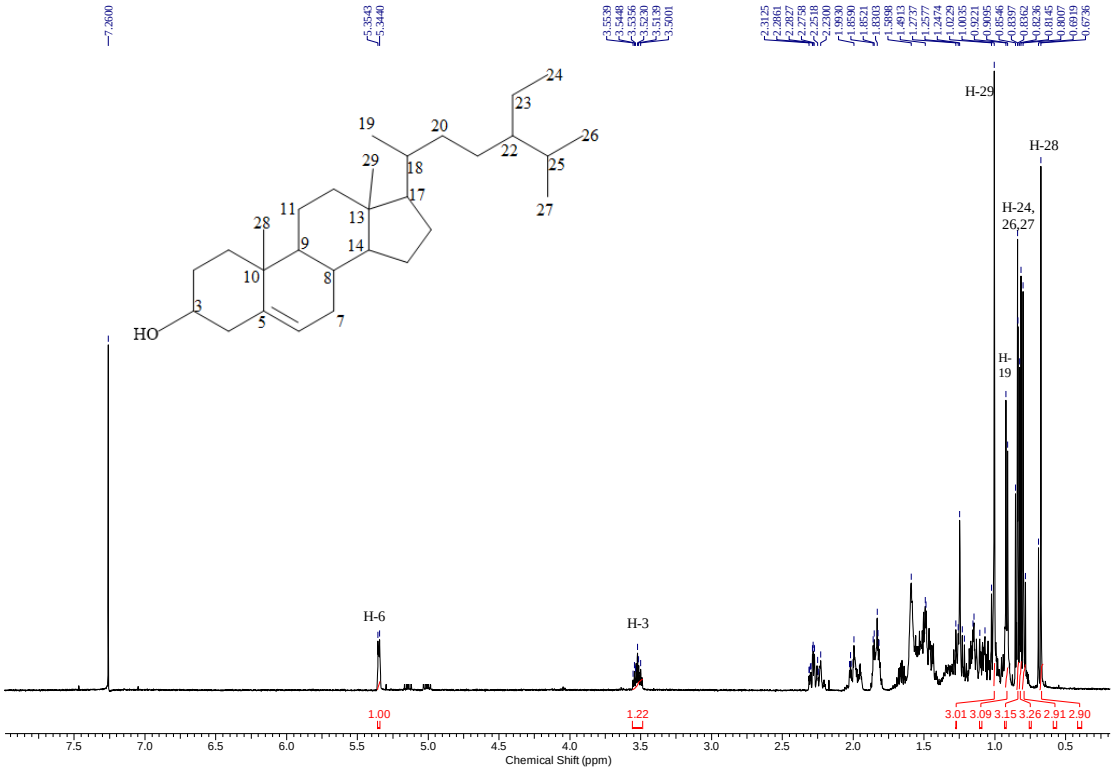


Figure 4.23. ^{1}H-NMR spectrum of compound **ASBD5**

The ^{13}C-NMR spectrum of **ASBD5** (Figure 4.24) showed 29 signals, including six methyl carbons (CH_3) at δ 19.1, 12.1, 19.9, 19.5, 18.9, and 11.9, assigned to C-19, C-24, C-26, C-27, C-28, and C-29, accordingly. Eleven methylene carbons (CH_2) were appeared at δ 37.3 (C-1), 31.7 (C-2) 42.4 (C-4), 32.0 (C-7), 21.2 (C-12), 39.8 (C-15), 26.1 (C-16), 28.3 (C-16), 34.0 (C-20), 26.1 (C-21), and 23.1 (C-23). Three quaternary carbons displayed at δ 140.7, 36.6, and 42.3, attributed to C-5, C-10, and C-13, respectively. An isolated signal at δ 71.9 was assigned to C-3 due to the presence of a hydroxyl group. The spectral data ^{1}H and ^{13}C in Table 4.3 are consistent with the reported literature values of *β*-sitosterol (**101**), which previously isolated from *Rubus suavissimus* (Sai, Chaturvedula, & Prakash, 2012).

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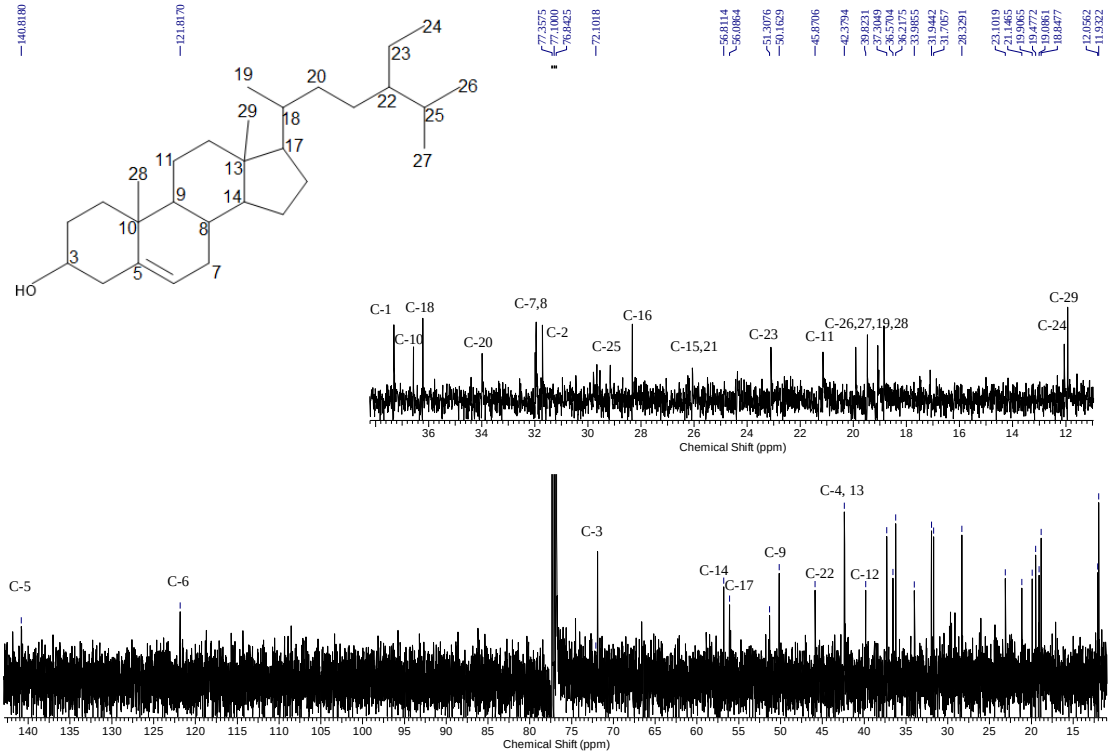


Figure 4.24. ^{13}C NMR spectrum of compound **ASBD5**

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Table 4.6

^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C (125 MHz, $CDCl_3$) spectral data of compound ***ASBD5*** *and β – sitosterol (**101**)*

	Compound ASBD5		***β*-sitosterol (101)**	
No	**δ^{1}H (ppm)**	**δ^{13}C (ppm)**	***δ^{1}H (ppm)**	***δ^{13}C (ppm)**
1		37.3		37.5
2		31.7		31.9
3	3.51 (1H, *tdd*, *J*= 4.6, 5.2, 6.3 Hz)	72.1	3.53	72.0
4		42.4		42.5
5		140.8		140.9
6	5.34 (1H, *t*, *J*=5.2 Hz)	121.8	5.36	121.9
7		32.0		32.1
8		32.0		32.1
9		50.2		50.3
10		36.6		36.7

(continued)

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Table 4.6 *(continued)*

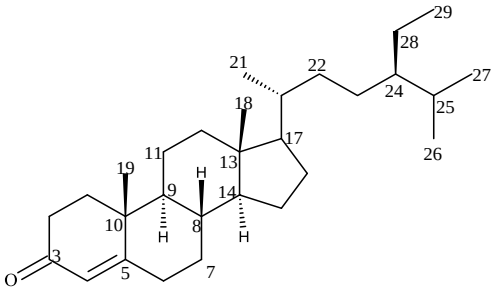
	Compound ASBD5		***β*-sitosterol (101)**	
No	**δ^{1}H (ppm)**	**δ^{13}C (ppm)**	***δ^{1}H (ppm)**	***δ^{13}C (ppm)**
11		21.1		21.3
12		39.8		39.9
13		42.4		42.6
14		56.8		56.9
15		26.1		26.3
16		28.4		28.5
17		56.2		56.3
18		36.2		36.3
19	0.92 (3H, *d*, *J*=6.5 Hz)	19.1	0.93	19.2
20		34.0		34.2
21		26.1		26.3
22		45.9		46.1
23		23.1		23.3
24	0.84 (3H, *t*, *J*= 7.5 Hz)	12.1	0.84	12.2
25		29.2		29.4
26	0.82 (3H, *d*, *J*= 4.6 Hz)	19.9	0.83	20.1
27	0.80 (3H, *d*, *J*= 6.3 Hz)	19.5	0.81	19.6
28	0.67 (3H, *s*)	18.9	0.68	19.0
29	1.0 (3H, *s*)	11.9	1.01	12.0

Note: * Measured at 600 MHz (^{1}H) and 150 MHz (^{13}C) in $CDCl_3$ (Sai, Chaturvedula, & Prakash, 2012)

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4.2.6 ASBD6: *β*-sitostenone (139)



Chromatographic purification of the fraction from CH_2Cl_2 extract by multiple CC yielded compound **ASBD6** as a white amorphous. The TLC analysis was non-active under UV and displayed a brown spot after spraying with *p*-anisaldehyde reagent, suggesting a steroid compound. The IR spectrum (Figure 4.25) displayed several absorption bands which were observed at 2955 cm^{-1} (sp^3 C-H stretching), 1679 cm^{-1} (carbonyl stretching), and 1465 cm^{-1}(C=C). Its molecular formula of $C_{29}H_{48}O$ was supported by its molecular ion peak at *m/z* 413.26709 $[M+H]^+$ analysed using ESI-MS technique (Figure 4.26).

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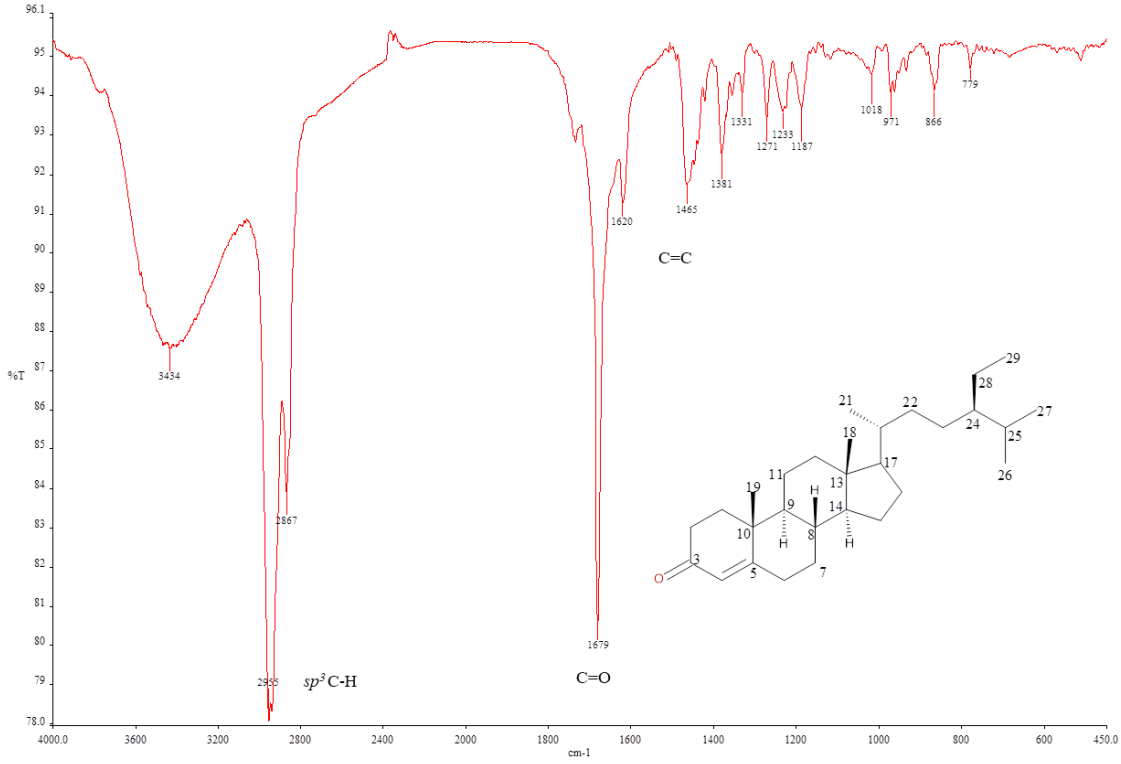


Figure 4.25. IR spectrum of compound **ASBD6**

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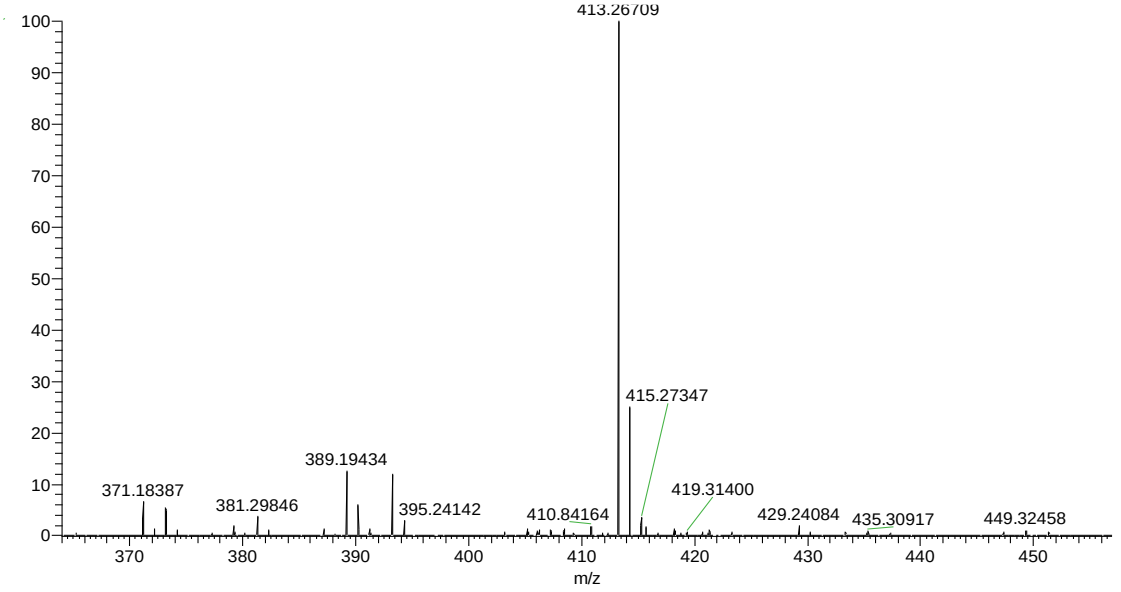


Figure 4.26. LC-MS spectrum of compound **ASBD6**

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The [1]H-NMR spectrum of **ASBD6** (Figure 4.27) showed two methyl singlet signals at δ 0.70 and 1.18 corresponding to H-18 and H-19, respectively. Other methyls were resonated at δ 0.91 (*d, J*=6.9 Hz, H-21), 0.82 (*d, J*=4.6 Hz, H-26), 0.80 (*d, J*=8.0 Hz, H-27) and 0.83 (*t*, J=7.5 Hz, H-29). Meanwhile, a singlet signal resonated at δ 5.72 and was assigned to proton located at C-4.

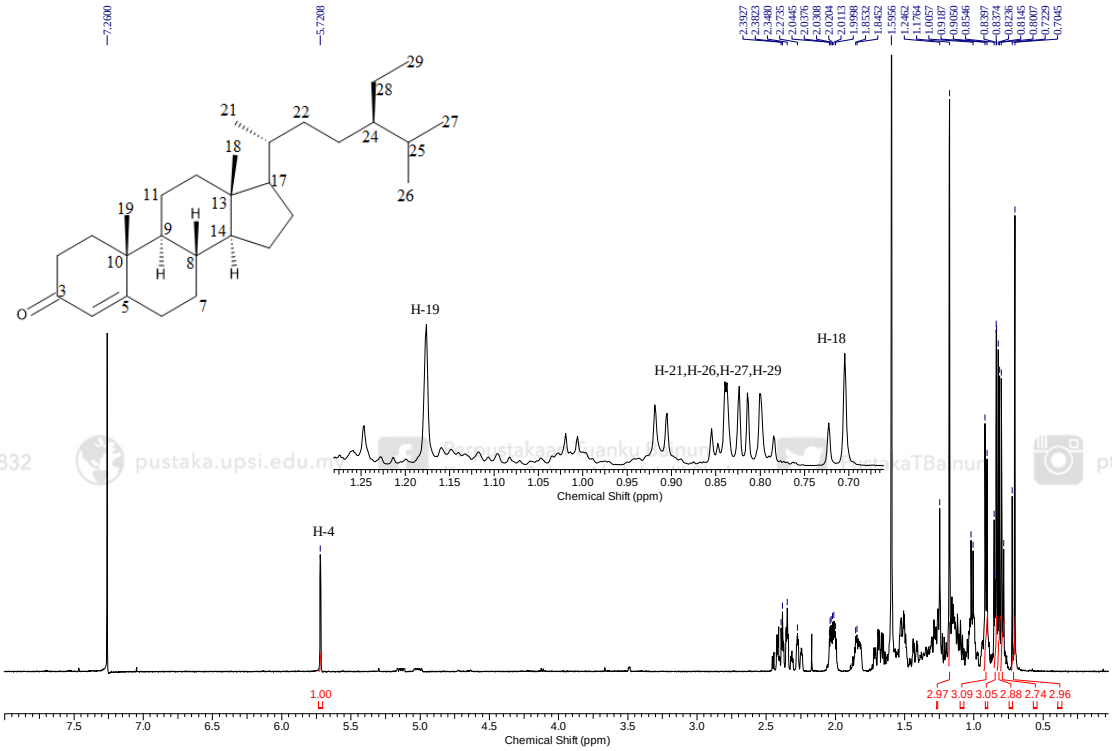


Figure 4.27. [1]H-NMR spectrum of compound **ASBD6**

The [13]C-NMR spectrum of **ASBD6** (Figure 4.28) exhibited 29 signals; consist of six methyl carbons (CH_3) at δ 12.2, 18.8, 19.1, 21.1, 19.9, and 12.0, assigned to C-18, C-19, C-21, C-26, C-27, and C-29, accordingly. Eleven methylene carbons (CH_2) were displayed at δ 36.2 (C-1), 34.1 (C-2), 33.9 (C-6), 33.0 (C-7), 21.2 (C-11), 38.7 (C-12), 24.3 (C-15), 28.3 (C-16), 35.8 (C-22), 26.1 (C-23), and 23.1 (C-28). Three quaternary carbons exhibited at δ 171.97, 39.7 and 42.5, attributed to C-5, C-10, and C-

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13, respectively. The spectral data of ^{1}H and ^{13}C-NMR of **ASBD6** in Table 4.7 are consistent with the reported literature values of β-sitostenone (**139**) which formerly obtained from *Spilanthes acmella* Murr. (Compositae) (Prachayasittikul et al., 2009).

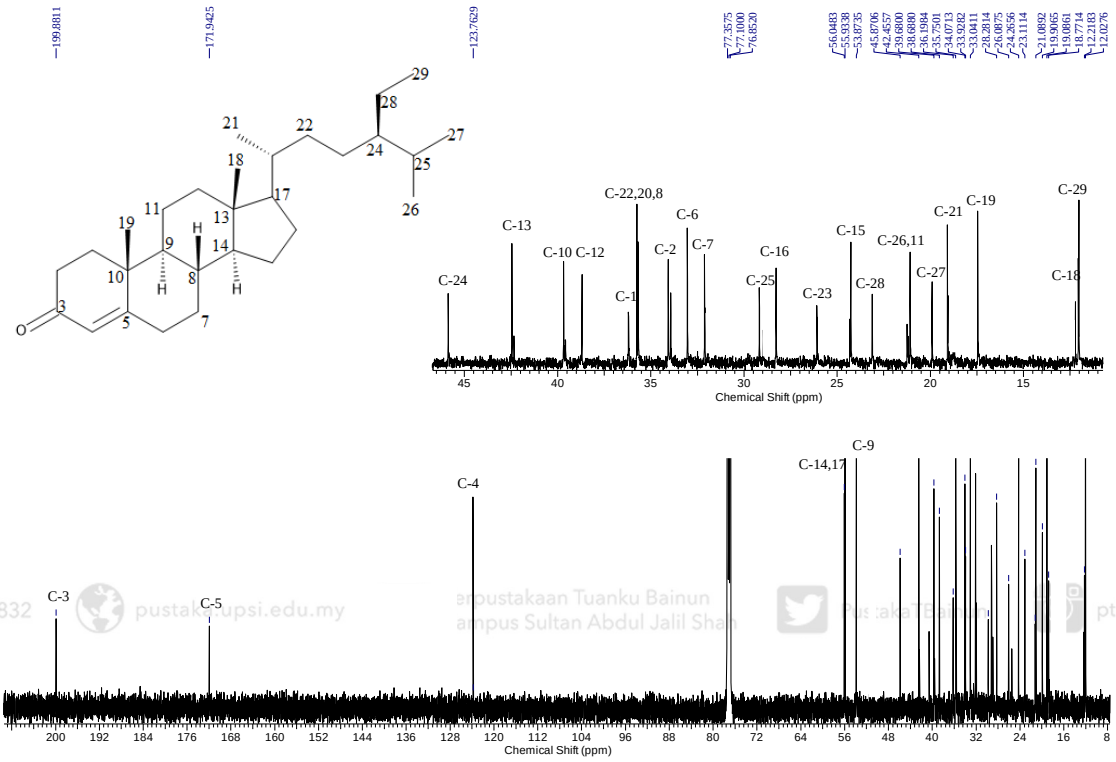


Figure 4.28. ^{13}C-NMR spectrum of Compound **ASBD6**

Table 4.7

*^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C (125 MHz, $CDCl_3$) spectral data of compound **ASBD6** and β-sitostenone (**139**)*

	Compound ASBD6		***β*-sitostenone (139)**	
No	**$\delta^{1}H$ (ppm)**	**$\delta^{13}C$ (ppm)**	****$\delta^{1}H$ (ppm)**	***$\delta^{13}C$ (ppm)**
1		36.2		36.1
2		34.1		33.9
3		199.9		199.6
4	5.72 (1H, *s*)	123.8	5.74 (1H, *s*)	123.7
5		171.9		171.6

(continued)

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Table 4.7 *(continued)*

	Compound ASBD6		***β*-sitostenone (139)**	
No	**δ^1H (ppm)**	**δ^{13}C (ppm)**	****δ^1H (ppm)**	***δ^{13}C (ppm)**
6		33.9		33.9
7		33.0		32.9
8		35.7		35.6
9		53.9		53.8
10		39.7		39.6
11		21.2		21.1
12		38.7		38.6
13		42.5		42.4
14		55.9		55.9
15		24.3		24.1
16		28.3		28.1
17		56.0		56.0
18	0.70 (3H, *s*)	12.2	0.71 (3H, *s*)	11.9
19	1.18 (3H, *s*)	18.8	1.18 (3H, *s*)	18.7
20		35.8		36.1
21	0.91 (3H, *d, J*=6.9 Hz)	19.1	0.93 (3H, *d, J*=6.6 Hz)	19.0
22		35.8		35.7
23		26.1		26.1
24		45.9		45.8
25		29.7		29.6
26	0.82 (3H, *d, J*=4.6 Hz)	21.1	0.84 (3H, *d, J*=6.8 Hz)	21.0
27	0.80 (3H, *d, J*=8.0 Hz)	19.9	0.82 (3H, *d, J*=6.8 Hz)	19.8
28		23.1		23.0
29	0.83 (3H, *t, J*=7.5 Hz)	11.4	0.85 (3H, *t, J*=7.2 Hz)	11.1

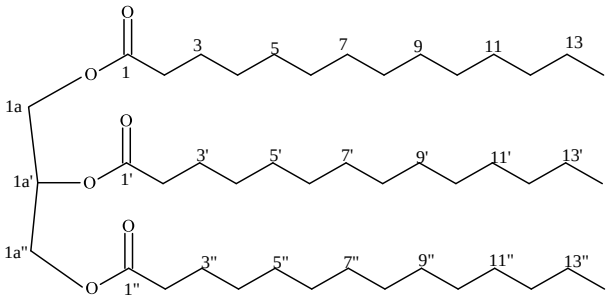
Note: * Measured ^{13}C in $CDCl_3$ (Prachayasittikul et al., 2009)

** Measured at 400 MHz (^{1}H) in $CDCl_3$ (Ara, Rahman, Rahman, Hasan, & Rashid, 2009)

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4.2.7 ASBD7: Saturated Triglyceride Fatty Acid (186)



Compound **ASBD7** (10.0 mg) was successfully isolated from a combination of fractions C1A-1 and C1A-2 from CH_2Cl_2 extract and obtained as a white solid. On the TLC analysis, the compound was observed as a dark spot under short wave UV (254 nm). The molecular formula of **ASBD7** is $C_{45}H_{86}O_6$ correspond with *m/z* 723.58582 $[M+H]^+$ (Figure 4.29). The IR spectrum displayed the maximum absorptions of the carbonyl group at 1738 cm^{-1} and C–O (stretching) band at 1176 cm^{-1} which characterized the ester groups (Tariq, Ali, Ahmad, Ahmad, & Zafar, 2011). The absorption band at 1470 cm^{-1} was attributed to CH_2, while the band at 2917 cm^{-1} showed the presence of C-H (stretching) (Figure 4.30).

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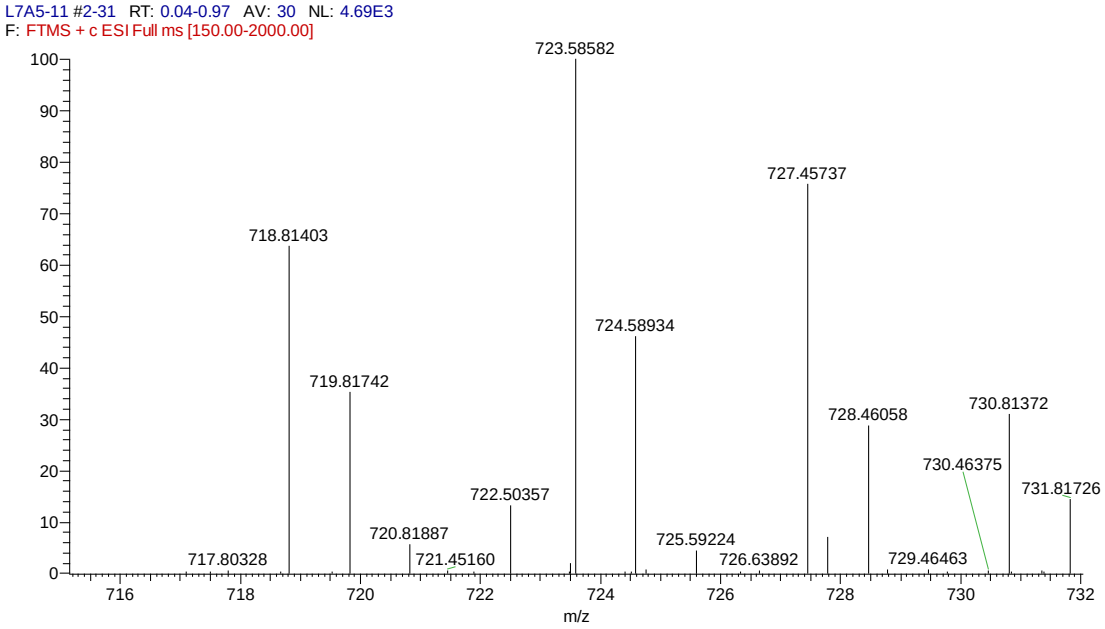


Figure 4.29. LC-MS spectrum of compound **ASBD7**

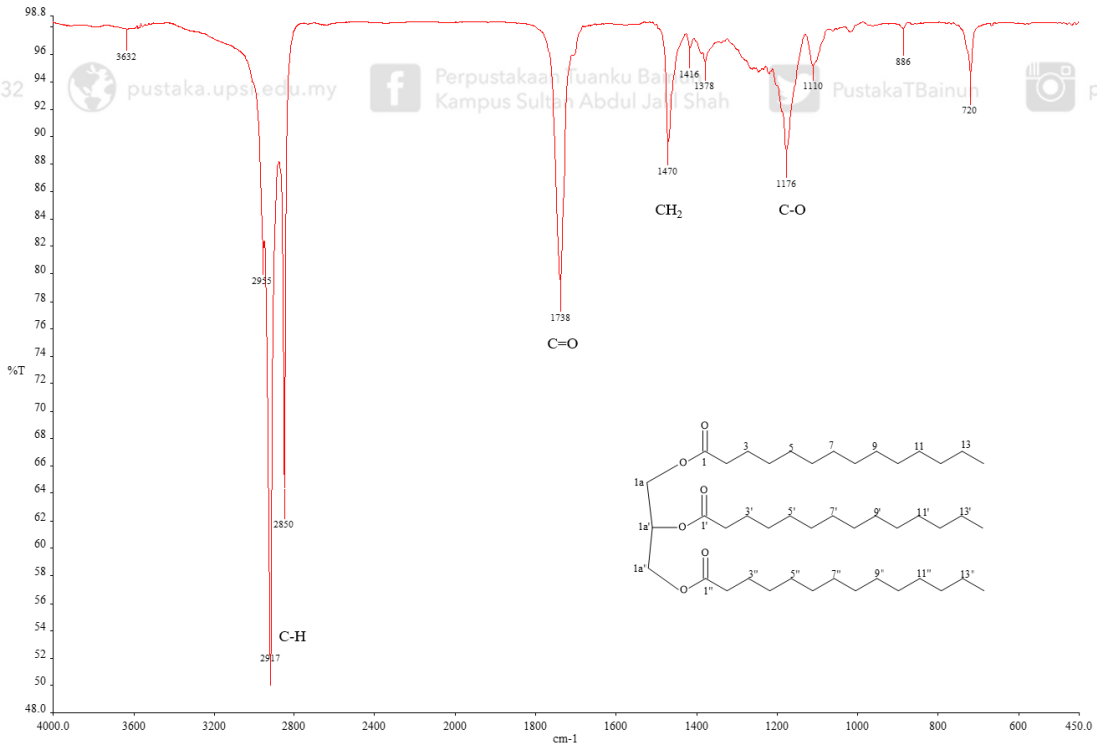


Figure 4.30. IR spectrum of compound **ASBD7**

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The ^{1}H-NMR spectrum of compound **ASBD7** was shown in Figure 4.31. A multiplet signal at δ 5.26 was assigned to H-1a′ position of the glycerol backbone. The presence doublet of doublet signals at δ 4.27 and 4.14 attributed to H-1a and H-1a″ positions with coupling constant values of 4.1, 4.0, 5.8 and 5.7, respectively. These signals indicated the presence of glycerol, and the assignments are interchangeable.

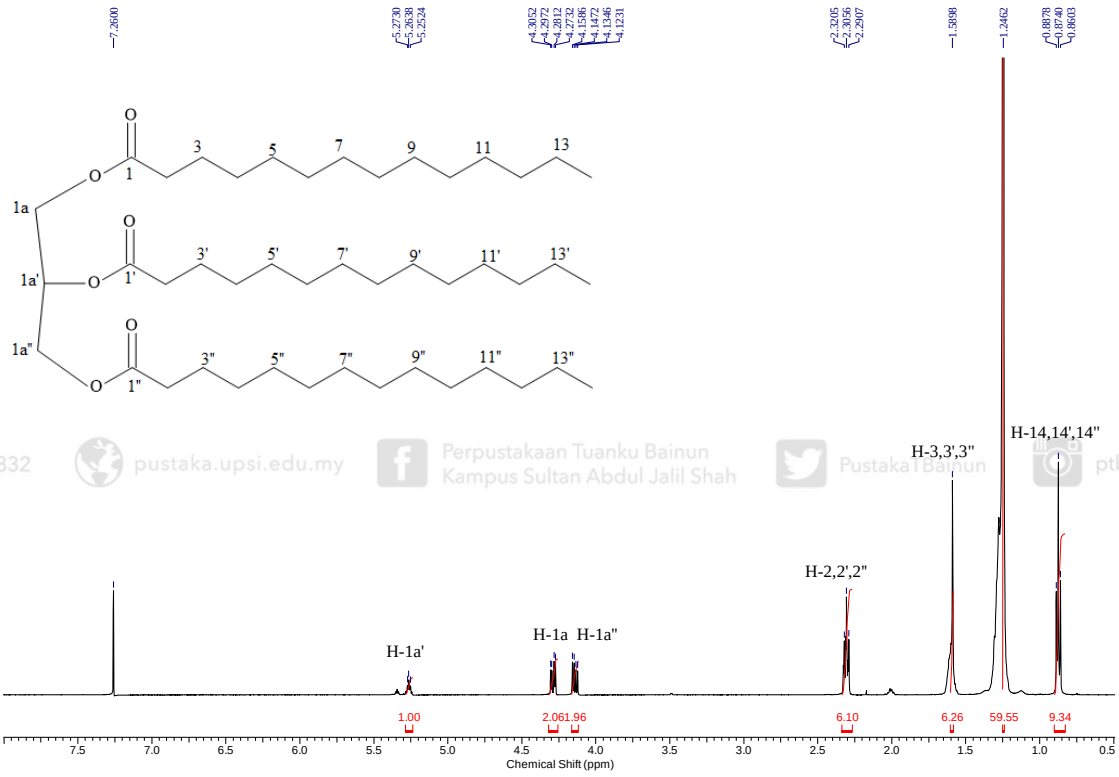


Figure 4.31. ^{1}H-NMR spectrum of compound **ASBD7**

The ^{13}C-NMR spectrum of compound **ASBD7** was displayed in Figure 4.32. This spectrum indicated three carbonyl carbons at δ 173.4 (C-1 and C-1″) and 173.0 (C-1′). Also, one methine carbon exhibited at δ 68.9, 38 methylene carbons were assigned at δ 34.1 (C-2, C-2′, C-2″), 24.9 (C-3, C-3′, C-3″), 29.2 - 29.8 (C-4, C-4′, C-4″; C-5, C-5′, C-5″; C-6, C-6′, C-6″; C-7, C-7′, C-7″; C-8, C-8′, C-8″; C-9, C-9′, C-9″; C-10, C-10′,

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C-10″; C-11, C-11′, C-11″), 32.0 (C-12, C-12′, C-12″), 22.8 (C-13, C-13′, C-13″), and 62.2 (C-1a, C-1a″) as well as three methyl carbons appeared at δ 14.2 (C-14, C-14′, C-14″).

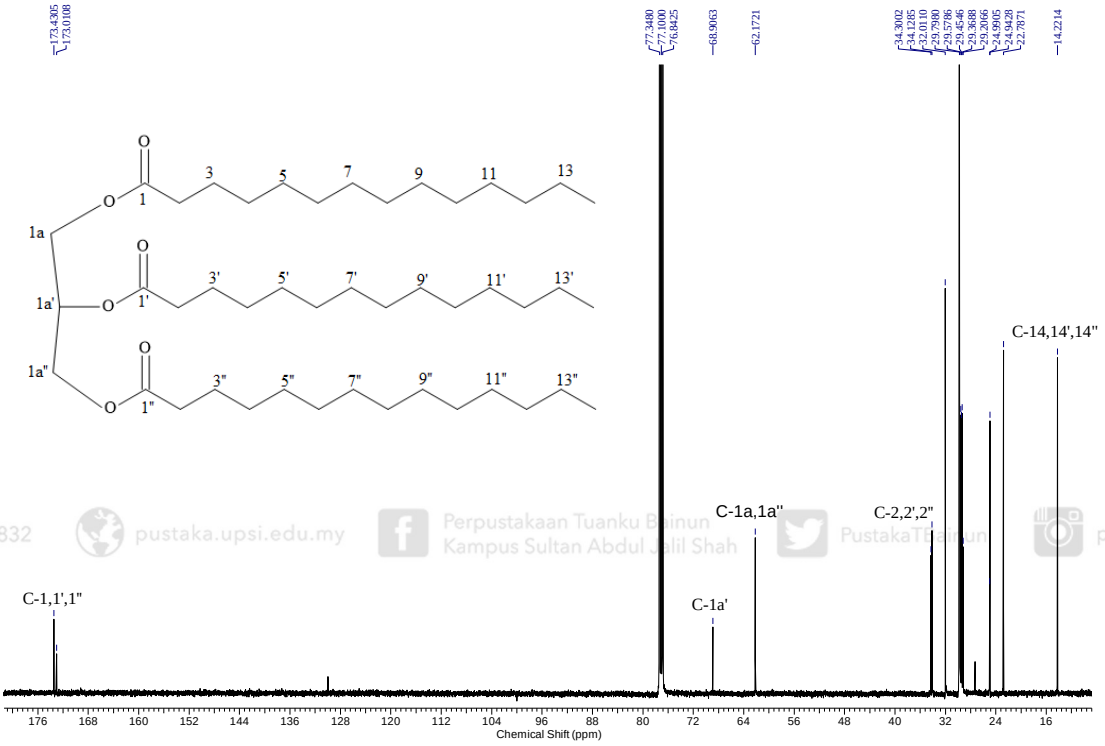


Figure 4.32. ^{13}C-NMR spectrum of compound **ASBD7**

Two dimensional NMR analyses provided additional evidence regarding the structure of compound **ASBD7**. The COSY spectrum (Figure 4.33) revealed the ^{1}H-^{1}H correlations between H-2 with H-3, H-1a′ with H-1a, and H-1a″ as well as H-13 with H-14. The HMQC spectrum in Figure 4.34 displayed direct correlation of proton with associated carbon, H-1a′ to C-1a′ (δ 68.9), H-1a, 1a″ to C-1a, 1a″ (δ 62.2), H-2, 2′, 2″ to C-2, 2′, 2″ (δ 34.1), H-3, 3′, 3″ to C-3, 3′, 3″ (δ 24.9) and H-14, 14′, 14″ to C-14, 14′, 14″ (δ 14.2), and thus confirmed the assignment of all ^{1}H and ^{13}C NMR signals.

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Heteronuclear multiple bond coherence or HMBC spectral (Figure 4.35) displayed the key ^{1}H-^{13}C long-range correlations, ^{3}J correlations from H-3, 3′, 3″ to C-1, 1′, 1″ (δ 173.4, 173.0) and ^{2}J to C-2, 2′, 2″ (δ 34.1) and C-4, 4′, 4″ (δ 29.2-29.8). At the same time, H-14, 14′, 14″ was observed to correlate ^{3}J with C-12, 12′, 12″ (δ 32.0), and ^{2}J with C-13, 13′, 13″ (δ 22.8) and H-2, 2′, 2″ correlate ^{2}J with C-3, 3′, 3″ (δ 24.9) and C-1, 1′, 1″ (δ 173.4, 173.0). The ^{1}H-^{1}H COSY and HMBC correlations of compound **ASBD7** was displayed in Figure 4.36

This compound was considered to know as saturated triglyceride fatty acid (FA) (**186**) due to the disappearance of olefinic carbon signals at range δ 127.0 until 103.3 in the ^{13}C-NMR spectrum. The content of saturated fatty acids could be verified by the ratio between the areas one of the signals from α glycerol hydrogens and the signal at δ 2.31 as well as a signal at δ 1.59 comparative to the β carbonyl hydrogens from all fatty acids esterified to the glycerol moiety (Barison et al., 2010).

The complete assignment of the 1D and 2D NMR of compound **ASBD7** were tabulated in Table 4.8. The present data were compared to the compound saturated triglyceride fatty acid (FA) (**186**) in the previous study carried out by Barison and co-workers (2010).

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Table 4.8

*^{1}H, ^{13}C and 2D-NMR spectral data of compound **ASBD7** and triglycerides (FA) (**186**)*

	Compound ASBD7			**Triglycerides F.A (186)**	
No	**δ^{1}H (ppm)**	**δ^{13}C (ppm)**	**HMBC**	**COSY**	***δ^{1}H (ppm)**
1,1″		173.4		H-2	
1		173.0		H-1	
2,2′,2″	2.31 (6H, *m*)	34.1	C-1,1″, C-3, C-4		2.28
3,3′,3″	1.59 (6H, *m*)	24.9	C-1,1″, C-4		1.59
4,4′,4″	1.27 (6H, br. *s*)	29.2-29.8		H-6	
5,5′,5″	1.27 (6H, br. *s*)	29.2-29.8		H-5, H-7	
6,6′,6″	1.27 (6H, br. *s*)	29.2-29.8		H-6	
7,7′,7″	1.27 (6H, br. *s*)	29.2-29.8			
8,8′,8″	1.27 (6H, br. *s*)	29.2-29.8			
9,9′,9″	1.27 (6H, br. *s*)	29.2-29.8			
10,10′,10″	1.27 (6H, br. *s*)	29.2-29.8			
11,11′,11″	1.27 (6H, br. *s*)	29.2-29.8		H-12	
12,12′,12″	1.27 (6H, br. *s*)	32.0		H-11	
13,13′,13″	1.27 (6H, br. *s*)	22.8			
14,14′,14″	0.87 (9H, *t*)	14.2	C-13, C-12		
1a	4.27 (2H, *dd*, *J*=4.1, 4.0 Hz)	62.2	C-,1a′,1a″, C-1		4.27
1a″	4.14 (2H, *dd*, *J*=5.8, 5.7 Hz)	62.2	C-1a, 1a′, C-1		4.12
1a′	5.26 (1H, *m*)	68.9			

Note: * Measured at 600 MHz (^{1}H) and 150 MHz (^{13}C) in $CDCl_3$ (Barison et al., 2010)

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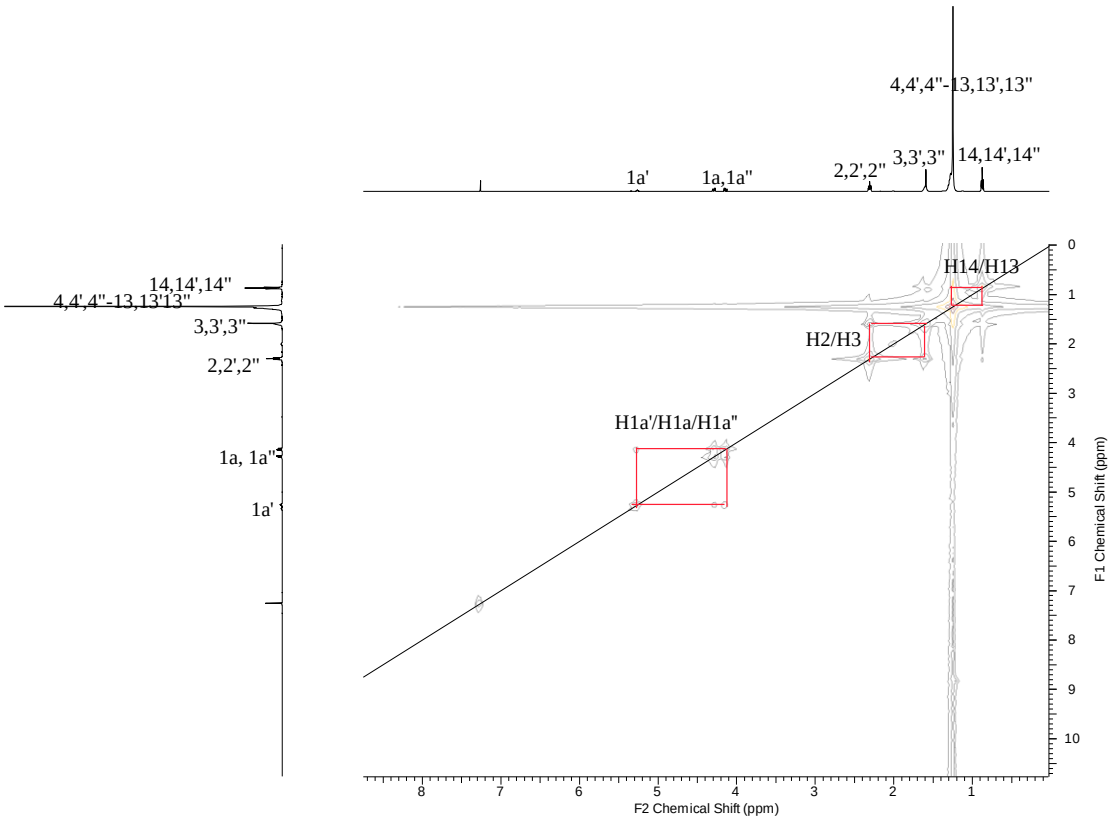


Figure 4.33. COSY spectrum of compound **ASBD7**

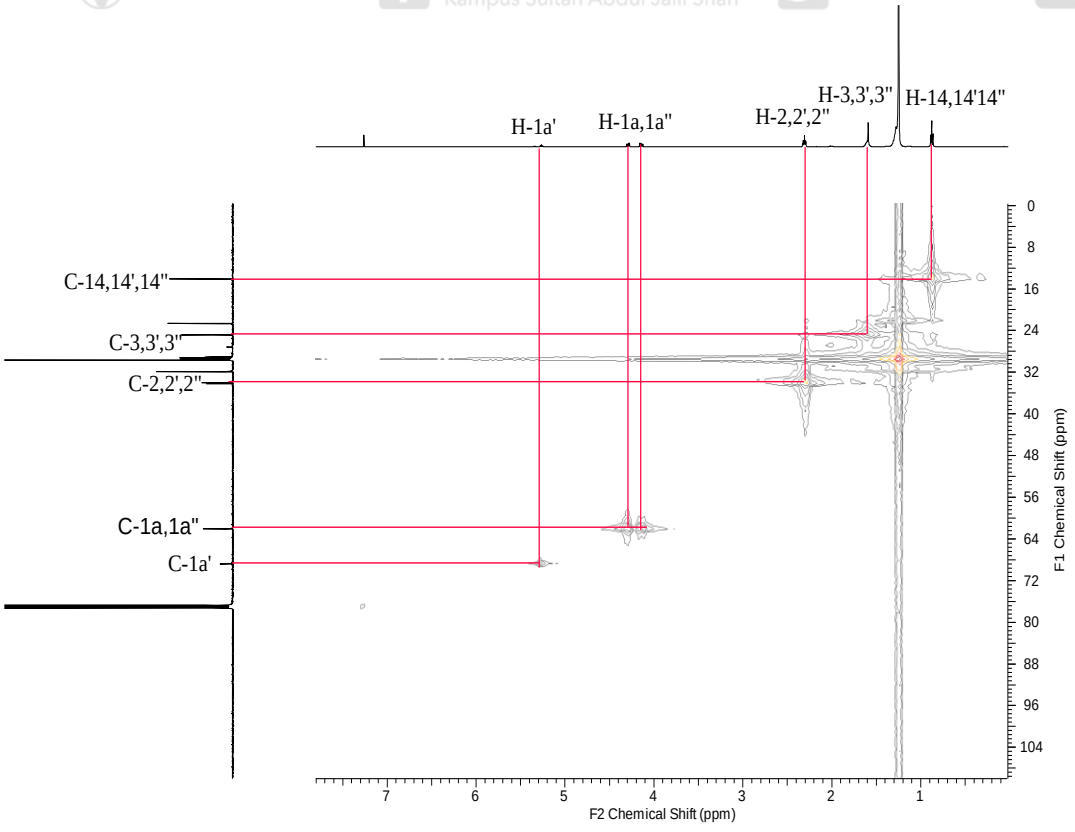


Figure 4.34. HMQC spectrum of compound **ASBD7**

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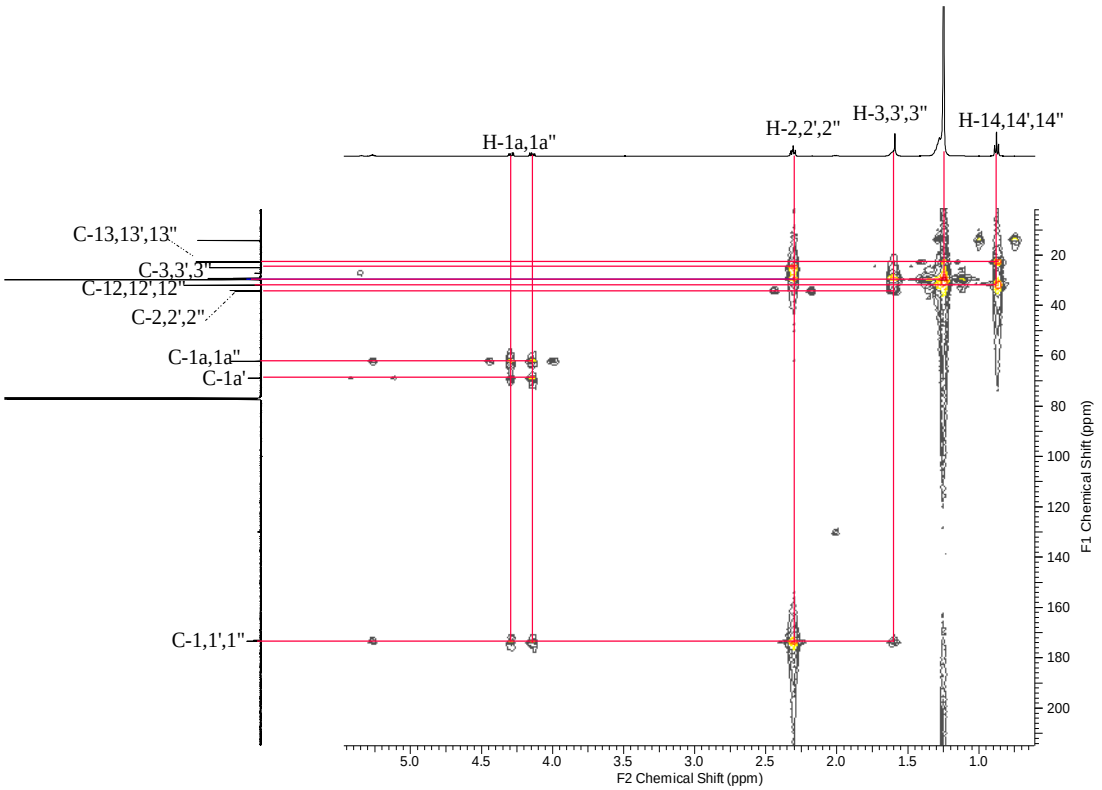


Figure 4.35. HMBC spectrum of compound **ASBD7**

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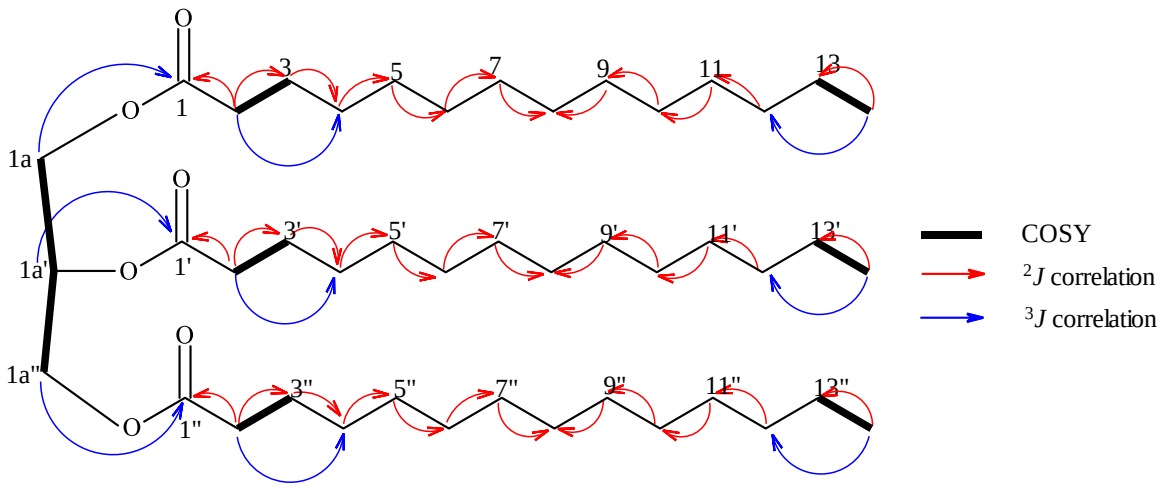
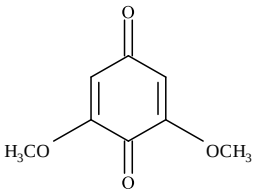


Figure 4.36. COSY and HMBC correlations of compound **ASBD7**

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4.2.8 ASBD8: 2,6-Dimethoxyquinone (187)



Compound **ASBD8** was isolated as orange-yellow needles from the bark part of the plant and well dissolved in CH_2Cl_2. This compound was active under short wave UV and appeared as a black spot. The spot was turned to brown colour when sprayed with *p*-anisaldehyde and heated it to 50°C using a hot plate. The IR spectrum (Figure 4.37) of **ASBD8** showed the absorption band at 1695 cm^{-1} attributed to the carbonyl (C=O) group, whereas band at 1592 and 1447 cm^{-1} assigned to C=C and alkane (CH_3), respectively. The UV spectrum in Figure 4.38 exhibited the maximum absorption at 285 nm. Its molecular formula of $C_8H_8O_4$ was supported by its molecular ion peak at *m/z* 169.04961 $[M+H]^+$ analysed using ESI-MS technique (Figure 4.39)

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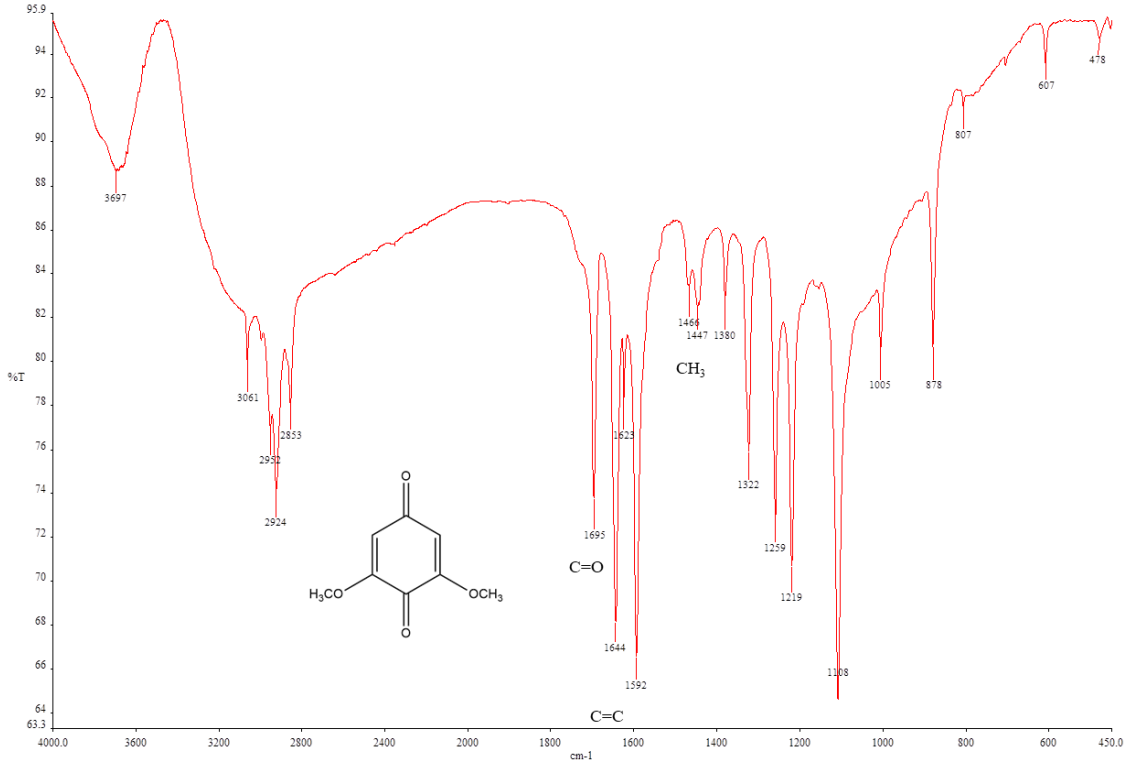


Figure 4.37. IR spectrum of compound **ASBD8**

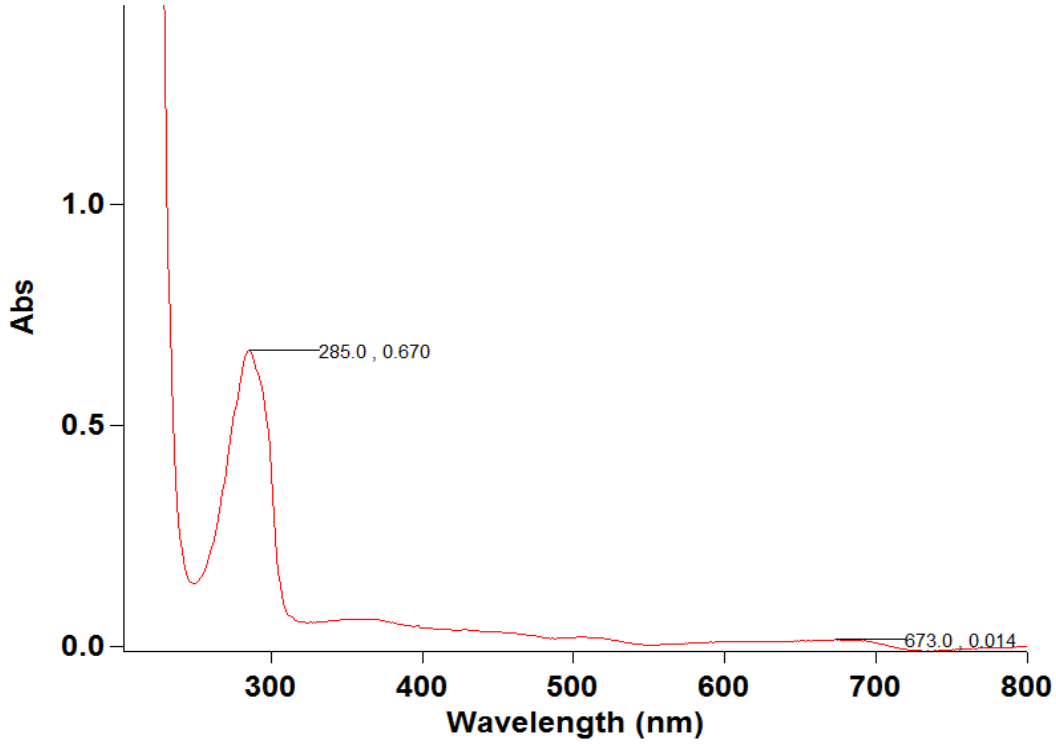


Figure 4.38. UV spectrum of compound **ASBD8**

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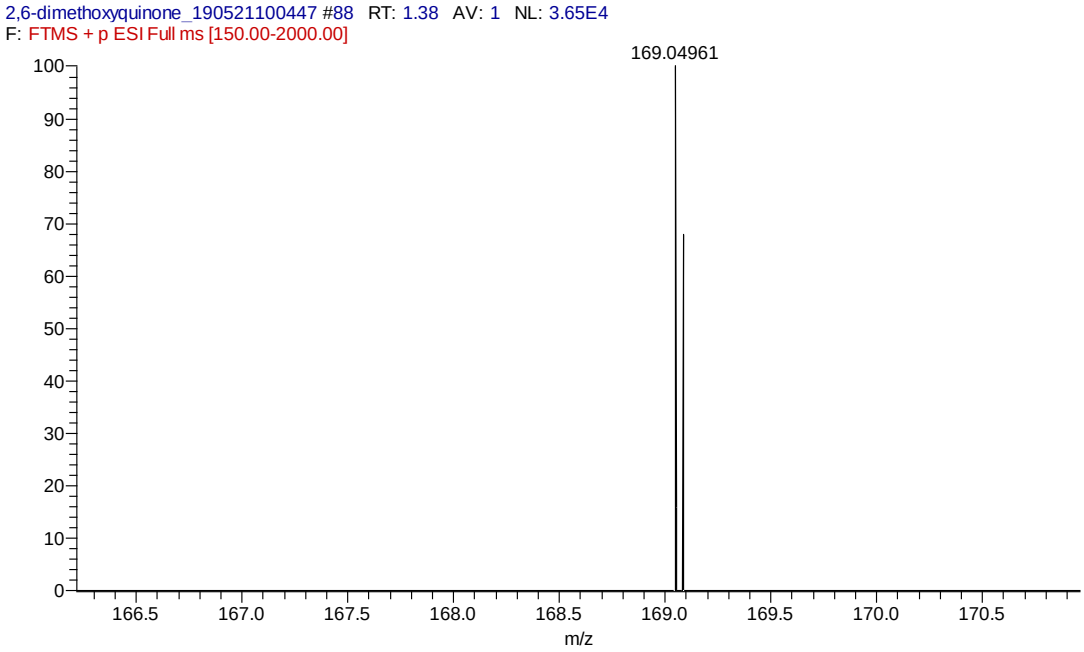


Figure 4.39. LC-MS spectrum of compound **ASBD8**

The ^{1}H-NMR spectrum ($CDCl_3$, 500 MHz) of compound **ASBD8** in Figure 4.40 displayed two proton signals. One resonance of olefinic proton appeared at δ 5.86 (*s*, 2H) attributed to H-3, and H-5. Two methoxyl group characteristics as a singlet signal appeared at δ 3.82 assigned to OCH_3-2 and OCH_3-6.

The ^{13}C-NMR spectrum ($CDCl_3$, 125 MHz) of compound **ASBD8** was displayed in Figure 4.41. The spectrum exhibited five signals of carbon. The signal at δ 56.6 assigned to a methoxy group, OCH_3-2, and OCH_3-6. The methine signals at δ 107.5 were attributed to C-3 and C-5, and δ 157.4 (C-2 and C-6). The carbonyl signals appeared at δ 176.8 (C-1) and δ 186.9 (C-4) due to the different environments surrounded them.

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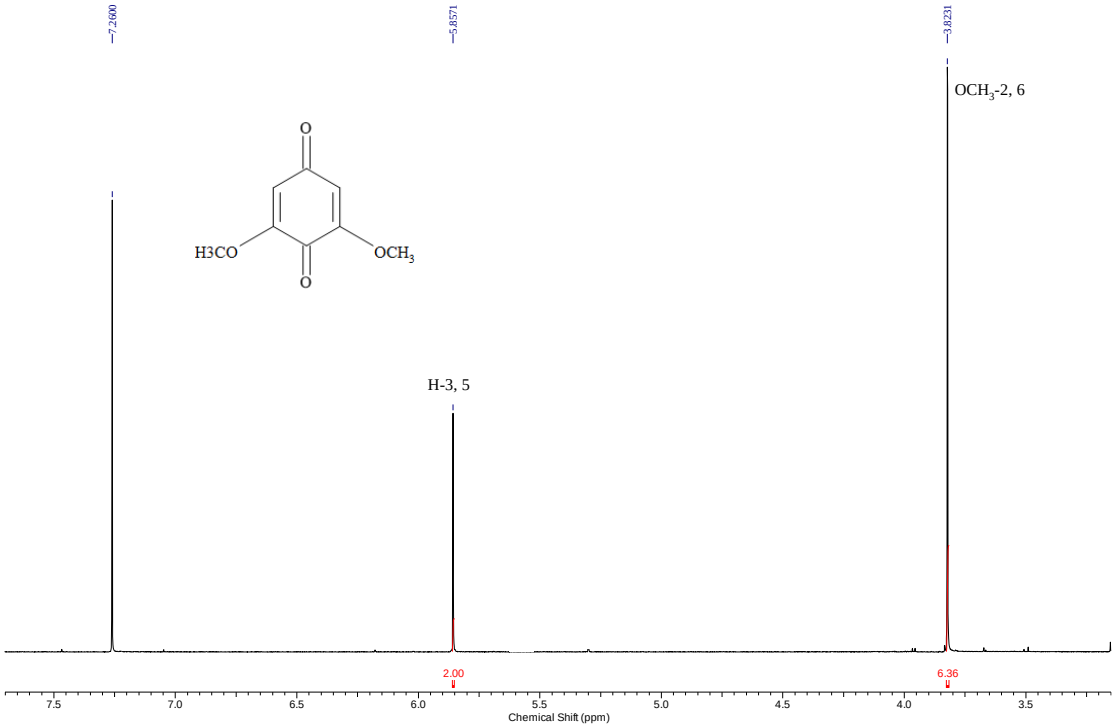


Figure 4.40. ^{1}H-NMR spectrum of compound **ASBD8**

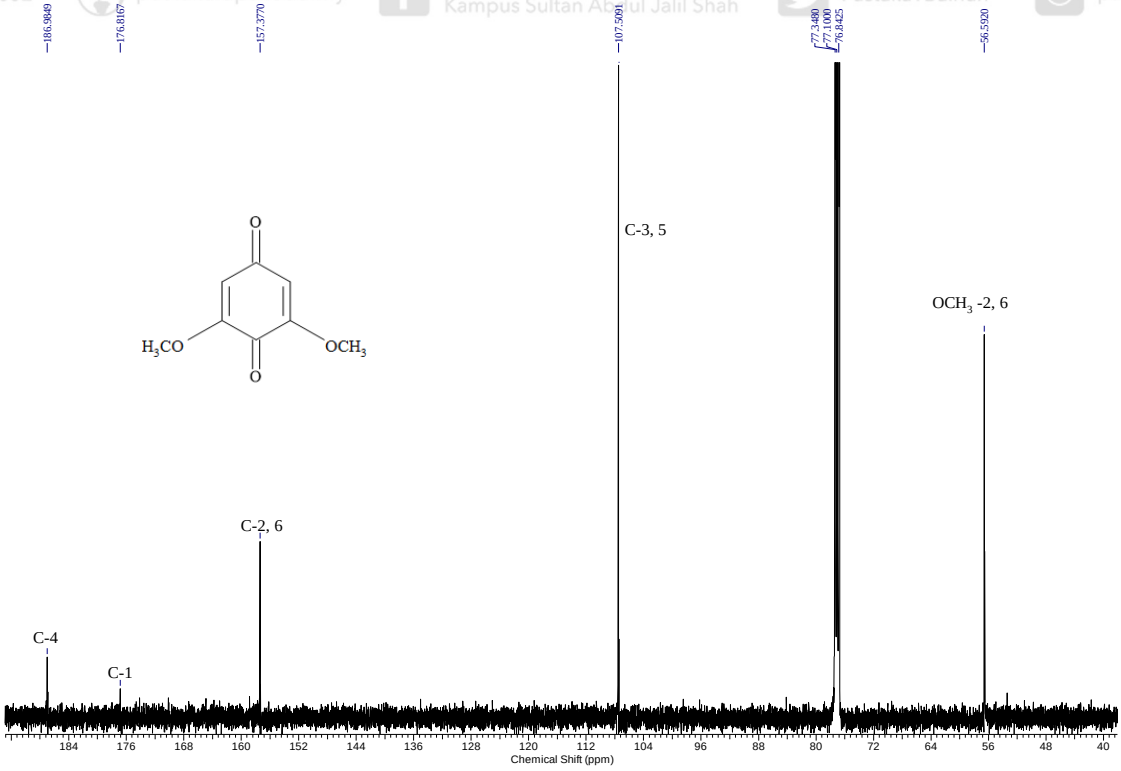


Figure 4.41. ^{13}C-NMR spectrum of compound **ASBD8**

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Table 4.9 showed the ^{1}H and ^{13}C NMR chemical shift values for compound **ASBD8** in $CDCl_3$. On the basis of the analysed spectral data and comparison with the literature, the structure of compound **ASBD8** was elucidated as 2,6-dimethoxyquinone (**187**) which was earlier isolated from *Achillea alpine* (Zhang et al., 2014).

Table 4.9

*^{1}H-NMR (500 MHz, $CDCl_3$) and ^{13}C (125 MHz, $CDCl_3$) spectral data of compound **ASBD8** and 2,6-dimethoxyquinone (**187**)*

	Compound ASBD8		**2,6-dimethoxyquinone (187)**	
No	**$\delta^{1}H$ (ppm)**	**$\delta^{13}C$ (ppm)**	***$\delta^{1}H$ (ppm)**	***$\delta^{13}C$ (ppm)**
1		176.8		176.7
2		157.4		157.3
3	5.86 (1H, *s*)	107.5	5.86 (*s*)	107.4
4		186.9		186.8
5	5.86 (1H, *s*)	107.5	5.86 (*s*)	107.4
6		157.4		157.3
OCH_3-2	3.82 (3H, *s*)	56.6	3.82 (*s*)	56.5
OCH_3-6	3.82 (3H, *s*)	56.6	3.82 (*s*)	56.5

Note: * Measured at 500 MHz (^{1}H) and 125 MHz (^{13}C) in $CDCl_3$ (Zhang et al., 2014)

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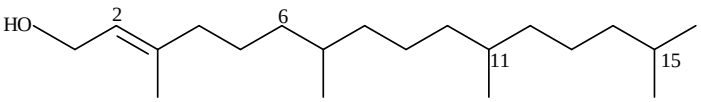
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4.3 Chemical Constituents Isolated from The Leaf of *A. subinterga*

Isolation and purification of leaf extract of *A. subintegr*a were carried out using column chromatography (CC), thin layer chromatography (TLC), washing and recrystallization technique to afford pure phytochemicals.

Purification of CH_2Cl_2 extract yielded 14 pure compounds. These compounds are including terpenes (**ASLD1**, **ASLD2**, **ASLD3**, **ASLD7**, **ASLD8**), steroids (**ASLD4**, **ASLD11**, **ASLD12**), three flavonoids (**ASLD6**, **ASLD13,** and **ASLD14**), alkane (**ASLD5**), a derivative of tocopherol (**ASLD9**) and fatty acid (**ASLD10**).

4.3.1 ASLD1: 3,7,11,15 – Tetramethyl – 2 – hexadecen – 1 – ol (Phytol) (74)



Compound **ASLD1** was isolated as a colourless oil and well dissolved in CH_2Cl_2. This compound was non-active UV where there is no spot appeared under short or long UV. The spot was turned purple colour when sprayed with *p*-anisaldehyde and heated it to 50^oC using a hot plate. The IR spectrum (Figure 4.42) of this compound showed the absorption bands at 3736 and 2927 cm^{-1} for O-H and sp^3 C-H stretching, respectively. Its molecular formula of $C_{20}H_{40}O$ was supported by its molecular ion peak at *m/z* 297.13068 $[M+H]^+$ analysed using ESI-MS technique (Figure 4.43).

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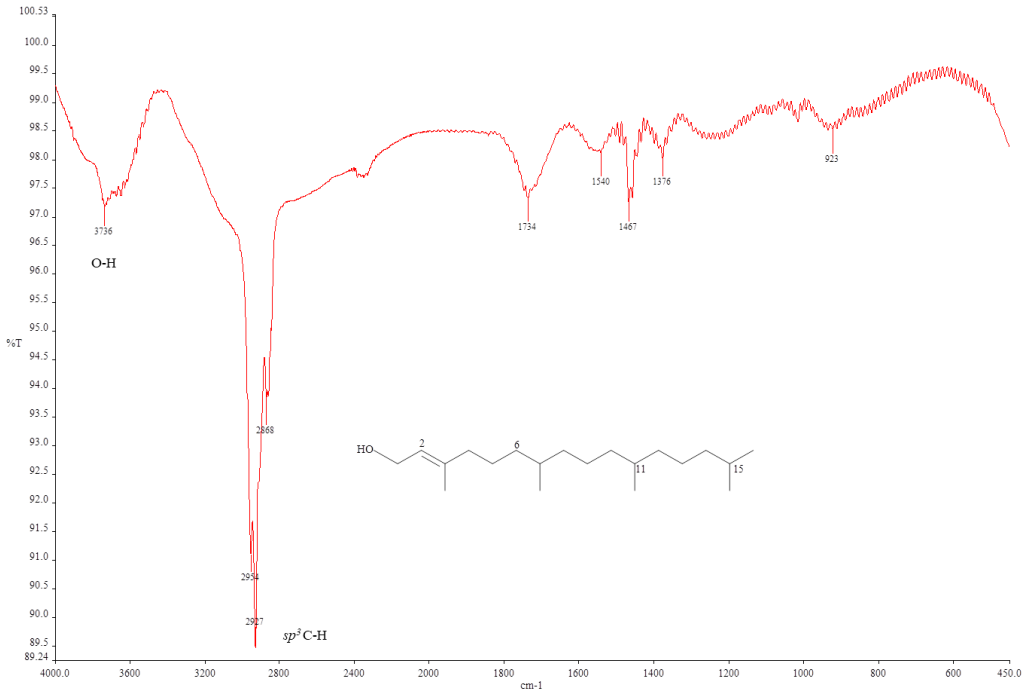


Figure 4.42. IR spectrum of compound **ASLD1**

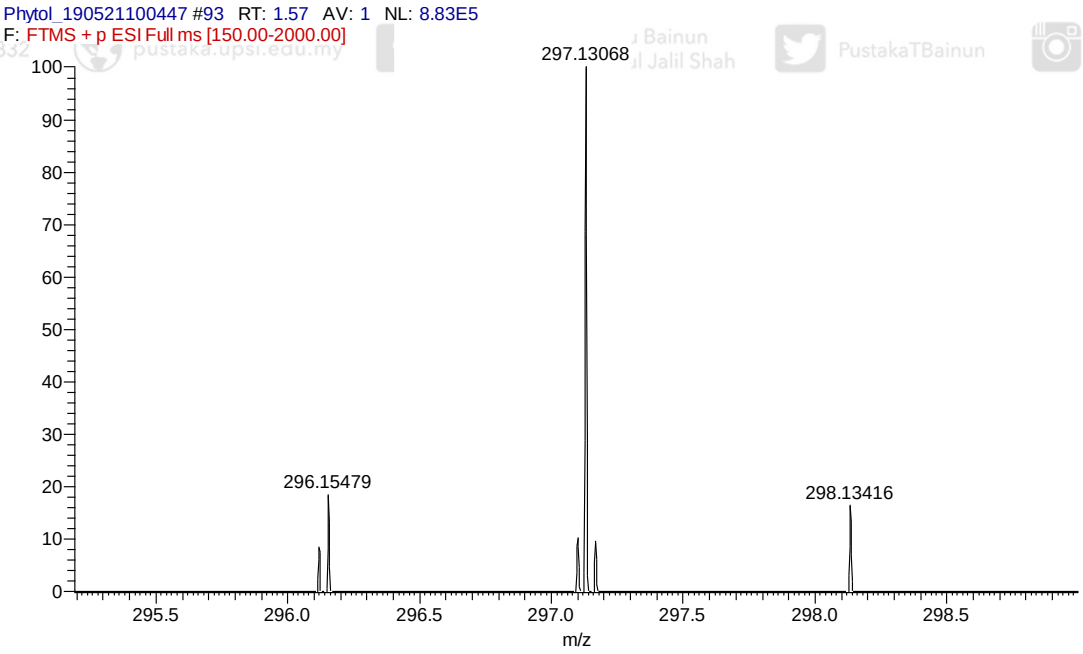


Figure 4.43. LC-MS spectrum of compound **ASLD1**

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The ^{1}H-NMR spectrum ($CDCl_3$, 500 MHz) of compound **ASLD1** in Figure 4.44 displayed resonances of an olefinic proton at δ 5.39 (H-2) and an oxygenated methylene characteristic at δ 4.15 (*d*, *J* = 6.9). Another methylene proton was observed at δ 1.97 (*t*, *J* = 5.7). The resonance of vinyl methyl proton was exhibited at δ 1.67, and four methyl groups appeared at δ 0.83 to 0.87.

The ^{13}C-NMR spectrum of **ASLD1** (Figure 4.45) displayed the presence of twenty carbons consist of five methyl carbons, ten methylenes, four methanes, and one quaternary carbon. The presence of the hydroxyl group represented the signals at δ 59.52 (C-1). One double bond carbon (C=C) was exhibited at δ 123.1 and 140.5 attributed to C-2 and C-3, accordingly. The four methyl carbons (C-16 to C-19) were resonated at δ 19.80 to 22.82. The data of ^{1}H and ^{13}C-NMR of this compound was tabulated at Table 4.10 and was in good agreement with 3,7,11,15 – tetramethyl – 2 – hexadecen – 1 – ol (phytol) (**74**) which reported in previous studies (Eldeen et al., 2016; Kashiwagi et al., 2005).

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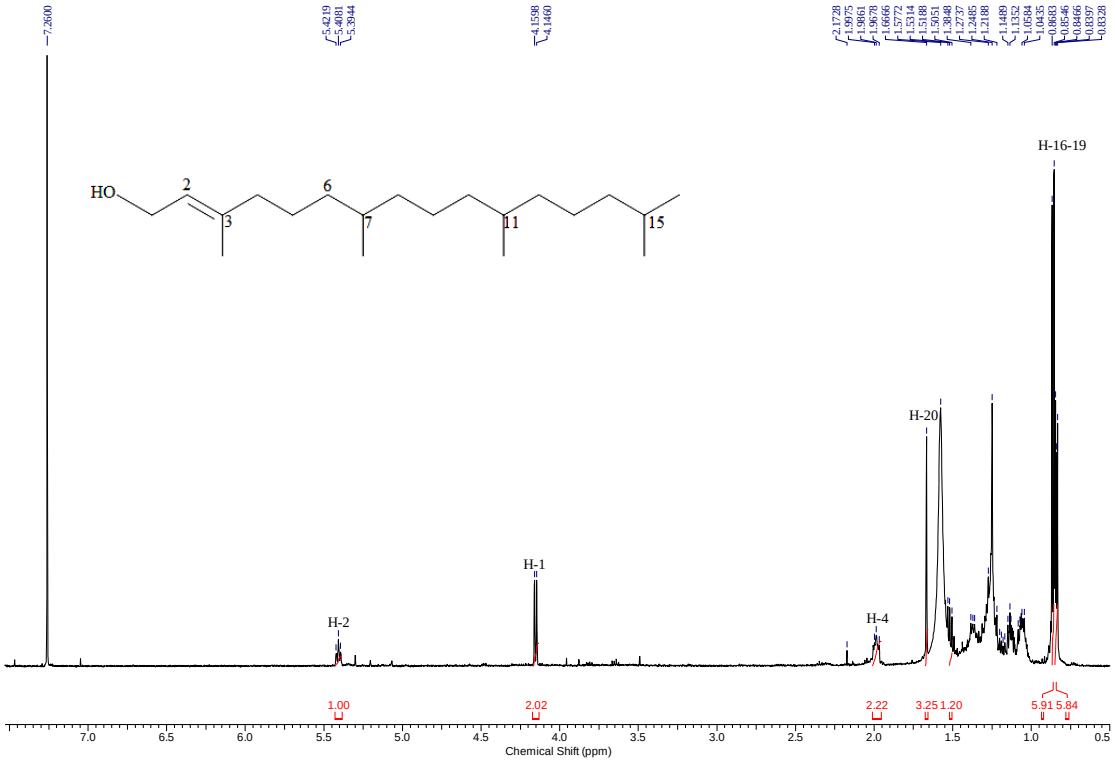


Figure 4.44. ^{1}H-NMR spectrum of compound **ASLD1**

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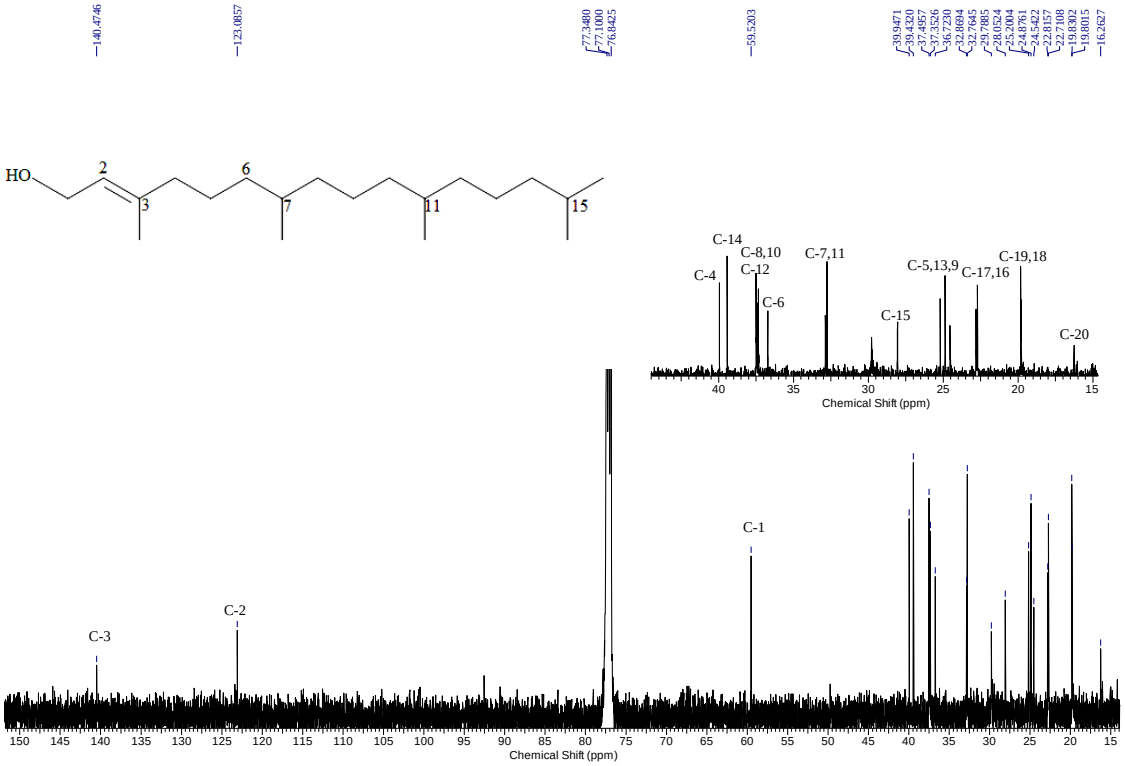


Figure 4.45. ^{13}C-NMR spectrum of compound **ASLD1**

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Table 4.10

^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C (125 MHz, $CDCl_3$) spectra data of compound ***ASLD1*** *and 3,7,11,15 – tetramethyl – 2 – hexadecen – 1 – ol (phytol) (**74**)*

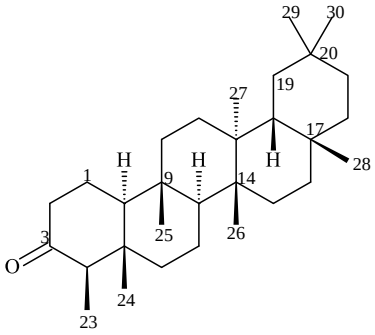
	Compound ASLD1		**Phytol (74)**	
No	**$\delta^{1}H$ (ppm)**	**$\delta^{13}C$ (ppm)**	***$\delta^{1}H$ (ppm)**	***$\delta^{13}C$ (ppm)**
1	4.14 (2H, *d*, *J*=7.6 Hz)	59.5	4.13	59.3
2	5.39 (1H, *t*, *J*=6.8 Hz)	123.1	5.39	123.2
3		140.5		140.0
4	1.97 (2H, *br. t*)	39.9	1.97	39.9
5		25.2		25.1
6		36.7		36.7
7		32.9		32.8
8		37.5		37.4
9		24.5		24.5
10		37.4		37.4
11		32.8		32.7
12		37.3		37.3
13		24.9		24.8
14		39.4		39.4
15	1.50 (1H, *m*)	28.1	1.50	28.0
16	0.85 (3H, *d*, *J*=6.9 Hz)	22.7	0.84	22.6
17	0.85 (3H, *d*, *J*=6.9 Hz)	22.8	0.84	22.7
18	0.83 (3H, *d*, *J*=6.9 Hz)	19.8	0.82	19.7
19	0.83 (3H, *d*, *J*=6.9 Hz)	19.8	0.83	19.7
20	1.67 (3H, *br. s*)	16.3	1.65	16.1

Note: *Measured at 400 MHz (^{1}H) and 100 MHz (^{13}C) in $CDCl_3$ (Kashiwagi, Mikagi, & Bisrat, 2005)

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4.3.2 ASLD2: Friedelin (188)



Chromatographic purification of ASLD L1A-12 has yielded compound **ASLD2** as a colourless needle (10.0 mg, 1.3%). The TLC of this compound was not active under short and long wave UV (254 and 365 nm) and turned to a purple colour when heat on hotplate after sprayed with a *p*-anisaldehyde reagent. The IR spectrum (Figure 4.46) revealed the presence of carbonyl group at 1716 cm^{-1}, C-H (sp^3) stretching band at 2928 cm^{-1}, and the presence of the CH_3 band at 1388 cm^{-1}. The LC-MS spectrum (Figure 4.47) of compound **ASLD2** showed a triterpenoid with a molecular formula $C_{30}H_{50}O$ which was deduced from the molecular ion peak at *m/z* 428.30719 $[M+H]^+$.

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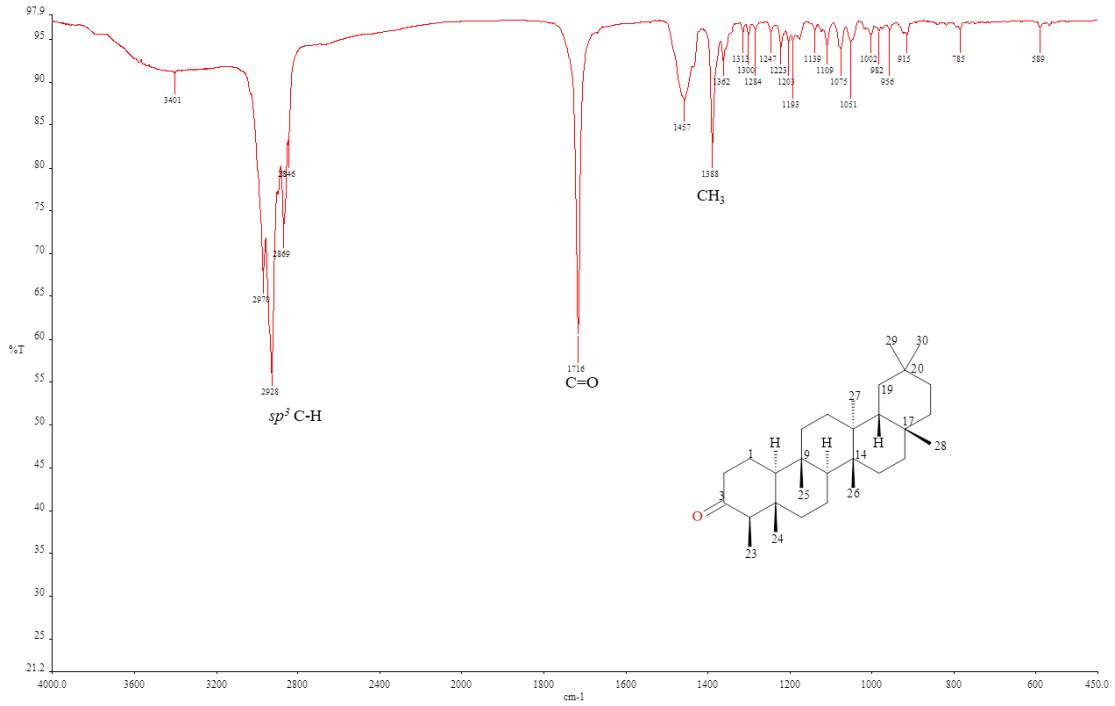


Figure 4.46. IR spectrum of compound **ASLD2**

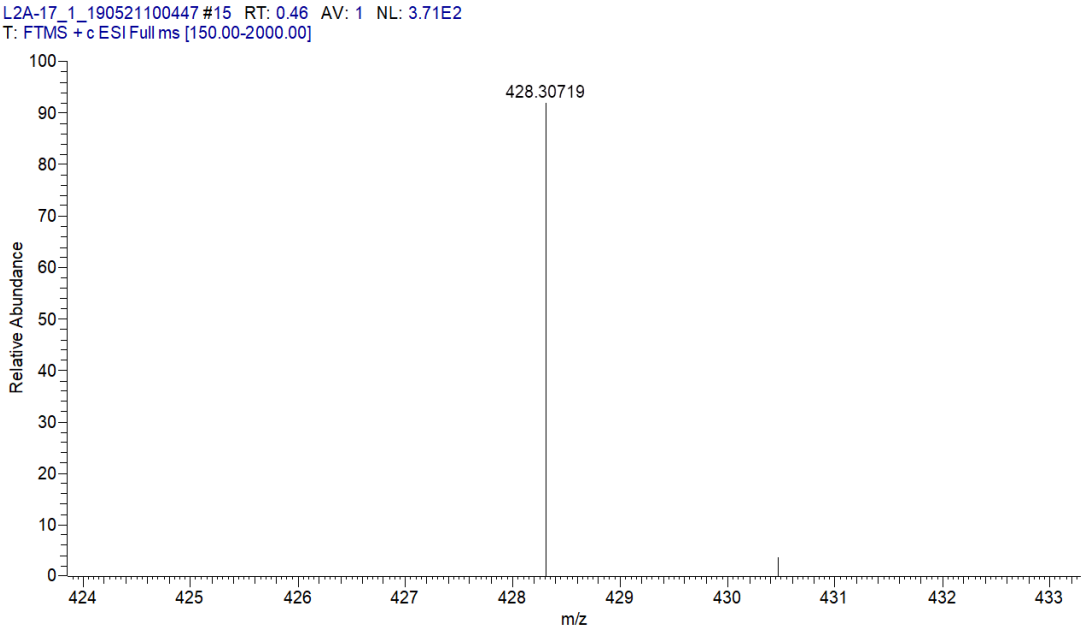


Figure 4.47. LC-MS spectrum of compound **ASLD2**

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The ^{1}H-NMR spectrum of compound **ASLD2** in Figure 4.48 revealed the presence of seven methyl groups appeared as singlets at δ 0.72 (H-24), 0.86 (H-25), 0.99 (H-26), 1.04 (H-27), 1.17 (H-28), 0.95 (H-29) and 1.00 (H-30). One methyl group was displayed as a doublet at δ 0.88 (J = 7.4 Hz) assigned to H-23. The spectrum also showed a multiplet signal at δ 2.37 (H-2), and the quartet signal at δ 2.24 (J= 6.9 Hz) belonged to H-4.

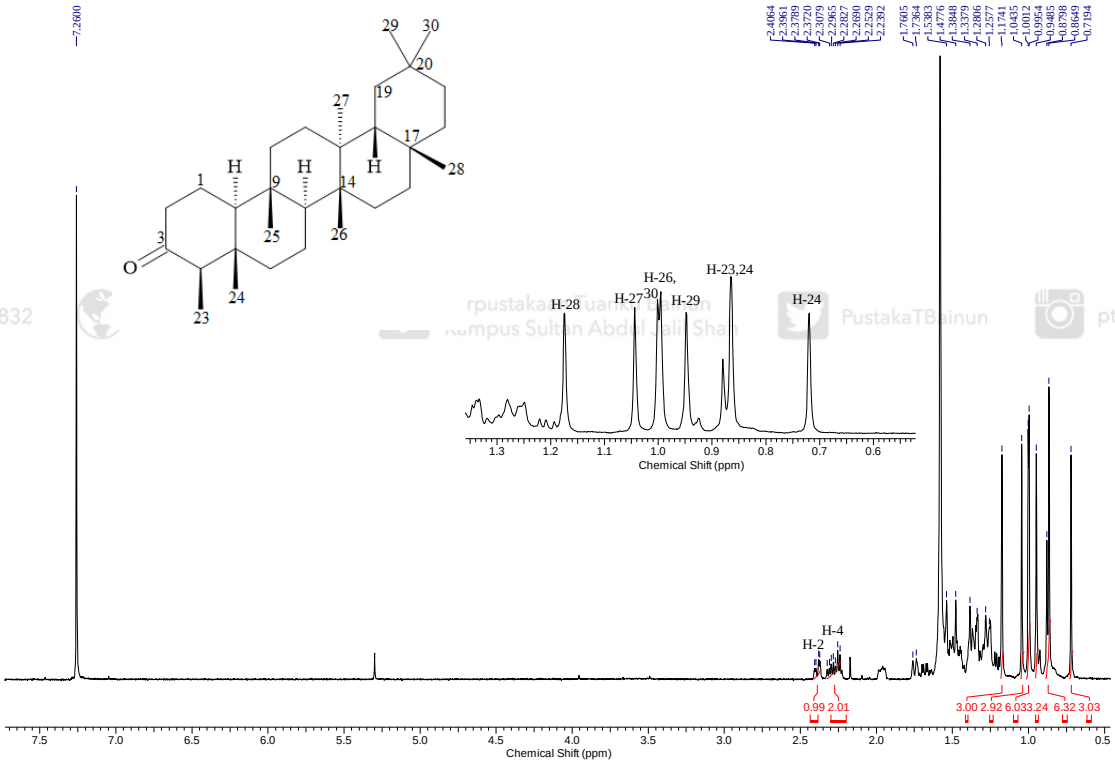


Figure 4.48. ^{1}H-NMR spectrum of compound **ASLD2**

The ^{13}C-NMR spectrum of **ASLD2** (Figure 4.49) indicated the presence of 30 signals corresponding to 30 carbons, which were classified into eight methyls, eleven methylene, four sp^3 methines, and seven quaternary sp^3 carbons. One of the quaternary carbon appeared at δ 213.5 was typical for a carbonyl group (C-3). A signal at δ 6.9

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showed the methyl carbon for C-23. Other seven methyl carbons were exhibited at δ 14.7 (C-24), 18.0 (C-25), 20.3 (C-26), 18.8 (C-27), 32.2 (C-28), 35.1 (C-29) and 31.9 (C-30). Table 4.11 shows the complete assignments of the NMR data of compound **ASLD2**.

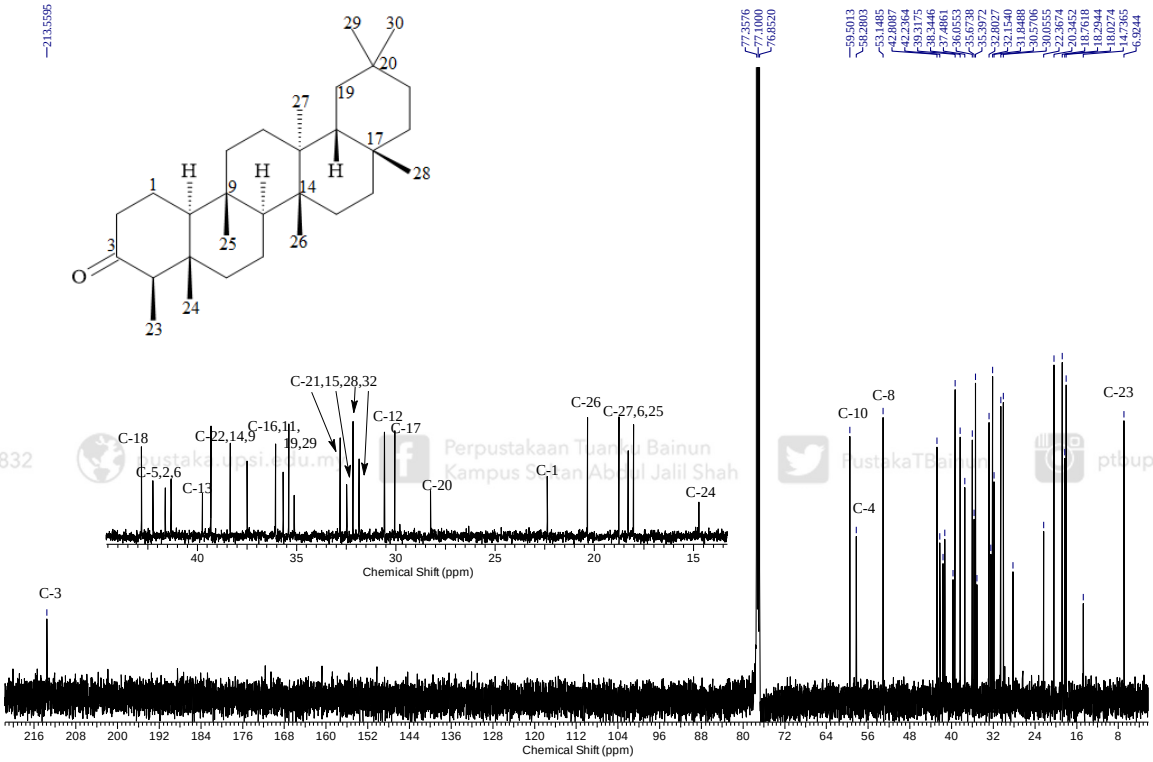


Figure 4.49. ^{13}C-NMR spectrum of compound **ASLD2**

By comparing the spectroscopic data of this compound with previously reported data, compound **ASLD2** was identified as friedelin (**188**), a compound that previously obtained from *Mallotus apelta* Muell. -Arg, (Euphorbiaceae) (Kiem et al., 2004) and from the leaf of *Maytenus robusta* (Celastraceae) (Sousa et al., 2012).

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Table 4.11

^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C (125 MHz, $CDCl_3$) spectra data of compound ***ASLD2*** *and friedelin (**188**)*

	Compound ASLD2		**Friedelin (188)**	
No	**$\delta^{1}H$ (ppm)**	**$\delta^{13}C$ (ppm)**	***$\delta^{1}H$ (ppm)**	***$\delta^{13}C$ (ppm)**
1		22.4		22.6
2	2.37 (2H, *m*)	41.6	2.36 (*m*)	41.9
3		213.5		213.4
4	2.24 (1H, *q*, *J* = 6.9 Hz)	58.3	2.24 (*q*, *J*=6.4 Hz)	58.6
5		42.2		42.5
6		41.3		41.7
7		18.3		18.6
8		53.1		53.5
9		37.5		37.8
10		59.5		59.9
11		35.7		36.0
12		30.6		30.9
13		39.7		40.1
14		38.3		38.7
15		32.5		32.8
16		36.1		36.4
17		30.1		30.4
18		42.8		43.2
19		35.4		35.7
20		28.2		28.5
21		32.8		33.1
22		39.3		39.6
23	0.88 (3H, *d*, *J* = 7.4 Hz)	6.9	0.87 (*d*, *J*=6.4 Hz)	7.1
24	0.72 (3H, *s*)	14.7	0.71 (*s*)	15.0
25	0.86 (3H, *s*)	18.0	0.85 (*s*)	18.3
26	0.99 (3H, *s*)	20.3	0.99 (*s*)	20.6
27	1.04 (3H, *s*)	18.8	1.03 (*s*)	19.0

(continued)

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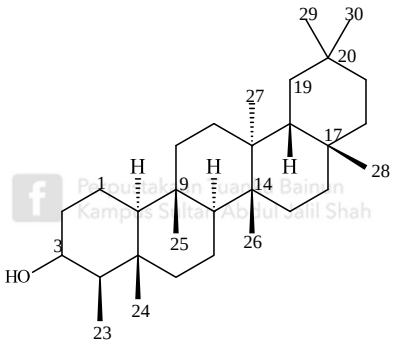
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Table 4.11 *(continued)*

	Compound ASLD2		**Friedelin (188)**	
No	**δ^1H (ppm)**	**δ^{13}C (ppm)**	***δ^1H (ppm)**	***δ^{13}C (ppm)**
28	1.17 (3H, *s*)	32.2	1.16 (*s*)	32.4
29	0.95 (3H, *s*)	35.1	0.94 (*s*)	35.4
30	1.00 (3H, *s*)	31.9	0.99 (*s*)	32.1

Note: * Measured at 300 MHz (^{1}H) and 75 MHz (^{13}C) in $CDCl_3$ (Kiem et al., 2004)

4.3.3 ASLD3: Epifriedelanol (189)



Purification of fraction L1A-12 from CH_2Cl_2 extract of leaf part had afforded compound epifriedelanol **ASLD3** (8.0 mg) as a colourless needle. The TLC of this compound was not active under short and long wave UV (254 and 365 nm) but turned to yellowish-orange colour when heat on hotplate after sprayed with a *p*-anisaldehyde reagent. The IR spectrum of **ASLD3** in Figure 4.50 exhibited the maximum absorption band of the hydroxyl group (O-H) at 3692 cm^{-1}, C-H (sp^3) stretching band at 2932 cm^{-1}, and the presence of CH_3 band at 1385 cm^{-1}. The ESI-MS of this compound (Figure 4.51) revealed a strong molecular ion peak at *m/z* 430.22055 $[M+H]^+$ and giving the molecular formula of $C_{30}H_{52}O$.

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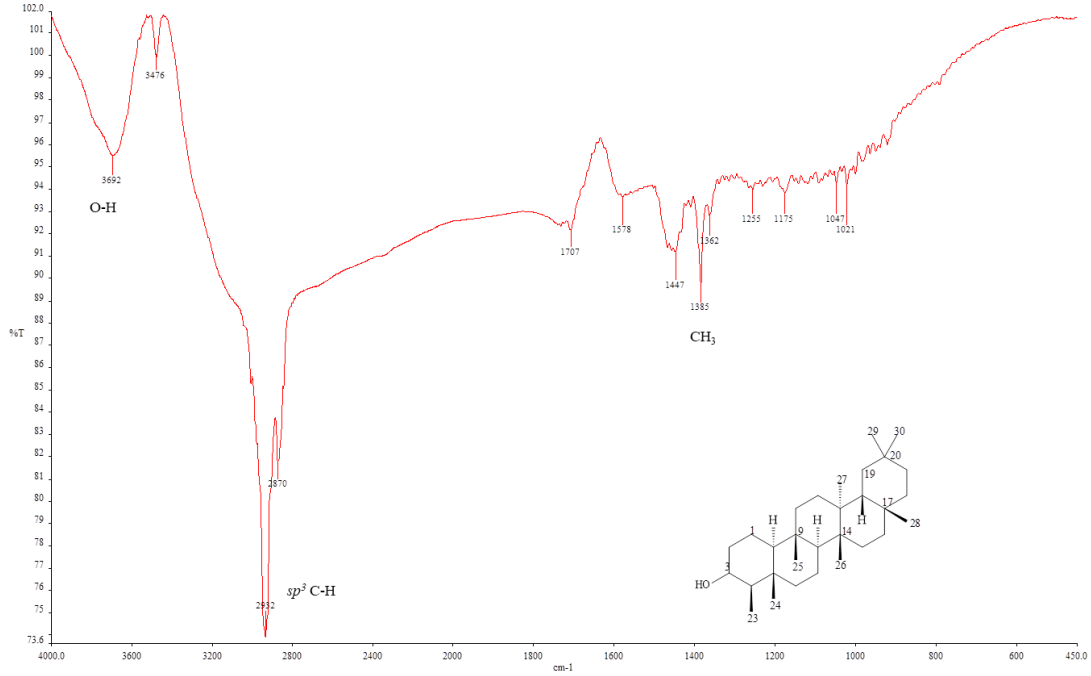


Figure 4.50. IR spectrum of compound **ASLD3**

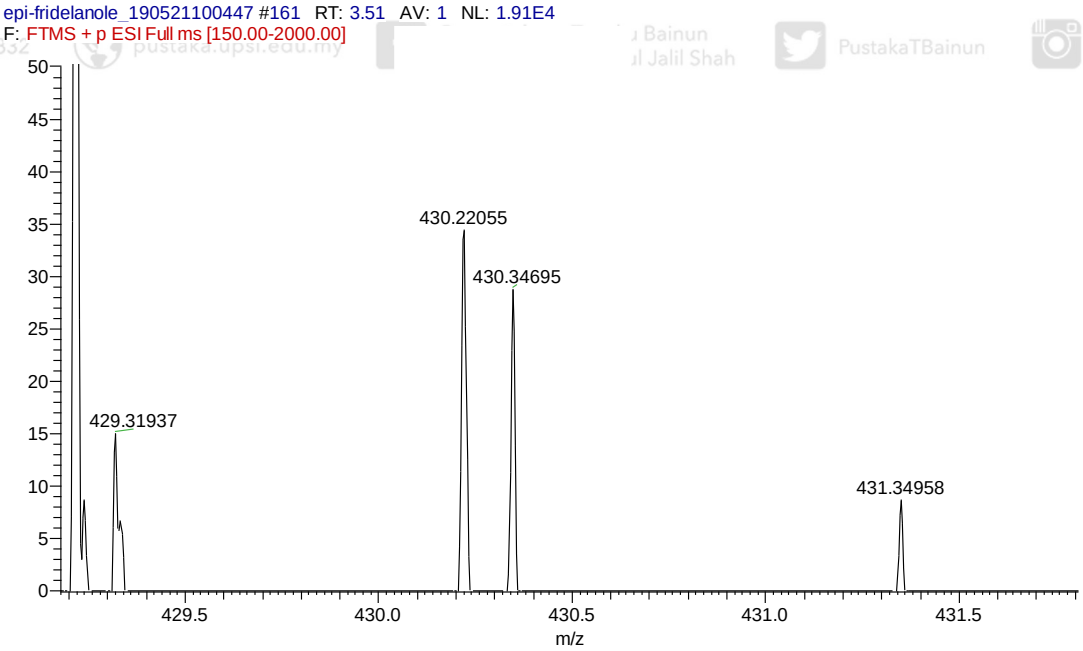


Figure 4.51. LC-MS spectrum of compound **ASLD3**

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The ^{1}H-NMR spectrum of **ASLD3** (Figure 4.52) was closely resembled the ^{1}H-NMR spectrum of compound friedelin (**188**) by existing proton under the hydroxyl residue at δ 3.73 attributed to H-3. In addition, the spectrum revealed eight methyl singlets at δ 0.93, 0.95, 0.85, 1.00, 0.98, 0.99, 0.94, and 1.16 belonged to the methyl protons at H-23, H-24, H-25, H-26, H-27, H-28, H-29 and H-30 respectively. The remaining 26 protons were unresolved absorption at δ 1.20 to 1.58.

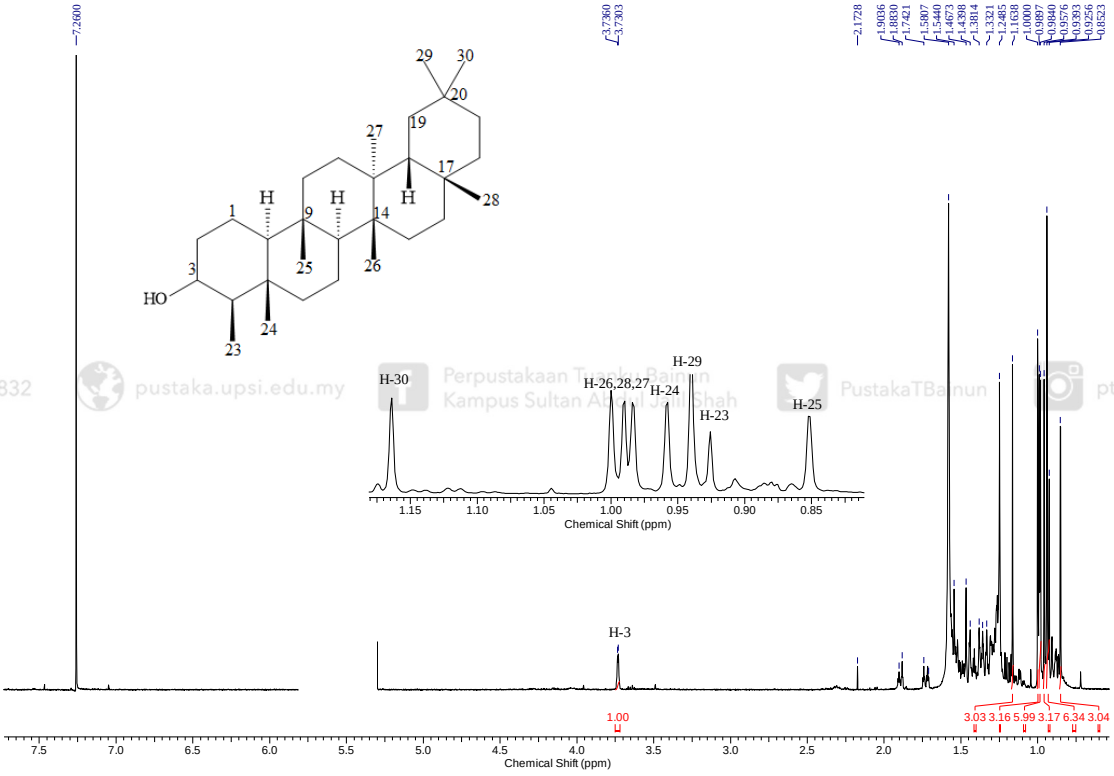


Figure 4.52. ^{1}H-NMR spectrum of compound **ASLD3**

The ^{13}C-NMR spectrum of **ASLD3** in Figure 4.53 exhibited thirty carbons which contain eight methyls, eleven methylene, five methine, and six quaternary carbons. This compound consists of oxygenated methine carbons, where C-3 resonated at δ 72.8, and C-4 appeared at δ 49.2. The signal at δ 11.7 has belonged to methyl carbon (C-23). Other seven methyl carbons were exhibited at δ 16.5, 18.3, 18.7, 20.2, 32.2,

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35.1, and 31.9 corresponded to C-24,C-25,C-26, C-27, C-28, C-29, and C-30, respectively. In addition, the signals at δ 37.1, 37.9, 38.4, 39.7, 29.8, and 28.3 were resonated to C-5, C-9, C-13, C-14, C-17, and C-20, accordingly. The signals at δ 53.2, 61.4, and 42.8 were assigned to C-8, C-10 and C-18. The remaining signals at δ15.8 (C-1), 35.4 (C-2), 41.8 (C-6), 17.6 (C-7), 35.2 (C-11), 30.7 (C-12), 32.8 (C-15), 36.1 (C-16), 35.6 (C-19), 32.4 (C-21), and 39.3 (C-22) were attributable to methylene carbons. Table 4.12 shows the complete assignments of the 1D-NMR data of compound **ASLD3**.

Compound **ASLD3** was identified as epifriedelanol (**189**) based on a comparison of its physical properties and spectral data with the compound previously isolated from the leaf of *Mallotus apelta* (Euphorbiaceae) (Kiem et al., 2004).

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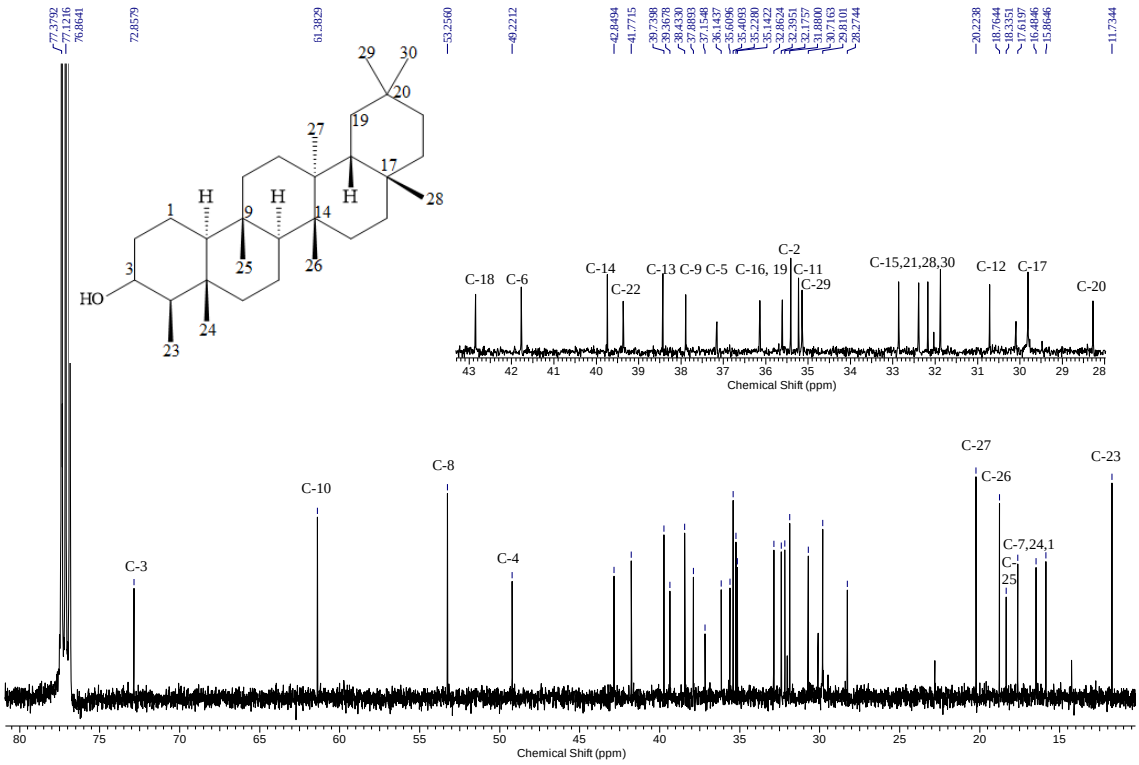


Figure 4.53. ^{13}C NMR spectrum of compound **ASLD3**

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Table 4.12

^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C (125 MHz, $CDCl_3$) spectra data of compound ***ASLD3*** *and epifriedelanol(**189**)*

	Compound ASLD3		**Epifriedelanol (189)**	
No	**$\delta^{1}H$ (ppm)**	**$\delta^{13}C$ (ppm)**	***$\delta^{1}H$ (ppm)**	***$\delta^{13}C$ (ppm)**
1		15.8		16.2
2		35.4		35.7
3	3.73 (1H, br *d*)	72.8	3.73 (br *d*)	73.1
4		49.2		49.6
5		37.1		37.5
6		41.8		42.1
7		17.6		17.9
8		53.2		53.6
9		37.9		38.2
10		61.4		61.8
11		35.2		35.6
12		30.7		31.0
13		38.4		38.7
14		39.7		40.0
15		32.8		33.2
16		36.1		36.5
17		29.8		30.0
18		42.8		43.2
19		35.6		35.9
20		28.3		28.7
21		32.4		32.7
22		39.3		39.7
23	0.92 (3H, *s*)	11.7	0.91 (*d*, *J*=7.0 Hz)	12.0
24	0.95 (3H, *s*)	16.5	0.93 (*s*)	16.8
25	0.85 (3H, *s*)	18.3	0.83 (*s*)	18.6
26	1.00 (3H, *s*)	18.7	0.98 (*s*)	19.0
27	0.98 (3H, *s*)	20.2	0.95 (*s*)	20.5

(continued)

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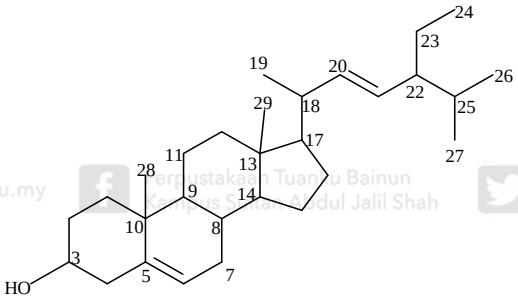
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Table 4.12 *(continued)*

	Compound ASLD3		**Epifriedelanol (189)**	
No	**δ^{1}H (ppm)**	**δ^{13}C (ppm)**	***δ^{1}H (ppm)**	***δ^{13}C (ppm)**
28	0.99 (3H, *s*)	32.2	0.97 (*s*)	32.5
29	0.94 (3H, *s*)	35.1	0.92 (*s*)	35.4
30	1.16 (3H, *s*)	31.9	1.14 (*s*)	32.2

Note: * Measured at 300 MHz (^{1}H) and 75 MHz (^{13}C) in $CDCl_3$ (Kiem et al., 2004)

4.3.4 ASLD4: Stigmasterol (102)



Compound **ASLD4** was obtained from the CH_2Cl_2 extract as a white amorphous and dissolved in CH_2Cl_2. This compound was not active under long (365 nm) and short wave UV (254 nm) but appeared as violet spot after derivatisation with a *p*-Anisaldehyde reagent. The IR spectrum (Figure 4.54) displayed several absorption bands that were observed at 3435 cm^{-1} (O-H group), 2960 and 2937 cm^{-1} (sp^3 C-H stretching), and 1641 cm^{-1} (C=C). In addition, bands at 1464 and 1053 cm^{-1} were corresponded to bending vibrations of CH_2 and cycloalkane, respectively. The molecular formula of compound **ASLD4** was $C_{29}H_{48}O$ as established from the LC-MS spectrum

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in accordance with the appearance of a significant peak at *m/z* 413.26266 $[M+H]^+$ by ESI-MS (Figure 4.55).

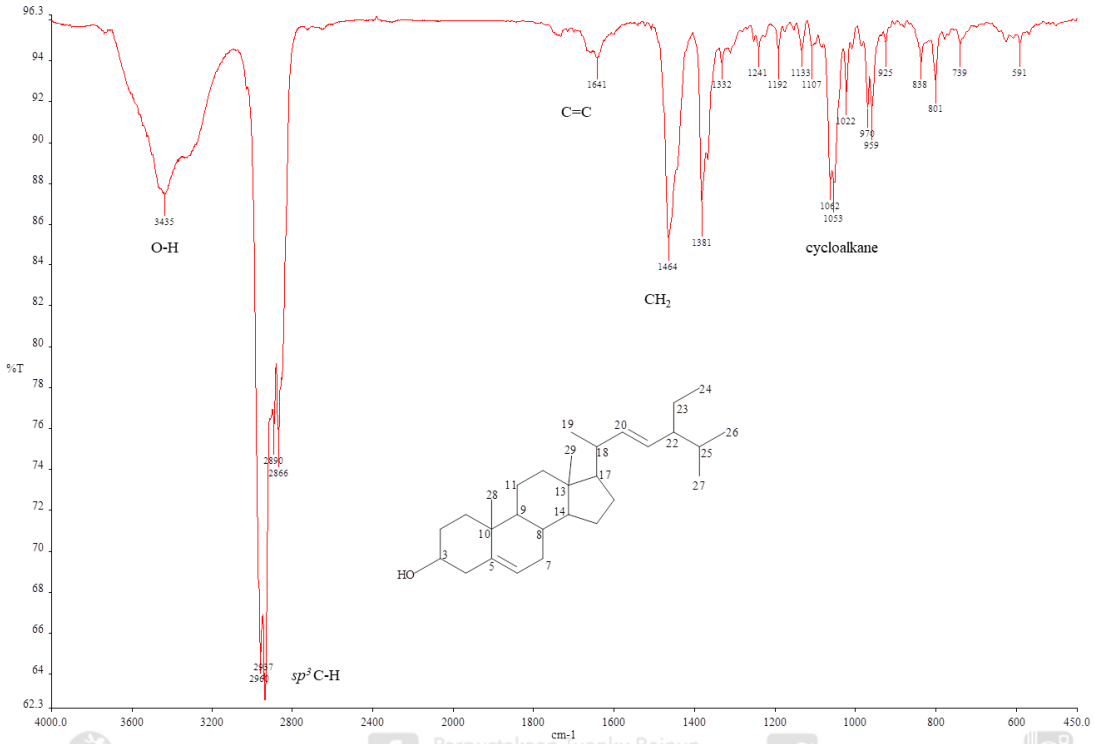


Figure 4.54. IR spectrum of compound **ASLD4**

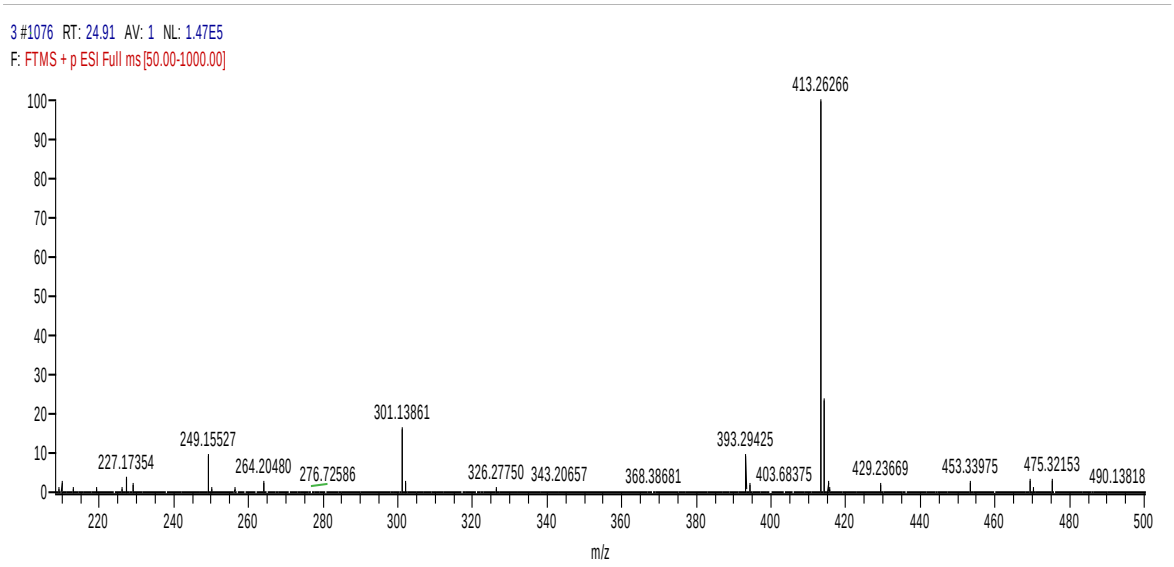


Figure 4.55. LC-MS spectrum of compound **ASLD4**

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The ^{1}H-NMR spectrum of compound **ASLD4** can be referred to in Figure 4.56. The spectrum displayed 48 protons with the presence of a hydroxyl group at the same carbon of proton at δ 3.52 as a multiplet signal. Two methyl groups at δ 0.69 and 1.02 as singlet signal corresponding to H-28 and H-29, respectively. Besides, three methyl groups were observed at δ 0.91, 0.82, and 0.80, which attributable to H-19, H-26, and H-27, respectively. Meanwhile, a multiplet signal corresponding to the methyl group at δ 0.80 was assigned as H-24. The olefinic proton at H-6 was observed at δ 5.34 as a triplet. Two exo-olefinic protons appeared as a pair of multiplet at δ 4.98 and 5.12 were belonged to H-20 and H-21, respectively.

The ^{13}C-NMR spectrum (Figure 4.57) of compound **ASLD4** revealed twenty-nine signals comprising of six methyl carbons (CH_3) at δ 21.2, 12.0, 19.9, 19.4, 18.8 and 12.3, which assigned to C-19, C-24, C-26, C-27, C-28 and C-29, respectively. Three quarternary carbons displayed at δ 140.8, 36.2, and 42.2 were assigned to C-5, C-10, and C-13, respectively. Nine methylene carbons (CH_2) observed at δ 37.3, 32.0, 42.3, 31.7, 21.1, 39.7, 24.4, 29.0, and 25.5 were belonged to C-1, C-2, C-4, C-7, C-11, C-12, C-15, C-16, and C-23, respectively. Furthermore, four olefinic carbons displayed at δ 140.8, 121.8, 138.4, and 129.3 were identified to C-5, C-6, C-20, and C-21, respectively. Meanwhile, an isolated signal at δ 71.9 was attributable to C-3 due to the existence of a hydroxyl group.

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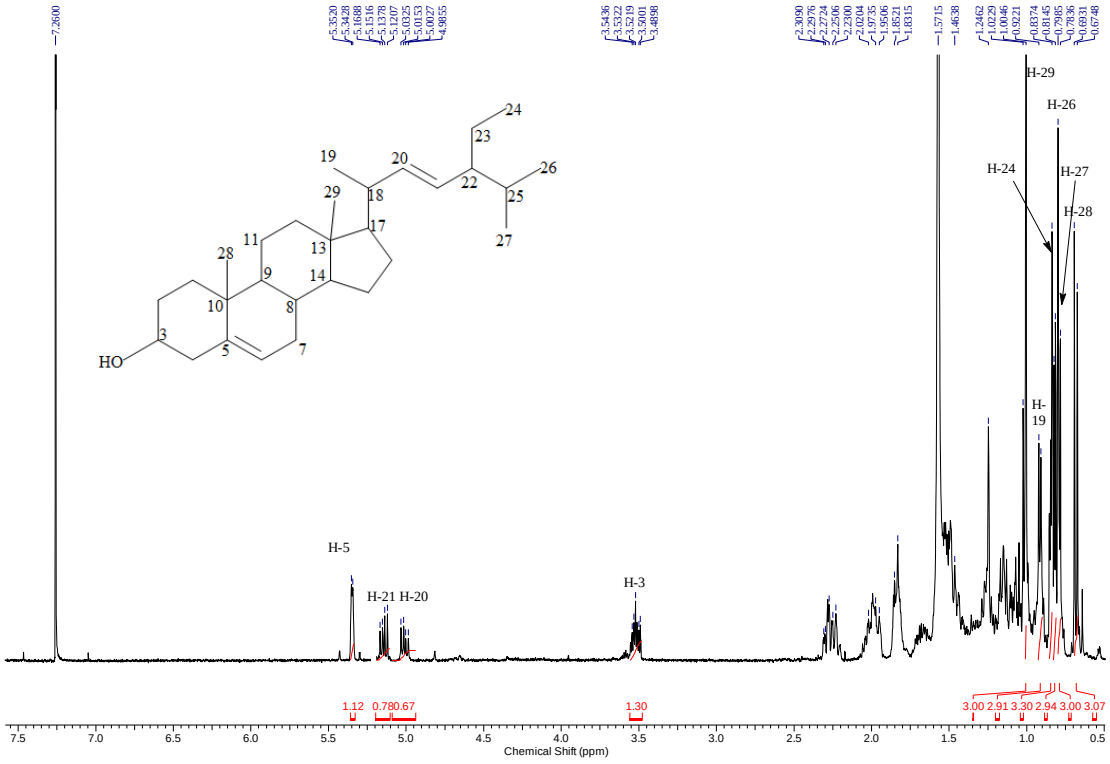


Figure 4.56. ^{1}H-NMR spectrum of Compound **ASLD4**

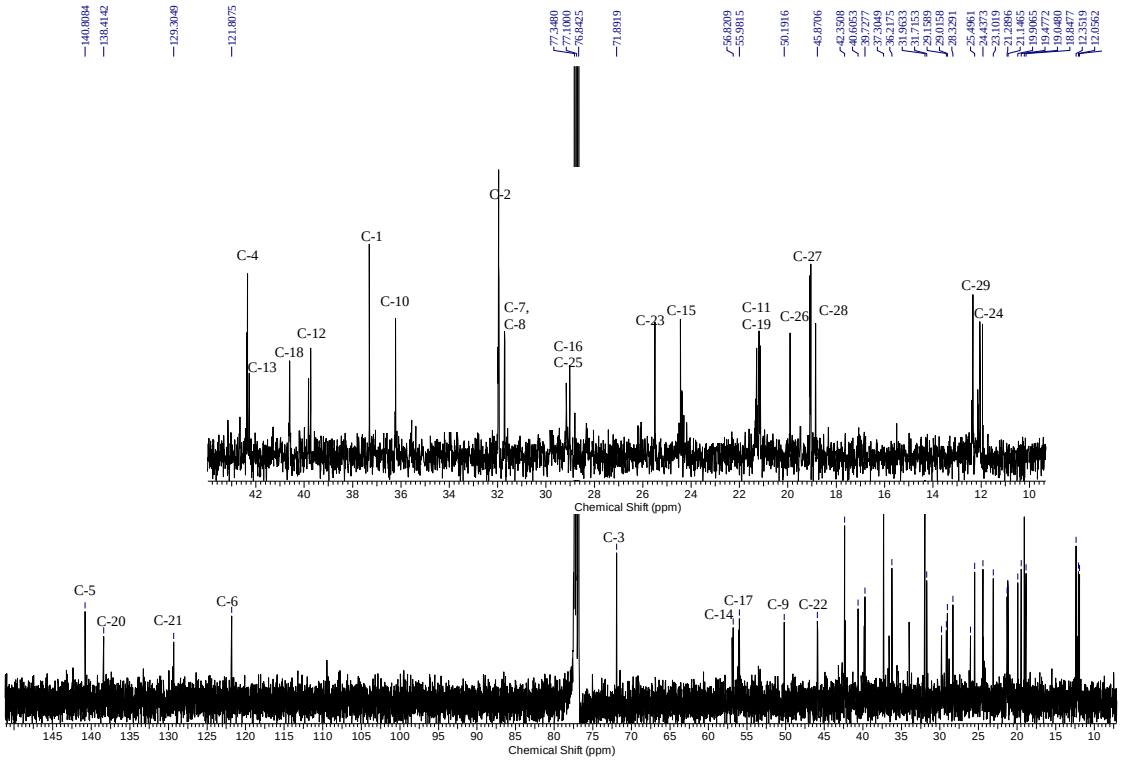


Figure 4.57. ^{13}C-NMR of Compound **ASLD4**

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The assignment of ^{1}H and ^{13}C-NMR of compound **ASLD4** was presented in Table 4.13. Therefore, compound **ASLD4** was identified as stigmasterol (**102)**, a compound which was isolated previously from aqueous extract of *Rubus suavissimus* leaf (Sai et al., 2012).

Table 4.13

*^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C (125 MHz, $CDCl_3$) spectra data of compound **ASLD4** and stigmasterol (**102**)*

	Compound ASLD4		**Stigmasterol (102)**	
No	**$\delta^{1}H$ (ppm)**	**$\delta^{13}C$ (ppm)**	***$\delta^{1}H$ (ppm)**	***$\delta^{13}C$ (ppm)**
1		37.3		37.6
2		32.0		32.1
3	3.52 (1H, *m*)	71.9	3.51	72.1
4		42.3		42.4
5		140.8		141.1
6	5.34 (1H, *t*)	121.8	5.31	121.8
7		31.7		31.8
8		31.7		31.8
9		50.2		50.2
10		36.2		36.6
11		21.1		21.5
12		39.7		39.9
13		42.2		42.4
14		56.8		56.8
15		24.4		24.4
16		29.0		29.3
17		56.0		56.2
18		40.6		40.6
19	0.91 (3H, *d*, *J*=6.5 Hz)	21.2	0.91	21.7
20	4.98 (1H, *m*)	138.4	4.98	138.7

(continued)

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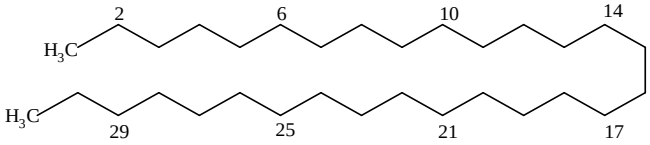
Table 4.13 *(continued)*

	Compound ASLD4		**Stigmasterol (102)**	
No	**δ^1H (ppm)**	**δ^{13}C (ppm)**	***δ^1H (ppm)**	***δ^{13}C (ppm)**
21	5.12 (1H, *m*)	129.3	5.14	129.6
22		45.9		46.1
23		25.5		25.4
24	0.83 (3H, *t*, *J*= 7.5 Hz)	12.0	0.83	12.1
25		29.2		29.6
26	0.82 (3H, *d*, *J*= 4.6 Hz)	19.9	0.82	20.2
27	0.80 (3H, *d*, *J*= 6.3 Hz)	19.4	0.80	19.8
28	0.69 (3H, *s*)	18.8	0.71	18.9
29	1.02 (3H, *s*)	12.3	1.03	12.2

Note: * Measured at 600 MHz (^{1}H) and 150 MHz (^{13}C) in $CDCl_3$ (Sai et al., 2012)

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4.3.5 ASLD5: Hentriacontane (190)



Compound **ASLD5** was isolated as a white amorphous from the first column of the leaf part. This compound was non-active toward a short and long wave of UV-vis. However after sprayed with *p*-anisaldehyde reagent and heat up to 50°C, it turned into purplish pink. The IR spectrum of this compound exhibited the absorptions of aliphatic alkane C-H (stretching) at 2955, 2915, and 2847 cm^{-1} and CH_2 (bending) at 1461 cm^{-1} (Quintanilla-Licea et al., 2012). This IR spectrum was shown in Figure 4.58. The molecular formula of this compound was suggested as $C_{31}H_{64}$ and supported by its

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molecular ion peak at *m/z* 437.21381 $[M+H]^+$ analysed using ESI-MS technique (Figure 4.59).

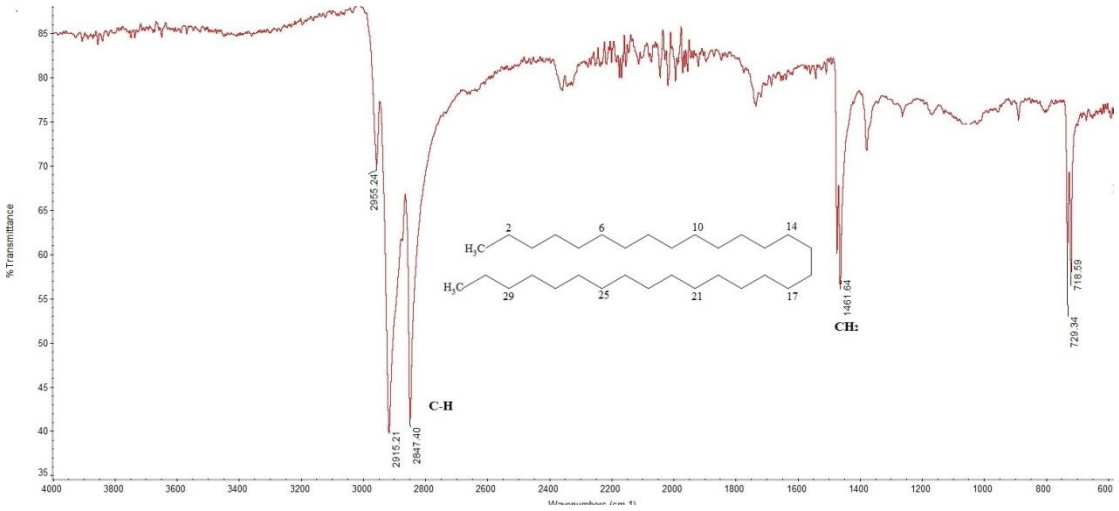


Figure 4.58. IR spectrum of compound **ASLD5**

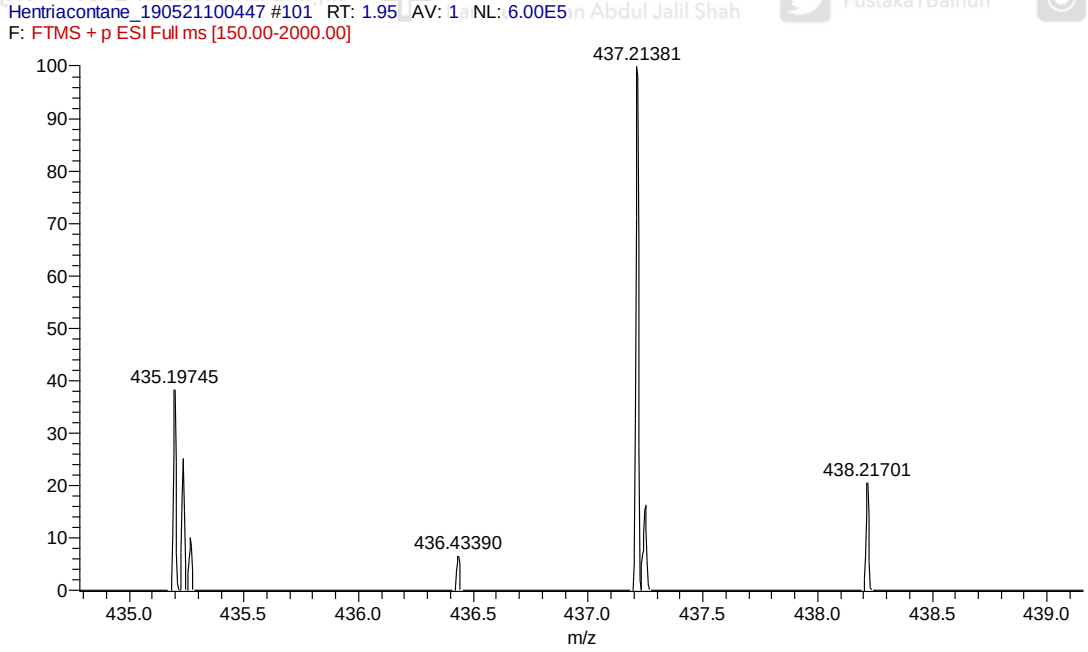


Figure 4.59. LC-MS spectrum of compound **ASLD5**

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The ^{1}H-NMR spectrum ($CDCl_3$, 500MHz) of this compound displayed a sharp and tall peak at δ 1.25 represent the presence of the methylene group. The resonance at δ 0.88 showed the existence of methyl protons. The spectrum was shown in Figure 4.60.

The ^{13}C-NMR spectrum of **ASLD5** in Figure 4.61 revealed five-carbon signals; consist of methyl carbons which appeared at δ 14.23 resonated to C-1 and C-31. The signal at δ 22.80 was represented to C-2 ad C-30. In addition, the signals of methylene carbons also appeared at δ 29.40 (C-4 and C-28), δ 29.70 (C-5 to C-27), and δ 32.02 belonged to C-3 and C-29. The comparison of spectral data of this compound **ASLD5** and values of hentriacontane (**190**) is tabulated in Table 4.14.

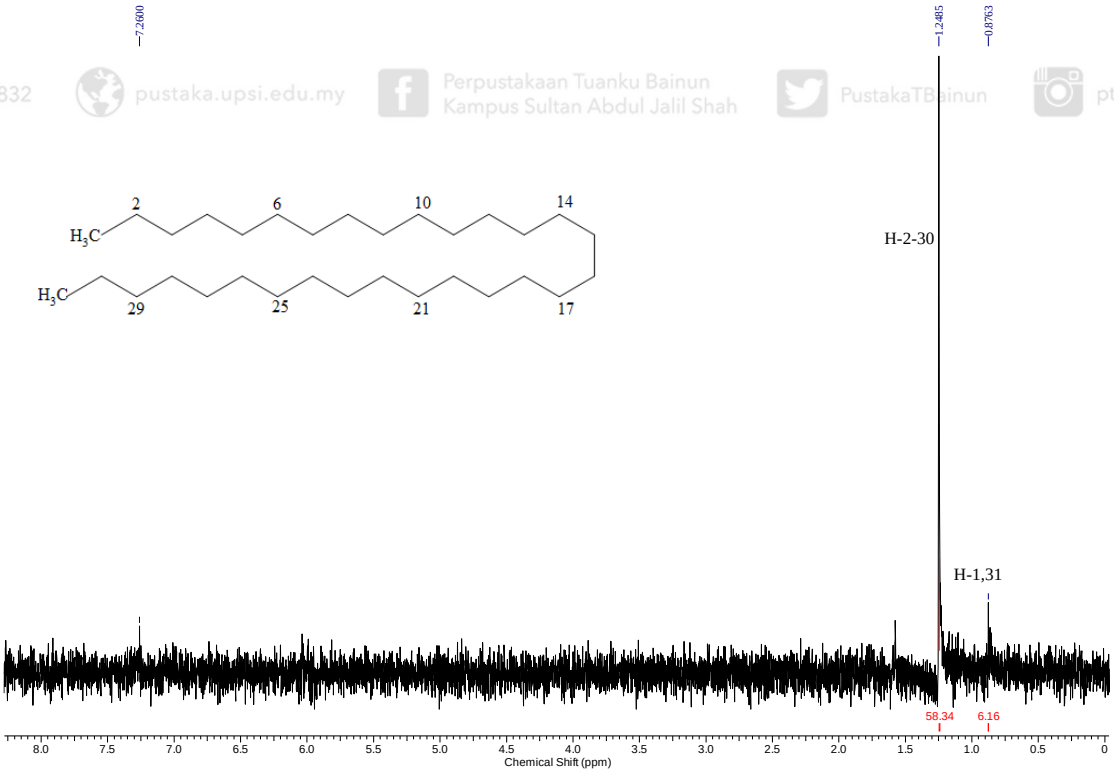


Figure 4.60. ^{1}H-NMR spectrum of Compound **ASLD5**

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Figure 4.61. ^{13}C-NMR spectrum of compound **ASLD5**

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Table 4.14

*^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C (125 MHz, $CDCl_3$) spectra data of compound **ASLD5** and hentriacontane (**190**)*

	Compound ASLD5		**Hentriacontane (190)**	
No	**δ^{1}H (ppm)**	**δ^{13}C (ppm)**	***δ^{1}H (ppm)**	***δ^{13}C (ppm)**
1, 31	0.88 (6H, *s*)	14.2	0.88	14.1
2, 30	**1.25	22.8	**1.27	22.7
3, 29		32.0		31.9
4, 28		29.4		29.4
5 – 27		29.7		29.7

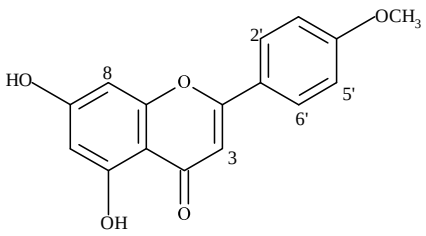
Note: ** 58H, overlapped

* Measured at 400 MHz (^{1}H) and 100 MHz (^{13}C) in $CDCl_3$ (Quintanilla-Licea et al., 2012)

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4.3.6 ASLD6: 5,7-dihydroxy-4′-methoxyflavone (39)



Compound **ASLD6** was afforded as a yellow amorphous. This compound was appeared as a black spot under short wave UV (254 nm) and not active under long-wave UV (365 nm). The spot was changed to yellow fluorescence after derivatisation with *p*-anisaldehyde and heated it to 50ºC. The UV spectrum of this compound (Figure 4.62) showed the maximum absorptions at 270 and 314 nm. The IR spectrum revealed the strong absorption at 3271 cm^{-1} represented the occurrence of the hydroxyl group. In addition, absorption bands at 2926 and 1606 cm^{-1} attributed to sp^3 C-H (stretching) and C=C group, respectively. The IR spectrum was displayed in Figure 4.63. Its molecular formula was suggested as $C_{16}H_{12}O_5$ and supported by its molecular ion peak at *m/z* 283.06128 $[M-H]^-$ analysed using ESI-MS technique (Figure 4.64).

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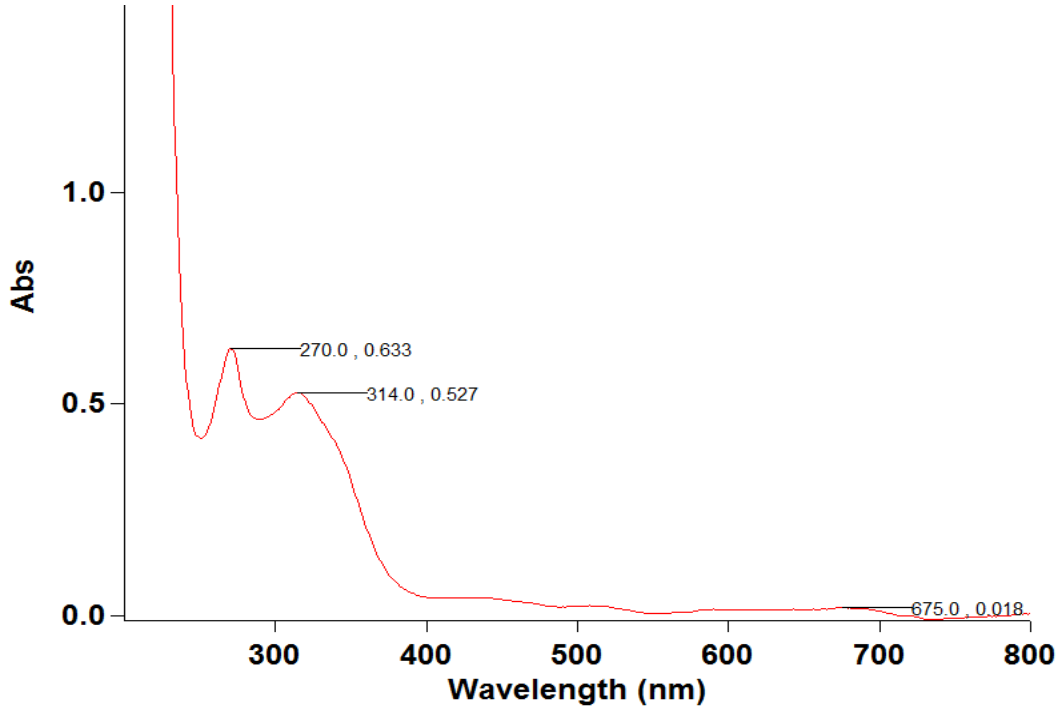


Figure 4.62. UV spectrum of compound **ASLD6**

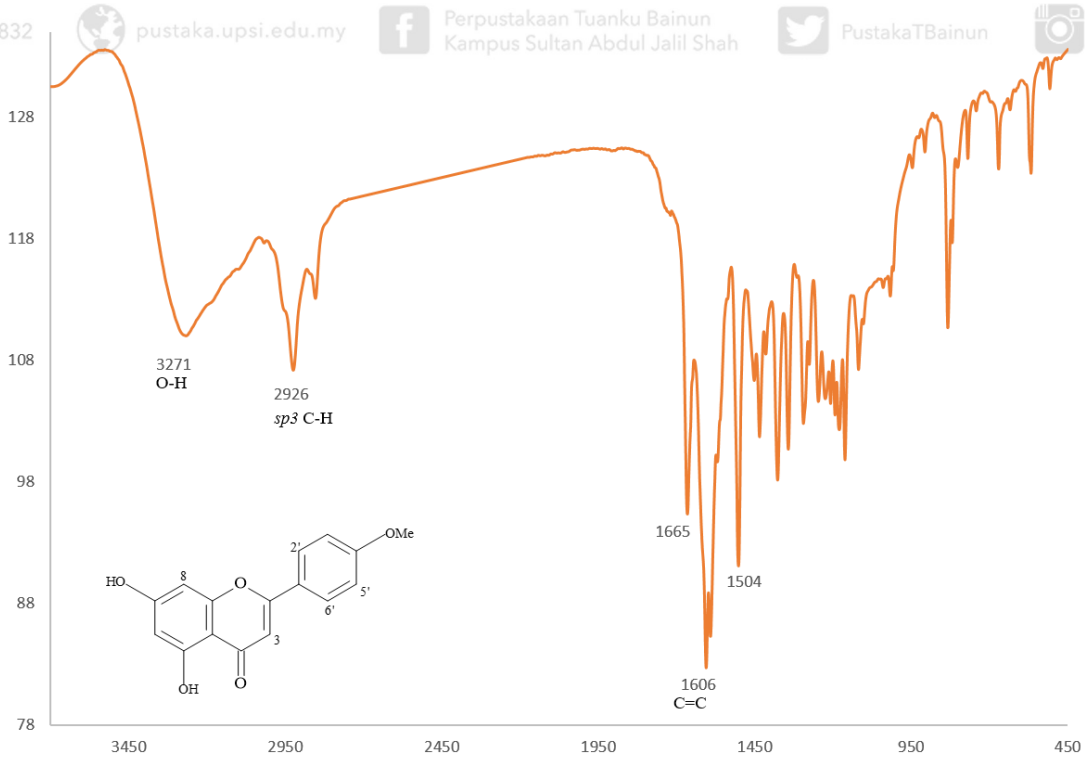


Figure 4.63. IR spectrum of compound **ASLD6**

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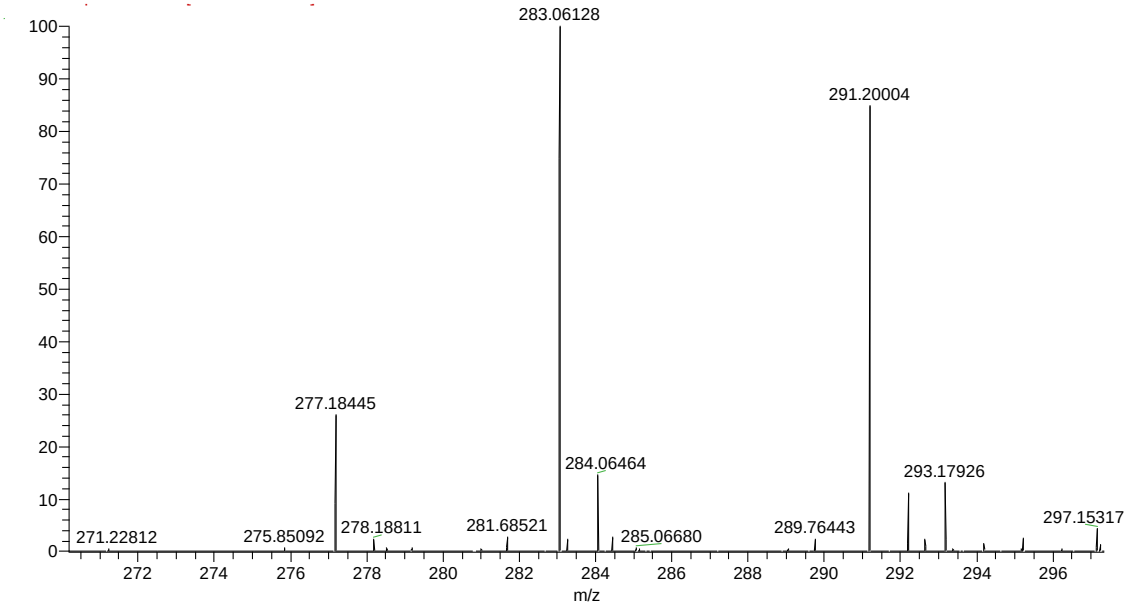


Figure 4.64. LC-MS spectrum of compound **ASLD6**

The ^{1}H-NMR spectrum ($CDCl_3$ + CD_3OD) in Figure 4.65 displayed resonances of one singlet of methoxyl group at δ 3.88 assigned to OCH_3-4′. A singlet corresponding to an aromatic proton, H-3 was observed at δ 6.56. Furthermore, two doublets of the aromatic proton were exhibited at δ 6.37 (H-6, *J*= 2.3 Hz) and δ 6.48 (H-8, *J* = 2.3 Hz). Another signal displayed as two doublets which characteristic of *ortho*-proton; signal at δ 6.94 (2H, *J* = 8.1 Hz) assigned to H-3′ and H-5′ as well as δ 7.78 (2H, *J* = 8.6 Hz) attributed to H-2′ and H-6′. A broad singlet corresponded to the hydroxyl group was observed at δ 12.81.

On the basis of the analysed spectral data and comparison with the literature, the structure of compound **ASLD6** was elucidated as 5,7-dihydroxy-4′-methoxyflavone (**39**) which was formerly isolated from flower of *Chromolaena odorata (Eupatorium odoratum)* (Suksamrarn et al., 2004).

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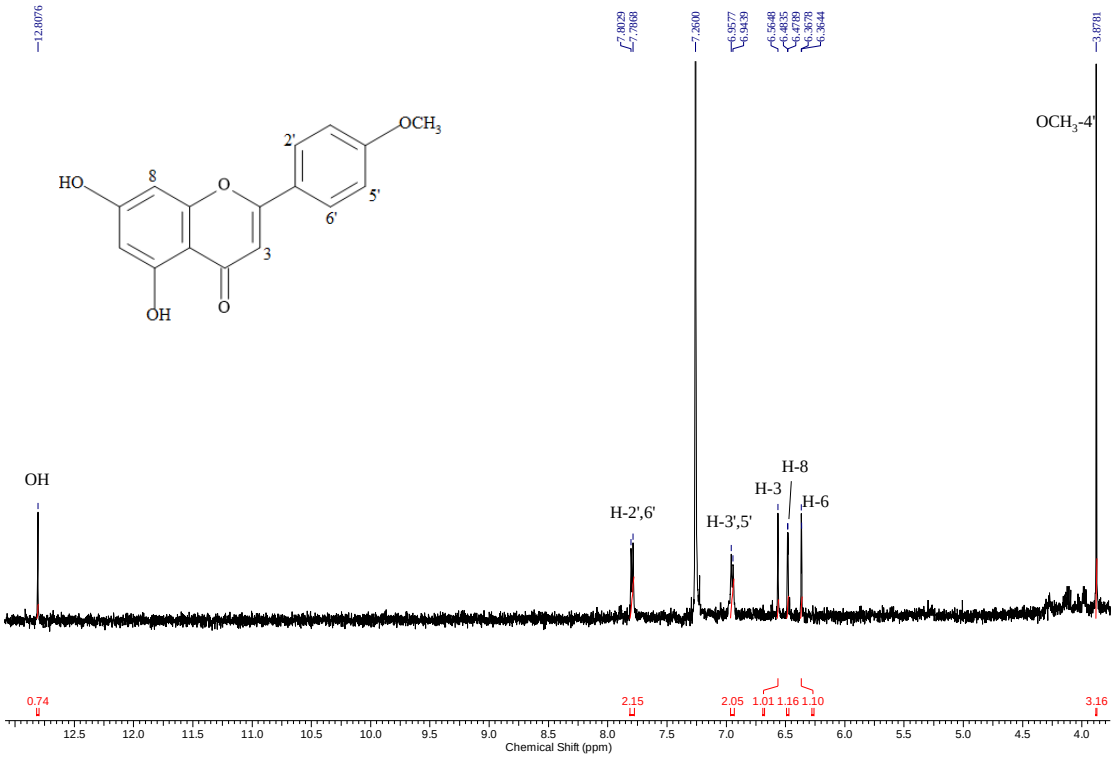


Figure 4.65. ^{1}H-NMR spectrum of compound **ASLD6**

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Table 4.15

*^{1}H NMR (500 MHz, $CDCl_3$+CD_3OD) spectral data of compound **ASLD**6 and 5,7-dihydroxy-4′-methoxyflavone (**39**)*

	Compound ASLD6	**5,7-dihydroxy-4′-methoxyflavone (39)**
No	**δ^{1}H (ppm)**	***δ^{1}H (ppm)**
1		
2		
3	6.56 (1H, *s*)	6.51 (*s*)
4		
5		
6	6.37 (1H, *d*, *J*=2.3 Hz)	6.22 (*d*, *J*=1.9 Hz)
7		
8	6.48 (1H, *d*, *J*=1.7 Hz)	6.38 (*d*, *J*=1.9 Hz)
9		
10		

(continued)

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Table 4.15 *(continued)*

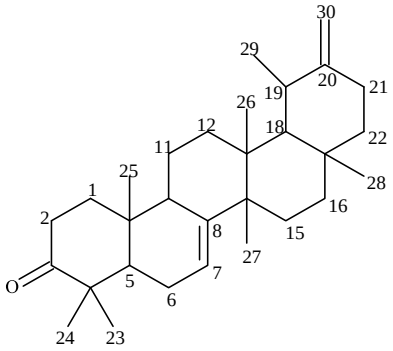
	Compound ASLD6	**5,7-dihydroxy-4′-methoxyflavone (39)**
No	**δ^1H (ppm)**	***δ^1H (ppm)**
1′		
2′	7.78 (1H, *d, J*=8.6 Hz)	7.79 (*d, J*=8.8 Hz)
3′	6.94 (1H, *d, J*=8.1 Hz)	6.96 (*d, J*=8.8 Hz)
4′		
5′	6.94 (1H, *d, J*=8.1)	6.96 (*d, J*=8.8 Hz)
6′	7.78 (1H, *d, J*=8.6)	7.79 (*d, J*=8.8 Hz)
OH-5	12.81 (1H, *s*)	
OH-7		
OCH_3-4′	3.88 (3H, *s)*	3.84 (*s*)

Note: * Measured at 400 MHz (^{1}H) in $CDCl_3$ + CD_3OD (15:1) (Suksamrarn et al., 2004)

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4.3.7 ASLD7: Aquilene A (191)



Purification of CH_2Cl_2 crude extract of *A. subintegra* leaf (L1A-11) has afforded compound **ASLD7**. Compound **ASLD7** was obtained as a white needle (15.0 mg, 0.75%). On the TLC analysis, the compound was non-active under a short wave (254 nm) and long wave UV (365 nm), however, it changed into purple colour upon mild heating (50^oC) after sprayed with a *p*-anisaldehyde reagent. The IR spectrum (Figure 4.66) revealed the presence of carbonyl group at 1703 cm^{-1}, C-H (sp^3) stretching band at 2916 cm^{-1}, and the presence of the CH_3 band at 1386 cm^{-1}. The LC-MS spectrum revealed a significant molecular ion peak at *m/z* 423.19754 $[M+H]^+$ and its molecular formula was determined as $C_{30}H_{46}O$ (Figure 4.67).

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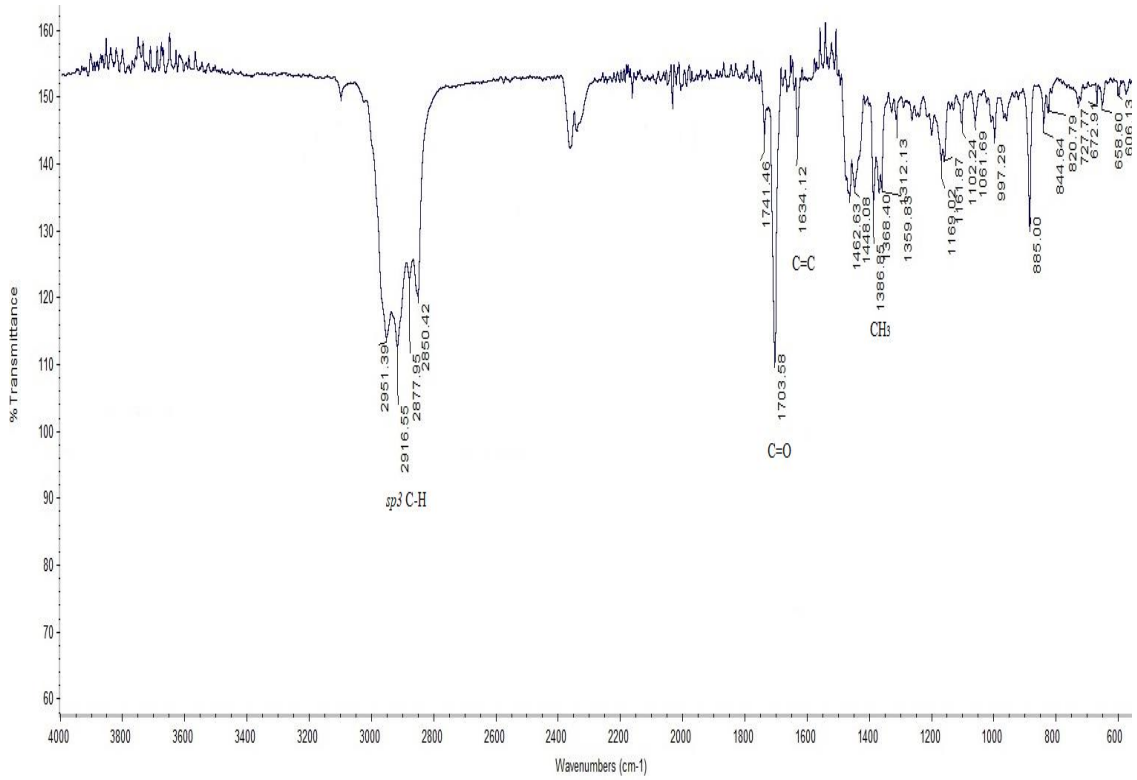


Figure 4.66. IR spectrum of compound **ASLD7**

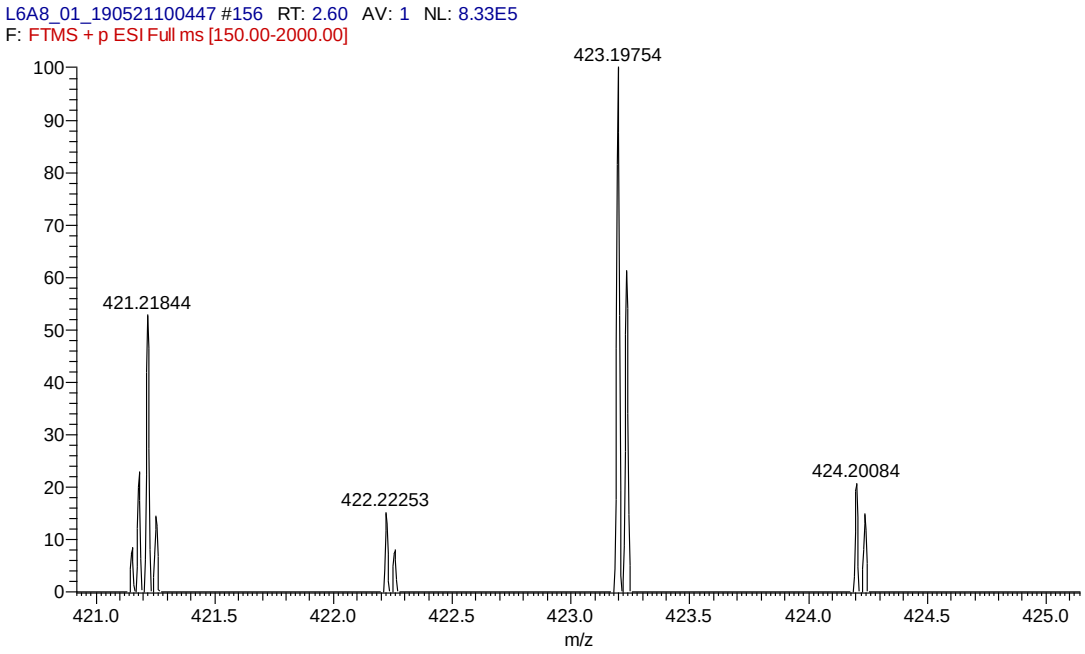


Figure 4.67. LC-MS spectrum of compound **ASLD7**

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The ^{1}H-NMR spectrum of compound **ASLD7** was presented in Figure 4.68. This spectrum showed 46 protons with the presence of six methyl singlets at δ 1.04, 1.11, 1.00, 0.81, 1.01, and 1.06 corresponding to H-23, H-24, H-25, H-26, H-27, and H-28, respectively. A methyl doublet resonated at δ 0.91 (*J*= 6.3 Hz) assigned to H-29. The spectrum also exhibited two singlets resonated at δ 4.66 and 4.83, representing the exo-cyclic double bond proton, H-30a, and H-30b, respectively. A doublet with a coupling constant of 3.5 Hz resonated at δ 5.30 was attributed to an olefinic proton at H-7.

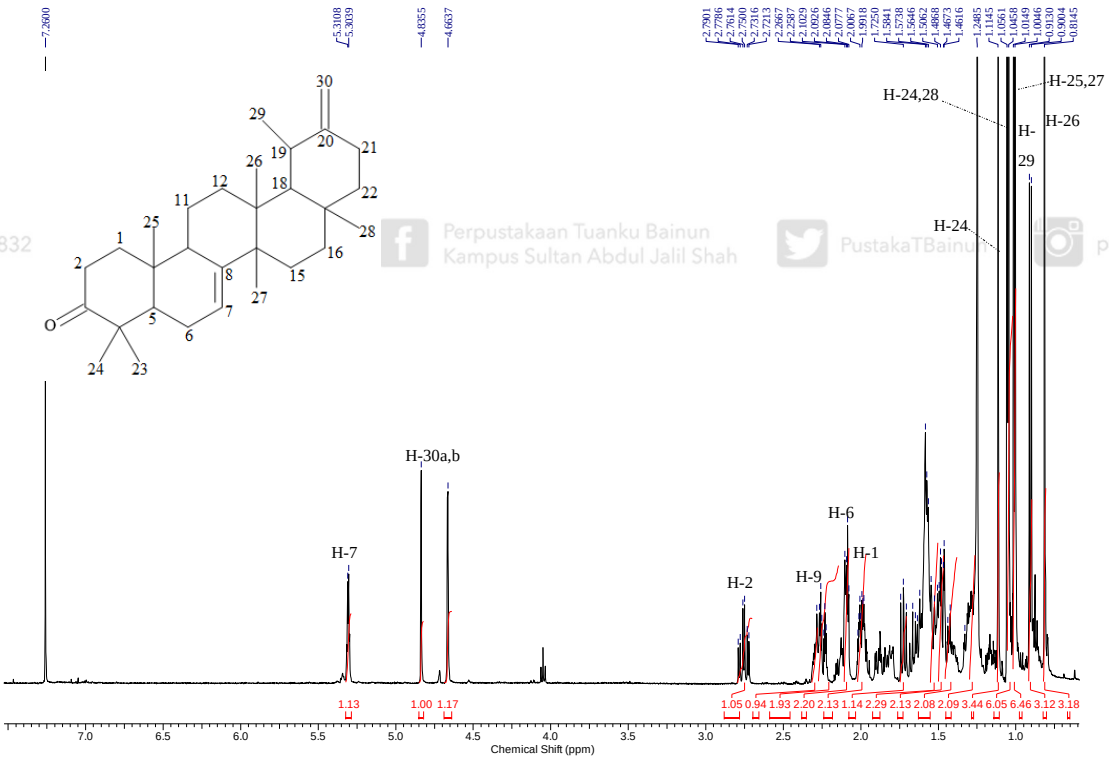


Figure 4.68. ^{1}H-NMR spectrum of compound **ASLD7**

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The ^{13}C-NMR and DEPT spectrum of **ASLD7** (Figure 4.69 and Figure 4.70) revealed 30 signals corresponding to 30 carbons. The spectrum showed the presence of seven methyls, ten methylene, five methine and eight quaternary carbons. The signal at δ 217.2 representing the carbonyl group (C-3), while another seven quaternary carbons positioned at δ 47.9, 146.1, 36.2, 51.3, 43.6, 36.6 and 159.1 attributed to C-4, C-8, C-10, C-13, C-14, C-17 and C-20, respectively. In addition, seven methyls signal appeared at δ 24.4, 21.7, 12.8, 22.1, 27.5, 18.3 and 29.4 assigned to C-23, C-24, C-25, C-26, C-27, C-28 and C-29, accordingly.

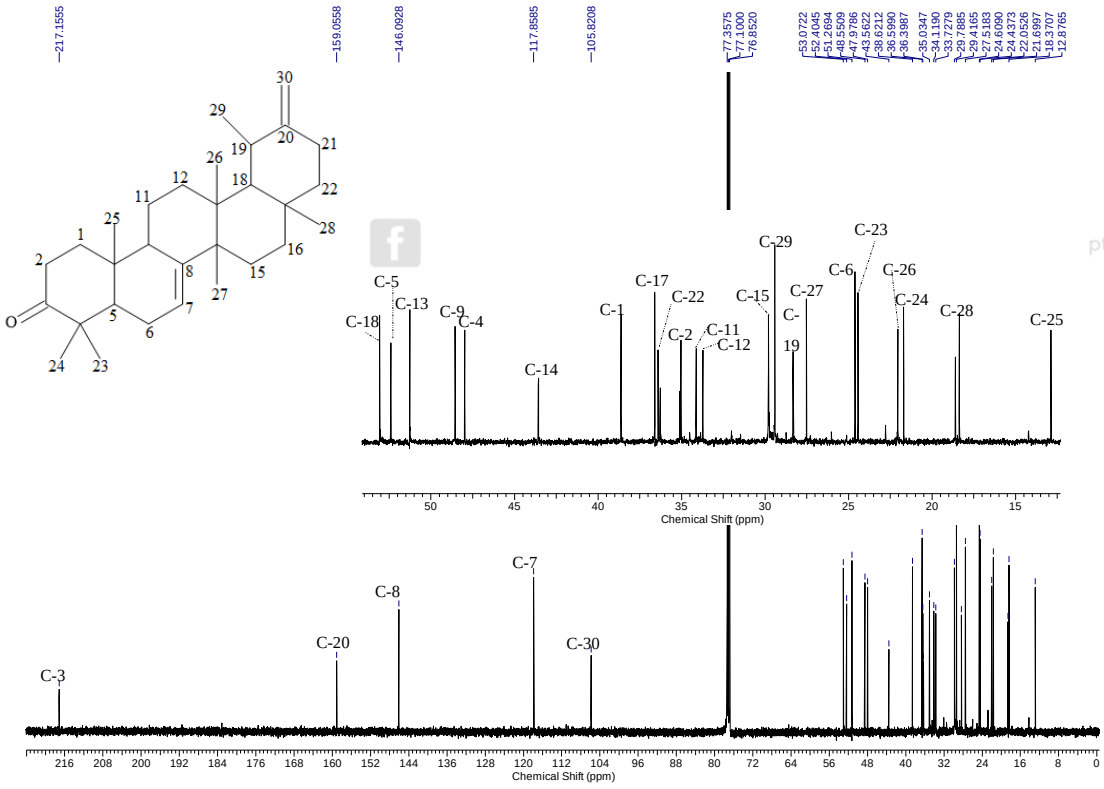


Figure 4.69. ^{13}C-NMR spectrum of compound **ASLD7**

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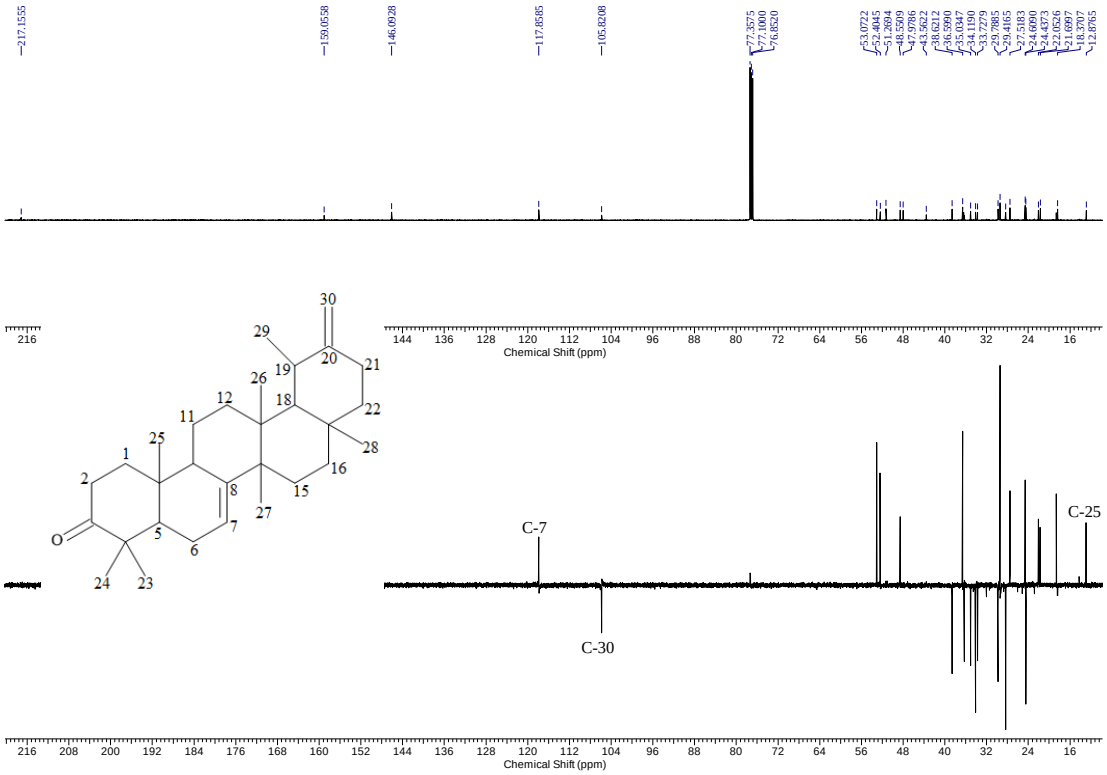


Figure 4.70. DEPT spectrum of compound **ASLD7**

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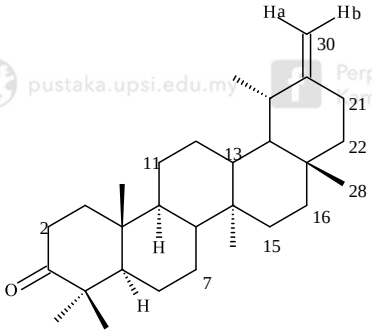
In 2D NMR experiments; COSY, HMBC and HMQC data analyses used to confirm the assignments of carbon and proton in the molecule. Based on the COSY spectrum in Figure 4.71, there were correlations between H-1/H-2, H-5/H-6, H- 6/H-7, H-16/H-15 and H-22/H-21. The position of an olefinic proton H-7 was confirmed by 2D TOCSY assignment in Figure 4.72. TOCSY is an extension of the COSY assignment were showed the protons unbroken chain of couple spins in the molecule, linked by a set of the cross peak. The spectrum showed the correlation between H-7 with H-1, H-2, H-5 and H-6. The HMQC spectrum of **ASLD7** is shown in Figure 4.73. The structure of **ASLD7** was further confirmed by the HMBC spectrum, as shown in Figure 4.74 and Figure 4.75.

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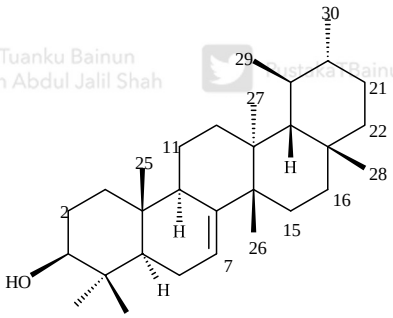
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The HMBC long-range correlations furnished correlation of H-5 with C-4/ C-24/ C-23; H-23 with C-3/ C-4/ C-5/ C-6/ C-24; H-24 with C-3/ C-4/ C-5/ C-23; H-25 with C-1/ C-9/ C11; H-26 with C-18/ C-14/ C-13/ C-12; H-27 with C-14/ C-13/ C-8; H-28 with C-16/ C-17/ C-18/ C-22; H-29 with C-18/ C-19/ C-20; and H-30 with C-20. The correlations of HMBC and COSY of **ASLD7** were shown in Figure 4.76.

The structure of **ASLD7** is closest to the taraxastenone (**194**), which has been reported from the aerial parts of *Wissadula periplocifolia* (Teles et al., 2014). However, the structure **ASLD7** has an additional double bond at C-7 and C-8, similar to the compound bauerenol (**195**) from *Tabernaemontana longipes* (Carothers et al., 2018).



(**194**)



(**195**)

From the analysis of its spectral data summarized in Table 4.16, compound **ASLD7** was established as a new compound, identified as 4,4,6a,6b,8a,12,14a-heptamethyl-11-methylene-2,4a,5,7,8,9,10,12,12a,13,14,14b-dodecahydro-1H-picen-3-one or trivially named as aquilene A (**191**).

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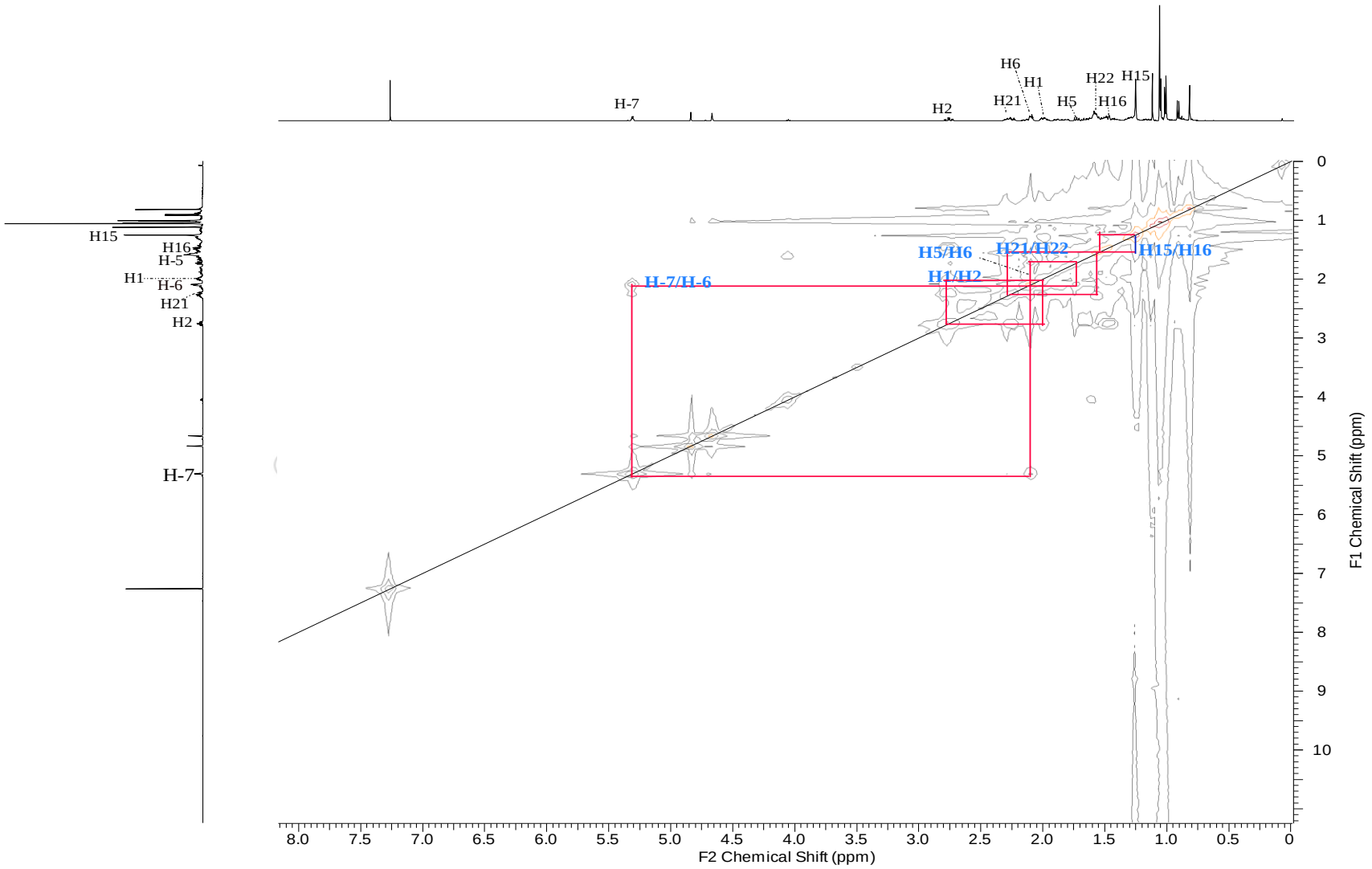


Figure 4.71. COSY spectrum of compound **ASLD7**

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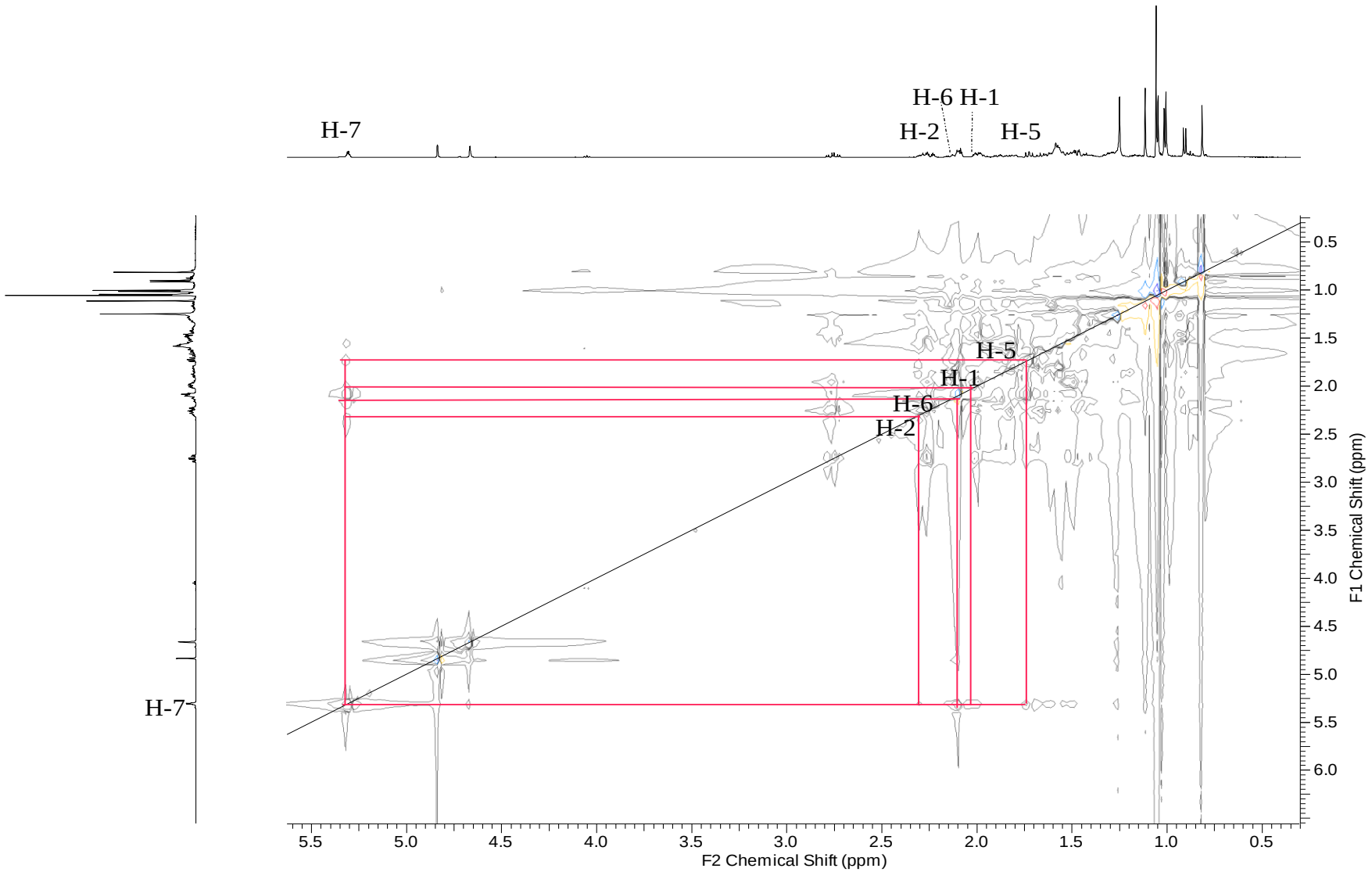


Figure 4.72. TOCSY spectrum of compound **ASLD7**

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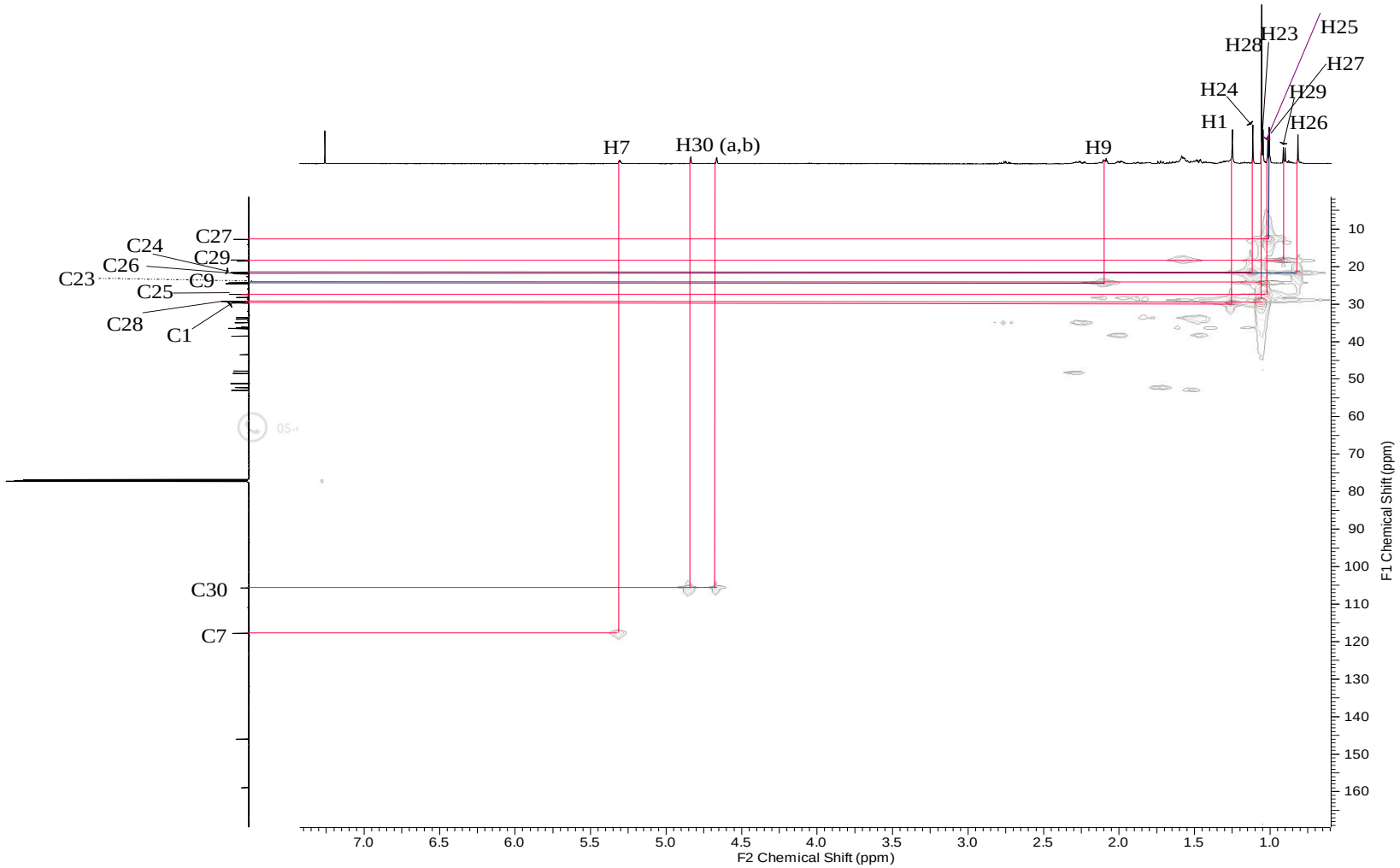


Figure 4.73. HMQC spectrum of compound **ASLD7**

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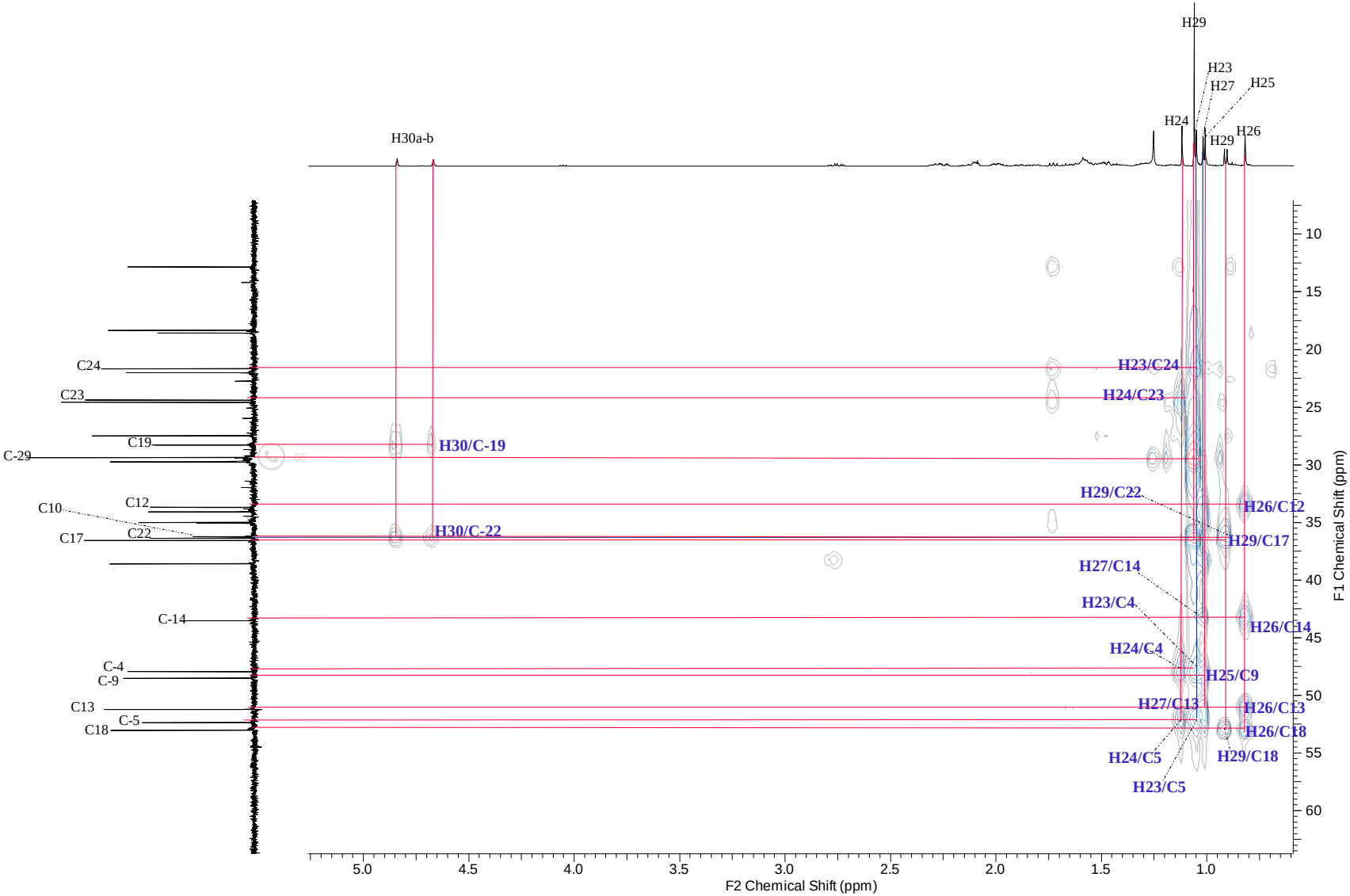


Figure 4.74. HMBC spectrum of compound **ASLD7**

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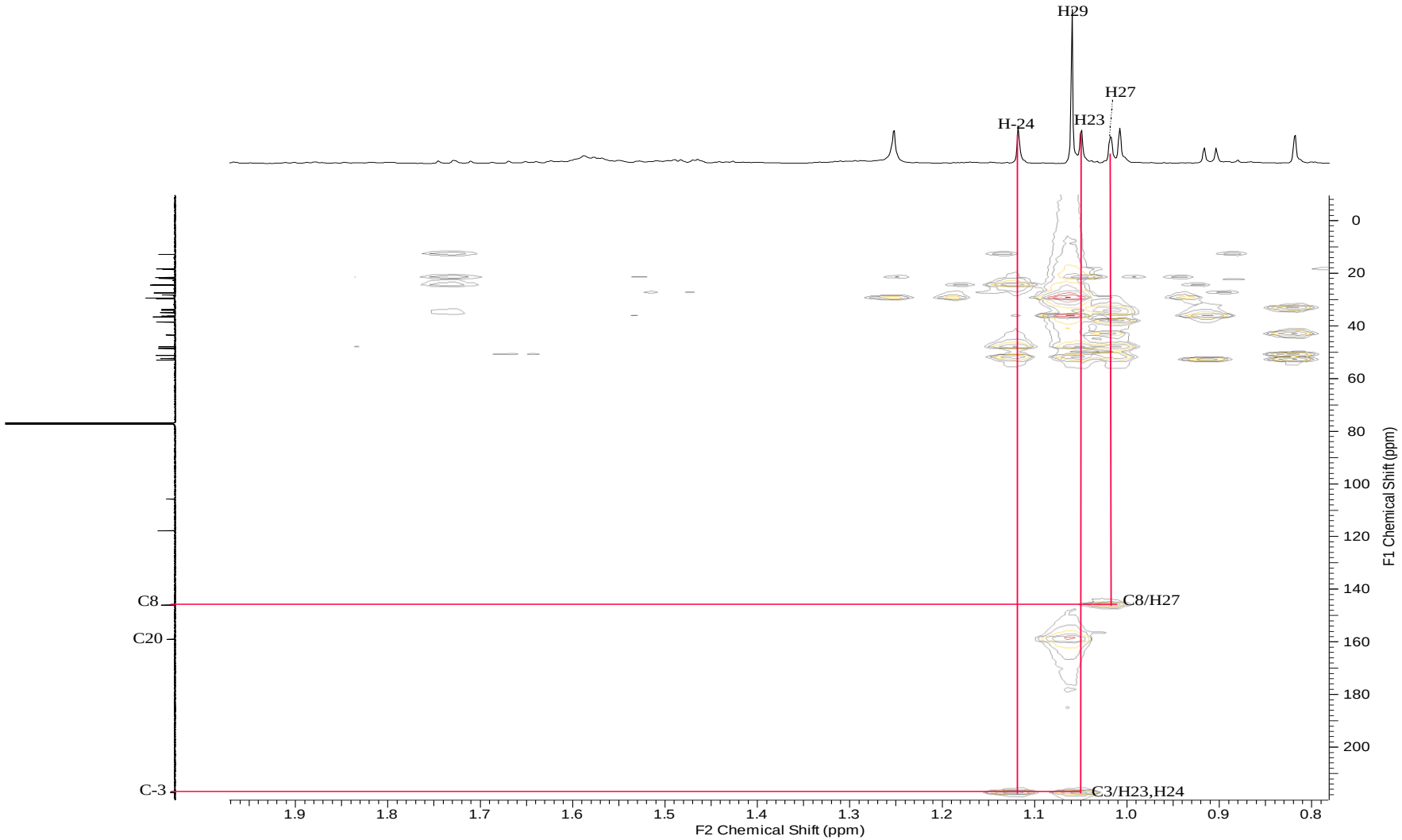


Figure 4.75. Expanded HMBC spectrum of compound **ASLD7**

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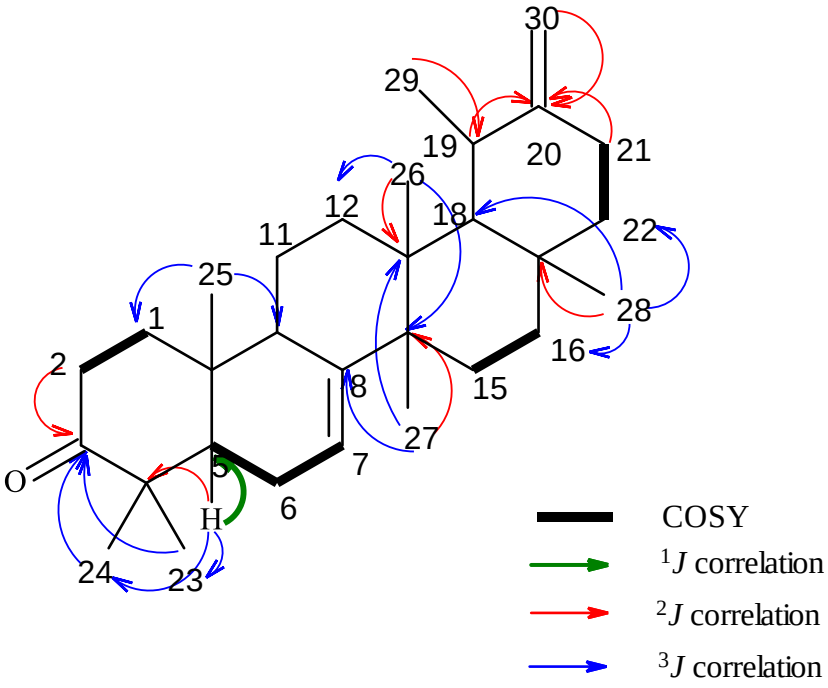


Figure 4.76. COSY, ^{1}H and ^{13}C correlations observed in HMBC spectrum of **ASLD7**

Table 4.16

*^{1}H (500 MHz, CDCl3), ^{13}C (125 MHz, CDCl3), and 2D-NMR spectral data of compound **ASLD7 (191)***

No	**δ^{1}H (ppm)**	**δ^{13}C (ppm)**	**HMBC**	**COSY**	**δ^{13}C (ppm) (Reference)**
1	2.00 (2H, *m*)	38.6		H-2	39.6*
2	2.76, 2.28 (2H, *m*)	35.0	C-3	H-1	34.1*
3		217.2			218.1*
4		47.9			47.4*
5	1.71 (1H, *m*)	52.4	C-24, C-23, C -4	H-6	55.0*
6	2.10 (2H, *m*)	24.6		H-5, H-7	24.1**
7	5.30 (1H, *d*, *J* = 3.5 Hz)	117.9		H-6	116.5**
8		146.1			145.4**
9	2.30 (1H, *m*)	48.6			48.6**

(continued)

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Table 4.16 *(continued)*

No	δ^1H (ppm)	δ^{13}C (ppm)	HMBC	COSY	δ^{13}C (ppm) (Reference)
10		36.2			35.0**
11	1.40 (2H, *m*)	34.1			32.5**
12	1.45 (2H, *m*)	33.7			31.6**
13		51.3			39.3*
14		43.6			42.1*
15	1.25 (2H, *m*)	29.7		H-16	26.7*
16	1.55 (2H, *m*)	18.6		H-15	16.7**
17		36.6			34.1*
18	1.51 (1H, *m*)	53.1			55.0**
19	1.80 (1H, *m*)	28.3	C-20		35.3**
20		159.1			154.5*
21	2.25 (2H, *m*)	35.1	C-20		29.3**
22	1.47 (2H, *m*)	36.4			37.8**
23	1.04 (3H, *s*)	24.4	C-3, C-24, C-4, C-5, C-6		25.6*
24	1.11 (3H, *s*)	21.7	C-3,C-23, C-4, C-5		21.0*
25	1.00 (3H, *s*)	12.8	C-1, C-9, C-11		13.1**
26	0.81 (3H, *s*)	22.1	C-12, C-13, C-14, C-18		23.6**
27	1.01 (3H, *s*)	27.5	C-14,C-13, C-8		22.8**
28	1.06 (br. *s*)	29.4	C-16,C-17, C-18, C-22		19.5*
29	0.91 (3H, *d*, J = 6.3 Hz)	18.3	C-18, C-19		25.5*
30a	4.66 (1H, *s*)	105.8	C-20		107.3*
30b	4.83 (1H, *s*)				

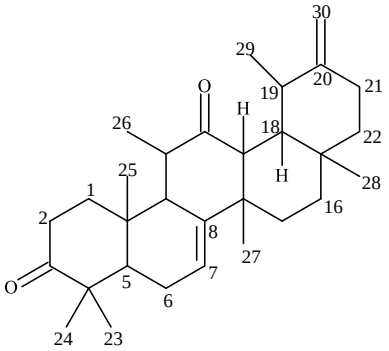
Note: * Measured at 100 MHz (^{13}C) in $CDCl_3$ (Teles et al., 2014)

** Measured at 125 MHz (^{13}C) in $CDCl_3$ (Carothers et al., 2018)

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4.3.8 ASLD8: Aquilene B (192)



Purification of the CH_2Cl_2 crude extract of *A. subintegra* leaf (L1A-11) using multiple CC has afforded compound **ASLD8**. Compound **ASLD8** was obtained as a white amorphous (5.0 mg, 0.25%). On the TLC analysis, the compound was non-active under short (254 nm) and long wave UV (365 nm), but it changed to purple colour upon mild heating (50^{o}C) after sprayed with the *p*-anisaldehyde reagent. The IR spectrum of **ASLD8** (Figure 4.77) disclosed the presence of a carbonyl (C=O) group at 1704 cm^{-1}. In addition, C-H (sp^3) stretching was observed at band 2957 cm^{-1} and the presence of C=C group at 1630 cm^{-1} and CH_3 band at 1369 cm^{-1}. The LC-MS spectrum revealed a significant molecular ion peak at *m/z* 437.23420 $[M+H]^+$ and its molecular formula was determined as $C_{30}H_{44}O_2$ (Figure 4.78).

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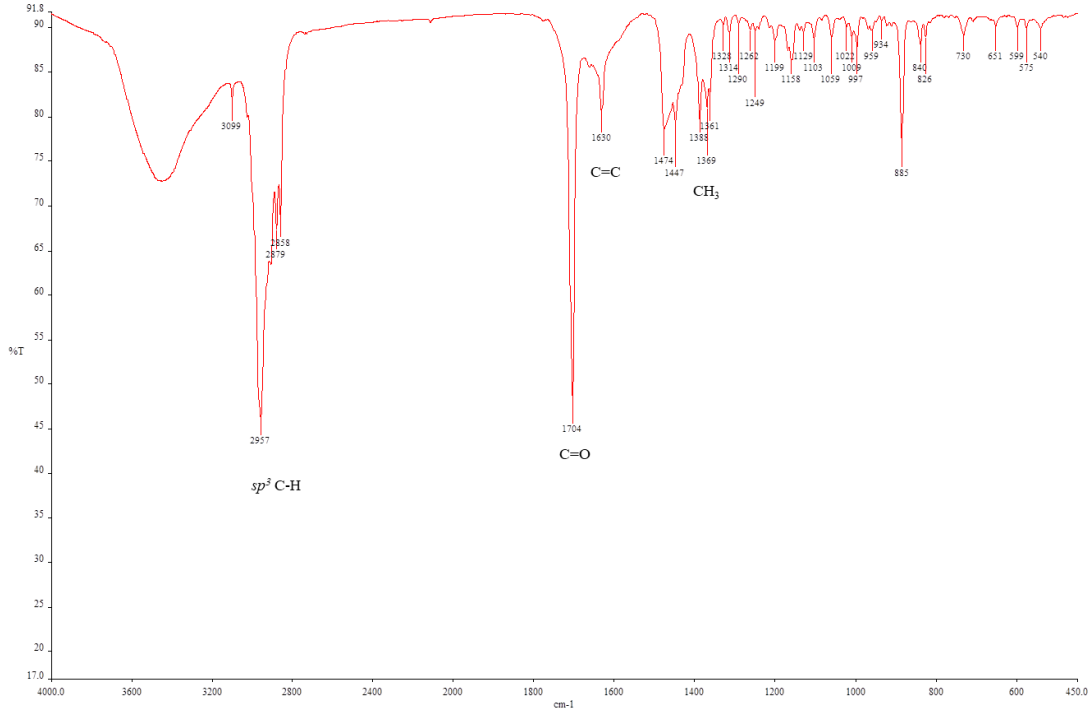


Figure 4.77. IR spectrum of compound **ASLD8**

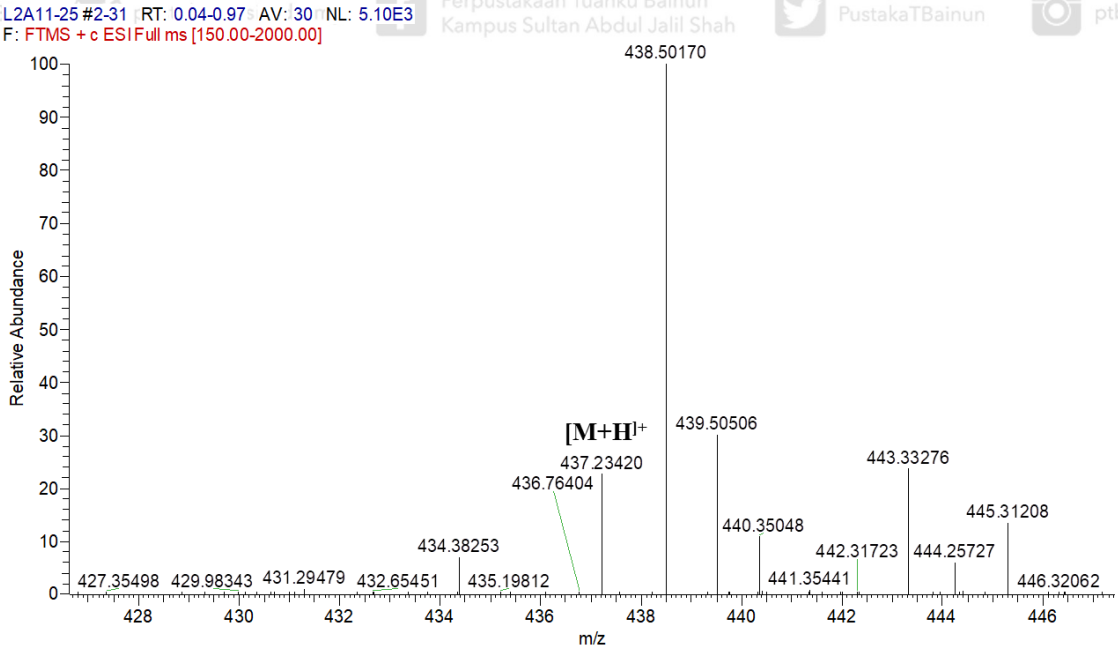


Figure 4.78. LC-MS spectrum of compound **ASLD8**

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The ^{1}H-NMR spectrum of compound **ASLD8** was presented in Figure 4.79. This spectrum showed 46 protons with the presence of six methyl singlets at δ 1.04, 1.11, 1.00, 0.81, 1.01 and 1.05 corresponding to H-23, H-24, H-25, H-26, H-27 and H-29, respectively. One methyl doublet was located at δ 0.91 (*J*=6.9 Hz) attributed to H-28. A triplet with a coupling constant J = 4.0 Hz resonated at δ 5.30 was attributed to an olefinic proton at H-7. The spectrum also exhibited two singlets resonate at δ 4.66 and 4.83, representing the exocyclic double bond proton, H-30a and H-30b. In addition, a doublet proton at δ 3.48 (1H, *J* = 5.15 Hz) resonates to H-13.

The ^{13}C-NMR spectrum of **ASLD8** (Figure 4.80) revealed 30 signals corresponding to 30 carbons. The spectrum showed the presence of seven methyls, ten methylene, five methine and eight quaternary carbons. The signal at δ 217.2 and 217.0 representing the carbonyl group (C-3 and C-12), while another seven quaternary carbons positioned at δ 47.9, 148.7, 36.2, 51.3, 43.6, 36.6 and 158.0 attributed to C-4, C-8, C-10, C-13, C-14, C-17 and C-20, respectively. In addition, seven methyls signal appeared at δ 24.4, 21.6, 12.8, 22.0, 27.5, 18.3 and 28.3 assigned to C-23, C-24, C-25, C-26, C-27, C-28 and C-29, accordingly.

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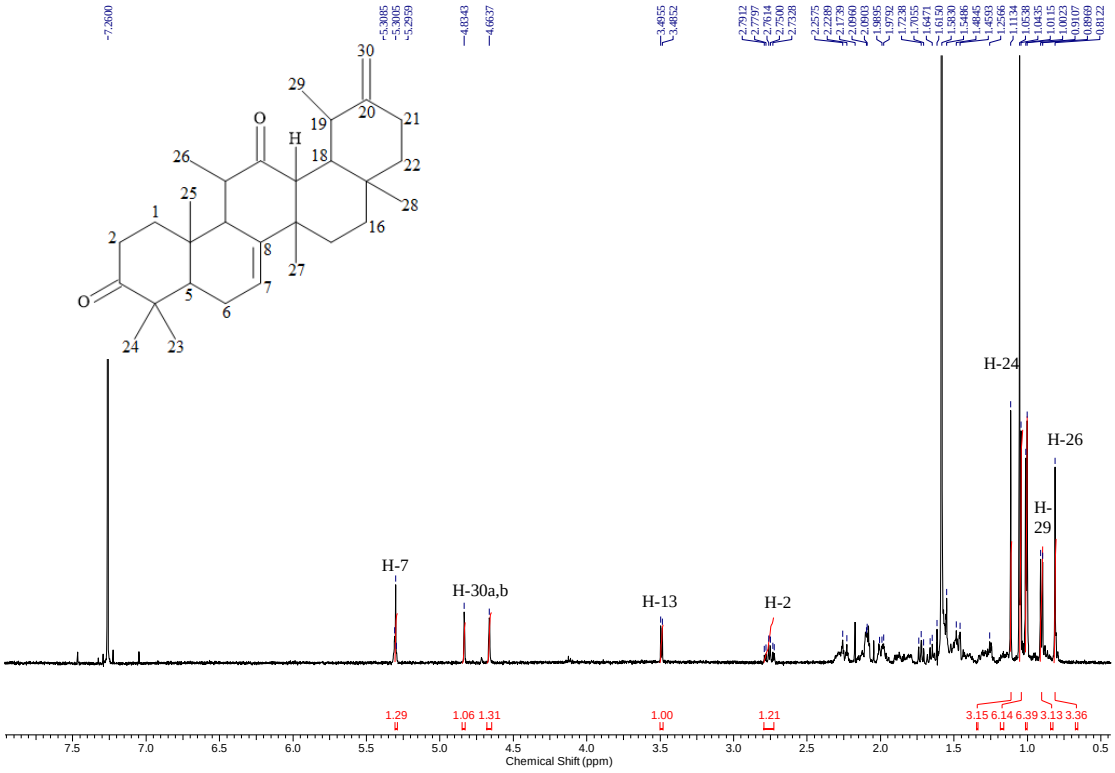


Figure 4.79. ^{1}H-NMR spectrum of compound **ASLD8**

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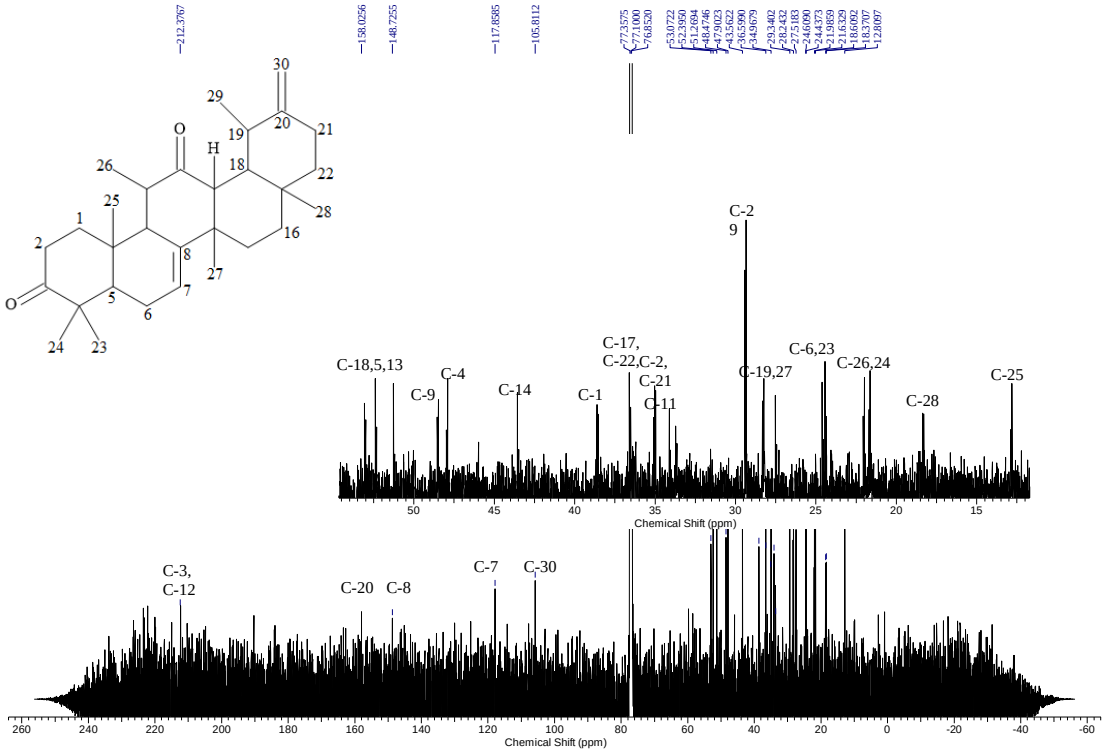


Figure 4.80. ^{13}C-NMR spectrum of compound **ASLD8**

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In 2D NMR experiments; COSY, HMBC and HMQC data analyses helps confirm the assignments of carbon and proton in the molecule. Based on the COSY spectrum in Figure 4.81, there were correlations between H-1/H-2 and H-5/H-6. The HMQC spectrum of **ASLD8** is shown in Figure 4.82. The spectrum showed the correlation H-14 to its belonging carbon C-14 (δ 51.3). This signal was deshielded due to the presence of the carbonyl group at C-12. The structure of **ASLD8** was further confirmed by the HMBC spectrum, as shown in Figure 4.83.

The HMBC long-range correlations furnished correlation of H-26 with C-14/ C-12 (^3J) and C-13 (^2J); H-23 with C-3/ C-24 (^3J); H-24 with C-3/ C-23 (^3J); H-27 with C-15 (^2J), C-14/ C-16 (^3J) and C-8 (^4J) ; H-26 with C-14/ C-13/ C-12; H-29 with C-20 (^3J) and H-30 with C-19/ C-29. The correlations of HMBC and COSY of **ASLD8** were presented in Figure 4.84.

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From the analysis of its spectral data shown in Table 4.17, compound **ASLD8** was established as a new compound, identified as 6b-hydroxy-4,4,7,8a,12,12b,14a-heptamethyl-11-methylene- 1,4,4a,5,6b,7,8,8a,9,10,11,12,12a,12b,13,14,14a,14b-octadecahydro picen- 3(2H)-one or trivially named as aquilene B (**192**). This compound has similarity with the previous (**ASLD7**), except the additional carbonyl group at C-12. Thus compound **ASLD**8 possess same backbone as **ASLD7** with additional functional group carbonyl at C-12 make it slightly differ with previous.

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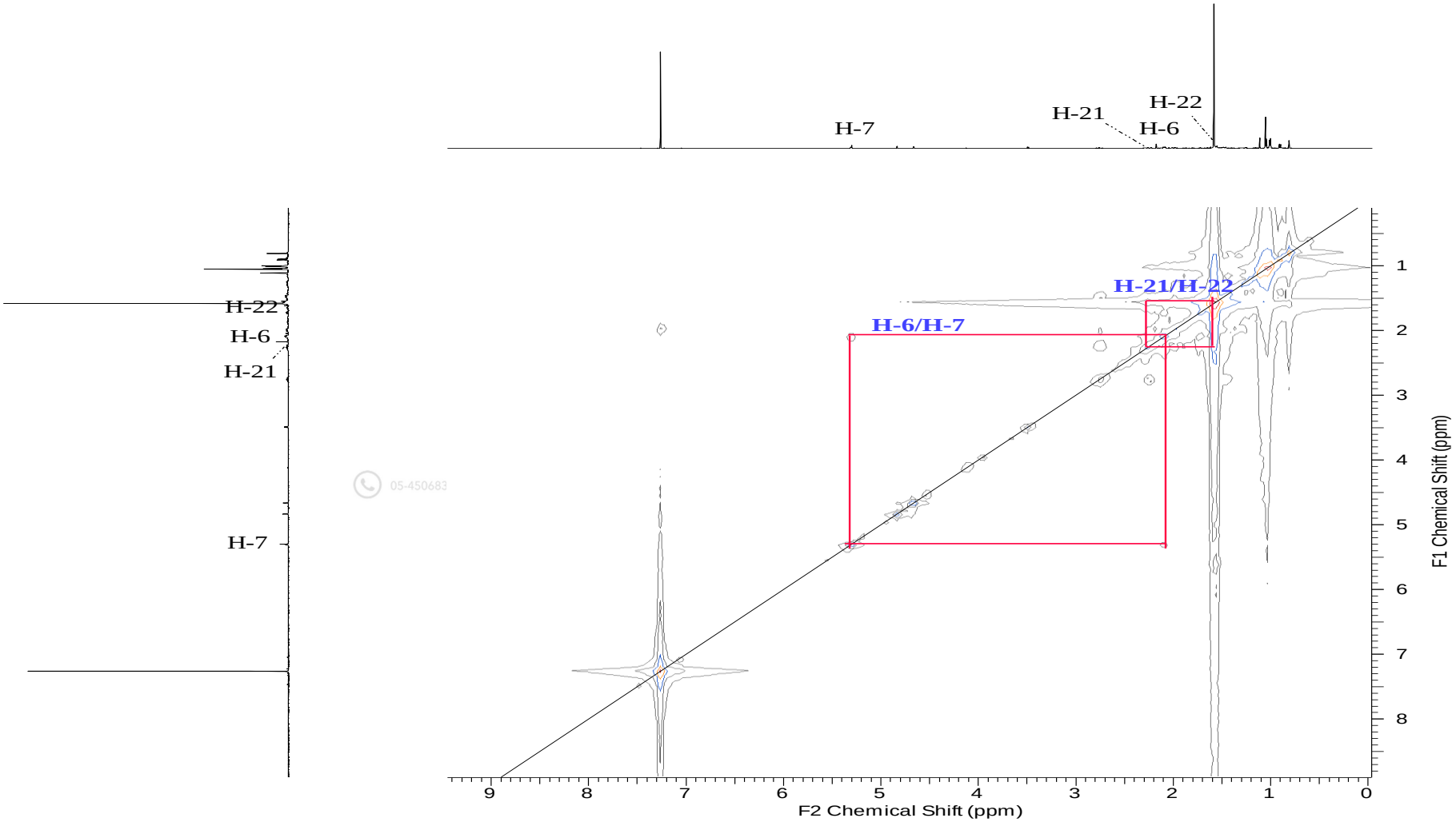


Figure 4.81. COSY spectrum of compound **ASLD8**

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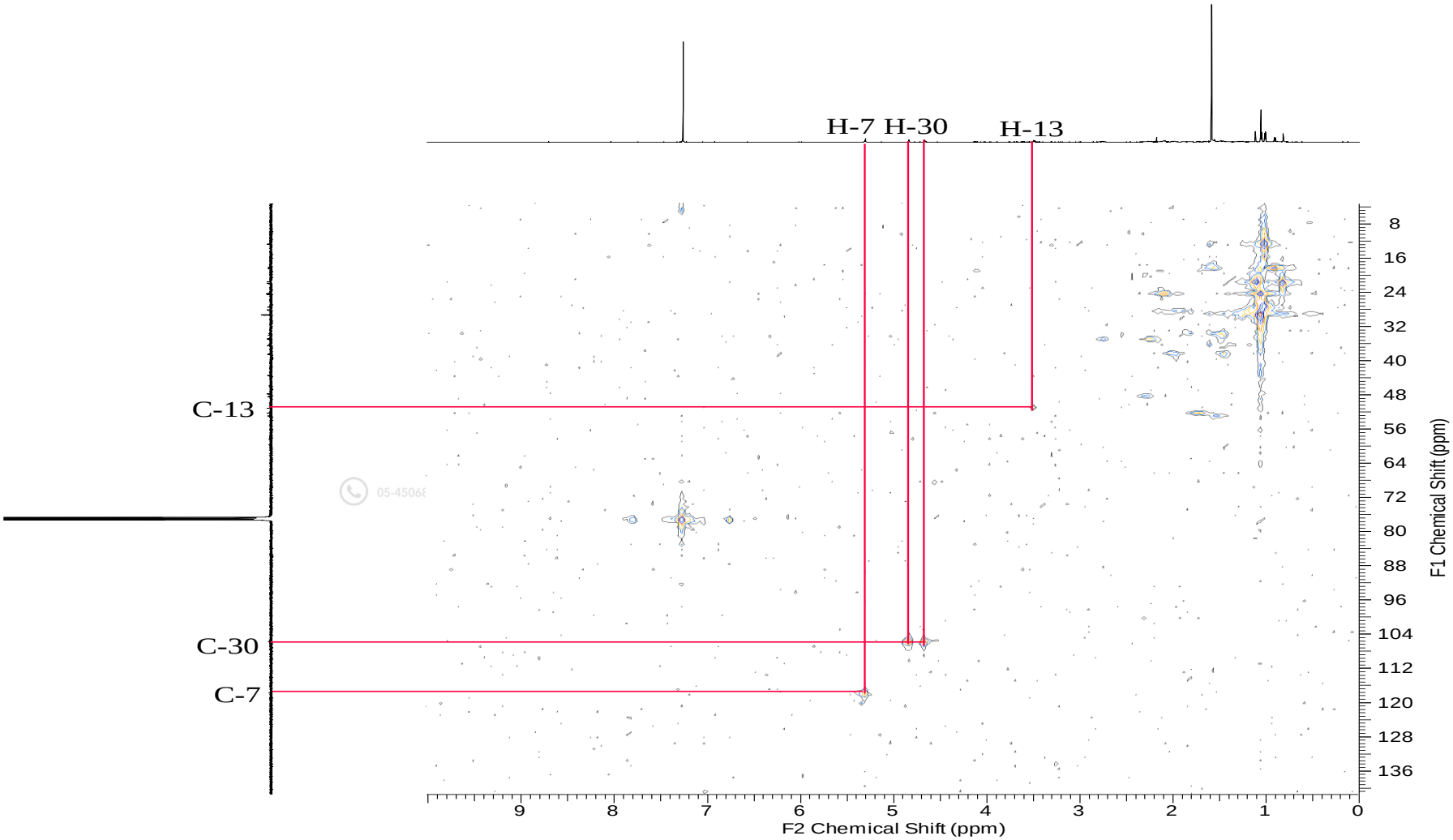


Figure 4.82. HMQC spectrum of compound **ASLD8**

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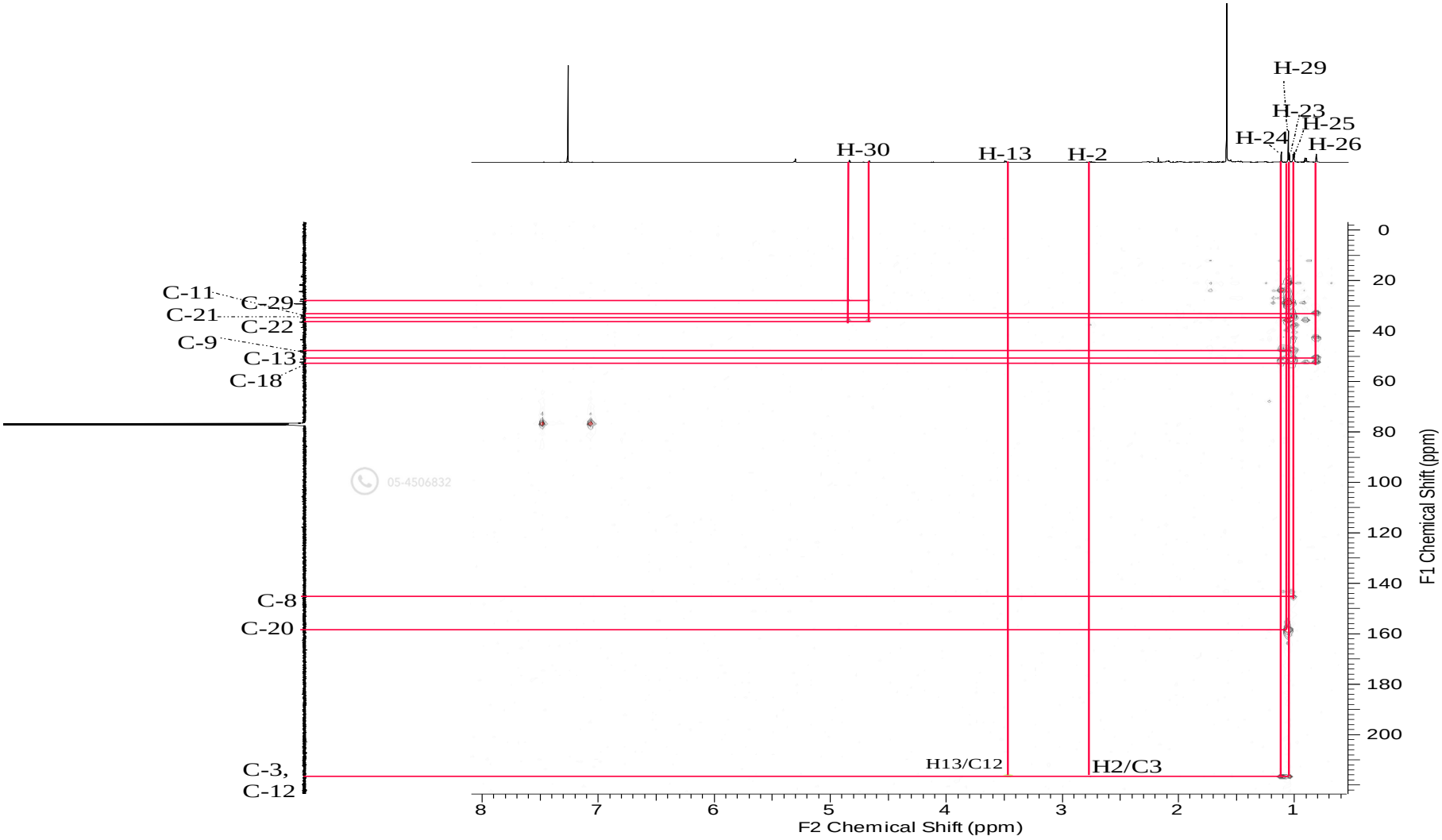


Figure 4.83. HMBC spectrum of compound **ASLD8**

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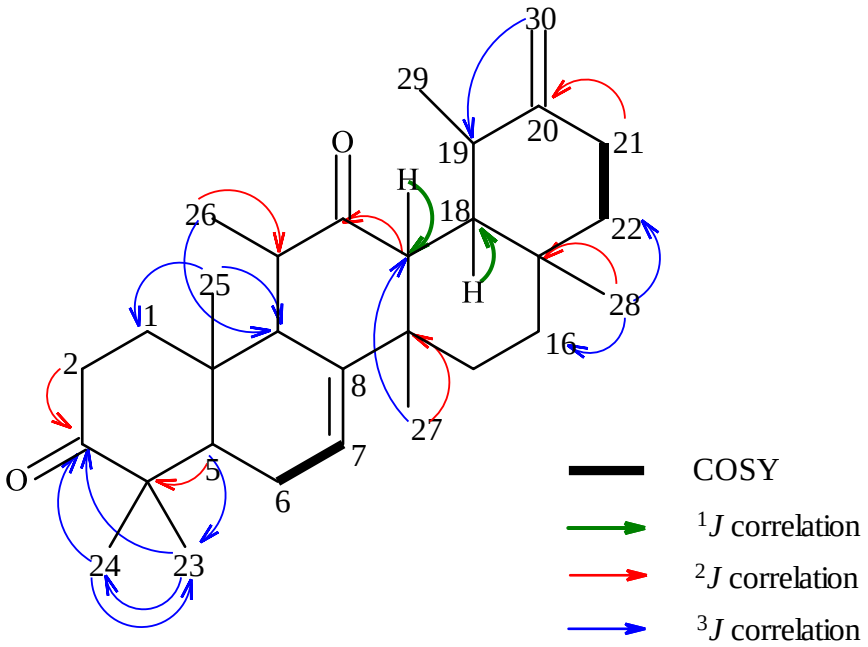


Figure 4.84. COSY, ^{1}H and ^{13}C correlations observed in HMBC spectrum of **ASLD7**

Table 4.17

^{1}H (500 MHz, $CDCl_3$), ^{13}C (125 MHz, $CDCl_3$), and 2D-NMR spectral data of compound ***ASLD8 (192)***

No	δ^{1}H (ppm)	δ^{13}C (ppm)	HMBC	COSY
1	1.97 (2H, *m*)	38.6		
2	2.76, 2.30 (2H, *m*)	35.1	C-3	
3		217.2		
4		47.9		
5	1.71 (1H, *m*)	52.4		
6	2.10 (2H, *m*)	24.6		H-7
7	5.30 (1H, *d*, *J* = 4.0 Hz)	117.9		H-6
8		148.7		
9	2.27 (1H, *m*)	48.5		
10		36.2		
11	1.43 (2H, *m*)	34.1		
12				
13	3.48 (*d*, *J* = 5.15 Hz)	51.3	C-12	

(continued)

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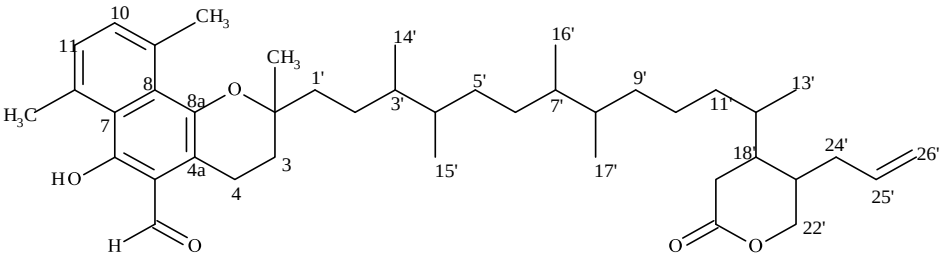
Table 4.17 *(continued)*

No	δ^{1}H (ppm)	δ^{13}C (ppm)	HMBC	COSY
14		43.6		
15	1.25 (2H, *m*)	29.4		
16	1.65 (2H, *m*)	18.6		
17		36.6		
18	1.51 (1H, *m*)	53.1		
19	1.80 (1H, *m*)	28.3		
20		158.0		
21	2.25 (2H, *m*)	35.0		H-22
22	1.54 (2H, *m*)	36.5		H-21
23	1.04 (3H, *s*)	24.4	C-3,C-24,	
24	1.11 (3H, *s*)	21.6	C-3, C-23	
25	1.00 (3H, *s*)	12.8	C-1, C-9, C-11	
26	1.01 (3H, *s*)	27.5	C-13, C-9, C-11	
27	0.81 (3H, *s*)	22.0	C-7, C-13	
28	1.05 (3H, *s*)	29.3	C-16,C-17, C-18, C-22	
29	0.91 (3H, *d*, *J*= 6.9 Hz)	18.3		
30a	4.66 (1H, *s*)	105.8	C-19, C-29	
30b	4.83 (1H, *s*)			

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4.3.9 ASLD9: Subintegranol (193)



Purification of fraction L1A-11 from CH_2Cl_2 extract of leaf part has afforded compound **ASLD9** (8.0 mg, 0.42%) as a yellowish oil. The TLC of this compound showed a single black spot under short wave UV (254 nm) and non-active under long UV (365 nm). The spot changed to yellowish-orange colour when heated on the hotplate after sprayed with the *p*-anisaldehyde reagent.

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The IR spectrum (Figure 4.85) exhibited the absorption bands at 3705, 2927 and 1637 cm^{-1} ascribable to hydroxyl, *sp3* C-H (stretching) and conjugated double bond, respectively. Besides, the absorption band which assignable to the carbonyl group was observed at 1737 cm^{-1}. The ESI-MS of this compound (Figure 4.86) was revealed a strong molecular ion peak at *m/z* 647.38440 $[M+H]^+$ and suggested the molecular formula of $C_{42}H_{62}O_5$.

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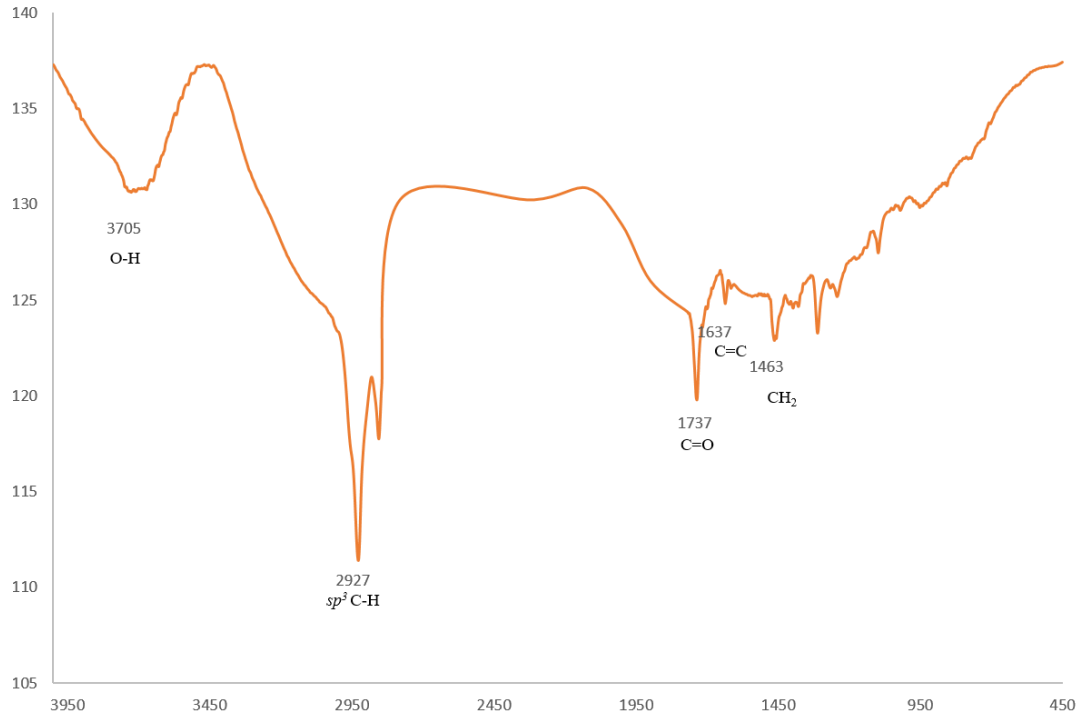


Figure 4.85. IR spectrum of compound **ASLD9**

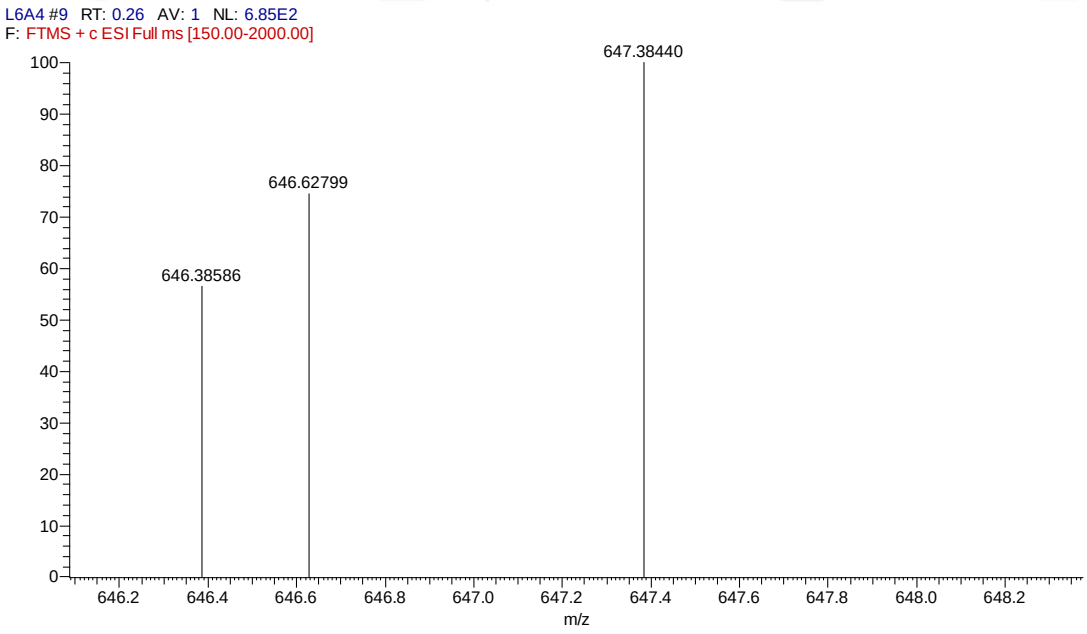


Figure 4.86. LC-MS spectrum of compound **ASLD9**

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The [1]H-NMR spectrum of compound **ASLD9** was demonstrated in Figure 4.87. This spectrum showed 62 protons with the presence of one methyl singlet at δ 1.21 (3H, H-2), and a bunch of 15 protons resonated at range δ 0.84 to 0.86 corresponding to H-13′, H-14′, H-15′, H-16′ and H-17′. Two methylene protons at δ 1.83 (*ddd*, *J* = 6.9, 7.4 and 6.3 Hz, H-3) were coupled with the adjacent methylene protons at δ 3.02 (*t*, *J* = 7.4 and 5.7 Hz, H-4). In addition, two doublets in the aromatic region, δ 7.21 (*d*, *J* = 6.9 Hz) and δ 7.28 (*d*, *J* = 7.4 Hz) attributed to H-10 and H-11, respectively. Three olefinic protons with *cis*, *trans* and *geminal* characteristic resonated at δ 5.34 (*J* = 15.5, 10.9 and 2.9 Hz) attributable to H-25′, H-26a′ and H-26b′. Two singlet protons appeared at δ 12.10, and 10.18 belonged to a hydroxyl group at C-6 and aldehyde group at C-5a, respectively.

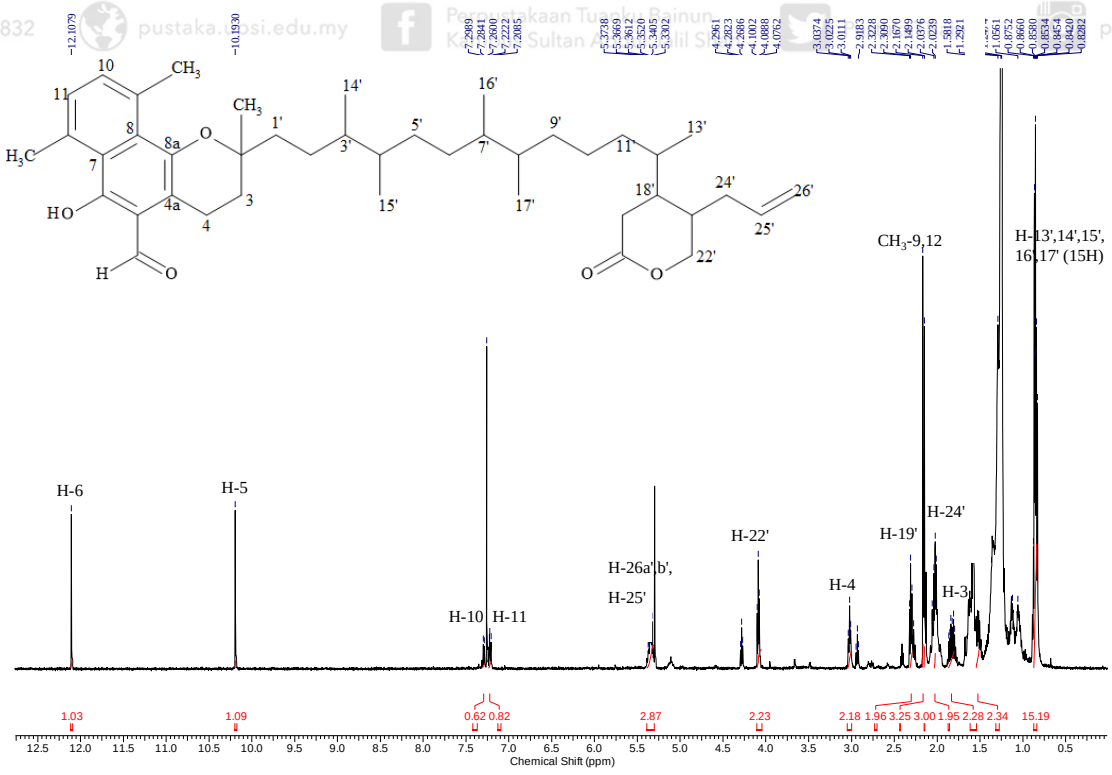


Figure 4.87. [1]H-NMR spectrum of compound **ASLD9**

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The ^{13}C-NMR spectrum of **ASLD9** (Figure 4.88) displayed 42 signals corresponding to 42 carbons. The DEPT spectrum in Figure 4.89 showed the presence of eight methyls, thirteen methylene and eleven methine carbons.

The signal at δ 194.1 representing the carbon for aldehyde group (C-5a), while the signal at δ 174.2 belonged to carbonyl for ester group (C-20′). Eight quaternary carbons positioned at δ 75.2, 117.6, 114.5, 124.2, 126.9, 144.1, 138.6 and 125.6 attributed to C-2, C-4a, C-5, C-7, C-8, C-8a, C-9 and C-12, respectively. Another carbon at δ 155.9 was attached with OH group at position C-6. Furthermore, eight methyl signals appeared at δ 23.7, 13.3, 11.1, 14.2, 22.7, 22.8, 19.8, 19.7 assigned to CH_3-2, CH_3-9, CH_3-12, C-13′, C-14′, C-15′, C-16′ and C-17′, accordingly. Two methine carbons in aromatic region, resonated at δ 129.0 (C-11) and 128.5 (C-10). Another methine carbons appeared at δ 32.8, 29.7, 29.7, 26.2, 28.3, 25.1, 28.8, 130.1 belonged to C-3′, C-4′, C-7′, C-8′, C-12′, C-18′, C-23′ and C-25′, respectively.

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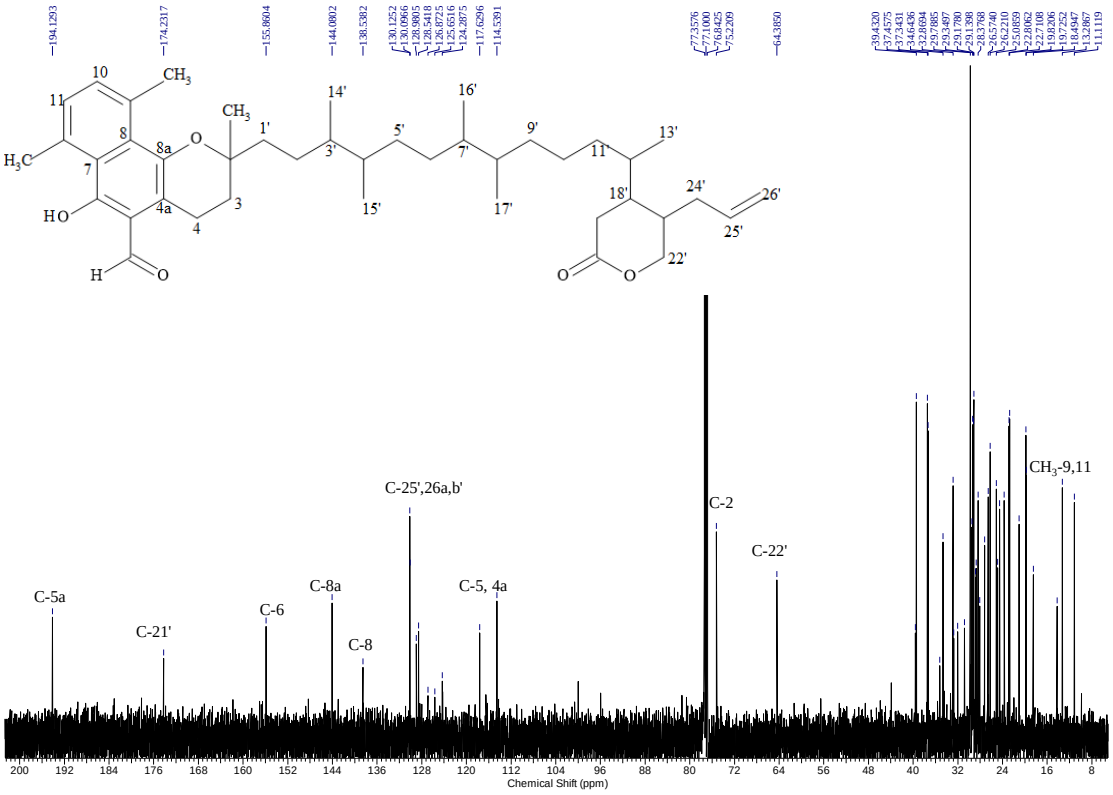


Figure 4.88. ^{13}C-NMR spectrum of compound **ASLD9**

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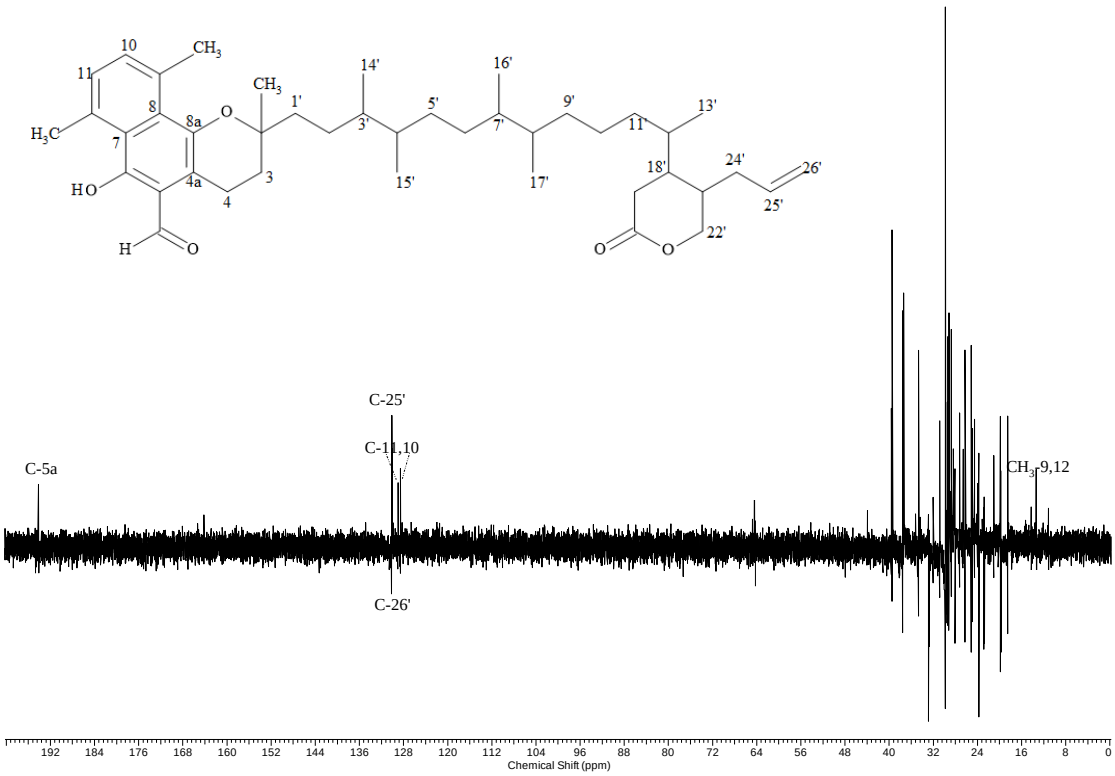


Figure 4.89. DEPT spectrum of compound **ASLD9**

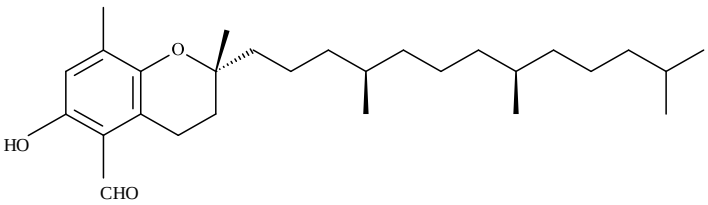
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In 2D NMR experiments; COSY, HMBC and HMQC data analyses were used to confirm the assignments of carbon and proton in the molecule. Based on the COSY spectrum displayed in Figure 4.90, there were correlations between H-3/H-4, H-23′/H-24′ and H-25′/H-26′, H-16′/H-15′. The HMQC spectrum of **ASLD9** is shown in Figure 4.91. The confirmation structure of **ASLD9** was extended by the HMBC spectrum, as shown in Figure 4.92.

The HMBC spectrum showed correlations between H-4 with C-2, C-5, C- 8a (^{3}J); C-3 and C-4 (^{2}J). Besides, H-5a was observed to correlate ^{2}J with C-5, C-6 (^{3}J) and C-7 (^{4}J), and OH-6 was showed the correlation with C-5, C-6 and C-7. Furthermore, H-19′ showed the correlation with C-20′, C-18′, C-23′ and C-24′; H-22′ correlated with C-24′, C-23′ and C-20′; and H-24′ correlated with C-23′, C-25′, C-26′. The summary correlations of HMBC and COSY of **ASLD9** were shown in Figure 4.93.

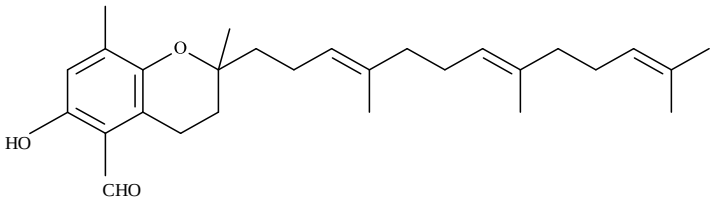
The tocopherol's backbone of **ASLD9** has similarity with reported 5-formyl-δ-tocophero (**196**) (Alsabil et al., 2017), 5-formyl-δ-tocotrienol (**197**) the stem bark of *Garcinia virgata* (Merza et al., 2004) and prunusol A (**198**) from *Prunus grayana* (Osawa, Kumazawa, & Kawakishi, 1991).



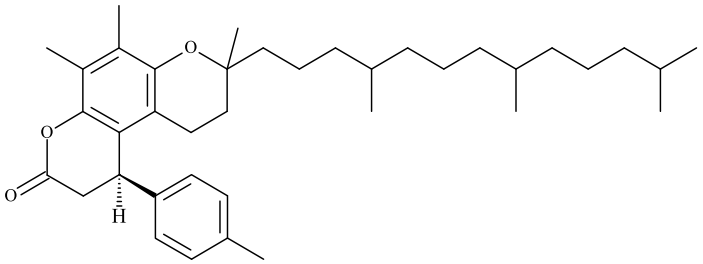
(**196**)

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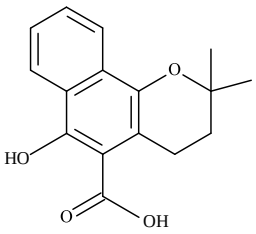


(**197**)

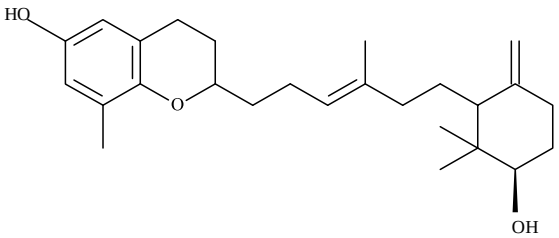


(**198**)

However, this compound had additional benzene ring at C-7 and C-8, which resemble the compound 6-Hydroxy-2,2-dimethyl-3,4-dihydro-2H-benzo[h]chromene-5-carboxylic acid (**199**) (Naik, 2018). In addition, this compound was suggested to have cyclic/lactone ring attached to the long-chain, which is quite similar to the compound walsurol (**200**) (Birringer et al., 2018).



(**199**)



(**200**)

Thus, from the analysis of its spectral data tabulated in Table 4.18, compound (**193**) was established as a new compound, identified as 2-[12-(5-allyl-2-oxo-tetrahydropyran-4-yl)-3,4,7,8-tetramethyl-tridecyl]-6-hydroxy-2,7,10-trimethyl-3,4-dihydrobenzo[h]chromene-5-carbaldehyde or trivially named as subintegranol.

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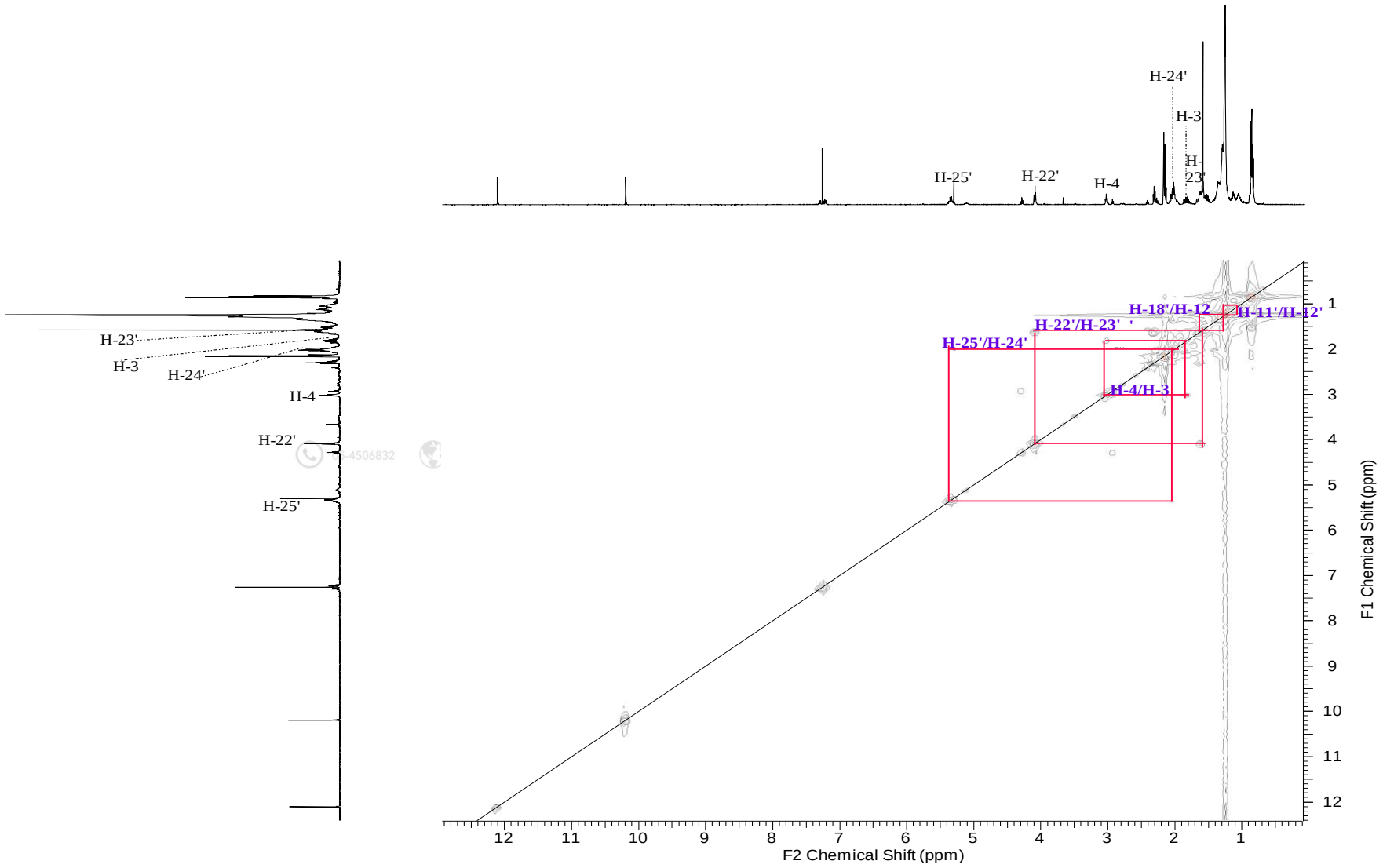


Figure 4.90. COSY spectrum of compound **ASLD9**

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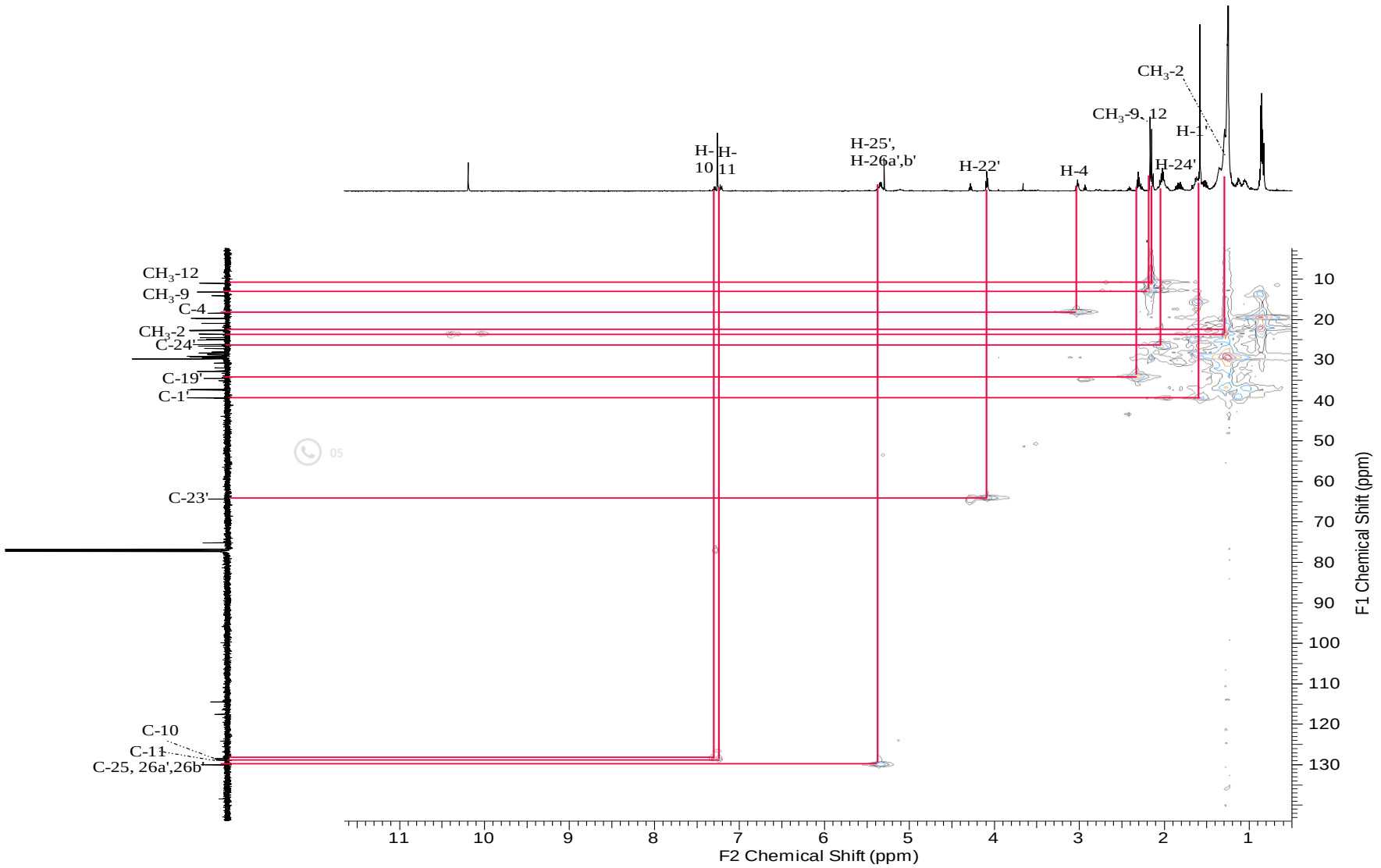


Figure 4.91. HMQC spectrum of compound **ASLD9**

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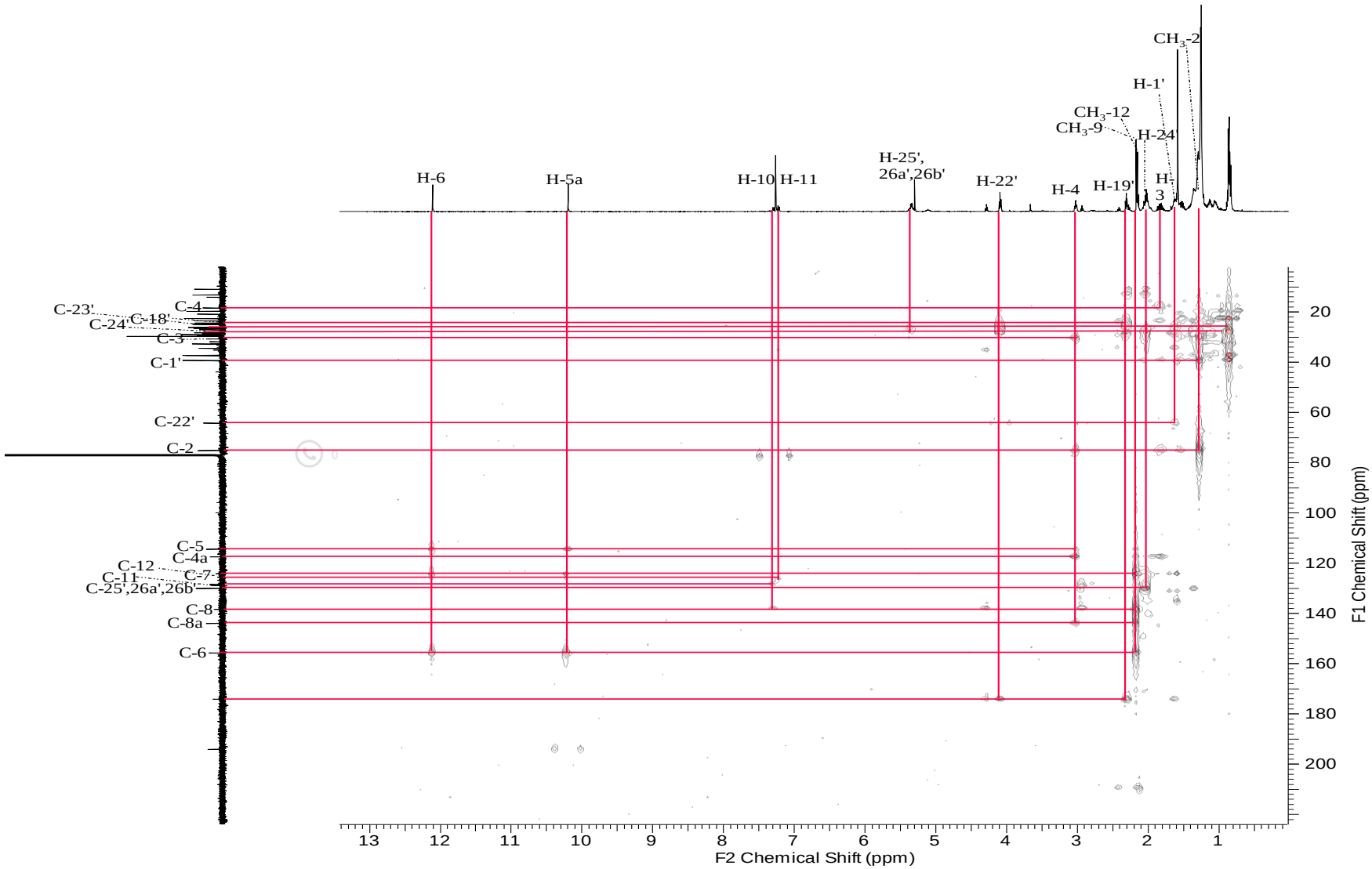


Figure 4.92. HMBC spectrum of compound **ASLD9**

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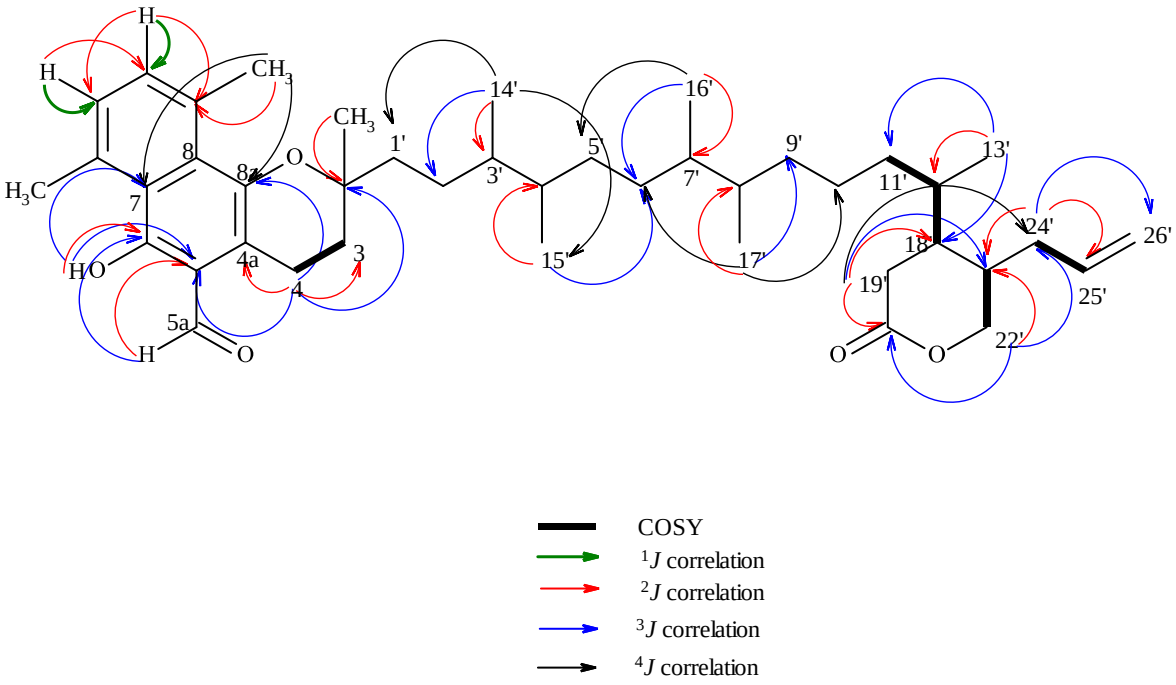


Figure 4.93. COSY, ^{1}H and ^{13}C correlations observed in HMBC spectrum of **ASLD9**

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Table 4.18

^{1}H (500 MHz, $CDCl_3$), ^{13}C (125 MHz, $CDCl_3$), and 2D-NMR spectral data of compound ***ASLD9 (193)***

No	$\delta^{1}H$ (ppm)	$\delta^{13}C$ (ppm)	HMBC	COSY	$\delta^{13}C$ (ppm) (Reference)
1					
2		75.2			75.3*
2-CH_3	1.25 (3H, *s*)	23.7			24.2*
3	1.83 (2H, *ddd, J*= 6.9, 7.4, 6.3 Hz)	30.8		H-4	30.8*
4	3.02 (2H, *t*)	18.5	C-2,C-3,C-4a, C-5, C-8a	H-3	19.7*
4a		117.6			119.8*
5		114.5			115.3*
5a	10.18 (1H, *s*)	194.1	C-5, C-6, C-7		194.2**
6	12.10 (1H, *s*)	155.9	C-5, C-6, C-7		157.1**
7		124.2			123.6*
8		126.9			125.4*
8a		144.1			143.0*
9		138.6			140.2**
9-CH_3	2.16 (3H, *s*)	13.3	C-7, C-9, C-8		12.0*
10	7.28 (1H, *d, J* = 7.4 Hz)	128.5	C-9, C-11		
11	7.21 (1H, *d, J* = 6.9 Hz)	129.0	C-10		
12		125.6			123.6*
12-CH_3	2.15(3H, *s*)	11.1	C-6, C-7, C-8		12.1*
1′	1.25 (2H, *m*)	39.6			39.3*
2′	0.98 (2H, *m*)	37.3			21.0*
3′	1.40 (1H, *m*)	32.8			37.4*
4′	1.26 (1H, *m*)	29.7			37.4*
5′	1.53 (2H, *m*)	28.7			24.5*
6′	1.14 (2H, *m*)	37.5			24.5*

(continued)

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Table 4.18 *(continued)*

No	δ^{1}H (ppm)	δ^{13}C (ppm)	HMBC	COSY	δ^{13}C (ppm)
7′	1.36 (1H, *m*)	29.7			37.4*
8′	1.26 (1H, *m*)	26.2			32.7*
9′	1.25 (2H, *m*)	23.7			37.4*
10′	1.25 (2H, *m*)	24.8			24.7*
11′	1.05 (2H, *m*))	37.4		H-12′	39.4*
12′	1.29 (1H, *m*)	28.3		H-11′, H-18′	
13′	0.85 (3H, *d, J*=2.3 Hz)	14.2	C-12′, C-11′, C-18′		
14′	0.86 (3H, *d, J*=4.6 Hz)	22.7	C-1′, C-2′, C-3′, C-15′		
15′	0.85 (3H, *d, J*=2.3 Hz)	22.8	C-4′, C-6′		
16′	0.84 (3H, *d, J*=1.7 Hz)	19.7	C-5′, C-6′, C-7′		
17′	0.86 (3H, *d, J*=4.6 Hz)	19.8	C-9′, C-6′, C-8′, C-10′		
18′	1.64 (1H, *m*)	25.1		H-12′	
19′	2.30 (2H, *m*)	34.6	C-20′, C-18′, C-23′, C-24′		
20′		174.2			
21′					
22′	4.08 (2H, *t*)	64.4	C-20′, C-23′, C-24′	H-23′	
23′	1.61 (1H, *m*)	28.8		H-22′	
24′	2.03 (2H, *m*)	26.2	C-23′, C-25′, C-26′	H-25′	
25′	5.34 (1H, *m*)	130.1		H-24′	
26a′	5.34 (1H, *m*)	130.1			
26b′	5.34 (1H, *m*)				

Note: * Measured at 50 MHz (^{13}C) in $CDCl_3$ (Osawa et al., 1991), ** Measured at 125 MHz (^{13}C) in $CDCl_3$ (Merza et al., 2004)

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4.3.10 ASLD10: Saturated Triglyceride Fatty Acid (186)

Purification of CH_2Cl_2 of extract from fractions L1A-12 using several column chromatography practices was afforded compound **ASLD10** as white amorphous with mass 8.0 mg (0.39%). The spectroscopic data of NMR, as well as LC-MS, were correspondent with compound **ASBD7**, which obtained from the bark of *A. subintegra*. Therefore, this compound was concluded as saturated triglyceride fatty acid (**186**).

4.3.11 ASLD11: *β*-sitosterol (101)

Purification of CH_2Cl_2 of extract from fractions L1A-14 using several column chromatography practices and washing technique was afforded compound **ASLD11** as white needles with mass 10.0 mg (0.45%). The spectroscopic data of ^{1}H and ^{13}C-NMR, as well as LC-MS, equivalent with compound **ASBD5**, which obtained from the bark of *A. subintegra*. Therefore, this compound was concluded as *β*-sitosterol (**101**).

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4.3.12 ASLD12: *β*-sitostenone (139)

Compound **ASLD12** was obtained from repeated column chromatography from fraction L1A-13. The compound was a white needle with mass 10.0 mg (0.10%). The spectroscopic data of ^{1}H, ^{13}C-NMR, LC-MS and its physical appearance was similar to *β*-sitostenone (**142**) which also found from the bark.

4.3.13 ASLD13: 5-Hydroxy-7,4′-dimethoxyflavone (36)

Compound **ASLD13** was acquired as yellow amorphous with mass 10.0 mg (0.45%) from fraction L1A-14. The physical properties and spectral data, ^{1}H and ^{13}C NMR, and LC-MS were identical with 5-hydroxy-7,4′-dimethoxyflavone (**36**) which was isolated from the bark of the plant.

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4.3.14 ASLD14: 5,3′-Dihydroxy-7,4′-dimethoxyflavone (41)

Compound A**SLD14** was obtained from repeated column chromatography from fraction L1A-14. The compound was a yellow needle with mass 10.0 mg (0.45%). The spectroscopic data of ^{1}H, ^{13}C-NMR, LC-MS and its physical appearance was alike 5,3′-dihydroxy-7,4′-dimethoxyflavone (**41**) which also found from the bark.

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4.4 Biological Activity

In our continuing interest of pharmacological properties of *A. subintegra* plant, several bioactivity studies have been done. These are anti-lipase, anticancer and antioxidant activities. The anti-lipase activity was evaluated by using a colorimetric assay. Meanwhile, the antioxidant and anticancer activities were carried via DPPH radical scavenging and WST-8/CCK-8 cytotoxic assays, respectively. All biological activities have done towards crude extracts and pure phytochemicals.

4.4.1 Anti-lipase Activity

Lipases or triacylglycerol acylhydrolase (EC 3.1.1.3) are the water-soluble enzyme, which hydrolyses the esters of long-chain aliphatic acids from glycerol at the interface between aqueous and organic phase (Stoytcheva, Montero, Zlatev, León, & Gochev, 2012).

The regulation of fatty acid and triglyceride accessibility in biological responses rely on the activity of lipolytic enzymes in fatty acid metabolism in adipose tissue. The classification of numerous genes associated with lipid metabolism has yielded a rich pool of possible targeted drugs in obesity treatment and other metabolic syndromes (Roh & Jung, 2012).

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Pancreatic lipase (PL) is an enzyme which formed in the pancreas and secreted into the small intestine (Ahmed et al., 2018; Lee et al., 2010). Thus, PL is one of the essential enzymes which hydrolysis 50-70% ester bonds of dietary fats to form monoglycerides and free fatty acids (FFA) (Ado et al., 2013; Garza, Milagro, Boque, Campion, & Martinez, 2011). Therefore, the activity of PL could be as the primary target in controlling obesity due to its ability to increase the weight of the body (Ahmed et al., 2018). In addition, PL usually used in most studies for the evaluation of the efficiency of natural compounds as antiobesity inhibitors (Lee et al., 2010).

Colorimetric assay is one of the most practical methods based on the quantification of lipolysis products. The colorimetric assay measures colour after free fatty acids converted to copper soap with the colour reagent. Then, evaluated by spectrophotometric analysis. Abundant studies have improvised the methods by decreasing the incubation stage, remove the phase transfer step and selected the suitable colour developing reagents and organic solvent. Thus, this method more sensitive and suitable for determination free fatty acid (Kwon & Rhee, 1986; Stoytcheva et al., 2012).

In this study, the anti-lipase activity of crude extracts and selected isolated compounds were evaluated using colorimetric assay introduced by Kwon and Rhee (1986) with slight modification. In addition, olive oil has been choosing as a natural substrate and porcine pancreatic lipase (PPL) as a positive control. All crude extract of *A. subintegra* and PPL were tested at concentration 1000 µg/mL.

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The development colour of fatty acid determined the observation of lipase activity in the solvent phase (Kwon & Rhee, 1986). The more colour of fatty acid developed; the higher of lipase activity, meanwhile, the less colour develop showed, the lower activity of lipase. Figure 4.94 displayed the percentage (%) lipase activity of crude extract toward PPL (positive control).

The bar graph in Figure 4.94 showed that most of the crude extracts could inhibit the activity of PPL by comparing the value of positive control. The hexane extract from the leaf (ASLH) showed the highest inhibition with 53.8% reduction. Then, continued with methanol extract of bark (ASBM) with 52.7% inhibition of lipase activity. Meanwhile, the CH_2Cl_2 extract of leaf and bark showed moderate lipase inhibition, and MeOH extract of leaf showed minimal inhibition. In addition, the statistical analysis paired t-test by SPSS (Appendix B, Table 2) of bark and leaf crude extract of *A. subintegra* has performed with significant different $*p<0.05$. From the result, MeOH and *n*-Hex crude extract showed a significant difference between bark and leaf part, respectively. Meanwhile, there is no significant different discovered for CH_2Cl_2 for both bark and leaf of the plant.

Thus, the deficiency of inhibition effect could be explained by the diverse bioactive content compared to potent extracts, or synergistic/antagonistic cause between bioactive compounds in the extract (Martinez-Gonzalez et al., 2017). The crude extract with high inhibitory effect might seem because of the presence of polyphenolic compounds which rich in biological properties. In the previous study indicated that polyphenolic constituents from *molokheiya* leaf as a critical role in reducing diet-induced obesity and oxidative stress (Wang et al., 2011). Also, the

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polyphenolic, including flavonoid group, able to inhibit lipase cope with fat absorption to prevent obesity problem (Garza et al., 2011).

The previous study had screened anti-lipase property onto 44 natural extracts and orlistat as a positive control at concentration 100 µg/mL; the result showed that orlistat well-inhibited lipase activity with 42.0% inhibition (Roh & Jung, 2012). By comparing this data, the ASLH and ASBM were inhibited lipase activity higher than orlistat (positive control). It could have happened might due to the concentration of ASLH and ASBM were ten times more than orlistat.

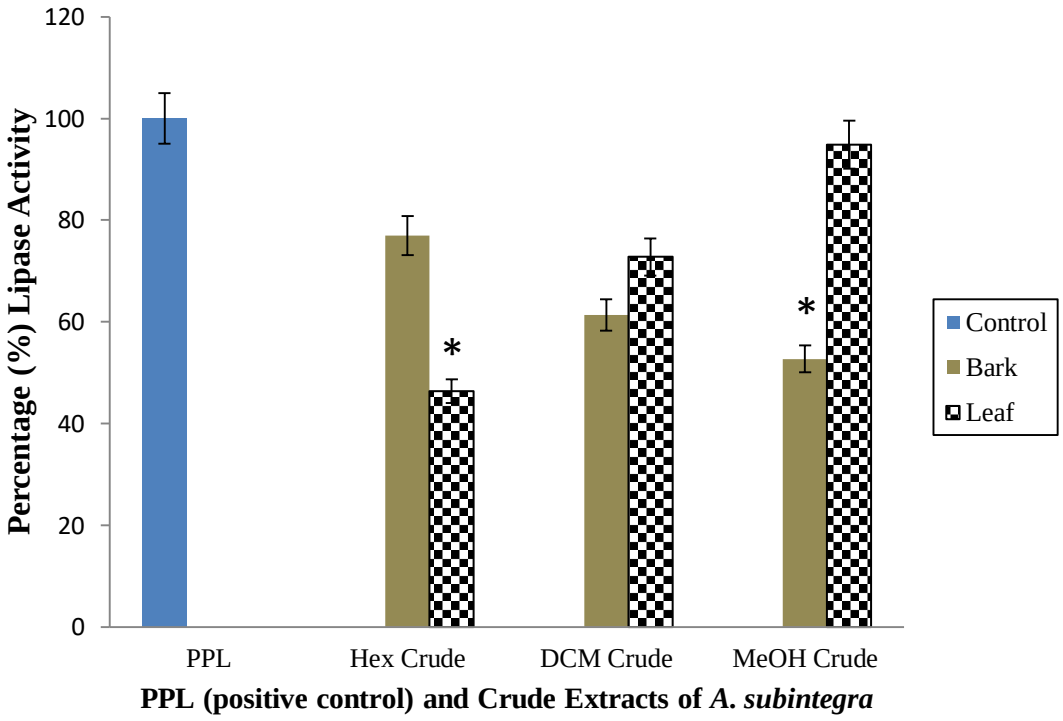


Figure 4.94. The percentage of lipase activity (U/mL) of six crude extracts from *A. subintegra* and PPL (positive control). Results stated as mean ± SD (n=3), *p<0.05. Hex, DCM and MeOH represented *n*-hexane, dichloromethane and methanol crude extracts, respectively.

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This finding was in accordance with the previous study, which indicated that *Aquilaria* species have a pharmacological effect such as in Alzheimer disease, diabetes mellitus and antioxidant properties (Jok et al., 2015). The prior discovery of laxative properties in *Aquilaria* leaves believes that it may contain active polyphenolic compounds such as mangiferin and genkwanin *5-0-β*-primeveroside (Hara et al., 2008; Ito et al., 2012). Thus, the anti-lipase property of *A. subintegra* crude extract might associate with the presence of polyphenolic compounds (Ibrahim et al., 2018).

The anti-lipase activity of selected phytochemicals exhibited in Figure 4.95. These phytochemicals were tested by colorimetric assay at final concentrations 1 mg/mL, 0.5 mg/mL and 0.25 mg/mL. Among the tested compounds, compound (**36**) has exhibited the strongest lipase inhibitor, which inhibited the lipase activity at concentration 1 mg/mL and 0.5 mg/mL. Besides, flavonoids compound (**37**) and compound (**41**) displayed their capability in lipase activity inhibition, ranging from 6% to 53%. Another promising lipase inhibitor was from the steroid group, compound (**101**), which displayed percentage inhibition up to 51%.

The percentage activity of PPL disclosed in a bar graph (Figure 4.95). This bar graph represented the mean (n=3) value of the lipase activity of each compound. The absence of bar graph for compound (**36)** (at 1.0 mg/mL and 0.5 mg/mL) and compound (**101)** (at 1.0 mg/mL) represented the 100% inhibition of lipase activity or no lipase activity was detected. The statistical analysis (one way ANOVA) (Appendix B, Table 3) indicated that compounds 5-hydroxy-7,4′-dimethoxyflavone and β-sitosterol (**101**) showed the significant different $*p<0.05$ at concentration 1mg/mL, respectively.

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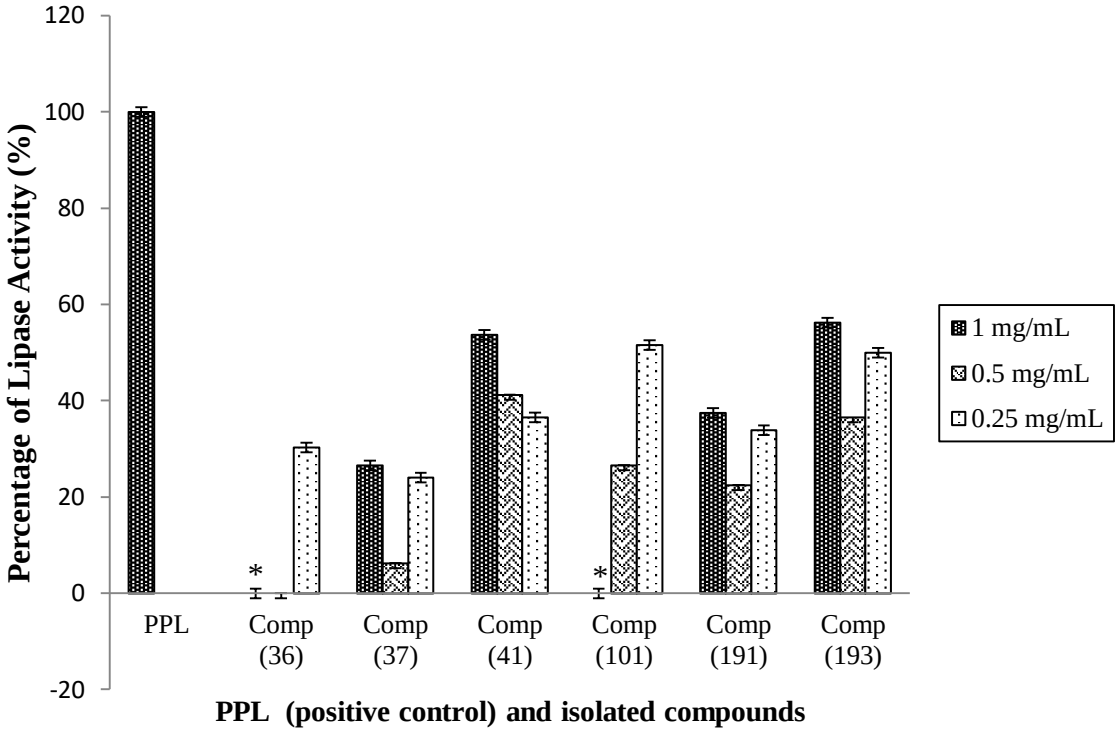


Figure 4.95. Percentage (%) lipase activity of isolated compounds and PPL (positive control). Results stated as mean ± SD (n=3), **p*<0.05

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This result was following the previous study, which reported that the bark of *A. subintegra* showed a promising inhibitor toward pancreatic lipase activity (Ibrahim et al., 2018). Moreover, flavonoid compounds were acknowledged to own numerous biological properties such as pancreatic lipase inhibitor (Sergent et al., 2012), anti-inflammatory (Liu et al., 2013) and as acetylcholinesterase inhibitor on Alzheimer's treatment (Bahrani et al., 2014).

An earlier study indicated that flavonols such as kaemferol (**201**) and quercetin (**12**) were potent to inhibit pancreatic lipase activity (Ado et al., 2013; Sergent et al., 2012), as well as antioxidant and α-glucosidase inhibitor (Zhang et al., 2015).

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This study exhibited interesting finding which the compound (**36**), (**37**) and (**41**) derived from flavone group showed their beneficial in the anti-lipase activity. The previous study indicated that the presence of hydroxyl in position 3′ and methoxyl in position 4′ of flavonoid was support lipase inhibition (Buchholz & Melzig, 2015). In addition, Wu and Tian (2018) suggested that the inhibition of lipase activity enhanced by increasing the number of the hydroxyl group of flavone.

This finding also associated with an earlier study which showed that flavone has few biological properties. For instance, 7,8-dihydroxyflavone, luteolin (Djeradi et al., 2014), 7,3′-dihydroxyflavone, 7,3′-dimethoxy-3-hydroxyflavone and 3,6-dihydroxyflavone (Park et al., 2006) exhibited antioxidant property. In addition, another study reported that genkwanin possess few pharmacological effects including antioxidative, radical scavenging and antimicrobial, whereas genkwanin 5-*O*-primeveroside revealed laxative effect (Hara et al., 2008). Furthermore, compound (**36**) which isolated from *Combretum zeyheri* also exhibited antifungal property (Mangoyi et al., 2015).

On the other hands, compound (**101**) also showed the inhibitory effect of lipase activity and thus suggested as an anti-lipase agent. This result was in accordance with the previous study, which revealed that *β*-sitosterol is the most widespread vegetable-derived phytosterols in the diet (Paniagua-P´erez et al., 2005). This compound possesses a similar structure to the dietary fat, which does substantial competition in the gastrointestinal area; in the meantime, it can diminish the absorption of fat (Chidrawar et al., 2011). In addition, *β*-sitosterol (**101**) also exhibited a few biological effects, such as antioxidant and antidiabetic (Karan, Mishra, Pal, & Mondal, 2012).

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However, Ong and friends (2016) revealed that compound (**101**) showed as a weak PPL inhibitor by comparing with orlistat, and it explained by few factors including the concentration of substrate and enzyme were varied during the experiment.

Interestingly, two new compounds, aquilene A (**191**) and subintegranol (**193**) demonstrated a moderate inhibitory effect of lipase activity. The percentage inhibition of lipase activity was ranging from 43.8% to 77.6%. Thus, these compounds seem worthy of consideration as potential therapeutic agents in lipase inhibitory.

4.4.2 Molecular Docking

Molecular docking has proven as an essential tool of computer-aided drug design. This application widely used in the analysis of molecular recognition events including binding energies, molecular interactions and induced conformational changes and comprises of bioactive small-molecule libraries (Ferreira, Santos, Oliva, & Andricopulo, 2015). Molecular docking is performed on structure-based rational drug design to identify correct conformations of small molecule ligands and also to estimate the strength of the protein-ligand interaction, usually one receptor and one ligand (Kumalo, Bhakat, & Soliman, 2015; Meng, Zhang, Mezei, & Cui, 2011).

In this study, YASARA software has used. Through YASARA, the cluster conformation was ranked with binding energy. The more durable binding indicated when more positive energies shown in the docking prediction result. Meanwhile, negative energies showed no binding. Their result was possible to show the binding of

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ligand toward PPL active and non-active site. In addition, the LigPlot+ program was illustrated specific interaction between ligand and protein; this allowed 2D demonstrated into an automated and straightforward mode of contact between ligand-protein complexes (Laskowski & Swindells, 2011).

The docking process involves two necessary steps: prediction of the ligand conformation as well as its position and orientation within these sites (usually referred to as pose) and assessment of the binding affinity. These two steps are related to sampling methods and scoring schemes, respectively (Meng et al., 2011).

In the docking study, the crystal structure of the ternary PPL-CLP-TGME complex (PDB-ID: 1ETH) operated as the receptor. The PPL consists of two folding units, the N-terminal (residues 1-336) and C-terminal (residue 337-449) domains with co-lipase attached to the C-terminal domain. Meanwhile, N-terminal domain is catalytically active which consist of the catalytic triad, aspartic acid (177), histidine (264) and serine (153), where serine (153) is central for the hydrolytic activity of the enzyme (Buchholz & Melzig, 2015).

Usually, this application studies the binding modes of ligands by utilizing prior knowledge of binding sites, or it is known as focus docking. However, in this study, blind docking was carried out for substrate and potential inhibitor since the inhibitor may inhibit at the active site or not at the active site. We substantiated the results obtained from the lipase assay result with docking studies.

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4.4.2.1 Docking Result of Isolated Compound

This study correlates in silico data derived from in vitro experimental data of the anti-lipase assay, thus sought to correlate the binding energies obtained from docking of isolated compounds into PPL to investigate their interaction mechanisms. The structure-based rational design used to identify the best phytochemical as a potent inhibitor of pancreatic lipase (PL). PL has discovered as the prime target for obesity therapy in numerous studies. (Garza et al., 2011; Hirose et al., 2013; Wu et al., 2014; Martinez-Gonzalez et al., 2017).

Table 4.19 showed the summary binding residues that interact with the ligands (isolated compound). Each ligand corresponds to the different location of residues, respectively. Figure 4.96 disclosed the interaction between ligands and the enzyme. This figure showed that flavone compounds (**36, 37** and **41**) were bound tightly into the catalytic site of the enzyme, Serine (Ser153) and Histidine (His264) (Martinez-Gonzalez et al., 2017). Meanwhile, compound (**101**) binds outside the catalytic site. Table 4.20 showed the distance of hydrogen bonding formed by ligand. There is showed compound (**36**), (**37**) and (**41**) consist of one hydrogen bond between ether (C-O-C) position and residue Ser153, respectively. In flavone, the primary functional group involved in hydrogen bond interaction was oxygen atom of ketonic (C-O-C) at heterocyclic pyrane ring. Meanwhile, there is no hydrogen bond presence for compound **(101)**.

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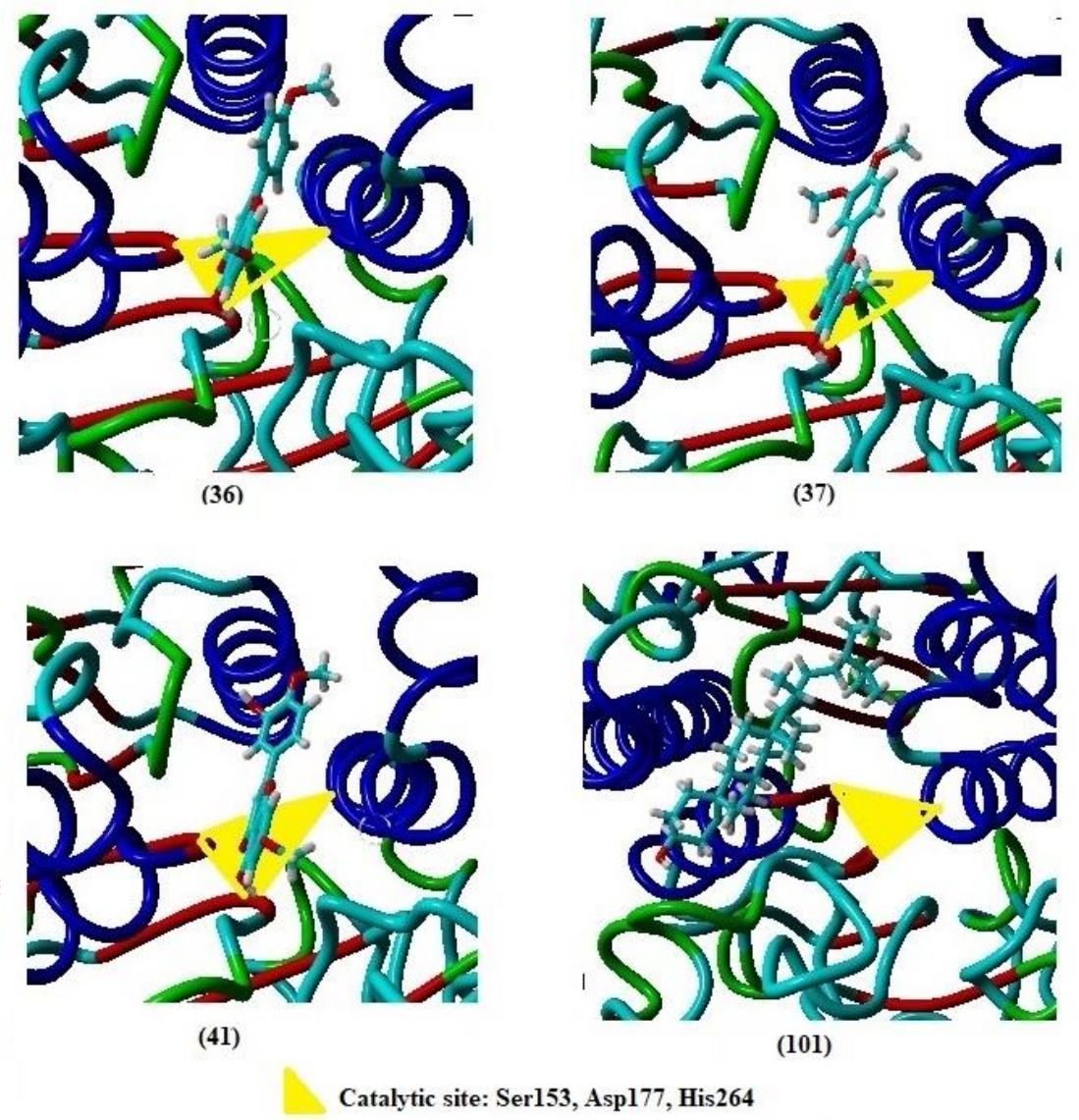


Figure 4.96. Ligand molecules docked onto PPL receptor. Ligands are shown in stick model meanwhile active site of PPL shown in yellow colour. (**36**) 5-hydroxy-7,4′-dimethoxyflavone, (**37**) luteolin-7,3′,4′-trimethyl ether, (**41**) 5,3′-dihydroxy-7,4′-dimethoxyflavone and (**101**) *β*-sitosterol.

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Table 4.19

Binding energies and residues involved in binding of the isolated compound with PPL

Compound	Binding Energy (kcal/mol)	Interacting residues
36	8.624 kcal/mol	Phe78, Ile79 ,Asp80, Tyr115 His152, Ser153, Ala179, Glu180 ,Pro181, Phe216, Arg257, Val260, Ala261, His264, Leu265
37	8.9310 kcal/mol	His76, Gly77 ,Phe78, Asp80, Trp86, Tyr115, His152, Ser153, Ala179, Pro181, Phe216, Arg257, Val260, Ala261, His264, Leu265
41	8.809 kcal/mol	Phe78, Ile79, Asp80, Tyr115, Ser153, Ala179, Glu180, Pro181, Ile210, Phe216, Arg257, Val260, Ala261, His264, Leu265
101	8.892 kcal/mol	Phe78, Ile79, Asp80, Tyr115, Ser153, Pro181, Ile210, Phe216, Trp253, Thr256, Arg257, Val260, Ala261, Leu265

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Table 4.20

Hydrogen bonds between isolated compounds and PPL

Compound	Residue	Distance (Å)
36	Ser153	5.76
37	Ser153	5.67
41	Ser153	5.95
101	N/A	N/A

Note: N/A: not available

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Docking result showed the flavone compounds possess strong bonding might occur due to the presence of one hydrogen donor/acceptor and intense interaction with Ser153 (Ahmed et al., 2018). Ser153 possibly an essential residue of the catalytic site in the execution of inhibition activity and could change the protein's structure (Veeramachaneni et al., 2015). This situation could be explained that flavone compounds were competitive component by considering the interaction of inhibitor with the substrate-enzyme binding near the catalytic site (Martinez-Gonzalez et al., 2017). Thus, flavone compounds may compete with the substrate for the active site of the enzyme and would change the reaction rates (Köhler & Wünsch, 2007; Martinez-Gonzalez et al., 2017).

The inhibitory mechanism of flavones to the PPL considered being a non-covalent interaction. Non-covalent interaction between phenolic compounds and the enzyme was hydrophobic and might be stabilized with hydrogen bonding, as well as may alter the enzyme molecular conformation and decrease the catalytic activity at once (Wu et al., 2014; Martinez-Gonzalez et al., 2017). The binding energy principally contributed by hydrophobic interactions, and the specificity of the interaction might cause by electrostatic force; therefore, affinity constant is self-reliant of the ionic force (Dhananjayan, 2015).

On the other hands, compound (**101**) was predicted as a non-competitive inhibitor. The compound was inhibited allosterically toward enzyme which might not obstruct the catalytic site of the enzyme. Hence, it might be stimulating target in the promotion of natural ligands and drugs which may bind reversibly or irreversibly (Köhler & Wünsch, 2007).

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Thus, in silico molecular docking study suggests that 5-hydroxy-7,4′-dimethoxyflavone (**36**), luteolin-7,3′,4′,-trimethyl ether (**37**) and 5,3′-dihydroxy-7,4′-dimethoxyflavone (**41**) were preferential as a competitive inhibitor, with strong binding affinity to the lipase receptor. This finding aligned to the previous study, which reported that flavonoid owned inhibitory effect toward adipocytes as well as lipolytic actions in primary adipose cells of rat (Mohammed, Al-numair, & Balakrishnan, 2015).

In conclusion, this experimental data indicated that flavonoid and steroid compound possessed as potential anti-lipase agents related to the reduction of fatty acid absorption. Thus, 5-hydroxy-7,4′-dimethoxyflavone (**36**) promises the highest inhibition and as a competitive inhibitor. Also, *β*-sitosterol (**101**) might highlight as a promising inhibitor due to inhibiting allosterically toward PPL. Nevertheless, extensive study is desirable to investigate the effect of bioactive compounds from *A. subintegra* in animal experiment.

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4.4.3 Antioxidant Activity

Free radicals are unstable chemical, resulted from an imbalance of the generation of Reactive Oxygen Species (ROS) and the antioxidant protection conferred by enzymatic systems; it comprises more than one unpaired electron and cause damage to other molecules by extracting electrons from them in order to attain stability (Morales & Paredes, 2014).

Antioxidants are compound that can impede the oxidation process by preventing the proliferation of oxidizing chain reactions (Karou et al., 2005). A growing interest in finding bioactive compounds from medicinal plants as candidates to prevent oxidative damage might due to their diversity of chemical structure and synergistic actions acts as powerful antioxidants (Morales & Paredes, 2014). Thus, there have few quick methods developed to evaluate antioxidant activity such 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), oxygen radical absorbance capacity (ORAC) and photochemiluminescence (PCL) (Zhang & Tsao, 2016).

4.4.3.1 DPPH Free Radical Scavenging Assay

Antioxidant activity has determined using DPPH (2, 2-diphenyl-1-picrylhy- drazyl) free-radical scavenging assay. DPPH itself is an organic radical with purple colour and reducing into pale yellow hydrazine by antioxidant agents (Ray et al., 2014). DPPH assay universally applied for the evaluation of scavenging ability of antioxidant

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compounds toward the radical DPPH through the donation of hydrogen at absorption 515 nm (Seyoum, Asres, & El-fiky, 2006; Liu et al., 2018).

Figure 4.97 displayed the percentage of inhibition H-donor activity of hexane, CH_2Cl_2 and methanol extract of bark and leaf as measured using DPPH scavenging assay with different concentrations. From the graph (Figure 4.98), we can see that at concentration 500 µg/mL, three extracts, ASLM, ASBD and ASBM showed the highest inhibition. These extracts scavenged about more than 90% DPPH free radicals, strictly to the positive control, ascorbic acid. Also, the trend plots showed that the DPPH free radical scavenging capacity of the crude extract was upsurge by increasing their concentration (Naidu et al., 2012).

The antioxidant activity determined by calculating the IC_{50} values interpreted as the concentration of crude extract required to scavenge 50% of DPPH free radicals. The IC_{50} values of the tested of the sample given in Table 4.21. The result showed that methanol extract from the leaf (ASLM) the lowest IC_{50} value, 29.56 ± 6.37 µg/mL and closed to ascorbic acid (4.27 ± 0.74 µg/mL). Thus, ASLM extract was assumed to have the most antioxidant property as its lowest minimum inhibitory concentration (Saad et al., 2015). In addition, CH_2Cl_2 extract of bark (ASBD) also showed significant scavenging activity with IC_{50} value 112.48 ± 2.06 µg/mL. Meanwhile hexane crude of bark (ASBH) exhibited as a weak antioxidant activity. It could be explained by the presence of non-polar compounds which have no or less able to act as free radical scavenge (Koleva, Van Beek, Linssen, De Groot, & Evstatieva, 2002).

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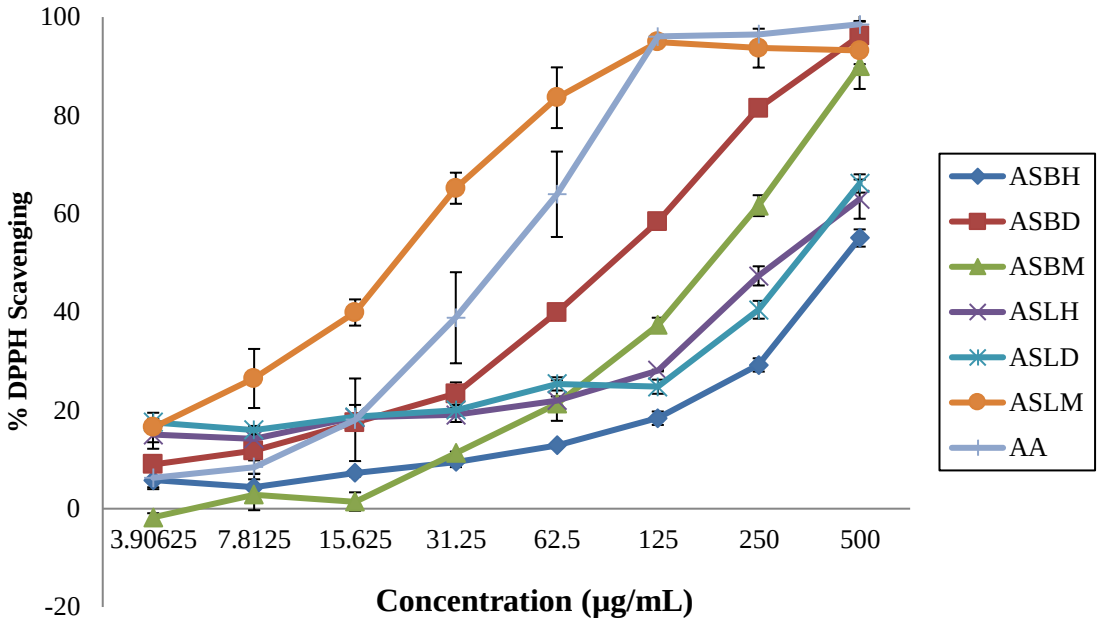


Figure 4.97. The percentage of inhibition of DPPH radicals by different concentration of extracts in comparison to ascorbic acid standard. Data expressed as mean ± SD, n=3. ASBH: *A. subintegra* bark n-hex; ASBD: *A. subintegra* bark CH_2Cl_2; ASBM: *A. subintegra* bark MeOH; ASLH: *A. subintegra* leaf n-hex; ASLD: *A. subintegra* leaf CH_2Cl_2; ASLM: *A. subintegra* leaf MeOH; AA: ascorbic acid

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Table 4.21

The DPPH radical scavenging activity of crude extracts of A. subintegra in comparison with the ascorbic acid standard

Part	**Crude extract**	**IC_{50} (µg/mL)**
Bark	Hexane (ASBH)	478.33 ± 2.24
	CH_2Cl_2 (ASBD)	112.48 ± 2.06
	Methanol (ASBM)	204.48 ± 7.00
Leaf	Hexane (ASLH)	305.83 ± 16.96
	CH_2Cl_2 (ASLD)	356.68 ± 34.24
	Methanol (ASLM)	29.56 ± 6.37
Positive control	Ascorbic Acid (AA)	4.27 ± 0.74

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Five isolated compounds were selected to test for DPPH free radical scavenging assay. The IC_{50} value for each compound was tabulating in Table 4.22. The results showed that IC_{50} values for all compounds were over 500 µg/mL. Thus, this finding indicated that isolated compounds need a high concentration in order to scavenge DPPH free radicals.

Furthermore, Figure 4.98 displayed the percentage inhibition of DPPH radical scavenging of phytochemicals at different concentrations. The plotted graph showed that all isolated compounds were possessed low ability in scavenging DPPH free radicals compare to ascorbic acid. Thus, this study did not find a significant difference between level concentration among the compounds.

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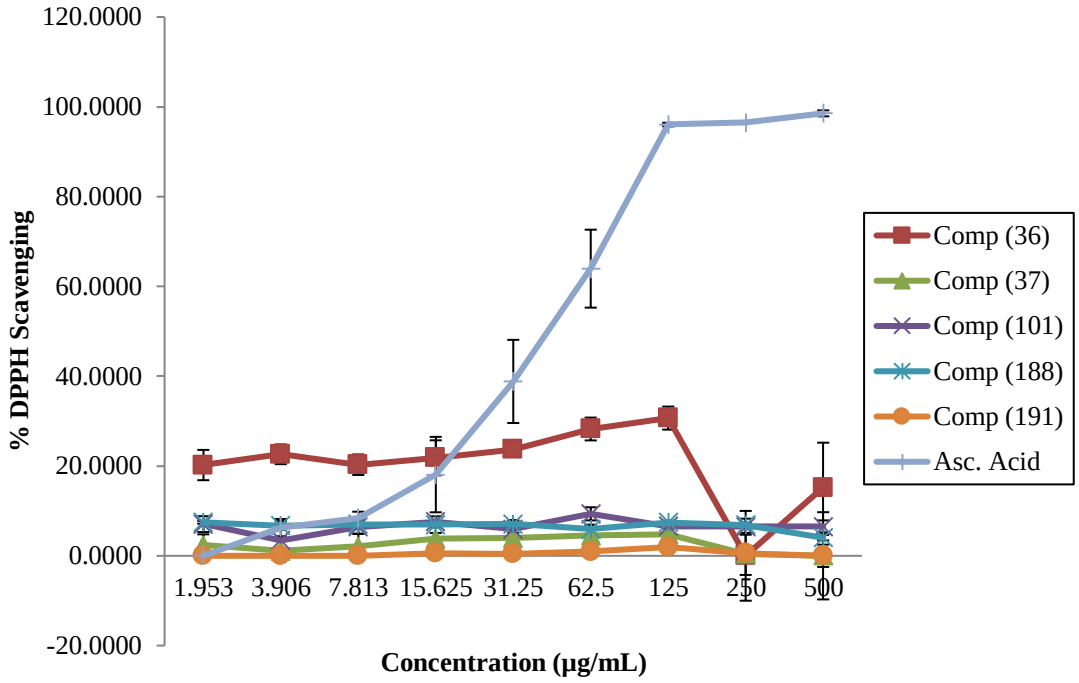


Figure 4.98. The percentage of inhibition of DPPH radicals by different concentration of the isolated compound in comparison to ascorbic acid standard. Data expressed as mean ± SD, n=3.

Table 4.22

The DPPH radical scavenging activity of isolated compounds of A. subintegra in comparison with the ascorbic acid standard

Isolated compound	**Value of IC_{50} (µ/mL)**
5-Hydroxy-7,4′-dimethoxyflavone (**36**)	> 500
Luteolin -7,3′,4′-trimethyl (**37**)	> 500
β-sitosterol (**101**)	> 500
Friedelin (**188**)	> 500
Aquilene A (**191**)	> 500
Ascorbic Acid (Control)	4.27 ± 0.74

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In particular, compound (**36**) exhibited the most potent antioxidative activity among the isolated compound; however, it is scavenging ability less than 30%. The property of compound (**36**) might be due to the presence of hydroxyl group in the phenolic (flavonoid) compounds' structure which may offer essential component as a scavenger (Pourmorad, Hosseinimehr, & Shahabimajd, 2006). Meanwhile, compound (**37**) showed the weakest free radical scavenger. Unfortunately, this finding has been unable to demonstrate a significant antioxidant property of compound (**37**) as a previous finding (Liu et al., 2018). Thus, the result might be associated by the excessive methoxyl group in the structure where the methylation of the hydroxyl group substantially reduces the scavenging activity of the compound (Teffo, Aderogba, & Eloff, 2010).

In addition, the rest compounds, *β*-sitosterol (**101**), friedelin (**188**) and aquilene A (**191**) showed weak antioxidant activity. Although both β-sitosterol (**101**) and friedelin (**188**) in previously have reported having anti-inflammatory and analgesic effects (Selegato et al., 2017), unfortunately, in current finding, they did not exhibit as a potential free radical scavenger.

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4.4.4 Anticancer Activity

The following biological test was done on *in vitro* cytotoxicity of MCF-7 breast cancer cell line using Cell Counting Kit-8 (CCK-8). CCK-8 allow convenient assay by developing water-soluble tetrazolium salt WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] produces a water-soluble formazan dye upon reduction in the presence of an electron carrier. WST-8 is reduced by dehydrogenases in cells to produce formazan (yellow colour), which is soluble in the tissue culture medium. The amount of formazan dye caused by the activity of dehydrogenases in cells is directly related to the number of living cells. The CCK-8 has higher detection sensitivity compared to 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and other tetrazolium salts (Hassan et al., 2012).

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In this study, the cytotoxicity effect of crude extract of leaf and bark on MCF-7 breast cancer cell investigated in six serial concentrations ranging from 3.125 to 100 µg/mL. The median inhibitory concentration (IC_{50}) values calculated for each crude extract, and the values are given in Table 4.23.

Based on non-linear regression analysis on the cell inhibition by the crude extracts (Appendix E), it shows that ASLM and ASBM managed to inhibit 50 % of cell growth at 24.30 ± 0.07 µg/mL (R^2 = 0.8911) and 17.46 ± 0.08 µg/mL (R^2 = 0.8501), respectively. This result seems to be consistent with the previous study, which indicated that the methanolic fruit extract of *A. subintegra* demonstrated higher anticancer activity against MCF-7's breast cancer cell (Nurhanan et al., 2017).

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The other crude extracts, ASLH and ASLD, showed low IC_{50} values 4.36 ± 0.14 µg/mL and 13.93 ± 0.17 µg/mL indicating potential cytotoxicity towards MCF-7 cells, however, their R^2 values, are mediocre due in no small error on the raw data. Therefore, this finding suggested that ASLH and ASLD showed low cytotoxicity effect. Meanwhile, ASBH and ASBD showed no inhibition of MCF-7 cells, as their lowest R^2 score, as shown in Table 4.23.

Table 4.23

Cytotoxicity of the crude extract against MCF-7's breast cancer cell with an IC_{50} value

Crude Extract	**IC_{50} values derived from non-linear regression analysis**	
	MCF-7 (µg/mL)	**R^2**
ASBH	7.93 ± 0.29	0.2823
ASBD	12.94 ± 0.13	0.5780
ASBM	17.46 ± 0.08	0.8501
ASLH	4.36 ± 0.14	0.6512
ASLD	13.93 ± 0.17	0.6097
ASLM	24.30 ± 0.07	0.8911

Note: ASBH = *A. subintegra* bark n-Hex, ASBD = *A. subintegra* bark CH_2Cl_2, ASBM = *A. subintegra* bark MeOH, ASLH = *A. subintegra* leaf n-Hex, ASLD = *A. subintegra* leaf CH_2Cl_2, ASLM = *A. subintegra* leaf MeOH.

Six selected compounds evaluated for cytotoxicity effect against MCF-7's breast cancer cell. Table 4.24 exhibited the IC_{50} values of isolated compounds against MCF-7's breast cancer cells. Compound 5-hydroxy-7,4′-dimethoxyflavone (**36**) showed the most cytotoxicity effect, where could inhibit 50 % of cell growth at 38.48 ± 0.06 µg/mL. Also, compound *β*-sitostenone (**139**) showed the IC_{50} value of 33.27 ± 0.08 µg/mL.

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However, the other compounds, aquilene A (**191**), *β*-sitosterol (**101**), 5,3′-dihydroxy-7,4′-dimethoxyflavone (**41**) and saturated triglyceride (**186**) unable to inhibit the cell growth; as a result a low and unreliable R^2 score. Meanwhile, compound luteolin-7,3′,4′-trimethyl ether (**37**) was not able to generate IC_{50} values. Thus, the result suggested that these compounds needed higher concentration to increase the ability to inhibit the growth of MCF-7's breast cancer cells (Waluyo & Pasaribu, 2017).

Table 4.24

Cytotoxicity of the isolated compound against MCF-7's breast cancer cell with IC_{50} value

Compound	**IC_{50} values derived from non-linear regression analysis**	
	MCF-7	**R^2**
36	38.48 ± 0.06	0.8833
37	Not converged	Not available
41	> 100	0.01616
101	59.82 ± 0.58	0.1364
139	33.27 ± 0.08	0.7621
186	10.54 ± 0.32	0.2495
191	5.76 ± 0.44	0.1924

The cytotoxic activity, however, could not be done for the remaining phytochemicals in this study as the little amounts available were used for compound identification, leaving no material for any other analyses.

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CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

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5.1 Conclusion

Phytochemical studies of the bark and leaf of *Aquilaria subintegra* have successfully isolated 22 compounds in which three of them are new. Eight compounds were obtained from the bark, including four flavones, two steroids, one triglyceride and one quinone. The leaf of *A. subintegra* afforded 14 compounds. Including three flavonoids, three steroids, five triterpenes, one diterpene, one alkane and one tocopherol derivative. These phytochemical findings of *A. subintegra* were summarized in Figure 5.1.

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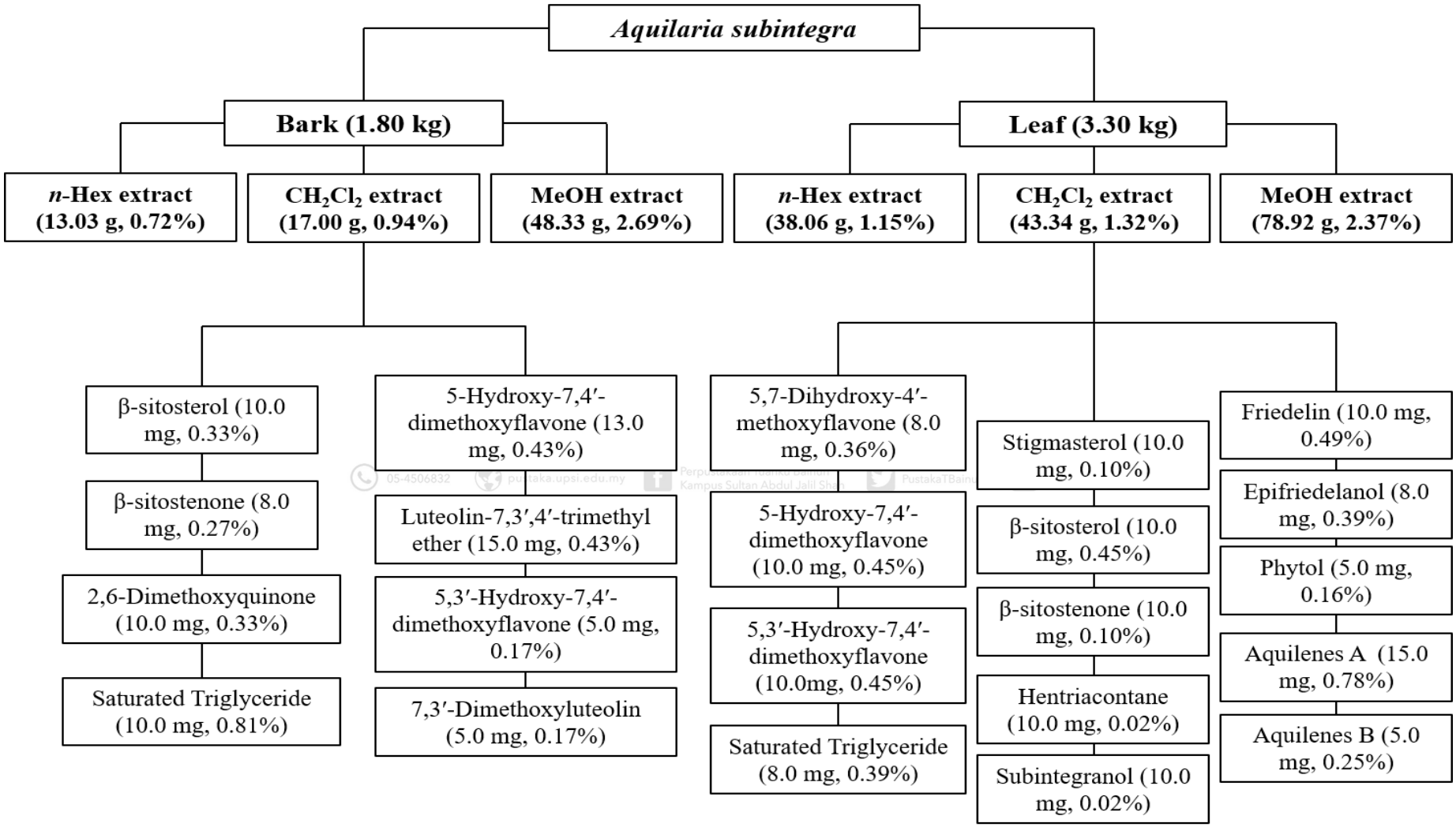


Figure 5.1. Summary phytochemicals isolated from bark and leaf of *A. subintegra*

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The present studies report on the isolation and identification of the phytochemicals from *A. subintegra*. To the best our knowledge, all known isolated compounds, as well as new compounds, were obtained in the first time for *A. subintegra.*

The antioxidant activity was examined using DPPH free radical scavenging. The antioxidant properties of crude extracts showed that the MeOH leaf extract of *A. subintegra* was the most potent free radical scavenger with the IC50 value of 29.56 ± 6.37 µg/mL. However, there is no significant effect from isolated compounds toward DPPH free radical scavenging assay.

The *in vitro* cytotoxicity experiment of MCF-7 breast cancer cell line has been done using Cell Counting Kit-8 (CCK-8). The result exhibited that MeOH extract of bark and leaf possess the most cytotoxicity effect with IC_{50} value of 24.30 ± 0.07 and 17.46 ± 0.08 µg/mL, respectively. Furthermore, 5-hydroxy-7,4′-dimethoxyflavone (**36**) owned the most anticancer property with IC_{50} value, 38.48 ± 0.06 µg/mL. However, the remaining compounds showed no cytotoxicity effect against MCF-7's breast cancer cell.

The anti-lipase activity was carried out by colorimetric assay with some modification. As for the crude extracts, intense anti-lipase activity was shown by hexane crude of leaf and moderate effect showed by CH_2Cl_2 extract from leaf and bark of the plant. The anti-lipase results of the isolated compounds exhibited that 5-hydroxy-7,4′-dimethoxyflavone (**36**) was the potent lipase inhibitor among the others.

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In a continuing study of the mechanism of the isolated compound in anti-lipase activity, one computational study was approached using YASARA software. The molecular docking was performed to directly determine the potential mode of the isolated compound toward the target protein. Docking study showed the flavonoid compounds (**36**, **37** and **41**) performed as a competitive inhibitor in which they were docked close to the catalytic site (Ser153, Asp177 and His264) of an enzyme. Meanwhile, *β*-sitosterol (**101**) predicted as a non-competitive inhibitor as it binds allosterically toward enzyme.

The combined data of wet experimental and computational study will help us to a better understanding of the structure-activity relationship between active compound and their binding site on the protein molecule (Skrt, Benedik, Podlipnik, & Ulrih, 2012). Yet, the innovation of experimental of the relationship between small molecules and receptor is still needed; the accuracy and low cost of computational study may convey docking application to another stage (Meng et al., 2011).

5.2 Recommendations for Future Study

The present study makes several noteworthy contributions to extending knowledge about *A. subintegra* and its potential biological properties. However, there has some limitations in this work, especially a small amount of mass of isolated compound for other desired study. Since this study has done on bark and leaf parts, therefore, in future will elucidate another part such as fruits or root, which may contain different constituents from this finding.

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As the mass of isolated compounds was a small amount, we also recommended synthesizing marker compounds with biological values to increase the number of samples. In addition, the active compounds and new compound derived from this part of the plant will continue with an extensive experiment such as pharmacological, toxicological, clinical test and afterwards developed as a new medicinal drug. Furthermore, the bioactive compound will subject to the structure-activity relationship in other biological activities to better understanding their mechanisms.

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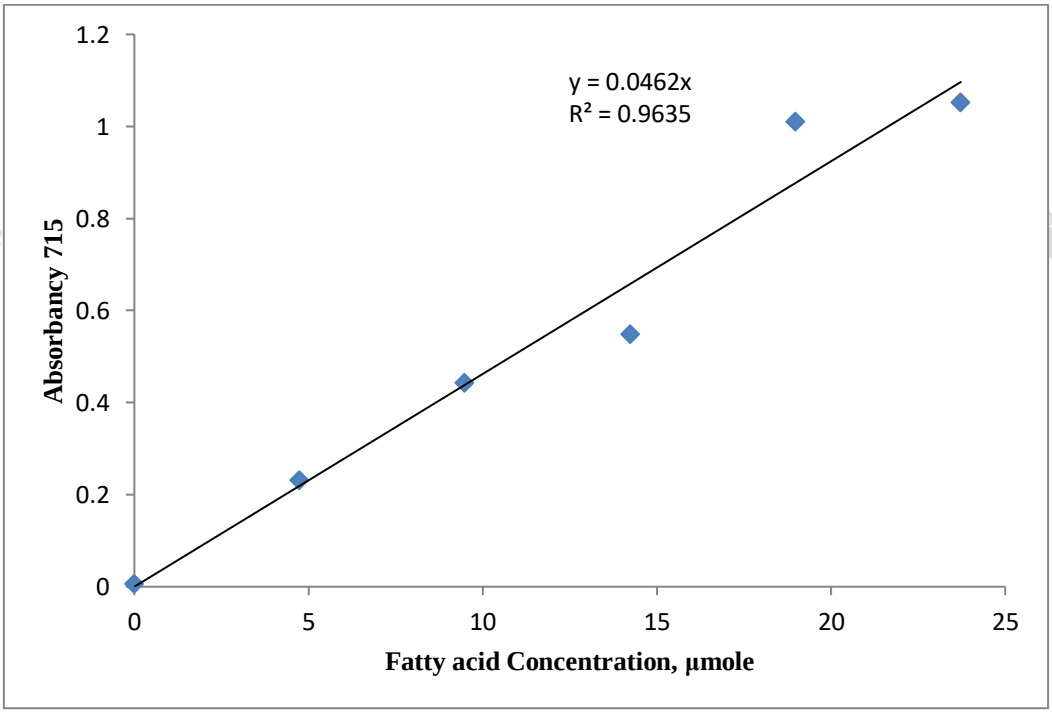
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APPENDICES

APPENDIX A

Standard Curve of Free Fatty Acid



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APPENDIX B

Anti-lipase activity of crude extract and isolated compounds of *A. subintegra*

Table 1: Descriptive data of anti-lipase activity for crude extract bark and leaf of *A. subintegra*

Group Statistics

	Treatment Group	N	Mean	Std. Deviation	Std. Error Mean
HEX	BARK	3	.5797	.08615	.04974
	LEAF	3	.3490	.07716	.04455
DCM	BARK	3	.4617	.05237	.03023
	LEAF	3	.5483	.04005	.02313
MEOH	BARK	3	.3967	.00462	.00267
	LEAF	3	.7143	.05050	.02916

Table 2: T-test analysis of anti-lipase activity for crude extract bark and leaf of *A. subintegra*

		Levene's Test for Equality of Variances		t-test for Equality of Means		
		F	Sig.	t	df	Sig. (2-tailed)
HEX	Equal variances assumed	.078	.794	3.455	4	.026
	Equal variances not assumed			3.455	3.952	.026
DCM	Equal variances assumed	.577	.490	-2.277	4	.085
	Equal variances not assumed			-2.277	3.743	.090
MEOH	Equal variances assumed	10.877	.030	-10.850	4	.000
	Equal variances not assumed			-10.850	2.033	.008

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Table 3: ANOVA analysis on the anti-lipase activity of isolated compounds of *A.subintegra*

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Comp **36**	Between Groups	.173	2	.087	27.801	.001
	Within Groups	.019	6	.003		
	Total	.192	8			
Comp **37**	Between Groups	.002	2	.001	.811	.488
	Within Groups	.007	6	.001		
	Total	.008	8			
Comp **101**	Between Groups	.138	2	.069	34.664	.001
	Within Groups	.012	6	.002		
	Total	.150	8			
Comp **193**	Between Groups	.020	2	.010	1.785	.246
	Within Groups	.034	6	.006		
	Total	.054	8			
Comp **191**	Between Groups	.001	2	.001	.193	.830
	Within Groups	.021	6	.004		
	Total	.023	8			
Comp **41**	Between Groups	.005	2	.002	1.151	.377
	Within Groups	.013	6	.002		
	Total	.018	8			

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Multiple Comparisons (Tukey HSD)

Dependent Variable	(I) Concentrations	(J) Concentrations	Mean Difference (I-J)	Std. Error	Sig.
Comp **36**	1 mg/ml	0.5 mg/ml	-.25933*	.04556	.003
		0.25 mg/ml	-.31967*	.04556	.001
	0.5 mg/ml	1 mg/ml	.25933*	.04556	.003
		0.25 mg/ml	-.06033	.04556	.434
	0.25 mg/ml	1 mg/ml	.31967*	.04556	.001
		0.5 mg/ml	.06033	.04556	.434
Comp **101**	1 mg/ml	0.5 mg/ml	.03367	.02706	.473
		0.5 mg/ml	-.23533*	.03645	.002
		0.25 mg/ml	-.28367*	.03645	.001
	0.5 mg/ml	1 mg/ml	.23533*	.03645	.002
		0.25 mg/ml	-.04833	.03645	.433
	0.25 mg/ml	1 mg/ml	.28367*	.03645	.001
		0.5 mg/ml	.04833	.03645	.433

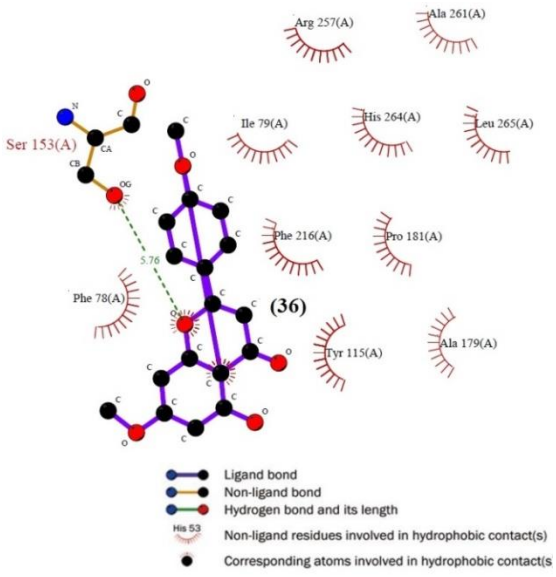
*. The mean difference is significant at the 0.05 level.

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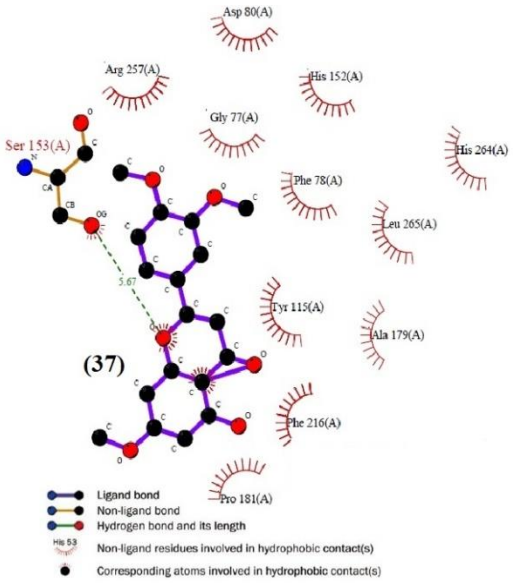
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APPENDIX C

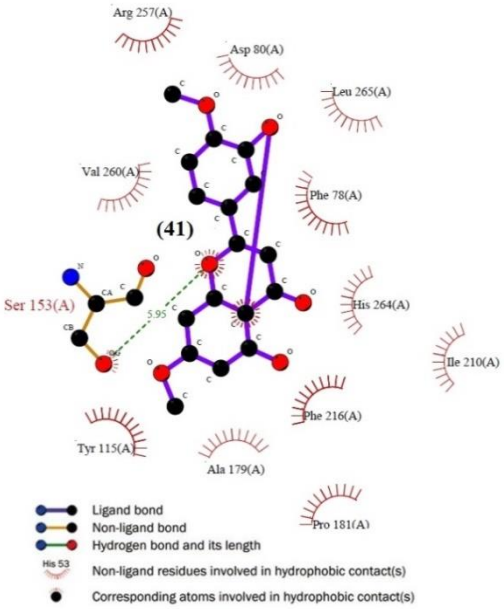
2-D illustration of docking study using Ligplot+



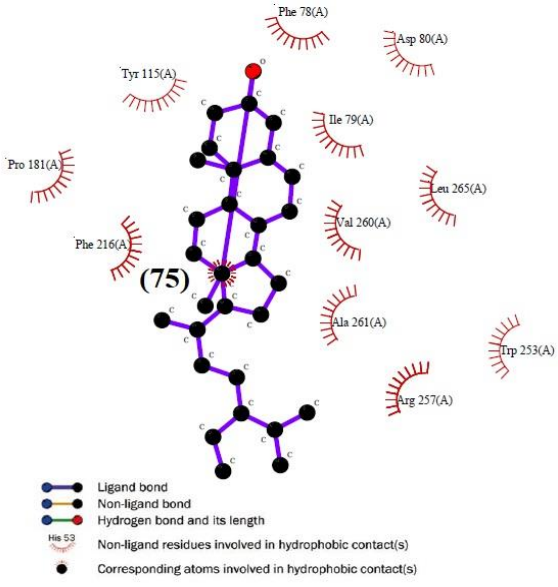
5-Hydroxy-7,4′-dimethoxyflavone (**36**)



Luteolin 7,3′,4′-trimethyl ether (**37**)



5,3′-Dihydroxy-7,4′-dimethoxyflavone (**41**)



β – sitosterol (**101**)

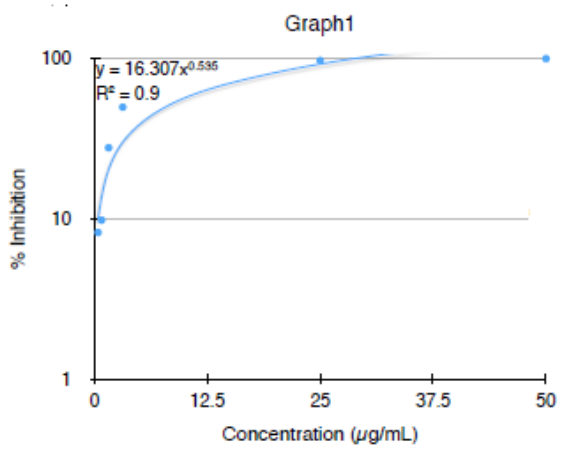
2-D illustration of the amino acid residues interacting with ligand were created by use Ligplot+, ligands represented as a chemical formula.

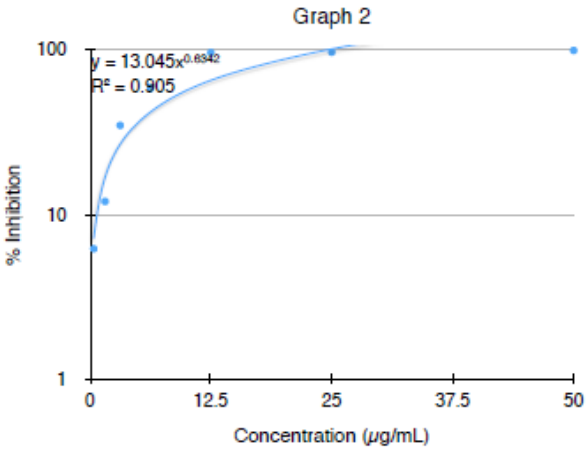
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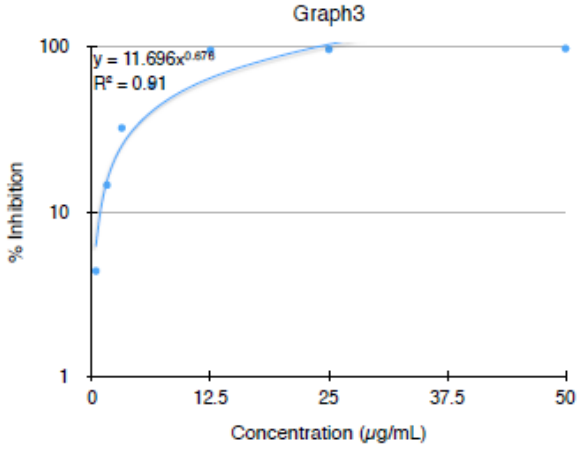
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APPENDIX D

Standard Curve of Antioxidant Acyivity





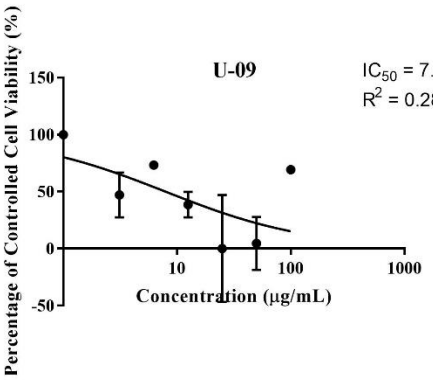


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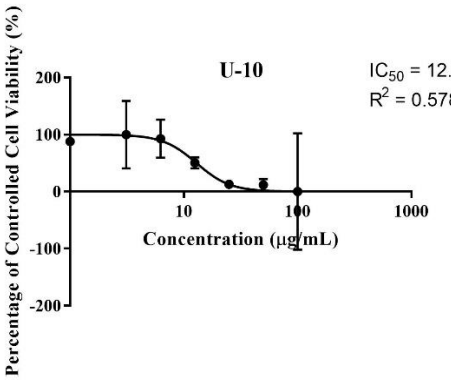
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APPENDIX E

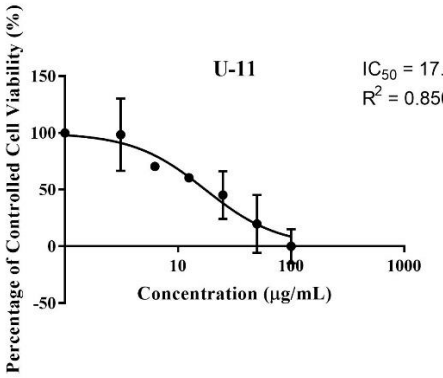
Dose-response Plot Based on Non-linear Regression Analysis



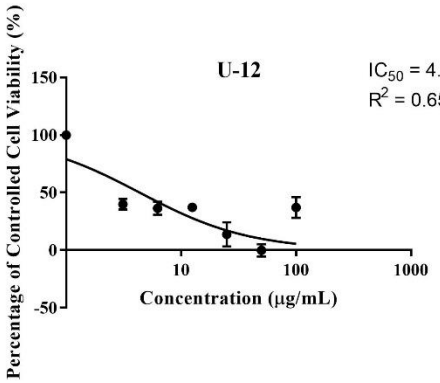
ASBH



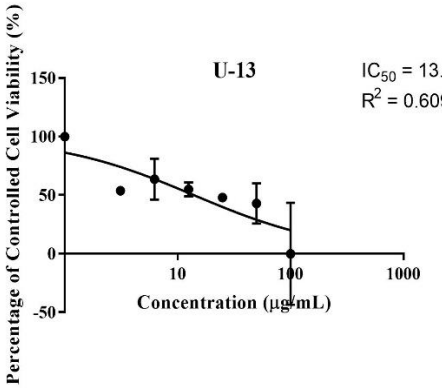
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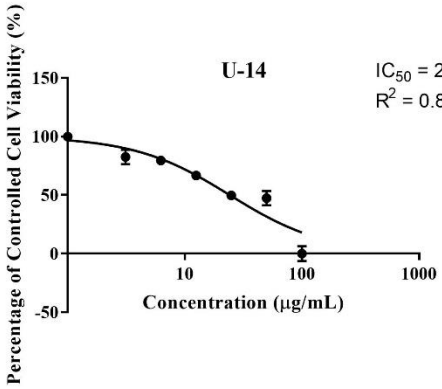
ASBM



ASLH



ASLD

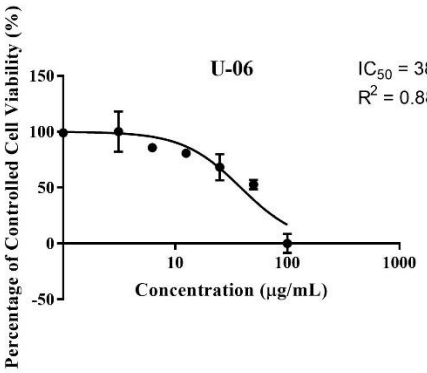


ASLM

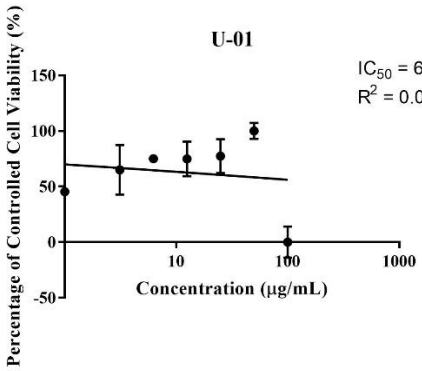
E1: Dose-response plot based on non-linear regression analysis of crude extracts against MCF-7 cell. Experiment conducted in triplicate set (n=3, ± s.d)

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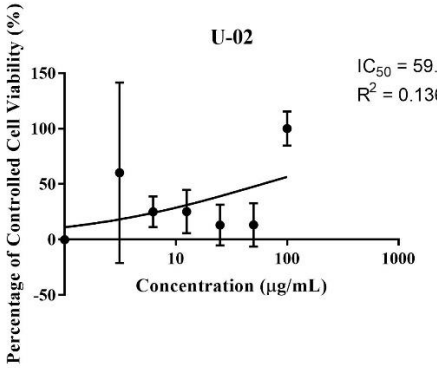
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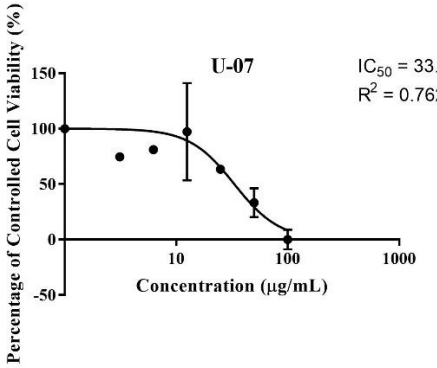
(36)



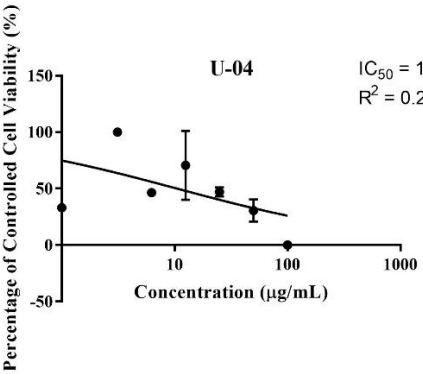
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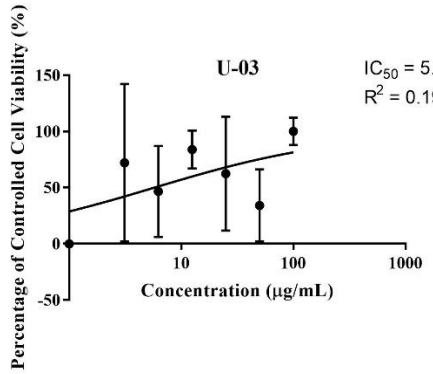
(101)



(139)



(186)



(191)

E2: Dose-response plot based on non-linear regression analysis of isolated compounds against MCF-7 cell. Experiment conducted in triplicate set (n=3, ± s.d)

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APPENDIX F

Publication(s) to be derived from this work

1. **Title: Evaluation of anti-lipase activity of leaf and bark extracts from *Aquilaria subintegra* and *A. malaccensis* (Published)**

   Authors: Mastura Ibrahim, Saripah Salbiah Syed Abdul Azziz, Chee Fah Wong, Wan Nor Izzati Wan Mohamad Din, Wan Rusmawati Wan Mahamod, Yuhanis Mhd Bakri, Mohamad Syahrizal Ahmad, Rozita Yahaya, Nor Hadiani Ismail, Wan Mohd Nuzul Hakimi Wan Salleh.

   Journal: *Marmara Pharmaceutical Journal* (SCOPUS)

   Year/Vol./Page: 2018; 22(1): 91-95

   Publisher: Marmara University, Faculty of Pharmacy, Turkey.

2. **Title: Flavones and steroids from leaf and bark of *Aquilaria subintegra* (Published)**

   Authors: Mastura Ibrahim, Saripah Salbiah Syed Abdul Azziz, Chee Fah Wong, Fauziah Abdullah, Yuhanis Mhd Bakri.

   Journal: *Chemistry of Natural Compound* (ISI Indexed)

   Year/Vol./Page: 2020; 56(3): 537-538

   Publisher: Springer, United State.

3. **Title: Interactions of Flavone and Steroid from *A. subintegra* as Potential Inhibitors for Porcine Pancreatic Lipase (Accepted)**

   Authors: Mastura Ibrahim, Saripah Salbiah Syed Abdul Azziz, Chee Fah Wong, Yuhanis Mhd Bakri, Fauziah Abdullah.

   Journal: *Current Computer-Aided Drug Design* (ISI Indexed)

   Publisher: Bentham Science Publishers, United Arab Emirates.

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APPENDIX G

Conference Attendance

1. Conference: **ICEMS 2018 & 6th IPCSM 2018**
   Venue: Universiti Pendidikan Sultan Idris
   Date: 7th – 8th November 2018
   Title: Flavonoids and Triterpenes from *Aquilaria subintegra* and Its Antioxidant activity (ORAL)
   Authors: Mastura Ibrahim, Saripah Salbiah Syed Abdul Azziz, Chee Fah Wong, Norazian Mohd Hassan

2. Conference: **International Malaysian French Scientific Conference 2017**
   Venue: Universiti Malaya
   Date: 18th – 19th December 2017
   Title: Anti-lipase activity of isolated compounds from A. *subintegra* (POSTER)
   Authors: Mastura Ibrahim, Saripah Salbiah Syed Abdul Azziz, Chee Fah Wong, Nor Hadiani Ismail, Mohamad Syahrizal Ahmad, Yuhanis Mhd Bakri, Wan Rusmawati Wan Mahamod, Fauziah Abdullah, Norsyuhada Alias

4. Conference: **International Conference on Natural Products (ICNP) 2016**
   Venue: Permai Hotel, Kuala Terengganu, Terengganu
   Date: 15th -17th March 2016
   Title: Phytochemical and Biological Studies of *Aquilaria subintegra* (ORAL)
   Authors: Mastura Ibrahim, Saripah Salbiah Syed Abdul Azziz, Wong Chee Fah, Hasimah Alimon, Rozita Yahaya, Nor Hadiani Ismail, Humera Naz, Wan Rusmawati Wan Mahamod, Aisyah Mohamad Sharif, Mohamad Syahrizal Ahmad

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