Addis Ababa University

College of Natural and Computational Sciences

Department of Chemistry

MSc Thesis

Phytochemical Studies on *Premna schimperi* Engl. (Lamiaceae).

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A thesis submitted to the Department of Chemistry, College of Natural and Computational Sciences, in partial fulfillment of the requirement for the degree of Master of Science in Chemistry.

September 2021

Declaration

I, the undersigned, declare that this MSc. thesis is my original work and has not been presented for any degree in any university and that all sources of materials used for this project have been duly acknowledged.

Name: Mohammedamin Nasir

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This MSc. thesis has been submitted for examination with our approval as university advisors:

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Date and place of submission: Department of Chemistry
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Acknowledgements

I want to express my deepest gratitude to my advisors Dr. Estifanos Ele Yaya and Prof. Wendimagegn Mammo for their guidance, patience and scientific support during conducting this work. Again, I would like to acknowledge Dr. Estifanos Ele Yaya for conducting the GC-MS analysis of some compounds and essential oils and Prof. Wendimagegn Mammo for running the NMR spectra of all compounds including 1D and 2D NMR.

I want to extend my gratitude to Dr. Yonas Chebude for running FT-IR spectra of all compounds. I sincerely thank my family for their encouragements and unconditional support. I also thank my friends Dr. Melaku Assefa, Mr. Tadele Tamenu, Mr. Asaminew Yerango and all staff members of the Department Chemistry for their great help in this work.

I gratefully acknowledge Addis Ababa University for sponsorship and Department of Chemistry for providing laboratory space, required chemicals and apparatus to conduct this research.

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Abstract

In this study, attempts were made to isolate secondary metabolites from the solvent extracts of the dried leaves of *Premna schimperi* Engl. The essential oil obtained by hydrodistillation of the dried leaves of *P. schimperi* was also analyzed by GC-MS. A total of six compounds were isolated from the solvent extracts, of which *ent*-8β,12α-epidioxy-12β-hydroxylabda-9(11),13-dien-15-oic acid γ-lactone (**71**), 3,5,5'-trihydroxy-6,7,3',4'-tetramethoxyflavone (**157**) and 3,5,7,5'-tetrahydroxy-6,3',4'-trimethoxyflavone (**158**) were isolated from the chloroform extract, while a straight chain primary alcohol (**165**) was isolated from the petroleum ether extract, and luteolin (**159**) and *p*-hydroxycinnamic acid (**164**) were isolated from the ethyl acetate portion of the methanol extract. Per acetylation of compound **157** using acetic anhydride and pyridine gave triacetate **157-Ac**. The GC-MS analysis of the essential oil revealed 36 compounds, of which the major compositions were caryophyllene (**9**), caryophyllene oxide (**10**), terpinen-4-ol (**33**), α-curcumene (**166**), eugenol (**167**), β-sesquiphellandrene (**168**), γ-gurjunene (**169**), 2-isopropyl-5-methyl-9-methylene-bicyclo[4.4.0]dec-1-ene (**170**), γ-eudesmol (**171**). The antioxidant activities of the MeOH extract of the leaves of *P. schimperi* and compound **71** were assessed using the DPPH radical scavenging assay. The MeOH extract reduced the stable DPPH radical by 95.3% whereas compound **71** reduced the stable DPPH radical by 62.2% at 100 µg/mL, which suggested that they are potential radical scavengers.

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Lists of Abbreviations and Acronyms

bs	Broad singlet
^{13}C-NMR	Carbon-13 Nuclear Magnetic Resonance
δ	Chemical shift
$CHCl_3$	Chloroform
CC	Column Chromatography
J	Coupling constant
^{o}C	Degree Celsius
Acetone-d_6	Deuterated Acetone
$CDCl_3$	Deuterated Chloroform
DEPT-135	Distortionless Enhancement by Polarization Transfer 135
d	Doublet
dd	Doublet of doublets
ddd	Doublet of doublets of doublet
EtOAc	Ethyl acetate
FTIR	Fourier Transform Infrared Spectroscopy
Fr.	Fraction
GC-MS	Gas Chromatography-Mass Spectrometry
g	Gram
mg	Milligram
mL	Milliliter
Hz	Hertz
h	Hour
IR	Infrared Spectroscopy
L	Liter
MSD	Mass Selective Detector
MeOH	Methanol
m	Multiplet
NIST	National Institute of Standards and Technology
NMR	Nuclear Magnetic Resonance
ppm	Parts Per Million
PTLC	Preparative Thin Layer Chromatography
^{1}H-NMR	Proton Nuclear Magnetic Resonance
q	Quartet
RT	Retention time
TLC	Thin Layer Chromatography
UV-Vis	Ultraviolet and Visible Spectroscopy
λ	Wavelength
DPPH	2,2-Diphenyl-1-picrylhydrazyl

1. Introduction

Plants synthesize a wide variety of phytochemicals that play roles in performing their normal physiological functions and to protect themselves from microbial pathogens and animal herbivores.[1] These phytochemicals are classified as primary and secondary metabolites.

Primary metabolites such as basic sugars, amino acids, nucleosides and small organic acids are associated with essential cellular functions which serve the plants as component of growth and energy metabolism. They are directly involved in normal growth, development and reproduction of plants under any condition. Secondary metabolites such as terpenoids, alkaloids, phenylpropanoids, polyketides, quinones and cyanogenic glycosides are formed by metabolism and are no longer used in the formation of new cells in plants. They are species specific and may not increase the plant's fitness but they might be useful for survival and reproduction.[2,3] Whereas, secondary metabolites play a major role in defensive and attractive interactions between plants and their environment. Although they are not directly involved in growth and development of plants, they are often accumulated in high concentration (1-3% of fresh weight); and may show high toxicity; and biological effects on other organisms.[4]

Secondary metabolites are natural products (NPs) that have low molecular weight with diverse chemical structures and biological activities. They are produced by living organisms such as plants, animals and microorganisms. The discovery of secondary metabolites have been driven by the findings that they are useful and valuable agents in pharmaceuticals, agriculture, and cosmetics and food industries.[5] Chemical investigation of the secondary metabolites motivated the development of separation techniques, spectroscopic approaches for elucidation of chemical structures and synthetic methodologies.[6] Modern drugs and natural products are closely linked through the use of traditional medicines and natural poisons.[7] Plants are one of the most abundant sources of the modern drugs.[8] Still large number of plant extracts such as concoctions, poultices, decoctions or pastes are in use in many countries including China, India, and most of Africa for treatment of diseases, cuts, wounds and burns.[1] Chemical investigation of the traditional medicinal plants are the fundamental sources for most of the pharmaceutical medicines.[7]

1.1. The genus *Premna*

The word '*Premna*' comes from the Greek word 'premnon', meaning 'tree stump', which refers to the short and twisted trunks of *P. serratifolia* L., the first collected species of this genus.[9] Formerly, the genus *Premna* L. belonged to the family Verbenaceae, but has been reclassified in to Lamiaceae.[10] Mostly, species in the genus *Premna* are small trees or shrubs rarely lianas and pyroherbs,[9] and usually their morphology is dependent on geographical area.[11] There are about 200 *Premna* species and they are mainly flowering plants distributed in tropical and sub-tropical Asia, Australia, Africa and the Pacific Islands.[12]

1.2. Traditional uses of plants belonging to the genus *Premna*

Premna species are known medical plants used for treatment of many diseases such as pyogenic infections, trauma, fracture, dysentery, hemorrhoids, toothache, skin disease and rheumatic arthritis. In addition, the phytochemical studies of reported compounds showed to exhibit anti-malaria, anti-cancer, anti-inflammation and anti-tuberculosis properties.[13,14]

1.3. Chemical constituents of the genus *Premna*

1.3.1. Essential oils

The genus *Premna* is known to be poor in their content of essential oils.[9] The leaves of some *Premna* species were analyzed by GC-MS and some of the compounds identified are given in Figure 1. The GC-MS analysis of the essential oil of the leaves of *P. cambodiana* revealed 72 compounds, of which sesquiterpenes α-copaene (**17**, 23.3%), (E)-caryophyllene (**52**, 12.8%), and δ-cadinene (**56**, 5.5%) are the most abundant constituents.[10] The essential oil of the leaves of *P. mucronata* Roxb afforded 1-octen-3-ol (**48**, 9.6%), linalool (**20**, 5.5%), methyl salicylate (**46**, 2.9%) and (E)-caryophyllene (**52**, 2.9%) as major constituents.[15] The essential oil of *P. chevalieri* afforded two monoterpenes; α-pinene (**37**, 12.2%) and β-pinene (**42**, 16.8%) and two sesquiterpenes; (E)-caryophyllene (**52**, 31.5%) and α-humulene (**54**, 7.5%) as the major components.[10] *P. corymbosa* (syn. *P. integrifolia, P. serratifolia*) was found to be rich in phytol (**45**, 27.3%), α-humulene (**54**, 14.2%), spathulenol (**55**, 12.1%) and 1-octen-3-ol (**48**, 8.2%).[16] The essential oils of the leaves of *P. flavescens* from two sites

afforded (E)-caryophyllene (**52**) and *trans*-β-elemene (**51**) as the major components.[10] The essential oil of the leaves of *P. maclurei* afforded (E)-caryophyllene (**52**, 30.7%), α-humulene (**54**, 5.3%), δ-cadinene (**56**, 8.4%), spathulenol (**55**, 6.8%), and caryophyllene oxide (**10**, 12.3%).[10] The essential oil of the leaves of *P. mekongensis* afforded α-pinene (**37**) and (E)-caryophyllene (**52**).[10] The essential oil of the leaves of *P. puberula* was dominated by two sesquiterpene hydrocarbons, namely α-copaene (**17**, 5.3%) and alloaromadendrene (**53**, 4.1%) and three oxygenated sesquiterpenoids, namely caryophyllene oxide (**10**, 21.2%), spathulenol (**55**, 7.7%) and humulene epoxide II (**49**, 4.7%) as the major components.[10] The essential oil of the leaves of *P. tomentosa* was rich in two sesquiterpene hydrocarbons, namely (E)-caryophyllene (**52**, 22.0%) and germacrene D (**15**, 11.4%).[10] However the report by Narayan and Muthana in 1953 identified limonene (**19**, 57.8%), (E)-caryophyllene (**52**, 17.2%) as the major constituents of the species.[17] The essential oil of the leaves of *P. coriacea, P. latifolia*, *P. quadrifolia*, and *P. odorata* are rich in sesquiterpene hydrocarbons, while *P. barbata*, *P. latifolia*, and *P. angolensis* are rich in low molecular weight alcohols such as 1-octen-3-ol (**48**).[10]

Figure 1: Chemical structures of some compounds identified from the essential oils of plants belonging to the genus *Premna.*

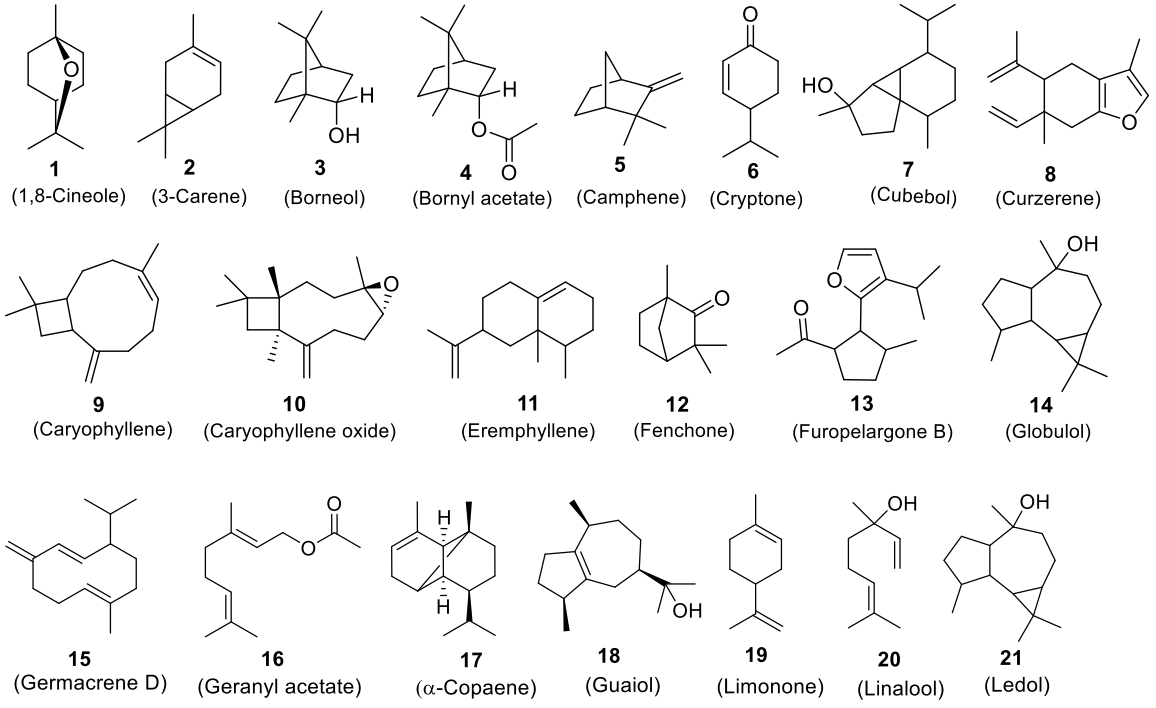
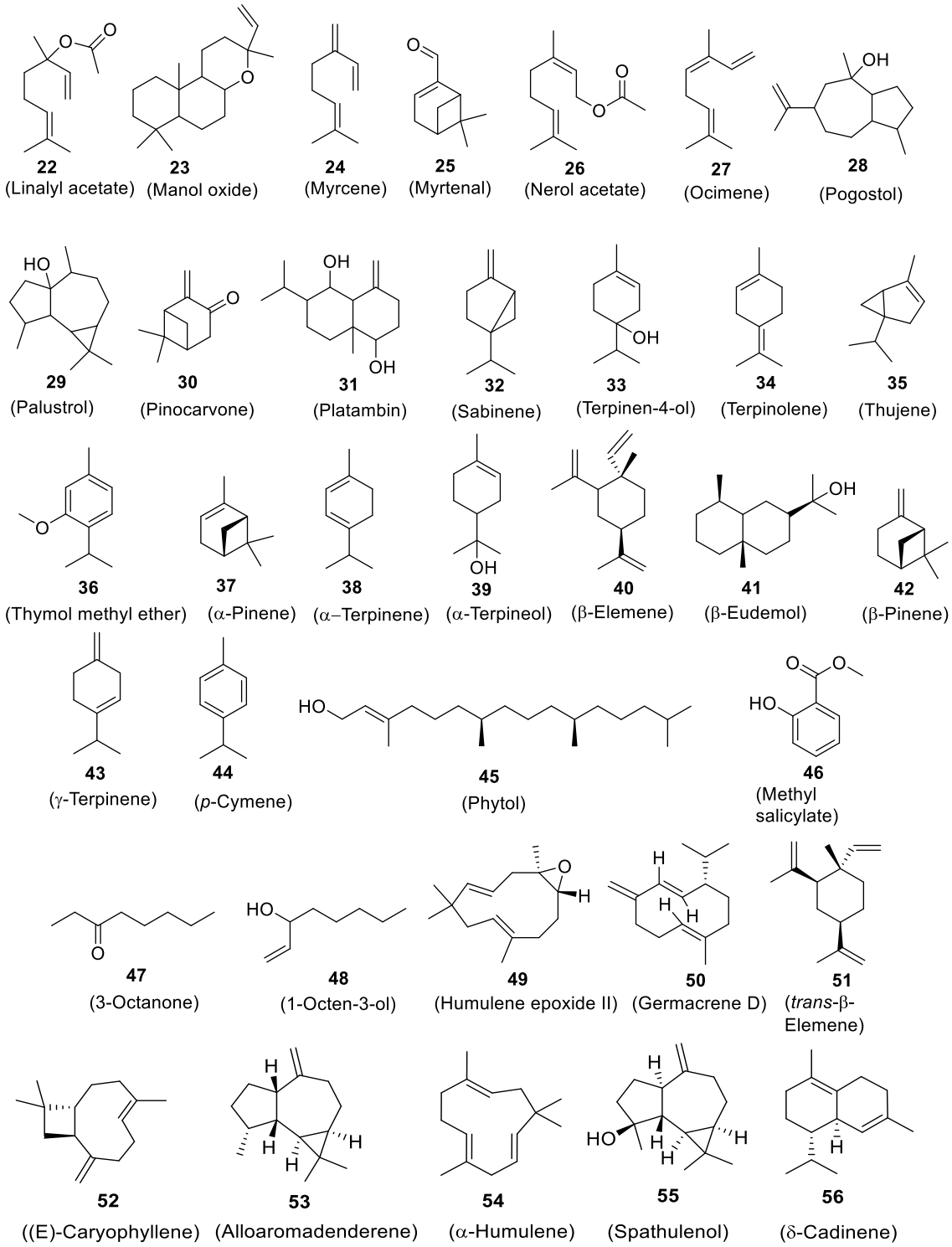


Figure 1 continued

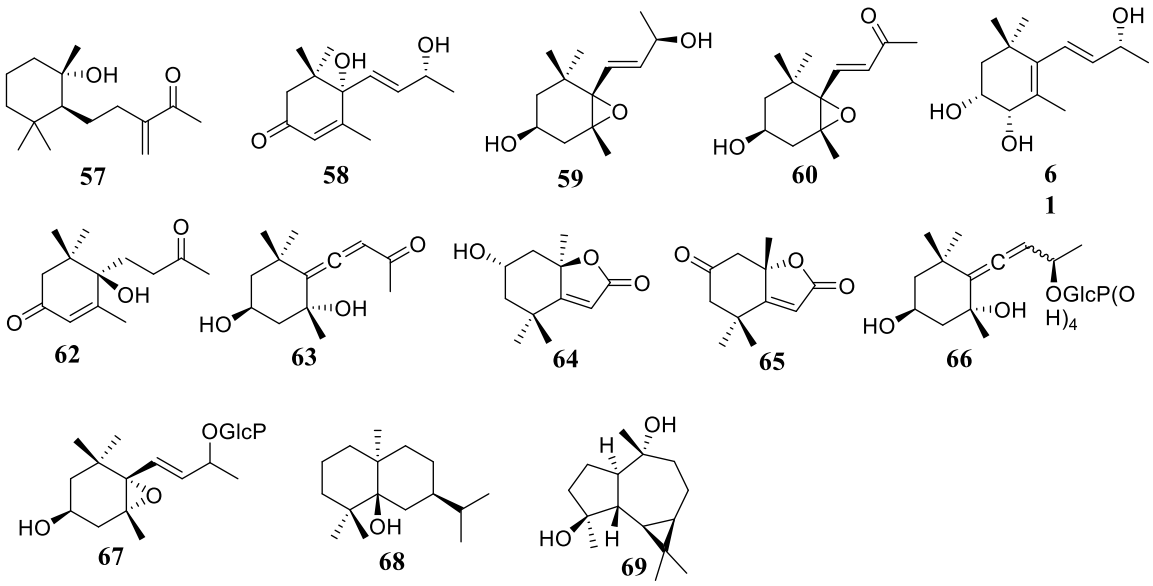


1.3.2. Chemical structures of some compounds reported from the genus *Premna.*

More than 250 compounds were isolated from the genus *Premna*. The major classes of the compounds reported from the genus were sesquiterpenes, diterpenoids, flavonoids,

iridoid glycosides, xanthones, phenylethanoid glycosides, chalcones, triterpenoids and lignans.[9] Figure 2 shows the structures of mono- and sesquiterpenes isolated from *Premna* species.

Figure 2: Structures of mono- and sesquiterpenes isolated from the genus *Premna*.



The sesquiterpene, 7-α-hydroxy-6,11-cyclofarnes-3(15)-en-2-one (**57**), was reported from the aerial parts of *P. oligotricha* in 1993.[18] Blumenol A (**58**), (3S,5R,6S,7E,9R)-5,6-epoxy-3,9-dihydroxy-7-megastigmene (**59**), 3β-hydroxy-5α,6α-epoxy-7-15 megastigmen-9-one (**60**), ixerol B (**61**), (-)-dehydrovomifoliol (**62**), (3S,5R)-dihydroxy-6S,7-megastigmadien-9-one (**63**), loliotide (**64**), (+)-dehydrololiolide (**65**) were obtained from *P. microphylla* Turcz.[19] The leaves of *P. subscandens* MERR afforded 7-(3,5-dihydroxy-1,1,5-trimethylcyclohexylidene)-9-methylprop-8-enyl-9-*O*-D glucopyranoside (**66**) and 3-hydroxy-5,6-epoxy-β-ionol-9-*O*-β-D-glucopyranoside (**67**).[20] 4β,5β-Dihydroxy-10-epi-eudesmane (**68**) and 4β, 10β-dihydroxyaromadendrane (**69**) were isolated from the root and twigs of *P. obtusifolia*.[21]

Figure 3 shows the diterpenes reported from *Premna* species. Thus, *ent*-12-oxolabda-8,13(16)-dien-15-oic acid (**70**), *ent*-8β,12α-epidioxy-12β-hydroxylabda-9(11),13-dien-15-oic acid γ-lactone (**71**), (5R,8R,9S,10R)-12-oxo-*ent*-3,13(16)-clerodien-15-oic acid (**72**) and 16α-hydroxy-cleroda-3,13(14)-Z-dien-15,16-olide (**73**) were reported from *P. oligotricha*.[22, 23] The leaves of *P. tomentosa* afforded (5R,6R,7R,8S,9R,10R)-6-*O*-(*trans*-cinnamoyl)-7-hydroxy-12-oxo-3,13(16)-clerodien-15-oic acid methyl ester

(**74**), (5R,6R,7R,8S,9R,10R)-6-*O*-(*cis*-cinnamoyl)-7-hydroxy-12-oxo-3,13(16)-clerodien-15-oic acid methyl ester (**75**) and (5R,6R,7R,8S,9R,10R)-7-*O*-(*trans*-cinnamoyl)-6-hydroxy-12-oxo-3,13(16)-clerodien-15-oic acid methyl ester (**76**).[24] Salvicaranaldehyde (**96**), ferruginol (**83**), *O*-methyl-ferruginol (**84**), 12-hydroxyabieta-8(14),9(11),12-trien-7-one (**85**), royleanone (**86**), montbretrol (**88**), 11,12-dihydroxy-8,11,13-icetexatrien-1-one (**115**), taxodion (**90**), salviasperanol (**117**), 5,6-dihydro-6α-hydroxysalviasperanol (**118**), arucadiol (**93**), abietatrien-1-β-ol (**80**), abietatrien-1β,12-diol (**81**), 5α,11,12-trihydroxy-6-oxa-abieta-8,11,13-trien-7-one (**91**), α,11,12-trihydroxy-7β,20-epoxy-8,11,13-abietatriene (**92**), obtusinone A (**107**), obtusinone B (**108**), obtusinone C (**109**), isopimara-7,15-dien-1β,3β-diol (**101**), 13-epi-5,15-rosadien-3α,11β-diol (**103**) and lambertic acid (**82**) were reported from the root and twigs of *P. obtusifolia*.[25] 6α,11,12,16-Tetrahydroxy-7-oxo-abieta-8,11,13-triene (**78**), 11,12-dihydroxy-6,8,11,13-icetexatetraen-1-one (**116**), 1β,3α,8β-trihydroxypimara-15-ene (**99**), 2β,19-dihydroxypimara-7,15-diene (**100**) were obtained from the root bark of *P. integrifolia*.[26] Obtusinone D (**122**) and obtusinone E (**123**) are dimeric icetexane diterpenoids obtained from the root extracts of *P. obtusifolia*.[27] Sirutekkone (**110**) was reported from *P. herbacea*.[28] Latifolionol (**121**) was obtained from *P. latifolia* Roxb.[29] 5,6,10-Trihydroxy-7-isopropyl-1,1,4a-trimethyl-2,3,4,4a-tetrahydro phenanthren-9(1*H*)-one (**89**), was reported from the roots of *P. obtusifolia*.[30] 7α,12-Dihydroxy-8,12-abietadiene-11,14-dione (**87**) was obtained from the roots of *P. obtusifolia*.[31] 11,12,16-Trihydroxy-2-oxo-5-methyl-10-demethyl-abieta-1(10),6,8,11,13-pentene (**94**) was obtained from the methanolic extract of the leaves, root bark and root wood of *P. serratifolia*.[32] 13-Formyl-11,14-dihydroxypodocarpa-8,11,13-triene (**97**), sandaracopimar-15-en-1α,8β-diol (**104**), sandaracopimar-15-en-1β,8α,12β-triol (**105**) and 6α,11,14,16-tetra-*O*-acetylabieta-8,1,13-trien-7-one (**79**) were reported from the *P. latifolia* Roxb.[33] 8,11,13-Icetexatriene-10,11-12,16-tetrol (**111**), 8,11,13-icetexatriene-10,11,16-triol (**112**), 8,11,13-icetexatriene-7,10,11,16-tetrol (**113**) and 7,10-epoxy-8,11,13-icetexatriene-11,12,16-triol (**114**) were obtained from the stem bark of *P. tomentosa*.[34] Premnalatifolin A (**124**) was obtained from *P. latifolia*.[35]

Figure 3: Structures of diterpenes and dimerized diterpenes isolated from genus *Premna*.

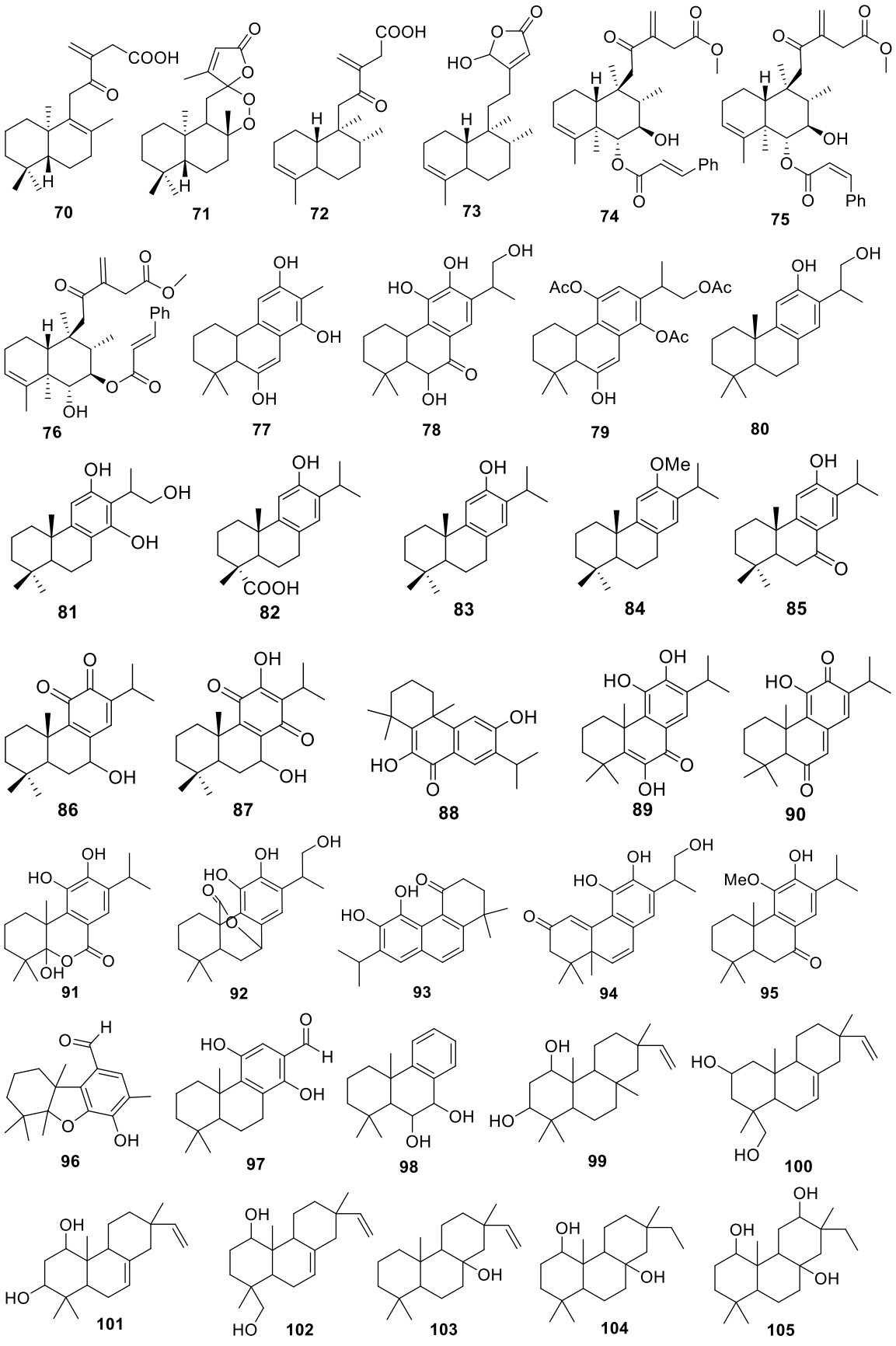
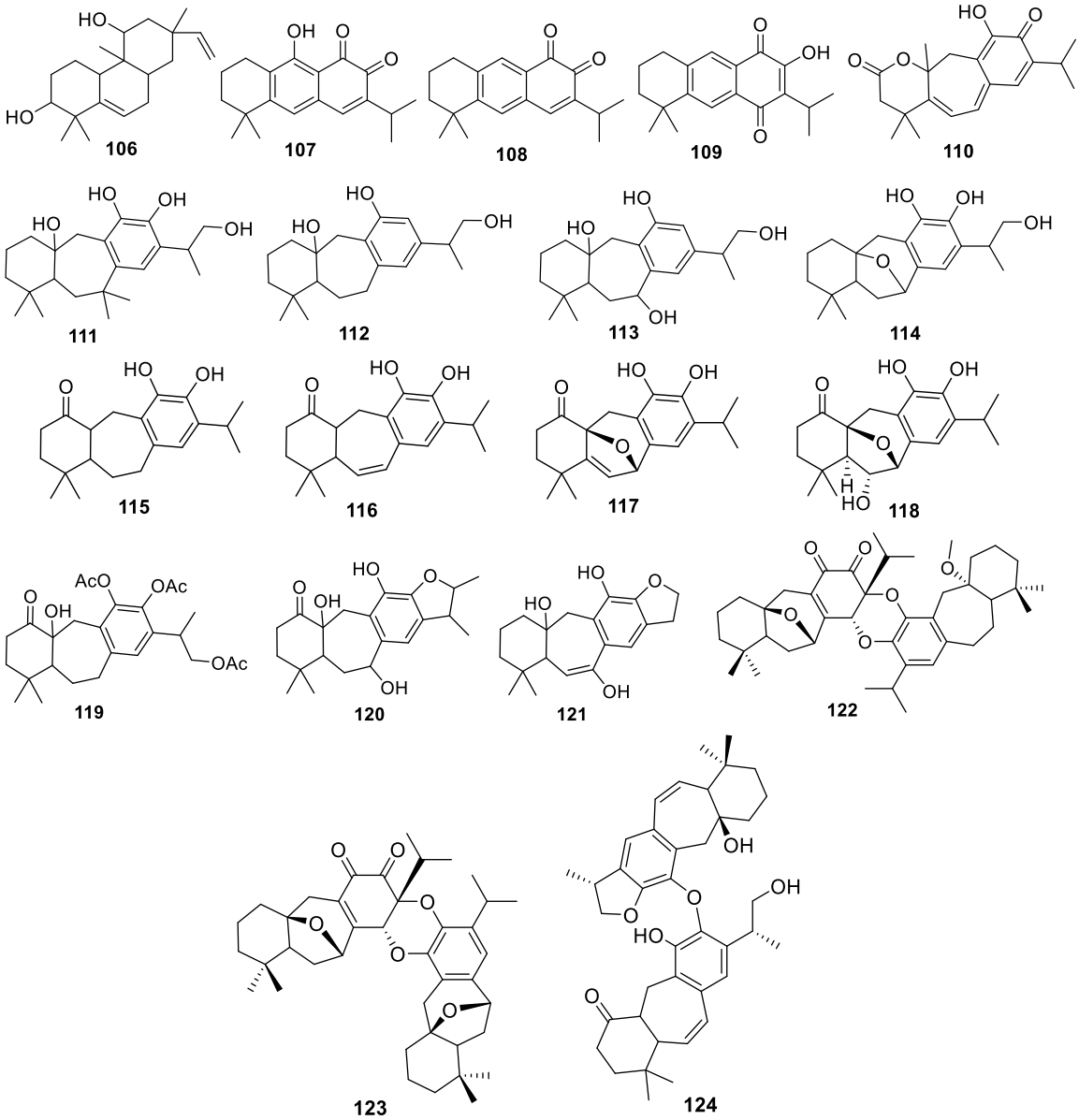
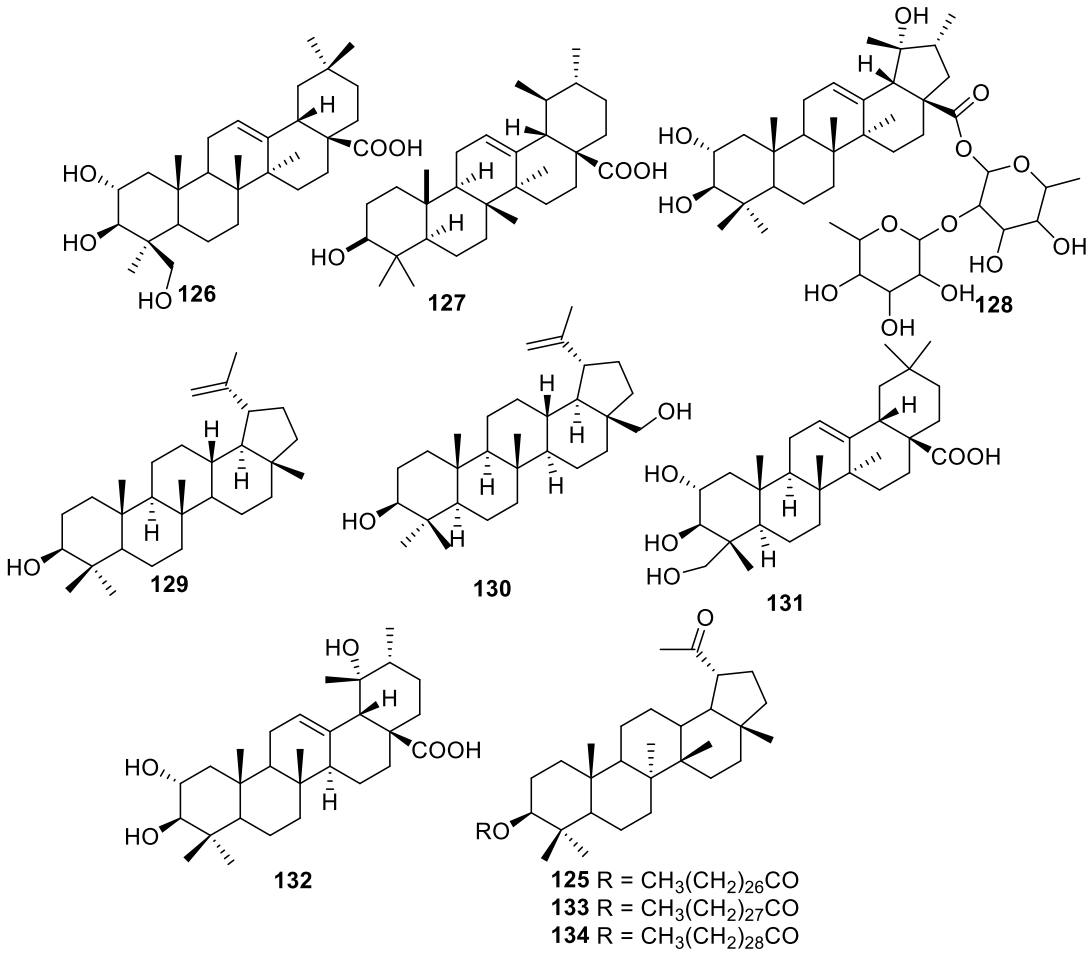


Figure 3 continued



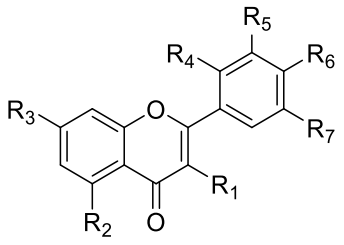
Some higher terpenes (triterpenes) were reported from the genus *premna* (Figure 4). For example, lupeol (**129**) and betulin (**130**) were obtained from the root of *P. tomentosa*.[36] *P. foetida* afforded ursolic acid (**127**).[37] Arjunolic acid (**131**) was obtained from the leaves of *P. microphylla*.[38] Tormentic acid (**132**) was reported from the leaves of *P. microphylla* Turcz.[19] Hyptatic acid (**126**) and 28-*O*-α-L-rhamnopyranosyl(1→2)-β-D-glucopyranoside tormentic acid ester (**128**) were reported from the leaves of *P. microphylla*.[39] Lupeo1 octacosanoate (**125**), lupeol noraacosanoate (**133**) and lupeol melissate (**134**) were reported from the stems of *P. fulva* Craib.[40]

Figure 4: Structures of some triterpenes isolated from the genus *Premna*.



Albadawi *et al*.[41] isolated and characterized five flavonoids (**135–139**) from the aerial parts of *P. resinosa* (Hochst.). Figure 5 shows the structures of these compounds.

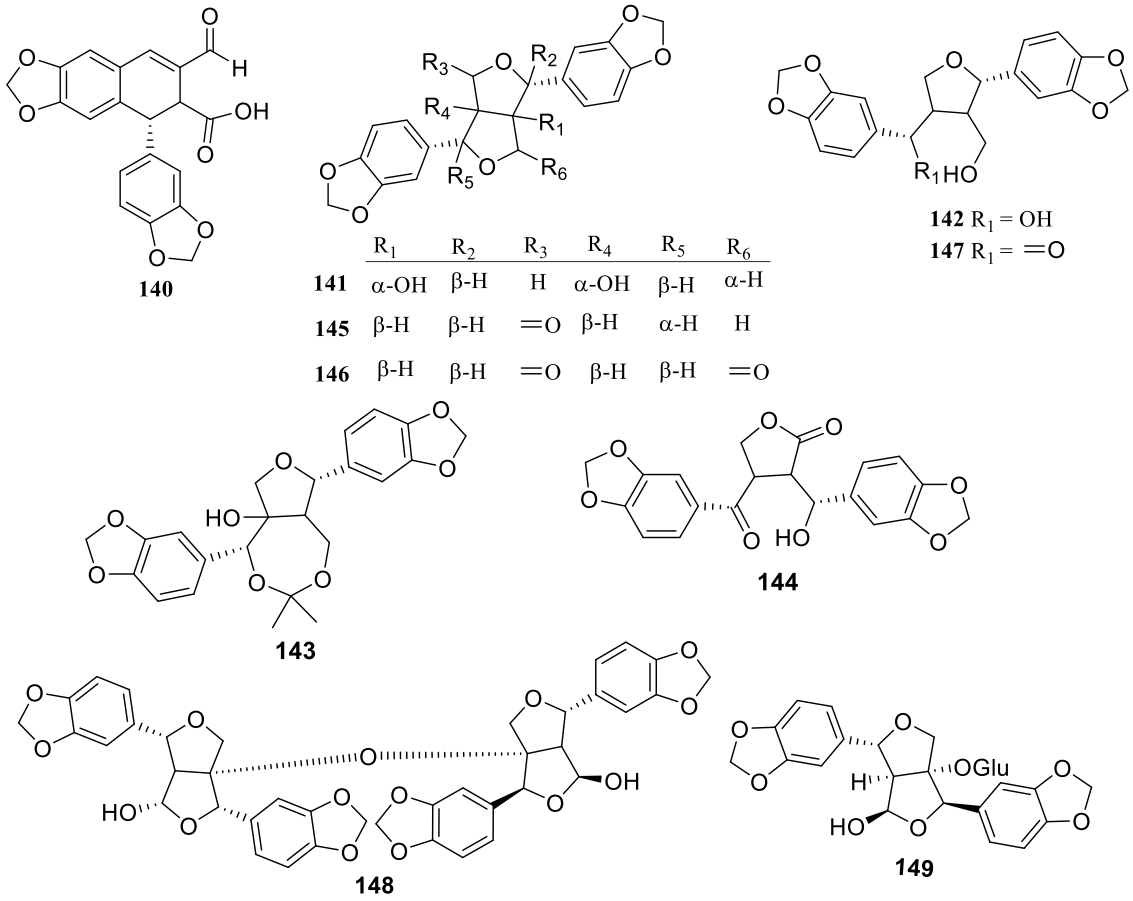
Figure 5: Structures of flavonoids reported from *Premna resinosa*.



	R_1	R_2	R_3	R_4	R_5	R_6	R_7
135	OH	OH	OH	H	OH	OH	H
136	OMe	OH	OH	H	OH	OH	H
137	OH	OH	OH	H	H	OH	H
138	OMe	OH	OH	H	H	OH	H
139	OMe	OH	OMe	H	OMe	OH	OH

Figure 6 shows the structures of lignans obtained from *Premna* species. Woo *et al*.[42] isolated and characterized eight lignans, namely premnan A (**140**), premnan B (**141**), tauntangyiol C (**142**), premnan C (**143**), and the lignoids (**144 – 147)** from the wood of *P. serratifolia*, a traditional cosmetic plant in Myanmar. Yadev *et al*.[43] reported the furofuran lignans premnadimer (**148**) and 4β-hydroxyasarinin-1-*O*-β-glucopyranoside (**149**) from the stem bark of *P. integrifolia*.

Figure 6: Structure of lignans isolated from *Premna* species.



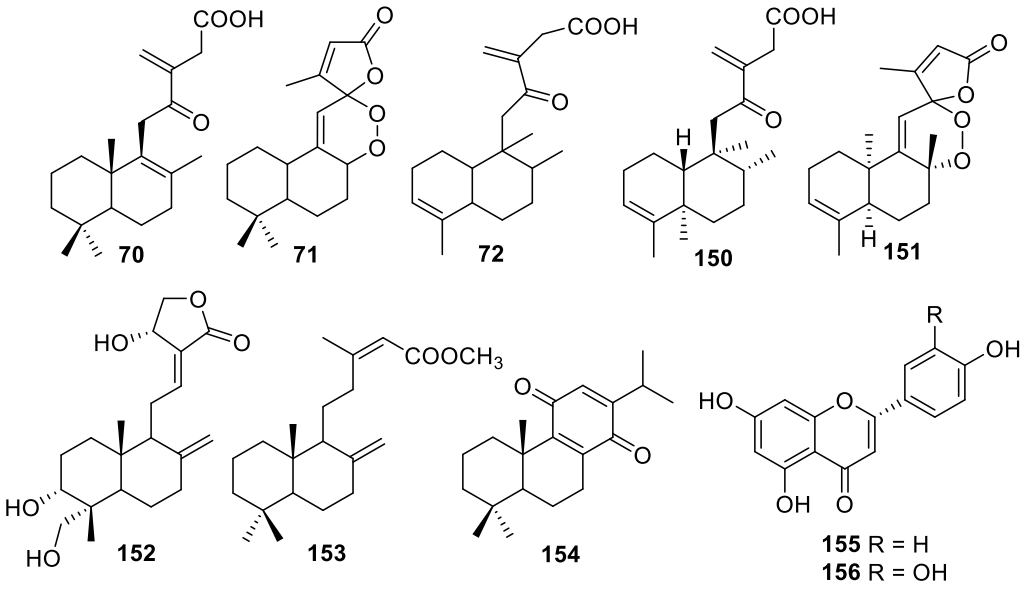
	R_1	R_2	R_3	R_4	R_5	R_6
141	α-OH	β-H	H	α-OH	β-H	α-H
145	β-H	β-H	=O	β-H	α-H	H
146	β-H	β-H	=O	β-H	β-H	=O

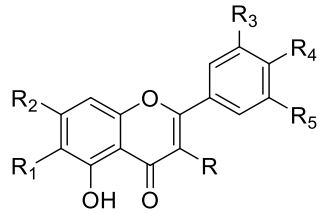
1.4. Phytochemical studies on *Premna* species from Ethiopia.

There are literature reports on the phytochemical investigation of *P. oligotricha*, *P. resinosa* and *P. schimperi* growing in Ethiopia.[44] The studies revealed the presence of diterpenoids, flavanones and flavanols.[44, 45] The phytochemical studies of some reported compounds showed biological activities including anti-oxidant, anti-bacterial, anti-inflammatory, cytotoxicity, anti-cancer and hepatoprotective.[45, 46] Figure 7 shows the structures of diterpenes and flavonoids reported from *P. schimperi* Engl., *P. resinosa* and *P. oligotricha.*

Thus, 16-hydroxy-clerod-3,13(14)-diene-15,16-olide (**70**), *ent*-8β,12α-epidioxy-12β-hydroxylabda-9(11),13-dien-15-oic acid γ-lactone (**71**),1 (5R,8R,9S,10R)-12-oxo-*ent*-3,13(16)-clerodien-15-oic acid (**72**), *ent*-12-oxolabda-8,13(16)-dien-15-oic acid (**73**) were reported from *P. oligotricha*, while andrographolide (**152**), luteolin (**159**), quercetin (**159**), kaempferide (**161**), *ent*-12-oxolabda-8,13(16)-dien-15-oic acid (**73**) were reported from *P. schimperi*, and 3,5,5'-trihydroxy-6,7,3',4'-tetramethoxyflavone (**157**), 3,5,7,5'-tetrahydroxy-6,3',4'-trihydroxyflavone (**158**), and diterpene (**149**) were reported from *P. oligotricha*, while naringenin (**155**), eriodictyol (**156**), pachypodol (**162**), chrysosplenol-D (**163**) and flavonoids (**135-139**) were obtained from *P. resinosa*.[22,23,41,44]

Figure 7: Structures of diterpenes and flavonoids reported from *Premna schimperi*, *Premna resinosa* and *Premna oligotricha.*





	R	R_1	R_2	R_3	R_4	R_5
157	OH	OMe	OMe	OMe	OMe	OH
158	OH	OMe	OH	OMe	OMe	OH
159	H	H	OH	OH	OH	H
160	OH	H	OH	OH	OH	H
161	OH	H	OH	H	OMe	H
162	OMe	OMe	OH	OMe	OH	H
163	OMe	OMe	OMe	OH	OH	H

2. Objectives of the work

2.1. General objective

The general objective of this research has been the isolation and characterization of secondary metabolites from the leaves of *Premna schimperi* Engl.

2.2. Specific objectives

The specific objectives of this work have been:

- GC-MS analysis of the essential oil obtained from the leaves of *P. schimperi* by hydrodistillation.
- Solvent extraction of the dried leaves of *P. schimperi*.
- Isolation of compounds from the solvent extracts of the leaves of *P. schimperi* by using silica gel column chromatography and preparative TLC.
- Characterization of the isolated compounds by using spectroscopic techniques such as NMR, UV-vis and IR.

3. Results and Discussion

In the course of this study, attempts were made to isolate and characterize secondary metabolites from the solvent extracts of the leaves of *P. schimperi* Engl. Thus, six compounds (Figure 8), namely; *ent*-8β,12α-epidioxy-12β-hydroxylabda-9(11),13-dien-15-oic acid γ-lactone (**71**), 3,5,5′-trihydroxy-6,7,3′,4′-tetramethoxyflavone (**157**), 3,5,7,5′-tetrahydroxy-6,3′,4′ trimethoxyflavone (**158**), straight chain primary alcohol **165**, luteolin (**159**) and *p*-hydroxycinnamic acid/*p*-coumaric acid (**164**) were isolated and characterized. The dried leaves were also hydrodistilled to extract the essential oil which was subjected to GC-MS analysis. The major constituents of the essential oil (Figurev 9) were caryophyllene (**9**), caryophyllene oxide (**10**), terpinen-4-ol (**33**), α-curcumene (**166**), eugenol (**167**), β-sesquiphellandrene (**168**), γ-gurjunene (**169**), 2-isopropyl-5-methyl-9-methylene-bicyclo[4.4.0]dec-1-ene (**170**), γ-eudesmol (**171**).

Figure 8: Structures of compounds isolated from the solvent extracts of the leaves of *Premna schimperi*.

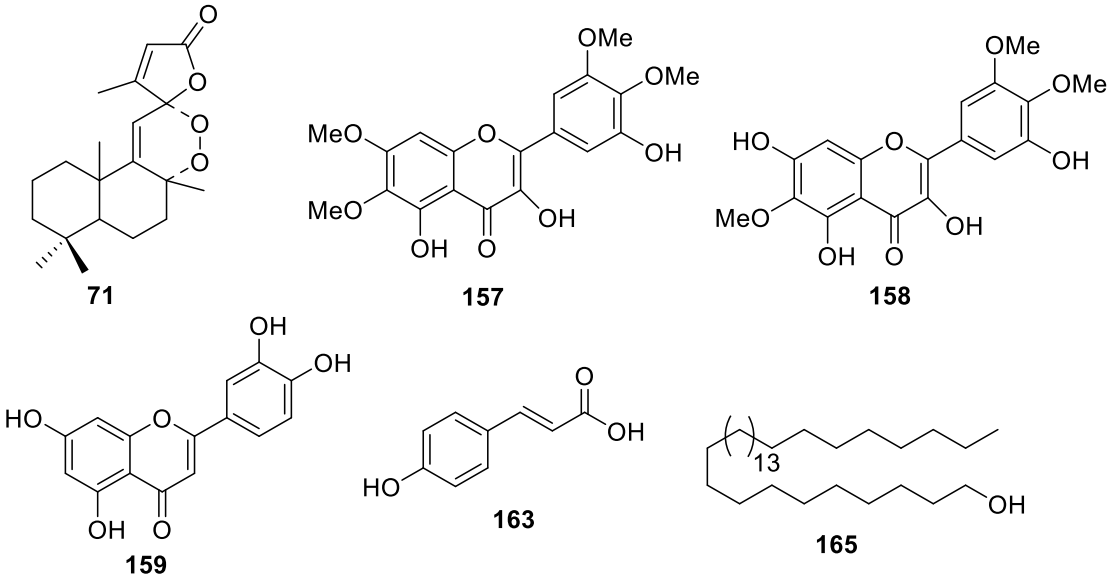
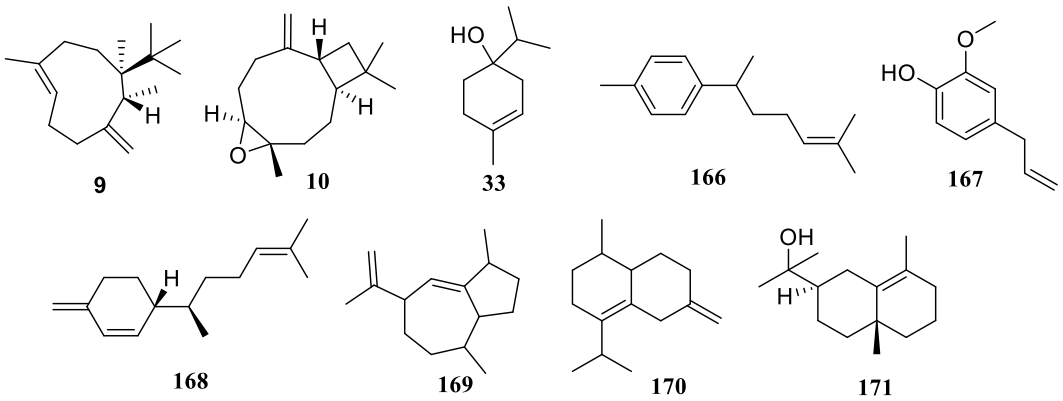


Figure 9: Structures of the major constituents the essential oil of the leaves of *Premna schimperi*.



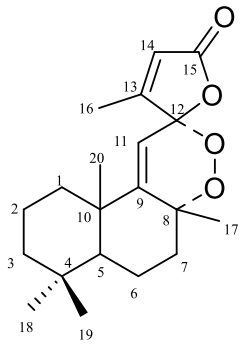
3.1. Phytochemical studies on *Premna schimperi.*

The leaves of *P. schimperi* Engl. were collected from Yabelo city, Borena Zone, Oromia regional state, Ethiopia in May 2019. The plant specimen (voucher specimen No. M002) was identified and deposited at the National Herbarium, College of Natural and Computational Sciences, Addis Ababa University. The extracts of the plant were phytochemically studied as described in the following sections.

3.2. Extraction, fractionation and isolation of compounds from the leaves of *Premna schimperi.*

The ground dried leaves of *P. schimperi* were first extracted with petroleum ether and then with chloroform and finally with methanol. The methanol extract was then fractionated between water and ethyl acetate. All solvent extracts were subjected to column chromatography over silica gel and fractionated as described in the Experimental section. The chloroform extract afforded compounds **71**, **157** and **158**, while compound **165** was isolated from the petroleum ether extract. Compounds **159** and **164** were isolated from the ethyl acetate portion of methanol extract.

3.2.1. Characterization of compound 71.



71

Compound **71** was obtained as white crystals (m. p. 214-215^{o}C) from the chloroform extract of the dried leaves of *P. schimperi* as described in Section 4.1. The IR spectrum (Appendix 1) of **71** showed a very strong absorption band at 1764 cm^{-1} suggesting the presence of an α,β-unsaturated lactone while the weak absorption band at 1659 cm^{-1} suggested the presence of a double bond. In addition, the weak bands at 1239 and 1241 cm^{-1} can be attributed to C-O bending vibrations while the weak bands at 1457 and 1438 cm^{-1} can be due to C-H bending vibrations.

The ^{1}H-NMR spectrum (Appendix 2) of compound **71** showed four singlets at δ 0.91, 0.94 1.23 and 1.69 attributable to H-18, H-19, H-20 and H-17, respectively, and a doublet at δ 1.98 (3H, *J* = 2.0 Hz) which was assigned to H-16. The downfield signal at δ 5.23 (1H, *s*) is due to the olefinic methine proton at C-11 while the most downfield quartet at δ 5.98 (*J* = 2.0 Hz) can be assigned to the olefinic methine proton (H-14) α-to the lactone carbonyl which exhibited allylic coupling to the methyl protons at C-16. In addition, the ^{1}H-NMR spectrum, coupled with the HSQC spectrum (Appendix 5), revealed the presence of five methyl, five aliphatic methylene and three methine groups as shown in Table 1.

The ^{13}C-NMR spectrum (Table 2 and Appendix 3) together with the DEPT-135 spectrum (Appendix 4) displayed 20 carbon resonances corresponding to five methyl groups (δ 13.08, 21.24, 25.38, 26.37 and 32.99), five methylene groups (δ 16.56, 19.04, 26.39, 40.39 and 41.87), three methine groups (δ 40.39, 111.24, 121.05) and seven quaternary carbon atoms (δ 33.60, 38.47, 78.94, 107.19, 162.43, 163.13 and 169.52). The downfield aliphatic quaternary carbon resonance at δ 78.94 can be assigned to the

oxygenated carbon atom C-8. The signals at δ 111.24, 121.05, 162.43 and 163.13 indicated the presence of two double bonds. The most downfield quaternary carbon signal at δ 169.52 indicated the presence of an α,β-unsaturated carbonyl carbon of a lactone moiety. The presence of a ketal in the structure was evident from the quaternary carbon resonance at δ 107.19 which can be assigned to C-12.

Table 1: The ^{1}H-NMR data (δ_{ppm}) of compound **71**.

71 ($CDCl_3$, 400 MHz)	Lit.[22] ($CDCl_3$, 400 MHz)
1.20 (1H, *m*, H-1)	1.26 (1H, *ddd*, H-1)
1.85 (1H, *m*, H-1)	1.85 (1H, *m*, H-1)
1.50-1.60 (2H, *m*, H-2)	1.50-1.60 (2H, *m*, H-2)
1.18 (1H, *m*, H-3)	1.22 (1H, *ddd*, H-3)
1.48 (1H, *m*, H-3)	1.50 *m* (1H, *m*, H-3)
1.57 (1H, *m*, H-5)	1.57 (1H, *m*, H-5)
1.50-1.60 (2H, *m*, H-6)	1.50-1.60 (2H, *m*, H-6)
1.65 (2H, *m*, H-7)	1.65 (2H, *m*, H-7)
5.23 (1H, *s*, H-11)	5.23 (1H, *s*, H-11)
5.98 (1H, *q*, *J* = 2.0, H-14)	5.98 (1H, *q*, *J* = 2.0, H-14)
1.98 (3H, *d*, *J* = 2.0, H-16)	2.01 (3H, *d*, *J* = 2.0, H-16)
1.69 (3H, *s*, H-17)	1.70 (3H, *s*, H-17)
0.91 (3H, *s*, H-18)	0.91 (3H, *s*, H-18)
0.94 (3H, *s*, H-19)	0.94 (3H, *s*, H-19)
1.23 (3H, *s*, H-20)	1.23 (3H, *s*, H-20)

Table 2: The ^{13}C-NMR data (δ_{ppm}) of compound **71**.

C	**71** ($CDCl_3$, 100MHz)	Lit.[22] ($CDCl_3$, 100MHz)
1	40.39	403
2	19.04	19.0
3	41.87	41.8
4	33.60	33.5
5	43.69	43.6
6	16.56	16.5
7	26.39	26.3
8	78.94	78.1
9	163.13	163.1
10	38.47	38.4
11	111.24	111.2
12	107.19	107.1
13	162.43	162.4
14	121.05	121.0
15	169.52	169.5
16	13.08	13.0
17	25.38	25.3
18	32.99	32.9
19	21.24	21.2
20	25.66	25.6

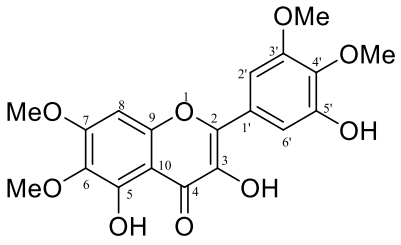
The HMBC spectrum (Table 3 and Appendix 6) showed the correlations of H-18 (δ 0.91) to C-19 (δ 21.24), C-4 (δ 33.60), C-3 (δ 41.87) and C-5 (δ 43.69) and H-19 (δ 0.94) to C-18 (δ 32.99), C-4 (δ 33.60), C-3 (δ 41.87) and C-5 (δ 43.69) which indicated that C-18 and C-19 are attached to C-4 (δ 33.60). The correlations of H-20 (δ 1.23) to C-10 (δ 38.47), C-1 (δ 40.39), C-5 (δ 43.69) and C-9 (δ 163.13) suggest that C-20 is attached to C-10 (δ 38.47). The correlations of H-17 (δ 1.69) to C-7 (δ 26.39), C-8 (δ 78.94) and C-9 (δ 163.13) were used to confirm that C-17 is attached to C-8 (δ 78.94). The correlations of H-16 (δ 1.98) to C-12 (δ 107.19), C-14 (δ 121.05) and C-13 (δ 162.43) suggest that C-16 is attached to C-13 (δ 162.43). In addition, the HMBC correlations of H-11 (δ 5.23) to C-20 (δ 25.66), C-10 (δ 38.47), C-8 (δ 78.94), C-12 (δ 107.19), C-13 (δ 162.43) and C-9 (δ 163.13) and H-14 (δ 5.98) to C-16 (δ 13.08), C-12 (δ 107.19), C-13 (δ 162.43) and C-15 (δ 169.52) were used to establish the structure of compound **71**.

Table 3: The HMBC data of compound **71**.

	^{13}C (δ_{ppm})	
	^{3}J	^{2}J
H-11	25.6, 38.47, 78.94	107.19, 163.13
H-14	13.08, 107.19	162.43, 169.52
H-16	107.19, 121.05	162.43
H-17	26.39, 163.13	78.94
H-18	21.24, 41.87, 43.69	33.60
H-19	32.99, 41.87, 43.69	33.60
H-20	40.39, 43.69, 163.13	38.47

The spectroscopic data of **71** allowed for the identification of the compound as *ent*-8β,12α-epidioxy-12β-hydroxylabda-9(11),13-dien-15-oic acid γ-lactone, a compound which was previously isolated from Ethiopian *P. oligotricha* species *by* Solomon Habtemariam.[22] Comparison of the ^{1}H- and ^{13}C-NMR spectral data of compound **71** with those reported in the literature for *ent*-8β,12α-epidioxy-12β-hydroxylabda-9(11),13-dien-15-oic acid γ-lactone revealed a very close agreement (Tables 1 and 2). This is the first report of compound **71** from *P. schimperi* Engl.

3.2.2. Characterization of compound 157.



157

Compound **157** was isolated as a yellowish solid from the chloroform extract of the leaves of *P. schimperi* as described in Section 4.9.3. The UV spectrum (MeOH) (Appendix 7) showed absorption maxima at 255, 267, 340 and 358 nm, which indicated a flavone moiety.[47] The IR spectrum (Appendix 8) revealed absorption bands at 3537, 3436 and 3256 cm^{-1} indicating the presence of hydroxyl groups. The bands at 2993 and 2922 cm^{-1} are due to C-H stretching vibrations. The strong absorption band at 1655 cm^{-1} indicated the presence of an α,β-unsaturated carbonyl group and the band at 1587 cm^{-1} indicated the presence of double bond. The bands at 1493, 1465 and 1434 cm^{-1} are due to C-H bending vibrations and the bands at 1215 and 1279 cm^{-1} can be attributed to C-O bending vibrations.

Table 4: The ^{1}H-NMR data (δ_{ppm}) of compound **157**.

157 ($CDCl_3$, 400 MHz)	Lit.[44] (C_5D_5N, 400 MHz)
6.55 (1H, *s*, H-8)	6.80 (1H, *s*, H-8)
7.45 (1H, *d*, *J* = 2.0 Hz, H-2′)	8.26 (1H, *d*, *J* = 2.0 Hz, H-2′)
7.50 (1H, *d*, *J* = 2.0 Hz, H-6′)	7.85 (1H, *d*, *J* = 2.0 Hz, H-6′)
4.01 (3H, *s*, OMe-6)	3.99 (3H, *s*, OMe-6)
3.99 (3H, *s*, OMe-7)	3.82 (3H, *s* OMe-7,)
3.95 (3H, *s*, OMe-3′)	3.88 (3H, *s* OMe-3,)
3.98 (3H, *s*, OMe-4′)	3.99 (3H, *s*, OMe-4′)
5.96 (1H, *s*, OH-5′)	---
6.76 (1H, *s*, OH-3)	---
11.63 (3H, *s*, OH-5)	13,12 (1H, *s*, OH-5)

The ^{1}H-NMR spectrum (Table 4 and Appendix 9) of compound **157** showed an ABX spin system[48] in the aromatic region and four singlets at δ 3.95, 3.98, 3.99 and 4.01, which can be assigned to the methoxy groups at C-3′, C-4, C-7 and C-6, respectively. The one-proton singlets at δ 5.96 (1H, *s*) and 6.76 (1H, *s*) are attributed to the hydroxyl groups at C-5′ and C-3 respectively, and the most downfield broad singlet at δ 11.63 (1H) is assignable to the chelated hydroxy group at C-5. The singlet at δ 6.55 can be assigned to H-8 of the 5,6,7-trisubstituted ring-A. The *meta*-coupled doublets (*J* = 2.0

Hz) at δ 7.45 and 7.50 are attributable to H-2′ and H-6′, respectively, of the 3′,4′,5′-trisubstituted ring-B.

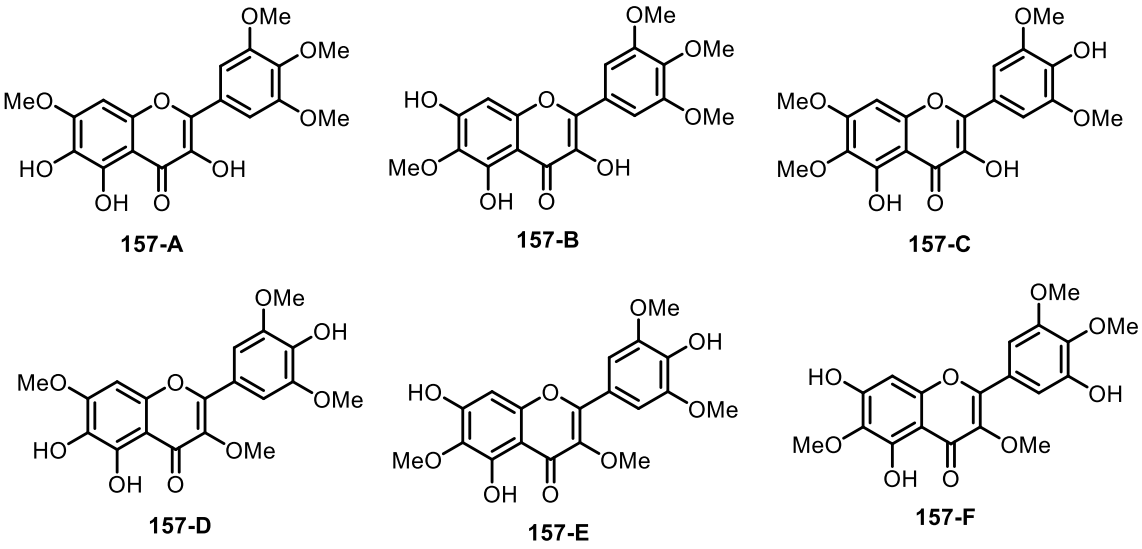
The ^{13}C-NMR spectrum (Appendix 10) of **157** revealed 19 carbon signals of which twelve are due to quaternary carbon atoms, three are due to aromatic methine carbon atoms and four are due to methyl groups as evidenced by the DEPT-135 (Appendix 11) and HSQC (Appendix 12) spectra. The signals at δ 56.08, 56.41, 60.95 and 61.12 revealed the presence of four methoxy groups. It is worth noting that the carbon shifts of two of the methoxy groups appeared below δ 60.0 (56.08 and 56.41), whereas, the signals due to the other two methoxy groups appeared above δ 60.0 (60.95 and 61.12) suggesting that only one *ortho* position of the two former methoxy-containing carbons is substituted, while both of the *ortho* positions of the latter two methoxy-containing carbons are substituted.[49] Thus, the two former methoxy groups must be attached to C-7 (δ 159.42) and C-3′ (δ 152.45), but not at C-6 and C-4′ while the latter two methoxy groups must be attached to C-6 (δ 132.23) and C-4′ (δ 137.30), but not at C-7 and C-3′. The remaining fifteen carbon resonances at δ 90.75 (C-8), 104.38 (C-10), 104.51 (C-2′), 107.55 (C-6′), 126.31 (C-1′), 132.23 (C-6), 136.23 (C-3), 137.30 (C-4′), 144.96 (C-2), 149.26 (C-5′), 151.37 (C-5), 152.19 (C-9), 152.45 (C-3′), 159.42 (C-7) and 175.40 (C-4) indicated that **157** is a trihydroxytetramethoxyflavone.

Table 5: The ^{13}C-NMR data (δ_{ppm}) of compound **157**.

C	**157** ($CDCl_3$, 100 MHz)
2	144.96
3	136.23
4	175.40
5	151.37
6	132.23
7	159.42
8	90.75
9	152.19
10	104.38
1′	126.31
2′	104.51
3′	152.45
4′	137.30
5′	149.26
6′	107.55
6-OMe	60.95
7-OMe	56.41
3′-OMe	56.08
4′-OMe	61.12

Several alternative structures can be drawn for compound **157** based on the ^{1}H- and ^{13}C-NMR data as shown in Figure 10. However, structure **157** is most plausible based on the arguments mentioned below.

Figure 10: Alternative structures of compound **157.**

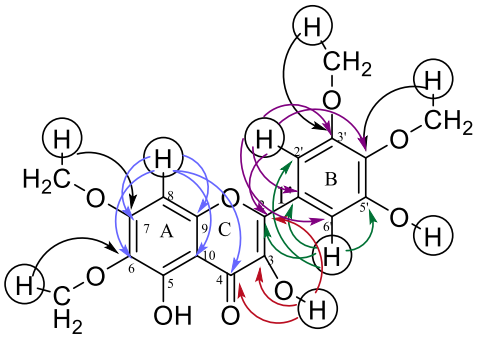


The HMBC spectrum (Table 6, Figure 11 and Appendix 13) showed correlations of H-8 (δ 6.55) to the carbon resonances at δ 159.42 and 132.23, and the correlations of these two carbons to the two methoxy resonances at δ 3.99 and 4.01. Such correlations can occur only if the methoxy groups are placed at C-6 and C-7. In addition, the correlations of H-2′ (δ 7.45) to the carbon resonances at δ 137.30 and 152.45, and the correlations of these two carbons to the two remaining methoxy groups at δ 3.98, and 3.95 can occur only if the methoxy groups are placed at C-3′ and C-4′. Thus, it can be concluded that there are no methoxy groups at C-3 and C5′. The HMBC correlations of C-3 OH (δ 6.76) to C-3 (δ 136.23), C-2 (δ 144.96) and C-4 (δ 175.40) indicates that this OH group is directly attached to C-3. Figure 11 summarizes all HMBC correlations observed.

Table 6: The HMBC data of compound **157**.

	^{13}C (δ_{ppm})	
	^{3}J	^{2}J
C-6 OMe	132.23	
C-7 OMe	159.42	
C-3′ OMe	152.45	
C-4′ OMe	137.30	
H-8	104.38, 132.23	152.45, 159.42
H-2′	137.23, 144.96, 107.55	126.31, 152.45
H-6′	144.96, 104.51	126.31, 149.26
C-3 OH	144.96, 175.40	136.23

Figure 11: The HMBC correlations of compound **157.**



Compound **157** was acetylated as described in Section 3.2.3 to observe the acetylation shifts of the hydroxylated and other carbon atoms. It is known that acetylation of free hydroxyl groups produces marked changes in the ^{13}C-NMR shifts of flavonoids and this can be used to detect the positions of hydroxyl groups on the aromatic ring. After acetylation of compound **157**, the signals of C-5, C-5′ and C-3 were shifted upfield by 9.31, 5.29 and 2.88 ppm, respectively, which indicated that C-5, C-5′ and C-3 are the hydroxyl-bearing carbon atoms of compound **157**. The signals of C-6′, C-6, C-10 and C-2 were moved downfield by 8.25, 7.63, 6.92 and 8.81 ppm, respectively, which suggested that C-6′, C-6, C-10 and C-2 are *ortho* to the hydroxyl-bearing carbon atoms. The signals due to C-8, C-9 and C-2′ were moved downfield by 7.43, 1.20 and 5.55 ppm, respectively, indicating that C-8, C-9 and C-2′ are *para*-to the hydroxyl-bearing carbon atoms. The signals due to C-1′, C-7, C-9, and C-3′ showed slight changes from δ 126.31 to 124.66, 159.42 to 158.11, 152.19 to 153.39 and 152.45 to 153.77, respectively, suggesting that C-1′, C-7, C-9, and C-3′ are *meta*-to the hydroxyl-bearing carbon atoms.[50]

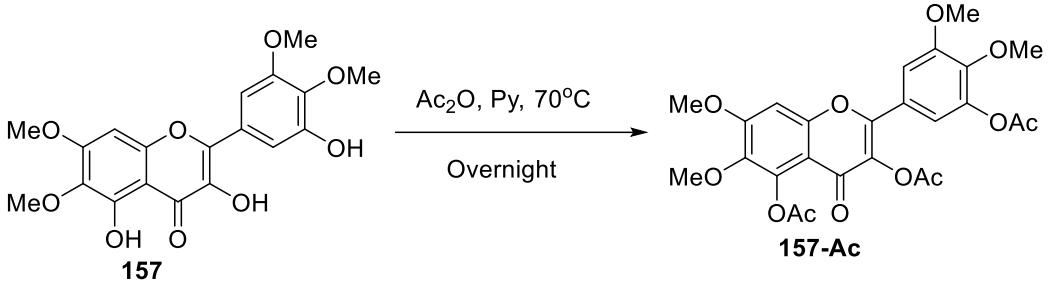
The above spectroscopic data in conjunction with comparison of the data with those reported in the literature allowed for the identification of compound as: 3,5,5'-trihydroxy-6,7,3',4'-tetramethoxyflavone **157**. This is the first report of compound **157** from *P. schimperi* Engl.

3.2.3. Characterization of compound 157-Ac.

The peracetylation of **157** was achieved by reaction with acetic anhydride and pyridine at 70^{o}C (Scheme 1). The UV spectrum (Appendix 14) of compound **157-Ac** revealed absorption maxima at 262 and 318 nm, as is characteristic of a flavone skeleton.[47] The

IR spectrum (Appendix 15) showed a C-H stretching band at 2937 cm^{-1}. The spectrum also showed bands at 1776 and 1648 cm^{-1} due to ester carbonyl groups and an α,β-unsaturated carbonyl group respectively. The band at 1461 cm^{-1} is due to C-H bending, while the band at 1200 cm^{-1} corresponds to C-O bending vibration.

Scheme 1: Synthesis of compound **157-Ac**.



The ^{1}H-NMR spectrum (Table 7 and Appendix 16) of **157-Ac** revealed three-proton singlets at δ 2.35, 2.38 and δ 2.50 due to the methyl protons of the three phenolic acetyl groups. The singlets at δ 3.88, 3.94, 3.95 and 4.00 are attributable to the methoxy groups at C-6, C-3′, C-4′ and C-7, respectively. The singlet at δ 6.89 is due to H-8 of the 5,6,7-trisubstituted ring-A, while the broad singlets at δ 7.18 and 7.28 are assignable to H-6′ and H-2′, respectively, of the 3′,4′5′-trisubstituted ring-B. The ^{13}C-NMR spectrum (Table 8 and Appendix 17) of **157-Ac** coupled with the DEPT-135 spectrum (Appendix 18) revealed twenty-five carbon resonances corresponding to three methyl carbons (δ 20.59, 20.73 and 20.99) of the phenolic acetyl groups, four methoxy groups (δ 56.34, 56.55, 60.90 and 61.58) three methine carbon atoms (δ 98.18, 110.06 and 115.80) and fifteen quaternary carbon resonances (δ 111.30, 124.66, 133.35, 139.86, 142.06, 142.57, 143.57, 143.97, 153.39, 153.77, 158.11, 168.00, 168.83, 169.37 and 170.10), of which four are due to carbonyl carbons and seven are due to oxygenated aromatic carbons. Hence, based on the above spectroscopic data and by comparing these with data reported in the literature,[44] the structure of compound **157-Ac** was confirmed to be 3,5,5′-triacetoxy-6,7,3′,4′-tetramethoxyflavone.

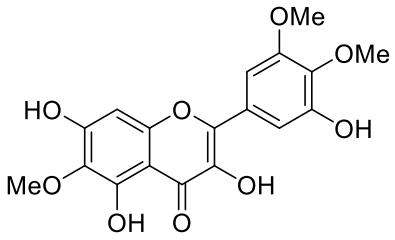
Table 7: The ^{1}H-NMR data (δ_{ppm}) of compound **157-Ac**.

157-Ac ($CDCl_3$, 400MHz)	Lit.[44] ($CDCl_3$, 400MHz)
6.89 (1H, *s*, H-8)	6.88 (1H, *s*, H-8)
7.18 (1H, *s*, H-6′)	7.15 (1H, *d*, H-6′)
7.28 (1H, *s*, H-2′)	7.24 (1H, *d*, H-2′)
3.88 (3H, *s*, OMe-6)	3.89 (3H, *s*, OMe-6)
4.00 (3H, *s*, OMe-7)	3.99 (3H, *s*, OMe-7)
3.94 (3H, *s*, OMe-3′)	3.92 (3H, *s*, OMe-3′)
3.95 (3H, *s*, OMe-4′)	3.91 (3H, *s*, OMe-4′)
2.35 (3H, *s*, 5′-$COCH_3$)	2.32 (3H, *s*, $COCH_3$)
2.38 (3H, *s*, 3-$COCH_3$)	2.36 (3H, *s*, $COCH_3$)
2.50 (3H, *s*, 5-$COCH_3$)	2.37 (3H, *s*, $COCH_3$)

Table 8: The ^{13}C-NMR data (δ_{ppm}) of **157-Ac**.

C	**157-Ac** ($CDCl_3$, 100MHz)	Lit.[44] ($CDCl_3$, 100MHz)
2	153.77	153.7
3	133.35	133.8
4	170.10	170.1
5	142.06	142.0
6	139.86	139.8
7	158.11	158.1
8	98.18	98.1
9	153.39	153.4
10	111.30	111.2
1′	124.66	124.6
2′	110.06	110.0
3′	153.77	153.5
4′	142.57	143.5
5′	143.97	143.9
6′	115.80	115.8
6-OMe	61.58	61.5
7-OMe	56.55	56.5
3′-OMe	56.34	56.2
4′-OMe	60.90	60.9
5-$COCH_3$	169.37, 20.99	169.7, 20.9
3-$COCH_3$	168.38, 20.73	168.8, 20.7
5′-$COCH_3$	168.00, 20.59	168.0, 20.5

3.2.4. Characterization of compound 158.



158

Compound **158** was obtained as a yellowish solid from the chloroform extract as described in Section 4.9.3. The UV spectrum (MeOH) (Appendix 19) showed

absorption maxima at 254, 262, 301,339 and 362 nm, which is characteristic of a flavonoid skeleton.[47]

The IR spectrum of **158** (Appendix 20) showed strong absorption bands at 3469, 3412 and 3332 cm^{-1} indicating the presence of hydroxyl groups. The strong absorption bands at 2994 and 2923 cm^{-1} are due to C-H stretching vibrations and the band at 1656 cm^{-1} indicated the presence of a conjugated carbonyl group. The strong absorption bands at 1493 and 1445 cm^{-1} are due to C-H bending vibrations and the bands at 1200 and 1228 cm^{-1} correspond to C-O bending vibrations.

The ^{1}H-NMR spectrum (Table 9 and Appendix 21) of compound **158** showed three-proton singlets at δ 3.95, 3.98 and 3.89 which can be assigned to the methoxy groups at C-3′, C-4′ and C-6, respectively. The aromatic proton singlet at δ 6.69 is assignable to H-8 of the 5,6,7-trisubstituted ring-A. The aromatic proton singlets at δ 7.55 and 7.54 are attributable to H-2′ and H-6′ of ring-B. The broad singlets at δ 8.18 and 8.22 are due to the hydroxyl groups at C-5′ and C-7, respectively, and the singlet at δ 9.25 is assignable to the hydroxyl group at C-3. The most deshielded singlet at δ 12.27 can be attributed to the strongly chelated OH group at C-5.

Table 9: The ^{1}H-NMR data (δ_{ppm}) of compound **158**.

158 (Acetone-d_6, 400 MHz)	Lit.[44] (C_5D_5N, 400 MHz)
6.69 (1H, *s*, H-8)	6.89 (1H, *s*, H-8)
7.54 (1H, *s*, H-2′)	8.21(1H, *d*, *J* = 2. 0 Hz, H-2′)
7.49 (1H, *s* ,H-6′)	7.84 (*d*, *J* = 2.0 Hz, H-6′)
3.89 (3H, *s*, OMe-6)	3.95 (3H, *s*, OMe-6)
3.95 (3H, *s*, OMe-3′)	3.88 (3H, *s*, OMe-3′)
3.98 (3H, *s*, OMe-4′)	3.97 (3H, *s*, OMe-4′)
8.18 (1H, *s*, OH-5′)	---
8.22 (1H, *bs*, OH-7)	---
9.25 (1H, *bs*, OH-3)	---
12.27 (1H, *s*, OH-5)	13.30 (1H, *s*, OH-5)

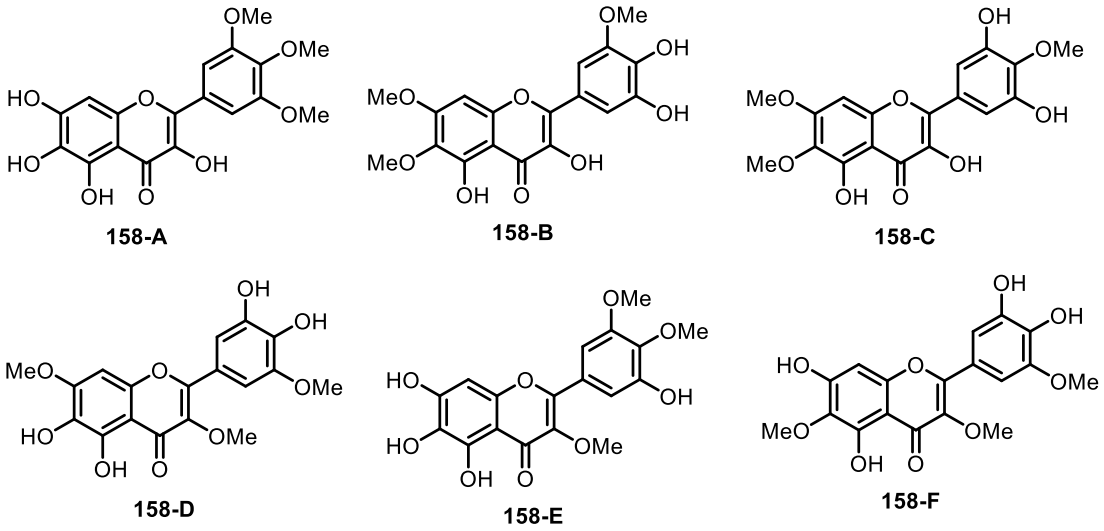
The ^{13}C-NMR spectrum (Table 10 and Appendix 22) together with the DEPT-135 (Appendix 23) and HSQC spectra (Appendix 24) revealed eighteen carbon resonances corresponding to three methoxy groups (δ 55.54 (C-3′), 59.9 (C-6) and 59.95 (C-3′)), three methine carbon atoms (δ 93.8 (C-8), 103.76 (C-2′) and 108.99 (C-6′)), and twelve quaternary carbon atoms of which nine are oxygenated and one is a conjugated carbonyl carbon.

Table 10: The ^{13}C-NMR data (δ_{ppm}) of compound **158**.

C	**158** (Acetone-d_6, 100 MHz)
2	145.45
3	136.35
4	176.18
5	151.64
6	130.87
7	157.21
8	93.80
9	152.26
10	103.79
1	126.45
2	103.76
3	153.11
4	138.26
5	150.40
6	108.99
6-OMe	59.89
3′-OMe	55.54
4′-OMe	59.98

Several alternative structures can be drawn for compound **158** based on the ^{1}H- and ^{13}C-NMR data as shown in Figure 12. However, structure **158** is most plausible based on the arguments presented below. The ^{13}C-NMR spectrum revealed an OMe resonance at δ 55.54 indicating that this methoxy group should be attached to C-3′/C-5′, but not to C-4′. On the other hand, the OMe resonances at δ 59.89 and 59.98 suggest that these two OMe groups should be attached to C-6 and C-4′, but not at C-7 and C-3′.[49]

Figure 12: Alternative structures of compound **158**.



The HMBC spectrum (Figure 13) showed correlations of H-8 (δ 6.69) to the carbon resonance at δ 130.87, and the correlation of this carbon atom to the methoxy resonance

at δ_H 3.89 (δ_C 59.89). This can occur only if the methoxy group is placed at C-6, but not at C-3 or C-7. H-2′ (δ 7.54) revealed correlations to the carbon resonances at δ 153.11 and 138.26. These two carbon signals are also correlated to the two methoxy signals at δ_H 3.95 (δ_C 55.54) and 3.98 (δ_C 59.98). This can occur only if the methoxy groups are placed at C-3′ and C-4′. The OH signal at δ 12.25 showed correlations to C-10 (δ 103.79), C-6 (δ 130.87), and C-5 (δ 151.64) in agreement with its placement at C-5. On the other hand, the signal due to C-5′ OH (δ 8.18) revealed correlations to C-6′ (δ 108.99), C-4′ (δ 138.26) and C-5′ (δ 150.40). In addition, the HMBC correlations of H-8 (δ 6.69) to C-10 (103.79), C-6 (δ 130.87), C-9 (δ 152.26) and C-7 (δ 157.21) and the correlations of H-2′ (δ 7.54) to C-6′ (δ 108.99), C-1′ (δ 126.45), C-4′ (δ 138.26), C-2 (δ 145.45) and C-7 (δ 157.21) were used to deduce **158** as most plausible. Figure 14 and Table 11 summarize the HMBC correlations observed.

Figure 13: The HMBC spectrum of compound **158** in $(CD_3)_2CO$.

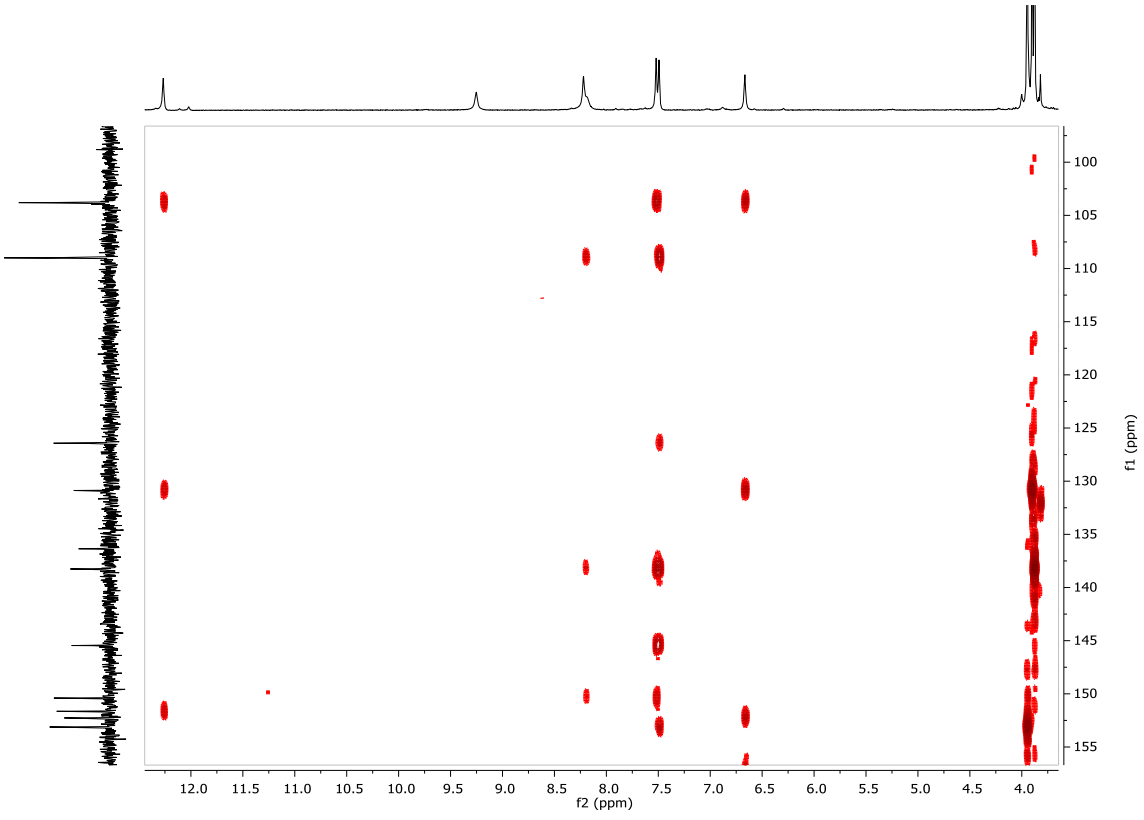


Figure 14: The HMBC correlations of compound **158.**

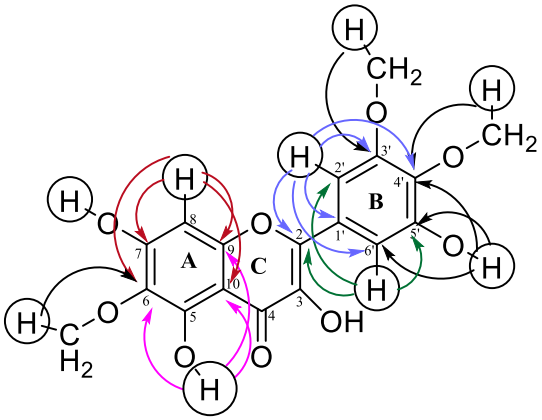
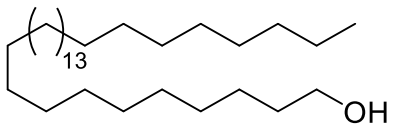


Table 11: The HMBC data of compound **158**.

	^{13}C (δ_{ppm})	
	^{3}J	^{2}J
C-6 OMe	130.87	
C-3′ OMe	153.11	
C-4′ OMe	138.26	
H-8	130.87, 103.79	157.21, 152.26
H-2′	145.45, 138.26, 108.99	153.11, 126.45
H-6′	145.45, 103.76	150.40
C-5 OH	130.87, 103.79	151.64
C-5′ OH	108.99, 138.26	150.40

The above spectroscopic data and comparison of these with those reported in the literature allowed for the identification of compound **158** as 3,5,7,5'-tetrahydroxy-6,3',4'-trimethoxyflavone, previously reported from *P. oligotricha* by S. Habtemariam.[44] This is the first report of compound **158** from *P. schimperi* Engl.

3.2.5. Characterization of compound 165.



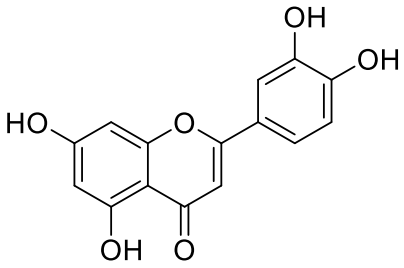
Compound **165** was obtained as white solid from petroleum ether extract of the leaves of *P. schimperi* as described in Section 4.10 and had a melting point of 76.5-78 ºC.

The IR spectrum (Appendix 25) showed a stretching band at 3405 cm^{-1} due to the presence of an OH group. The spectrum also revealed aliphatic C-H stretching bands at 2922 and 2853 cm^{-1}, C-O stretching band at 1644 cm^{-1}, C-H bending bands at 1240 and 1461 cm^{-1} and C-C stretching band at 719 cm^{-1}.

The ^{1}H-NMR spectrum (Appendix 26) showed a triplet at δ 0.80 (3H, *t*, *J* = 8.0 Hz) due to protons of a terminal methyl group. The unresolved broad signal centered at δ 1.30 integrated for 52 protons and suggested the presence of 26 methylene groups. The multiplet at δ 1.60 integrated for four protons and corresponds to two methylene groups. The most deshielded protons signal appeared as a triplet at δ 3.68 (2H, *J* = 8.0 Hz) and suggested the presence of a hydroxymethylene group. It was evident from the ^{1}H-NMR spectrum that compound **165** is a straight-chain alcohol. The molecular formula of $CH_3(CH_2)_{30}OH$ can be deduced for **165** from the ^{1}H-NMR spectrum.

The ^{13}C-NMR spectrum (Appendix 27) of **165** showed signals at δ 14.12, 22.70, 25.74, 29.37, 29.44, 29.62, 29.67, 29.71, 31.93, 32.82 and 63.10. The most downfield signal at δ 63.10 is due to the oxymethylene group while the most upfield carbon resonance at δ 14.1 is due to the terminal methyl group. Although the total number of carbon atoms could not be discerned from the ^{13}C-NMR spectrum due to signal overlapping, structure **165** was tentatively assigned to this compound based on the above spectroscopic data. This is the first report of compound **165** from *P. schimperi*.

3.2.6. Characterization of compound 159.



159

Compound **159** was obtained as yellow solid from the ethyl acetate soluble portion of the methanol extract after crystallization from acetonitrile as described in Section 4.11. The UV spectrum (EtOH) (Appendix 28) showed absorption maxima at 255, 268, 292 and 352 nm, which showed the presence of a flavonoid moiety.[47] The IR spectrum (Appendix 29) showed a strong absorption band at 3426 cm^{-1} indicating the presence of hydroxyl groups. In addition, absorption bands were observed due to C-H stretching (2923 cm^{-1}), carbonyl stretching (1655 cm^{-1}), C-H bending (1455 cm^{-1}) and O-C bending (1265 and 1246 cm^{-1}). The presence of C-C double bonds was also evident from the band at 1615 cm^{-1}.

The ^{1}H-NMR spectrum of **159** (Table 12 and Appendix 30) revealed the presence of six aromatic proton resonances, of which two are *meta*-coupled doublets (J = 2.0 Hz) at δ 6.26 and 6.54 and can be attributable to H-6 and H-8, respectively, of the 5,7-disubstituted ring-A. The singlet at δ 6.60 is assignable to the H-3 on ring-C. The remaining three aromatic proton resonances appeared at δ 7.00 (*d*, J = 2.0 Hz), 7.52 (*dd*, J = 8.4, 2.0 Hz) and 7.48 (*d*, J = 8.4 Hz*)* and were assigned to H-5′, H-2′ and H-6′, respectively, of the 3′,4′-disubstituted ring-B. The most downfield singlet at δ 13.04 is assignable to the strongly chelated OH group at C-5.

Table 12: The ^{1}H-NMR data (δ_{ppm}) of compound **159**.

159 (Acetone-d_6, 400 MHz)	Lit.[51] (DMSO-d6, 400 MHz)
6.26 (1H, *d*, J = 2.0 Hz, H-6)	6.12 (1H, *s*, H-6)
6.54 (1H, *d*, J = 2.0 Hz, H-8)	6.40 (1H, *s*, H-8)
6.60 (1H, *s*, H-3)	6.52 (1H, *s*, H-3)
7.00 (1H, *d*, J = 8.4 Hz, H-5′)	6.87 (1H, *s*, H-5′)
7.48 (1H, *dd*, J = 8.4, 2.0 Hz, H-6′)	7.23 (1H, *s*, H-6′)
7.52 (1H, *d*, J = 2.0 Hz, H-2′)	7.23 (1H, *s*, H-2′)
13.04 (1H, *s*, OH-5)	---

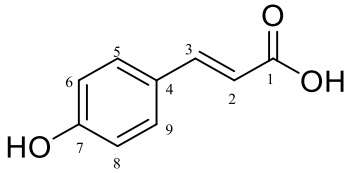
Table 13: The ^{13}C-NMR data (δ_{ppm}) of compound **159**.

C	**159** (Acetone-d_6, 100 MHz)	Lit.[51] (DMSO-d6, 100 MHz)
2	164.25	164.82
3	103.30	102.92
4	182.16	182.07
5	162.48	161.48
6	98.81	99.51
7	164.00	164.44
8	93.79	94.57
9	157.89	57.73
10	104.44	103.85
1′	122.84	121.83
2′	115.75	116.48
3′	145.62	145.88
4′	149.24	150.04
5′	113.23	113.46
6′	119.25	119.59

The ^{13}C-NMR spectrum (Table 13 and Appendix 31) together with the DEPT-135 spectrum (Appendix 32) of **159** revealed fifteen carbon resonances corresponding to six methine groups at δ 93.79 (C-8), 98.81(C-6), 103.30 (C-3), 113.23 (C-5′), 115.75 (C-2′) and 119.25 (C-6′) and nine quaternary carbon resonances at δ 104.44 (C-10), 122.84 (C-1′), 145.62 (C-3′), 146.00 (C-7), 149.24 (C-4′), 157.89 (C-9), 162.48 (C-5),

164.25 (C-2) and 182.16 (C-4). The most shielded carbon resonances at δ 93.79 and 98.81 are attributable to C-8 and C-6 respectively. The most downfield quaternary carbon resonance at δ 182.16 was assigned to the carbonyl carbon at C-4. Based on the above spectroscopic data and in comparison with the data reported in the literature, the structure of **159** was identified as 3′,4′,5,7-tetrahydroxyflavone (Luteolin).[51] Luteolin was previously reported from the leaves of *P. schimperi.*[44]

3.2.7. Characterization of compound 164.



164

Compound **164** was obtained as light-yellow solid (m. p. 210-212 ºC) from the ethyl acetate soluble portion of the methanol extract after crystallization from acetonitrile as described in Section 4.11. The UV spectrum (EtOH) (Appendix 33) showed absorption maxima at 211, 224, 292, 299 and 309 nm. The IR spectrum (Appendix 34) showed absorption bands at 3327 cm^{-1} (OH), 2928 cm^{-1} (C-H), 1672 cm^{-1} (carbonyl), 1601 cm^{-1} (C-C double bond), 1450 cm^{-1} (C-H bending), 1245 and 1212 cm^{-1} (O-C bending).

The ^{1}H-NMR spectrum (Table 14 and Appendix 35) revealed four aromatic proton resonances of a 1,4-disubstituted aromatic ring at δ 6.91 (2H, *d, J* = 8.8 Hz) and 7.56 (2H, *d, J* = 8.8 Hz) due to H-6/H-8 and H-5/H-9, respectively. The presence of a *trans* double bond was evident from the proton resonances at δ 6.35 (1H, *d,* H-2) and 7.63 (1H, *d, J* = 16 Hz, H-3). The chemical shifts of the olefinic proton signals suggest the presence of an α,β-unsaturated carbonyl moiety and the broad signal at δ 9.20 is due to the acidic proton of the carboxylic acid.

Table 14: The ^{1}H-NMR data (δ_{ppm}) of compound **164**.

164 (Acetone-d_6, 400 MHz)	Lit.[52] (CD_3OD, 500 MHz)
6.35 (1H, *d, J* = 16 Hz, H-2)	6.28 (1H, *d, J* = 15.9 Hz, H-2)
6.91 (2H, *d, J* = 8.8 Hz, H-6, H-8)	6.79 (2H, *d, J* = 2.2, *J* = 8.8 Hz, H-6, H-8)
7.56 (2H, *d, J* = 8.8 Hz, H-5, H-9)	7.44 (2H, *d, J* = 8.6 Hz, H-5, H- 9)
7.63 (1H, *d, J* = 16 Hz, H-3)	7.58 (1H, *d, J* = 15.9 Hz, H-3)
9.20 (1H, *broad*, COOH)	---

The ^{13}C-NMR spectrum (Table 15 and Appendix 36) and the DEPT-135 spectrum (Appendix 37) revealed seven carbon resonances of which four are due to methine carbon atoms (δ 114.82 (C-2), 115.81 (C-6, C-8), 130.05 (C-5, C-9) and 144.82 (C-3)) and three are due to quaternary carbon atoms. The signal at δ 167.57 is attributable to the conjugated carbonyl carbon (C-1) while the signals at δ 159.64 and 126.16 are assigned to C-7 and C-4, respectively. Thus, compound **164** was identified as *p*-hydroxycinnamic acid. The spectroscopic data generated for **164** showed close agreement with those reported for *p*-hydroxycinnamic acid.[52] This is the first report of compound **164** from *P. schimperi* Engl.

Table 15: The ^{13}C-NMR data (δ_{ppm}) of compound **164**.

C	**164** (Acetone-d_6, 100 MHz)	Lit.[52] (CD_3OD, 125 MHz)
1	167.57	171.20
2	114.82	115.82
3	144.82	146.49
4	126.16	126.26
5	130.05	131.05
6	115.81	116.79
7	159.64	161.16
8	115.81	116.79
9	130.05	131.06

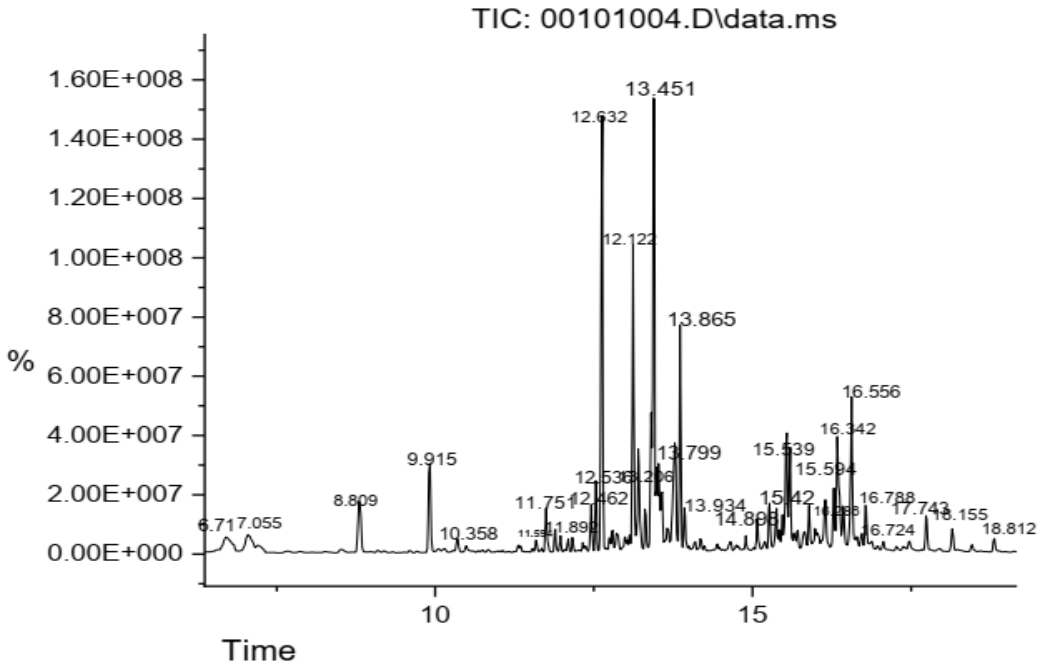
3.3. Characterization of the essential oil from the dried leaves of *Premna schimperi*

Hydrodistillation of the dried leaves of *P. schimperi* as described in Section 4.6 afforded an essential oil which was analyzed by using an Agilent Technology 7820A GC system coupled with an Agilent Technology 5977E MSD system equipped with an autosampler. Figure 15 shows the GC-MS chromatogram of the essential oil. Retention indices of the essential oil components were calculated by using the van den Dool and Kratz relationship[53] (Equation 1).

$$I = 100n + 100\left(\frac{R_{t(unknown)} - R_{t(n)}}{R_{t(n+1)} - R_{t(n)}}\right) \qquad \text{Equation 1}$$

where *I* represents the retention index of the analyte, *n* represents the number of carbon atoms of the *n*-alkane eluting immediately before the analyte, $R_{t(unknown)}$ is the retention time of the analyte, $R_{t(n)}$ and $R_{t(n+1)}$ represent the retention times of the reference *n*-alkanes eluting immediately before and after the analyte, respectively.

Figure 15: The GC-MS chromatogram of the essential oil from the leaves of *Premna schimperi.*



The GC-MS analysis of the essential oil revealed 36 compounds (Table 16). Characterization was done by matching the mass spectra with those of reference compounds recorded in NIST 2014 mass spectral library and confirmed by the retention indices obtained from a series of *n*-alkanes. The major components of the essential oil having qualities greater than 80% and area percentage greater than 2 were identified. These major compounds were caryophyllene (**9**), caryophyllene oxide (**10**), terpinen-4-ol (**33**), α-curcumene (**166**), eugenol (**167**), β-sesquiphellandrene (**168**), γ-gurjunene (**169**), 2-isopropyl-5-methyl-9-methylene-bicyclo[4.4.0]dec-1-ene (**170**), γ-eudesmol (**171**). The essential oil composition of the leaves of *P. schimperi* has not been previously reported.

Table 16: Compounds identified from the essential oil of the dried leaves of *Premna schimperi*.

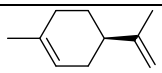
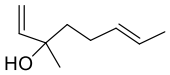
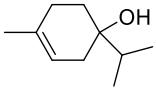
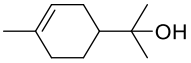
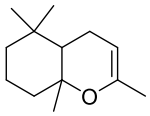
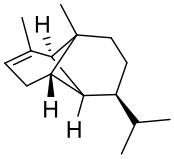
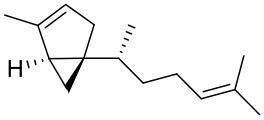
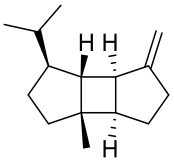
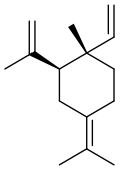
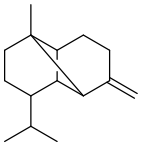
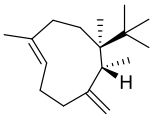
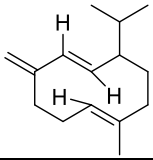
Pk	Rt	Area %	Compound name	Structure	Q	*I*
1	6.7121	1.8257	D-Limonene		99	1055.6
2	7.0554	1.171	Eucalyptol		76	861.6
3	8.8086	2.2042	Linalool		88	840.5
4	9.9151	2.8912	Terpinen-4-ol		93	872.5
5	10.3527	0.4586	α-Terpineol		93	800
6	11.5939	0.3212	1a,2,5,5-Tetramethyl-*trans*-1a,4a,5,6,7,8-hexahydro-γ-chromene		96	996.5
7	11.7505	1.1214	α-Copaene		99	803.8
8	11.8928	0.5251	7-epi-Sesquithujene		98	8014.8
9	11.9759	0.3533	(-)-β-Bourbonene		99	821.2
10	12.1684	0.2975	γ-Elemene		97	835.9
11	12.4623	1.1165	β-Ylangene		99	858.6
12	12.6319	10.7252	Caryophyllene		99	871.7
13	12.8662	0.7358	Germacrene D		89	889.2

Table 16 continued

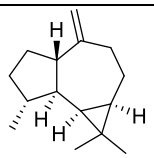
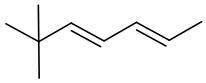
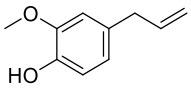
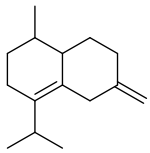
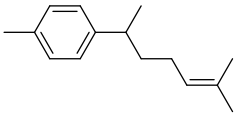
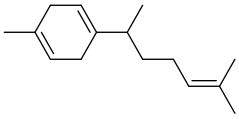
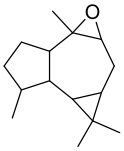
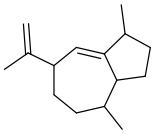
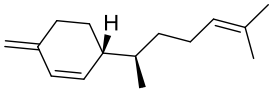
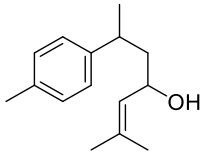
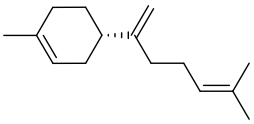
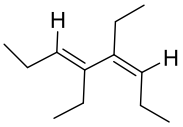
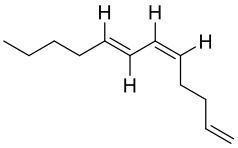
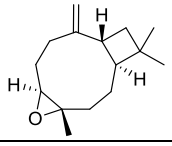
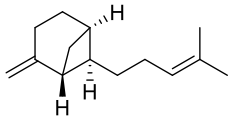
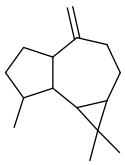
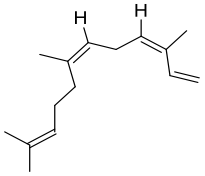
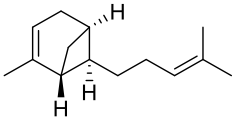
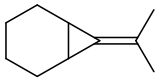
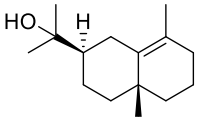
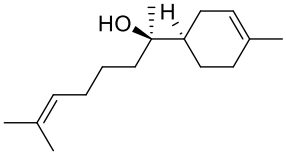
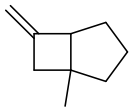
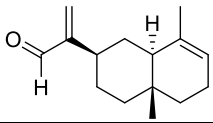
24	12.9961	0.5243	Alloaromadendrene		90	900.67
15	13.0643	0.3465	6,6-Dimethylhepta-2,4-diene		76	906.3
16	13.1224	6.8519	Eugenol		93	911.2
17	13.2075	3.0656	2-Isopropyl-5-methyl-9-methylene-bicyclo[4.4.0]dec-1-ene		86	918.2
18	13.4505	16.38	α-Curcumene		93	938.5
19	13.5722	1.684	β-Curcumene		97	48.7
20	13.6675	0.8699	Isoaromadendrene epoxide		70	956.6
21	13.7793	4.5476	γ-Gurgujene		89	966
22	13.8651	4.6537	β-Sesquiphellandrene		95	973.1
23	13.9342	0.9455	2-Methyl-6-(*p*-tolyl)hept-2-en-4-ol		78	978.8
24	14.8977	0.3015	β-Bisabolene		87	865.7
25	15.4265	0.4726	4,5-Diethyl-3,5-octadiene		35	1308.4
26	15.5386	4.2929	1, Z-5, E-7-Dodecatriene		50	1318.7
27	15.5935	2.6743	Caryophyllene oxide		87	1326.6

Table 16 continued

28	15.7124	0.5065	*trans*-γ-Bergamotene		76	1334.4
29	15.823	0.7124	Aromandendrene		98	1340.8
30	15.8954	1.0336	(Z, Z)- α-Farnesene		86	1350.8
31	16.15	1.7195	*trans*-α-Bergamotene		84	1374.1
32	16.2857	1.3812	7-(1-Methylethylidene)-bicyclo[4.1.0]heptane		74	1386.5
33	16.342	4.4341	γ-Eudesmol		96	1391.6
34	16.4361	1.1611	Bisabolol		90	1500.9
35	16.5653	4.022	1-Methyl-6-methylenebicyclo[3.2.0] heptane		50	1501.8
36	16.7884	1.0183	α-Costol		84	1503.9

Pk = peak number, RT = retention time (min), Q=quality, *I*=retention index

3.4. DPPH radical scavenging assay of the MeOH extract of the dried leaves of *Premna schimperi* and compound 71.

The DPPH radical scavenging assay is a simple method of determining the antioxidant activities by measuring the absorbance of stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical at 517 nm.[54] The radical scavenging activity of the MeOH extract of the leaves of *P. schimperi* and compound **71** were determined using DPPH according to the procedure described by Hoque *et al*.[55] The detailed procedure is given in Section 4.8.

Table 17 and Table 18 show the results obtained for antioxidant activities of the MeOH extract and compound **71**, respectively, in relation to ascorbic acid standards. The MeOH extract and compound **71** reduced the stable DPPH radical by 95.3% and 62.2% at 100 µg/mL, respectively, which suggested that they are potential radical scavengers. The presence of flavonoids should be responsible for the high antioxidant activity of the dried leaves of *P. schimperi*.

Table 17: Radical scavenging activities of the MeOH extract of the dried leaves of *Premna schimperi* and ascorbic acid standards determined using the DPPH assay.

Concentration (µg/mL)	% DPPH Inhibition	
	MeOH extract of the dried leaves of *P. schimperi*	Ascorbic acid standard
100.00	95.3% ± 0.88	96.09 ± 0.16
50.00	91.3 ±1.24	96.26 ± 0.06
25.00	88.6 ±0.45	96.06 ± 0.12
12.50	80.5 ±0.41	96.06 ± 0.12
6.25	61.3 ± 1.24	91.30 ± 0.31
3.13	34.9 ± 0.89	54.06 ± 1.27
1.56	27.1 ± 0.49	35.00 ± 0.52

The results are reported as mean ± SD of triplicates.

Table 18: Radical scavenging activities of compound **71** and ascorbic acid standards determined using the DPPH assay.

Concentration (µg/mL)	% DPPH Inhibition	
	Compound **71**.	Ascorbic acid standard
100.00	62.2 ± 0.57	96.09 ± 0.16
50.00	59.7 ± 0.47	96.26 ± 0.06
25.00	45.0 ± 0.81	96.06 ± 0.12
12.50	35.5 ± 0.40	96.06 ± 0.12
6.25	25.9 ± 0.77	91.30 ± 0.31
3.13	21.6 ± 0.62	54.06 ± 1.27
1.56	17.4 ± 0.81	35.00 ± 0.52

The results are reported as mean ± SD of triplicates.

4. Experimental

4.1. Chemicals

All chemicals and solvents were purchased from commercial sources and were used without further purification.

4.2. Chromatography

Column chromatography was performed using silica gel 60 (Merck), particle size 0.063 – 0.200 (70 – 230 mesh ASTM). Preparative thin-layer chromatography (PTLC) was carried out on acid-washed silica gel with 254 nm fluorescent indicator (Sigma-Aldrich, Germany) pre-coated on glass (20 × 20 cm). Thin-layer chromatography (TLC) was performed on Merck Kieselgel 60 F254 precoated plates and were visualized under UV light at 254 and 365 nm. TLC plates were also developed by spraying with ceric ammonium molybdate- reagent followed by heating until colored spots appeared.

4.3. Instruments

Melting point were recorded with a METTLER TOLEDO FP82HT hot stage coupled with a METTLER TOLEDO FP90 central processor. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker Avance 400 NMR spectrometer at 400.13 and 100.6 MHz, respectively. The proton and carbon chemical shifts (δ) were reported in parts per million (ppm), coupling constants were reported in hertz (Hz) and splitting patterns were designated as singlet (*s*), doublet (*d*), triplet (*t*) quartet (*q),* doublet of doublets (*dd*), doublet of doublets of doublets (*ddd*) and multiplet (*m*). The FT-IR spectra of compounds were recorded on a Perkin-Elmer Spectrum 65 IR spectrometer in the range of 4000-200 cm^{-1} as KBr pellets. UV-vis spectra were recorded on a UV-T60 spectrophotometer. Gas chromatography-mass spectrometry (GC-MS) experiments were done on Agilent Technologies 7820A GC system coupled with Agilent Technologies 5977E MSD, USA.

4.4. Plant material

Premna schimperi Engl. (Figure 16, Synonym: *Premna viburnoides* A. Rich) is used as traditional medical plant in Ethiopia.[56] It is a spreading shrub to tree up to 7-10 m high.

The branches are pale brown to grey and it grows in secondary forest, grassy meadows and along paths in forests at altitude of 1350-2500 m. In Ethiopia, it is found in Tigray, Gonder, Gojam, Welega, Shewa, Arsi, Harerge, Sidamo, Gamo Gofa, Kefa and Ilubabor Administrative Regions. It also occurs in Sudan, Kenya and Tanzania. The local names of *P. schimperi* Engl. are *Chocho* (Amharic); *Urgeessa* (Afaan Oromo); *Tulangi* (Sidama); *Taitos* (Gimira); *Tumo* (Kafa).[57]

The leaves of *P. schimperi* were collected from Yabelo City, Borana Zone, Oromia regional state, Ethiopia in May 2019. A voucher specimen number M002 was identified and deposited at the National Herbarium, Addis Ababa University, Ethiopia.

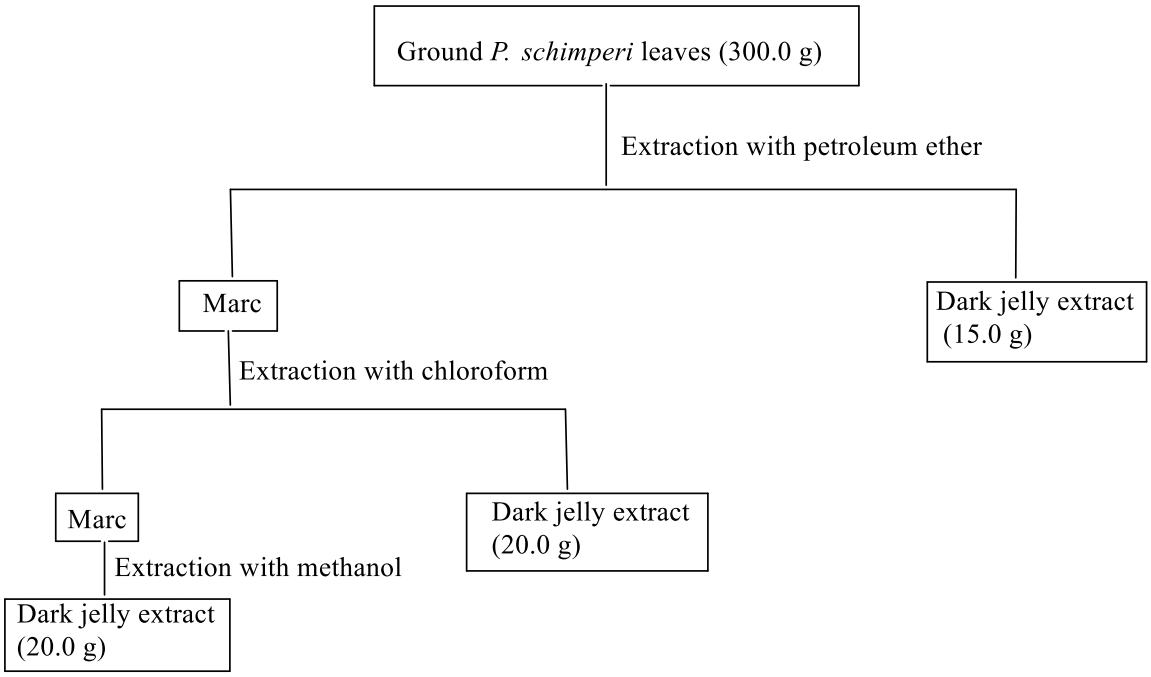
Figure 16: *Premna schimperi* Engl.

4.5. Solvent extraction

To the air-dried and ground leaves of *P. schimperi* (300.0 g) placed in a 3 L Erlenmeyer flask was added petroleum ether (1.0 L) and the mixture was allowed to shake on mechanical shaker at room temperature for 24 h. The mixture was filtered and another 1.0 L of the solvent was added to the marc and the same procedure was repeated for another 24 h. The solvent was removed from the combined extract by rotary evaporation to afford a dark jelly material (15.0 g). Following the same procedure, the marc left after extraction with petroleum ether was extracted with chloroform (2 x 1.0 L) to yield a dark jelly extract (20.0 g). Finally, the marc left after extraction with

chloroform was extracted with methanol (2 x 1.0 L) to afford a dark jelly extract (20.0 g). The methanol extract was further partitioned between water and ethyl acetate and to give the ethyl acetate extract (150.0 mg). Figure 17 shows a flow diagram for the extraction of the powdered leaves of *P. schimperi.*

Figure 17: Flow diagram for the extraction of the leaves of *Premna schimperi*.



4.6. Extraction of the leaves of *Premna schimperi* by hydrodistillation.

To a 5 L round bottomed flask containing ground leaves of *P. schimperi* (250.0 g), water (2.0 L) was added and a Clevenger apparatus, which was connected to a condenser, was attached to the flask. The content of the flask was heated to boiling on a heating mantle and hydrodistillation was carried out for 5 h. The essential oil (0.7 mL) was collected, dried over anhydrous $MgSO_4$, filtered and stored until analysis by GC-MS.

4.7. Characterization of the essential oil.

A solution of the essential oil in DCM was analyzed using an Agilent Technology GC-MS. The chromatographic separation was performed on a DB-1701 micro-column (30 m long, 0.25 mm internal diameter and 0.25 μm particle size) at a pressure of 8 psi and a flow rate of 1 mL/min. Ultra-pure helium (99.999%) was used as carrier gas at constant flow. Agilent G4567A autosampler was used to inject 1 μL of the sample with

a splitless injection mode into the inlet heated to 275 ºC with a total run time of 29.33 min. Oven temperature was programmed at 60 ºC with the hold-time of 2 min and the column temperature was increased at rate of 10 ºC/min until the temperature reached 200 ºC, and the heating was continued at the rate of 3 ºC/min until the temperature reached 240 ºC. The mass spectra were not collected during the first 4 min of the solvent delay. The transfer line temperature was maintained at 280 ºC and the ion source temperature was kept at 230 ºC. The ionization energy of the EI source was 70 eV. Mass spectral data were collected from 40 – 650 m/z. Characterization was done by matching the mass spectra with those of reference compounds recorded in NIST 2014 mass spectral library and confirmed by the retention indices obtained from a series of n-alkanes. The retention indices of the essential oil components were calculated by using the van den Dool and Kratz relationship[53] (Equation 1).

$$I = 100n + 100\left(\frac{R_{t(unknown)} - R_{t(n)}}{R_{t(n+1)} - R_{t(n)}}\right) \qquad \text{Equation 1}$$

where *I* represent the retention index of the analyte, *n* represent the number of carbon atoms of the *n*-alkane eluting immediately before the analyte, $R_{t(unknown)}$ is the retention time of the analyte, $R_{t(n)}$ and $R_{t(n+1)}$ represents the retention times of the reference *n*-alkanes eluting immediately before and after the analyte, respectively.

4.8. DPPH radical scavenging assay of the MeOH extract of the dried leaves of *Premna schimperi* and compound 71.

The radical scavenging assay of the MeOH extract of the dried leaves of *P. schimperi* and compound **71** were determined using DPPH according to the procedure described by Hoque *et al*.[55] The MeOH extract (10.0 mg) was dissolved in MeOH (10.0 mL). Seven different concentrations (500.00, 250.00, 125.00, 62.50, 31.30, 15.63 and 7.81 µg/mL), of the extract were prepared by diluting the stock solution (1.0 mg/mL) with MeOH. To the seven solutions (1.0 mL each), 0.004% DPPH in MeOH (4.0 mL) was added to make 100.00, 50.00, 25.00, 12.50, 6.25, 3.13 and 1.56 µg/mL solutions. The mixtures were shaken and allowed to stand at room temperature for 30 min. The absorbances of the solutions were then recorded at 517 nm using a UV-vis spectrophotometer. The antioxidant activity of each concentration was measured in relation to ascorbic acid standards. All measurements were carried out in triplicates and

the ascorbic acid standards were also analyzed following the same procedure. The percentage DPPH inhibition was then calculated using Equation 2.

$$\% \text{ DPPH inhibition} = \frac{A_{control} - A_{extract}}{A_{control}} \times 100 \qquad \text{Equation 2}$$

Where, $A_{control}$ is the absorbance of DPPH solution in MeOH and $A_{extract}$ is the absorbance of the test sample in MeOH plus DPPH solution.

4.9. Fractionation of the chloroform extract.

The chloroform extract (14.0 g) was dissolved in chloroform, adsorbed on silica gel (14.0 g) and fractionated over the column of silica gel using a gradient of $CHCl_3$ in pet. ether (PE) (PE:$CHCl_3$, 100:0→0:100) and then $CHCl_3$:MeOH, 100:0→70:30, v/v). A total of 120 fractions were collected. Fractions were then combined in to 4 groups based on their TLC profile as shown in Table 19.

Table 19: The combined fractions of the chloroform extract.

Fractions	Eluent	Combined fractions	Mass (g)	Designation
1	PE:$CHCl_3$ (100:0→70:30)	1-10	2.0	N_1
2	PE:$CHCl_3$ (70:30→40:60)	11-30	2.6	N_2
3	PE:$CHCl_3$ (40:60→0:100)	31-80	3.6	N_3
4	$CHCl_3$:MeOH (100:0→70:30)	81-120	3.5	N_4

4.9.1. Fractionation of N_1.

Fraction N_1 was chromatographed over a column of silica gel and eluted using a gradient of chloroform in petroleum ether (0:100→100:0) to yield a total of 20 fractions which were combined in to 9 groups based on their TLC profiles as shown in Table 20. Fraction 3 was recrystallized from hexane to give white crystals (11.0 mg). Fraction 4 was recrystallized from methanol to afford white crystals (19.0 mg). These two crystals were found to be similar on the bases on TLC profile and NMR data. Then, these crystals were combined to give compound **71** (30.0 mg)**.**

Table 20: Fractionation of N_1.

Fractions	Eluent	Volume (mL)	Mass (mg)	Number of spots on TLC
1	PE:EtOAc (100:0)	15	70	2
2	PE:EtOAc (90:10)	15	80	2
3	PE:EtOAc (80:20)	10	60	3
4	PE:EtOAc (70:30)	15	100	2
5-6	PE:EtOAc (60:40)	30	700	1
7-11	PE:EtOAc (50:50)	40	200	5
12	PE:EtOAc (40:60)	12	180	4
13-19	PE:EtOAc (25:75)	60	100	2
20	PE:EtOAc (0:100)	15	150	6

4.9.2. Fractionation of N_2.

Fraction N_2 was chromatographed over a column of silica gel using a gradient of ethyl acetate in chloroform (0:100→100:0) to yield a total of 65 fractions which were combined in to 9 groups based on their TLC profiles as shown in Table 21.

Table 21: Fractionation of N_2.

Fractions	Eluent	Volume (mL)	Mass (mg)	Number of spots on TLC
1-12	PE:EtOAc (100:0)	110	300	5
13-14	PE:EtOAc (90:10)	20	150	5
15	PE:EtOAc (80:20)	10	170	4
16-21	PE:EtOAc (70:30)	50	120	4
22-32	PE:EtOAc (60:40)	100	80	3
33-36	PE:EtOAc (50:50)	30	119	3
37-40	PE:EtOAc (40:60)	30	137	4
41-53	PE:EtOAc (25:75)	120	140	4
54-65	PE:EtOAc (100:0)	90	300	5

4.9.3. Fractionation of N_3.

Fraction N_3 was subjected to column chromatography over silica gel using a gradient of ethyl acetate in chloroform (0:100→100:0, v/v) to yield 60 fractions which were combined in to 9 groups based on their TLC profiles as shown in Table 22. Fractions 7-14 was chromatographed over a column of silica gel using $CHCl_3$:EtOAc (90:10, v/v) as eluent to afford compound **157** (30.0 mg). Crystallization of fractions 21-24 from methanol gave compound **158** (5.0 mg)**.** Figure 18 shows the flow diagram for the isolation of compounds **71**, **157** and **158**.

Table 22: The combined fractions of N_3.

Fractions	Volume (mL)	Mass (mg)	Number of spots on TLC
1-2	20	180	4
3-6	30	293	4
7-14	70	300	5
15-20	50	290	4
21-24	30	295	5
25-28	30	285	2
29-33	40	200	2
34-40	60	340	4
41-60	90	400	8

Figure 18: Flow diagram for the isolation of compounds **71**, **157** and **158**.

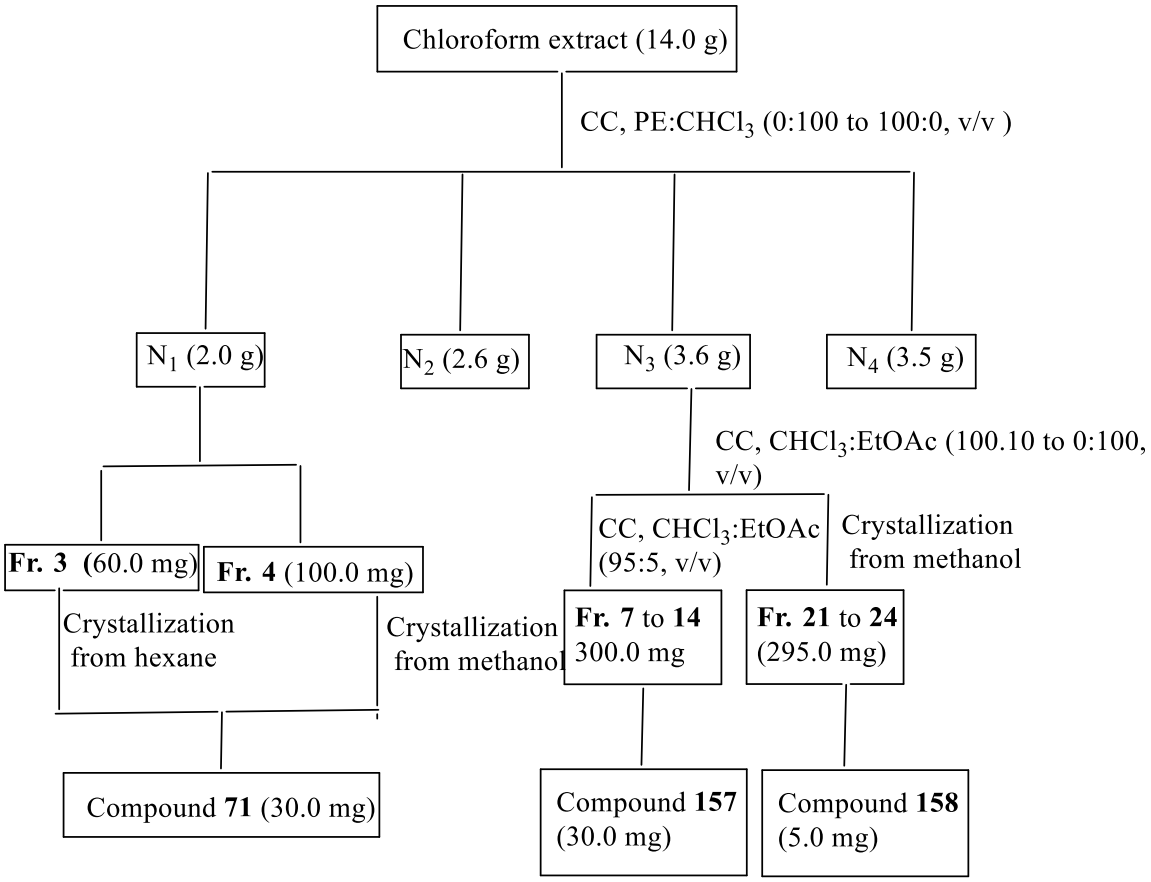


Figure 19: TLCs of compounds **157** ($CHCl_3$:EtOAc/5:1), **158** $CHCl_3$:EtOAc/4:1) and **71** (pet. ether:EtOAc/4:0.5).

TLC of Compound **157** | TLC of Compound **158** | TLC of Compound **71**

4.10. Fractionation of the petroleum ether extract.

The petroleum ether extract (10.0 g) was subjected to column chromatography over silica gel and eluted with a gradient of chloroform in petroleum ether (0:100→100:0, v/v). A total of 120 fractions were collected and combined in to seven groups based on their TLC profiles as shown in Table 23. Fractions 11-30 were combined and chromatographed over a column of silica gel using PE:$CHCl_3$ (95:5, v/v) and gave compound **165** as a white amorphous powder.

Table 23: Fractionation of the petroleum ether extract

Fractions	Eluent	Volume (mL)	Mass (g)	Number of spots on TLC
1-10	PE:$CHCl_3$ (95:5)	130	2.5	9
11-30	PE:$CHCl_3$ (95:5)	180	1.7	8
31-65	PE:$CHCl_3$ (95:5)	300	1.0	8
66-80	PE:$CHCl_3$ (95:5)	110	0.7	5
81-93	PE:$CHCl_3$ (95:5)	150	0.3	5
94-111	PE:$CHCl_3$ (95:5)	180	0.25	4
112-120	PE:$CHCl_3$ (95:5)	160	0.01	4

4.11. Fractionation of the methanol extract.

The methanol extract (10.0 g) was fractionated between water and ethyl acetate using a separatory funnel. The ethyl acetate soluble portion (150.0 mg) was dissolved in ethyl

acetate and adsorbed on to silica gel (150.0 mg) and was chromatographed on a column of silica gel and eluted with $CHCl_3$:EtOAc (50:50, v/v). A total of 40 fractions were collected and combined in to five groups based on TLC profiles as shown in Table 24. Fractions 13-20 was crystallized from acetonitrile to afford compound **164**. Similar crystallization of fractions 32-40 from acetonitrile afforded compound **159**.

Table 24: Fractionation of the ethyl acetate fraction of the methanol extract

Fractions	Eluent	Volume (mL)	Mass (mg)	Number of spots on TLC
1-11	$CHCl_3$:EtOAc (50:50)	120	4.0	2
12	$CHCl_3$:EtOAc (50:50)	15	10.0	2
13-20	$CHCl_3$:EtOAc (50:50)	150	18.0	2
21-31	$CHCl_3$:EtOAc (50:50)	100	25.0	2
32-40	$CHCl_3$:EtOAc (50:50)	90	30.0	2

4.12. Per acetylation of compound 157

To a 10 mL round bottomed flask, compound 1**57** (27.0 mg), pyridine (1.4 mL) and dried acetic anhydride (5.0 mL) were added. The mixture was heated at 70 ºC following the progress of the reaction by TLC ($CHCl_3$:EtOAc/5:1). After completion of the reaction (ca 12 h), the mixture was allowed to cool to room temperature and poured in to a beaker containing crushed ice. The precipitate was collected by filtration and was washed with distilled water exhaustively and dried to yield the per acetate of **157** (**157-Ac**) as yellowish solid (34.0 mg, 97%).

4.13. Physical and spectroscopic data of compounds isolated from the dried leaves of *Premna schimperi.*

***ent*-8β,12α-Epidioxy-12β-hydroxylabda-9(11),13-dien-15-oic acid γ-lactone (71):** White crystals, m. p. 214-215ºC, Lit.[22] m. p. 216 ºC; $[\alpha]_D$-140 ($CHCl_3$, c 0.1), Lit.[22] $[\alpha]_D$-128 ($CHCl_3$, c 0.1)' UV (EtOH, λ_{max}, nm): 227; IR_v (KBr, cm^{-1}): 2996, 2955, 2926, 2906, 1659, 1457, 1438, 1241, 1239; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.91 (3H, *s*, H-18), 0.94 (3H, *s*, H-19), 1.23 (3H, *s*, H-20), 1.69 (3H, *s*, H-17), 2.00 (3H, *d*, *J* = 2.0 Hz, H-16), 5.23 (1H, *s*, H-11), 5.98 (1H, *q*, *J* = 2.0 Hz, H-14); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 13.08 (C-16), 16.56 (C-6), 19.04 (C-2), 21.24 (C-19), 25.38 (C-17), 25.66 (C-20), 26.39 (C-7), 32.99 (C-18), 33.60 (C-4), 38.47 (C-10), 40.39 (C-1), 41.87 (C-3), 43.69 (C-5), 78.94 (C-8), 107.19 (C-12), 111.24 (C-11), 121.05 (C-14), 162.43 (C-13), 163.13 (C-9), 169.52 (C-15).

3,5,5′-Trihydroxy-6,7,3′,4′-tetramethoxyflavone (157): Yellowish solid, m. p. 210-211 ºC, Lit.[51] m. p. 211 ºC; UV (MeOH, λ_{max}, nm): 255, 267, 340, 358; IR_{ν} (KBr, cm^{-1}): 3537, 3436, 3256, 2993, 2922, 1655, 1587, 1493, 1434, 1279, 1215; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.95 (3H, *s*, C-3′ OMe), 3.98 (3H, *s*, C-4′ OMe), 3.99 (3H, *s*, C-7 OMe), 4.01 (3H, *s*, C-6 OMe), 11.63 (1H, *s*, OH-5), 7.45 (1H, *d*, *J* = 2.0 Hz, H-2′), 7.50 (1H, *d*, *J* = 2.0 Hz, H-6′); ^{13}C- NMR (100 MHz, $CDCl_3$): δ 144.96 (C-2), 136.23 (C-3), 175.40 (C-4), 151.37 (C-5), 132.23 (C-6), 159.42 (C-7), 90.75 (C-8), 152.19 (C-9), 104.38 (C-10), 126.31 (C-1′), 104.51 (C-2′), 152.45 (C-3′), 137.30 (C-4′), 149.26 (C-5′), 107.55 (C-6′), 60.95 (C-6), 61.12 (C-4′), 56.08 (C-3′), 56.41 (C-7).

3,5,5′-Triacetoxy-6,7,3′,4′-tetramethoxyflavone (157-Ac): Yellowish solid; m. p. > 300 ºC: UV (MeOH, λ_{max}, nm): 262, 318; IR_{ν} (KBr, cm^{-1}): 2937, 1776, 1648, 1461, 1200; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 2.35 (3H, *s*, $COCH_3$), 2.38 (3H, *s*, $COCH_3$), 2.50 (3H, *s*, $COCH_3$), 3.88 (3H, *s*, C-6 OMe), 3.94 (3H, *s*, C-4′ OMe), 3.95 (3H, *s*, C-3′ OMe), 4.01 (3H, *s*, C-7 OMe), 6.89 (1H, *s*, H-8), 7.18 (1H, *s*, H-6′), 7.28 (1H, *s*, H-2′); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 56.34 (C-3′ OMe), 56.55 (C-7 OMe), 60.90 (C-4′ OMe), 61.58 (C-6 OMe), 98.18 (C-8), 110.06 (C-2′), 111.30 (C-10), 115.80 (C-6′), 124.66 (1′), 133.35 (3), 139.86 (C-6), 142.06 (C-5), 143.57 (C-4′), 143.97 (C-9), 153.39 (C-3′), 153.77 (C-2), 158.11 (C-7), 170.1, Ac (168.00, 168.83, 169.37, 20.59, 20.73 and 20.99).

3,5,7,5′-Tetrahydroxy-6,3′,4′ trimethoxyflavone (158): Yellowish solid, m. p. 152-153 ºC, Lit.[44]; m. p. 53 ºC; UV (MeOH, λ_{max}, nm): 254, 262, 301, 339, 362; IR_{ν} (KBr, cm^{-1}): 3469, 3412, 3332, 2994, 2923, 1656, 1493, 1445, 1228, 1200; ^{1}H-NMR (400 MHz, Acetone-d_6): δ 3.95 (3H, ***s***, C-3′ OMe), 3.98 (3H, *s*, C-4′ OMe), 3.89 (3H, *s*, C-6 OMe), 6.67 (1H, *s*, H-8), 7.55 (1H, *d*, *J* = 2.0 Hz, C-2′), 7.45 (1H, *d*, *J* = 2.0 Hz, H-6′); ^{13}C- NMR (100 MHz, Acetone-d_6): δ 55.54 (C-3′ OMe), 59.89 (C-6 OMe), 59.98 (C-4′ OMe), 93.80 (C-8), 103.76 (C-2′), 103.79 (C-10), 108.99 (C-6′), 126.43 (C-1′), 130.87 (C-6), 136.35 (C-3), 138.26 (C-4′), 145.45 (C-2), 150.40 (C-5′),151.64 (C-5), 152.26 (C-9), 153.11 (C-3′) and 157.21 (C-7), 176.18 (C-4).

Straight chain primary alcohol (165): White solid, m. p. 76.5-78 ºC; IR_{ν} (KBr, cm^{-1}): 3405, 2922, 2853, 1644, 1461,1245, 719; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.8 (3H, *t*,

J = 8.0 Hz), 1.3 (52H, *bs*), 1.6 (4H, *m*), 3.68 (2H, *t*, 8.0 Hz); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 63.12, 32.82, 31.93, 29.71, 29.67, 29.62, 29.44, 25.37, 22.74, 14.12.

Luteolin (159): Yellow amorphous powder; m. p. > 300 ºC; Lit.[58] m. p.325 ºC; UV (EtOH, λ_{max}, nm): 255, 268, 292, 352; IR_v (KBr, cm^{-1}): 3426, 2923, 1655, 1615, 1455, 1265, 1246; ^{1}H-NMR (400 MHz, Acetone-d_6): δ 6.26 (1H, *d*, *J* = 2.0 Hz, H-6), 6.54 (1H, *d*, *J* = 2.0 Hz, H-8), 6.60 (1H, *s*, H-3), 7.00 (1H, *d*, *J* = 8.4 Hz, H-5′), 7.48 (1H, *dd*, *J* = 8.4, 2.0 Hz, H-6′), 7.52 (1H, *d*, *J* = 2.0 Hz, H-2′), 13.04 (1H, *s*, H-5); ^{13}C-NMR (100 MHz, Acetone-d_6): δ 93.79 (C-8), 98.81(C-6), 103.30 (C-3), 104.44 (C-10), 113.23 (C-5′), 115.75 (C-2′), 119.25 (C-6′), 122.84 (C-1′), 145.62 (C-3′), 149.24 (C-4′), 157.89 (C-9), 162.48 (5), 146.00 (C-7), 164.25 (C-2), 182.16 (C-4).[51]

***p*-Hydroxycinnamic acid (164):** Light-yellow solid, m. p. 210-212 ºC; Lit.[59] m. p. 211.5 ºC; UV (EtOH, λ_{max}, nm): 211, 224, 292, 299, 309; IR_v (KBr, cm^{-1}): 3374, 2928, 1672, 1601, 1450, 1245, 1212; ^{1}H-NMR (400 MHz, Acetone-d_6): δ 6.35 (1H, *d*, *J* = 16.0 Hz, H-2), 6.91(2H, *d*, *J* = 8.8 Hz, H-6, H-8), 7.56 (2H, *d*, *J* = 8.8 Hz, H-5, H-9), 7.63 (1H, *d*, *J* = 16.0 Hz, H-3), 9.02 (*broad*, COOH); ^{13}C-NMR (100 MHz, Acetone-d_6): δ 114.82 (C-2), 115.81(C-6, C-8), 126.16 (C-4), 130.05 (C-5, C-9), 144.82 (C-3), 159.64 (C-7), 167.57 (C-1).

5. Conclusion

In the course of this research, the phytochemical studies of *P. schimperi* have been conducted. Conventional silica gel chromatographic techniques and preparative thin layer chromatography (PTLC) were used to isolate pure compounds from the extracts. The solvent extract of the dried leaves afforded six compounds. These compounds are *ent*-8β,12α-epidioxy-12β-hydroxylabda-9(11),13-dien-15-oic acid γ-lactone (**71**), 3,5,5'-trihydroxy-6,7,3',4'-tetramethoxyflavone (**157**), 3,5,7,5'-tetrahydroxy-6,3',4'-trimethoxyflavone (**158**), straight chain primary alcohol **164**, luteolin (**158**) and *p*-hydroxy cinnamic acid (**164**). Five of the compounds namely, compounds **71**, **157**, **158**, **164** and **165** are reported herein for the first time from *P. schimperi*. The isolated compounds were characterized based on their IR, UV and NMR spectral data and by comparison of these with data reported in the literature. The GC-MS analysis of the essential oil of the leaves of *P. schimperi* showed the presence of nine major compounds, namely caryophyllene (**9**), caryophyllene oxide (**10**), terpinen-4-ol (**33**), α-curcumene (**166**), eugenol (**167**), β-sesquiphellandrene (**168**), γ-gurjunene (**169**), 2-isopropyl-5-methyl-9-methylene-bicyclo[4.4.0]dec-1-ene (**170**), γ-eudesmol (**171**). The antioxidant activities of the MeOH extract of the leaves of *P. schimperi* and compound **71** were investigated using the DPPH radical scavenging assay. The MeOH extract and compound **71** reduced the stable DPPH radical by 95.3% and 62.2%, respectively, at 100 μg/mL, which suggested that they are potential radical scavengers.

6. References

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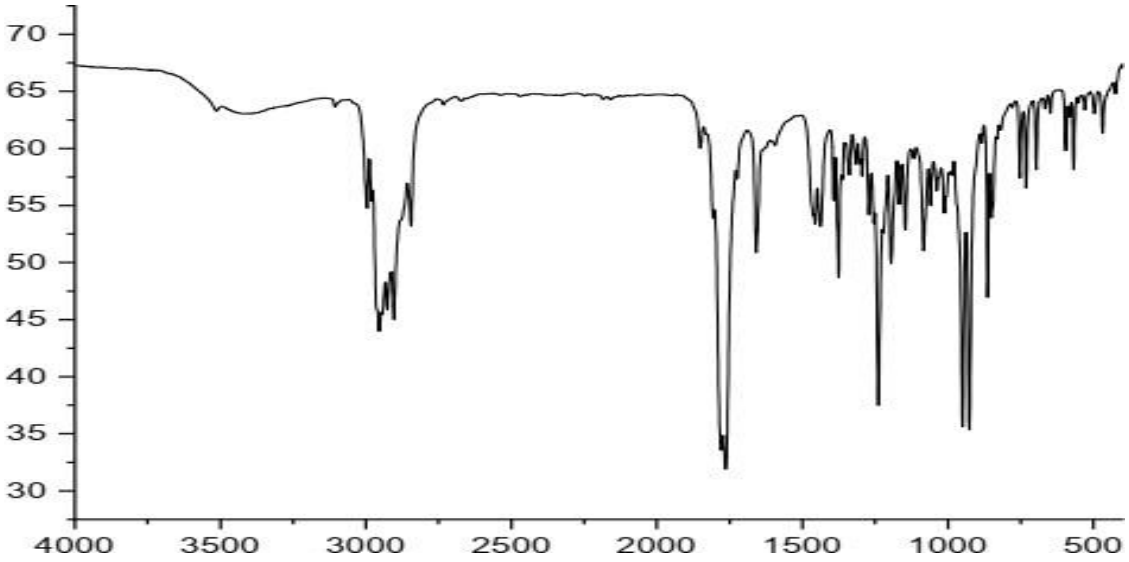
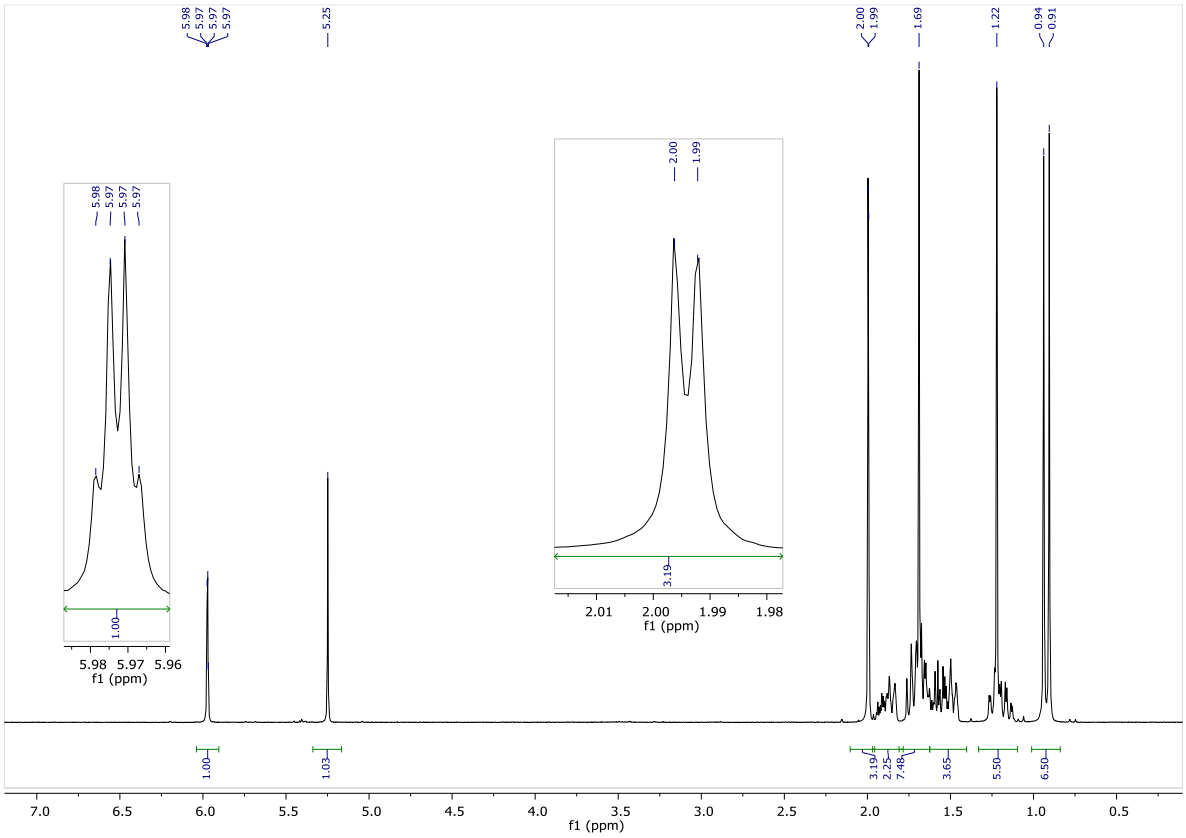
7. Appendices

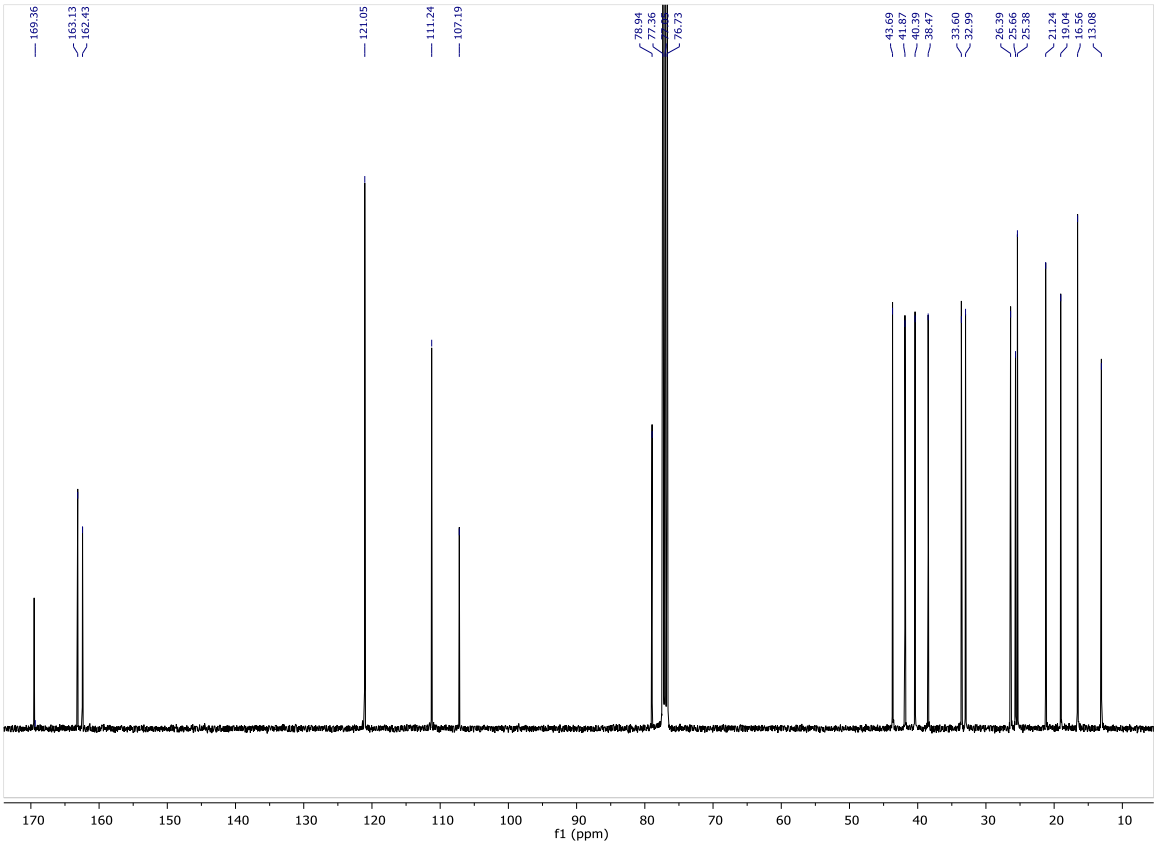
Appendix 1: The IR spectrum of compound **71**.



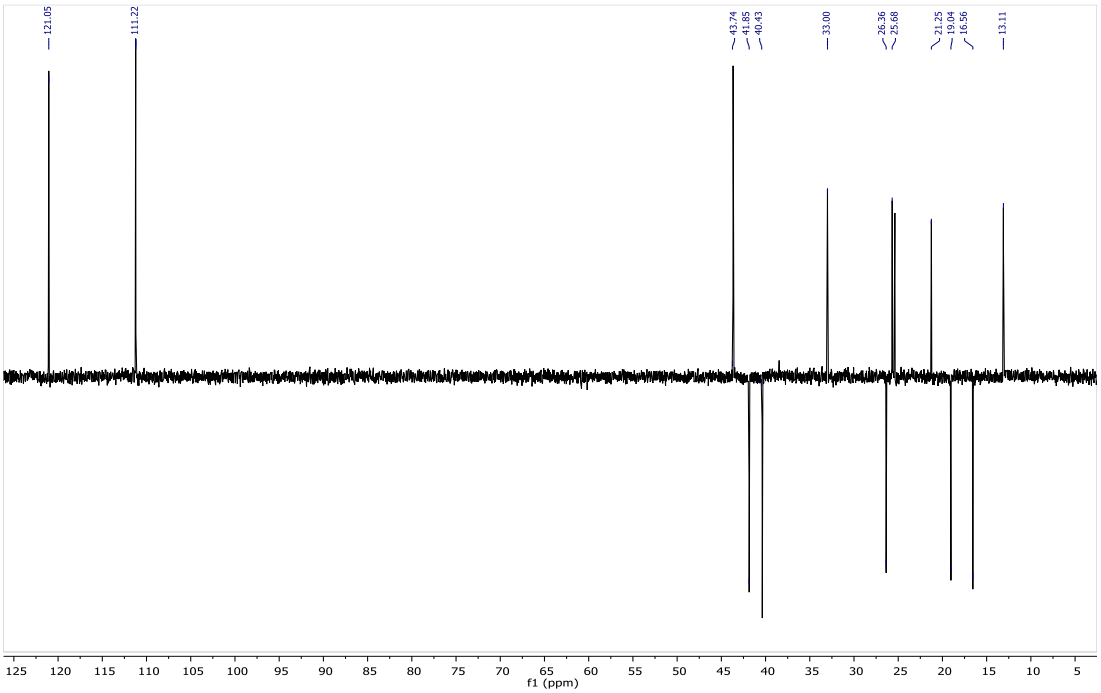
Appendix 2: The ^{1}H-NMR spectrum of compound **71** in $CDCl_3$.



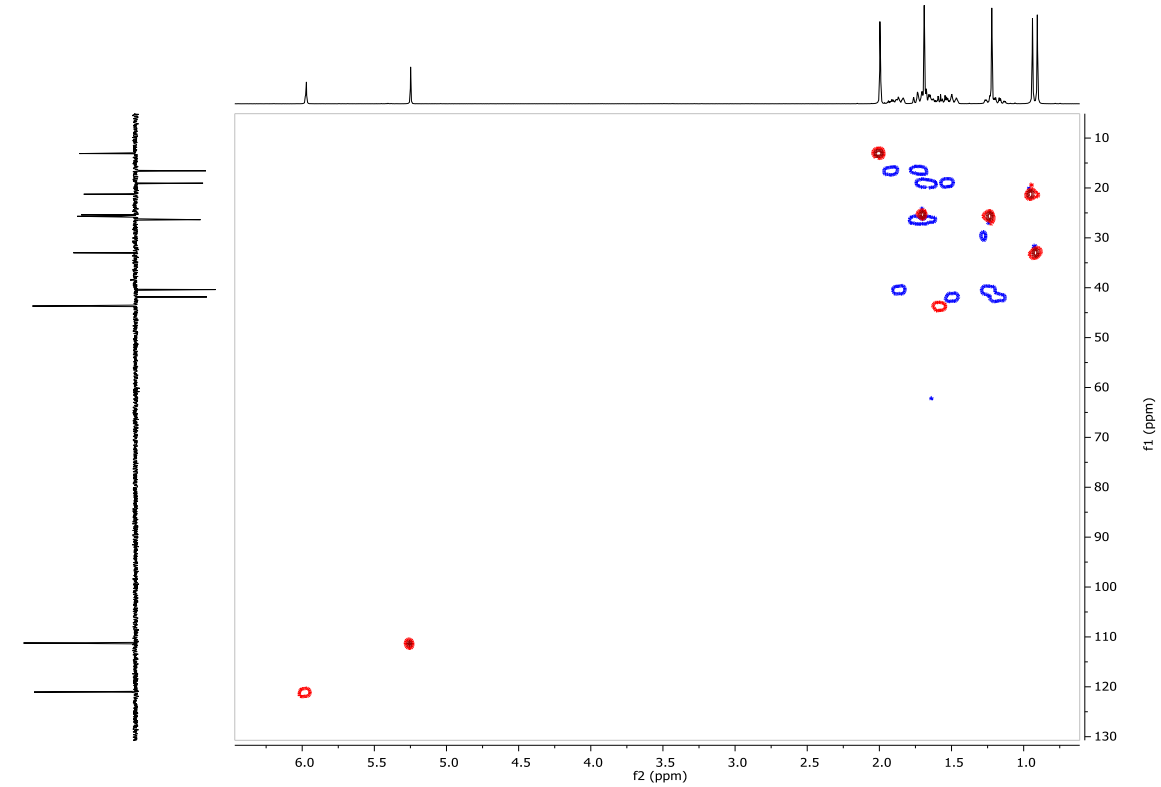
Appendix 3: The ^{13}C-NMR spectrum of compound **71** in $CDCl_3$.



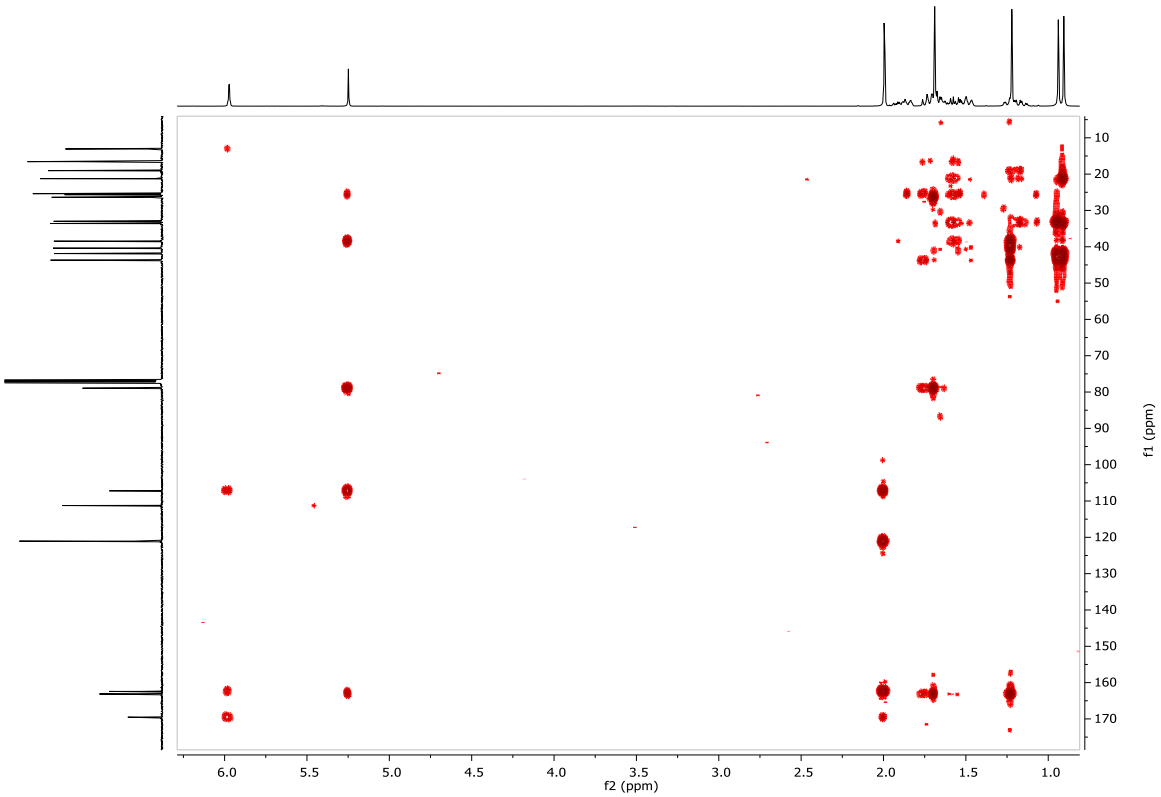
Appendix 4: The DEPT-135 NMR spectrum of compound **71** in $CDCl_3$.



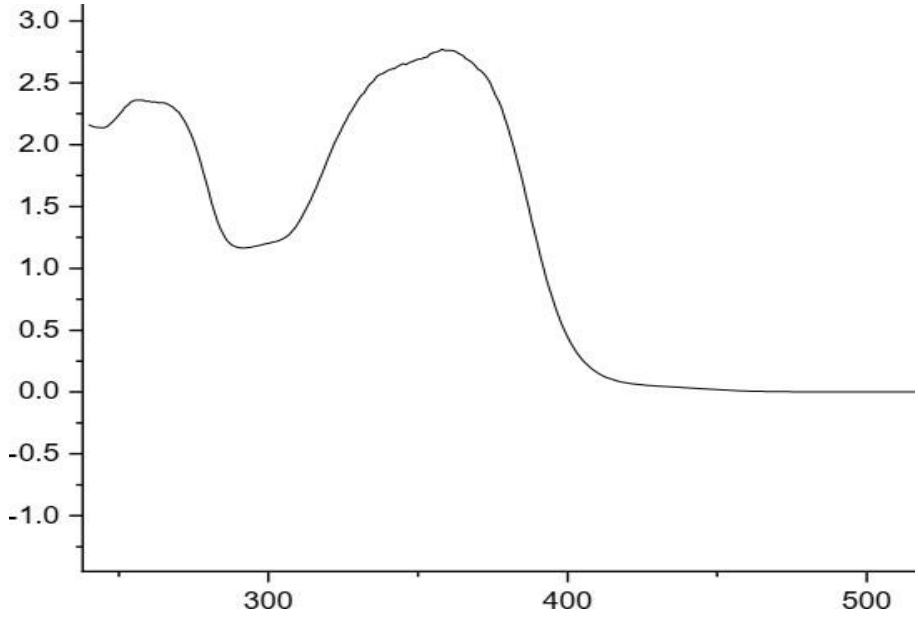
Appendix 5: The HSQC spectrum of compound **71** in $CDCl_3$.



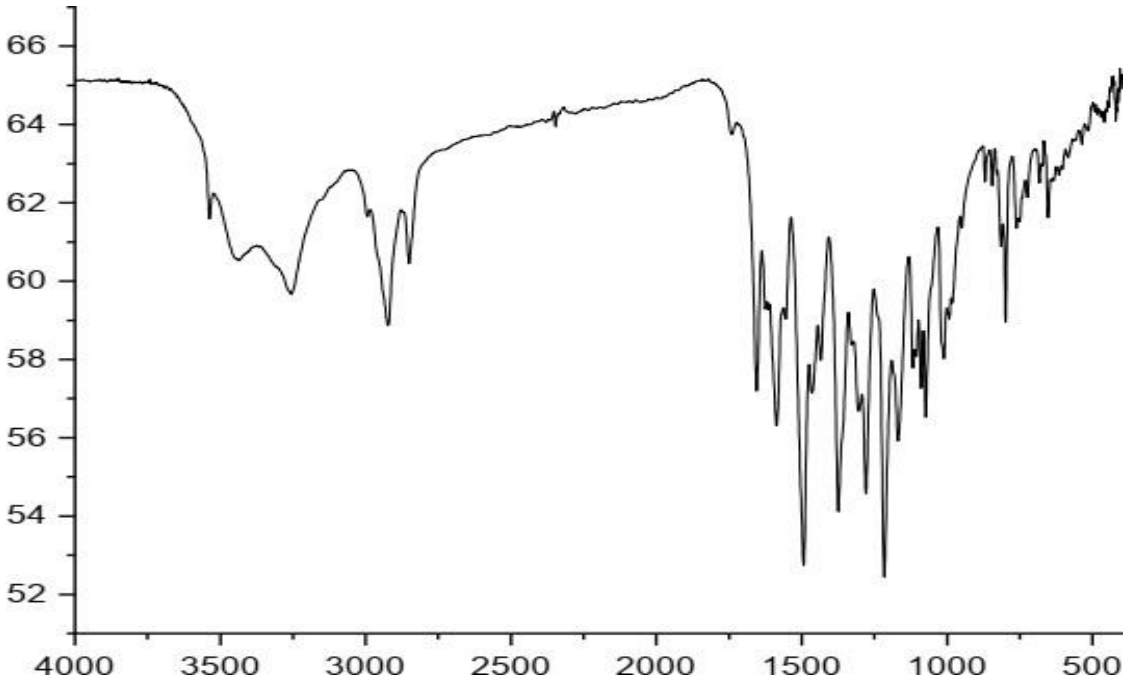
Appendix 6: The HMBC spectrum of compound **71** in $CDCl_3$.



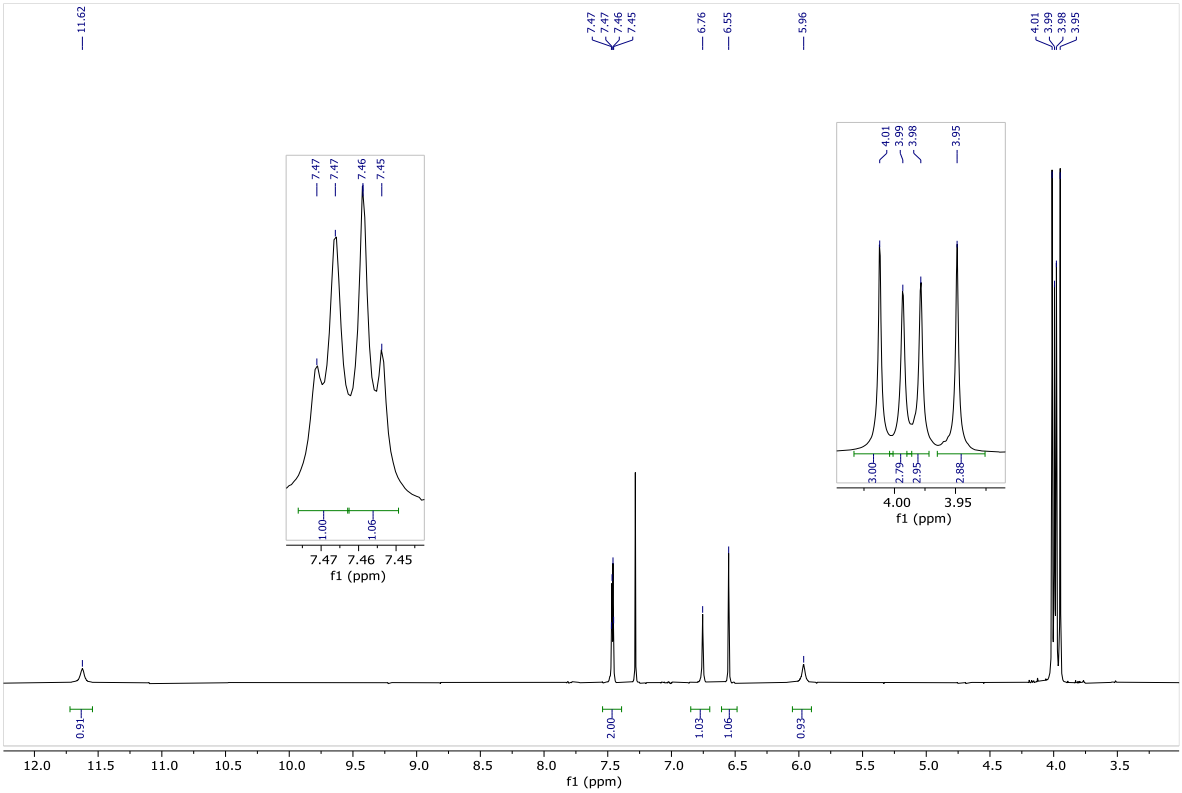
Appendix 7: The UV spectrum of compound **157** in MeOH.



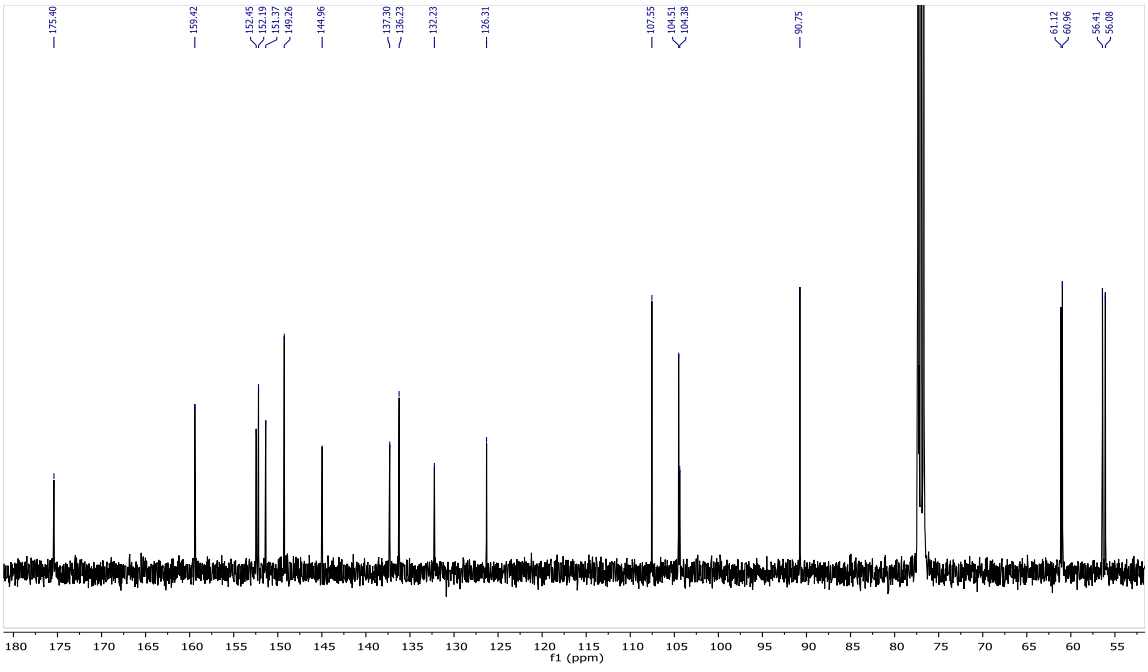
Appendix 8: The IR spectrum of compound **157**.



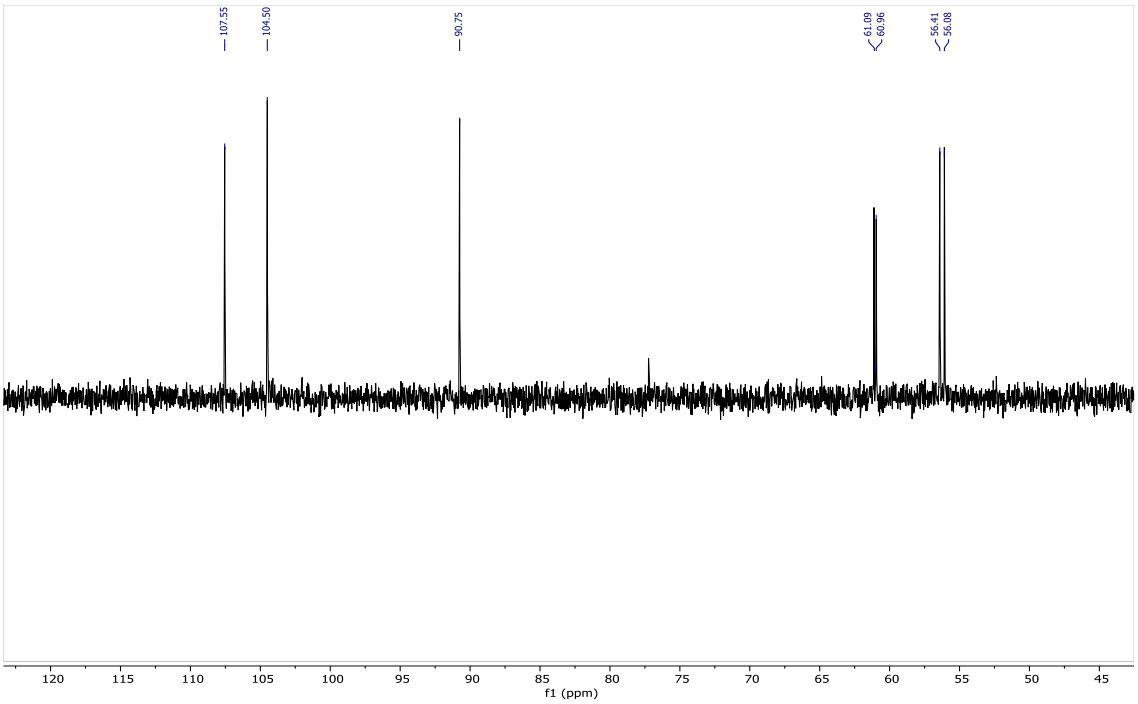
Appendix 9: The ^{1}H-NMR spectrum of compound **157** in $CDCl_3$.



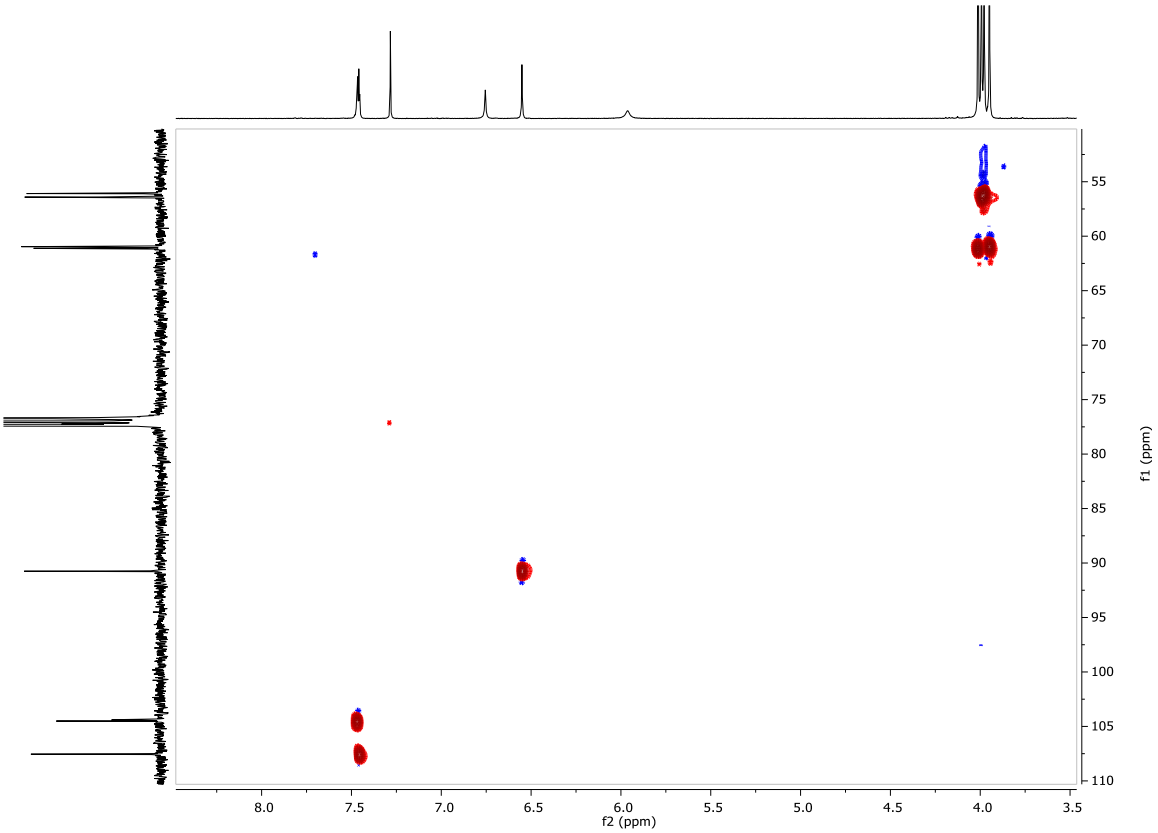
Appendix 10: The ^{13}C-NMR of compound **157** in $CDCl_3$.



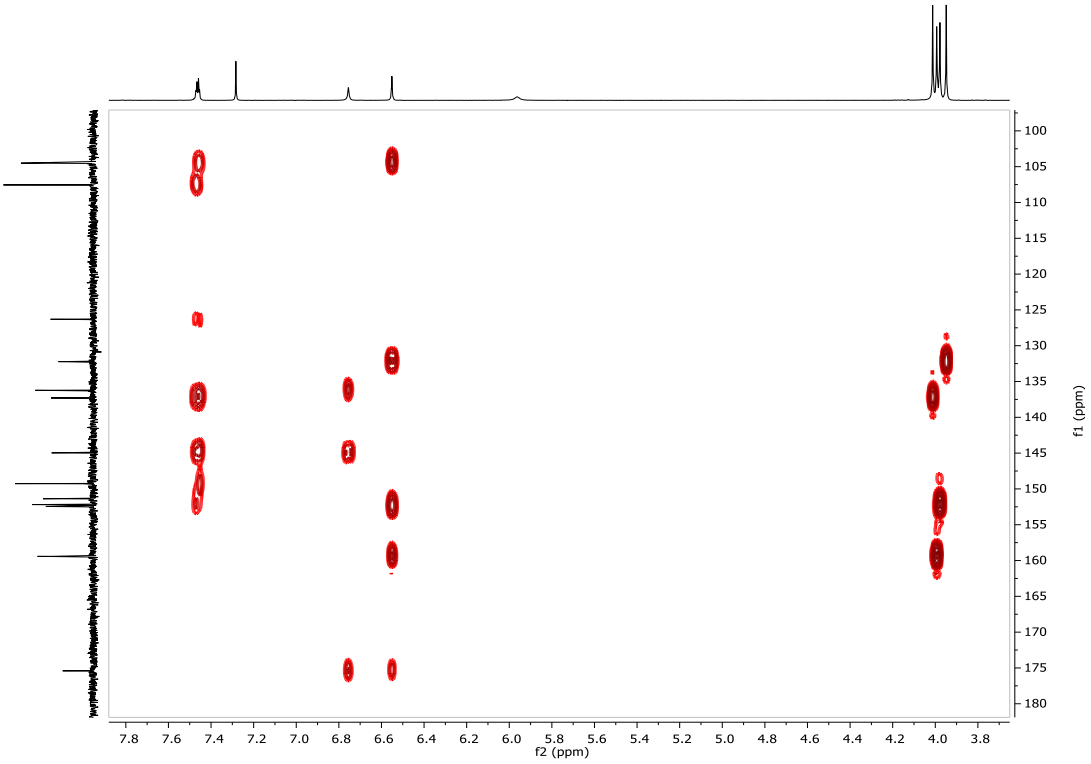
Appendix 11: The DEPT-135 NMR spectrum of compound **157** in $CDCl_3$.



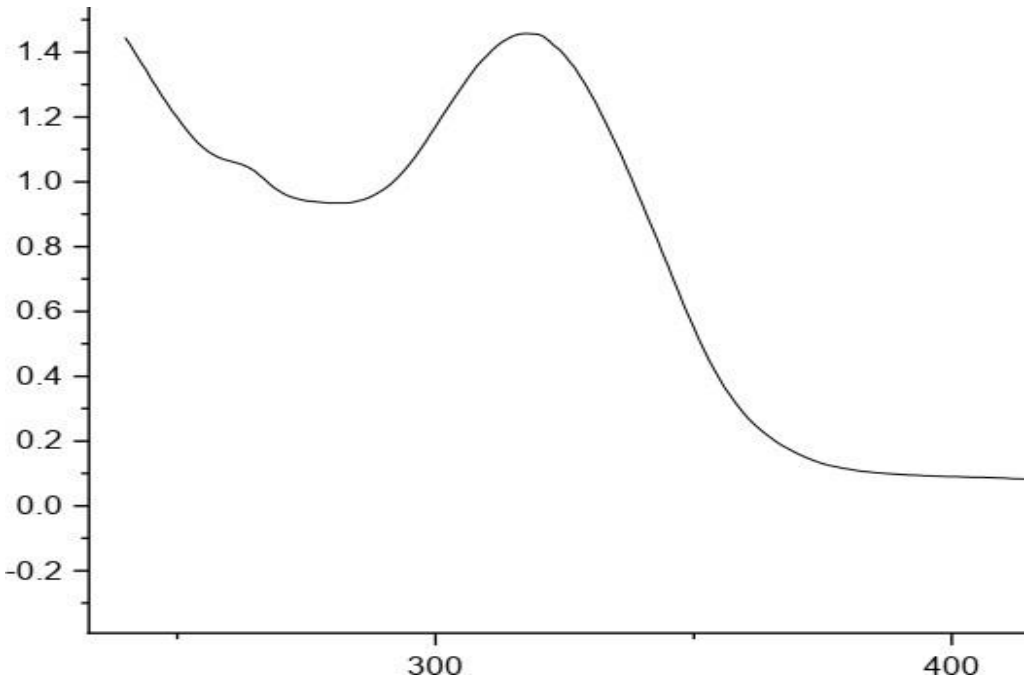
Appendix 12: The HSQC spectrum of compound **157** in $CDCl_3$.



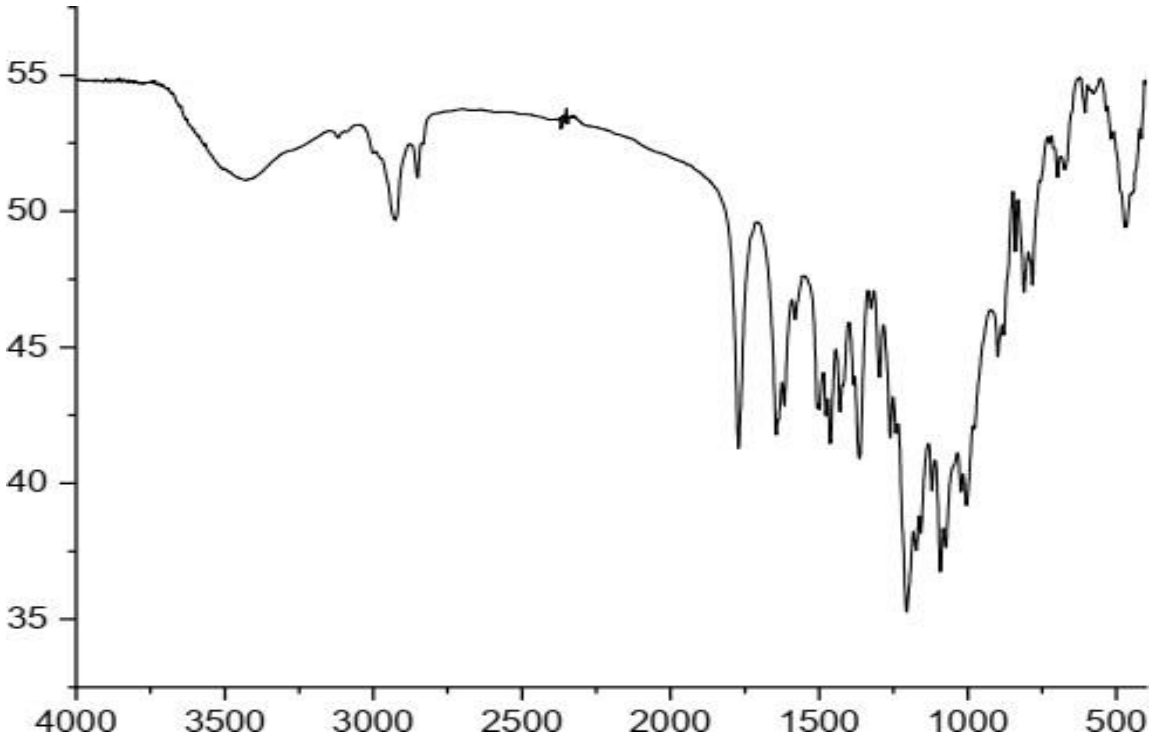
Appendix 13: The HMBC spectrum of compound **157** in $CDCl_3$.



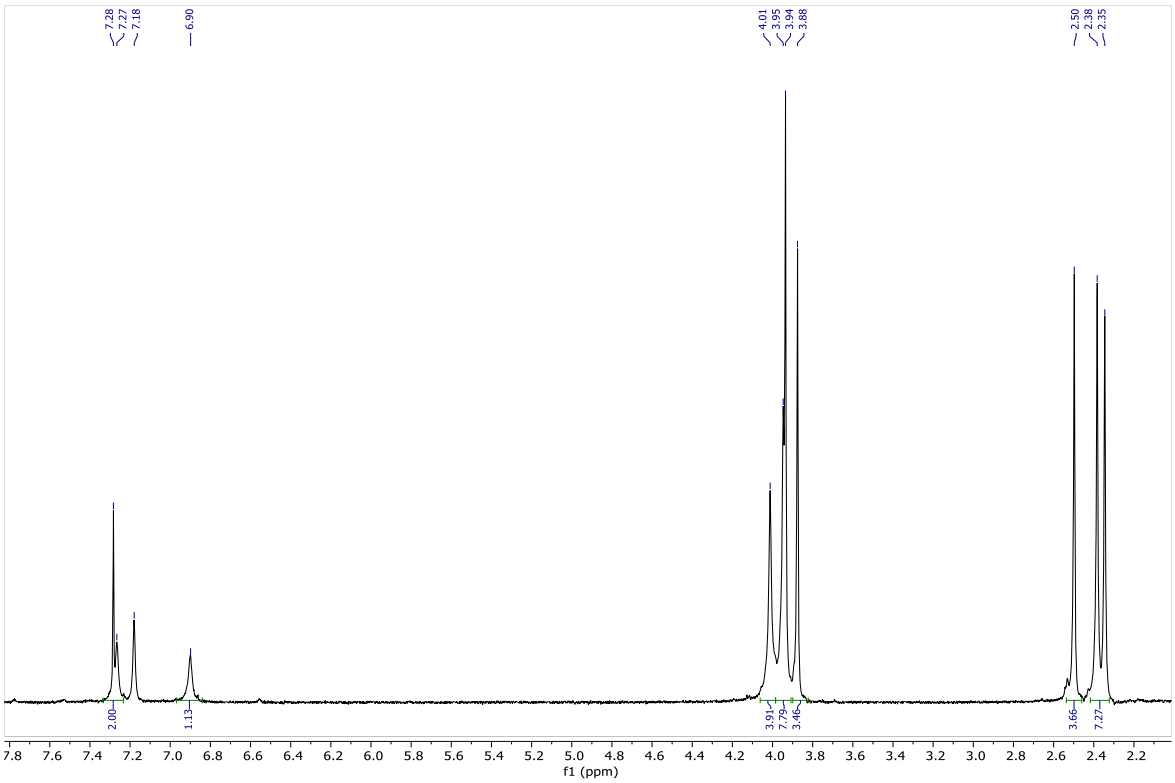
Appendix 14: The UV spectrum of compound **157-Ac** in MeOH.



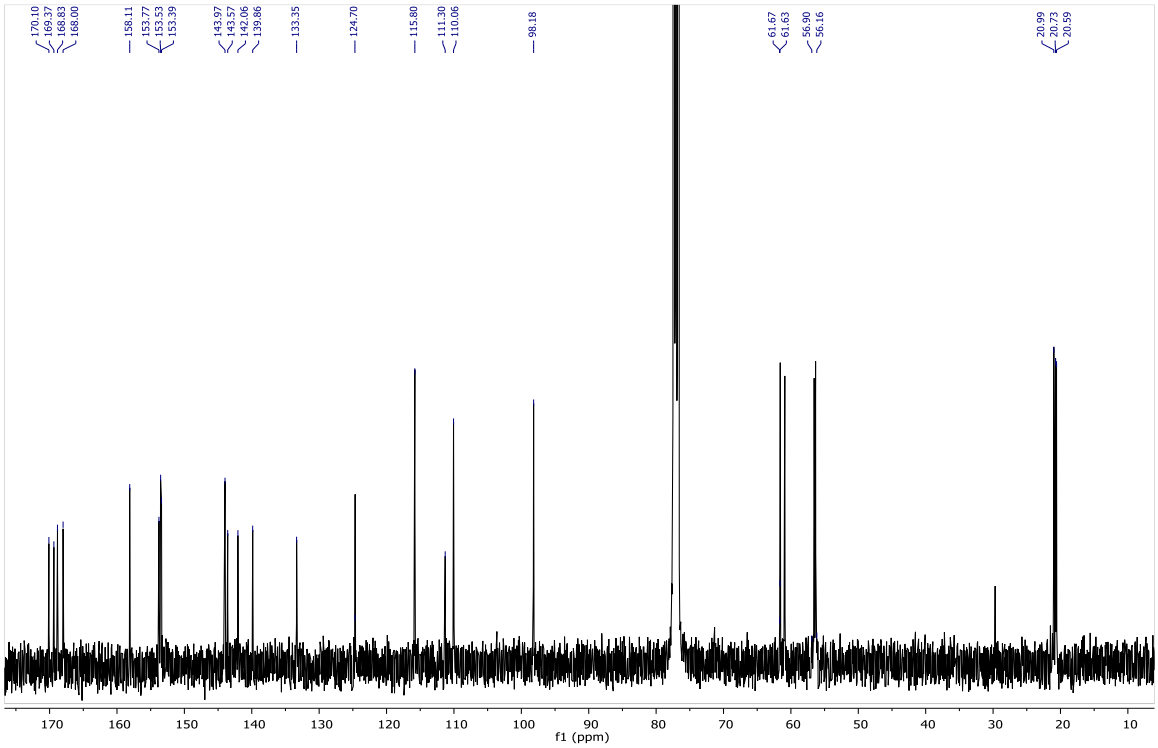
Appendix 15: The IR spectrum of compound **157-Ac**.



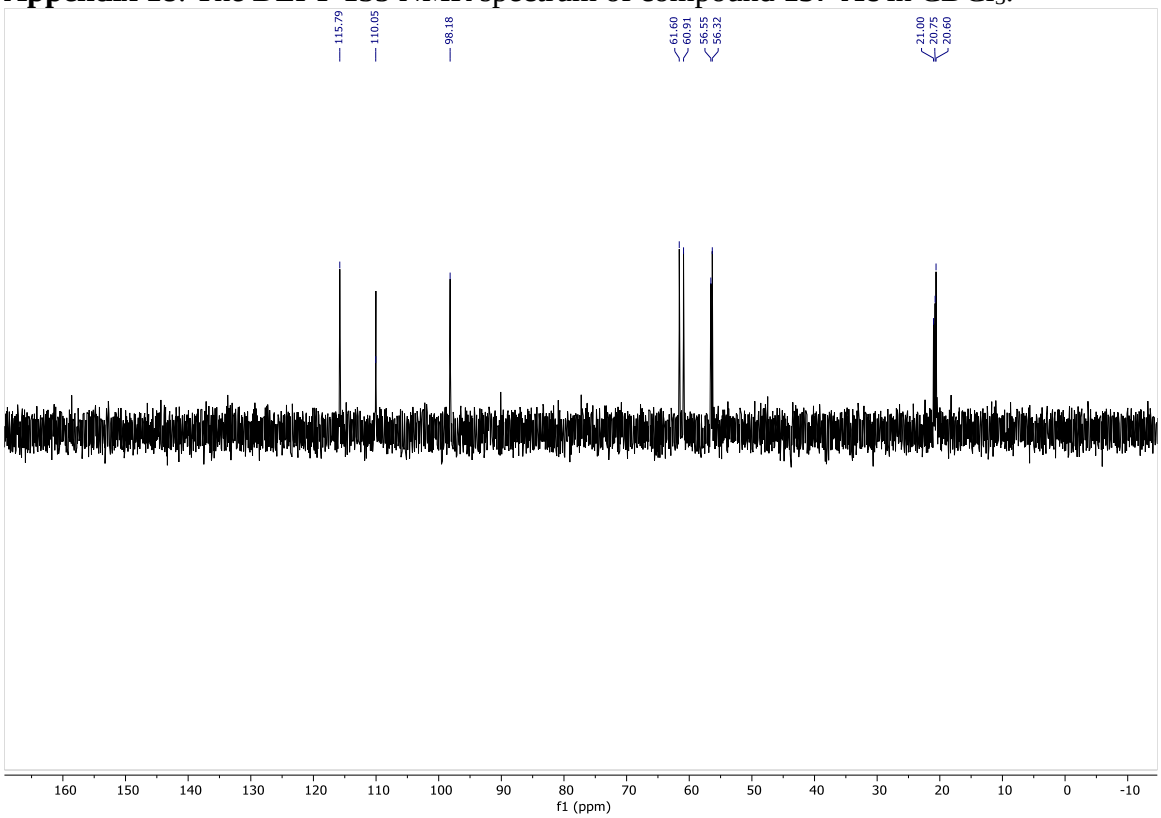
Appendix 16: The ^{1}H-NMR spectrum of compound **157-Ac** in $CDCl_3$.



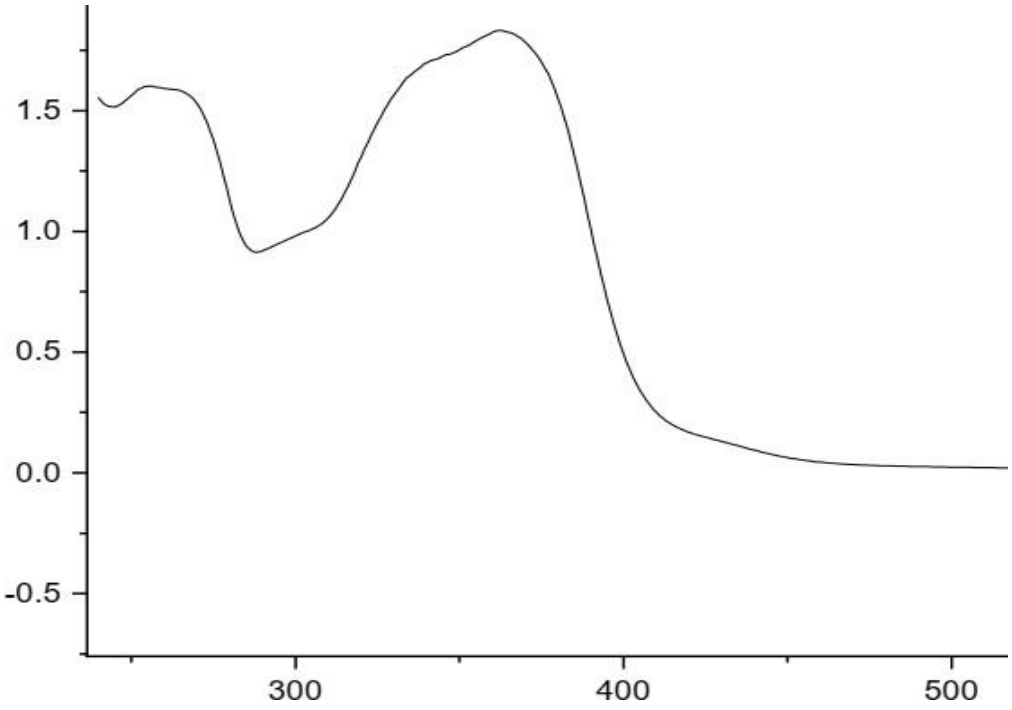
Appendix 17: The ^{13}C-NMR of compound **157-Ac** in $CDCl_3$.



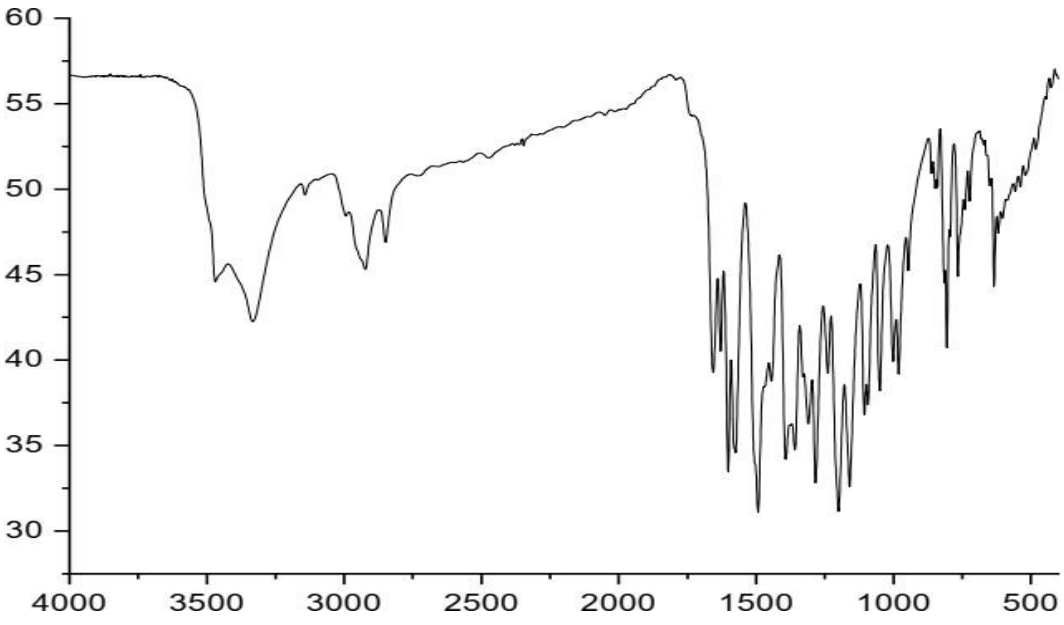
Appendix 18: The DEPT-135 NMR spectrum of compound **157-Ac** in $CDCl_3$.



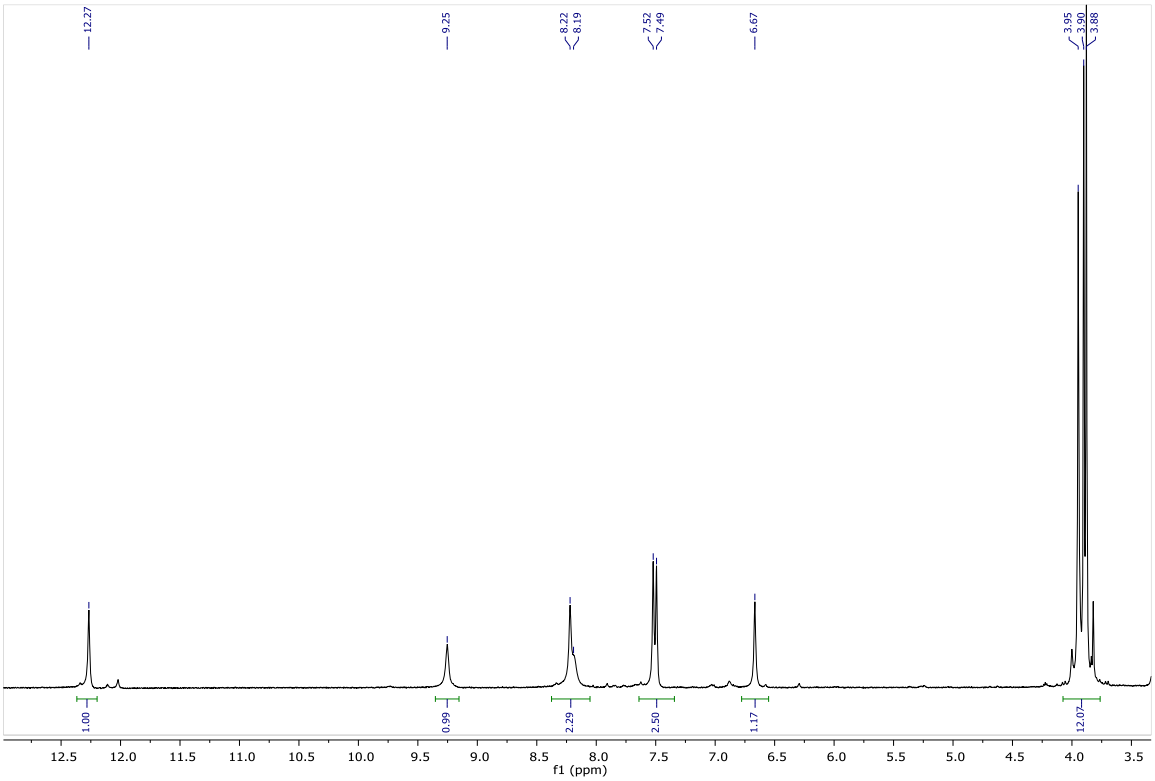
Appendix 19: The UV spectrum of compound **158** in MeOH.



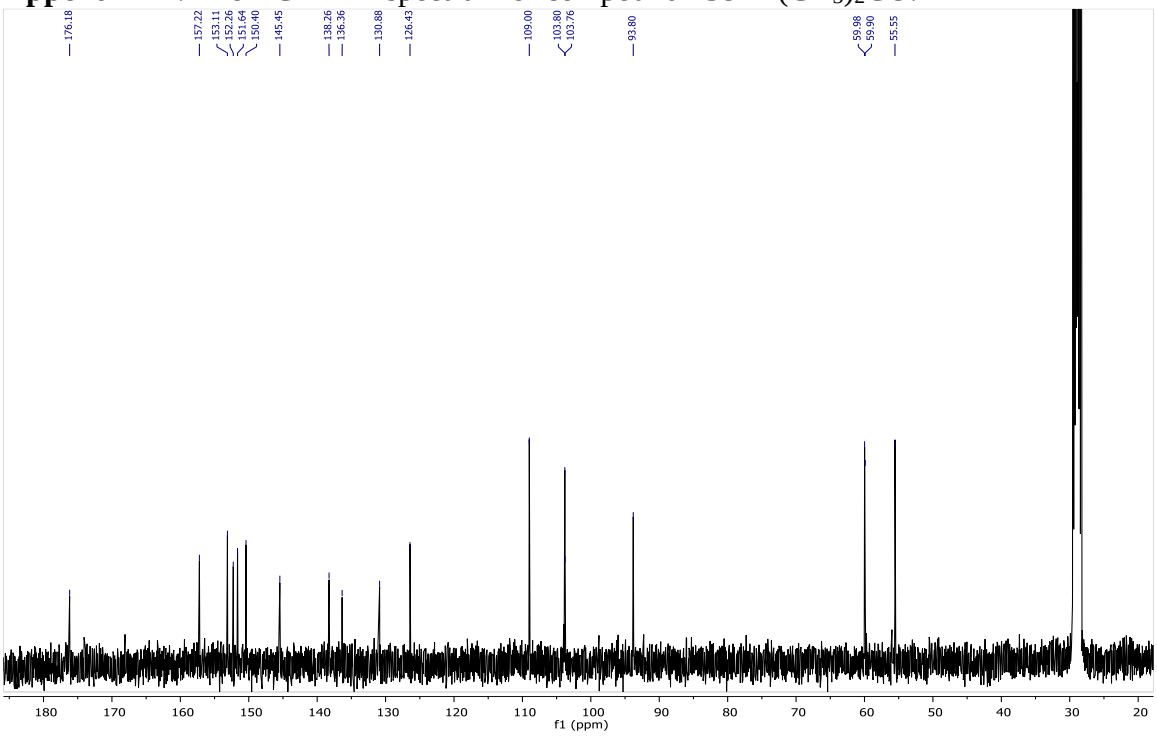
Appendix 20: The IR spectrum of compound **158**.



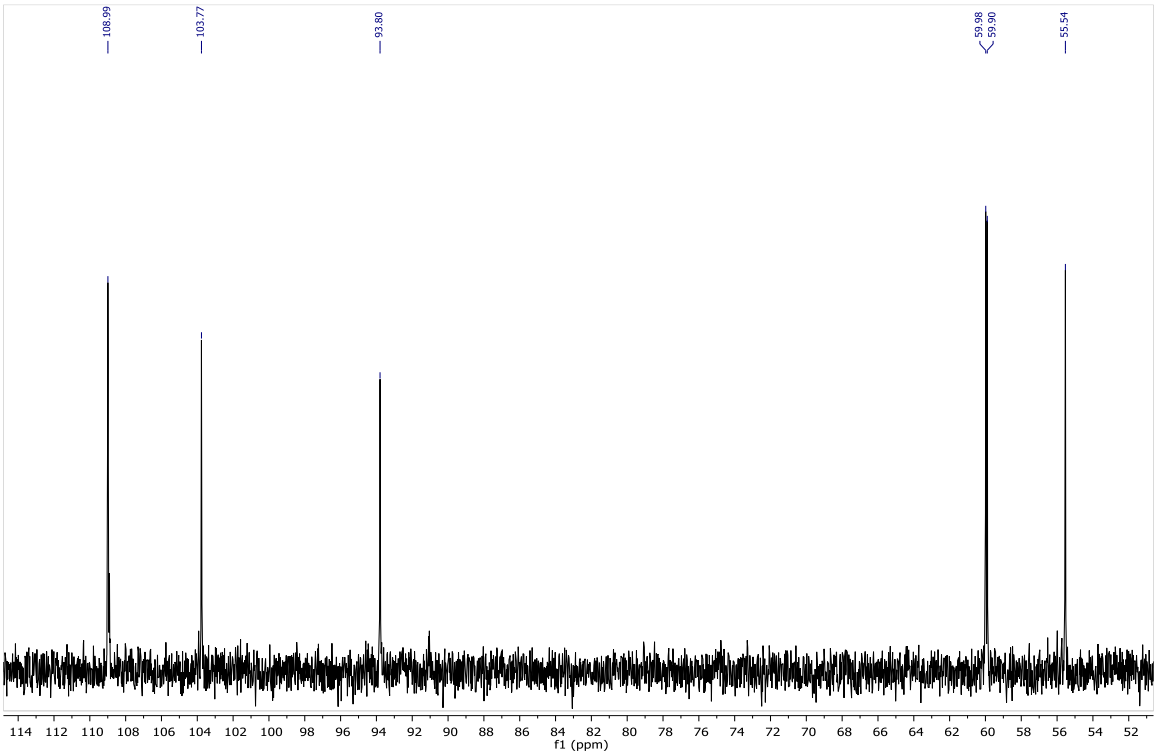
Appendix 21: The ^{1}H-NMR spectrum of compound **158** in $(CD_3)_2CO$.



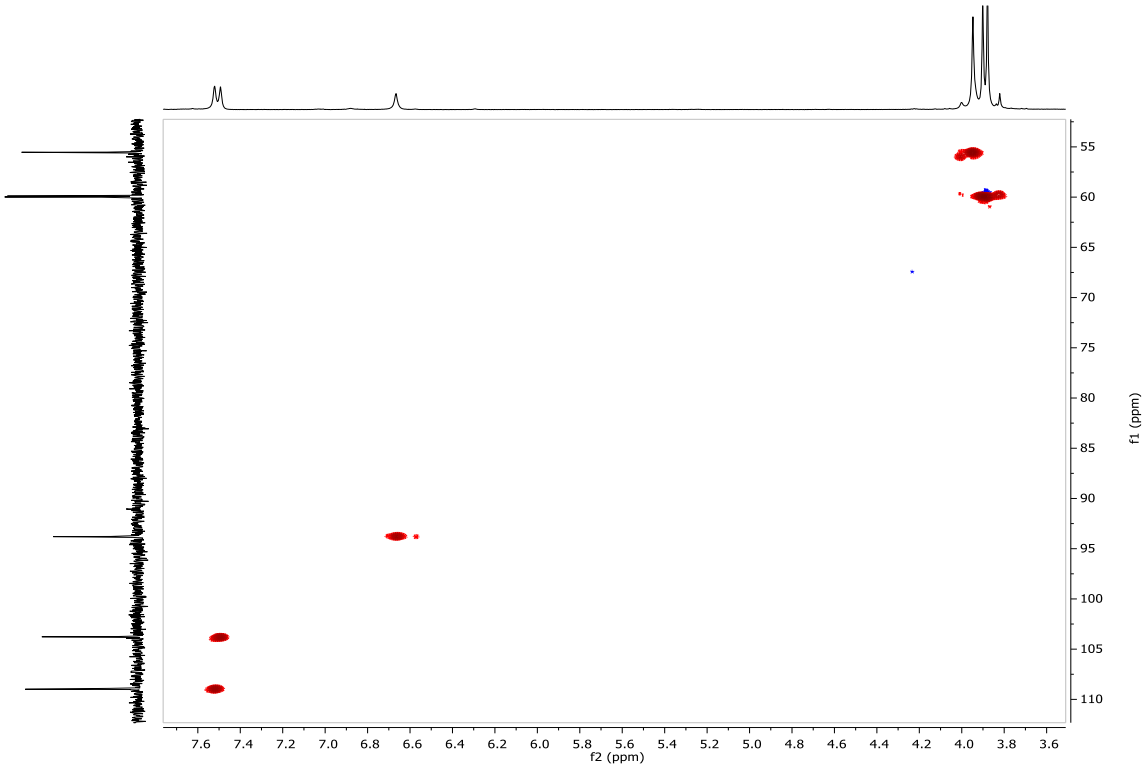
Appendix 22: The ^{13}C-NMR spectrum of compound **158** in $(CD_3)_2CO$.



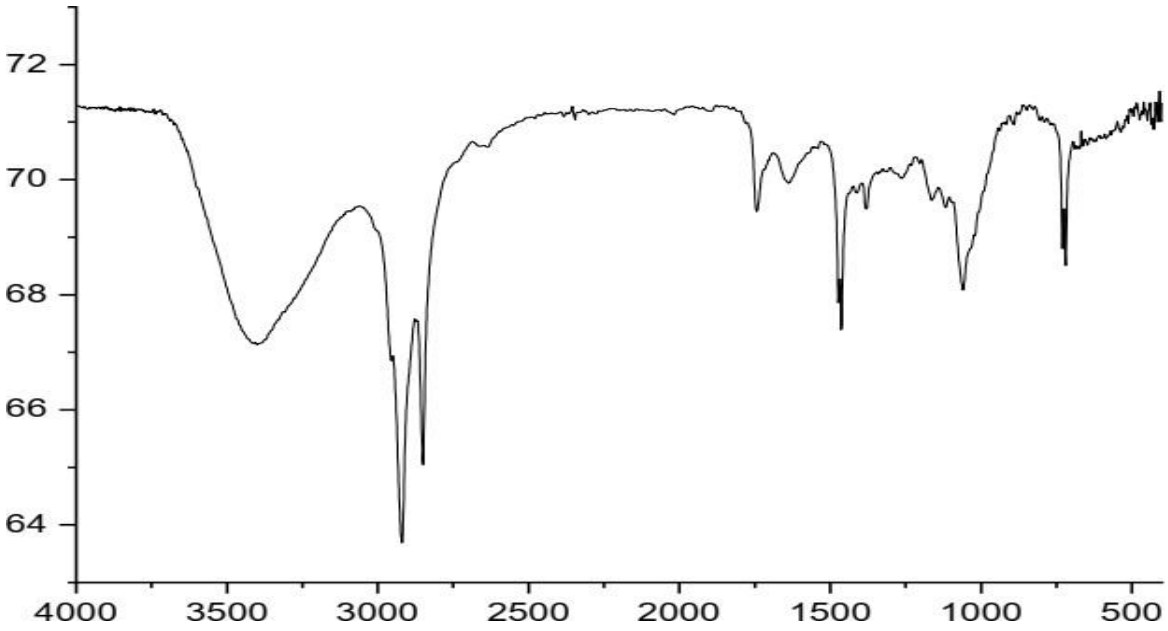
Appendix 23: The DEPT-135 of compound **158** in $(CD_3)_2CO$.



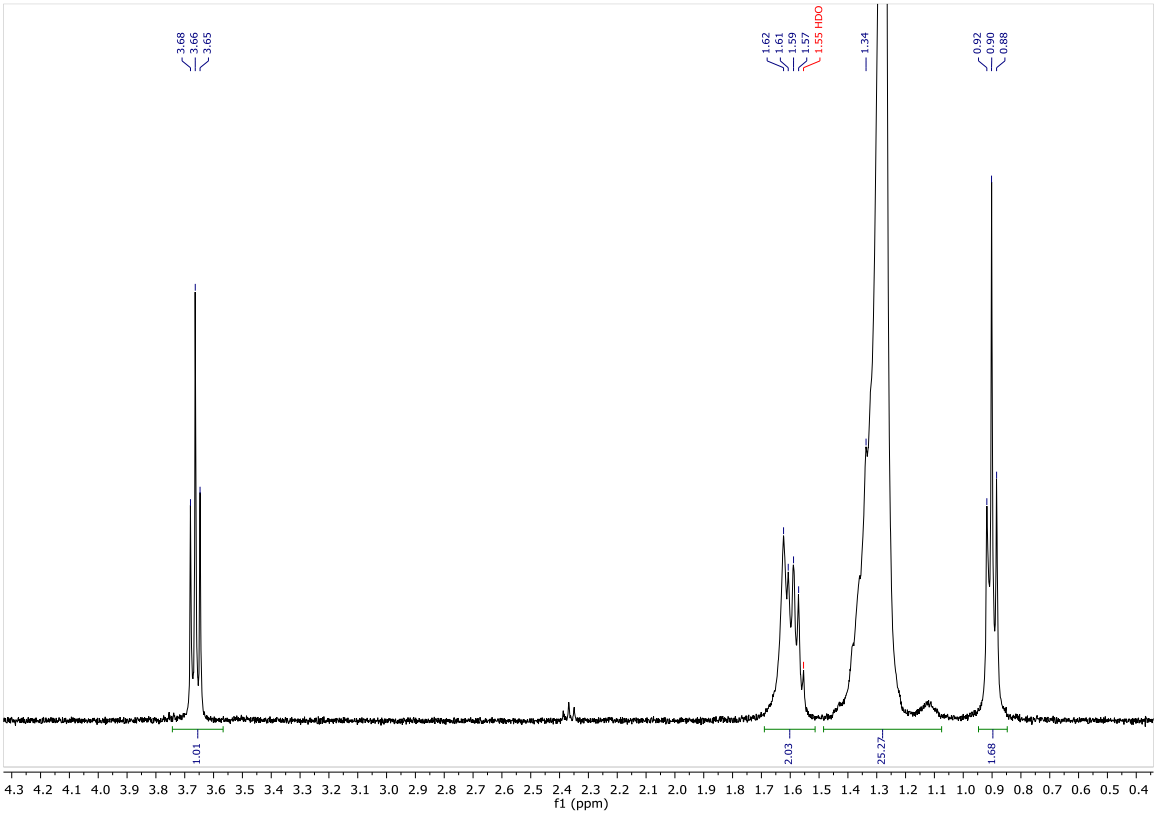
Appendix 24: The HSQC spectrum of compound **158** in $(CD_3)_2CO$.



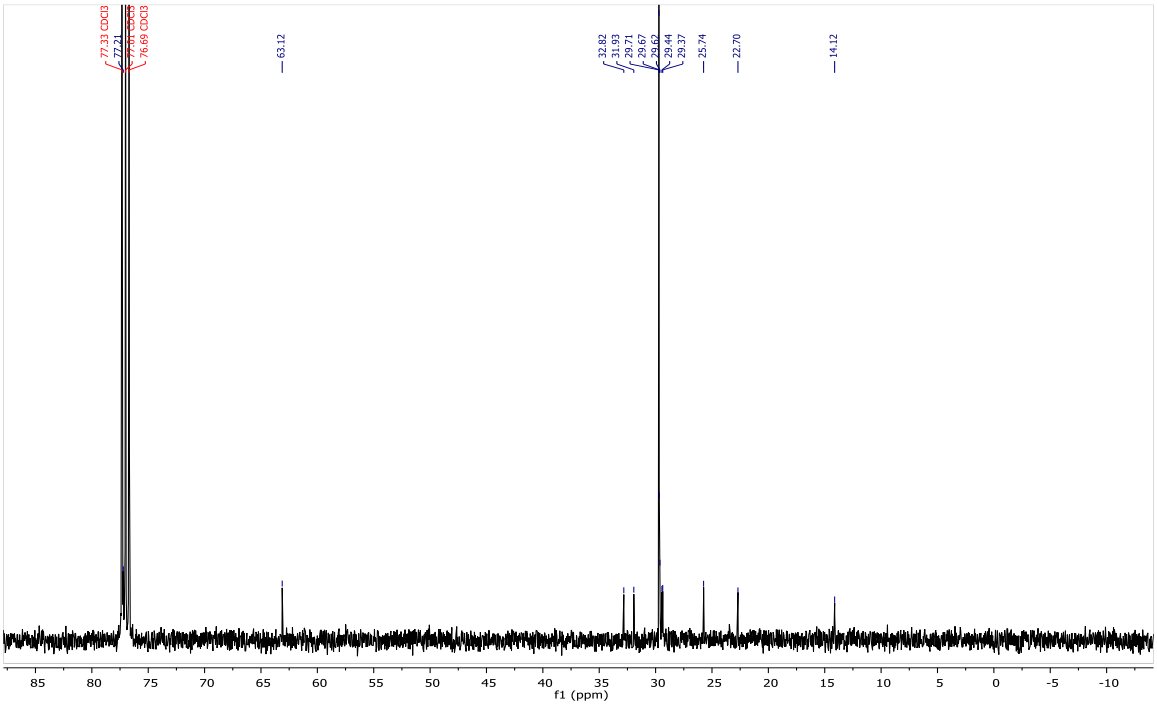
Appendix 25: The IR spectrum compound **165**.



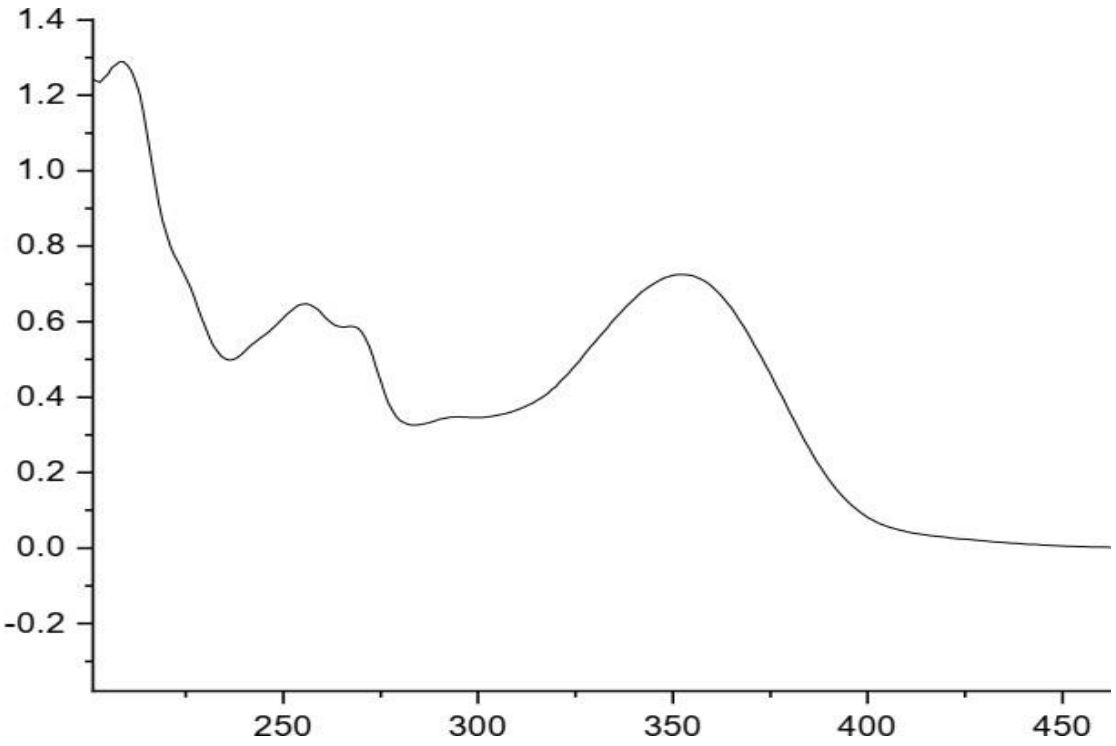
Appendix 26: The ^{1}H-NMR spectrum of compound **165** in $CDCl_3$.



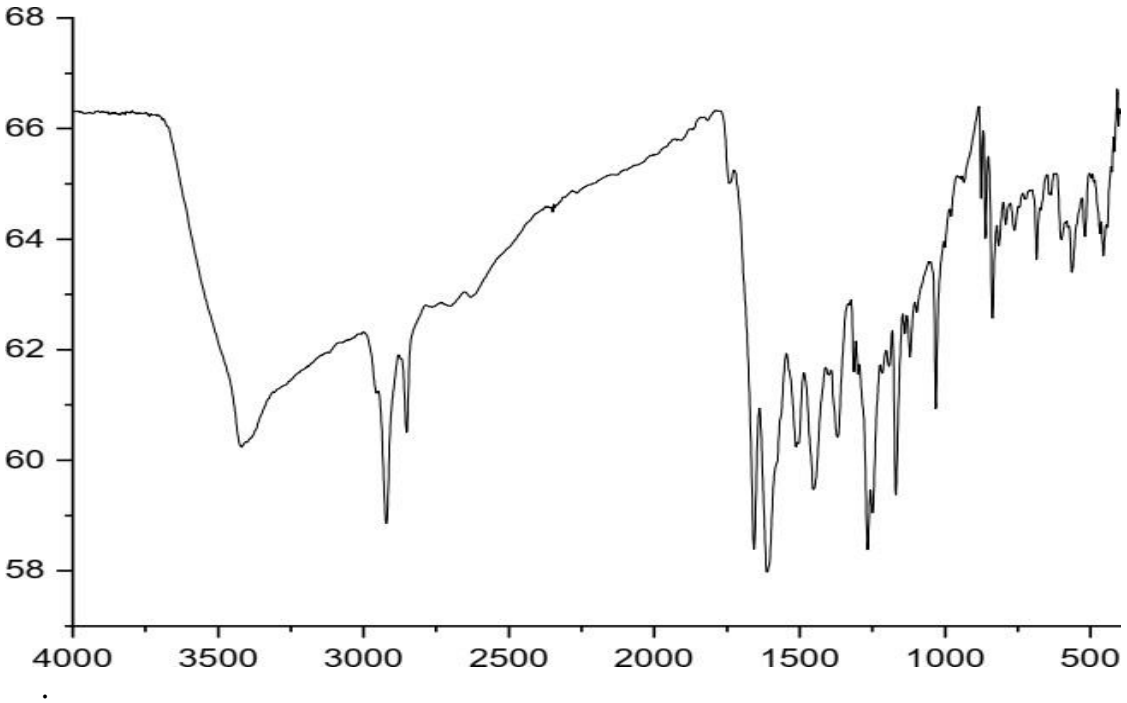
Appendix 27: The ^{13}C-NMR spectrum of compound **165** in $CDCl_3$.



Appendix 28: The UV spectrum of compound **159** in EtOH



Appendix 29: The IR spectrum of compound **159**.



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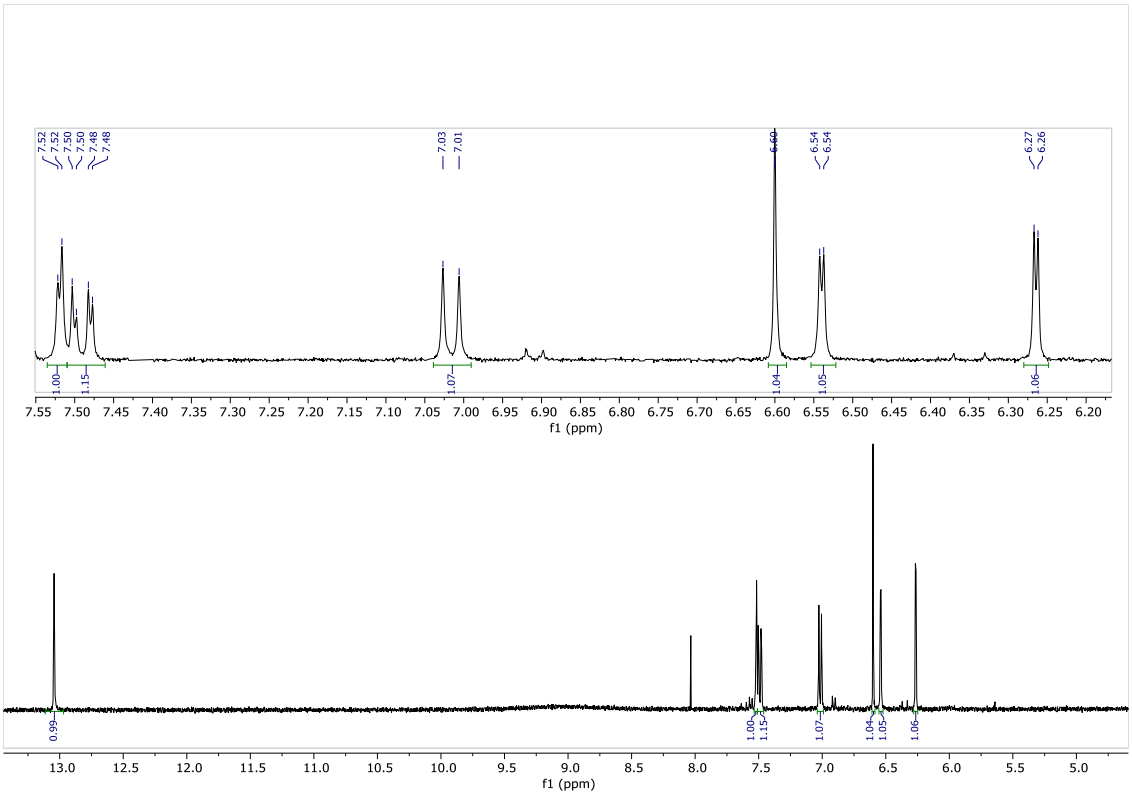
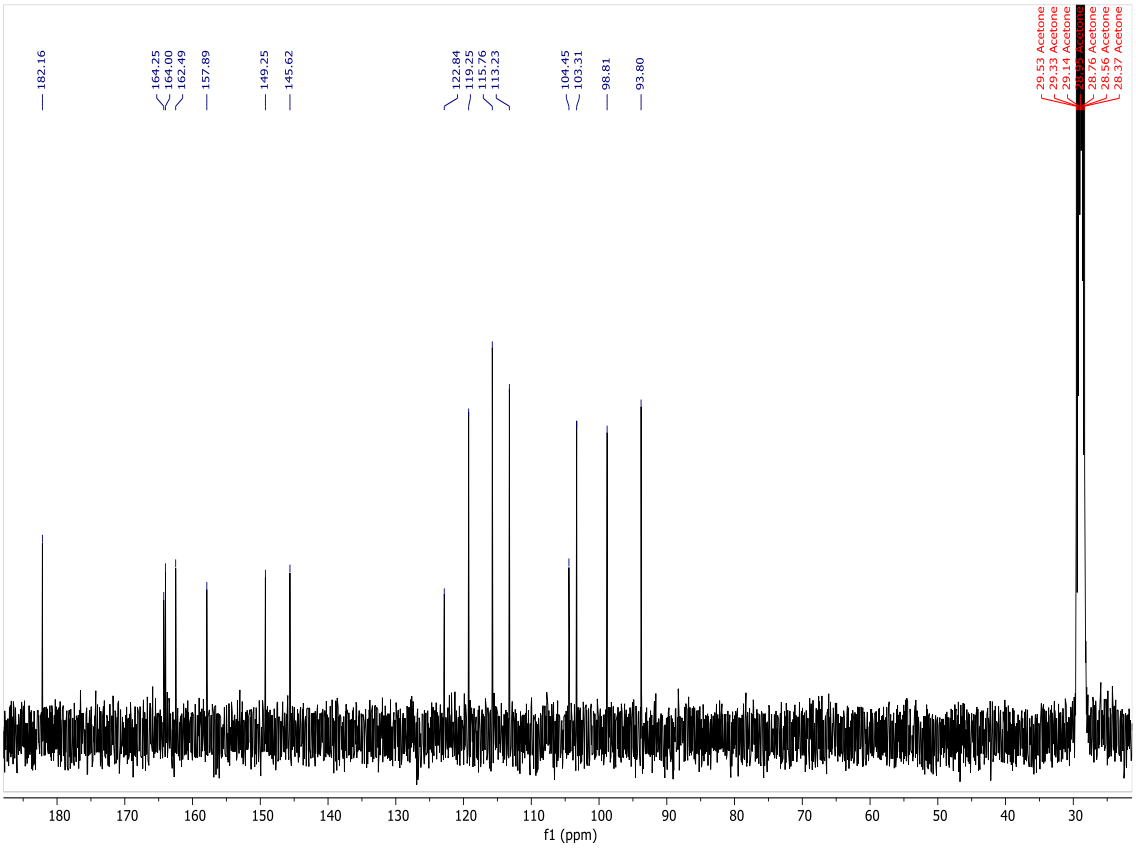
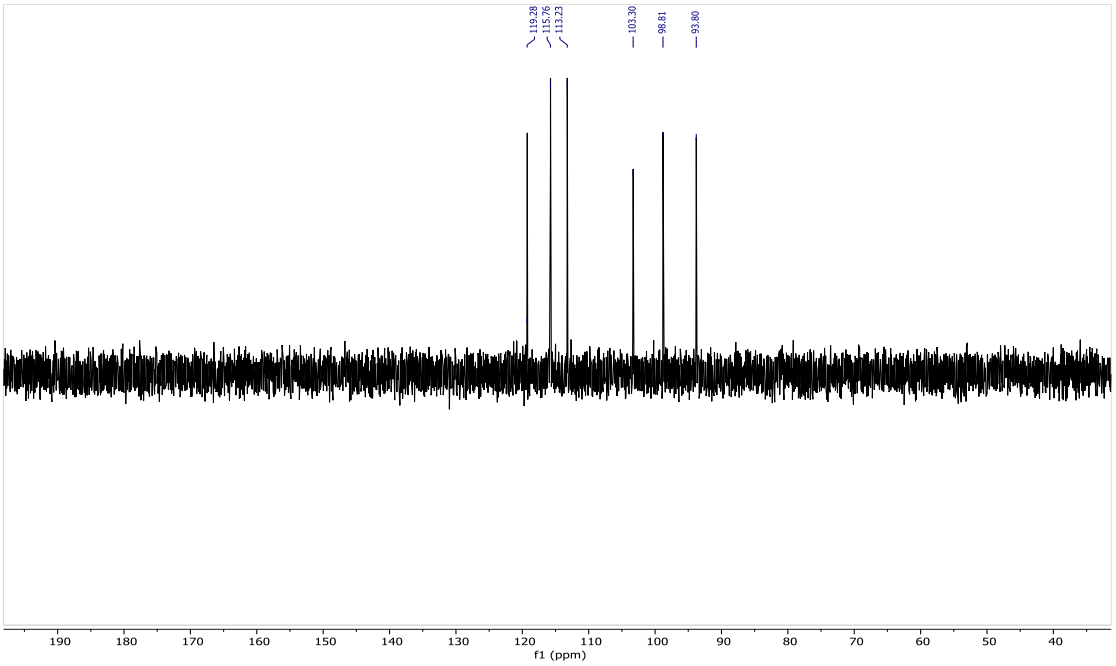
Appendix 30: The ^{1}H-NMR spectrum of compound **159** in $(CD_3)_2CO$.



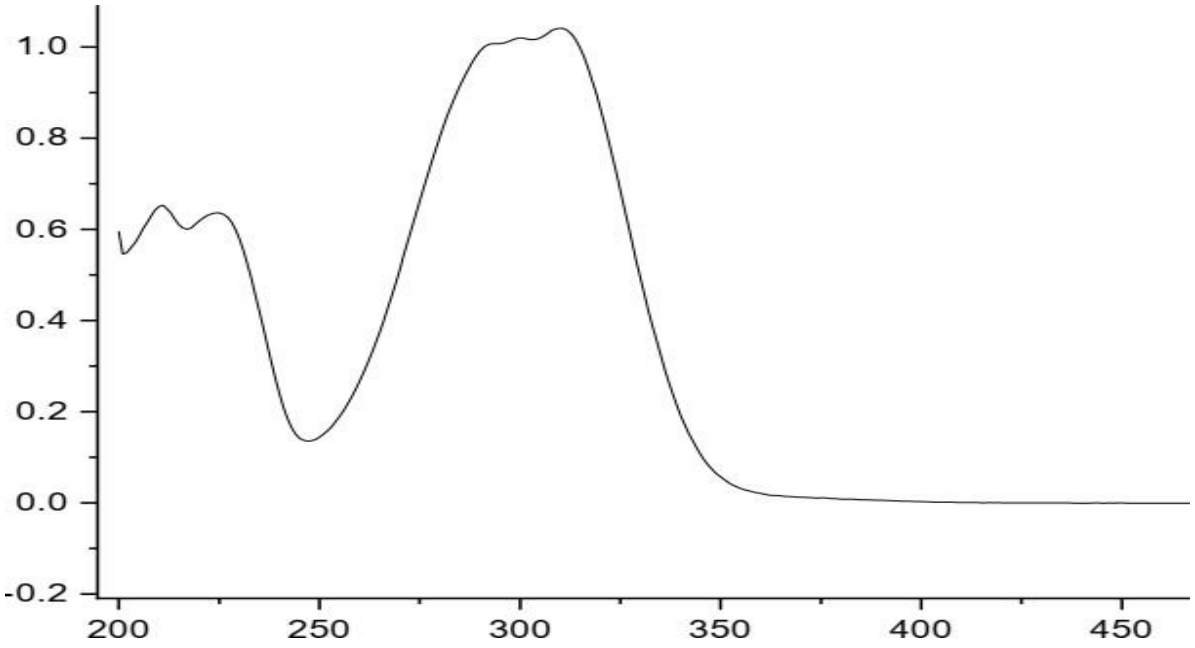
Appendix 31: The ^{13}C-NMR spectrum of compound **159** in $(CD_3)_2CO$.



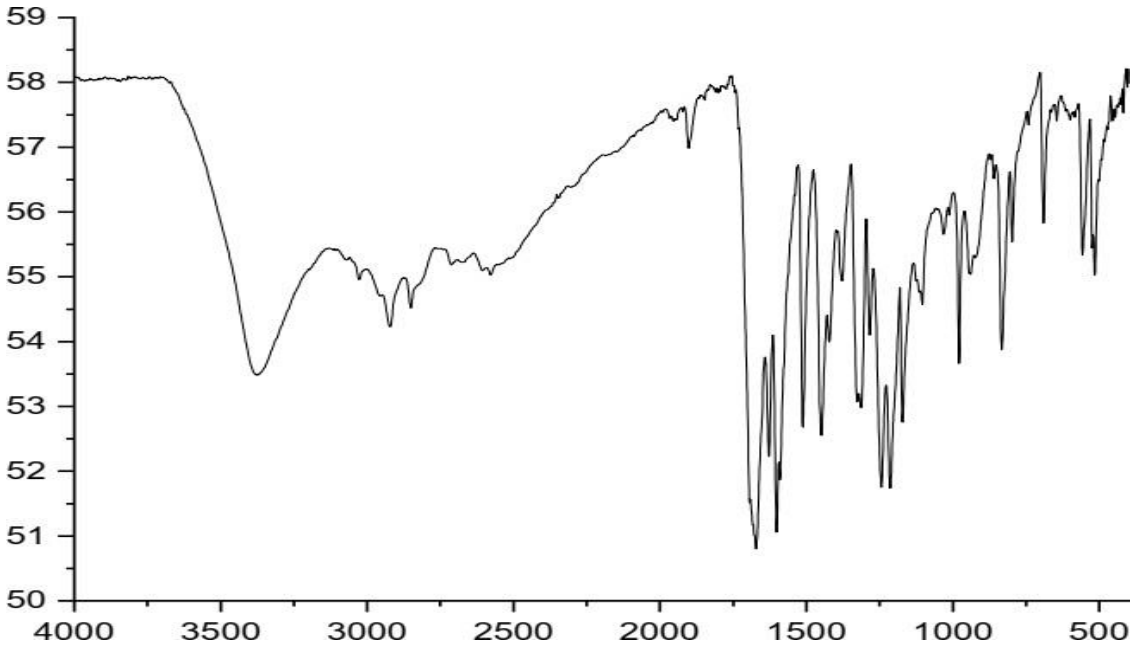
Appendix 32: The DEPT-135 NMR spectrum of compound **159** in $(CD_3)_2CO$.



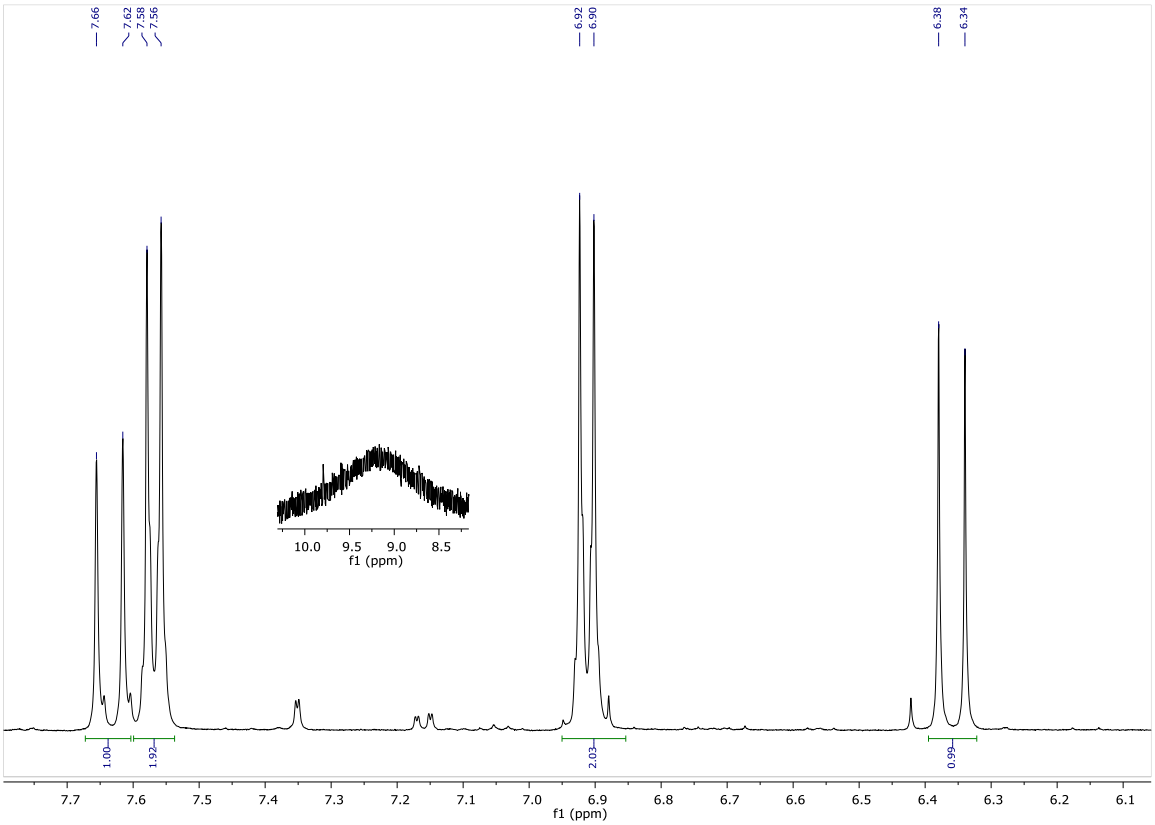
Appendix 33: The UV spectrum of compound **159** in EtOH.



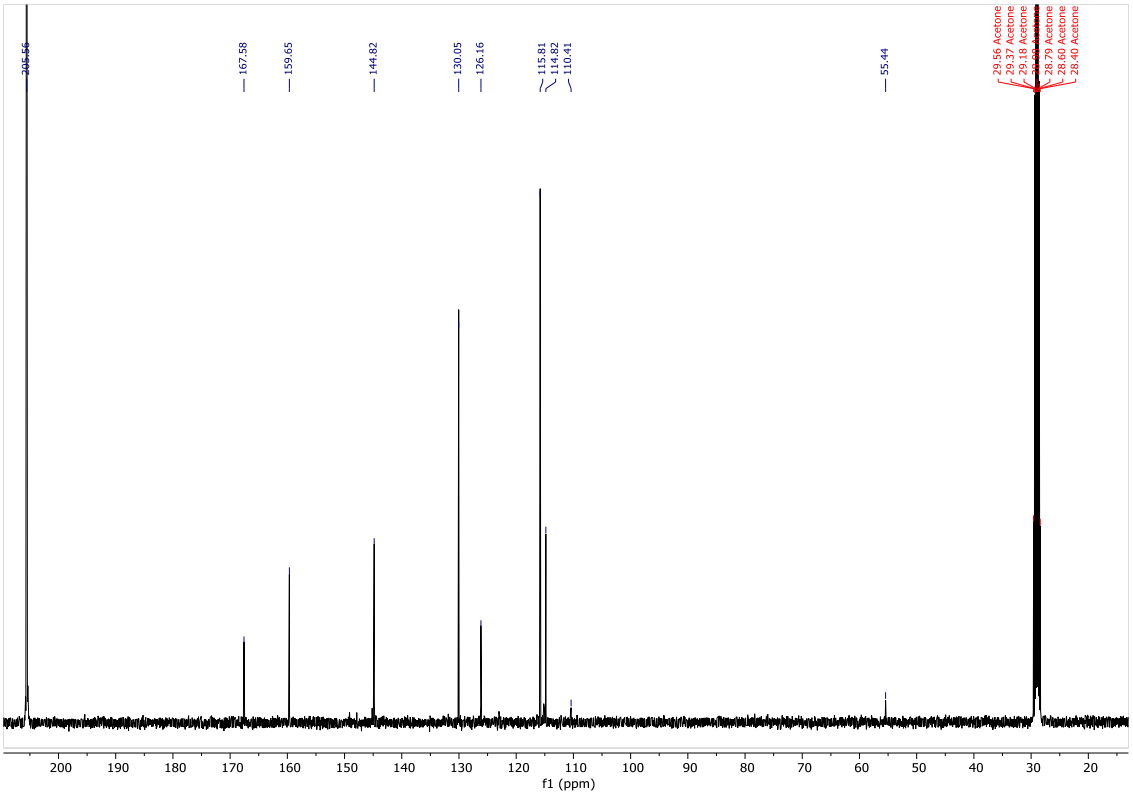
Appendix 34: The IR spectrum of compound **164**.



Appendix 35: The ^{1}H-NMR spectrum of compound **164** in $(CD_3)_2CO$.



Appendix 36: The ^{13}C-NMR spectrum of compound **164** in $(CD_3)_2CO$.



Appendix 37: The DEPT-135 spectrum of compound **164** in $(CD_3)_2CO$.