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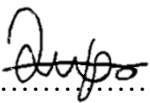
Secondary Metabolites from *Calophyllum soulattri* and *Calophyllum gracilentum* and Their Antibacterial Activity

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**Master of Science
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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.



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ABSTRACT

Antibiotic resistance has emerged as a substantial threat to global public health, necessitating an urgent investigation and evaluation of novel antimicrobial agents to combat this escalating issue. Plants are valuable resources for the development of novel pharmaceutical products. *Calophyllum* species are known for having excellent biological activities such as antimicrobial and antiviral due to their secondary metabolites, which led to the selection of two *Calophyllum* species, *Calophyllum soulattri* and *Calophyllum gracilentum* for this study. The stem bark of *C. soulattri* and *C. gracilentum* were studied for their phytochemical and biological activities. The powdered stem bark of both *Calophyllum* species were extracted at room temperature sequentially with hexane, ethyl acetate and methanol. Each of the extracts were filtered and solvents removed by rotary evaporator to give hexane, ethyl acetate and methanol extracts. The extracts were isolated using various chromatography techniques such as column chromatography and thin layer chromatography which led to the isolation of a new xanthone, soulaxanthone (**62**), along with four other known metabolites, euxanthone (**63**), calopolyanolide E (**19**), calanolide E (**3**) and friedelin (**60**). The structures of these compounds were identified and elucidated using spectroscopic techniques such as MS, IR, 1D and 2D NMR. The extracts showed inhibition against Gram-negative bacteria but showed no inhibition against Gram-positive bacteria. For compounds, there were no inhibition demonstrated against *B. cereus*, *L. plantarum* and *S. marcescens*. Ethyl acetate extract of *C. soulattri* demonstrated moderate activity against *P. aeruginosa* (MIC = 125 µg/mL). Meanwhile, the hexane extract from both plants exhibited moderate activity against *E. cloacae* (MIC = 250 µg/mL). For isolated compound, soulaxanthone (**62**) exhibited moderate activity against *P. aeruginosa*

with an MIC value of 25 µg/mL. Calopolyanolide E (**19**) and friedelin (**60**) demonstrated moderate activity against *E. cloacae* with the MIC values of 25 and 50 µg/mL, respectively. Both compounds showed bactericidal activity against *E. cloacae* with the similar value to ampicillin (MBC = 50 µg/mL). The significance of outcomes from this study is the isolation of a new xanthone, soulaxanthone from the stem bark of *C. soulattri*. For the future study, soulaxanthone could be isolated with higher percentage of yield by doing standardise method.

Keywords: *Calophyllum soulattri*, *Calophyllum gracilentum*, xanthone, soulaxanthone, antibacterial

Metabolit Sekunder daripada Calophyllum soulattri dan Calophyllum gracilentum dan Aktiviti Antibakterianya

ABSTRAK

Rintangan antibiotik telah muncul sebagai ancaman besar kepada kesihatan awam global, yang memerlukan penyelidikan segera dan penilaian agen antimikrob baharu untuk memerangi isu yang semakin meningkat ini. Tumbuhan adalah sumber yang berharga untuk pembangunan produk farmaseutikal baharu. Spesies Calophyllum terkenal dengan aktiviti biologi yang sangat baik seperti antimikrob dan antivirus disebabkan oleh metabolit sekundernya, yang membawa kepada pemilihan dua spesies Calophyllum, Calophyllum soulattri dan Calophyllum gracilentum untuk kajian ini. Kulit batang C. soulattri dan C. gracilentum telah dikaji untuk aktiviti fitokimia dan biologi mereka. Serbuk kulit batang kedua-dua spesies Calophyllum telah diekstrak pada suhu bilik secara berurutan dengan heksana, etil asetat dan metanol. Setiap ekstrak telah ditapis dan pelarut dikeluarkan oleh penyejat berputar untuk memberikan ekstrak heksana, etil asetat dan metanol. Ekstrak telah diasingkan dengan menggunakan pelbagai teknik kromatografi seperti kromatografi lajur dan kromatografi lapisan nipis yang membawa kepada pengasingan xanton baharu, soulaxanthone (62), bersama empat metabolit lain yang diketahui, euxanthone (63), calopolyanolide E (19), calanolide E (3) dan friedelin (60). Struktur sebatian ini dikenal pasti dan dijelaskan menggunakan teknik spektroskopi seperti MS, IR, 1D dan 2D NMR. Ekstrak menunjukkan perencatan terhadap bakteria Gram-negatif tetapi tidak menunjukkan perencatan terhadap bakteria Gram-positif. Untuk sebatian, tiada perencatan yang ditunjukkan terhadap B. cereus, L. plantarum dan S. marcescens. Ekstrak etil asetat C. soulattri menunjukkan aktiviti sederhana terhadap P.

aeruginosa (MIC = 125 $\mu\text{g/mL}$). Manakala, ekstrak heksana daripada kedua-dua tumbuhan menunjukkan aktiviti yang sederhana terhadap E. cloacae (MIC = 250 $\mu\text{g/mL}$). Manakala, untuk sebatian yang diasingkan, soulaxanthone (62) menunjukkan aktiviti yang sederhana terhadap P. aeruginosa dengan nilai MIC 25 $\mu\text{g/mL}$. Calopolyanolide E (19) dan friedelin (60) menunjukkan aktiviti sederhana terhadap E. cloacae dengan nilai MIC masing-masing 25 dan 50 $\mu\text{g/mL}$. Kedua-dua sebatian menunjukkan aktiviti bakteria terhadap E. cloacae dengan nilai yang serupa dengan ampicillin (MBC = 50 $\mu\text{g/mL}$). Kepentingan hasil daripada kajian ini ialah pengasingan xanton baharu, soulaxanthone daripada kulit batang C. soulattri. Untuk kajian masa depan, soulaxanthone boleh diasingkan dengan peratusan hasil yang lebih tinggi dengan melakukan kaedah piawai.

Kata kunci: Calophyllum soulattri, Calophyllum gracilentum, xanton, soulaxanthone, antibakteria

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
AMR	Antimicrobial resistance
ANOVA	Analysis of Variance
AR	Analytical Research
BaCl ₂	Barium chloride
br. s	Broad singlet
CDCl ₃	Deuterated chloroform
CD ₃ COCD ₃	Deuterated acetone
CH ₂	Methylene
CH ₃	Methyl
C ₂ H ₄	Ethylene
C=C	Alkene
COOH	Carboxyl group
COOMe	Methoxycarbonyl group
COSY	Correlation spectroscopy
C=O	Carbonyl group
DEPT	Distortionless Enhancement by Polarisation Transfer
DMSO	Dimethyl sulfoxide
d	Doublet
dd	Doublet of doublets

EIMS	Electron Ionisation Mass Spectroscopy
FeCl ₃	Iron(III) chloride
FTIR	Fourier-Transform Infrared Spectroscopy
GCC	Gravity Column Chromatography
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High-Performance Liquid Chromatography
HSD	Honestly Significant Difference
HSQC	Heteronuclear Single Quantum Coherence
HREIMS	High-Resolution Electron Ionisation Mass Spectroscopy
H ₂ SO ₄	Sulphuric acid
<i>J</i>	Coupling constant in Hertz
LC-MS	Liquid chromatography-Mass Spectroscopy
MBC	Minimum Bactericidal Concentration
MeOH	Methanol
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MSSA	Methicillin-susceptible <i>Staphylococcus aureus</i>
m	Multiplet
<i>m/z</i>	Mass-to-charge ratio
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Effect

OAc	Acetoxy group
OH ⁻¹	Hydroxide
-OH	Hydroxyl group
ppm	Part per million
q	Quartet
s	Singlet
TLC	Thin Layer Chromatography
t	Triplet
UV-Vis	Ultraviolet-Visible
1D NMR	One Dimension Nuclear Magnetic Resonance
2D NMR	Two Dimension Nuclear Magnetic Resonance
¹ H NMR	Proton Nuclear Magnetic Resonance
¹³ C NMR	Carbon Nuclear Magnetic Resonance
δ _C	Chemical shift of carbon
δ _H	Chemical shift of hydrogen
λ _{max}	Lambda max

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Antimicrobial resistance (AMR) is currently considered a significant global health menace, presenting substantial obstacles to prevent and treat chronic diseases (Prestinaci et al., 2015; Sharma et al., 2022). Numerous bacterial resistances should be a cause for concern, including the fact that it frequently leads to treatment failure and can have profound implications, particularly in critically ill patients (Tenover, 2006). The capability of bacteria to evolve resistance to antibacterial agents complicates the treatment of bacterial infections. Antimicrobial medicines that are efficient are essential for both curative and preventive therapies, protecting patients from possibly fatal diseases and allowing complex procedures such as chemotherapy and surgery to be performed securely. Therefore, the development of antibiotics is regarded as one of the most significant advances in modern science.

People have utilised plants to treat and cure illnesses since the beginning of civilisation (Jamshidi-Kia et al., 2018). Antimicrobials, including antivirals, antibiotics, antiparasitic and antifungals are medicines utilised to inhibit and cure infectivity in humans, animals and plants. The genus *Calophyllum* has been identified as a promising source of lead compounds for pharmacological research and development. *Calophyllum* species is rich in secondary metabolites with a distinct spectrum of pharmacological activities (Zailan et al., 2022). Xanthones, coumarins, triterpenes and flavonoids are examples of secondary metabolites that occur in this genus. Several studies have shown that

secondary metabolites from *Calophyllum* possess a broad range of interesting biological properties such as anti-inflammatory (Sundur et al., 2014), antibacterial (Aminudin et al., 2016), anticancer (Kurniawan et al., 2021), antioxidant (Abbas, 2019) and antiviral (Sundur et al., 2014). Numerous genus *Calophyllum* plants are utilised as folk medicines to cure health conditions such as infections, inflammation, malaria, tumours, eye diseases, and hypertension. In Vietnam, *Calophyllum inophyllum* seed oil has been utilised as a traditional medicine to cure insomnia, burns and rheumatic diseases (Nguyen et al., 2017). Zou et al. (2005) stated that the stem and bark of *Calophyllum membranaceum* have been used in Chinese folk medicine for the treatment of arthritis, wounds and lumbago. In Brazil, *Calophyllum brasiliense* is used to treat inflammation, ulcers and varicose haemorrhoids (Filho et al., 2009).

One of the species chosen for this study, *Calophyllum soulattri*, has been reported to possess numerous secondary metabolites such as soulamarin (Ee et al., 2011), soulattrin (Mah et al., 2011), phylattrin (Mah et al., 2012), airlanggins A and B (Tanjung et al., 2018). Previous study by Khan et al. (2002) stated that the methanol extract of leaves, stem and root barks from *C. soulattri* were fractionated with petrol, dichloromethane and ethyl acetate. 13 Gram-positive and 12 Gram-negative bacteria were used for antibacterial assay. All the extractives showed a range of activities against all the tested bacteria. Petrol fraction of the root bark demonstrated stronger antibacterial activity than the other fractions. Another species chosen for this study is *Calophyllum gracilentum*. However, *Calophyllum gracilentum* is a new species and there is no antimicrobial activity being done.

1.2 Genus *Calophyllum*

The genus *Calophyllum* is classified under the Calophyllaceae family. This genus consists of about 180 to 200 species, with some exclusively found only in the tropical rainforest of Sarawak, Malaysia (Lim et al., 2019). The habitat of the species ranges from drier regions at higher elevations to the lowlands' moist tropical rainforest (Gupta & Gupta, 2020). The Malay local name for the *Calophyllum* genus is “bintangur” (Whitemore, 1973). According to Gupta and Gupta (2020), the genus possesses an array of distinctive taxonomical characteristics, such as opposing leaves with closely parallel veins and the outer bark with red-coloured and diamond-shaped fissures.

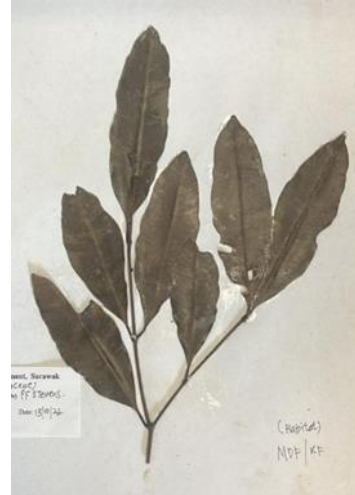
Calophyllum soulattri is a species of flowering plant which can be found in Southeast Asia, northern Australia and the Solomon Islands (Pelser et al., 2011 onward). It is a tall tree which can grow up to a height of 20 m and the length of branch-free stem is up to 35 m. The texture of its wood is slightly rough to rough, and uneven. Meanwhile, the direction of wood fibre is highly integrated and sometimes wavy (Kaliky et al., 2020).

Calophyllum gracilentum is one of the *Calophyllum* species that grows in Malaysia. The native range of this species is in Peninsular Malaysia (Kew, 2024). This tree normally grows in lowland dipterocarp forests and swampy areas. The leaves are elliptical with dark green colour. The flowers of this species are fragrant, with white or cream colour. It has round drupe fruits with hard shells (Kew, 2024).

Figure 1.1 below displays the leaves of *Calophyllum soulattri* (NParks Flora & Fauna Web, 2020) and *Calophyllum gracilentum*.



(A)



(B)

Figure 1.1: (A) The leaves of *Calophyllum soulattri*, (B) The leaves of *Calophyllum gracilentum*

1.3 Problem statement

Nowadays, the latest efficient antimicrobial compounds are important to be created as pathogens evolve resistance toward medicine. For instance, methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as the major general antimicrobial resistant bacteria (AMR) (Alghamdi et al., 2023). MRSA has developed resistance to beta-lactam drugs like penicillin (methicillin and oxacillin) and cephalosporin (Kırmusaoğlu 2017, Vestergaard et al., 2019; Nandhini et al., 2022). Medicinal plants are abundant in secondary metabolites and are widely utilised as drug in the pharmaceutical industry. Malaysia is rich in flora and fauna which have high biological activities and may have probable to be used as a drug. However, most of the flora and fauna in Malaysia have not yet been discovered. Numerous plants of the genus *Calophyllum* are used as traditional medicine. For example, seed oil from *Calophyllum inophyllum*, *Calophyllum apetalum* and *Calophyllum soulattri* has been used for leprosy, skin infection, rheumatic problems and pain (Watt, 2014; Sundur et al., 2016). Hence, it leads to investigating secondary metabolite and antibacterial activities of two *Calophyllum* species: *Calophyllum soulattri* and *Calophyllum gracilentum*. It is believed that the secondary metabolites from *Calophyllum* species possess various therapeutic activities and interact directly with cell membranes, receptors and nucleic acids, leading to antibacterial activities. These secondary metabolites can disrupt bacterial cell membranes, leading to leakage of cellular contents and cell death. Besides, they can inhibit essential bacterial enzymes and interfere with DNA replication and protein synthesis (Mitra et al., 2023). Studies have demonstrated the effectiveness of *Calophyllum* extracts against various bacterial strains, including both Gram-positive and Gram-negative bacteria (Aminudin et al., 2019; Abbas & Minarti, 2020). The antibacterial activities of the compounds and the extracts were tested against

five bacterial strains, *Bacillus cereus*, *Lactiplantibacillus plantarum*, *Enterobacter cloacae*, *Pseudomonas aeruginosa* and *Serratia marcescens*. These bacteria exhibited antibiotic resistance and can cause infections to humans, making them the important target for testing new antimicrobial agents.

1.4 Aim and Objectives of Research

Two *Calophyllum* species, *Calophyllum soulattri* and *Calophyllum gracilentum*, are selected for their phytochemical and biological studies. Below are the specific objectives needed to fulfil the study.

- a) To isolate secondary metabolites from the stem bark of *Calophyllum soulattri* and *Calophyllum gracilentum* via maceration technique.
- b) To characterise the secondary metabolites isolated from *Calophyllum soulattri* and *Calophyllum gracilentum* using several spectroscopic techniques such as NMR, FTIR, UV-Vis, GC-MS and LC-MS.
- c) To evaluate the antibacterial activities of crude extracts and isolated compounds from *Calophyllum soulattri* and *Calophyllum gracilentum* using agar well diffusion and dilution methods.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Natural products are metabolites of living organisms that have evolved continually in nature, and they are frequently structurally diverse and have distinct pharmacological and biological properties (Zhu et al., 2022). New medications are needed to fight pathogens that cause diseases, which are spreading worldwide and becoming major public health issues that harm both human health and sustainable economic development. *Calophyllum* has been identified as a possible source of main compounds for discovering and developing novel drugs.

2.2 Secondary Metabolites from *Calophyllum* spp.

Secondary metabolites or natural products are a diverse group of natural metabolic products that are unimportant for the producing organisms or vegetative growth. However, they are regarded as differentiation compounds that confer adaptive roles, such as defence compounds or signalling molecules in symbiosis, metal transport, ecological interactions and competition (Thirumurugan et al., 2018). Plant secondary metabolites are typically located in the plant's stems, roots, leaves or barks, depending on the type of secondary metabolites that are produced.

According to Mera et al. (2019), the classification of secondary metabolites has taken into a variety of factors, including chemical structure (the existence of rings or sugars), composition (the existence or non-existence of nitrogen), solubility in organic

solvents or water, and biosynthetic pathway. The biosynthetic pathway is the most generally utilised criterion for secondary metabolites classifying in plants.

The phytochemical investigation demonstrated that the genus *Calophyllum* possesses an extensive range of secondary metabolites, for instance coumarins (Ee et al., 2011), xanthenes (Morel et al., 2002), chromanones (Lemos et al., 2012) and triterpenes (Li et al., 2010) being the most common groups of phytoconstituents exist in the genus (Gupta & Gupta, 2020).

2.2.1 Coumarins

Coumarins (Figure 2.1) can frequently be found in the *Calophyllum* genus. Coumarins are part of a widely distributed group of plant metabolites called benzopyrones, which have over 1500 representatives in over 800 species (Tiwari & Rana, 2015). The coumarin structure is derived from cinnamic acid via trans-cis isomerisation of the side chain double bond, ortho-hydroxylation and lactonisation (Jain & Joshi, 2012).

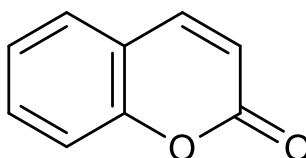


Figure 2.1: General structure of coumarin

A study by Reyes-Chilpa et al. (2004) revealed that hexane extract from *Calophyllum brasiliense* leaves yielded a coumarin named mammea A/BA (**1**). The hexane extracts experienced spontaneous precipitation, gaining a white powder. The compound

was obtained from the soluble part when the hexane extract was treated with dichloromethane. In 2009, Filho isolated the same compound from the same plant (Filho et al., 2009). This compound is characterised by the skeletons of 5,7-dioxycumarinic with alkyl or phenyl substituents at C-4 and prenyl or acyl substituents at C-6 or C-8.

Investigation on *Calophyllum wallichianum* stem bark yielded a coumarin named wallimarin T (**2**) and calanolide E (**3**) (Tee et al., 2018). The isolated wallimarin T from the chloroform extract was yielded as a white amorphous solid. The compound's UV-Vis spectrum revealed absorption at 281, 306, and 361 nm, suggesting a skeleton structure of coumarin. The IR spectrum indicated absorptions that are common signals for coumarins, which are at 1726, 1585, and 1433 cm^{-1} . ^1H NMR spectrum of calanolide E (**3**) showed signals that indicated the presence of a chromene unit, 4-propyldihydrocoumarin unit and additional isoprene unit. ^{13}C NMR and HSQC spectra of calanolide E (**3**) revealed the peaks assigned to the coumarin unit. One conjugated carbonyl carbon, one carbinolic carbon, two methyl groups and one peak were observed, which are in accordance with the 3-hydroxy-2-methyl-1-oxobutyl unit.

Calophyllum hosei stem bark was the subject of investigation by Daud et al. (2014), who discovered the isolated coumarin named hoseimarin (**4**). The ground bark of *C. hosei* was macerated two times with ethyl acetate for three days. The crude extract was subjected to a vacuum column chromatography over silica gel using hexane–ethyl acetate and ethyl acetate–methanol as eluting solvents to produce 20 fractions. Fraction seven was further purified via Sephadex LH-20 with methanol and afforded hoseimarin (**4**) as a pale-yellow gum. Due to the existence of carbonyl ($\text{C}=\text{O}$) and hydroxyl ($-\text{OH}$) moiety, the FTIR spectrum showed that it exhibited strong band absorptions (3220 cm^{-1} and 1710 cm^{-1}).

Meanwhile, five pyranocoumarins, caloforine A – E (**5** – **9**), were isolated from *Calophyllum scriblitifolium* bark by bioactivity-guided separation of the methanol extract (Ogasawara et al., 2022). Caloforine A (**5**) was yielded as an optically active colourless amorphous solid. The UV-Vis spectrum obtained from caloforine A (**5**) showed the existence of a coumarin chromophore. Meanwhile, the IR spectrum exhibited a significant absorption for the unsaturated ester C=O group at 1717 cm⁻¹. Caloforine B (**6**) was revealed to have a bigger molecular formula than caloforine A (**5**) by a CH₂ unit. Caloforine C (**7**) has an identical molecular formula as caloforine A (**5**). Meanwhile, caloforine D (**8**) and E (**9**) have an identical molecular formula to caloforine B (**6**).

Calophyllum soulattri stem bark was extracted and contributed to the isolation of a pyranocoumarin called soulamarin (**10**) (Ee et al., 2011). The air-dried stem bark of *C. soulattri* was ground to fine powder and extracted continuously in a Soxhlet apparatus with *n*-hexane (68-72 °C) and dichloromethane (40 °C) for 24 hours. Further purification of the *n*-hexane extract produced soulamarin (**10**) as yellowish oil. The presence of a pyran ring was observed from HMBC and COSY spectrum analysis. The pyran ring was attached at C-7 (non-oxygenated carbon) and C-8 (oxygenated carbon).

Meanwhile, a study by Joshi et al. (2013) led to the isolation of dipyrancoumarin, α -hydroxytomentolide A (**11**) from *Calophyllum apetalum* leaves. Powdered leaves were extracted with acetone at room temperature for 3 $\times$ 24 hours. The acetone extract was further purified several times and yielded compound **11** in the form of white needles. The characteristic signals proposed an inophyllolide skeleton in a dipyrancoumarin structure. According to the characteristic H-12 mesomerism at δ_H 6.85, which is about 0.3 ppm downfield of the correlating to H-8 signal for the inophyllolide series, the compound recognised as a member of the pseudoinophylloide class.

Ito et al. (2003) investigated constituents of the *Calophyllum brasiliense* stem bark and isolated three coumarins, identified as brasimarins A (**12**), B (**13**) and C (**14**). The dried stem bark of *C. brasiliense* was extracted with acetone at room temperature, and the solvent was evaporated under reduced pressure to produce acetone extract. The extract was chromatographed on a silica gel column eluted with hexane–acetone, acetone–methanol, and methanol continuously to give 13 fractions. Further purification of fourth fraction afforded compounds **12** and **13** in the form of light-yellow oil and colourless oil, respectively. Meanwhile, compound **14** was yielded as a colourless oil after the purification of fifth fraction. The existence of a partial structure of 4-propylcoumarin with a [5,6-b]-oriented dimethylpyran ring in the compound was suggested by the good resemblance of chemical shift values in the ^{13}C NMR of brasimarin A (**12**) with calanolide A, excluding the D ring carbons in formula (+)-calanolide A.

Four substituted coumarins, incrassamarin A (**15**), B (**16**), C (**17**) and D (**18**) were yielded from the *Calophyllum incrassatum* barks and leaves (Aminudin et al., 2016). Incrassamarin A was gained as a colourless needle-like crystal, incrassamarin B and D as pale yellow amorphous, incrassamarin C as a colourless crystal. The HREIMS spectrum of incrassamarin A (**15**) revealed the molecular ion peak at m/z 350.1161, and the molecular formula was concluded as $\text{C}_{21}\text{H}_{18}\text{O}_5$. The HREIMS spectrum of incrassamarin B (**16**) revealed the molecular ion peak at m/z 316.1309, and the molecular formula was deduced as $\text{C}_{18}\text{H}_{20}\text{O}_5$. ^1H NMR spectrum showed the presence of *n*-butyl as a side chain in incrassamarin B (**16**). Meanwhile, the incrassamarin C (**17**) possessed a molecular formula of $\text{C}_{16}\text{H}_{16}\text{O}_5$ which confirmed from the molecular ion peak displayed at m/z 288.0999 in the HREIMS spectrum correlating to the lack of C_2H_4 from incrassamarin B (**16**). This dissimilarity can be clarified through the ^1H NMR spectrum of incrassamarin C (**17**),

indicating the presence of a 4-ethyl group instead of 4-*n*-butyl. The incrassamarin D (**18**) was assigned the molecular formula of C₁₅H₁₄O₅. The ¹H NMR and ¹³C NMR spectra of incrassamarin D (**18**) were much the same to incrassamarin C (**17**) except for the substituent pattern at C-4.

The *Calophyllum membranaceum* leaves were extracted and contributed to the isolation of calopolyanolide E (**19**) (Shen et al., 2022). The compound had a molecular formula of C₂₅H₂₆O₆ concluded with a [M+H]⁺ ion at *m/z* 423.1808 by HRESIMS analysis. The compound's UV-Vis spectrum exhibited λ_{max} absorptions at 365, 311, 273 and 242 nm, indicating the existence of a coumarin skeleton. Besides, the absorption in IR spectrum observed at 2978 cm⁻¹ (C-H stretch), 1708 cm⁻¹ (conjugated C=O), 1625 cm⁻¹ (aromatic C=C) and 1132 cm⁻¹ (C-O stretch), which indicates the compound is a coumarin derivative. Figure 2.2 below shows the structures of isolated coumarins from *Calophyllum*.

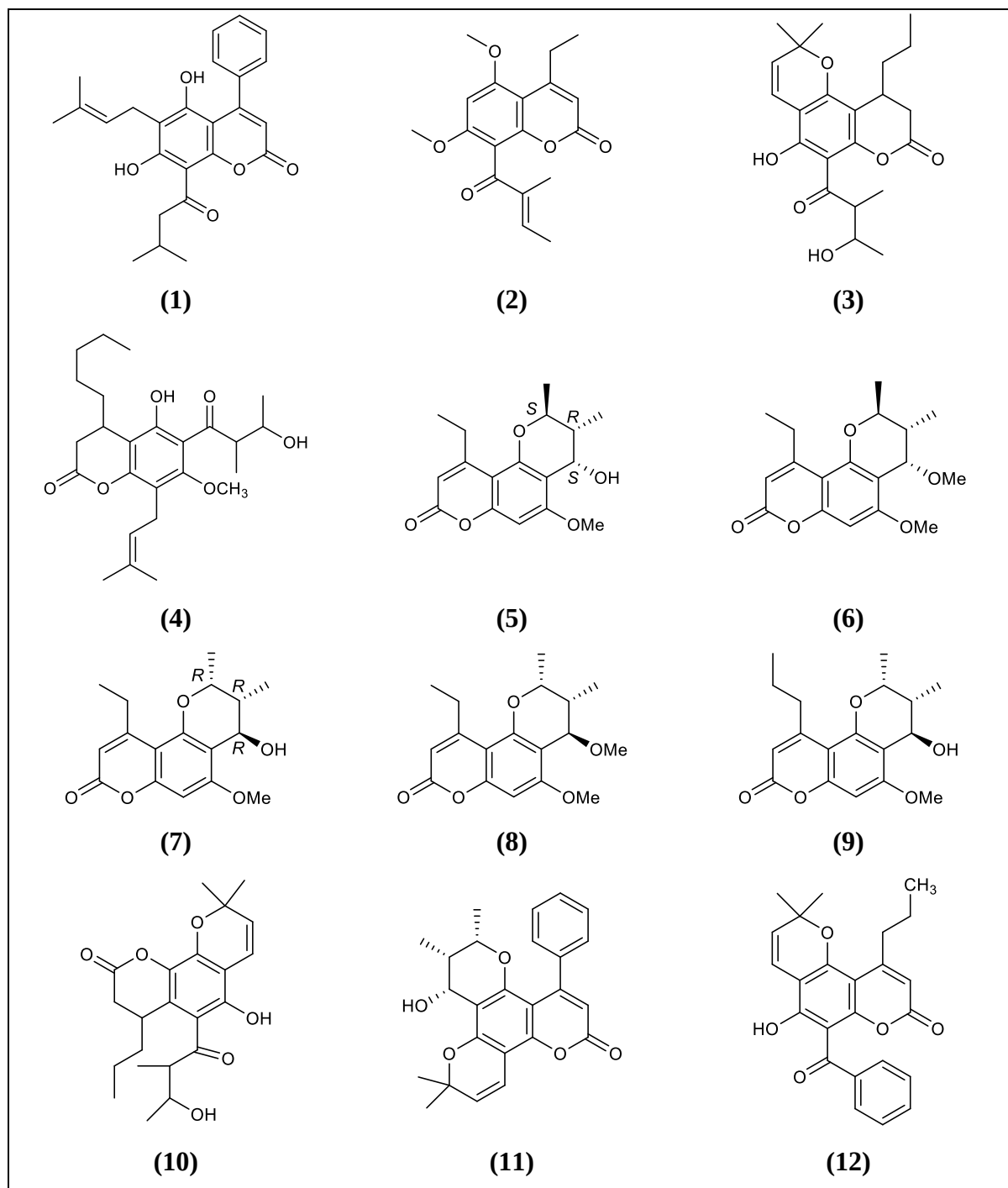


Figure 2.2: Structures of isolated coumarins from *Calophyllum*

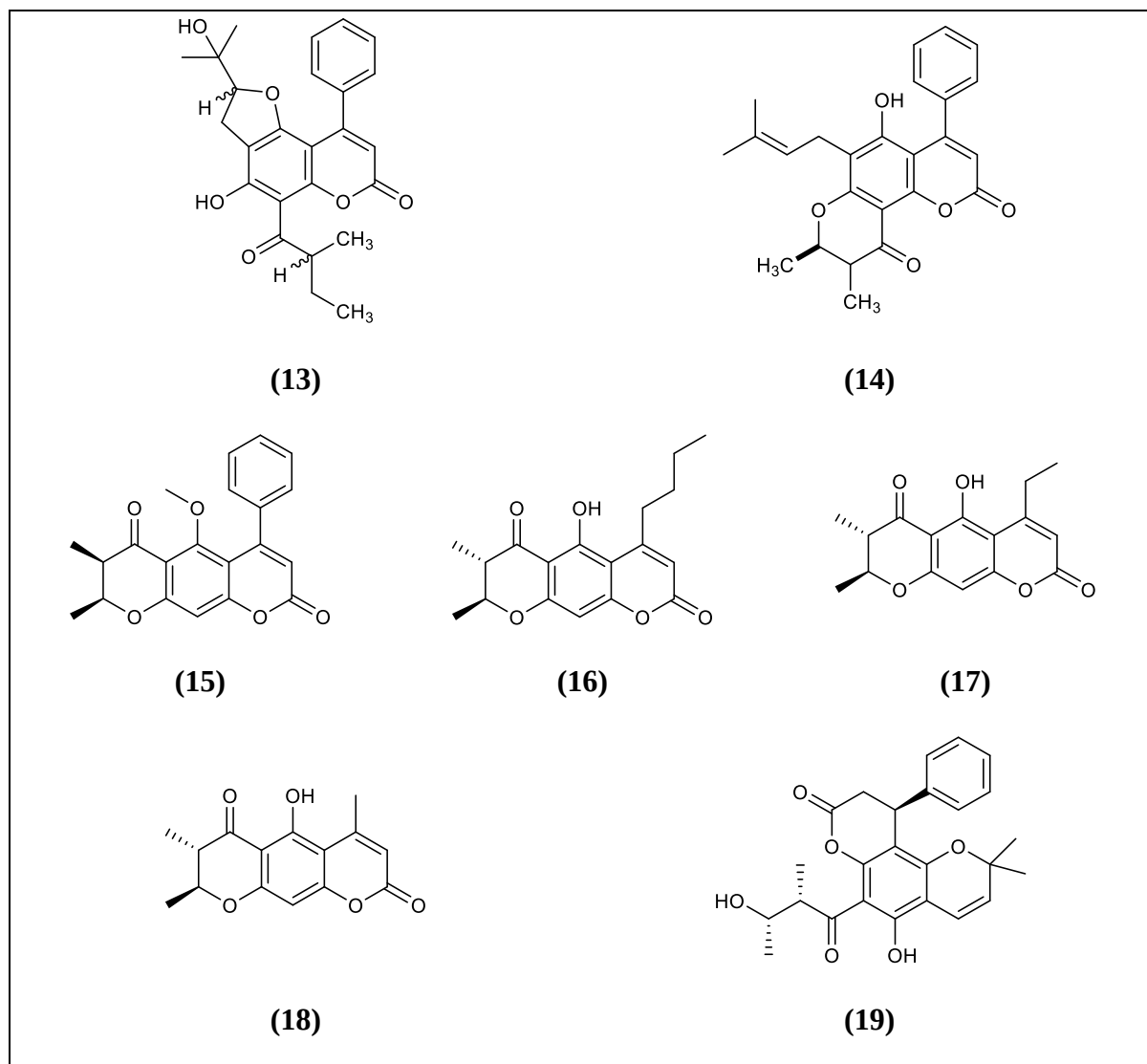


Figure 2.2: continued

2.2.2 Xanthenes

The xanthone or 9*H*-xanthen-9-one (Figure 2.3) contains a significant class of oxygenated heterocycles (Pinto et al., 2005). The word “xanthone” originates from the word for the colour yellow, “xanthos”, as the xanthone compounds are typically gained as yellow solids (Kurniawan et al., 2021). Xanthenes have been yielded from the wood and bark of numerous genus species.

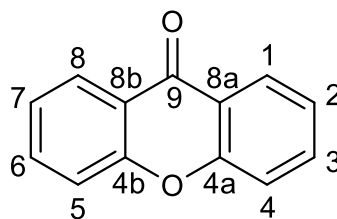


Figure 2.3: General structure of xanthone

Three xanthenes, named calaxanthenes (A-C) (**20** – **22**), were yielded from *Calophyllum calaba* roots (Laopian et al., 2019). The dried roots of *C. calaba* were extracted with dichloromethane at room temperature for seven days. The dichloromethane extract was further isolated by column chromatography to give four fractions. The second fraction was purified by Sephadex LH-20 column eluted with hexane–ethyl acetate to afford compound **22** in the form of white powder. The fourth fraction was purified by column chromatography to provide four subfractions. The first subfraction was further purified by chromatotron which afforded compounds **20** and **21** in the form of white powder and yellow viscous oil, respectively. The ^1H and ^{13}C NMR data of calaxanthone A (**20**) were incredibly alike to the identified xanthone, scriblitifolic acid. The contrast was located at C-4', where the carboxylic acid of scriblitifolic acid was substituted by methyl ester. The ^1H and ^{13}C NMR data of calaxanthone B (**21**) were displayed to be alike to calaxanthone A (**20**), except for unsubstituted at C-1 ring A. For calaxanthone C (**22**), the ^1H and ^{13}C NMR data were displayed to be exactly alike to calaxanthone B (**21**), except for the lack of ^1H NMR signal of the methyl-2-methylbutanoate substituent at C-6 and OCH_3 substituent at C-5 of calaxanthone C (**22**).

Balogun et al. (2019) reported the isolation of xanthone from *Calohyllum inophyllum*, 1,5-dihydroxyxanthone (**23**). The compound exhibited groups of proton

resonances in the aromatic region, which is the characteristic of oxygenated xanthone. The protons exhibited aromaticity and ortho-coupling, whereas the peaks corresponding to the -OH groups were suppressed at chemical shifts of 12.65 and 10.50 ppm. Besides that, Haerani et al. (2021) mentioned in their paper that two xanthenes, (2'S)-7-hydroxy caloxanthone B (**24**) and 1,3,6,7- tetrahydroxy-5-methoxy-4-(1',1'-dimethyl-2'-propenyl)-8-(3'',3''-dimethyl-2''-propenyl)-xanthone (**25**) were yielded from the Thai *Calophyllum inophyllum* root. Both compounds were obtained as yellow amorphous solids. The UV-Vis absorption of compound **25** at 266 and 330 nm characterised the xanthone chromophore. Besides, its IR spectrum revealed absorption at 3370 cm⁻¹ (O-H stretch), 1647 cm⁻¹ (conjugated C=O), and 1570 cm⁻¹ (aromatic C=C), which indicates the compound is a xanthone derivative. The ¹H and ¹³C NMR data for compound **25** demonstrated common signals for a xanthone skeleton with a conjugated C=O resonance at δ_C 183.0, a H-bonded -OH proton at δ_H 13.66 and an aromatic proton at δ_H 6.24. The NMR spectrum of compound **25** were alike to compound **24**. Both compounds also showed to have a similar structure according to the interpretation of 1D and 2D, with the dissimilarity only the presence of the doublet oxymethine at δ_H 4.55 and doublet CH₃ at δ_H 1.40 in place of the $\Delta^{2',3'}$ exomethylene resonances of compound **25**.

Mah et al. (2011) stated that one pyranoxanthone, soulattrin (**26**), was yielded from the *Calophyllum soulattri* stem bark. The powdered stem bark of *C. soulattri* was extracted successively in a Soxhlet apparatus with *n*-hexane, dichloromethane, ethyl acetate and methanol for 72 hours. Hexane and dichloromethane extracts were obtained after the extracts were evaporated to dryness under vacuum. Further purification of the dichloromethane extracts yielded compound **26**, which was gained as yellow crystals. The FTIR spectrum presented absorption of free hydroxyl, conjugated carbonyl, and aromatic

ring, indicating common xanthenes IR bands. The maximum absorptions of the UV-Vis spectrum were observed, which proposes a xanthone skeleton. The characteristic chemical shifts of the prenyl moiety were demonstrated in the proton NMR spectrum of the compound. The following year, Mah et al. (2012) successfully isolated diprenylated xanthone, phyllatrin (**27**). It was obtained in the form of a yellowish crystalline material from the dichloromethane extract. The UV-Vis spectrum and FTIR absorptions proved the existence of a xanthone functional group.

A few studies reported the isolation of a xanthone, jacareubin (**28**), from the heartwood of *Calophyllum brasiliense* (Pretto et al., 2004; Yasunaka et al., 2005). Yasunaka et al. (2005) also isolated another xanthone, named 1,3,5,6-tetrahydroxy-2-(3,3-dimethylallyl)xanthone (**29**) from the same plant. The structure of isolated compounds was determined using ^1H and ^{13}C NMR, IR spectroscopy, UV-Vis spectroscopies and mass spectrometry. Morel et al. (2002) reported that a xanthone, namely caledonixanthone E (**30**), yielded from the *Calophyllum caledonicum* stem bark. The powdered stem bark of *C. caledonicum* was sequentially extracted in a Soxhlet apparatus for 72 hours with hexane, dichloromethane, ethyl acetate and methanol. Concentration under reduced pressure afforded hexane-soluble, dichloromethane-soluble, ethyl acetate-soluble and methanol-soluble extracts. The chemical investigation of ethyl acetate-soluble extract resulted in the yielding of compound **30**. The compound was obtained as a yellow prism.

Nasir et al. (2011) reported that they isolated two xanthenes, known as nodusuxanthone (**31**) and trapezifolixanthone A (**32**), from the *Calophyllum nodosum* stem bark. The powdered stem bark of *C. nodosum* was extracted at room temperature consecutively with hexane, chloroform and finally methanol. Each of the extracts were filtered and solvents removed by rotary evaporator to give hexane, chloroform and

methanol extracts. The chloroform extract was fractionated by column chromatography eluting with different mixtures of hexane, chloroform and methanol to provide 168 fractions. Repeated column chromatographic separation of fractions 74-78 afforded compound **31**. Similar fractionation of the methanol extract with vacuum column chromatography and eluting with the same solvent systems gave 118 fractions. Further purification of fractions 87-97 yielded compound **32**. Both compounds were gained as yellow needle-shaped crystals. The UV-Vis spectrum of nodusuxanthone (**31**) displayed λ_{max} absorptions at 305, 284 and 247 nm, which suggested the presence of a xanthone skeleton, and the IR spectrum demonstrated a strong absorption at 1647 cm^{-1} for the chelated C=O group. The IR spectrum of trapezifolixanthone A (**32**) demonstrated a strong and broad absorption at 3463 cm^{-1} , suggesting an -OH group and another strong band at 1685 cm^{-1} allocated to a chelated C=O.

A known xanthone, trapezifolixanthone (**33**), was successfully yielded from the *Calophyllum soulattri* stem bark (Mah et al., 2012; Tanjung et al., 2018). The compound's isolation resulted from the dichloromethane extract's chemical investigation. The compound was obtained as a yellow crystal. Kawamura et al. (2012) reported from the heartwood of *Calophyllum symingtonianum*, they successfully isolated two major xanthone, 6-desoxyjacareubin (**34**) and 1,3,5-trihydroxy-2-(3-methylbut-2-enyl)xanthone (**35**). The air-dried sample meals were extracted under reflux with 350 mL of methanol four times. The extracted solution was filtered, and the solvent removed *in vacuo* ($30\text{ }^{\circ}\text{C}$) to afford residual methanol extract. The methanol extract was successively partitioned between *n*-hexane/water and ethyl acetate/water to afford *n*-hexane-soluble, ethyl acetate-soluble and ethyl acetate-insoluble after removing solvents, respectively. Both compounds

were yielded for the first time from *C. symingtonianum*. Compound **34** was yielded from *n*-hexane-soluble. Meanwhile, compound **35** was yielded from ethyl acetate-soluble.

The chemical investigation on the extraction of ethanol from the *Calophyllum inophyllum* twigs by Dai et al. (2010) led to the yielding of prenylated xanthenes, caloxanthone O (**36**) and caloxanthone P (**37**). Both compounds were gained as yellow powder. The IR spectrum exhibited free -OH (3560 cm^{-1}), chelated -OH (3160 cm^{-1}), conjugated C=O (1684 cm^{-1}) and aromatic ring ($1589, 1540\text{ cm}^{-1}$) absorptions. These data, along with those acquired from the UV-Vis spectrum which exhibited λ_{max} absorption at 321, 282, 252, 222 and 217 nm, were similar to the existence of a xanthone skeleton. Figure 2.4 shows the structures of isolated xanthenes from *Calophyllum*.

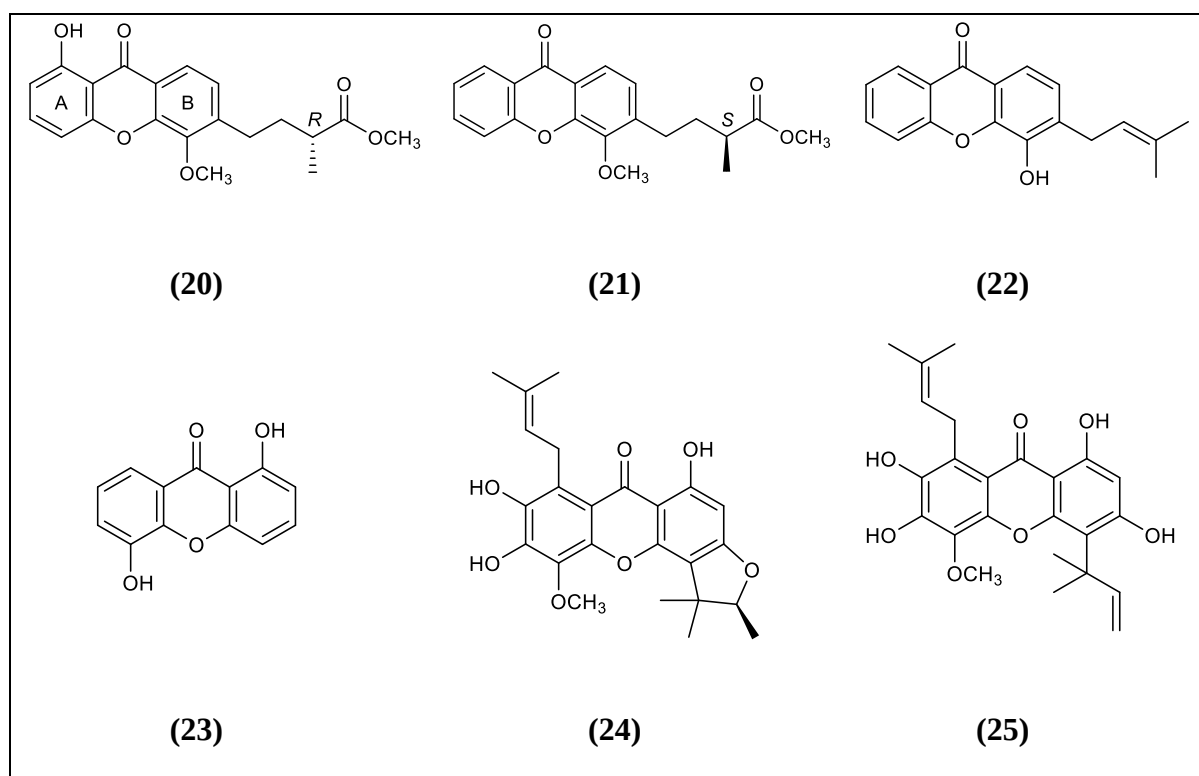


Figure 2.4: Structures of isolated xanthenes from *Calophyllum*

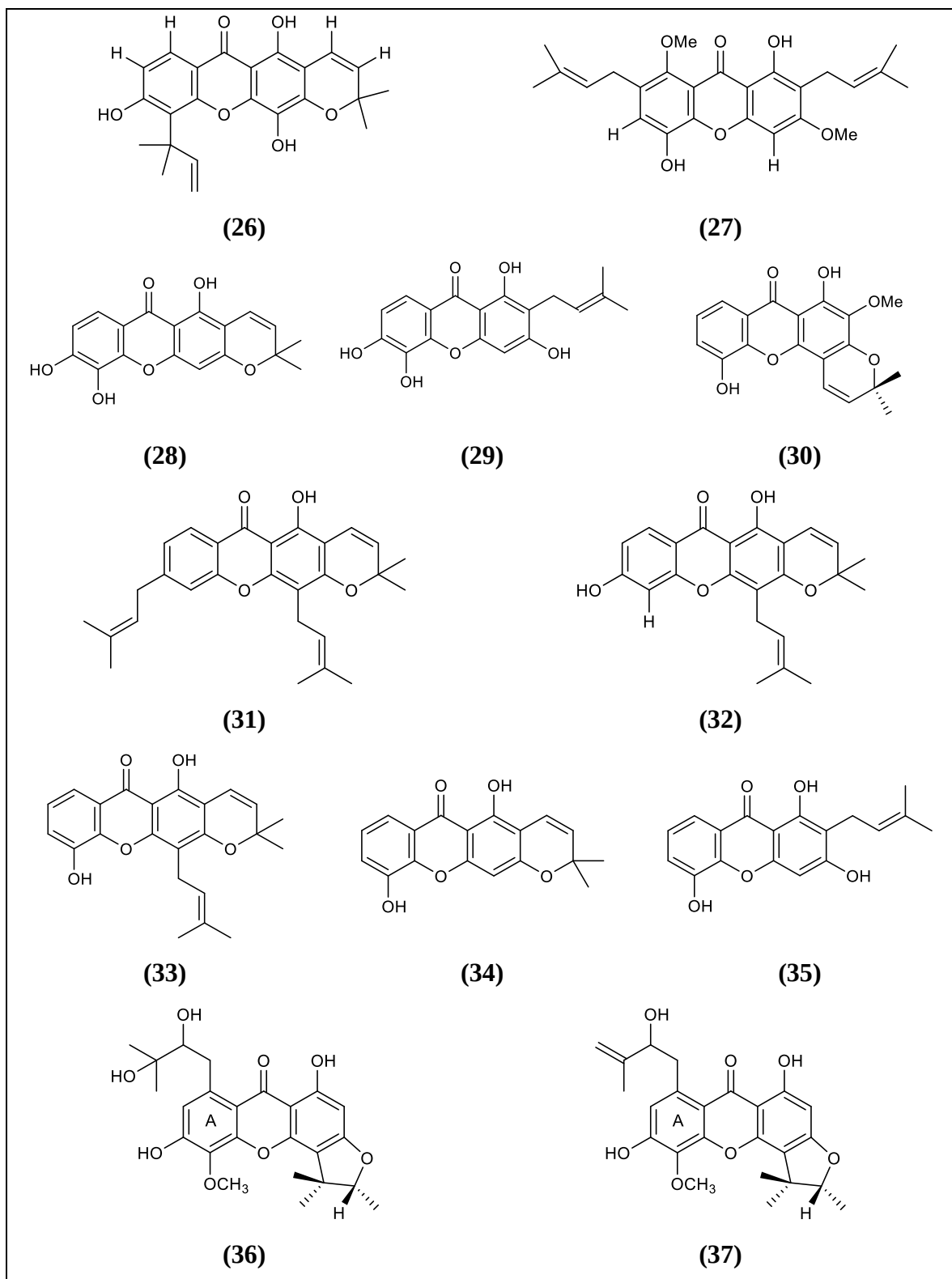


Figure 2.4: continued

2.2.3 Chromanones

Chromanone, also known as chroman-4-one, is an attachment of benzene nucleus with dihydropyran which associates with chromane, chromene, chromone and chromenone (Kamboj & Singh, 2022). A chromanone derivative, namely caloinophyllin A (**38**), was yielded from the *Calophyllum inophyllum* roots (Ponguschariyagul et al., 2018). The compound was gained as a yellow gum. The ^1H NMR experiment displayed a singlet signal at δ_{H} 12.22, signifying a chelated -OH moiety in the compound. The ^{13}C NMR spectrum specified this compound comprising six methyls, six methylenes, five methines, nine quaternary olefinic and one quaternary carbon.

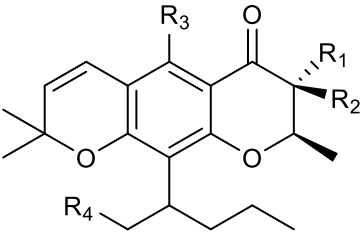
Cottiglia et al. (2004) reported the isolation of new two chromanone acids, brasiliensophyllic acid A (**39**) and isobrasiliensophyllic acid A (**40**) from the *Calophyllum brasiliense* bark. The ^{13}C NMR spectrum of compound **39** demonstrated signals comprising the compound to have 35 carbons, which were then classified into seven methyls, five methylenes, 11 methines and 12 quaternary carbons by DEPT NMR experiments. ^1H and ^{13}C NMR indicated a 2-carboxyl-1-phenylethyl substituent replacing a prenylated chromanone acid. Proton signals correlating to two methines established the existence of 2,3-dimethylchromanone ring which was highly substituted. An -OH proton contributing to a strong hydrogen bond was confirmed by the downfield that shifted the signal observed at δ 16.63 (s). The existence of an enol group was proven from a carbon signal observed at δ_{C} 190.7 (s), and two signals observed at δ_{C} 196.0 (s) and 199.1 (s) showed two unsaturated ketone C=O groups. Besides, five aromatic protons were observed in the ^1H NMR spectrum and implied a monosubstituted phenyl moiety. The proton NMR and COSY spectra demonstrated the existence of a vinyl proton (δ_{H} 4.54, *m*) bonded to two vinylic CH_3 groups (δ_{H} 1.39, s, 1.40, s), implying an isopent-2-enyl substituent. A signal of

a CH₃ group (δ_{H} 1.59, s) and two signals of terminal CH₂ (δ_{H} 4.38, br. s, 4.71, br. s) pointed to an isopropenyl group. NMR spectra and mass spectrometry of compound **40** were nearly alike to compound **39**. The major dissimilarities were because of the signal pattern of H-2 and H-3, the CH₃ groups at C-2 and C-3 (δ_{C} 16.3, 9.8, respectively) and the shift values of C-2, C-3 (δ_{C} 74.5, *d*, 40.5, *d*, respectively).

Hasanah et al. (2019) stated that two chromanone acids, calofolic acid B (**41**) and apetalic acid (**42**), were yielded from the *Calophyllum incrassatum* stem bark. Both compounds obtained were gained as yellow solids. The IR spectrum of apetalic acid (**42**) showed absorptions of 3436, 1622, 1598, 1496, 1446 and 1157 cm⁻¹ alike to calofolic acid B (**41**). Meanwhile, the ¹³C NMR spectrum of calofolic acid B (**41**) corresponded to chromanone acid. The ¹H NMR spectrum of apetalic acid (**42**) is very similar to calofolic acid B (**41**), and the significant contrast is the alkyl carboxylic group at C-10.

Dharmaratne et al. (1999) stated that the sodium carbonate, a soluble fraction of the *n*-hexane extract of *Calophyllum cordato-oblongum* stem bark yielded novel chromene acid, isocordato-oblongic acid (**43**). The IR spectrum of the compound demonstrated the existence of olefinic and C=O (ester) groups. The UV-Vis spectrum of the compound suggested the existence of a chromanone-chromene type chromophore, which is typical of cordato-oblongic acid and other chromene acids. Chromatographic fractionation of the acetone extract of the *Calophyllum blancoi* seeds isolated six pyranochromanone derivatives, as depicted in Table 2.1, which are apetalic acid (**44**), isoapetalic acid (**45**), apetalic acid methyl ester (**46**), apetalic acid 5-*O*-acetate (**47**), isoapetalic methyl ester (**48**) and isoapetalic acid 5-*O*-acetate (**49**) (Shen et al., 2004). All six compounds were gained as yellow oil.

Table 2.1: Structure of pyranochromanone derivatives

				
	R ₁	R ₂	R ₃	R ₄
Apetalic acid (44)	H	CH ₃	OH	COOH
Isoapetalic acid (45)	CH ₃	H	OH	COOH
Apetalic acid methyl ester (46)	H	CH ₃	OH	COOMe
Apetalic acid 5-O-acetate (47)	H	CH ₃	OAc	COOH
Isoapetalic methyl ester (48)	CH ₃	H	OH	COOMe
Isoapetalic acid 5-O-acetate (49)	CH ₃	H	OAc	COOH

A study on the *Calophyllum teysmannii* stem bark led to the yielding of caloteysmannic acid (**50**) (Lim et al., 2015). Compound **50** was gained as yellow cubic crystals. The UV-Vis absorption maxima at 367, 300, 271 and 214 nm indicated a pyranochromanone moiety common to isocalolongic acid (**54**), apetalic acid (**44**) and other chromanone derivatives.

Ha et al. (2012) mentioned that a chromanone acid named calodryobalanoic acid (**51**) was yielded from the *Calophyllum dryobalanoides* bark. The compound was obtained in the form of a yellowish needle. The UV-Vis spectrum which demonstrated absorption maxima at 364, 311, 299, 275, 268 and 227 nm suggested the existence of a chromanone-chromene type chromophore typical of chromanone acids. The HMBC plot proved the existence of a chromanone-chromene system in the compound. A study of hexane-soluble extract from the *Calophyllum caledonicum* stem bark by Hay et al. (2003) led to the yielding of a novel caledonic acid (**52**). Besides, two other known compounds, calolongic

acid (**53**) and isocalolongic acid (**54**), were isolated. All compounds were isolated as an amorphous solid. The structures of chromanone derivatives isolated from *Calophyllum* are depicted in Figure 2.5.

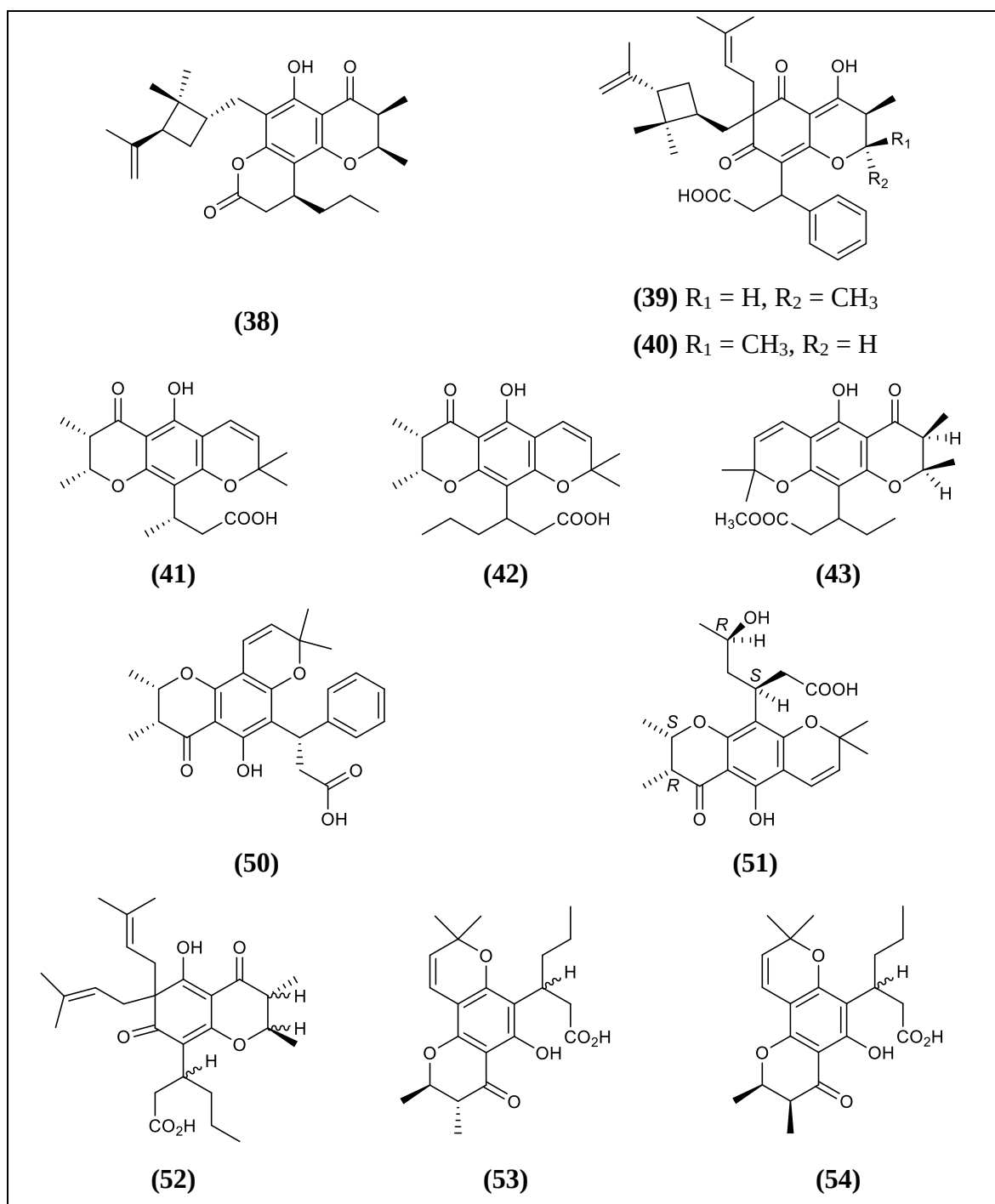


Figure 2.5: Structures of chromanone derivatives

2.2.4 Triterpenes

Zou et al. (2010) had successfully isolated a triterpenes isomer, namely 27- [(E)-*p*-coumaroyloxy]friedelin-28-carboxylic acid (**55**) and 27-[(Z)-*p*-coumaroyloxy]friedelin-28-carboxylic acid (**56**) from the *Calophyllum inophyllum* leaves. Both compounds were extracted as white amorphous powder with the molecular formula of C₃₉H₅₄O₆ deduced by HREIMS and NMR analysis. The IR spectrum of compounds demonstrated the appearance of -OH group, C=O group and an aromatic ring. A friedelin skeleton was indicated for compound **55**, with the (E)-*p*-coumaroyloxy group at C-27 and the free COOH group at C-28, validated by Nuclear Overhauser Effect (NOE) correlation signals. In the ¹H NMR spectrum of compound **56**, the existence of the (Z)-coumaroyl group was shown to be confirmed by ¹³C NMR signals and characteristic peaks at *m/z* 164.0473 (C₉H₈O₃), 147.0446 (C₉H₇O₂) and 119.0497 (C₈H₇O) in the HREIMS of the compound. Analysis of ¹H, ¹³C, DEPT, and EIMS data suggested that compound **56** also occupies a (Z)-*p*-coumaroyl group and a triterpene skeleton.

A study about the *Calophyllum inophyllum* leaves led to the isolation of three friedelane-type triterpenoids, 3,4-secofriedelan-3,28-dioic acid (**57**), 27-hydroxyacetate canophyllic acid (**58**) and 3-oxo-27-hydroxyacetate friedelan-28-oic acid (**59**) (Laure et al., 2005). All compounds were obtained as white amorphous powder. Absorption bands in the IR spectrum at 1699 cm⁻¹ and 3448 cm⁻¹ indicated the presence of acid functions for compound **57**. The spectral features demonstrated that compound **57** was a secotriterpene, with A ring-opened and two acid carboxylic functions at C-3 and C-28. The IR spectrum of compounds **58** and **59** showed the presence of C=O and -OH group, where compound **58** at 1702 cm⁻¹ (C=O stretch) and 3400 cm⁻¹ (O-H stretch), compound **59** at 1755 cm⁻¹ (C=O

stretch) and 3420 cm^{-1} (O-H stretch). The ^1H and ^{13}C NMR data of compound **58** exposed the existence of an acetate function.

Joshi et al. (2013) stated that the study on the *Calophyllum apetalum* leaves led to the yielding of known compounds, friedelin (**60**) and apetalactone (**61**). Apetalactone (**61**) was obtained as white crystals. Both compounds have been identified as triterpenes via comparison with literature data (Govindachari et. al., 1968; Cano et al., 2000). Figure 2.6 below shows the structures of triterpene derivatives isolated from *Calophyllum*.

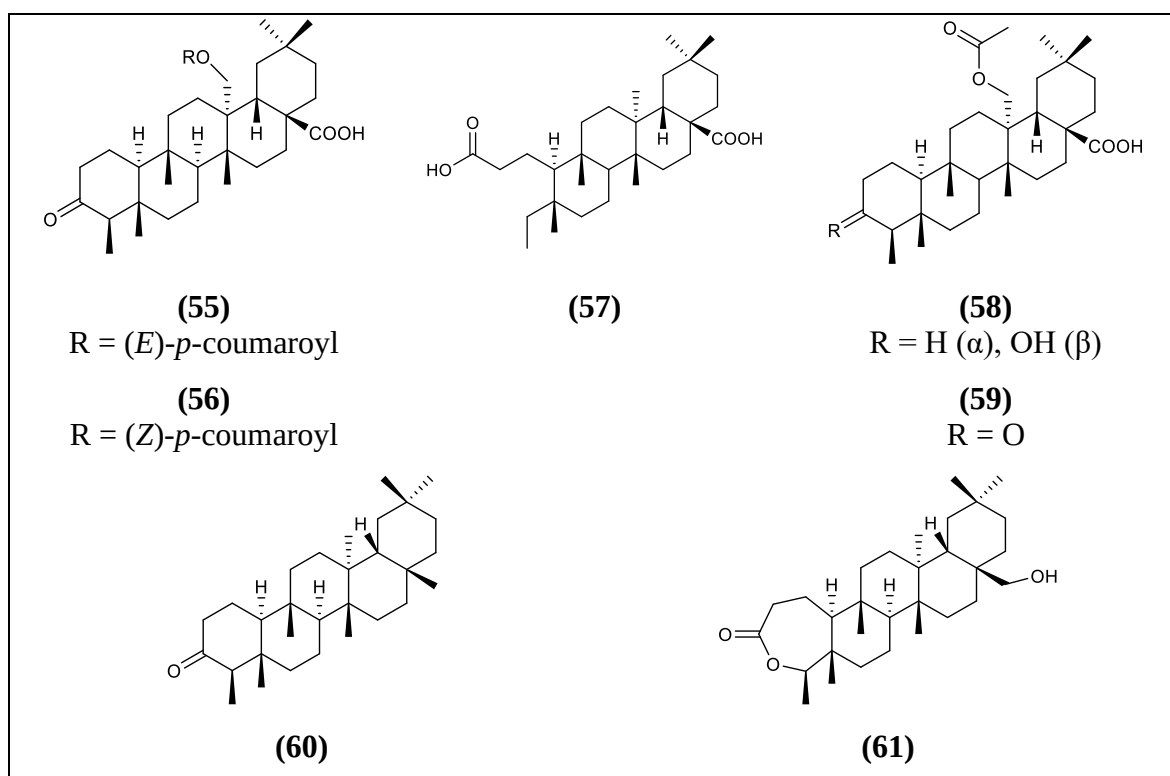


Figure 2.6: Structures of triterpene derivatives

2.3 Antibacterial Activity of Plant Extracts from *Calophyllum* spp.

The resistance in bacteria happens either by transmitting resistance genes from other bacteria or through gene mutation of pre-existing or acquired genes (Christaki et al.,

2020). Besides, it also may arise due to production of enzymes that break down the target agent and alter the bacterial envelope structure of composition (Aljeldah, 2022). The search for novel anti-infection medication has occupied many ethnopharmacology research groups during the last few decades. Traditionally, crude extracts from different medicinal plant parts such as stems, roots, fruits, flowers and twigs were commonly utilised to cure human illnesses (Khan et al., 2013).

Alkhamaiseh et al. (2011) mentioned that hexane and dichloromethane extracts from the bark of *Calophyllum rubiginosum* showed strong inhibition against two Gram-positive bacterial strains, *Bacillus cereus* and *Staphylococcus aureus*. In the following year, Alkhamaiseh et al. studied on the bark of *Calophyllum canum* (Alkhamaiseh et al., 2012). Similar antibacterial activity is observed from the study where hexane and dichloromethane extracts demonstrated strong inhibition against those two Gram-positive bacterial strains.

Aminudin et al. (2019) reported that the dichloromethane extract from the bark of *Calophyllum ferrugineum* exhibited moderate activity against Gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus* at 125 µg/mL. A study by Noh and Jong on the same *Calophyllum* species demonstrated that chloroform extract showed moderate activity against *Pseudomonas aeruginosa* and *Escherichia coli* (Noh & Jong, 2020).

A study on the antibacterial effects of the *Calophyllum inophyllum* bark showed that chloroform extract exhibited moderate activities against *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Bacillus subtilis* (Mishra et al., 2010). Kudera et al. (2017) mentioned that the ethanol extract from the leaves of *Calophyllum inophyllum* demonstrated moderate inhibitory activity against two Gram-positive bacteria, *Clostridium perfringens* and

Listeria monocytogenes at 128 µg/mL. The MIC results on the extract of *Calophyllum* species are tabulated in Table 2.2.

Table 2.2: Antibacterial activity of *Calophyllum* extracts

<i>Calophyllum</i> species	Part	Extract	MIC (µg/ml)	Strain	Reference
<i>Calophyllum canum</i>	Bark	Hexane	12.5	<i>B. cereus</i>	Alkhamaiseh et al. (2012)
			100	<i>S. aureus</i>	
		Dichloromethane	25 50	<i>B. cereus</i> <i>S. aureus</i>	
<i>Calophyllum ferrugineum</i>	Bark	Chloroform	112.5	<i>P. aeruginosa</i>	Noh & Jong (2020)
			112.5	<i>E. coli</i>	
	Bark	Dichloromethane	125 125	<i>B. subtilis</i> <i>S. aureus</i>	Aminudin et al. (2019)
<i>Calophyllum inophyllum</i>	Bark	Chloroform	100	<i>P. aeruginosa</i>	Mishra et al. (2010)
			100	<i>P. vulgaris</i>	
			125	<i>B. subtilis</i>	
	Leaves	Ethanol	128 128	<i>C. perfringens</i> <i>L. monocytogenes</i>	Kudera et al. (2017)
<i>Calophyllum rubiginosum</i>	Bark	Hexane	12.5	<i>B. cereus</i>	Alkhamaiseh et al. (2011)
			100	<i>S. aureus</i>	
		Dichloromethane	12.5 100	<i>B. cereus</i> <i>S. aureus</i>	

2.4 Antibacterial Activity of Secondary Metabolites from *Calophyllum* spp.

Medicinal plants possess numerous antimicrobial phytochemicals such as tannins, alkaloids, flavonoids and terpenoids (Talib & Mahasneh, 2010). *Calophyllum* species exhibit significant biological activity owing to their secondary metabolites. Previous studies demonstrated that secondary metabolites isolated from *Calophyllum* possess an

antimicrobial activity. Antimicrobial medicines are classified according to the microorganisms they predominantly target. Antibacterial, for example, is used against bacteria. A few prior studies showed that some of the isolated compounds from *Calophyllum* species exhibited antibacterial activity against a few bacterial strains.

Yasunaka et al. (2005) found that coumarin isolated from *Calophyllum brasiliense*, mammea A/B (**1**) has strong antibacterial activity against methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA) at 1 g/mL and 2 g/mL, respectively. Yasunaka et al. (2005) also did a study on *Calophyllum brasiliense* extracts. The extracts were evaluated for antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. The MIC values in Table 2.2 suggested that jacareubin (**28**) and 1,3,5,6-tetrahydroxy-2-(3,3-dimethylallyl) xanthone (**29**), which demonstrated stronger activity against *S. aureus*, are compounds in control of activity. Both compounds had moderate antibacterial activity against *Escherichia coli*. Tantapakul et al. (2016) reported that trapezifolixanthone (**33**) possessed strong to moderate antibacterial activity against eight different bacterial strains with the MICs of 4 to 64 µg/mL.

Cottiglia et al. (2004) discovered two new chromanone acids obtained from the *Calophyllum brasiliense* bark, brasiliensophyllic acid A (**39**) and isobrasiliensophyllic acid A (**40**), which showed strong antibacterial activity against *Staphylococcus epidermis* and *Bacillus cereus*. The isolated chromanone acids' potent antibacterial activity is most likely attributable to the existence of a cyclobutyl ring in compounds. Aminudin et al. (2016) studied the antibacterial activity of *Calophyllum symingtonianum* compounds against a few bacterial strains by using MIC in serial broth microdilution. The results demonstrated that isocordato-oblongic acid (**43**) demonstrated moderate inhibition against *Bacillus subtilis* bacteria at 62.5 µg/mL. The lipophilic character was enhanced due to the availability of

pentanoic acid moiety in the structure, hence facilitating the penetration of low lipid content of cell wall Gram-positive bacteria and reaching its active site. Table 2.2 shows the antibacterial activity of isolated compounds from *Calophyllum* species against several bacterial strains.

Table 2.3: Antibacterial activity of compounds isolated from *Calophyllum* species against several bacterial strains reported from 2000 to 2023

	Compound	MIC (µg/mL)	Strain	Reference
Coumarin	Mammea A/BA (1)	1	MSSA	Yasunaka et al. (2005)
		2	MRSA	
Xanthone	Jacareubin (28)	4	MSSA, MRSA	Yasunaka et al. (2005)
	1,3,5,6-tetrahydroxy-2-(3,3-dimethylallyl) xanthone (29)	1 4	MSSA MRSA	
	Trapezifolixanthone (33)	4	<i>M. luteus</i>	Tantapakul et al. (2016)
		4	<i>B. cereus</i>	
		64	<i>B. subtilis</i>	
		16	<i>S. aureus</i>	
		4	<i>S. epidermis</i>	
		32	<i>E. coli</i>	
		32	<i>S. typhimurium</i>	
Chromanone	Brasiliensophyllic acid A (39)	1	<i>B. cereus</i>	Cottiglia et al. (2004)
		16	<i>S. epidermis</i>	
	Isobrasiliensophyllic acid A (40)	1	<i>B. cereus</i>	
		16	<i>S. epidermis</i>	
	Isocordato-oblongic acid (43)	62.5	<i>B. subtilis</i>	Aminudin et al. (2016)

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant materials

Calophyllum soulattri (UiTM 3049) and *Calophyllum gracilentum* (UiTM 3048) stem bark were obtained from Sri Aman, Sarawak, Malaysia. The voucher specimens for each species were deposited in the Universiti Teknologi Mara Sarawak Herbarium and the samples were identified by the botanist. All collected plant samples were dried in the drying oven at 40 °C and ground into fine powder.

3.1.2 Chemicals

The AR grade chemical solvents, acetone, chloroform, ethyl acetate, hexane and methanol were utilised in the extraction, isolation and purification of secondary metabolites from *Calophyllum soulattri* and *Calophyllum gracilentum*. Silica gel 60 (MERCK 1.09385) with 230 – 400 mesh and Sephadex LH-20 were utilised in column chromatography technique. The deuterated solvents used in NMR analyses were acetone- d_6 and deuterated chloroform ($CDCl_3$). For LC-MS, methanol (HPLC grade) was used. Chemical reagents and materials used in the antibacterial assay were Mueller Hinton Agar (MHA), Mueller Hinton Broth (MHB), ampicillin, dimethyl sulfoxide (DMSO), barium chloride ($BaCl_2$), sulphuric acid (H_2SO_4), agar plate and 96-well plate.

3.2 Extraction and Isolation of Secondary Metabolites from *Calophyllum soulattri* and *Calophyllum gracilentum*

3.2.1 Preparation of crude extract

2.9 kg of *Calophyllum soulattri* and 2.1 kg of *Calophyllum gracilentum* stem bark were dried in the drying oven and crushed into fine powdered. The powdered stem bark of both *Calophyllum* was macerated three times in three different solvents of dissimilarity polarities, beginning with hexane, followed by ethyl acetate and lastly, methanol (3 × 5 L each). The macerated samples were filtered, and the solvents were concentrated using rotary evaporator to obtain crude extracts of hexane (*C. soulattri*, 147.7 g; *C. gracilentum*, 148.9 g), ethyl acetate (*C. soulattri*, 143.3 g; *C. gracilentum*, 144.1 g) and methanol (*C. soulattri*, 290.0 g; *C. gracilentum*, 332.5 g).

3.2.2 Isolation of secondary metabolites from *Calophyllum soulattri*

The isolation and purification of three crude extracts of *Calophyllum soulattri*, hexane (147.7 g), ethyl acetate (143.3 g) and methanol (290.0 g) extracts resulted in three compounds from coumarin and xanthone. One compound was isolated from hexane crude extract which was identified as calanolide E (**3**) (869.6 mg). Meanwhile, two compounds were successfully isolated from ethyl acetate crude extract which were identified as calopolyanolide E (**19**) (200.6 mg) and soulaxanthone (**62**) (191.1 mg).

The hexane extract of *Calophyllum soulattri* was chromatographed on silica column chromatography eluted with hexane-chloroform, chloroform-ethyl acetate and ethyl acetate-methanol continuously, resulting in 23 fractions. Fractions 1 – 7 were combined and fractionated using the gravity column chromatography technique, eluting

with hexane and acetone (7:3) on silica, affording 16 fractions. Calanolide E (**3**) was obtained after subfractions 5 – 10 were combined.

Besides that, the ethyl acetate extract of *Calophyllum soulattri* was introduced to vacuum liquid chromatography on silica eluted with hexane: chloroform: ethyl acetate: methanol which resulting in 27 subfractions. Fractions 1 – 6 were mixed and introduced to silica gel 60 eluted with a mixture of hexane and acetone (7:3), producing 14 fractions. The subfractions 4 – 8 from 14 fractions are then combined and chromatographed with column chromatography eluted with a mixture of hexane and acetone (7:3) producing 12 fractions. The subfractions 8 – 10 from 12 fractions were combined and purified, yielding calopolyanolide E (**19**).

In addition, fraction 16 from the ethyl acetate extract of *Calophyllum soulattri* was introduced to column chromatography with a combination of hexane and acetone (7:3), retrieving 13 fractions. Subfractions 1 and 2 were combined and fractionated using column chromatography eluting with hexane and acetone (7:3), producing 5 fractions. The first three vials from this fraction were combined and purified a few times using column chromatography on Sephadex LH-20. Final purification was carried out using centrifugal TLC eluted with a combination of hexane and ethyl acetate (8:2), yielding soulaxanthone (**62**).

Figure 3.1 and Figure 3.2 demonstrate the flowchart of the purification process of calanolide E (**3**), calopolyanolide E (**19**) and soulaxanthone (**62**).

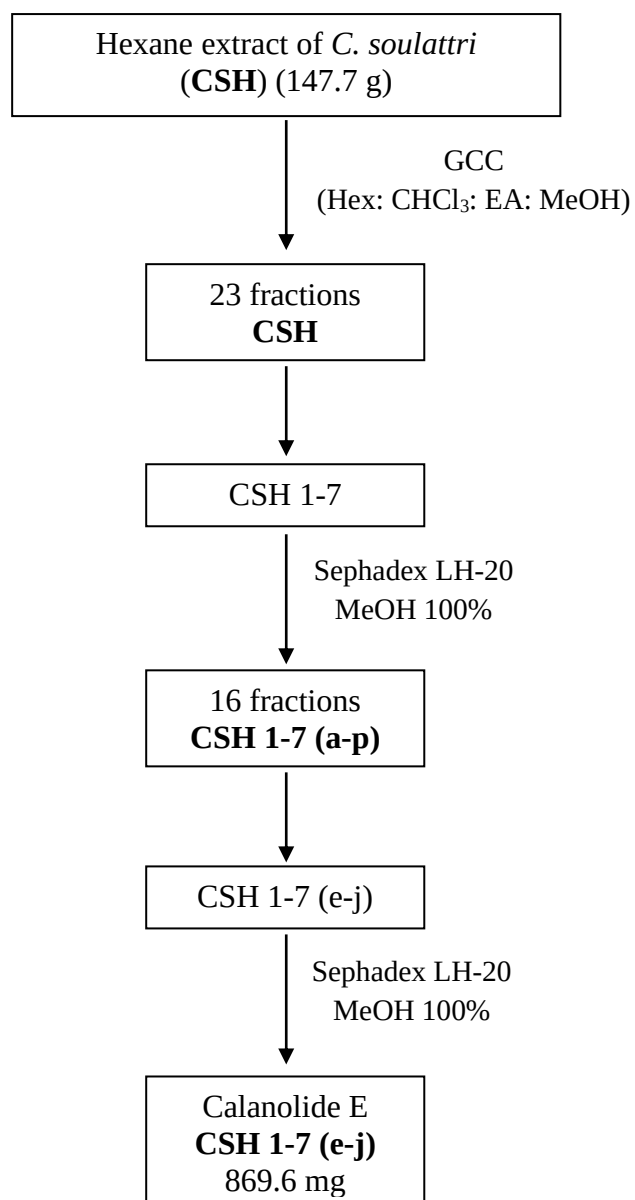


Figure 3.1: Flowchart of the purification process of calanolide E (3)

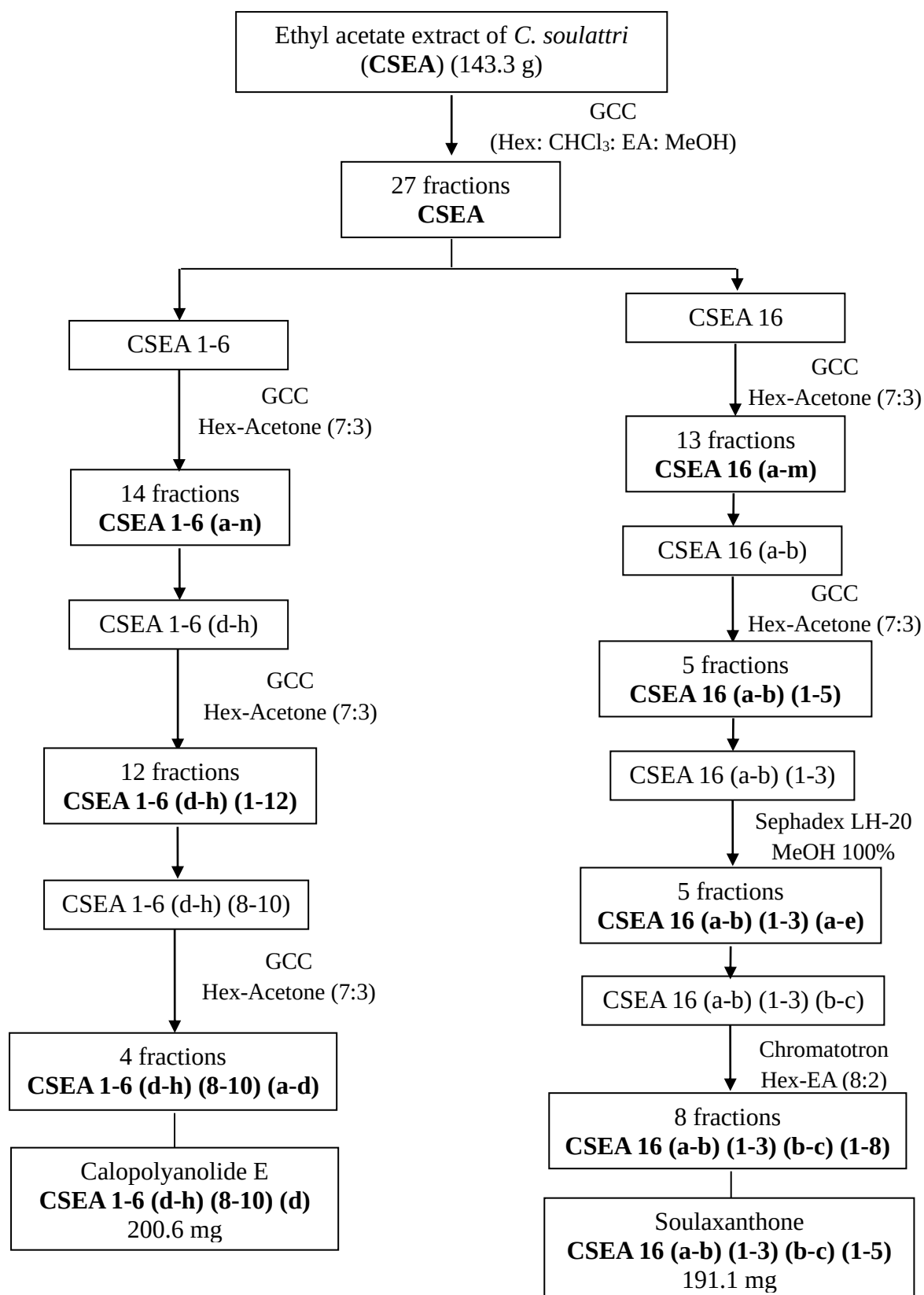


Figure 3.2: Flowchart of the purification process of calopolyanolide E (**19**) and soulaxanthone (**62**)

3.2.3 Isolation of secondary metabolites from *Calophyllum gracilentum*

The purification of three crude extracts from *Calophyllum gracilentum* which are hexane (148.9 g), ethyl acetate (144.1 g) and methanol (332.5 g) extracts led to the finding of two compounds. Euxanthone (**63**) (36.6 mg) was yielded from the hexane crude extract. Meanwhile, friedelin (**60**) (402.7 mg) was yielded from the crude extract of ethyl acetate.

The hexane extract of *Calophyllum gracilentum* undergoes column chromatography. Fraction 8 was introduced to silica gel 60 eluted with a mixture of hexane and acetone (8:2). The subfractions were then purified with Sephadex LH-20, producing euxanthone (**63**).

Besides that, the ethyl acetate extract of *Calophyllum gracilentum* was introduced to column chromatography on silica gel 60. Fraction 3 was purified using column chromatography, in which a mixture of acetone and methanol (5:5) was eluted on Sephadex LH-20, yielding friedelin (**60**).

Figure 3.3 and Figure 3.4 demonstrate the flowchart of the purification process of euxanthone (**63**) and friedelin (**60**).

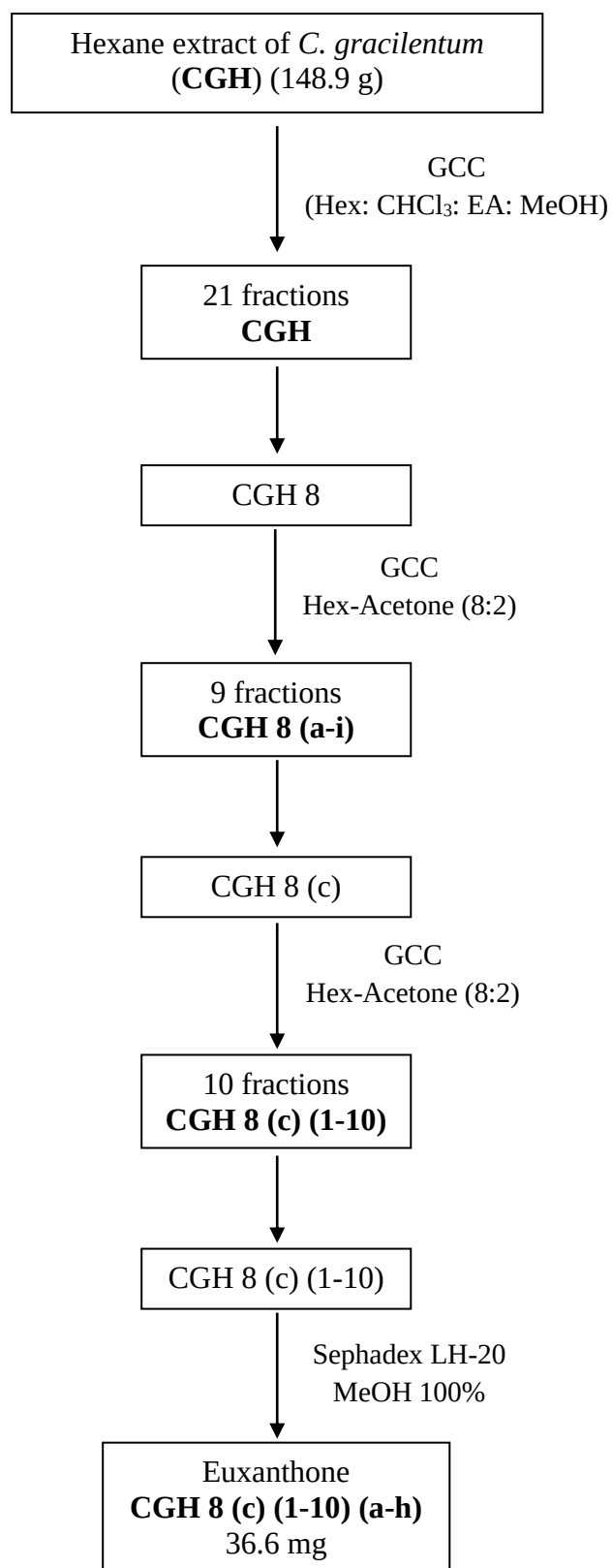


Figure 3.3: Flowchart of the purification process of euxanthone (63)

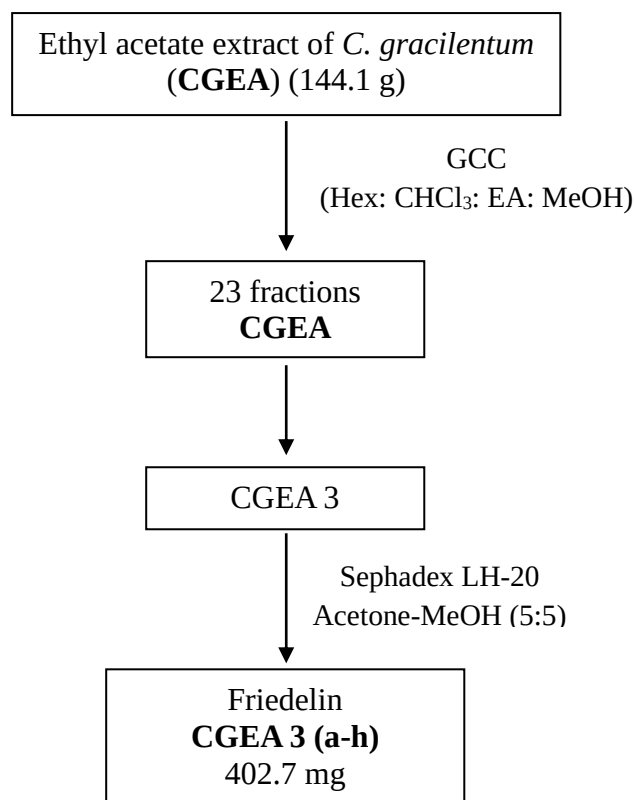


Figure 3.4: Flowchart of the purification process of friedelin (60)

3.3 Chromatographic Methods

3.3.1 Column chromatography

Column chromatography (Figure 3.5) was utilised to isolate mixtures of compounds by using a glass column packed with silica gel. The compound mixture separated when a mobile phase carried it via a stationary phase. The mobile phase for column chromatography is a solvent or mixture of solvents. Meanwhile, the stationary phase is the adsorbent. The adsorbent used was Merck's silica gel 60 (0.040 – 0.063 mm) that separated based on the polarity. The drying packing method was utilised for the sample preparation, which is mixing the sample dropwise with dry silica gel after dissolving it in a small amount of appropriate solvent. The sample that had been prepared was introduced to

the column, and then by gradient elution to increase polarity and isolate the compounds with different polarities. The collected fractions were analysed by Thin Layer Chromatography (TLC) after being concentrated using a rotary evaporator.

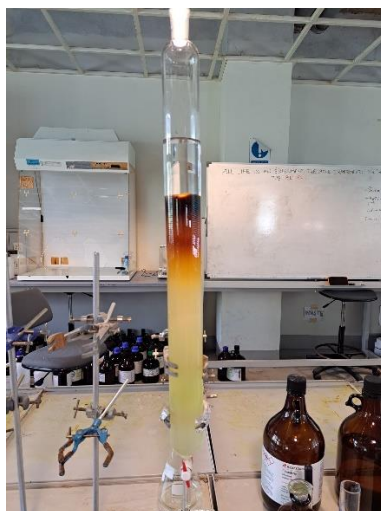


Figure 3.5: Column chromatography

3.3.2 Thin Layer Chromatography (TLC)

Thin-layer chromatography is a technique to separate the components of a mixture according to differences in their polarity. It was performed by using aluminium-backed plates coated with Merck Kieselgel 60 PF₂₅₄ as depicted in Figure 3.6. The mobile phase was an appropriate solvent system, and the stationary phase was a coated silica gel. First, a thin capillary spotter was utilised to spot the sample on the TLC plate. After that, a known composition of the solvent system was filled into a TLC chamber. Then, the lid of the chamber was closed and set away for a moment to make sure the solvent vapour had fully saturated the chamber. The TLC plate that had been spotted was then put inside the chamber to enable capillary action to cause the mobile phase to ascend on the plate. When

the solvent reached the solvent line, the TLC plate was removed from the TLC chamber and visualised under a UV lamp.

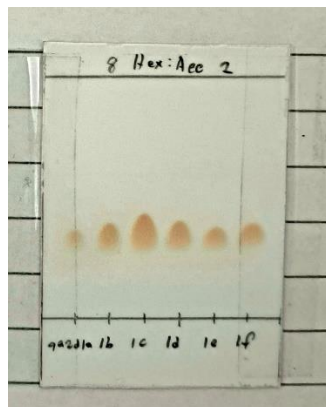


Figure 3.6: The developed TLC plate

3.4 Instrumentations

The 1D and 2D NMR were processed on JEOL JNM-ECZ 500 MHz and Bruker AV400III HD 400 MHz spectrophotometer using CDCl_3 and acetone- d_6 as solvent. The 1D NMR consisted of ^1H , ^{13}C and DEPT, while 2D consisted of COSY, HSQC and HMBC. LC-MS spectra were obtained using a Shimadzu CBM-20A spectrometer (the mobile phase consisted of a mixture of water: methanol (80:20) with a flow rate of 0.5 mL min^{-1} for an isocratic run of 10 minutes). The GC-MS spectra were recorded on a Shimadzu GCMS-QP 5050A spectrometer (column, SGE BPX5 $30 \text{ m} \times 0.25 \text{ mm I.D} \times 0.25 \text{ }\mu\text{m}$ film thickness, temperature, $200 \text{ }^\circ\text{C}$). The UV-Vis spectra were determined using an Agilent Cary 60 spectrophotometer. Infrared spectra were determined by attenuated total reflection (ATR) technique on a Thermo Scientific NICOLET iS10 spectrophotometer. The melting point of crystalline compounds were obtained using Stuart SMP3 Melting Point Apparatus. The rotary evaporator (Heidolph LABOROTA 4000 efficient) was used for the preparation of

crude extract. All the apparatus involved in biological assay work were autoclaved using EYELA autoclave MAC-601. Bioassay work was conducted in Biological Safety Cabinet Class II.

3.5 Antibacterial Assay

3.5.1 Preparation of media Mueller Hinton Agar (MHA)

In 1 L of distilled water, 34.0 g of MHA powder were dissolved. Then, the mixture was heated to boil with frequent stirring. The media was autoclaved at 121 °C for 15 minutes. Inside a biological safety cabinet, the sterilised MHA was poured into Petri dishes and solidified at room temperature.

3.5.2 Preparation of McFarland Standard and bacterial suspension

0.5 McFarland Standard was prepared by adding 0.05 mL of 1.0% barium chloride into a 15 mL centrifuge tube, followed by adding 9.95 mL of 1.0% sulphuric acid. Five bacterial strains, *Bacillus cereus* (ATCC 11778), *Lactiplantibacillus plantarum* (ATCC 14917), *Enterobacter cloacae* (ATCC 13047), *Pseudomonas aeruginosa* (ATCC 27853) and *Serratia marcescens* (ATCC 14756) were used in antibacterial assay. All bacterial strains were obtained from Molecular Genetic Laboratory, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak. After activation, the bacteria were prepared in 15 mL centrifuge tubes each in 10 mL of nutrient broth. The turbidity was compared by holding the bacterial sample and McFarland Standard tube up against the black and white bars printed on the enclosed card to obtain a bacterial suspension with a 1.5×10^8 CFU/mL.

3.5.3 Agar well diffusion method

The bacteria inhibition activities of the isolated compounds and extracts were carried out using the agar well diffusion method (Rivero-Pino et al., 2023). The tested bacteria strain (50 μ L) was spread on MHA plates. Aseptically, sterile micropipette tips were used to create wells with diameters of approximately 6 mm on the agar plates. 50 μ L of isolated samples were loaded in the agar wells. The agar plates were incubated for 24 hours at 37 °C. The extracts were screened at 1000 μ g/mL concentration, while 100 μ g/mL for isolated compounds. The antimicrobial activity of each sample was compared with positive control. Ampicillin was utilised as the positive control and DMSO as the negative control. The agar well diffusion experiment was performed in triplicate. The antibacterial activities of the sample were ascertained by measuring the diameter of the inhibitory zones on the agar surface around the wells. Diameter values of more than 10 mm were considered active against bacterial strains and continued to MIC (Ouchari et al., 2019).

3.5.4 Minimum Inhibitory Concentration (MIC)

The MIC evaluations were carried out by the broth micro-dilution method, where a 96-well flat-bottom microplate was used (Zamakshshari et al., 2022). Serial dilutions of the extracts and compounds were used for the assay, ranging from 1000 to 1.953 μ g/mL and 100 to 0.195 μ g/mL, respectively. Five microlitres of bacterial strains were added to each sample except for the control. DMSO was added as the negative control. In a different 96-well flat-bottom microplate, ampicillin was also evaluated and used as the positive control. After that, the plate was incubated for 24 hours at 37 °C. The lowest concentration that remained colourless was identified as the MIC value, which indicated the lowest concentration of the antimicrobial agent that prevents bacterial growth. All assays were

carried out in triplicate. MIC values were displayed as the mean value of each assay. A plant extract with the MIC value of less than 100 µg/mL showed high activity, while the MIC value of 100 to 500 µg/mL indicated moderate activity and more than 1000 µg/mL was considered weak antibacterial activity (Carneiro et al., 2017). For isolated compounds, the MIC value of less than 10 µg/mL showed high activity. Meanwhile, 10 to 50 and more than 100 µg/mL were displayed moderate and weak antibacterial activity, respectively (Kalli et al., 2021).

3.5.5 Minimum Bactericidal Concentration (MBC)

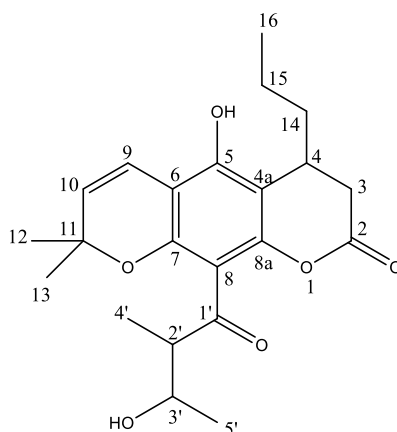
From each MIC broth tube without visible growth, the broth was aliquot and spread onto MHA plates. For ampicillin, the broth without visible growth was also aliquot and spread onto MHA plates. The MHA plates were incubated for 24 hours at 37 °C. The MBC experiment, which was adapted from Balouri et al. (2016), was performed in triplicate. It is indicated to as an MBC endpoint when 99.9% of the bacterial population is eliminated at the lowest antimicrobial agent concentration.

3.5.6 Statistical analysis

The data were analysed using one-way ANOVA with post-hoc Tukey HSD Test to determine the significance difference between samples and ampicillin. The significance level was set at $p < 0.05$. The antimicrobial testing in triplicate was reported as the mean $\pm$ standard error.

3.6 Physical and Spectral Data of Secondary Metabolites from *Calophyllum soulattri* and *Calophyllum gracilentum*

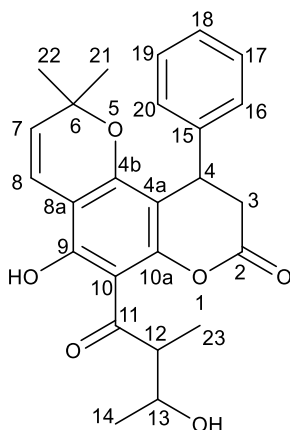
3.6.1 Calanolide E (3)



Yellowish oil. $C_{22}H_{28}O_6$. IR $\nu_{\max}$ (cm^{-1}): 3232 (broad, O-H stretch), 3058 (alkene C-H stretch), 2972 (C-H stretch), 1706 (conjugated C=O), 1621 and 1589 (aromatic C=C stretch), 1442 (C-H bend), 1117 (C-O stretch). UV-Vis (MeOH) $\lambda_{\max}$ (nm): 360, 270, 225. LC-MS (m/z): 388. ^1H NMR (500 MHz, $CDCl_3$) δ H: 6.50 (1H, d , $J = 10.3$ Hz, H-9), 5.41 (1H, d , $J = 10.3$ Hz, H-10), 4.52 (1H, m , $J = 3.4$ Hz, H-3'), 3.68 (1H, m , $J = 7.5$ Hz, H-4), 2.80 (1H, m , $J = 7.5$ Hz, H-3b), 2.72 (1H, m , $J = 7.5$ Hz, H-3a), 2.51 (1H, m , $J = 3.4$ Hz, H-2'), 1.85 (1H, m , $J = 8.0$ Hz, H-14a), 1.50 (1H, m , $J = 8.0$ Hz, H-14b), 1.40 (3H, s , H-12), 1.37 (3H, s , H-13), 1.35 (3H, d , $J = 6.3$ Hz, H-5'), 1.21 (1H, m , $J = 8.0$ Hz, H-15), 1.14 (3H, d , $J = 7.5$ Hz, H-4'), 0.85 (3H, t , $J = 7.5$ Hz, H-16). ^{13}C NMR (125 MHz, $CDCl_3$) δ C: 200.9 (C-1'), 179.5 (C-2), 162.4 (C-8a), 160.1 (C-5), 155.1 (C-7), 125.8 (C-10), 115.9 (C-9), 110.4 (C-4a), 101.1 (C-8), 101.0 (C-6), 78.1 (C-11), 76.2 (C-3'), 44.4 (C-2'), 38.3 (C-3), 35.3 (C-14), 30.2 (C-4), 28.4 (C-13), 28.3 (C-12), 21.0 (C-15), 16.5 (C-5'), 14.1 (C-

16), 9.6 (C-4'). The ^1H NMR and ^{13}C NMR spectral data are in accordance with the literature Silva et al., 2021.

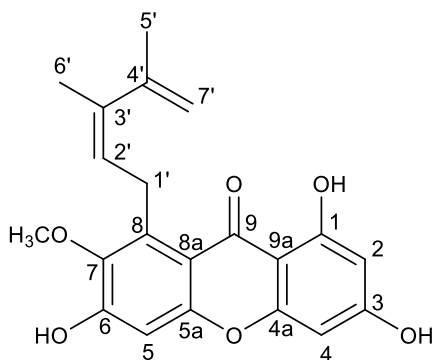
3.6.2 Calopolyanolide E (19)



Yellow crystal. $\text{C}_{25}\text{H}_{26}\text{O}_6$. Melting point: 171-172 °C IR ν_{max} (cm^{-1}): 3232 (broad, O-H stretch), 3061 (aromatic C-H stretch), 3032 (alkene C-H stretch), 2979 (C-H stretch), 1708 (conjugated C=O), 1626 and 1592 (aromatic C=C stretch), 1445 (C-H bend), 1132 (C-O stretch). UV-Vis (MeOH) λ_{max} (nm): 370, 300, 275, 222. GC-MS (m/z): 422. ^1H NMR (500 MHz, CDCl_3) δ H: 7.37 (2H, *d*, $J = 7.5$ Hz, H-16, H-20), 7.22 (2H, *m*, $J = 7.5$ Hz, H-17, H-19), 7.13 (1H, *t*, $J = 7.5$ Hz, H-18), 6.50 (1H, *d*, $J = 9.7$ Hz, H-8), 5.42 (1H, *d*, $J = 9.7$ Hz, H-7), 5.04 (1H, *t*, $J = 7.5$ Hz, H-4), 4.53 (1H, *m*, $J = 3.4$ Hz, H-13), 3.29 (2H, *m*, $J = 8.0$ Hz, H-3), 2.53 (1H, *m*, $J = 3.4$ Hz, H-12), 1.41 (3H, *s*, H-21), 1.37 (3H, *d*, $J = 6.9$ Hz, H-14), 1.19 (3H, *s*, H-22), 1.15 (3H, *d*, $J = 7.5$ Hz, H-23). ^{13}C NMR (125 MHz, CDCl_3) δ C: 200.8 (C-11), 178.3 (C-15), 161.8 (C-2), 159.7 (C-4b), 155.4 (C-9), 143.4 (C-10a), 128.0 (C-16, C-20), 127.9 (C-17, C-19), 126.0 (C-18), 126.0 (C-7), 115.8 (C-8), 111.2 (C-4a), 101.5 (C-8a), 101.2 (C-10), 78.4 (C-6), 76.2 (C-13), 44.4 (C-12), 36.7 (C-3),

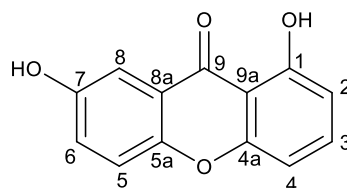
34.9 (C-4), 28.3 (C-21), 27.9 (C-22), 16.5 (C-14), 9.6 (C-23). The ^1H NMR and ^{13}C NMR spectral data are in accordance with the literature Shen et al., 2022.

3.6.3 Souluxanthone (62)



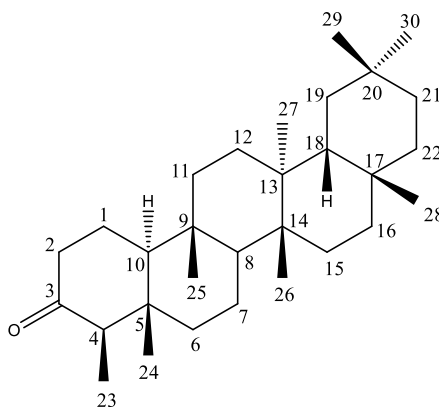
Yellowish oil. $\text{C}_{21}\text{H}_{20}\text{O}_6$. IR ν_{max} (cm^{-1}): 3318 (broad, O-H stretch), 3196 (aromatic-H stretch), 3085 (alkene C-H stretch), 2927 (C-H stretch), 1644 (conjugated C=O), 1605 (aromatic C=C stretch), 1274 (C-O stretch). UV-Vis (MeOH) λ_{max} (nm): 385, 285, 235, 205. LC-MS (m/z): 368. ^1H NMR (400 MHz, acetone- d_6) δ H: 13.47 (s, 1-OH), 9.66 (s, 6-OH), 6.84 (1H, s, H-5), 6.28 (1H, s, H-4), 6.19 (1H, s, H-2), 5.29 (1H, t, $J = 6.7$ Hz, H-2'), 4.81 (1H, s, H-7'), 4.67 (1H, s, H-7'), 4.12 (2H, d, $J = 6.1$ Hz, H-1'), 3.79 (s, 7-OCH₃), 1.83 (3H, s, H-6'), 1.62 (3H, s, H-5'). ^{13}C NMR (100 MHz, acetone- d_6) δ C: 182.8 (C-9), 165.5 (C-3), 164.9 (C-1), 158.0 (C-4a), 157.6 (C-5a), 156.3 (C-6), 149.4 (C-4'), 144.6 (C-7), 138.3 (C-8), 135.4 (C-3'), 124.6 (C-2'), 112.0 (C-8a), 110.2 (C-7'), 103.8 (C-9a), 102.8 (C-5), 98.8 (C-2), 93.8 (C-4), 61.4 (C-7a), 26.8 (C-1'), 17.8 (C-5'), 16.7 (C-6').

3.6.4 Euxanthone (63)



Yellow needle crystals. $C_{13}H_8O_4$. Melting point: 236-237 °C. IR $\nu_{\max}$ (cm^{-1}): 3303 (broad, O-H stretch), 3067 (aromatic C-H stretch), 2961 (C-H stretch), 1603 (conjugated C=O), 1478 (aromatic C=C stretch), 1230 (C-O stretch). UV-Vis (MeOH) $\lambda_{\max}$ (nm): 385, 285, 260, 235. LC-MS (m/z): 228. ^1H NMR (500 MHz, acetone- d_6) δ H: 12.68 (s, 1-OH), 8.98 (s, 7-OH), 7.67 (1H, t, J = 8.0 Hz, H-3), 7.58 (1H, d, J = 3.0 Hz, H-8), 7.49 (1H, d, J = 9.2 Hz, H-5), 7.42 (1H, dd, J = 9.2, 3.0 Hz, H-6), 6.98 (1H, d, J = 8.0 Hz, H-4), 6.75 (1H, d, J = 8.0 Hz, H-2). ^{13}C NMR (125 MHz, acetone- d_6) δ C: 182.1 (C-9), 161.9 (C-1), 156.5 (C-4a), 154.2 (C-7), 150.2 (C-5a), 137.0 (C-3), 125.3 (C-6), 121.0 (C-8a), 119.4 (C-5), 109.7 (C-8), 108.4 (C-9a), 108.4 (C-2), 106.9 (C-4). The ^1H NMR and ^{13}C NMR spectral data are in accordance with the literature Lizazman et al., 2023.

3.6.5 Friedelin (60)



White needle crystals. $C_{30}H_{50}O$. Melting point: 262-263 °C. IR $\nu_{\max}$ (cm^{-1}): 2925 and 2865 (C-H stretch), 1709 (C=O stretch), 1450 and 1384 (C-H bent). UV-Vis ($CHCl_3$) $\lambda_{\max}$ (nm): 288. LC-MS (m/z): 426. ^1H NMR (500 MHz, $CDCl_3$) δ H: 2.38, 2.26 (2H, *m*, H-2), 2.23 (1H, *q*, $J = 6.9$ Hz, H-4), 1.94, 1.72 (1H, *m*, H-1), 1.74, 1.26 (2H, *d*, $J = 11.5$ Hz, H-6), 1.52, 1.34 (2H, *m*, H-16), 1.52 (1H, *m*, H-18), 1.50 (1H, *m*, H-10), 1.49, 1.30 (2H, *m*, H-21), 1.49, 0.93 (2H, *m*, H-22), 1.48, 1.37 (2H, *m*, H-7), 1.46, 1.26 (2H, *m*, H-11), 1.45, 1.24 (2H, *m*, H-15), 1.37 (1H, *t*, $J = 8.6$ Hz, H-8), 1.37, 1.19 (2H, *m*, H-19), 1.37, 1.32 (2H, *m*, H-12), 1.16 (3H, *s*, H-28), 1.03 (3H, *s*, H-27), 0.99 (3H, *s*, H-26), 0.98 (3H, *s*, H-30), 0.93 (3H, *s*, H-29), 0.86 (3H, *d*, $J = 6.1$ Hz, H-23), 0.85 (3H, *s*, H-25), 0.70 (3H, *s*, H-24). ^{13}C NMR (125 MHz, $CDCl_3$) δ C: 213.3 (C-3), 59.6 (C-10), 58.3 (C-4), 53.2 (C-8), 42.9 (C-18), 42.2 (C-5), 41.6 (C-6), 41.4 (C-2), 39.8 (C-13), 39.3 (C-22), 38.4 (C-14), 37.5 (C-9), 36.1 (C-16), 35.7 (C-19), 35.4 (C-11), 35.1 (C-29), 32.8 (C-21), 32.5 (C-15), 32.2 (C-28), 31.9 (C-30), 30.6 (C-12), 30.1 (C-17), 28.2 (C-20), 22.4 (C-1), 20.3 (C-26), 18.7 (C-27), 18.3 (C-7), 18.0 (C-25), 14.7 (C-24), 6.9 (C-23). The ^1H NMR and ^{13}C NMR spectral data are in accordance with the literature Lizazman et al., 2023.

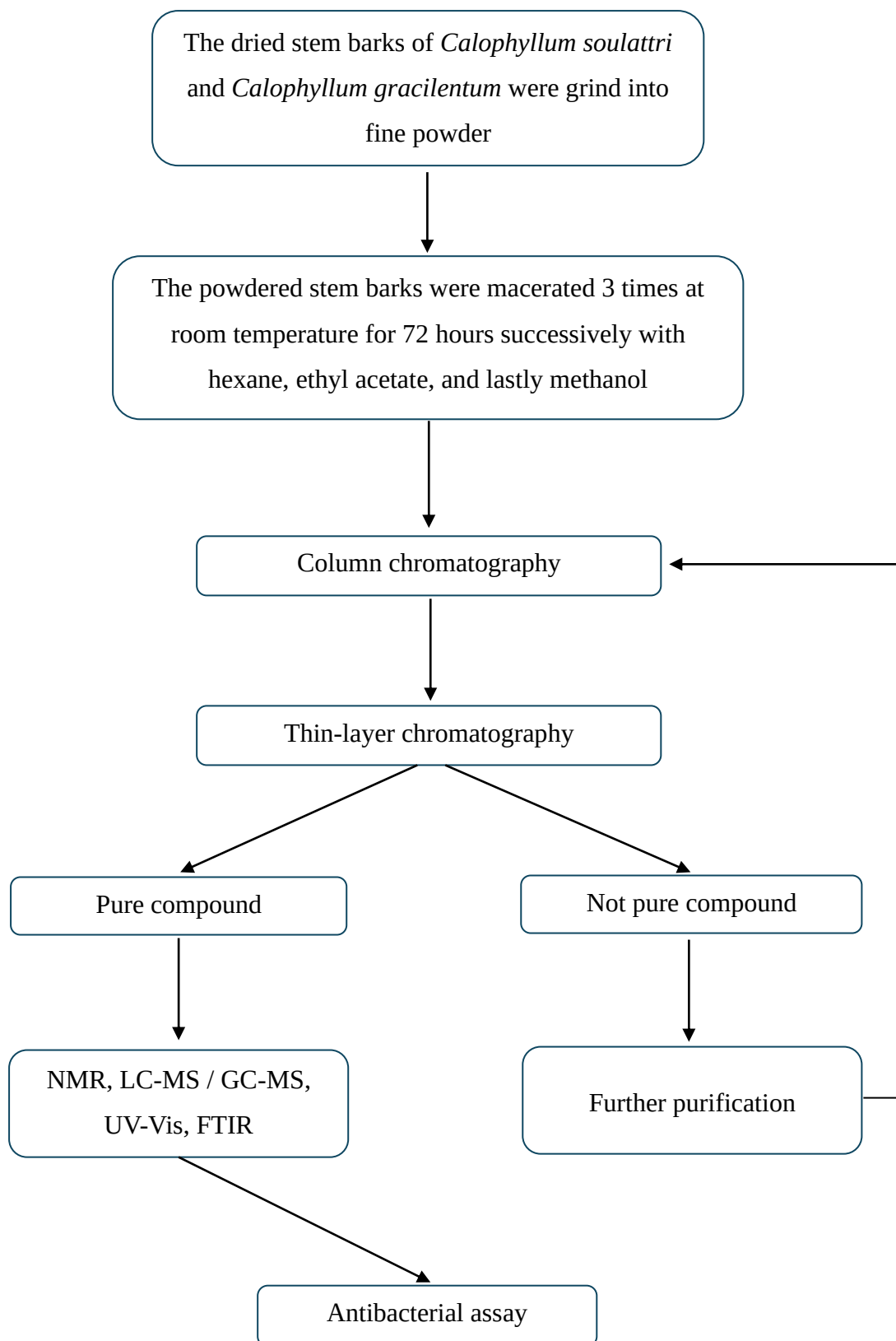


Figure 3.7: Flow diagram of the experimental

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation of Chemical Constituents from *Calophyllum soulattri*

4.1.1 Characterisation of calanolide E (3)

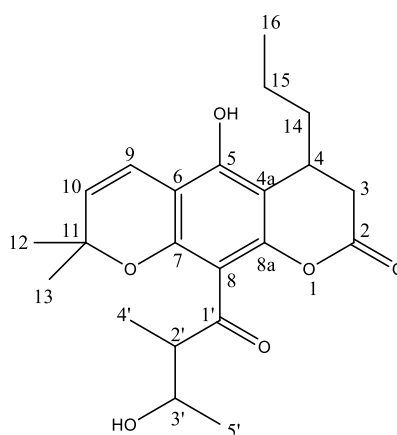


Figure 4.1: Structure of calanolide E (3)

Calanolide E (3) (Figure 4.1) was gained as yellowish oil. The LC-MS spectrum gave a molecular ion $[M^+]$ at m/z 388, which correlated to the molecular formula $C_{22}H_{28}O_6$. The FTIR spectrum demonstrated absorption bands at 3232 cm^{-1} (broad, O-H stretch), 3058 cm^{-1} (alkene C-H stretch), 2972 cm^{-1} (C-H stretch), 1706 cm^{-1} (conjugated C=O), 1621 and 1589 cm^{-1} (aromatic C=C stretch), 1442 cm^{-1} (C-H bend) and 1117 cm^{-1} (C-O stretch). The UV-Vis spectrum displayed λ_{max} absorptions at 360, 270 and 225, indicating the presence of a coumarin skeleton (Tee et al., 2018).

The interpretation of the ^{13}C and DEPT NMR spectrum showed five methines, three methylenes, five methyls and nine quaternary carbon moieties to be present in

compound **3**. Meanwhile, the ^1H NMR spectrum showed 13 proton signals that represent methine, methylene and methyl groups. All the protonated carbons were determined in regard to ^1J correlation peaks between protons and carbons in the HSQC NMR experiment.

The ^1H NMR spectrum displayed signals at δ 6.50 (H-9) and δ 5.41 (H-10), suggesting the existence of pyrano moiety in compound **3**. A pair of vinylic protons were adjacent to each other, as seen from the correlation peaks in the COSY NMR spectrum and had similar coupling constants at 10.3 Hz. The pyrano moiety was fused to positions C-6 and C-7 by referring to the HMBC NMR correlation contour. The HMBC NMR spectrum showed a ^3J correlation between the proton at δ 5.41 (H-10) and the non-oxygenated carbon at δ 101.0 (C-6). Meanwhile, ^2J and ^3J correlations were found between δ 6.50 (H-9) with the carbon at δ 101.0 (C-6) and δ 155.1 (C-7) respectively.

The proton signals observed at δ 2.51 (H-2'), δ 4.52 (H-3'), δ 1.14 (H-4') and δ 1.35 (H-5') indicated the existence of an isoprene unit. The HMBC NMR correlations (Figure 4.2) of δ 1.14 (H-4') with δ 76.2 (C-2'), δ 44.4 (C-3') and δ 200.9 (C-1') confirmed that this CH_3 proton was located at position C-2'. The coupling between H-2' with H-4' and H-3' with H-5' in the COSY NMR spectrum established the connectivity between δ 76.2 (C-2') with δ 9.6 (C-4') and δ 44.4 (C-3') with δ 16.5 (C-5'). The ^2J correlation of CH_3 proton at δ 1.35 (H-5') with the carbon at δ 44.4 (C-3') and ^3J correlation at δ 76.2 (C-2') confirmed its position at C-3'. One conjugated carbonyl carbon was discovered at δ 200.9 (C-1'), one carbinolic carbon at δ 44.4 (C-3'), two CH_3 groups at δ 9.6 (C-4') and δ 16.5 (C-5') as well as one peak at δ 76.2 (C-2') are in accordance with 3-hydroxy-2-methyl-1-oxobutyl unit.

An *n*-propyl group was observed to be fused to C-4, which was confirmed through long-range ^2J and ^3J correlations of proton at δ 1.50 (H-14) with the carbon at δ 30.2 (C-4) and δ 110.4 (C-4a) respectively. Besides, the presence of the *n*-propyl substituent was showed in the COSY NMR spectrum through cross-peaks between δ 1.50 (H-14) and δ 1.21 (H-15) and between δ 1.21 (H-15) and δ 0.85 (H-16).

Compound **3** was elucidated to be calanolide E previously isolated by Silva et al. (2021). The FTIR, LC-MS, 1D NMR and 2D NMR spectra of the compound are depicted in Figure 4.3 - 4.10. The NMR spectrum data of compound **3** are summarised in Table 4.1.

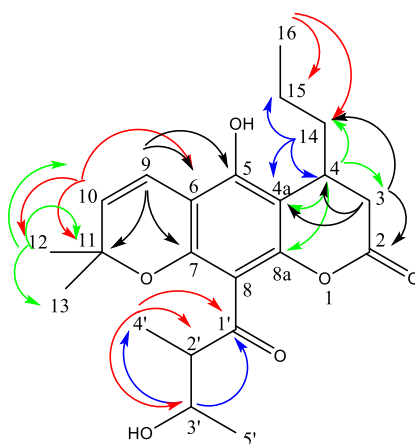
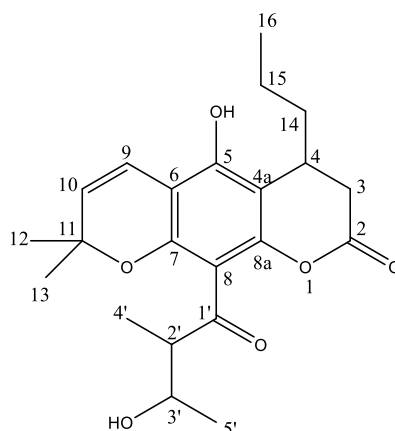


Figure 4.2: HMBC correlation in calanolide E (**3**)

Table 4.1: ^1H NMR (500 MHz, CDCl_3) and ^{13}C NMR (125 MHz, CDCl_3)

Assignments of calanolide E (3)



Position	^1H (δ)	^1H (δ)*	^{13}C (δ)	^{13}C (δ)*	DEPT	HMBC
2	-		179.5	178.6	-	-
3	2.72 (<i>d</i> , 1H, J = 7.5 Hz) 2.80 (<i>m</i> , 1H, J = 7.5 Hz)	2.43 (<i>m</i> , 1H) 2.68 (<i>m</i> , 1H)	38.3	38.6	CH_2	C-4, C-14, C-4a, C-2
4	3.68 (<i>m</i> , 1H, J = 7.5 Hz)	3.63 (<i>m</i> , 1H)	30.2	30.4	CH	C-14, C-3, C-4a, C-8a
4a	-	-	110.4	108.8	-	-
5	-	-	160.1	157.1	-	-
6	-	-	101.0	102.6	-	-
7	-	-	155.1	159.9	-	-
8	-	-	101.1	101.8	-	-
8a	-	-	162.4	159.8	-	-
9	6.50 (<i>d</i> , 1H, J = 10.3 Hz)	6.53 (<i>d</i> , 1H, J = 10.0 Hz)	115.9	115.6	CH	C-11, C-6, C-7, C-5
10	5.41 (<i>d</i> , 1H, J = 10.3 Hz)	5.39 (<i>d</i> , 1H, J = 10.0 Hz)	125.8	125.6	CH	C-12, C-11, C-6
11	-	-	78.1	78.1	-	-
12	1.40 (<i>s</i> , 3H)	1.32 (<i>s</i> , 3H)	28.3	27.9	CH_3	C-13, C-11, C-10
13	1.37 (<i>s</i> , 3H)	1.32 (<i>s</i> , 3H)	28.4	28.3	CH_3	C-12, C-11, C-10
14	1.85 (<i>m</i> , 1H, J = 8.0 Hz) 1.50 (<i>m</i> , 1H, J = 8.0 Hz)	1.40 (<i>m</i> , 2H)	35.3	35.5	CH_2	C-15, C-4, C-4a
15	1.21 (<i>m</i> , 2H, J = 8.0 Hz)	1.15 (<i>m</i> , 1H)	21.0	20.8	CH_2	C-16, C-4, C-14
16	0.85 (<i>t</i> , 3H, J =)	0.79 (<i>t</i> , 3H, J =)	14.1	14.0	CH_3	C-15, C-14

	7.5 Hz)	7.2 Hz)				
1'	-	-	200.9	199.6	-	-
2'	2.51 (<i>m</i> , 1H, <i>J</i> = 3.4 Hz)	2.45 (<i>m</i> , 1H)	44.4	45.6	CH	C-4', C-1'
3'	4.52 (<i>m</i> , 1H, <i>J</i> = 3.4 Hz)	4.11 (<i>m</i> , 1H)	76.2	78.8	CH	C-4', C-1'
4'	1.14 (<i>d</i> , 3H, <i>J</i> = 7.5 Hz)	1.13 (<i>d</i> , 3H, <i>J</i> = 6.3 Hz)	9.6	10.5	CH ₃	C-2', C-3', C-1'
5'	1.35 (<i>d</i> , 3H, <i>J</i> = 6.3 Hz)	1.47 (<i>d</i> , 3H, <i>J</i> = 6.3 Hz)	16.5	19.5	CH ₃	C-2', C-3'

*Silva et al. (2021)

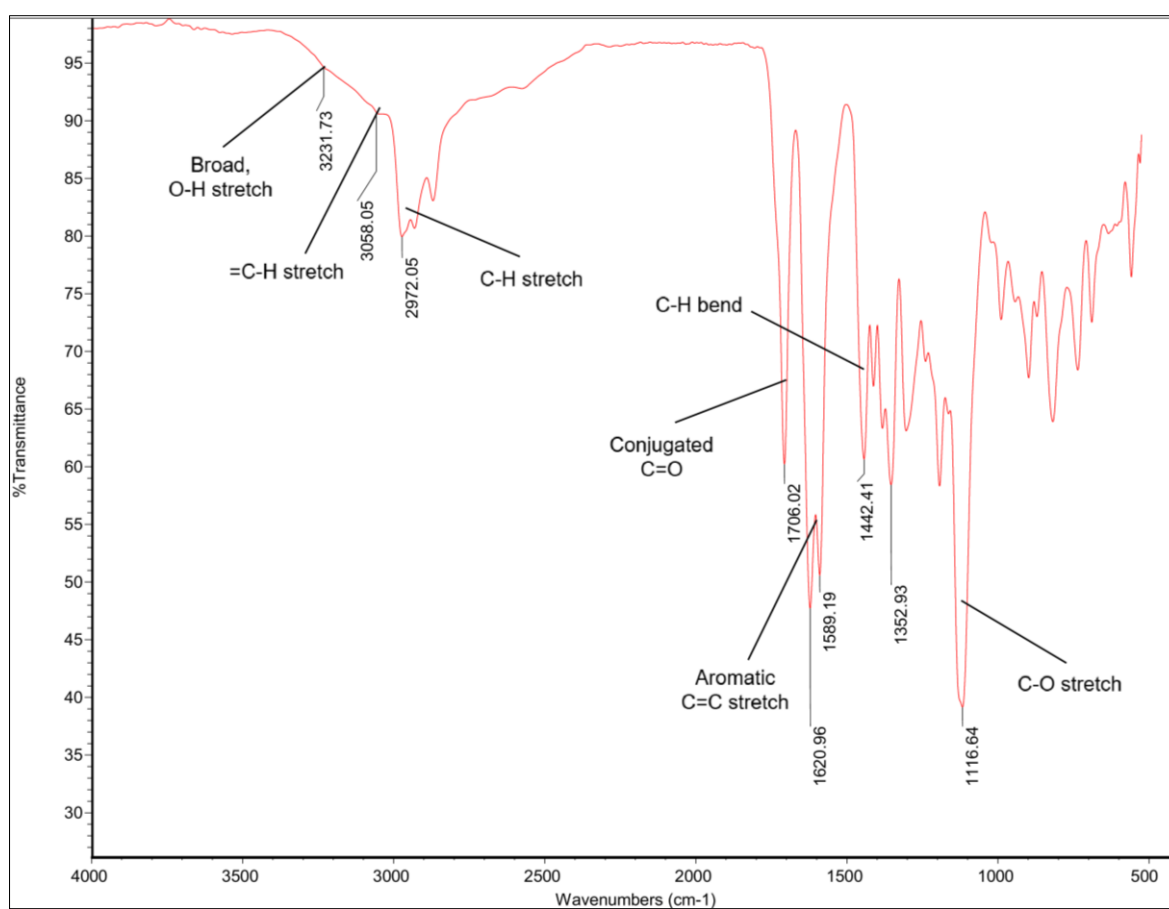
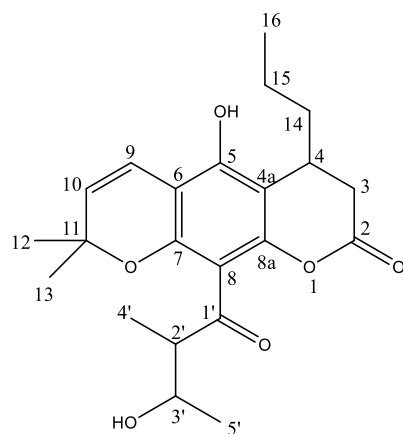


Figure 4.3: FTIR spectrum of calanolide E (3)

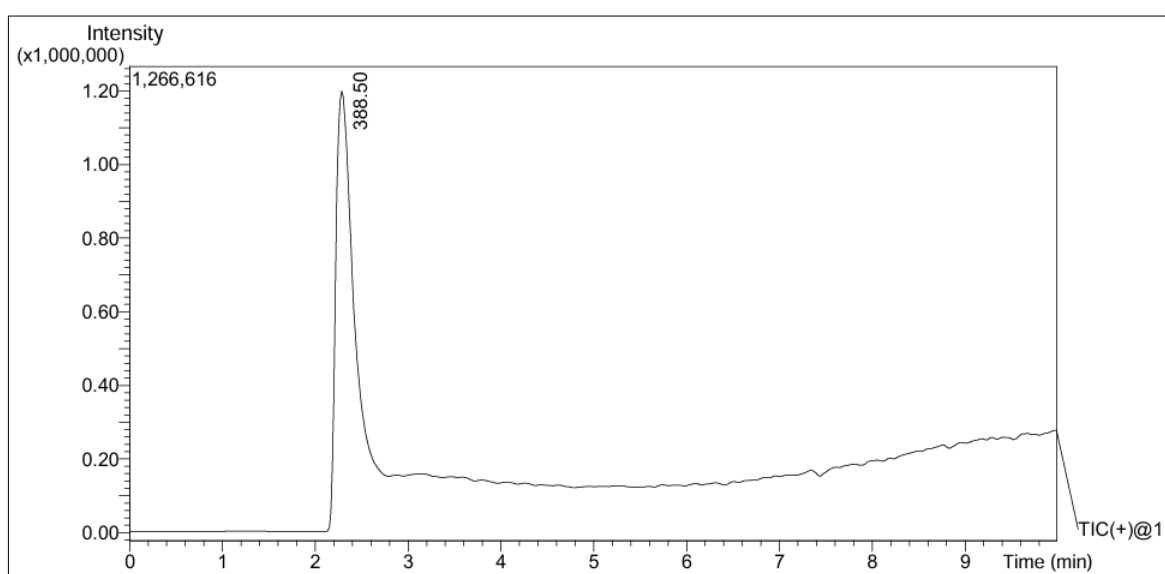
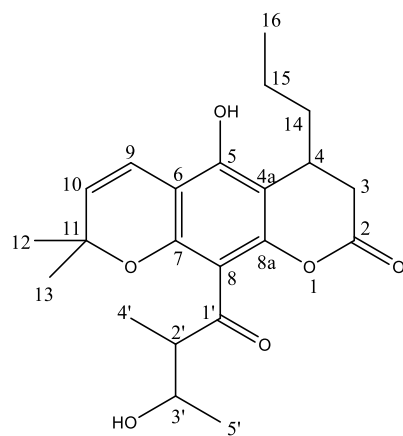


Figure 4.4: LC-MS spectrum of calanolide E (**3**)

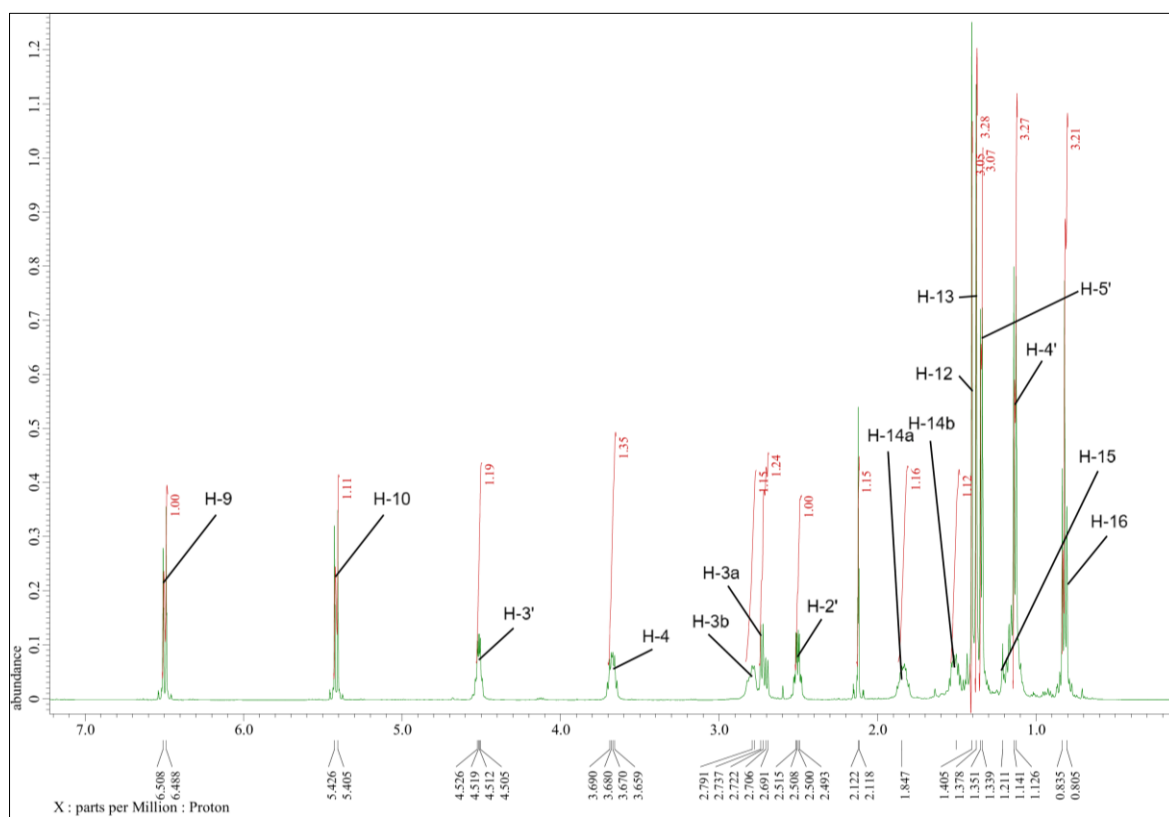
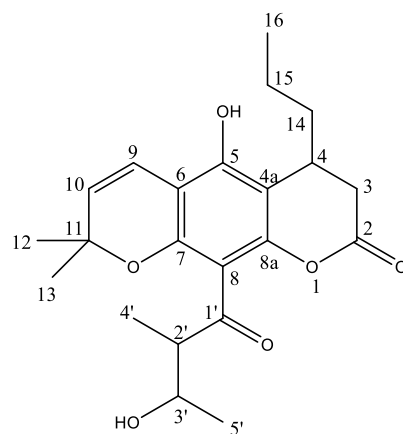


Figure 4.5: ^1H NMR spectrum of calanolide E (**3**) (500 MHz, CDCl_3)

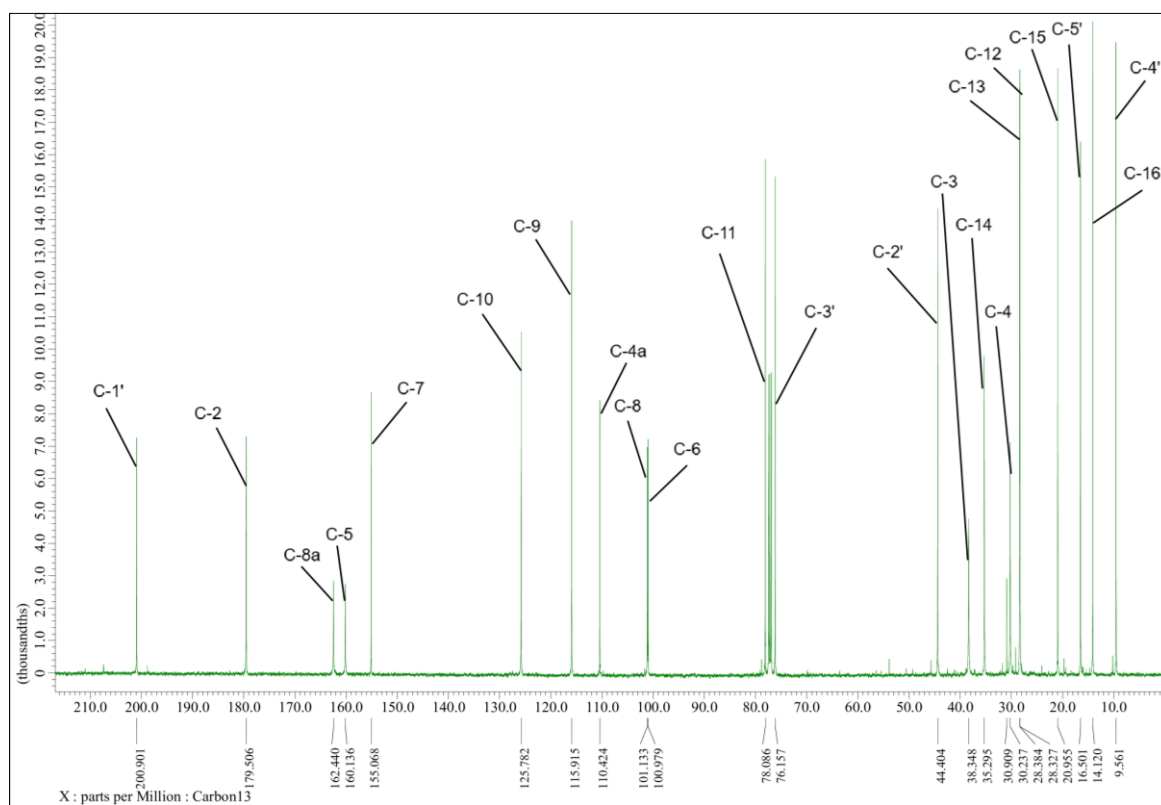
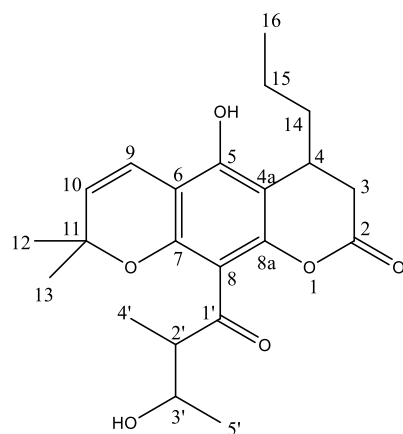


Figure 4.6: ^{13}C NMR spectrum of calanolide E (**3**) (125 MHz, CDCl_3)

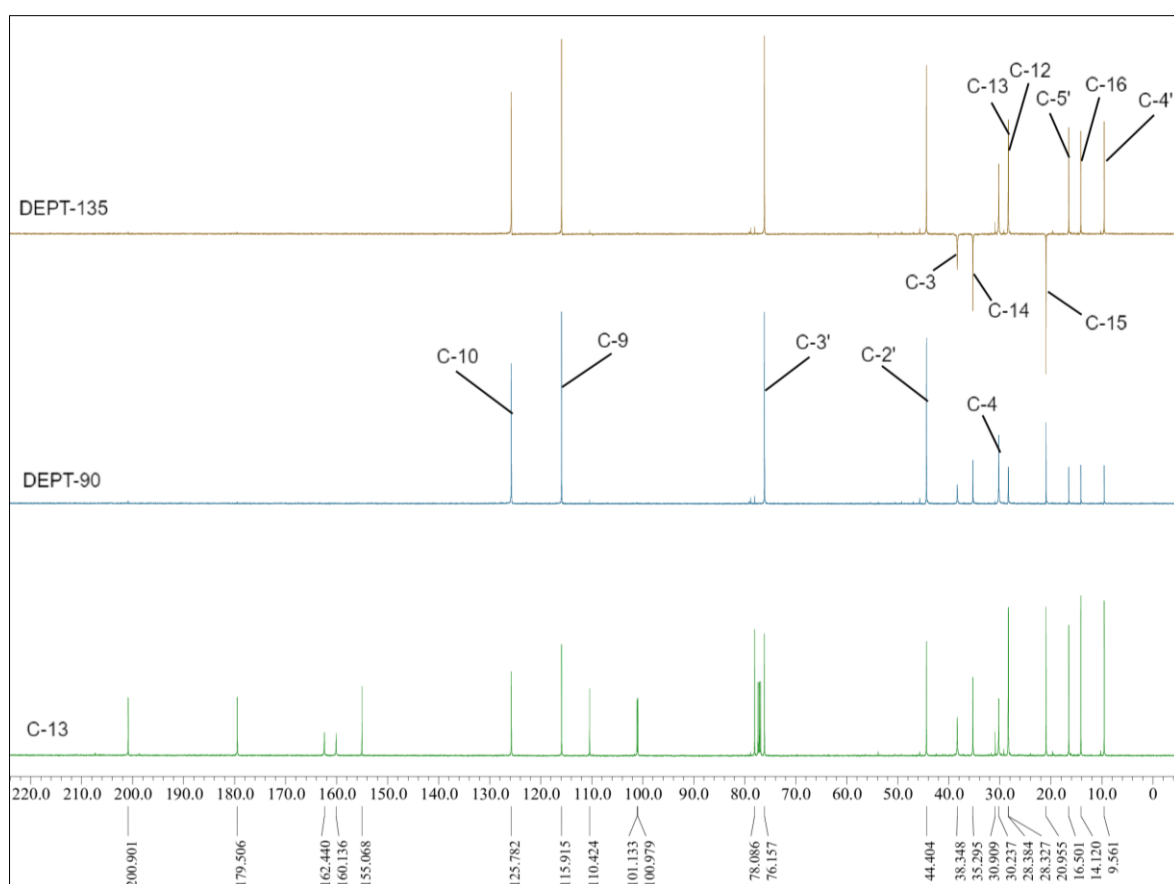
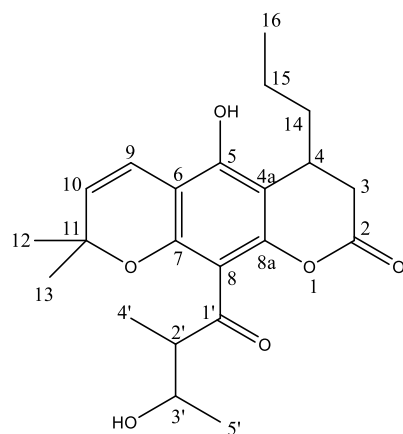


Figure 4.7: ^{13}C DEPT spectrum of calanolide E (**3**)

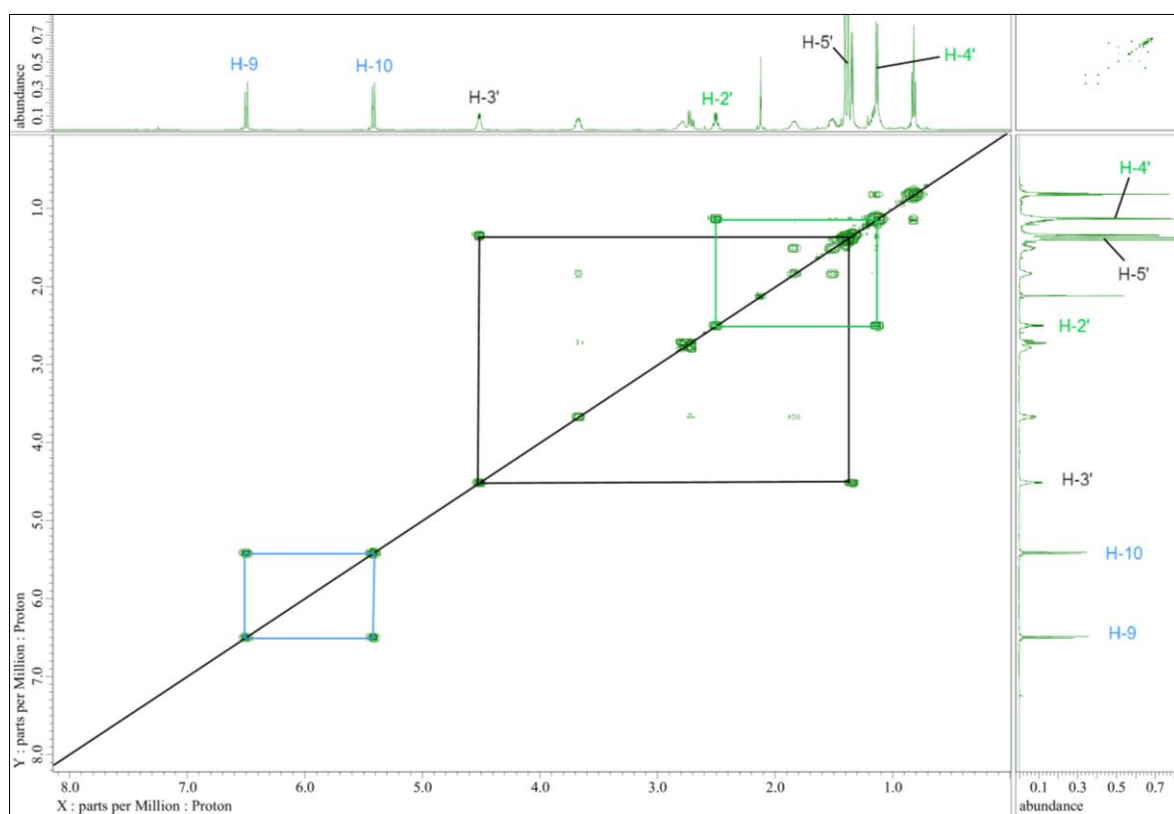
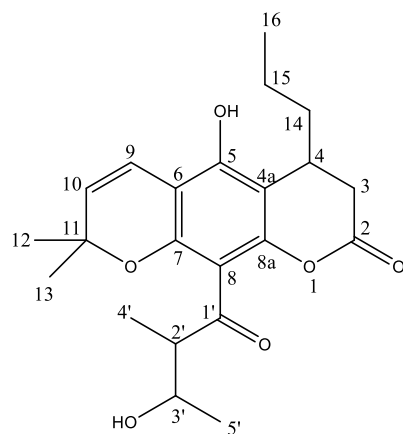


Figure 4.8: COSY spectrum of calanolide E (3)

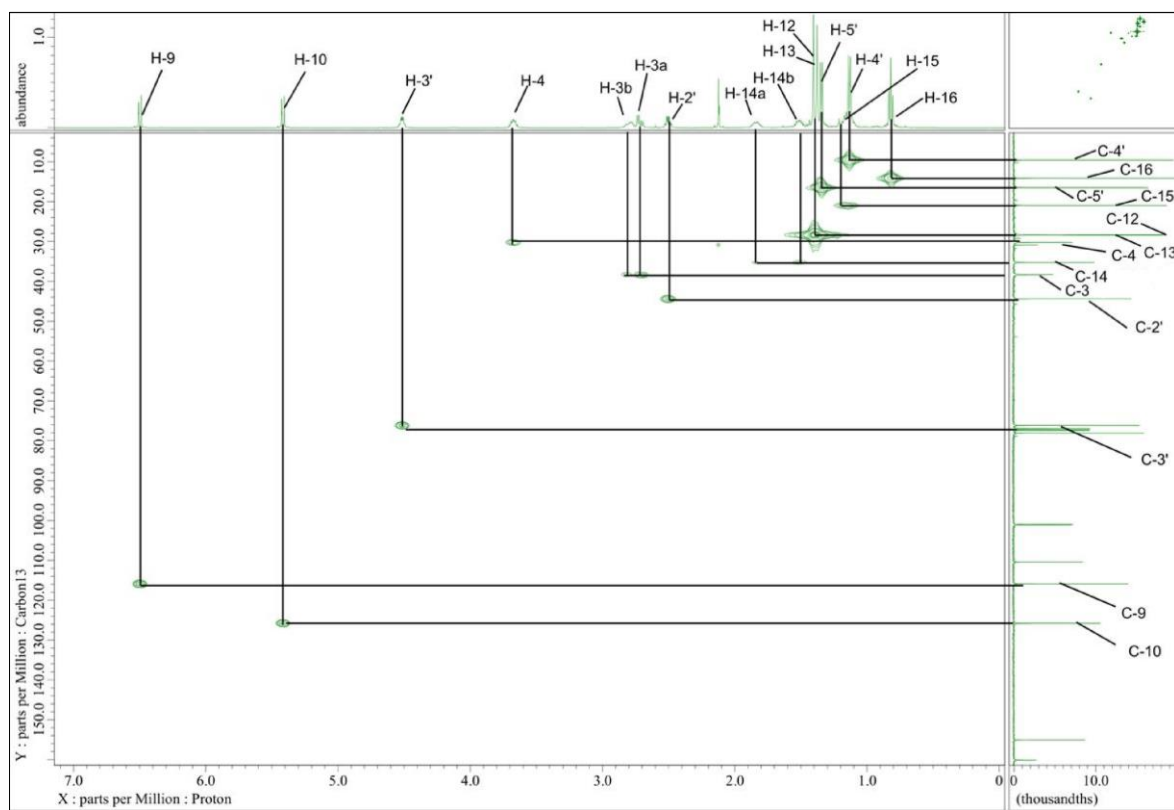
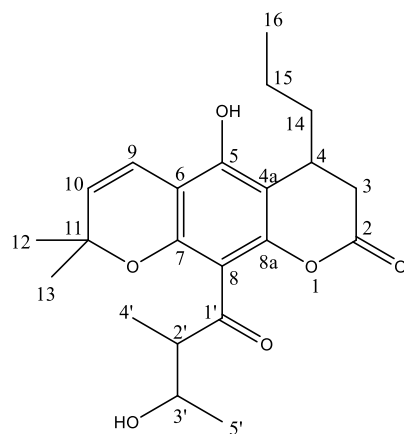


Figure 4.9: HSQC spectrum of calanolide E (3)

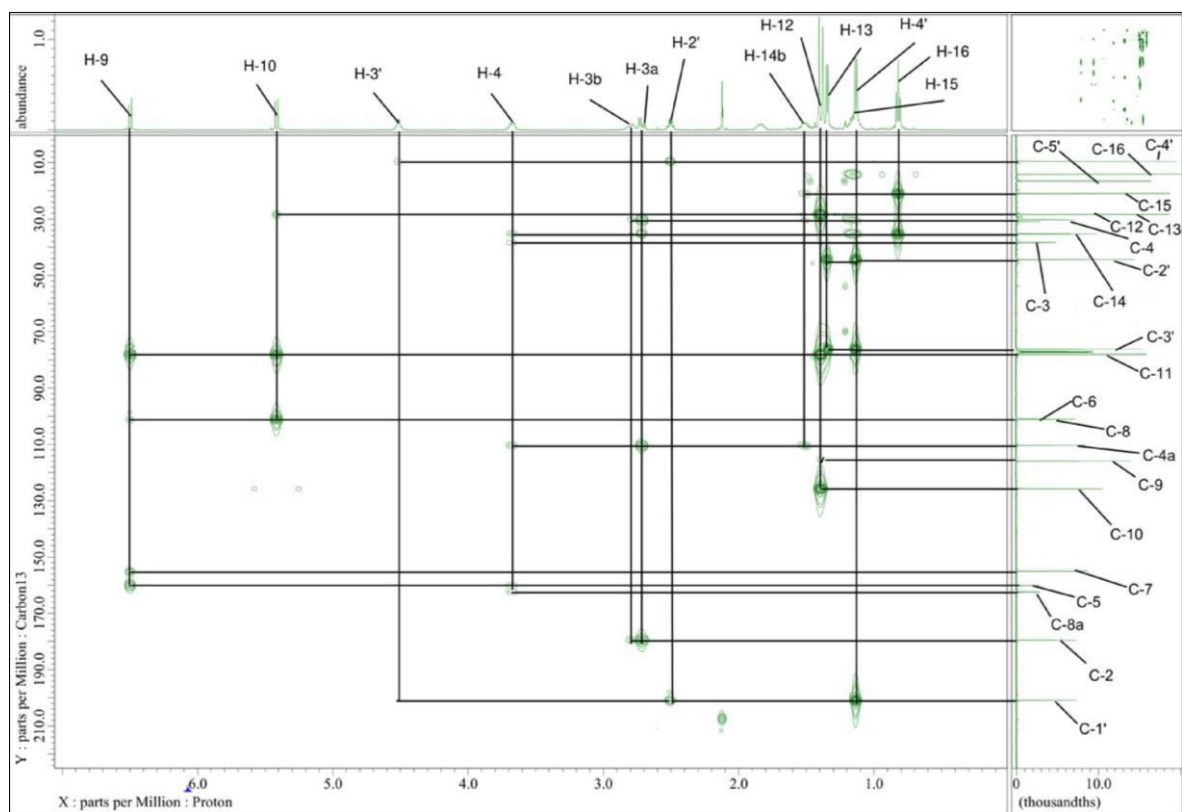
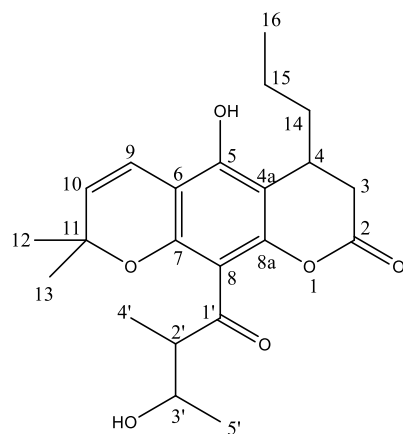


Figure 4.10: HMBC spectrum of calanolide E (3)

4.1.2 Characterisation of calopolyanolide E (**19**)

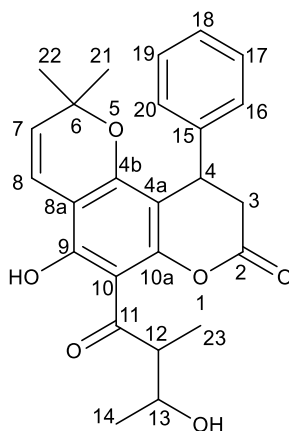


Figure 4.11: Structure of calopolyanolide E (**19**)

Calopolyanolide E (**19**) (Figure 4.11), which was previously found in 2022 (Shen et al., 2022), was isolated as yellow crystals with a melting point of 171-172 °C. The molecular ion $[M^+]$ peak observed at m/z 422 in the GC-MS spectrum corresponded to the $C_{25}H_{26}O_6$ molecular formula. The FTIR spectrum showed various values of absorbance, indicating the functional groups present in the compound. They are 3232 cm^{-1} (broad, O-H stretch), 3061 cm^{-1} (aromatic C-H stretch), 3032 cm^{-1} (alkene C-H stretch), 2979 cm^{-1} (C-H stretch), 1708 cm^{-1} (conjugated C=O), 1626 and 1592 cm^{-1} (aromatic C=C stretch), 1445 cm^{-1} (C-H bend) and 1132 cm^{-1} (C-O stretch). The UV-Vis spectrum displayed an observation of bands at 370, 300, 275 and 225 which are typical range for a coumarin skeleton (Zou et al., 2010).

Analysis of the ^1H NMR spectrum suggested the existence of a few substituent groups in the coumarin skeleton of compound **19**. A pyrano, phenyl and 3-hydroxy-2-methyl-1-oxobutyl moieties were seen. The ^1H NMR spectrum showed signals at δ 5.42 (H-7) and δ 6.50 (H-8), suggesting the presence of a pyrano moiety in compound **19**. A pair

of vinylic protons was observed adjacent to each other by the COSY NMR spectrum and had similar coupling constants at 9.7 Hz. The pyrano moiety was found to be attached to positions C-4b and C-8a by referring to the HMBC NMR correlation contour. The HMBC NMR spectrum showed a ^3J correlation between the proton at δ 5.42 (H-7) with the carbon at δ 101.5 (C-8a). Meanwhile, a ^3J correlation was found between δ 6.50 (H-8) with the carbon at δ 159.7 (C-4b).

The ^1H NMR signals at δ 7.37 (H-16 and H-20), δ 7.22 (H-17 and H-19) and δ 7.13 (H-18) suggested the presence of a monosubstituted phenyl group. These protons were observed next to each other, as seen from the correlation peaks in the COSY NMR spectrum, and their coupling constant values are listed in Table 4.2. Meanwhile, a triplet proton signal of aromatic proton at δ 7.13 (H-18) was placed in between two aromatic protons at δ 7.22 (H-17 and H-19). The phenyl group was attached at C-4. The attachment was established by the ^3J correlation of the proton at δ 7.37 (H-16 and H-20) with the carbon at δ 34.9 (C-4). Besides, the attachment was considered by ^2J and ^3J correlations of proton at δ 5.04 (H-4) with the carbons at δ 178.3 (C-15) and δ 128.0 (C-16 and C-20), respectively.

One conjugated carbonyl carbon was discovered at δ 200.8 (C-11), one carbinolic carbon at δ 76.2 (C-13), two CH_3 groups at δ 16.5 (C-14) and δ 9.6 (C-23) as well as one peak at δ 44.4 (C-12) are accordance with 3-hydroxy-2-methyl-1-oxobutyl moieties. The proton signals observed at δ 2.53 (H-12), δ 4.53 (H-13), δ 1.37 (H-14) and δ 1.15 (H-23) suggested the presence of an isoprene unit. The HMBC NMR correlations of δ 1.15 (H-23) with δ 44.4 (C-12), δ 76.2 (C-13) and δ 200.8 (C-11) confirmed that this CH_3 proton was located at position C-12. The coupling between H-12 with H-23 and H-13 with H-14 in the COSY NMR spectrum established the connectivity between δ 44.4 (C-12) with δ 9.6 (C-

23) and δ 76.2 (C-13) with δ 16.5 (C-14). ^2J correlation of CH_3 proton at δ 1.37 (H-14) with the carbon at δ 76.2 (C-13) and ^3J correlation at δ 44.4 (C-12) confirmed its position at C-13.

Compound **19** was identified as calopolyanolide E, previously isolated by Shen et al. (2022). The HMBC correlation of the compound is illustrated in Figure 4.12. The FTIR, GC-MS, 1D NMR and 2D NMR spectra of the compound are depicted in Figure 4.13 – 4.20. The NMR spectrum data of the compound are summarised in Table 4.2.

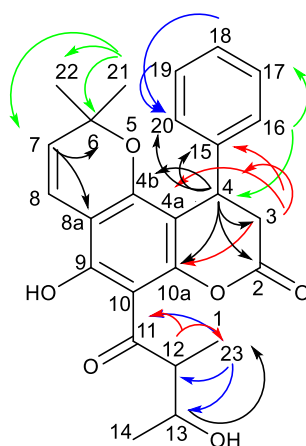
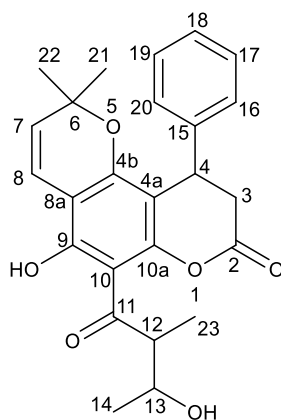


Figure 4.12: HMBC correlation in calopolyanolide E (**19**)

Table 4.2: ^1H NMR (500 MHz, CDCl_3) and ^{13}C NMR (125 MHz, CDCl_3)

Assignment of calopolyanolide E (19)



Position	^1H (δ)	^1H (δ)*	^{13}C (δ)	^{13}C (δ)*	DEPT	HMBC
2	-	-	161.8	159.5	-	-
3	3.29 (<i>m</i> , 2H, J = 8.0 Hz)	3.27 (<i>m</i> , 2H)	36.7	36.6	CH_2	C-4, C-4a, C-10a, C-15
4	5.04 (<i>t</i> , 1H, J = 7.5 Hz)	5.08 (<i>t</i> , 1H)	34.9	34.6	CH	C-3, C-4a, C-16 & C-20, C-10a, C-4b, C-2, C-15
4a	-	-	111.2	110.1	-	-
4b	-	-	159.7	159.3	-	-
6	-	-	78.4	79.1	-	-
7	5.42 (<i>d</i> , 1H, J = 9.7 Hz)	5.44 (<i>d</i> , 1H)	126.0	126.0	CH	C-6, C-8a
8	6.50 (<i>d</i> , 1H, J = 9.7 Hz)	6.58 (<i>d</i> , 1H)	115.8	115.6	CH	C-6, C-9, C-4b
8a	-	-	101.5	102.9	-	-
9	-	-	155.4	157.3	-	-
10	-	-	101.2	102.0	-	-
10a	-	-	143.4	143.3	-	-
11	-	-	200.8	199.3	-	-
12	2.53 (<i>m</i> , 1H, J = 3.4 Hz)	2.52 (<i>m</i> , 1H)	44.4	45.8	CH	C-23, C-11
13	4.53 (<i>m</i> , 1H, J = 3.4 Hz)	4.38 (<i>m</i> , 1H)	76.2	78.3	CH	C-23
14	1.37 (<i>d</i> , 3H, J = 6.9 Hz)	1.37 (<i>d</i> , 3H)	16.5	19.4	CH_3	C-12, C-13
15	-	-	178.3	178.5	-	-
16	7.37 (<i>d</i> , 1H, J = 7.5 Hz)	7.30 (<i>d</i> , 1H)	128.0	127.9	CH	C-4, C-8

17	7.22 (<i>m</i> , 1H, <i>J</i> = 7.5 Hz)	7.24 (<i>m</i> , 1H)	127.9	127.4	CH	C-16 & C-20
18	7.13 (<i>t</i> , 1H, <i>J</i> = 7.5 Hz)	7.17 (<i>t</i> , 1H)	126.0	125.9	CH	C-16 & C-20
19	7.22 (<i>m</i> , 1H, <i>J</i> = 7.5 Hz)	7.24 (<i>m</i> , 1H)	127.9	127.4	CH	C-16 & C-20
20	7.37 (<i>d</i> , 1H, <i>J</i> = 7.5 Hz)	7.30 (<i>d</i> , 1H)	128.0	127.9	CH	C-4, C-8
21	1.41 (<i>s</i> , 3H)	1.44 (<i>s</i> , 3H)	28.3	28.3	CH ₃	C-22, C-6, C-7
22	1.19 (<i>s</i> , 3H)	1.20 (<i>s</i> , 3H)	27.9	27.6	CH ₃	C-21, C-6, C-7
23	1.15 (<i>d</i> , 3H, <i>J</i> = 7.5 Hz)	1.09 (<i>d</i> , 3H)	9.6	10.2	CH ₃	C-12, C-13, C-11

*Shen al. (2022)

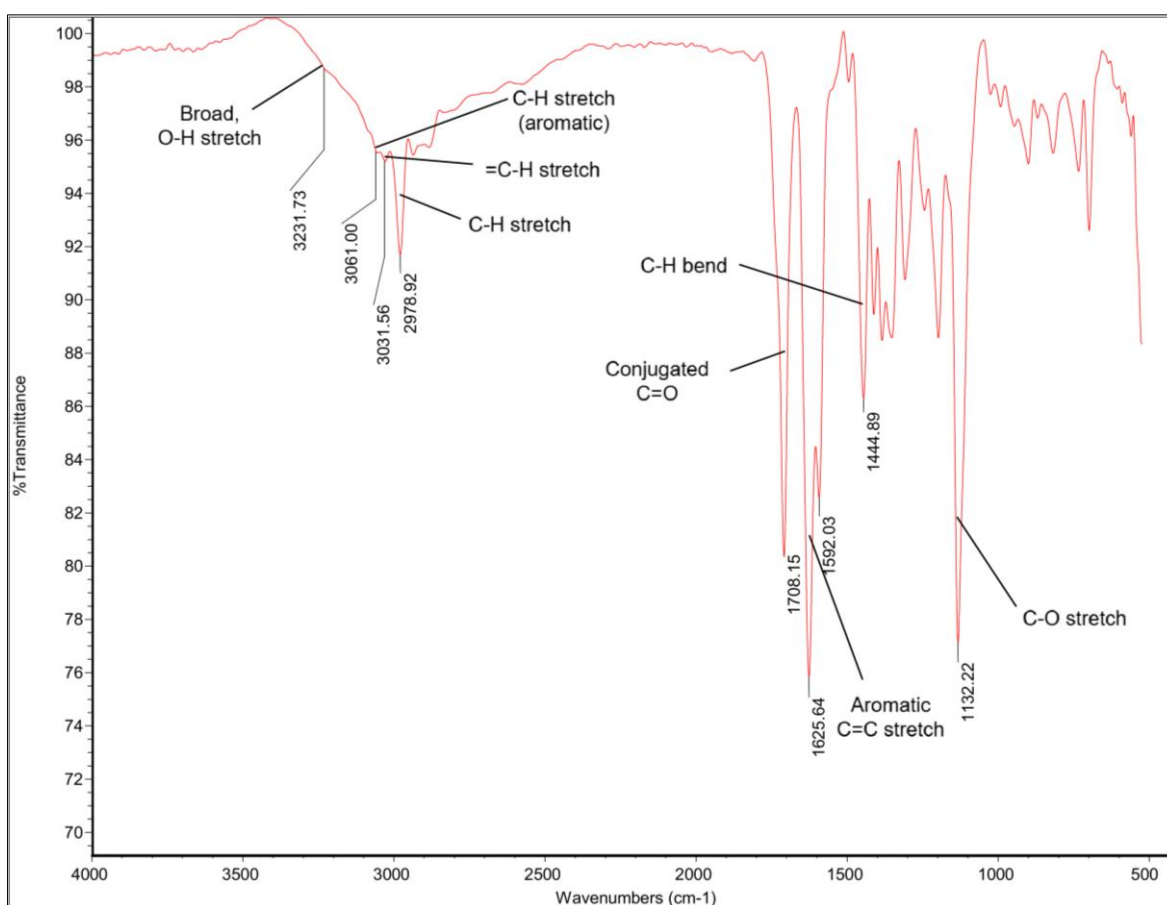
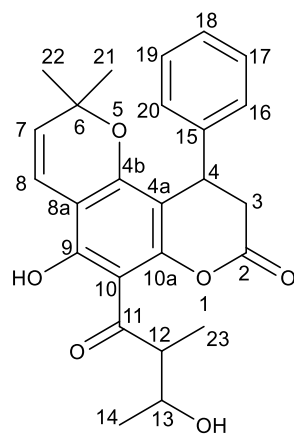


Figure 4.13: FTIR spectrum of calopolyanilide E (19)

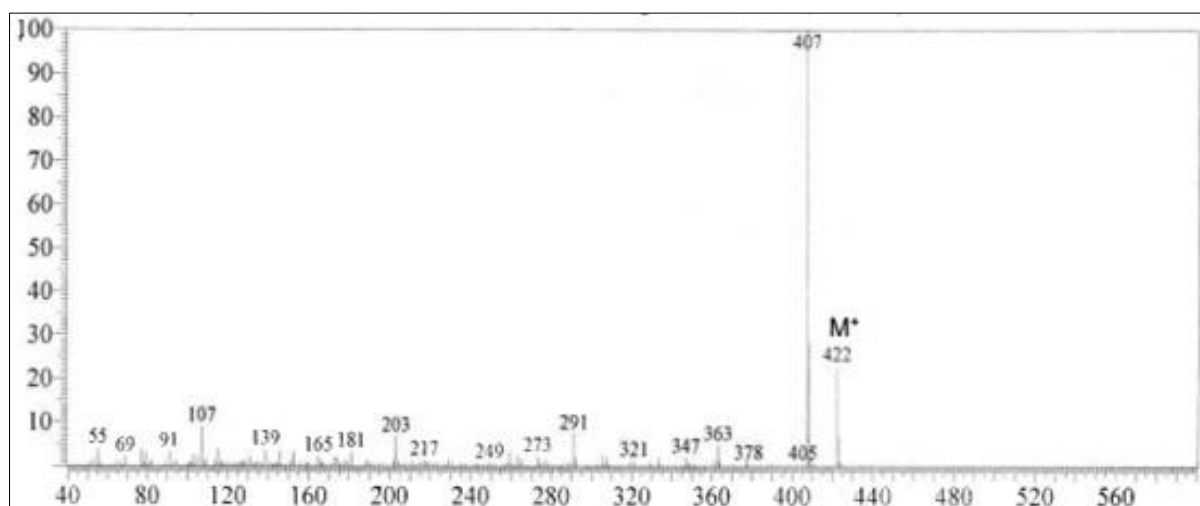
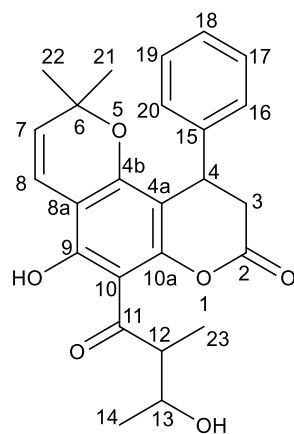


Figure 4.14: GC-MS spectrum of calopolyanolide E (**19**)

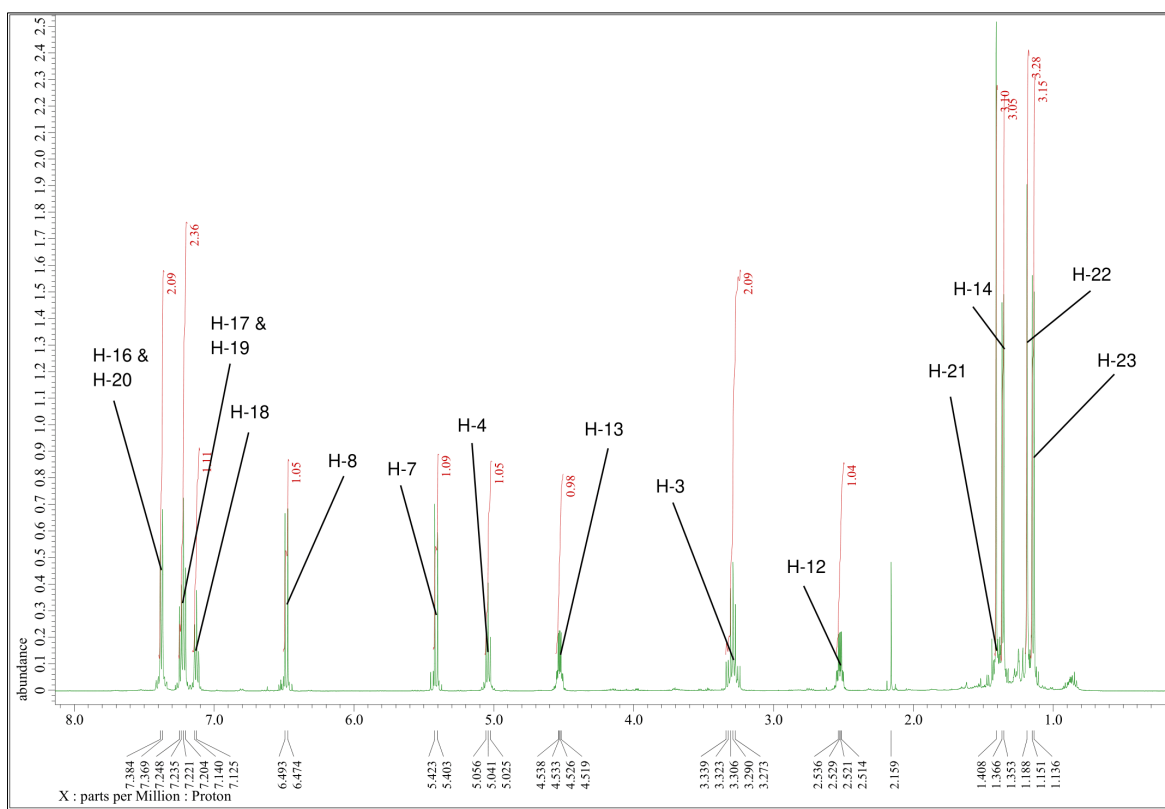
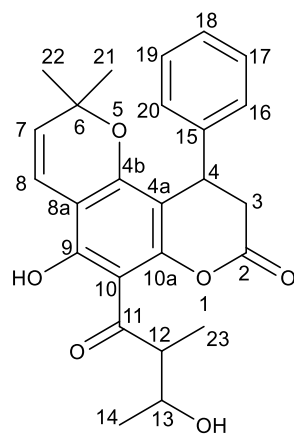


Figure 4.15: ^1H NMR spectrum of calopolyanolide E (**19**) (500 MHz, CDCl_3)

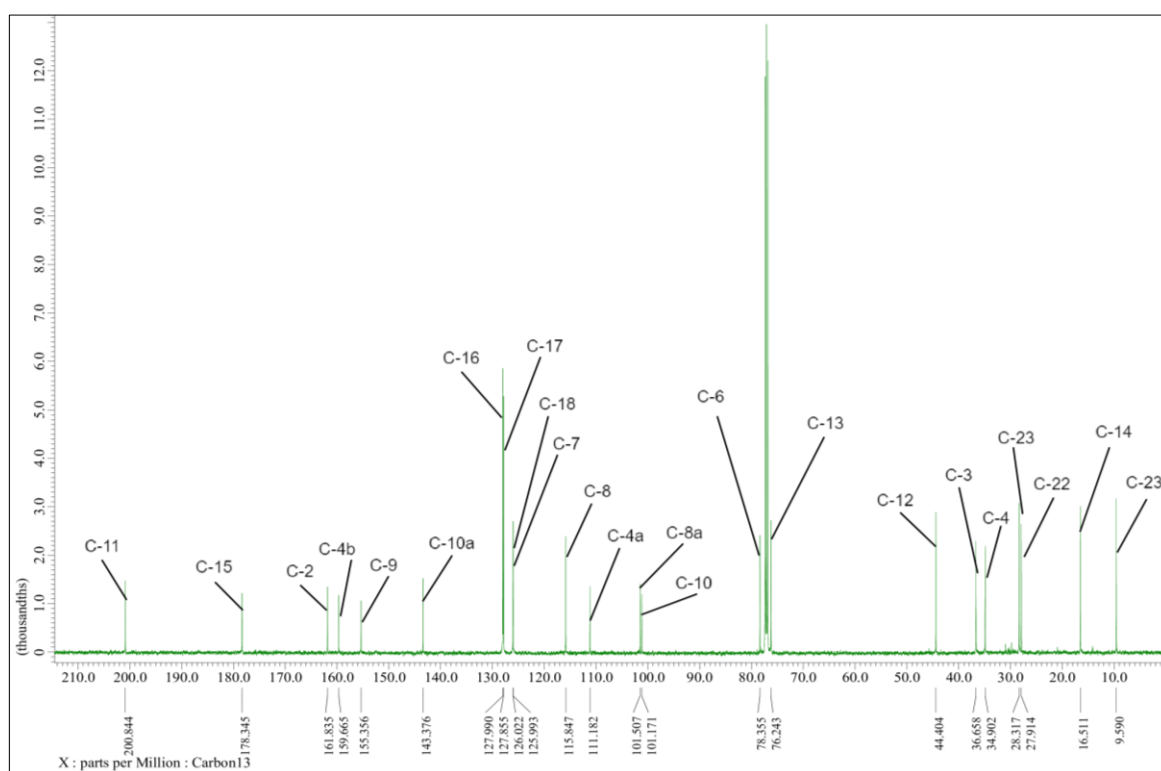
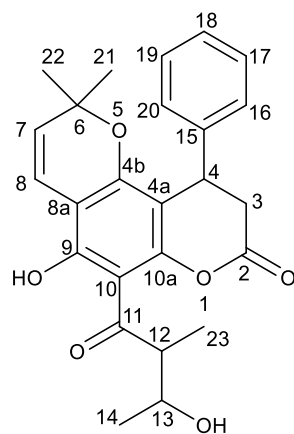


Figure 4.16: ^{13}C NMR spectrum of calopolyanilide E (**19**) (125 MHz, CDCl_3)

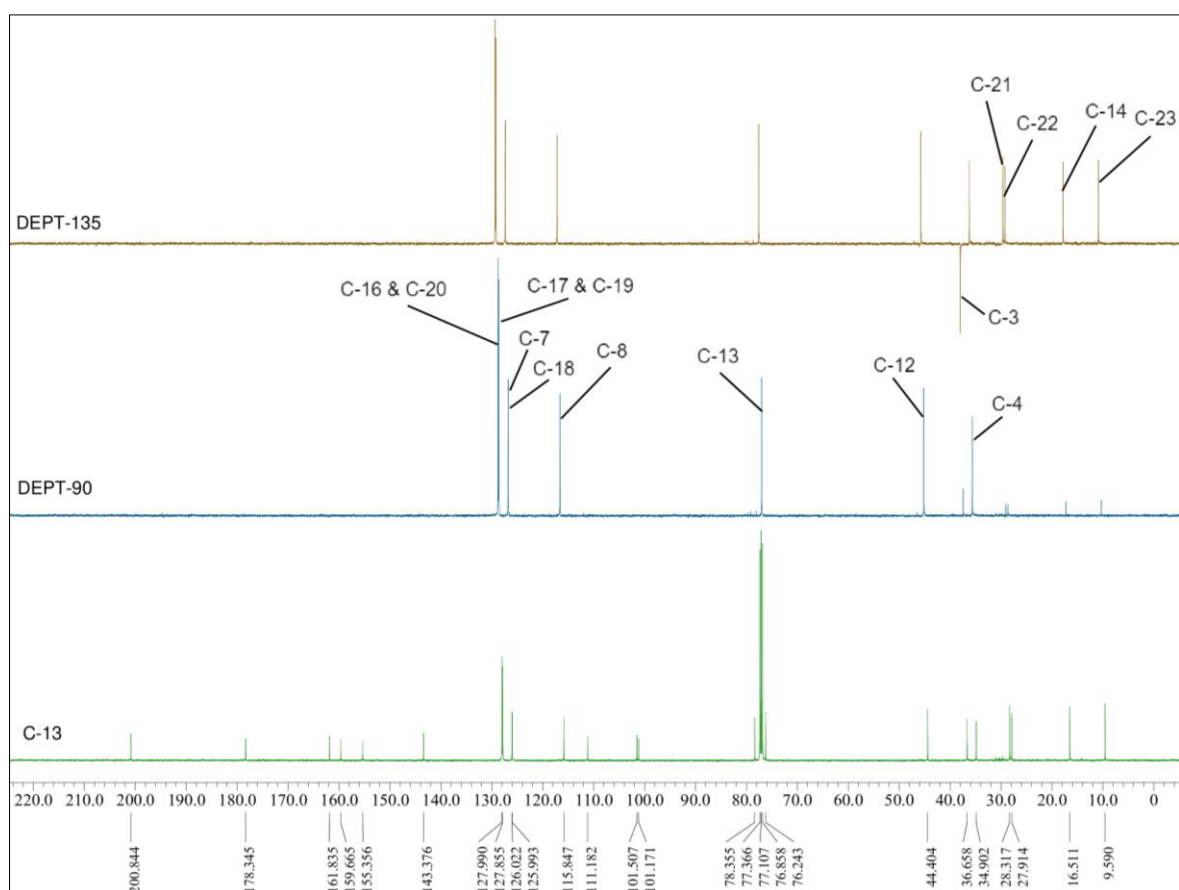
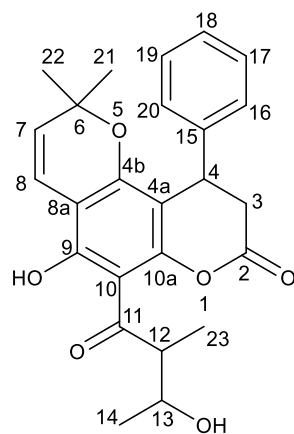


Figure 4.17: ^{13}C DEPT spectrum of calopolyanolide E (19)

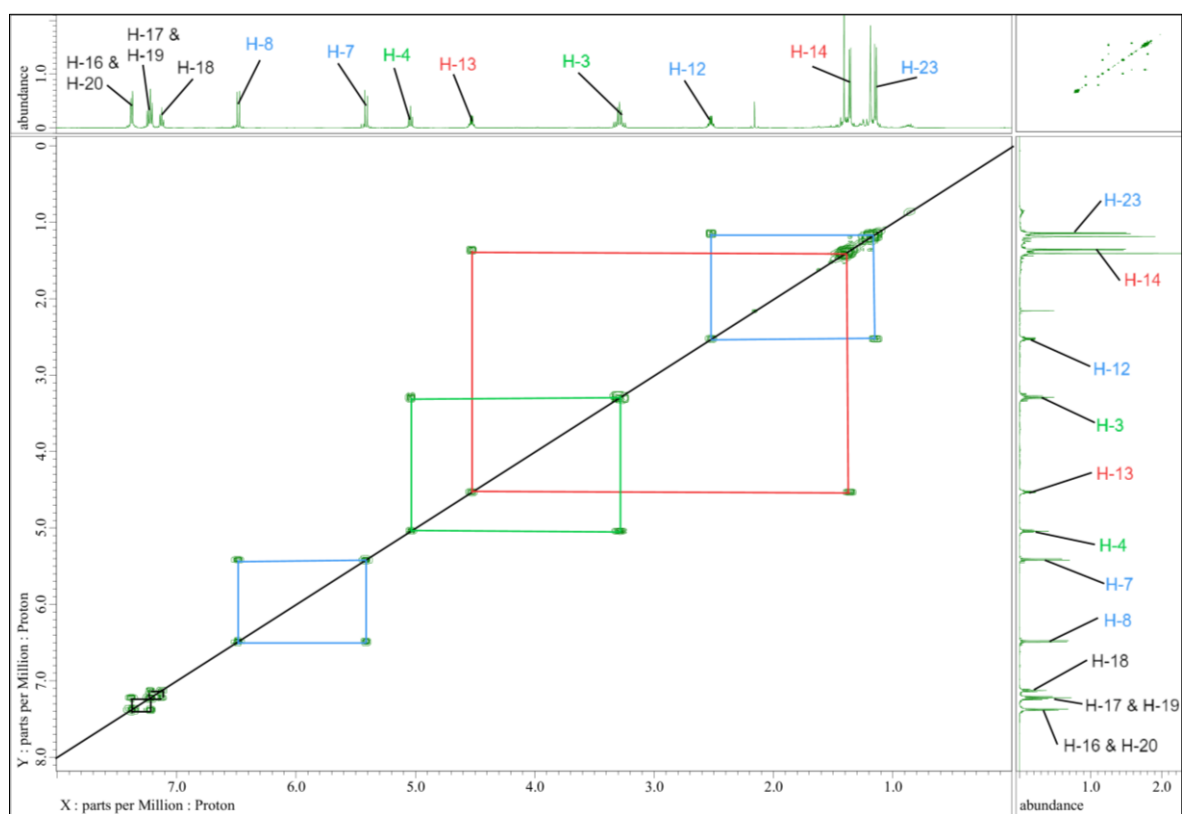
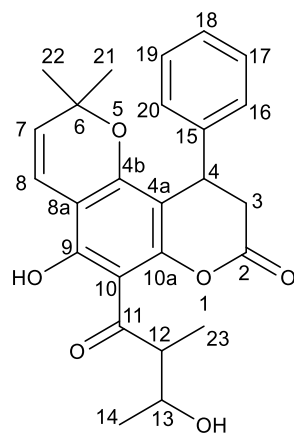


Figure 4.18: COSY spectrum of calopolyanolide E (19)

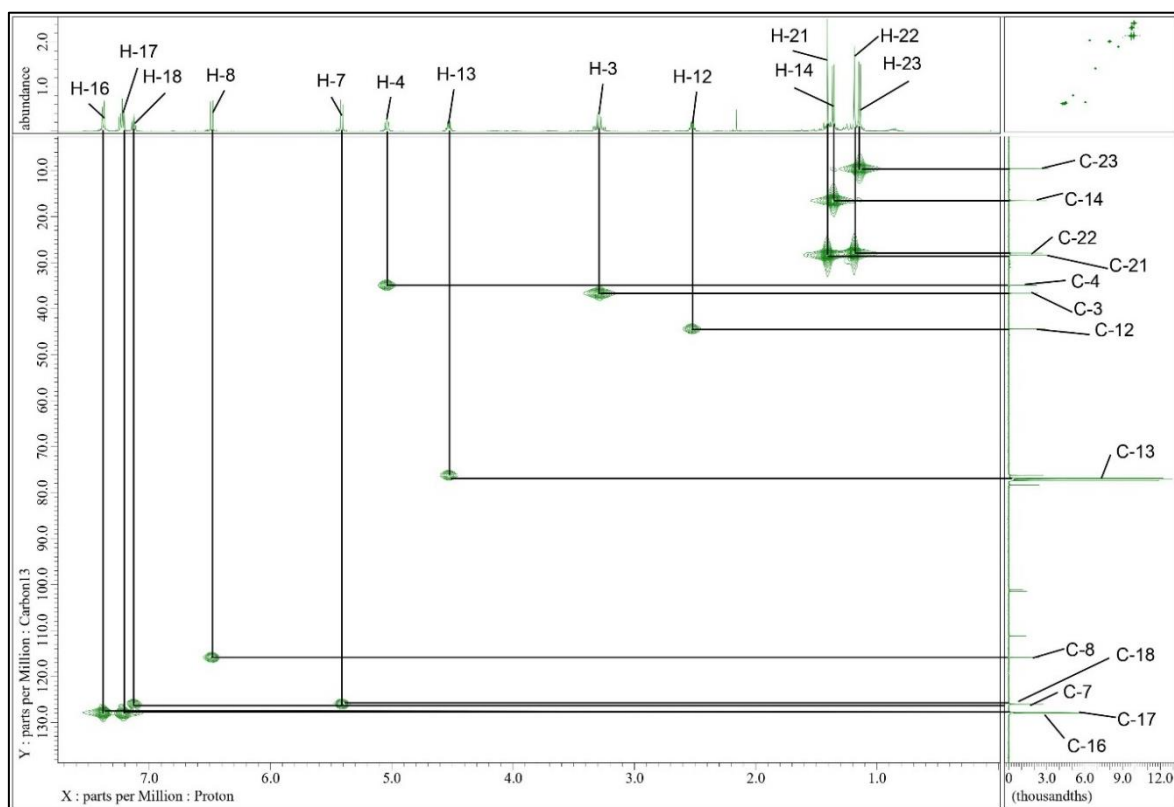
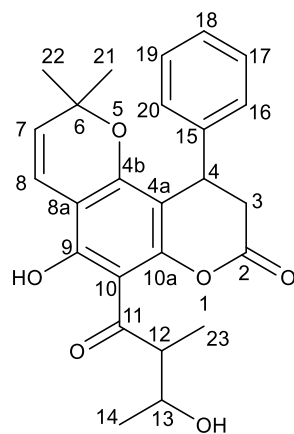


Figure 4.19: HSQC spectrum of calopolyanolide E (19)

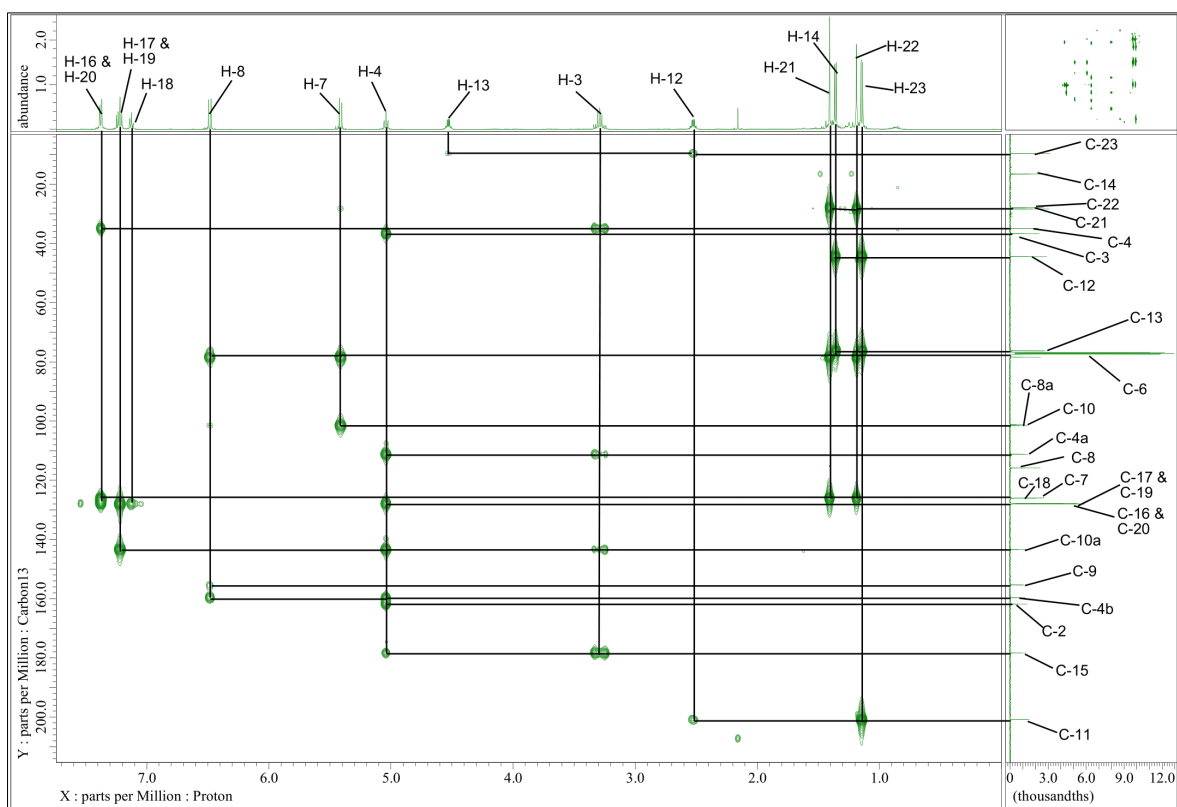
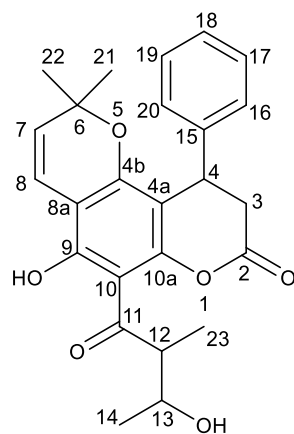


Figure 4.20: HMBC spectrum of calopolyanolide E (19)

4.1.3 Characterisation of soulaxanthone (62)

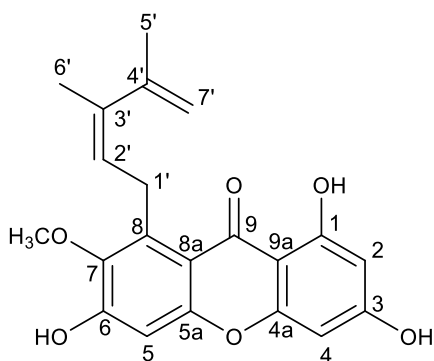


Figure 4.21: Structure of soulaxanthone (**62**)

The new xanthone, soulaxanthone (**62**) (Figure 4.21), was gained as yellow oil. The LC-MS spectrum gave a molecular ion [M^+] at m/z 368, which in accordance with the molecular formula $C_{21}H_{20}O_6$. The FTIR spectrum exhibited absorption bands at 3318 cm^{-1} (broad, O-H stretch), 3196 cm^{-1} (aromatic C-H stretch), 3085 cm^{-1} (alkene C-H stretch), 2927 cm^{-1} (C-H stretch), 1644 cm^{-1} (conjugated C=O), 1605 cm^{-1} (aromatic C=C stretch) and 1274 cm^{-1} (C-O stretch). The UV-Vis spectrum demonstrated λ_{max} absorptions at 385, 285, 235 and 205 nm which were consistent with the presence of a xanthone skeleton (Wei et al., 2011).

The interpretation of the ^{13}C and DEPT NMR spectrum displayed three methyls, two methylenes, four methines and 12 quaternary carbon moieties present in compound **62**. Meanwhile, the ^1H NMR spectrum displayed nine proton signals that represent methyl, methylene and methine groups. All the protonated carbons were determined based on ^1J correlation peaks between protons and carbons in the HSQC NMR experiment.

The presence of a singlet proton at δ 3.79 (7-OCH₃) in the ¹H NMR spectrum implied the existence of OCH₃ group in compound **62**. The position of the methoxy group was determined from the HMBC NMR spectrum, which exhibited a ³J correlation of the proton at δ 3.79 (7-OCH₃) with the carbon at δ 144.6 (C-7). A sharp singlet integrating as one proton in the ¹H NMR spectrum at low field region, δ 13.47 (1-OH), represented a chelated -OH group. It was attached to position C-1 as the HMBC NMR spectrum showed ²J and ³J correlations of the proton with three aromatic carbons, which are δ 164.9 (C-1), δ 98.8 (C-2) and δ 103.8 (C-9a).

The proton signals observed at δ 4.12 (H-1'), δ 5.29 (H-2'), δ 1.62 (H-5'), δ 1.83 (H-6') δ 4.67 (H-7') and δ 4.81 (H-7') suggested the presence of 3,4-dimethylpenta-2,4-dienyl moiety. Two CH₃ groups were observed at δ 17.8 (C-5') and δ 16.7 (C-6'), two CH₂ groups at δ 26.8 (C-1') and δ 110.2 (C-7'), as well as one CH group at δ 124.6 (C-2'). The HMBC NMR correlations of δ 1.62 (H-5') with δ 149.4 (C-4') and δ 110.2 (C-7') proved its position at C-4. Meanwhile, the ²J correlation of CH₃ proton at δ 1.83 (H-6') with δ 135.4 (C-3') and ³J correlation with δ 124.6 (C-2') proved the attachment of this CH₃ proton at C-3'. The CH₂ proton at δ 4.12 (H-1') and CH proton at δ 5.29 (H-2') were observed next to each other as seen from the correlation peaks in COSY NMR spectrum, and their coupling constant values are listed in Table 4.3. The 3,4-dimethylpenta-2,4-dienyl moiety was attached at C-8. It was concluded from the observation in the HMBC NMR spectrum, which showed ²J and ³J correlations of δ 4.12 (H-1') with δ 138.3 (C-8) and δ 112.0 (C-8a), respectively.

Hence, the structure of **62** was elucidated as demonstrated in Figure 4.21 and named soulaxanthone. The HMBC correlation of the compound is illustrated in Figure

4.22. The FTIR, LC-MS, 1D NMR and 2D NMR spectra of compound **62** are displayed in Figure 4.23 - 4.31. The NMR spectrum data of the compound are summarised in Table 4.3.

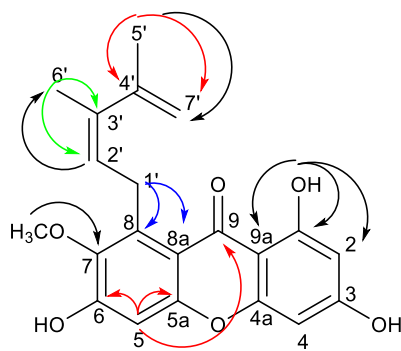
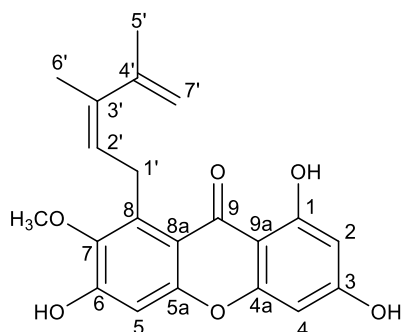


Figure 4.22: HMBC correlation in soulaxanthone (**62**)

Table 4.3: ^1H NMR (400 MHz, acetone- d_6) and ^{13}C NMR (100 MHz, acetone- d_6)

Assignment of soulaxanthone (62)



Position	^1H (δ)	^{13}C (δ)	DEPT	HMBC
1	-	164.9	-	-
2	6.19 (s, 1H)	98.8	CH	C-4, C-9a, C-1
3	-	165.5	-	-
4	6.28 (s, 1H)	93.8	CH	C-2, C-9a, C-4a, C-3
4a	-	158.0	-	-
5	6.84 (s, 1H)	102.8	CH	C-8a, C-7, C-6, C-5a
5a	-	157.6	-	-
6	-	156.3	-	-
7	-	144.6	-	-
8	-	138.3	-	-
8a	-	112.0	-	-
9	-	182.8	-	-
9a	-	103.8	-	-
1'	4.12 (d, 2H, $J = 6.1$ Hz)	26.8	CH_2	C-8a, C-2', C-3', C-8, C-7
2'	5.29 (t, 1H, $J = 6.7$ Hz)	124.6	CH	-
3'	-	135.4	-	-
4'	-	149.4	-	-
5'	1.62 (s, 3H)	17.8	CH_3	C-7', C-4'
6'	1.83 (s, 3H)	16.7	CH_3	C-2', C-3'
7'	4.67 (s, 1H) 4.81 (s, 1H)	110.2	CH_2	C-5'
1-OH	13.47 (s, 1H)	-	-	C-2, C-9a, C-1
3-OH	-	-	-	-
6-OH	9.66 (s, 1H)	-	-	-
7-OCH ₃	3.79 (s, 3H)	61.4	CH_3	C-7

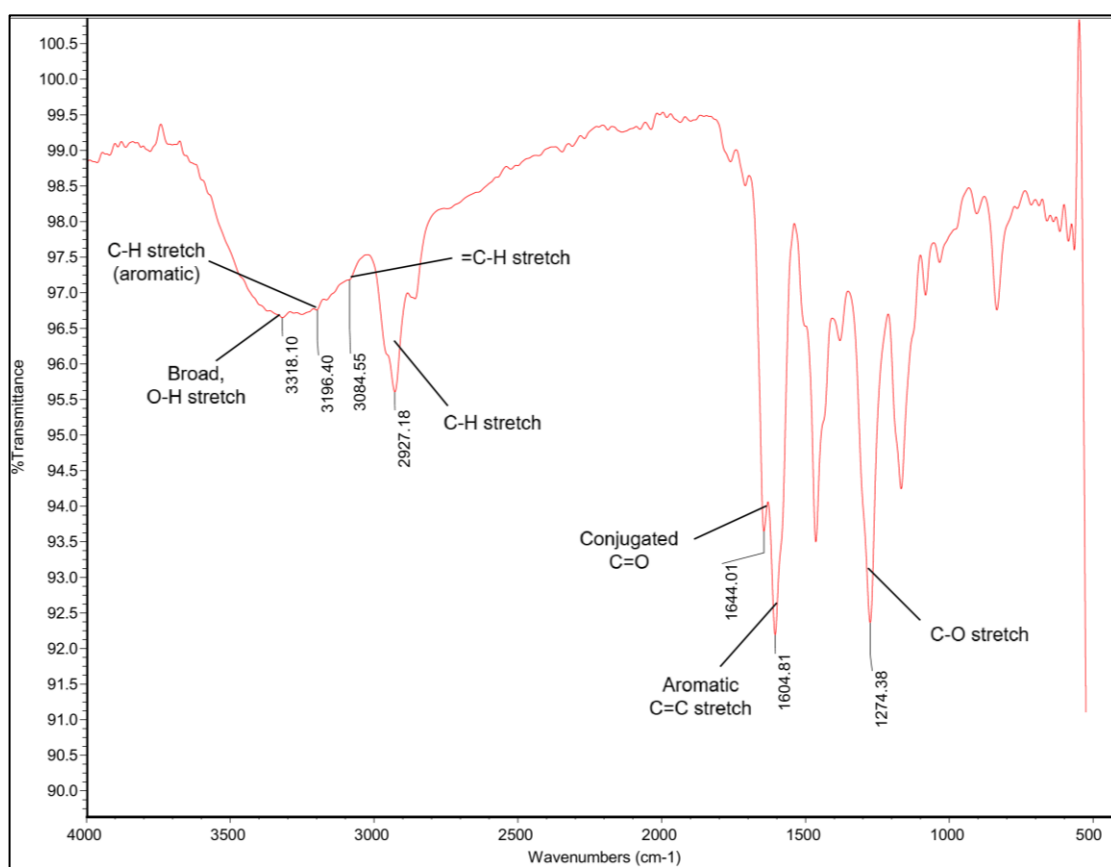
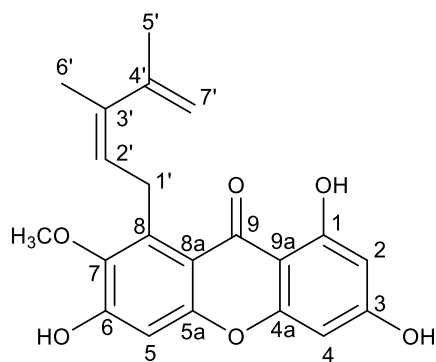


Figure 4.23: FTIR spectrum of souluxanthone (62)

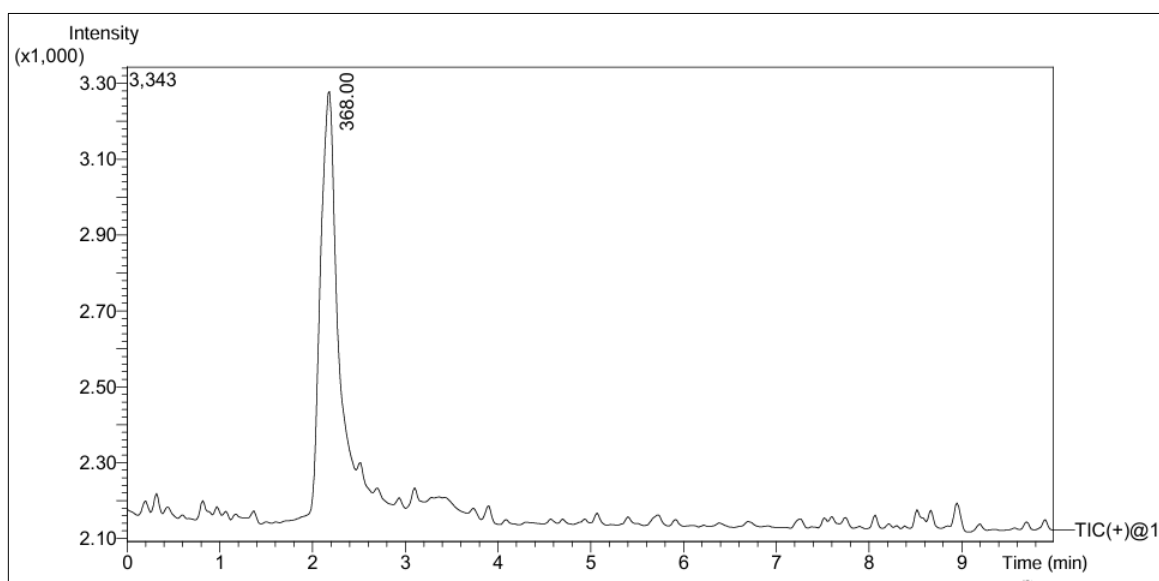
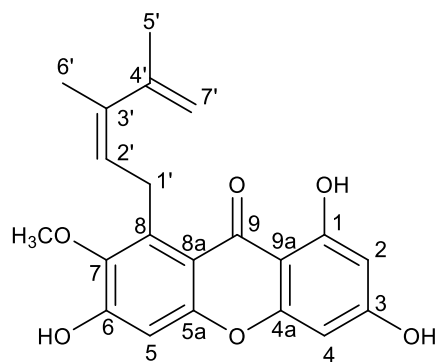


Figure 4.24: LC-MS spectrum of soulaxanthone (**62**)

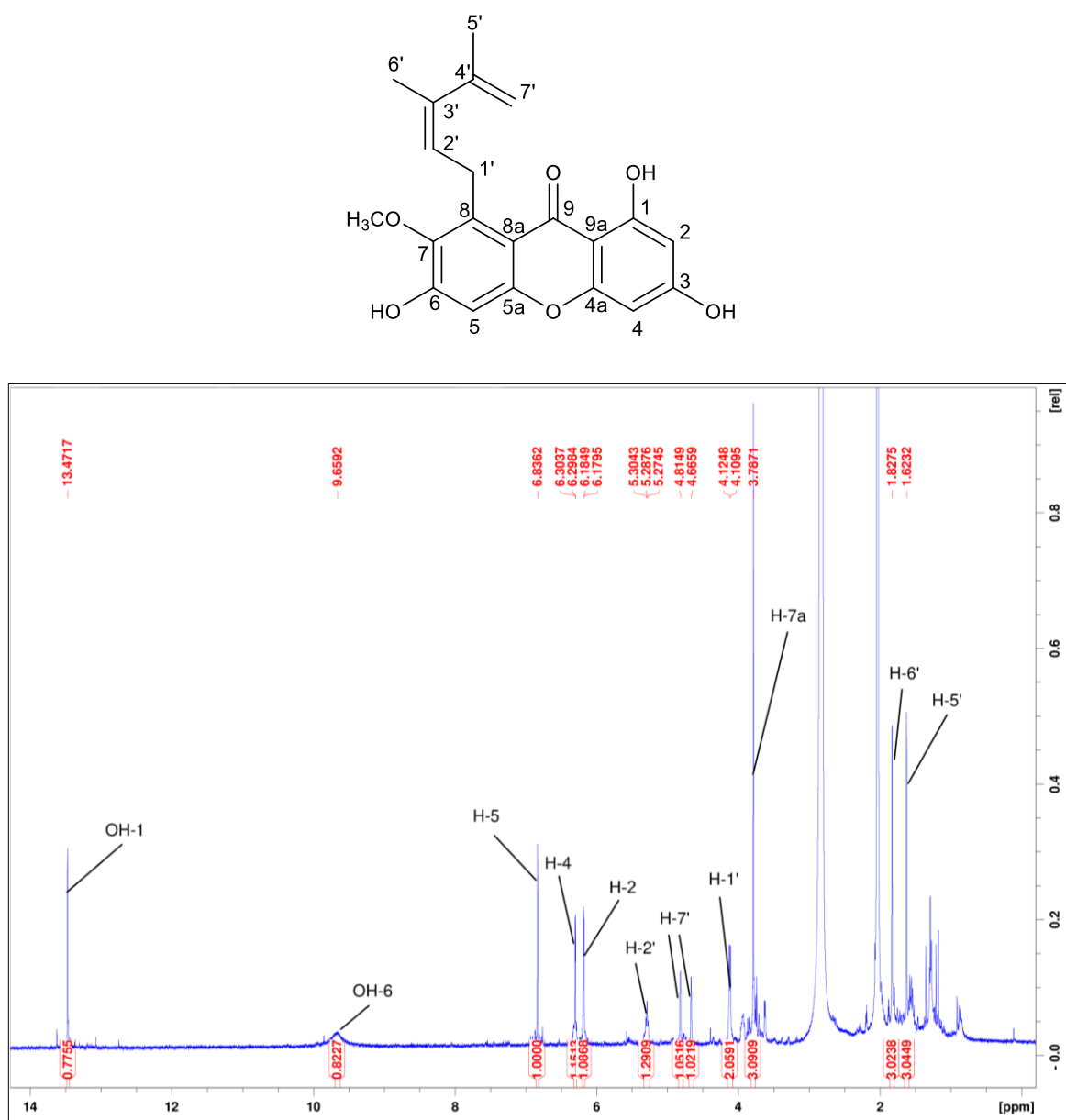


Figure 4.25: ^1H NMR spectrum of souluxanthone (**62**) (400 MHz, acetone- d_6)

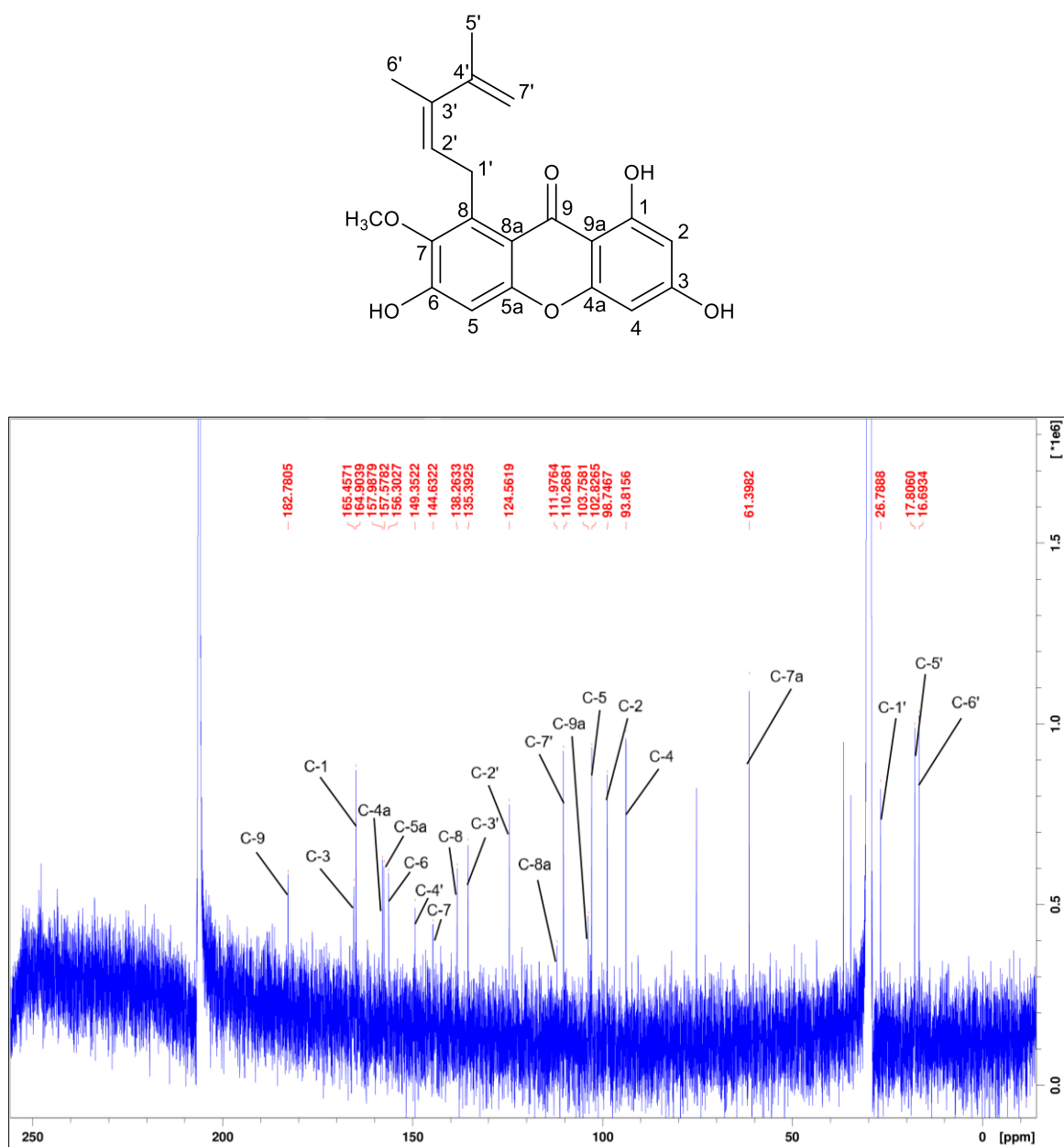


Figure 4.26: ^{13}C NMR spectrum of soulaxanthone (**62**) (100 MHz, acetone- d_6)

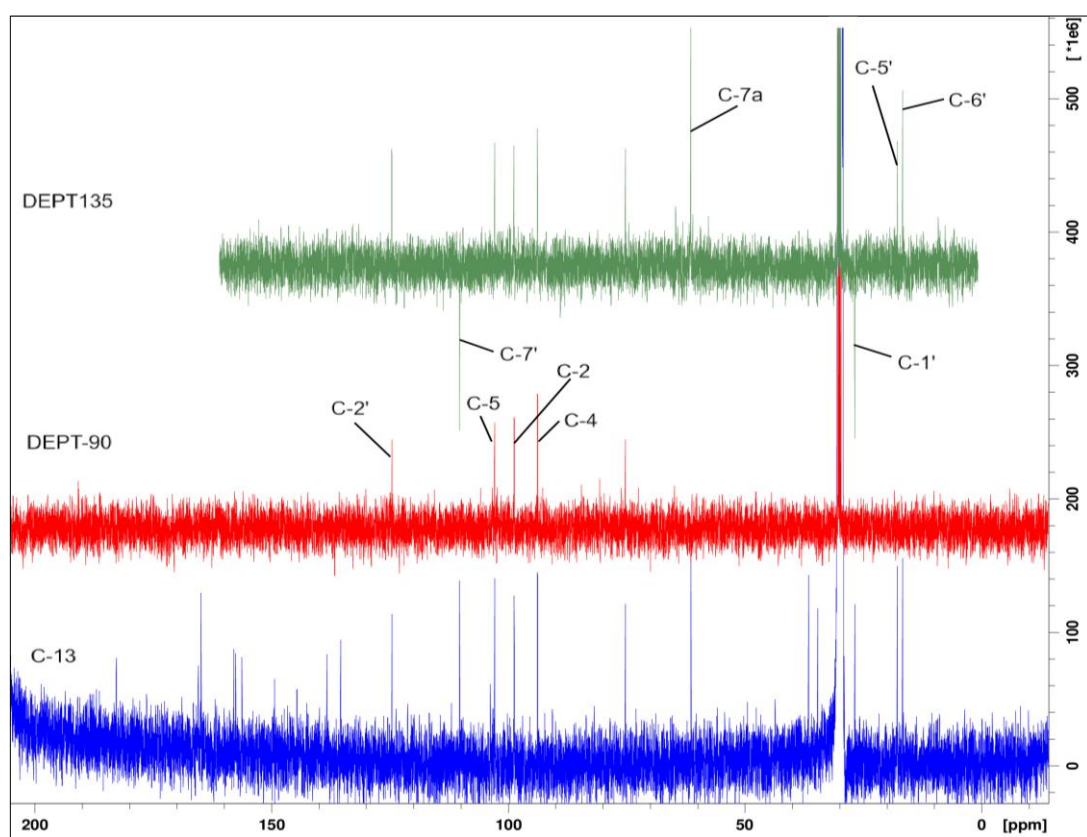
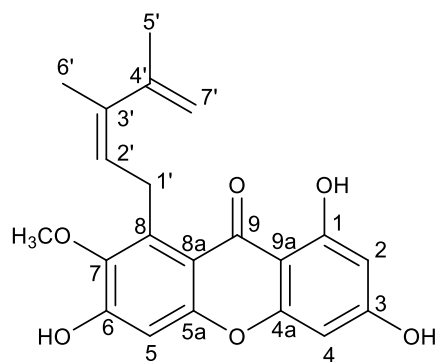


Figure 4.27: ^{13}C DEPT spectrum of soulaxanthone (62)

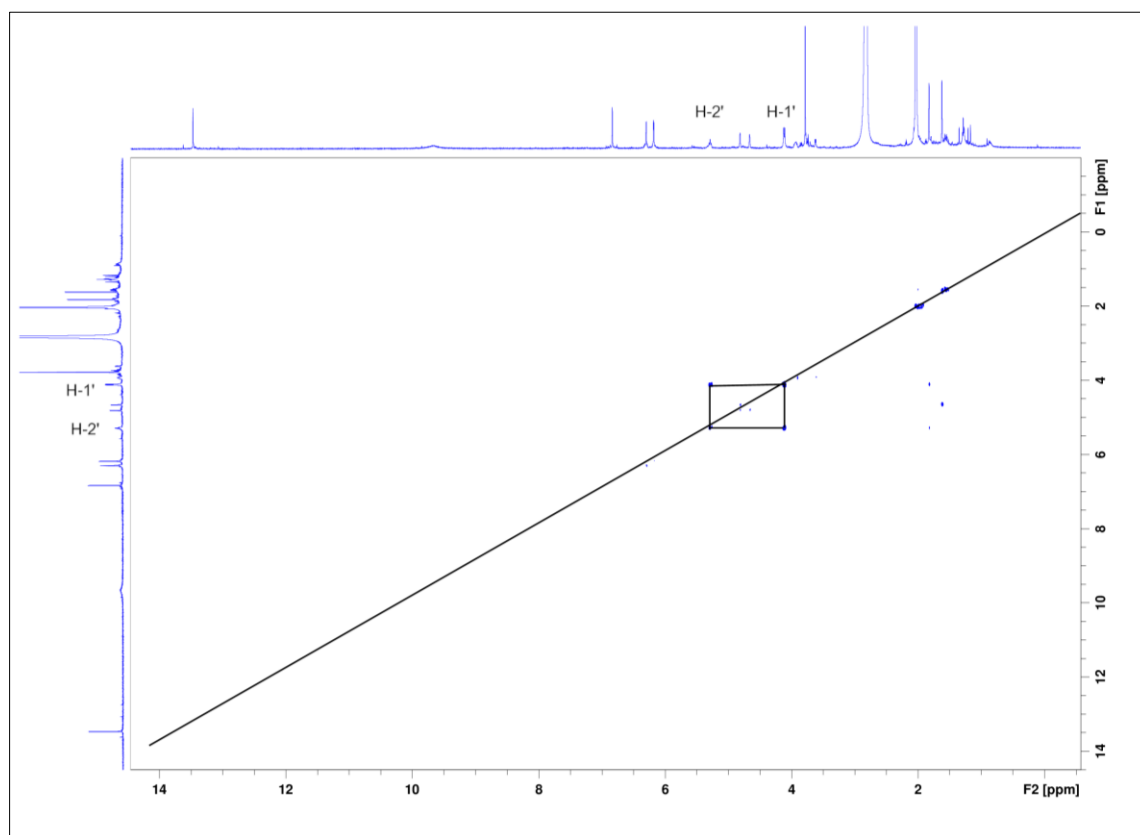
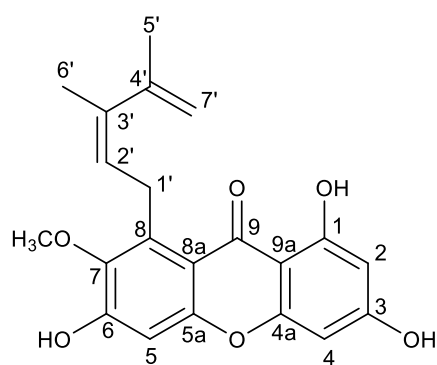


Figure 4.28: COSY spectrum of soulaxanthone (62)

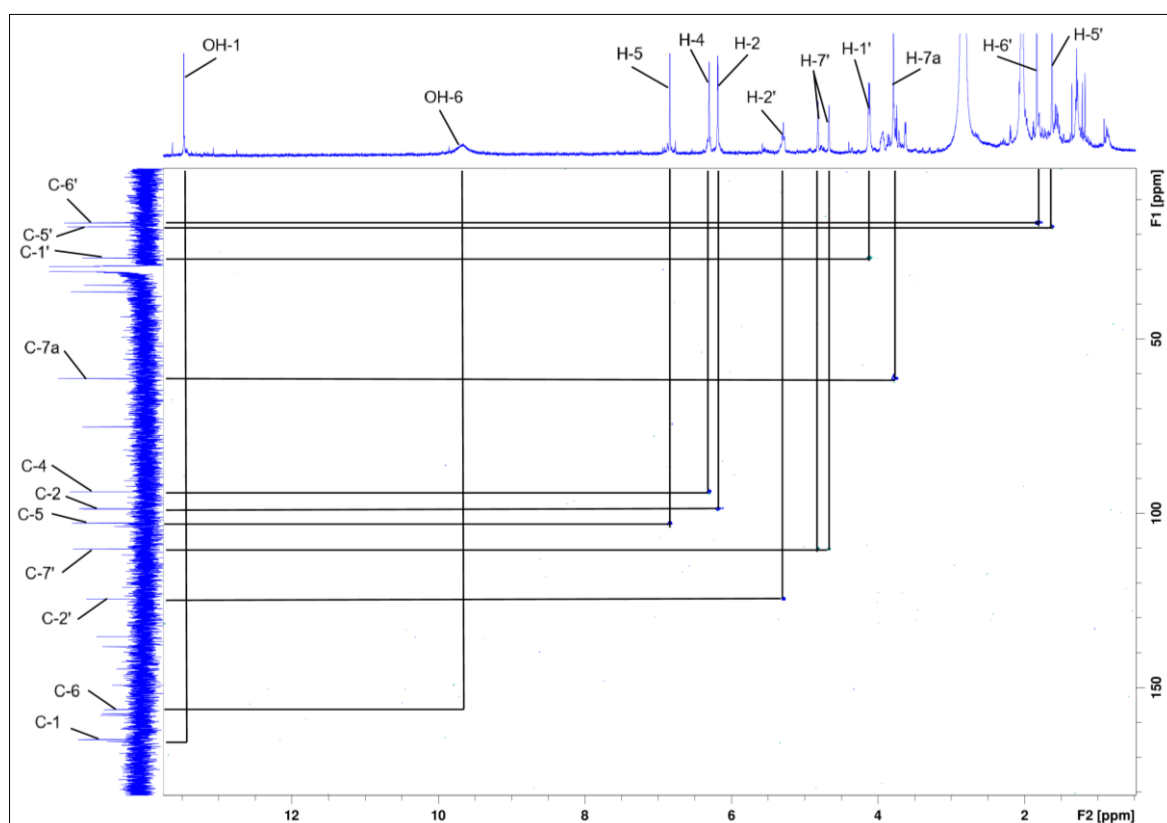
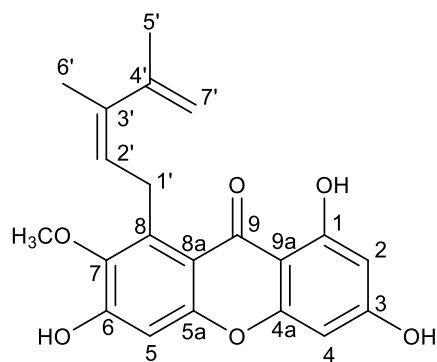


Figure 4.29: HSQC spectrum of soulaxanthone (**62**)

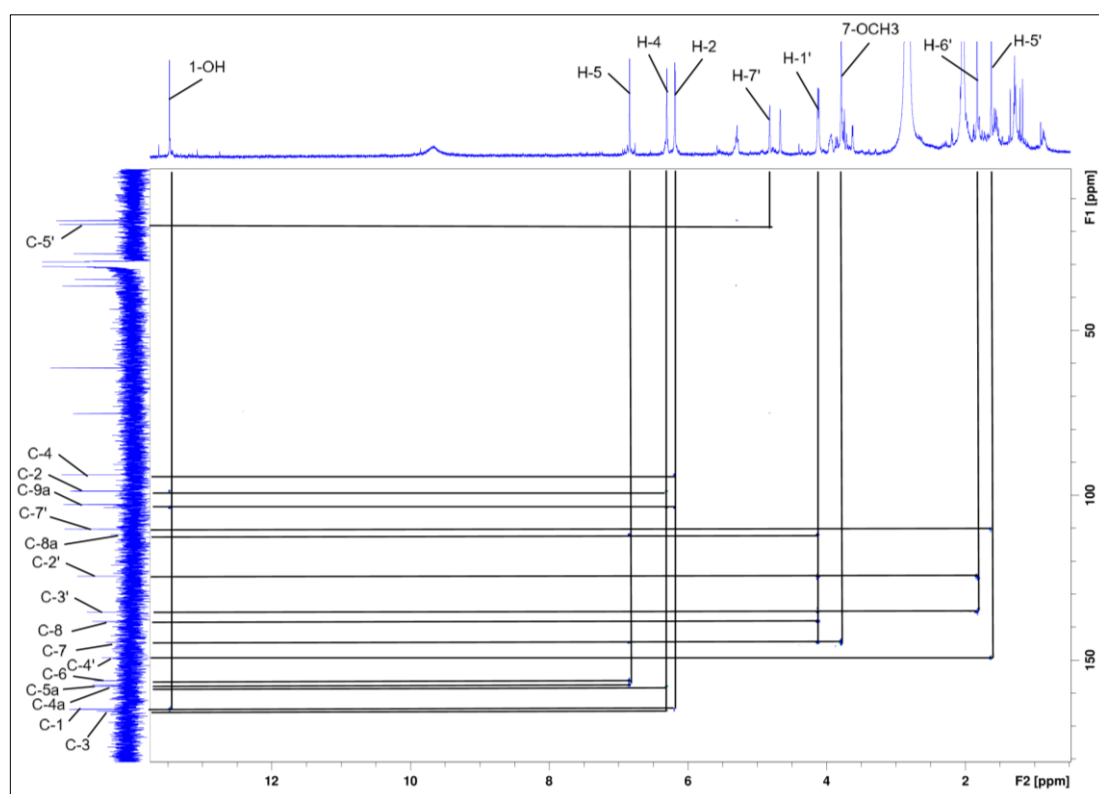
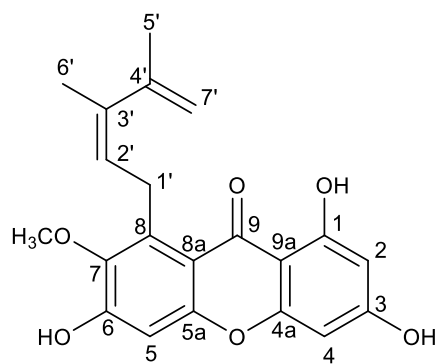


Figure 4.30: HMBC spectrum of souluxanthone (62)

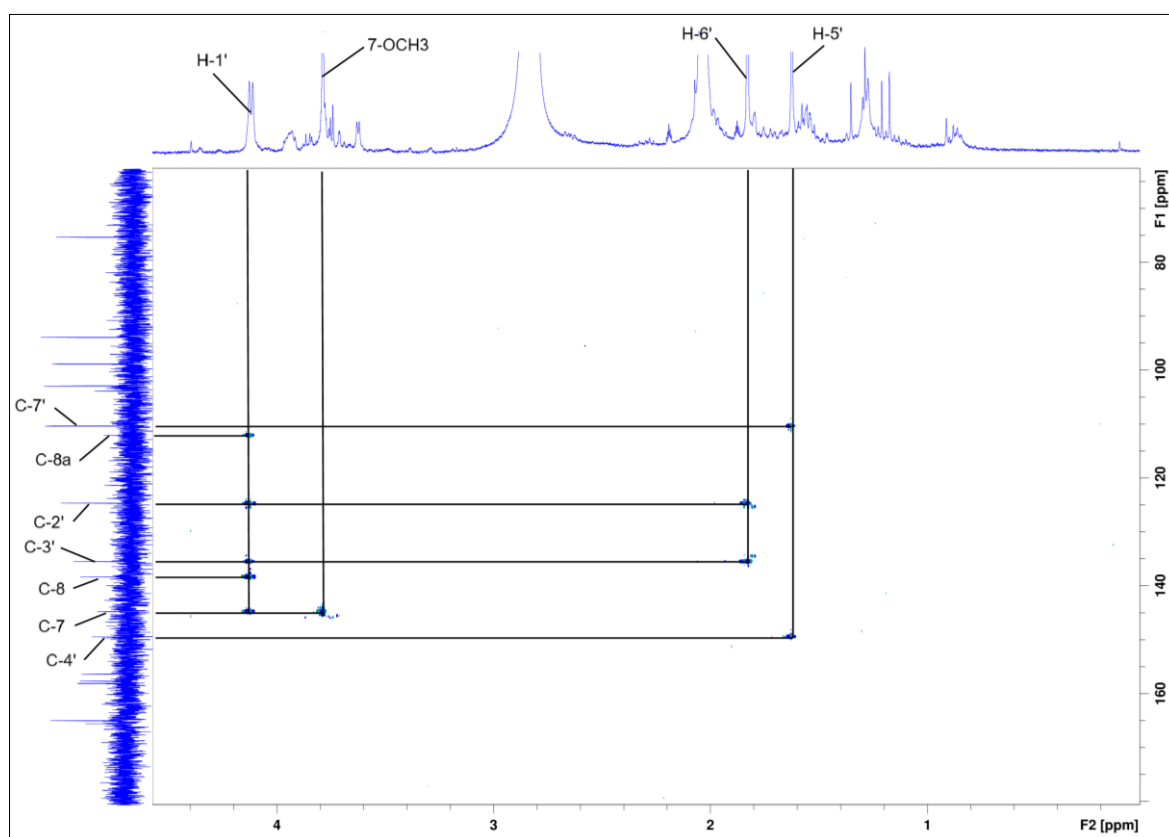
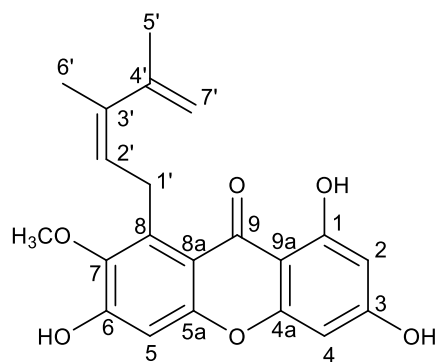


Figure 4.31: Expansion HMBC spectrum of soulaxanthone (**62**)

4.2 Isolation of Chemical Constituents from *Calophyllum gracilentum*

4.2.1 Characterisation of euxanthone (63)

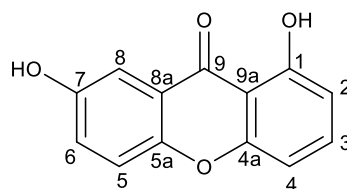


Figure 4.32: Structure of euxanthone (**63**)

The compound (**63**) (Figure 4.32) was yielded as yellow needle crystals with a melting point of 236-237 °C (literature: 235-236 °C, Lizazman et al., 2023). The LC-MS spectrum gave a molecular ion $[M^+]$ at m/z 228, which corresponded to the molecular formula $C_{13}H_8O_4$. The absorption bands in FTIR were observed at 3303 cm^{-1} (broad, O-H stretch), 3067 cm^{-1} (aromatic C-H stretch), 2961 cm^{-1} (C-H stretch), 1603 cm^{-1} (conjugated C=O), 1478 cm^{-1} (aromatic C=C stretch) and 1230 cm^{-1} (C-O stretch). The UV-Vis spectrum demonstrated absorption maxima at 385, 285, 260 and 235, which is owing to the compound having a highly conjugated system.

There were eight proton signals displayed in the ^1H NMR spectrum, with two highly deshielded proton signals at δ 12.68, which was allocated to a chelated hydroxyl proton (1-OH) and at δ 8.95, which was assigned to a free hydroxyl proton (7-OH). Another six aromatic protons gave signals with different multiplicities at δ 6.75 (*d*, H-2), δ 7.67 (*t*, H-3), δ 6.98 (*d*, H-4), δ 7.49 (*d*, H-5), δ 7.42 (*dd*, H-6) and δ 7.58 (*d*, H-8).

13 carbons were exhibited in the ^{13}C NMR spectrum, which was in accordance with the total of carbons in the suggested structure. A carbonyl group (C-9) was assigned to the most deshielded signal at δ 182.1. Also, four oxygenated aromatic carbons gave

comparatively more deshielded carbon signals at δ 161.9 (C-1), δ 156.5 (C-4a), δ 154.2 (C-7) and δ 150.2 (C-5a). The carbon signals that observed in the region below δ 140.0 were allocated to eight non-oxygenated aromatic carbons.

Therefore, compound **63** was elucidated to be euxanthone previously isolated by Lizazman et al. (2023). The HMBC correlation of the compound is illustrated in Figure 4.33. The FTIR, LC-MS, 1D NMR and 2D NMR spectra of the compound are depicted in Figure 4.34 – 4.41. The NMR spectrum data of this compound are summarised in Table 4.4.

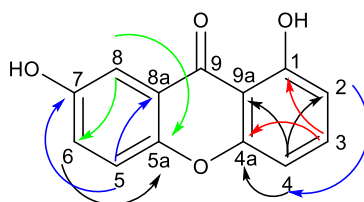
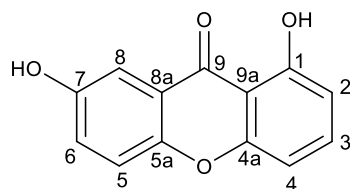


Figure 4.33: HMBC correlation in euxanthone (**63**)

Table 4.4: ^1H NMR (500 MHz, acetone- d_6) and ^{13}C NMR (125 MHz, acetone- d_6)

Assignment of euxanthone (63)



Position	^1H (δ)	^1H (δ)*	^{13}C (δ)	^{13}C (δ)*	DEPT	HMBC
1	-	-	161.9	161.9	-	-
2	6.75 (<i>d</i> , 1H, J = 8.0 Hz)	6.78 (<i>dd</i> , 1H, J = 8.5 Hz, 0.8 Hz)	108.4	108.3	CH	C-4
3	7.67 (<i>t</i> , 1H, J = 8.0 Hz)	7.71 (<i>t</i> , 1H, J = 8.3 Hz)	137.0	137.0	CH	C-4a, C-1
4	6.98 (<i>d</i> , 1H, J = 8.0 Hz)	7.02 (<i>dd</i> , 1H, J = 8.5 Hz, 0.8 Hz)	106.9	106.9	CH	C-9a, C-2, C-4a
4a	-	-	156.5	156.5	-	-
5	7.49 (<i>d</i> , 1H, J = 9.2 Hz)	7.54 (<i>d</i> , 1H, J = 9.0 Hz)	119.4	119.4	CH	C-8a, C-7
5a	-	-	150.2	150.2	-	-
6	7.42 (<i>dd</i> , 1H, J = 9.2 Hz, 3.0 Hz)	7.45 (<i>dd</i> , 1H, J = 9.2 Hz, 3.0 Hz)	125.3	125.3	CH	C-8, C-5a
7	-	-	154.2	154.2	-	-
8	7.58 (<i>d</i> , 1H, J = 3.0 Hz)	7.62 (<i>d</i> , 1H, J = 3.0 Hz)	109.7	109.7	CH	C-6, C-5a
8a	-	-	121.0	121.0	-	-
9	-	-	182.1	182.1	-	-
9a	-	-	108.4	108.3	-	-
1-OH	12.68 (<i>s</i> , 1H)	12.74 (<i>s</i> , 1H)	-	-	-	C-2
7-OH	8.98 (<i>s</i> , 1H)	9.15 (<i>s</i> , 1H)	-	-	-	-

*Lizazman et al. (2023)

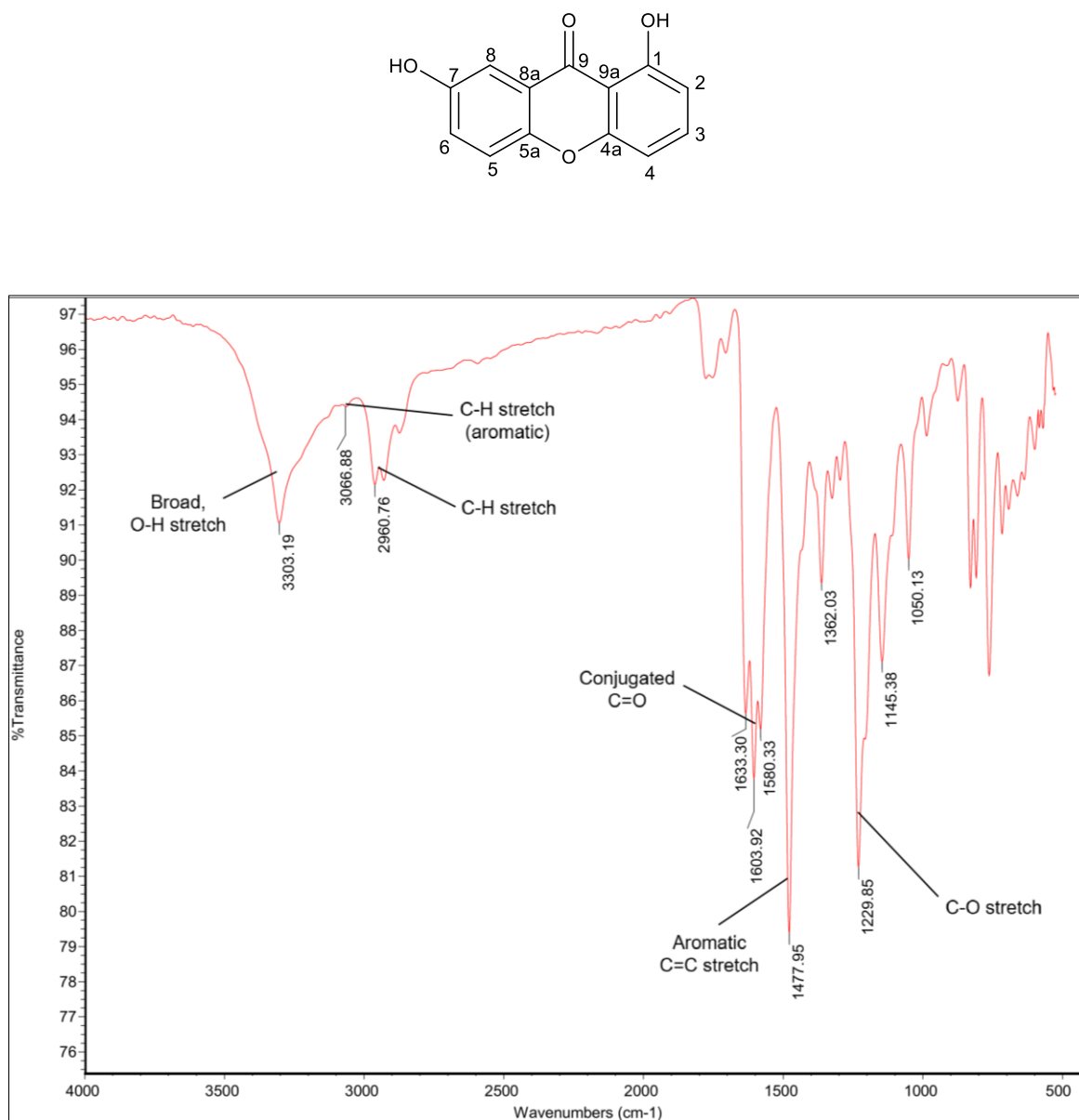


Figure 4.34: FTIR spectrum of euxanthone (63)

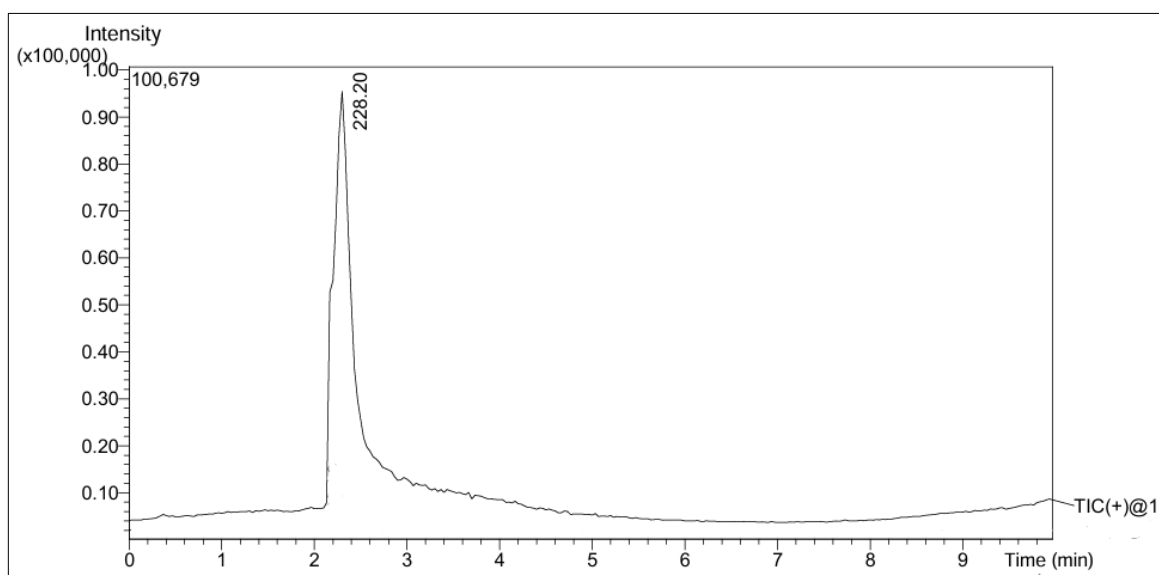
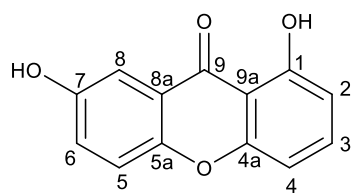


Figure 4.35: LC-MS spectrum of euxanthone (**63**)

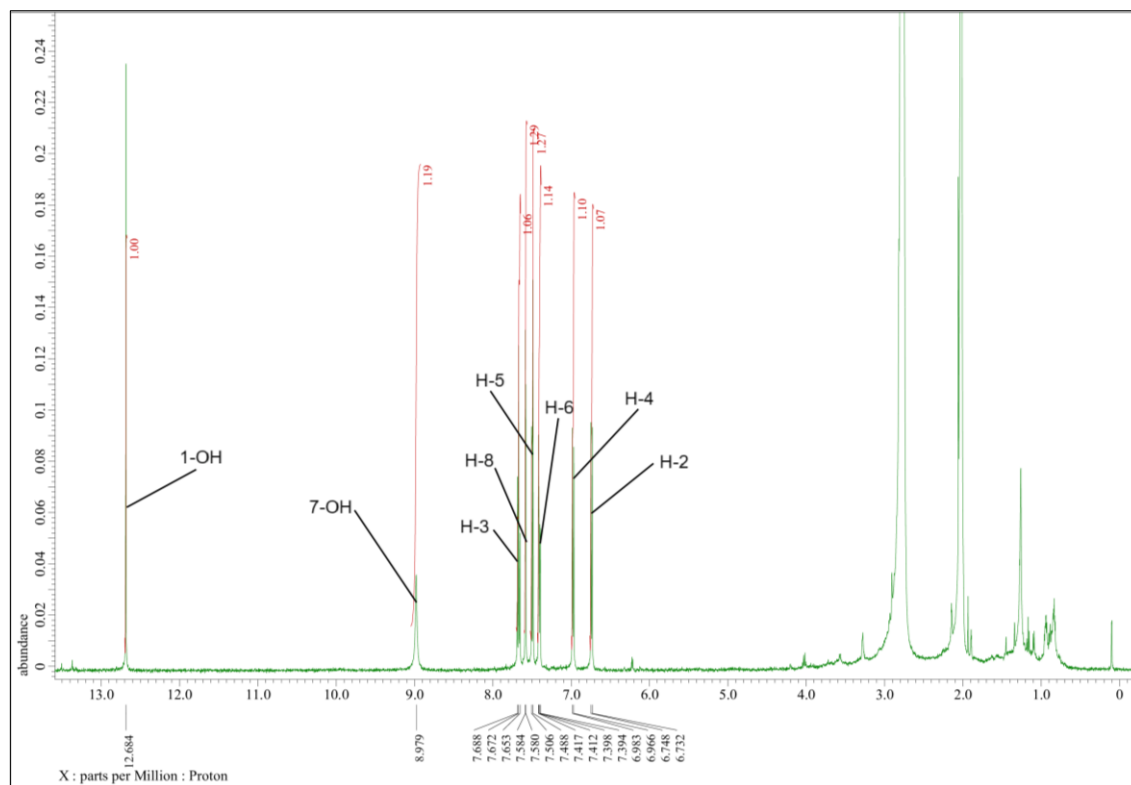
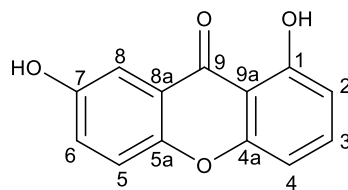


Figure 4.36: ^1H NMR spectrum of euxanthone (**63**) (500 MHz, acetone- d_6)

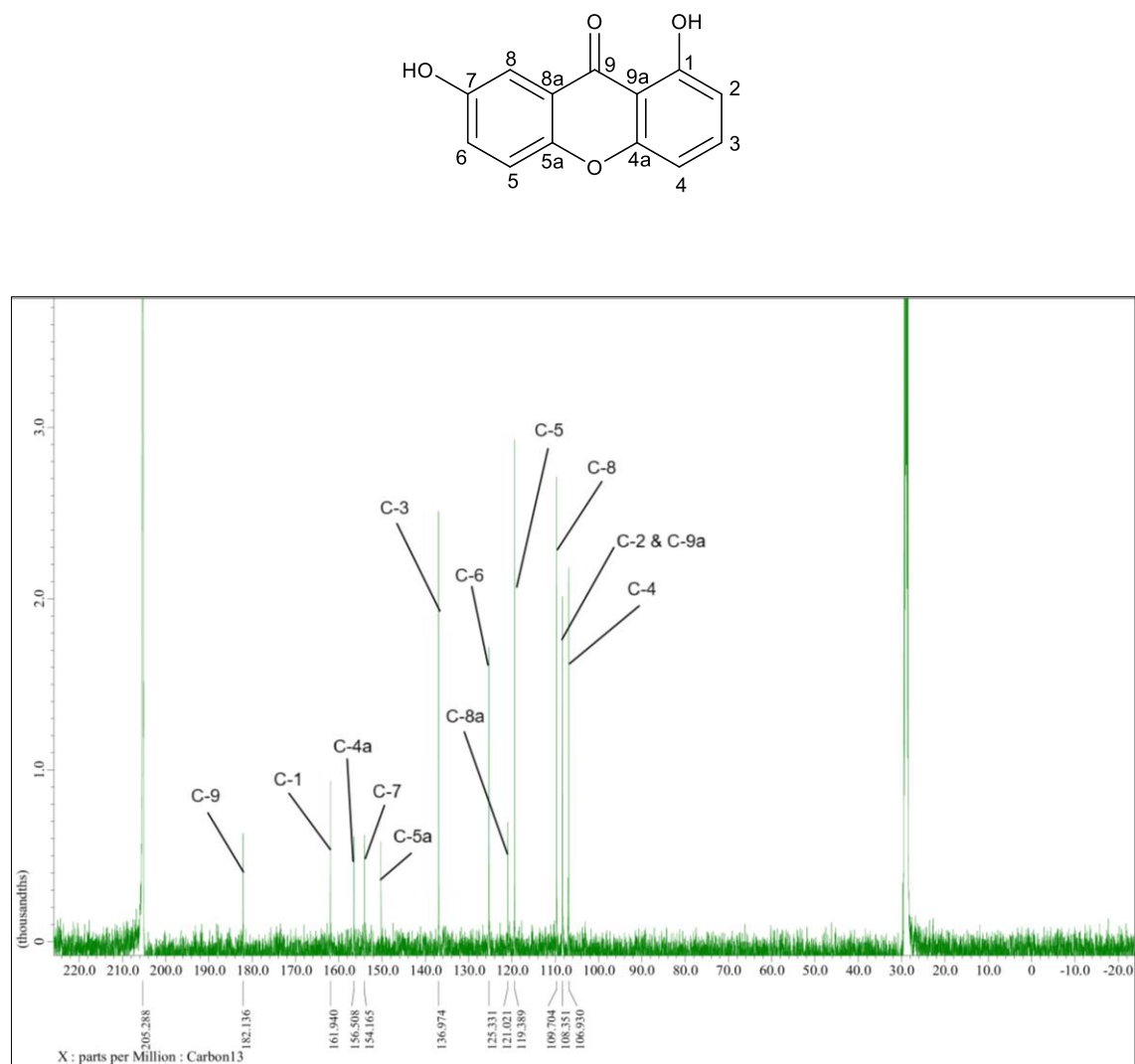


Figure 4.37: ^{13}C NMR spectrum of euxanthone (**63**) (125 MHz, acetone- d_6)

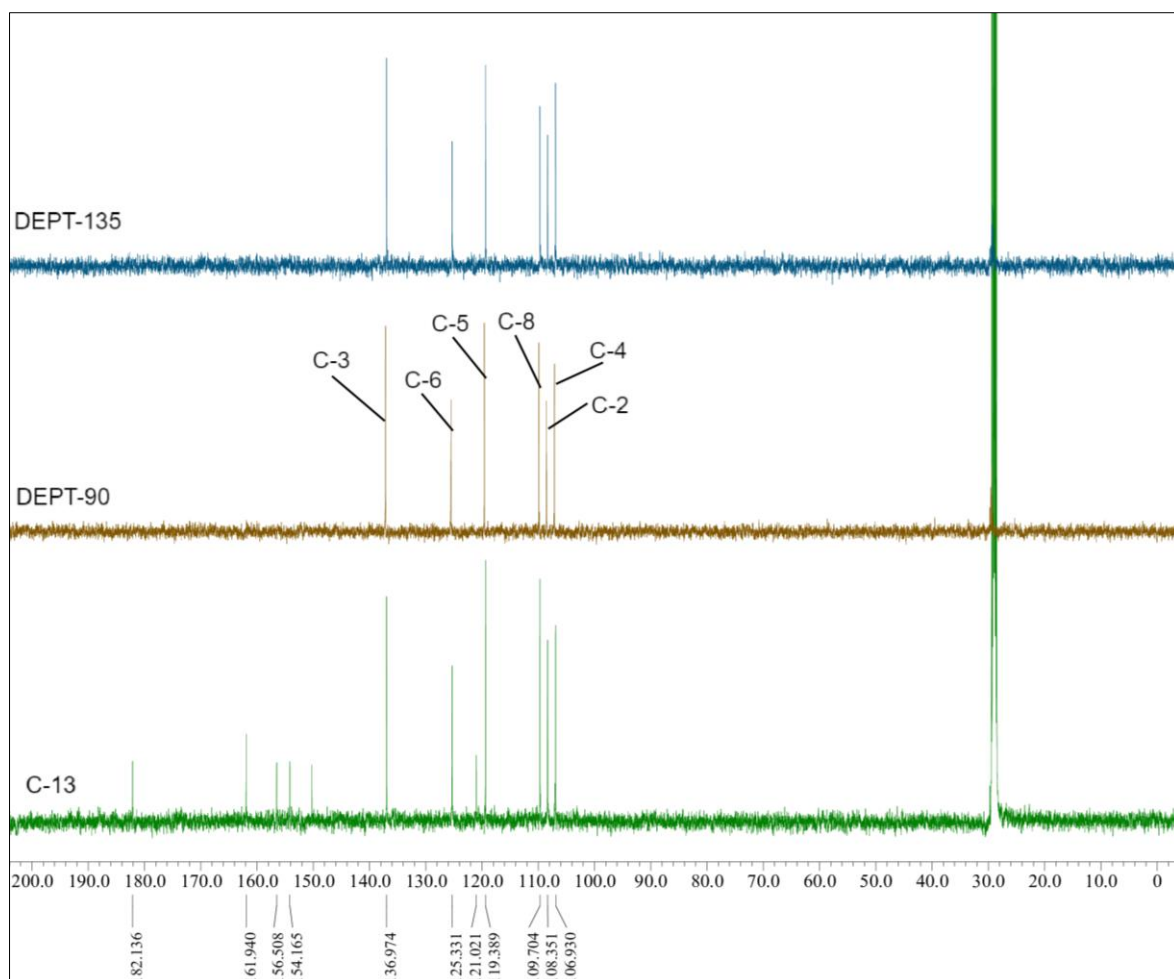
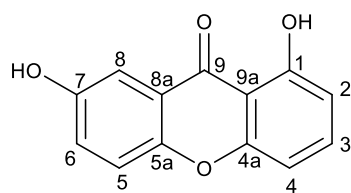


Figure 4.38: ^{13}C DEPT spectrum of euxanthone (63)

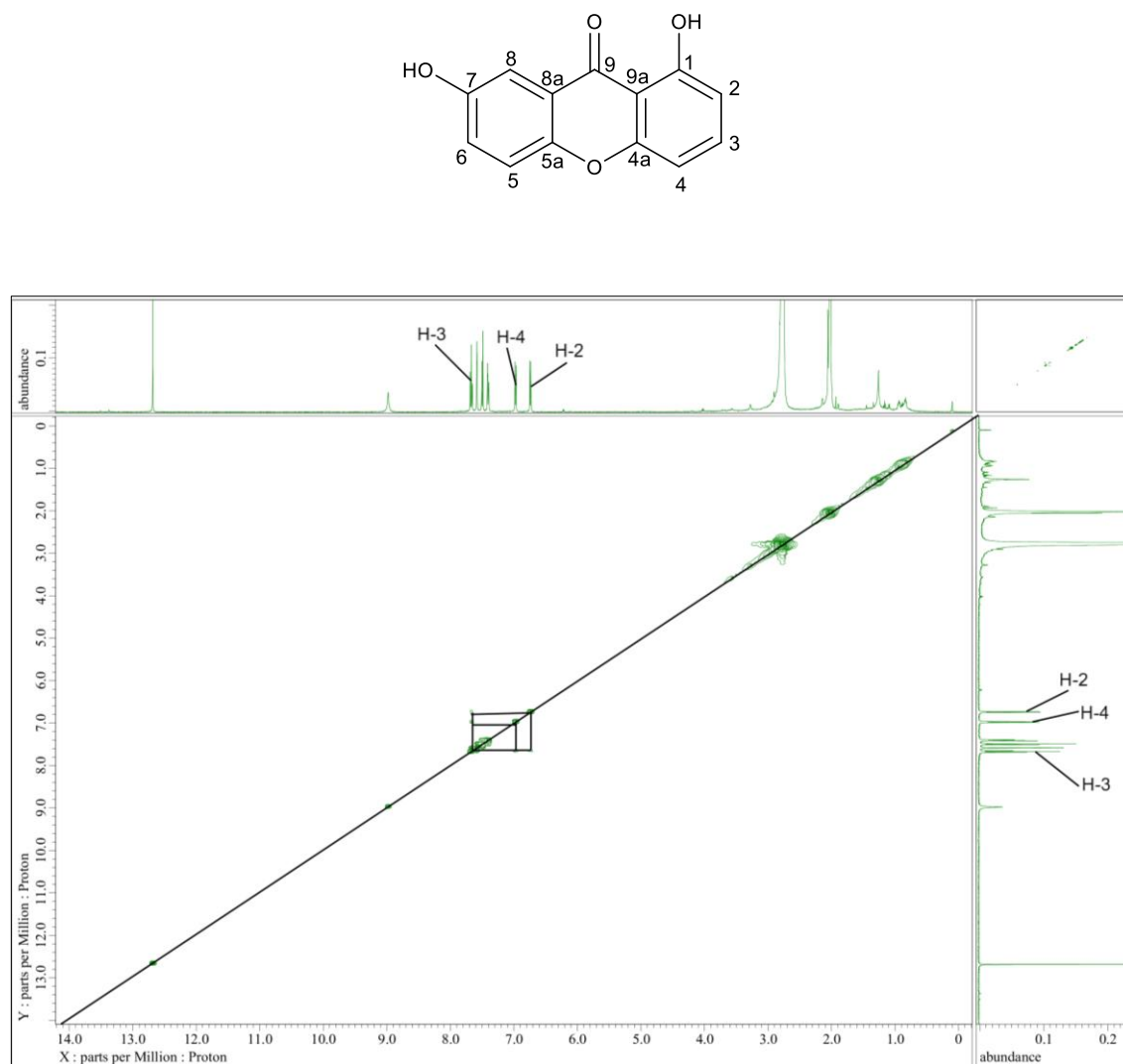


Figure 4.39: COSY spectrum of euxanthone (63)

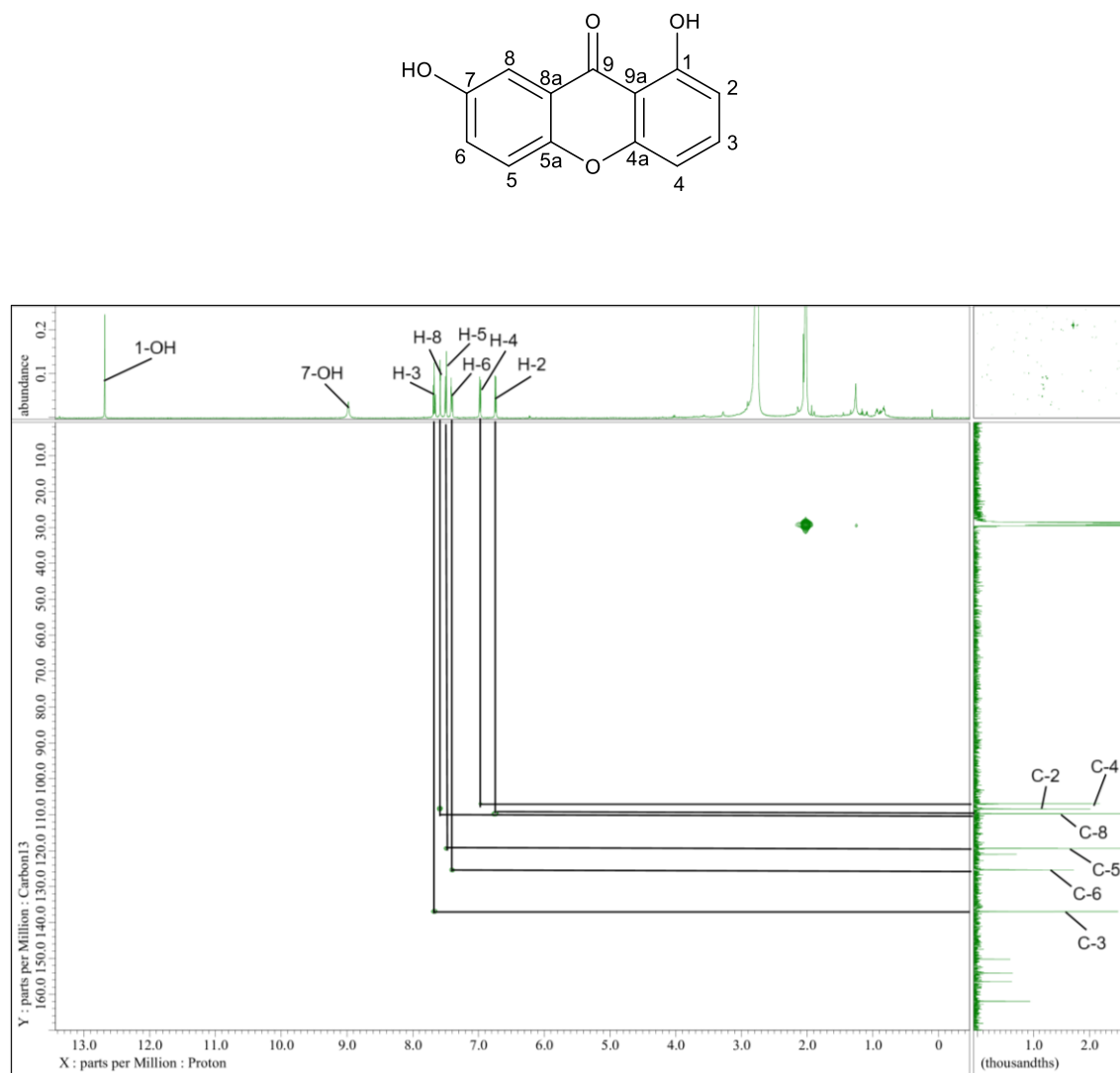


Figure 4.40: HSQC spectrum of euxanthone (63)



Figure 4.41: HMBC spectrum of euxanthone (63)

4.2.2 Characterisation of friedelin (60)

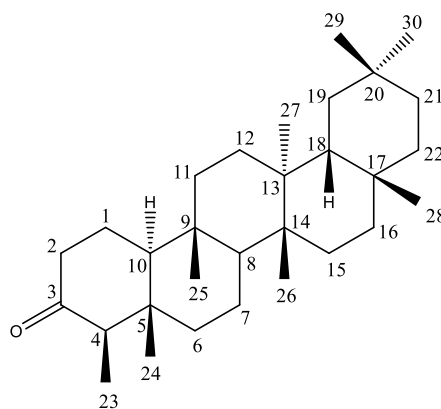


Figure 4.42: Structure of friedelin (**60**)

Compound (**60**) (Figure 4.42) was gained as white needle crystals with a melting point of 262-263 °C (literature: 260-261 °C, Lizazman et al., 2023). The LC-MS spectrum gave a molecular ion $[M^+]$ at m/z 426, which matched to the molecular formula $C_{30}H_{50}O$. The absorption bands in FTIR were demonstrated at 2925 and 2865 cm^{-1} (C-H stretch), 1709 cm^{-1} (conjugated C=O), 1450 and 1384 cm^{-1} (C-H bend). Meanwhile, the UV-Vis spectrum showed absorption maxima at 288 nm.

There were seven CH_3 singlets found at δ 0.70 (H-24), δ 0.85 (H-25), δ 0.99 (H-26), δ 1.03 (H-27), δ 1.16 (H-28), δ 0.93 (H-29) and δ 0.98 (H-30) in the ^1H NMR spectrum. Additionally, CH_3 with a doublet signal was observed at δ 0.86 (H-23). CH proton with a triplet signal was observed at δ 1.37 (H-8). Meanwhile, multiplet signals of CH were found at δ 2.23 (H-4), δ 1.50 (H-10) and δ 1.52 (H-18). CH_2 signals were observed at δ 1.94 and 1.72 (H-1), δ 2.38 and 2.26 (H-2), δ 1.74 and 1.26 (H-6), δ 1.48 and 1.37 (H-7), δ 1.46 and 1.26 (H-11), δ 1.37 and 1.32 (H-12), δ 1.45 and 1.24 (H-15), δ 1.52

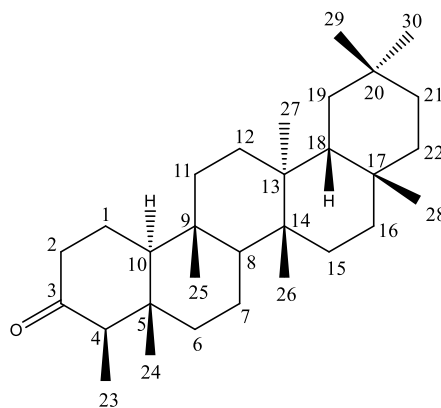
and 1.34 (H-16), δ 1.37 and 1.19 (H-19), δ 1.49 and 1.30 (H-21) and δ 1.49 and 0.93 (H-22).

In the ^{13}C NMR spectrum, 30 carbons were found. A C=O group which was highly deshielded was found at δ 213.3 (C-3). Eight CH_3 groups were observed at δ 6.9 (C-23), δ 14.7 (C-24), δ 18.0 (C-25), δ 20.3 (C-26), δ 18.7 (C-27), δ 32.2 (C-28), δ 35.1 (C-29) and δ 31.9 (C-30). Six quaternary carbons were demonstrated at δ 42.2 (C-5), δ 37.5 (C-9), δ 39.8 (C-13), δ 38.4 (C-14), δ 30.1 (C-17) and δ 28.2 (C-20). The rest of the carbons were observed at δ 22.4 (C-1), δ 41.4 (C-2), δ 58.3 (C-4), δ 41.6 (C-6), δ 18.3 (C-7), δ 53.2 (C-8), δ 59.6 (C-10), δ 35.4 (C-11), δ 30.6 (C-12), δ 32.5 (C-15), δ 36.1 (C-16), δ 42.9 (C-18), δ 35.7 (C-19), δ 32.8 (C-21) and δ 39.3 (C-22).

Both ^1H NMR and ^{13}C NMR data of compound **60** were observed to correspond with those literature values reported for friedelin (Lizazman et al., 2023). The ^1H NMR and ^{13}C NMR spectra of the compound are depicted in Figure 4.43 and Figure 4.44, respectively. Table 4.5 displayed the summary of NMR data for compound **60** in accordance with the literature values for friedelin.

Table 4.5: ^1H NMR (500 MHz, CDCl_3) and ^{13}C NMR (125 MHz, CDCl_3)

Assignments of friedelin (60)



Position	^1H (δ)	^{13}C (δ)	^1H (δ)*	^{13}C (δ)*
1	1.94, 1.72 (<i>m</i> , 1H)	22.4	1.98, 1.71 (<i>m</i> , 1H)	22.3
2	2.38, 2.26 (<i>m</i> , 2H)	41.4	2.40, 2.28 (<i>m</i> , 2H)	41.3
3	-	213.3	-	213.4
4	2.23 (<i>q</i> , 1H, $J = 6.9$ Hz)	58.3	2.26 (<i>q</i> , 1H, $J = 6.8$ Hz)	58.2
5	-	42.2	-	42.2
6	1.74, 1.26 (<i>d</i> , 2H, $J = 11.5$ Hz)	41.6	1.74, 1.28 (<i>d</i> , 2H, $J = 11.9$ Hz)	41.5
7	1.48, 1.37 (<i>m</i> , 2H)	18.3	1.50, 1.41 (<i>m</i> , 2H)	18.2
8	1.37 (<i>t</i> , 1H, $J = 8.6$ Hz)	53.2	1.41 (<i>t</i> , 1H, $J = 9.2$ Hz)	53.1
9	-	37.5	-	37.4
10	1.50 (<i>m</i> , 1H)	59.6	1.53 (<i>m</i> , 1H)	59.5
11	1.46, 1.26 (<i>m</i> , 2H)	35.4	1.48, 1.28 (<i>m</i> , 2H)	35.3
12	1.37, 1.32 (<i>m</i> , 2H)	30.6	1.34, 1.34 (<i>m</i> , 2H)	30.5
13	-	39.8	-	39.7
14	-	38.4	-	38.3
15	1.45, 1.24 (<i>m</i> , 2H)	32.5	1.47, 1.27 (<i>m</i> , 2H)	32.4
16	1.52, 1.34 (<i>m</i> , 2H)	36.1	1.58, 1.37 (<i>m</i> , 2H)	36.0
17	-	30.1	-	30.0
18	1.52 (<i>m</i> , 1H)	42.9	1.58 (<i>m</i> , 1H)	42.8
19	1.37, 1.19 (<i>m</i> , 2H)	35.7	1.39, 1.22 (<i>m</i> , 2H)	35.6
20	-	28.2	-	28.2
21	1.49, 1.30 (<i>m</i> , 2H)	32.8	1.51, 1.31 (<i>m</i> , 2H)	32.8
22	1.49, 0.93 (<i>m</i> , 2H)	39.3	1.51, 0.95 (<i>m</i> , 2H)	39.3
23	0.86 (<i>d</i> , 3H, $J = 6.1$ Hz)	6.9	0.89 (<i>d</i> , 3H, $J = 6.8$ Hz)	6.8
24	0.70 (<i>s</i> , 3H)	14.7	0.72 (<i>s</i> , 3H)	14.7
25	0.85 (<i>s</i> , 3H)	18.0	0.87 (<i>s</i> , 3H)	18.0

26	0.99 (s, 3H)	20.3	1.01 (s, 3H)	20.3
27	1.03 (s, 3H)	18.7	1.05 (s, 3H)	18.7
28	1.16 (s, 3H)	32.2	1.18 (s, 3H)	32.1
29	0.93 (s, 3H)	35.1	0.95 (s, 3H)	35.0
30	0.98 (s, 3H)	31.9	1.00 (s, 3H)	31.8

*Lizazman et al. (2023)

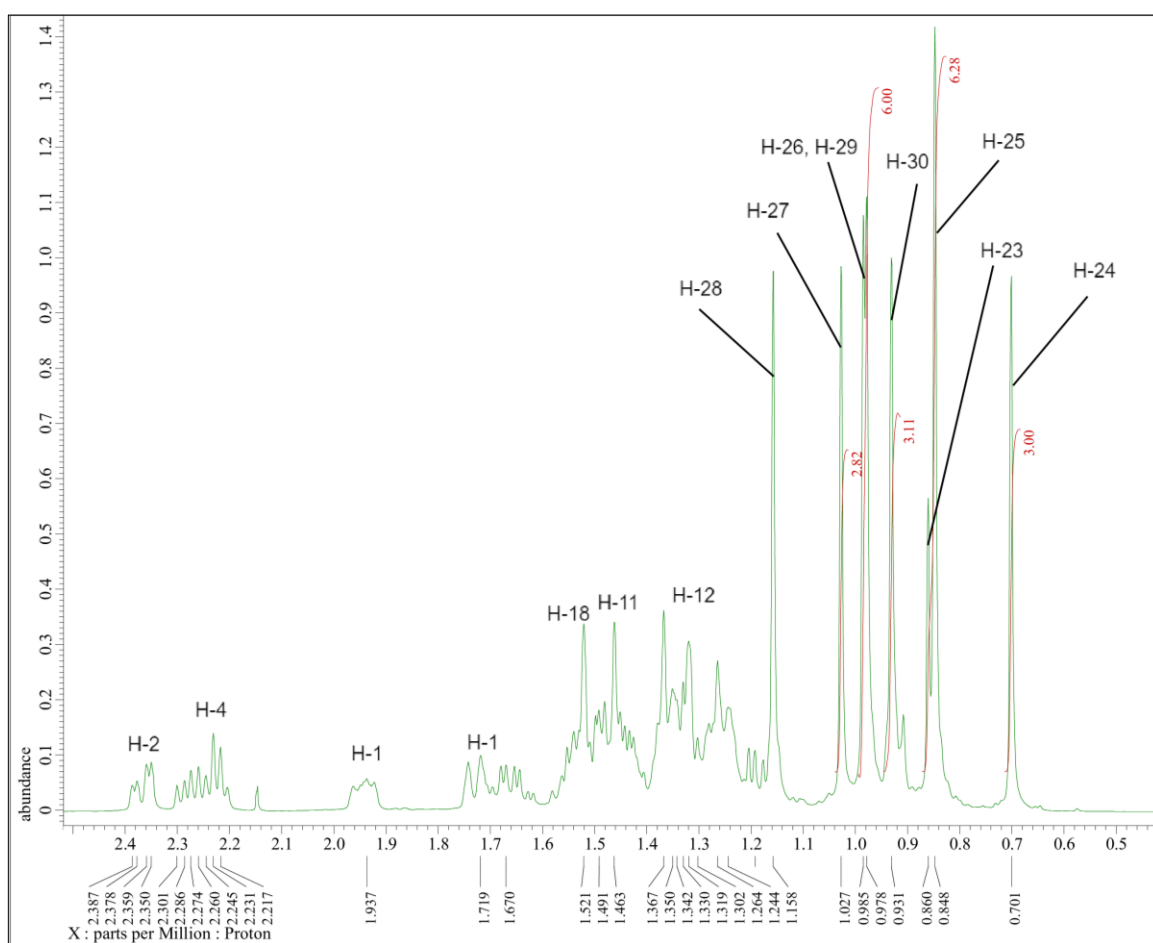
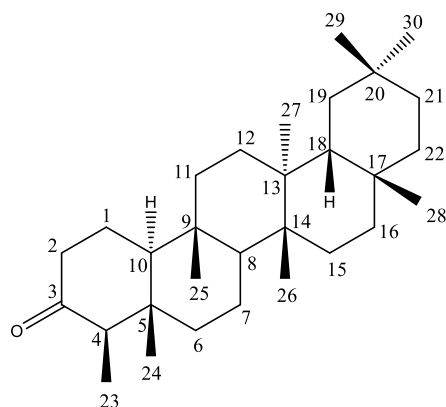


Figure 4.43: ^1H NMR spectrum of friedelin (**60**) (500 MHz, CDCl_3)

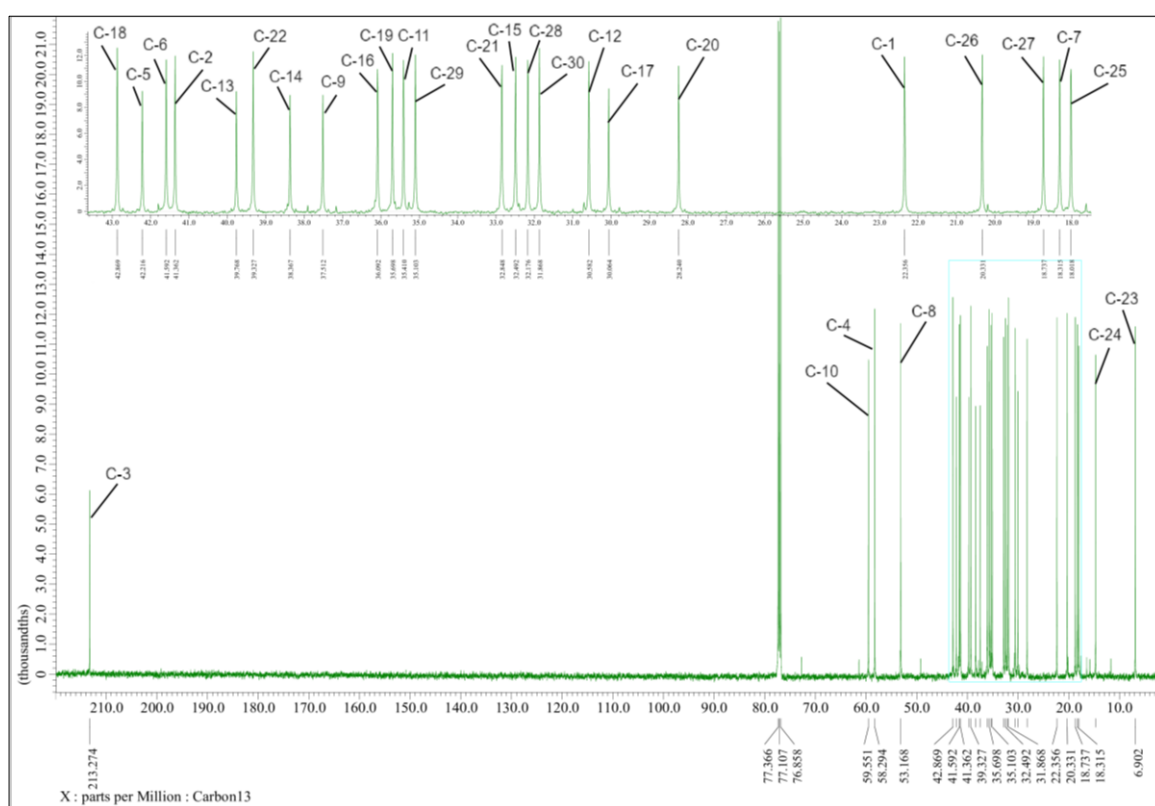
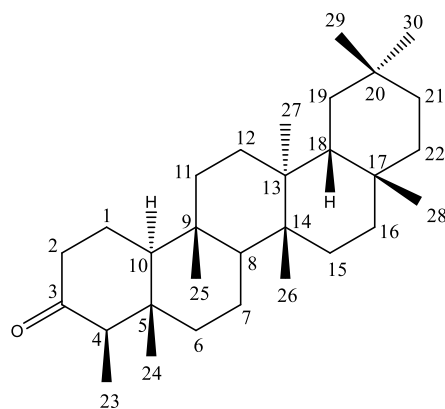


Figure 4.44: ^{13}C NMR spectrum of friedelin (**60**) (125 MHz, CDCl_3)

4.3 Antibacterial Activities

Crude extracts and isolated compounds of both *Calophyllum soulattri* and *Calophyllum gracilentum* were assessed by using agar well diffusion method and dilution method for their antibacterial activities. They were screened against five bacterial strains, which are *Lactiplantibacillus plantarum*, *Serratia marcescens*, *Enterobacter cloacae*, *Pseudomonas aeruginosa* and *Bacillus cereus*. The extracts and isolated compounds that showed an inhibition zone diameter of more than 10 mm in the agar well diffusion method was considered active (Ouchari et al., 2019) and proceeded to the dilution method. Ampicillin was utilised as a positive control for antibacterial assays. Crude extracts and isolated compounds' antibacterial activities were compared with the activities of ampicillin. The antibacterial screening on the extracts of *C. soulattri* and *C. gracilentum* showed that hexane extract for both plants demonstrated antibacterial activity against *E. cloacae* with inhibition zones of 13 mm and 11 mm, respectively. The hexane extract from *C. soulattri* exhibited inhibition against *S. marcescens* with inhibition zone of 15 mm, which shows no significant difference with ampicillin. Besides, extracts from *C. soulattri* (ethyl acetate and methanol) and *C. gracilentum* (hexane and ethyl acetate) demonstrated inhibition against *P. aeruginosa*. The activity of the hexane extract of *C. gracilentum* is excellent against *P. aeruginosa* as it produced the highest inhibition effect with an inhibition zone of 20 mm. Meanwhile, a new compound, soulaxanthone (**62**), demonstrated moderate inhibition against *P. aeruginosa* but showed no inhibition against all four bacterial strains. The calopolyanolide E (**19**) and friedelin (**60**) showed moderate inhibition against *E. cloacae*. Generally, crude extracts and isolated compounds were ineffective against *B. cereus* and *L. plantarum*. This is because of the thick cell wall owned by the Gram-positive bacteria, which is a significant component for its survival and ability to

infect humans (Romaniuk & Cegelski, 2015; Nikolic & Mudgil, 2023). All results were reported as mean $\pm$ standard error for three sample studies. The activities of crude extracts and isolated compounds against bacterial strains are tabulated in Table 4.6 and Table 4.7, respectively. Samples of pictures for agar well diffusion assay are presented in Figure 4.45.

The active extracts and compounds were further evaluated for their MIC and MBC activities. The MIC evaluations were performed by the broth micro-dilution method, which was adapted from Zamakshshari et al. (2022), where a 96-well flat-bottom microplate was used. Meanwhile, the MBC method was adapted from Balouiri et al. (2016). A plant extract with the MIC value of less than 100 $\mu\text{g/mL}$ showed high activity, while the MIC value of 100 to 500 $\mu\text{g/mL}$ indicated moderate activity and more than 1000 $\mu\text{g/mL}$ was considered weak antibacterial activity (Carneiro et al., 2017). Meanwhile, for isolated compounds, the MIC value of less than 10 $\mu\text{g/mL}$ showed high activity. Meanwhile, 10 to 50 and more than 100 $\mu\text{g/mL}$ were displayed moderate and weak antibacterial activity, respectively (Kalli et al., 2021). The result obtained showed that hexane extract for both plants demonstrated moderate activity against *E. cloacae* with the MIC value of 250 $\mu\text{g/mL}$. Only ethyl acetate extract of *C. soulattri* displayed activity against *P. aeruginosa* with the MIC value of 125 $\mu\text{g/mL}$. Hexane extract from both plant and ethyl acetate extract from *C. soulattri* demonstrated potential bactericidal activities with the value of 500 $\mu\text{g/mL}$ against *E. cloacae* and *P. aeruginosa*. Even though the hexane extract of both *Calophyllum* plants had shown activity against *E. cloacae*, *P. aeruginosa* and *S. marcescens*, none of the isolated compounds from these extracts have shown antibacterial activity against any bacterial strain. This is due to the synergetic effect of the compounds presents in the extracts. Soulaxanthone (**62**) demonstrated moderate activity against *P. aeruginosa* with the MIC value of 25 $\mu\text{g/mL}$. This can be clarified by the attachment of the -OH group to

position C-6 on compound **62**, which gives good ligand-protein interaction with topoisomerase IV by giving low binding energy and, as a result, leads to bacteriostatic action (Yele & Md, 2020; Helgesen et al., 2021). Calopolyanolide E (**19**) and friedelin (**60**) displayed moderate activity against *E. cloacae* with the MIC value of 25 µg/mL and 50 µg/mL, respectively. The MBC test showed that both calopolyanolide E (**19**) and friedelin (**60**) possess bactericidal activity against *E. cloacae* with the value of 50 µg/mL. A previous study stated that friedelin demonstrated good antimicrobial activity against *S. faecalis*, *S. typhi* and *E. cloacae* with the MIC value of 0.61 µg/mL (Kuethe et al., 2007). Besides, friedelin also showed good antibacterial activity against *S. aureus* (Ragasa et al., 2015). The results for the dilution method are tabulated in Table 4.8.

Table 4.6: Antibacterial activities of crude extracts

Sample (1 mg/mL)	Zone of inhibition (mm) of tested bacteria				
	Gram-positive bacteria		Gram-negative bacteria		
	<i>B. cereus</i>	<i>L. plantarum</i>	<i>E. cloacae</i>	<i>P. aeruginosa</i>	<i>S. marcescens</i>
CSH	7.00 ± 0.25*	8.00 ± 0.35*	13.33 ± 0.36*	8.00 ± 0.43*	15.33 ± 0.38
CSEA	7.33 ± 0.25*	7.67 ± 0.35*	9.00 ± 0.36*	11.00 ± 0.43*	8.33 ± 0.38*
CSM	7.00 ± 0.25*	8.33 ± 0.35*	8.67 ± 0.36*	12.00 ± 0.43*	8.33 ± 0.38*
CGH	7.00 ± 0.25*	7.67 ± 0.35*	11.00 ± 0.36*	20.33 ± 0.43*	8.67 ± 0.38*
CGEA	7.33 ± 0.25*	7.67 ± 0.35*	9.00 ± 0.36*	14.00 ± 0.43*	7.33 ± 0.38*
CGM	7.00 ± 0.25*	7.67 ± 0.35*	8.00 ± 0.36*	9.67 ± 0.43*	8.67 ± 0.38*
Ampicillin	23.67 ± 0.25	16.67 ± 0.35	20.67 ± 0.36	34.33 ± 0.43	16.00 ± 0.38
DMSO	NA	NA	NA	NA	NA

Each value represents mean ± SE; The (*) symbol denotes a significance difference between sample and standard drug ($p < 0.5$); CSH = *Calophyllum soulattri* (Hexane); CSEA = *Calophyllum soulattri* (Ethyl acetate); CSM = *Calophyllum soulattri* (Methanol); CGH = *Calophyllum gracilentum* (Hexane); CGEA = *Calophyllum gracilentum* (Ethyl acetate); CGM = *Calophyllum gracilentum* (Methanol); NA = No Activity



C. gracilentum (Hexane) against *P. aeruginosa*



Friedelin against *E. cloacae*

Figure 4.45: Samples of pictures for agar well diffusion assay

Table 4.7: Antibacterial activities of isolated compounds

Sample (0.1 mg/mL)	Zone of inhibition (mm) of tested bacteria				
	Gram-positive bacteria		Gram-negative bacteria		
	<i>B. cereus</i>	<i>L. plantarum</i>	<i>E. cloacae</i>	<i>P. aeruginosa</i>	<i>S. marcescens</i>
Soulaxanthone (62)	7.33 ± 0.25*	8.33 ± 0.35*	9.33 ± 0.36*	11.00 ± 0.43*	7.67 ± 0.38*
Euxanthone (63)	7.33 ± 0.25*	8.0 ± 0.35*	8.00 ± 0.36*	8.00 ± 0.43*	8.33 ± 0.38*
Calopolyanolide E (19)	7.00 ± 0.25*	8.67 ± 0.35*	11.00 ± 0.36*	8.33 ± 0.43*	8.00 ± 0.38*
Calanolide E (3)	7.67 ± 0.25*	7.67 ± 0.35*	9.33 ± 0.36*	8.33 ± 0.43*	7.33 ± 0.38*
Friedelin (60)	8.00 ± 0.25*	8.67 ± 0.35*	10.00 ± 0.36*	7.67 ± 0.43*	7.67 ± 0.38*
Ampicillin	16.67 ± 0.25	11.67 ± 0.35	15.67 ± 0.36	30.67 ± 0.43	11.33 ± 0.38
DMSO	NA	NA	NA	NA	NA

Each value represents mean ± SE; The (*) symbol denotes a significance difference between sample and standard drug ($p < 0.5$); NA = No Activity

Table 4.8: The MIC and MBC of crude extracts and isolated compounds

	<i>E. cloacae</i>		<i>P. aeruginosa</i>	
	MIC	MBC	MIC	MBC
Extracts				
<i>C. soulattri</i> (Hexane)	250 µg/mL	500 µg/mL	-	-
<i>C. gracilentum</i> (Hexane)	250 µg/mL	500 µg/mL	-	-
<i>C. soulattri</i> (Ethyl acetate)	-	-	125 µg/mL	500 µg/mL
Compounds				
Calopolyanolide E (19)	25 µg/mL	50 µg/mL	-	-
Friedelin (60)	50 µg/mL	50 µg/mL	-	-
Soulaxanthone (62)	-	-	25 µg/mL	>100 µg/mL
Positive control				
Ampicillin	25 µg/mL	50 µg/mL	3.13 µg/mL	50 µg/mL

(-) = Not tested

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Calophyllum is a genus of tropical flowering plants that has been broadly utilised for treating many diseases. They are known for having excellent biological activity due to their secondary metabolites, such as coumarins, xanthones, triterpenes and flavonoids. The phytochemical study on the *Calophyllum soulattri* and *Calophyllum gracilentum* stem bark led to the yielding of five compounds from three different classes which are xanthones, coumarins and triterpene. *Calophyllum soulattri* gave one new compound, namely, soulaxanthone (**62**), together with two known compounds, calanolide E (**3**) and calopolyanolide E (**19**). Meanwhile, *Calophyllum gracilentum* gave two compounds, namely, euxanthone (**63**) and friedelin (**60**). The antibacterial test results discovered that the crude extracts and isolated compounds displayed antibacterial activity against *E. cloacae* and *P. aeruginosa*. Among the extracts, hexane extracts from *C. soulattri* and *C. gracilentum* offer antibacterial activities against *E. cloacae*. Meanwhile, calopolyanolide E (**19**) and friedelin (**60**) exhibited moderate activities against *E. cloacae*. Soulaxanthone (**62**) exhibited moderate activity against *P. aeruginosa*. The compounds exhibited activity against Gram-negative bacteria, but inactive against Gram-positive bacteria. This is due to the Gram-positive bacteria possession on a thick cell wall, which is an essential component for its continuance and ability to infect humans. It is concluded that *Calophyllum* plants are potential source of main compounds for the pharmacological research and development.

5.2 Recommendations

The low yields of a new compound, soulaxanthone (**62**), has become the limitation of study. Since soulaxanthone demonstrated antibacterial activity, it is suggested to isolate more of the compound for future study by developing standardise method. The modern extraction methods such as microwave assisted extraction (MAE), supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE) are recommended to be applied due to their high extraction yields and stability of the target extracts. The ethyl acetate extract from *Calophyllum soulattri* can be fractionated by column chromatography eluting with different mixtures of hexane, chloroform, ethyl acetate and methanol. The middle fractions can be further separated with column chromatography eluting with solvent system of 70 % hexane and 30% acetone. Further purification of fractions can be done with column chromatography eluting with the same solvent system until two spots observed in TLC plate. Centrifugal column chromatography with the solvent system of 80% hexane and 20% ethyl acetate can be used in the final step for the purification to yield the compound.

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APPENDICES

Appendix A: Antibacterial Figure

Table 1: Agar well diffusion of isolated compounds and extracts on *Bacillus cereus*

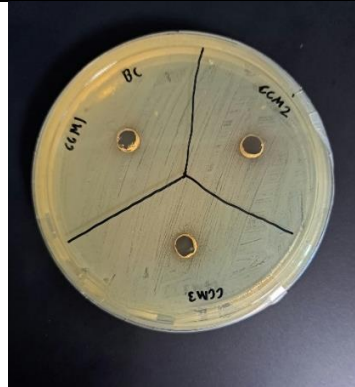
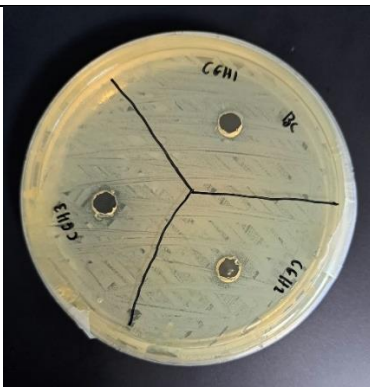
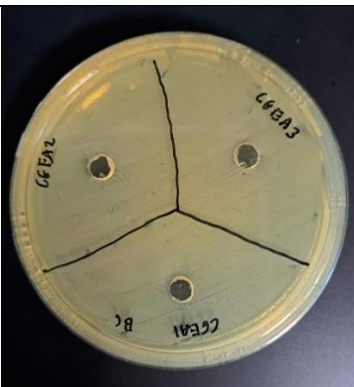
		
<i>C. soulattri</i> (Hexane extract)	<i>C. soulattri</i> (Ethyl acetate extract)	<i>C. soulattri</i> (Methanol extract)
		
<i>C. gracilentum</i> (Hexane extract)	<i>C. gracilentum</i> (Ethyl acetate extract)	<i>C. gracilentum</i> (Methanol extract)

Table 1: continued

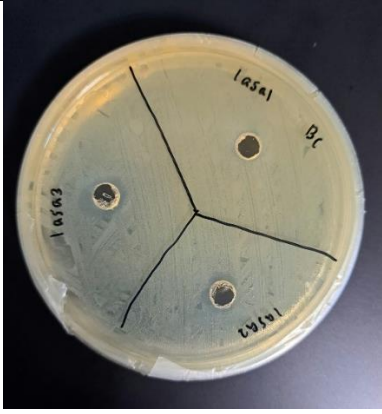
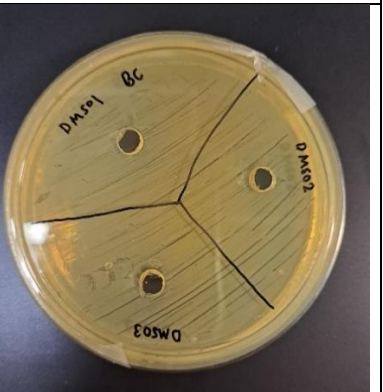
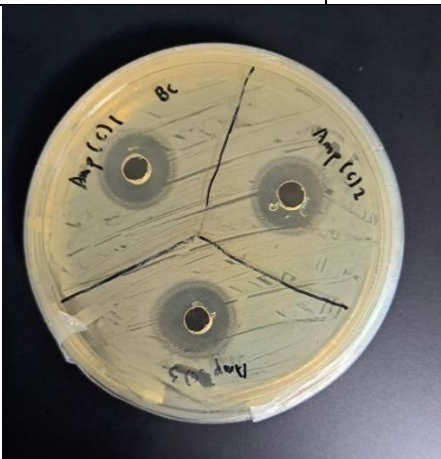
		
Calanolide E	Calopolyanolide E	Euxanthone
		
Soulaxanthone	Friedelin	DMSO
		
Ampicillin (Compound)	Ampicillin (Extract)	

Table 2: Agar well diffusion of isolated compounds and extracts on *Pseudomonas aeruginosa*

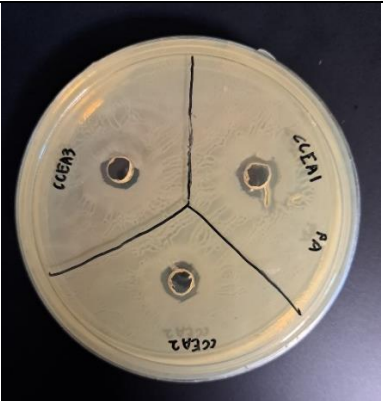
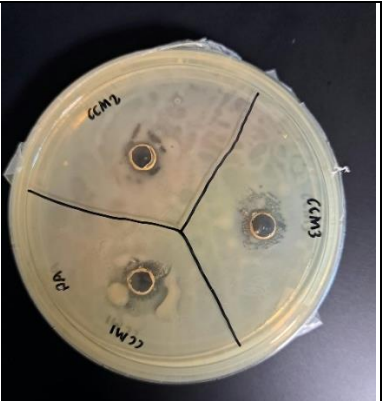
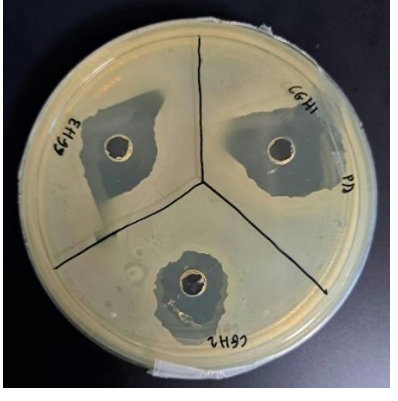
		
<i>C. soulattri</i> (Hexane extract)	<i>C. soulattri</i> (Ethyl acetate extract)	<i>C. soulattri</i> (Methanol extract)
		
<i>C. gracilentum</i> (Hexane extract)	<i>C. gracilentum</i> (Ethyl acetate extract)	<i>C. gracilentum</i> (Methanol extract)
		
Calanolide E	Calopolyanolide E	Euxanthone

Table 2: continued

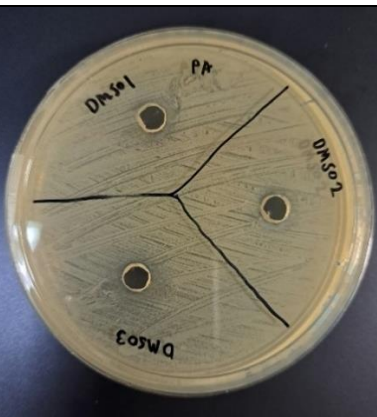
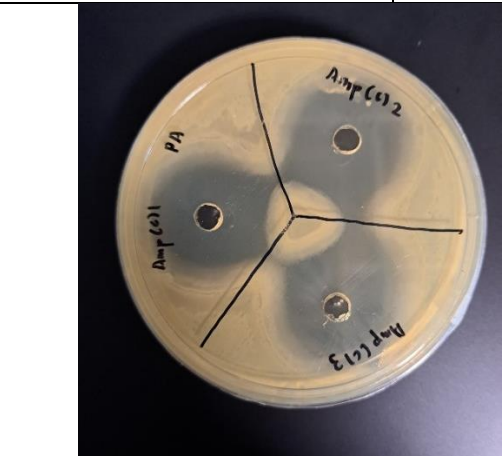
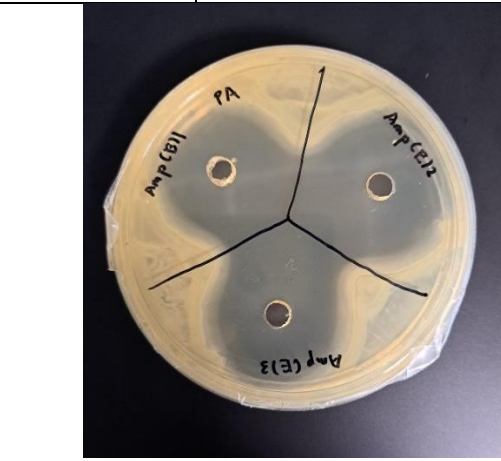
		
Souloxanthone	Friedelin	DMSO
		
Ampicillin (Compound)	Ampicillin (Extract)	

Table 3: Agar well diffusion of isolated compounds and extracts on *Lactiplantibacillus plantarum*

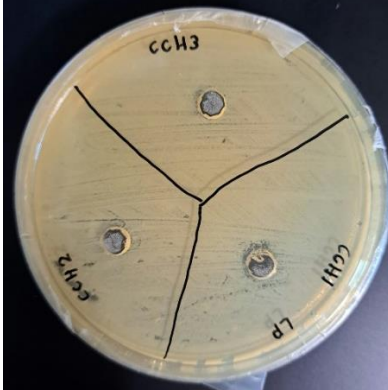
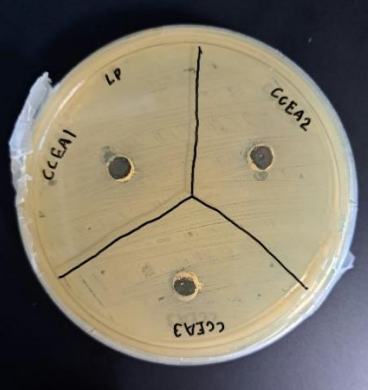
		
<i>C. soulattri</i> (Hexane extract)	<i>C. soulattri</i> (Ethyl acetate extract)	<i>C. soulattri</i> (Methanol extract)
		
<i>C. gracilentum</i> (Hexane extract)	<i>C. gracilentum</i> (Ethyl acetate extract)	<i>C. gracilentum</i> (Methanol extract)
		
Calanolide E	Calopolyanolide E	Euxanthone

Table 3: continued

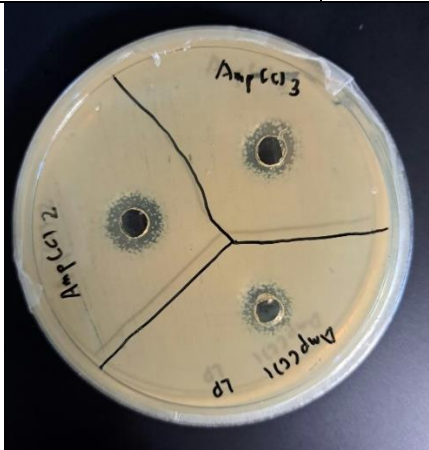
		
Souloxanthone	Friedelin	DMSO
		
Ampicillin (Compound)	Ampicillin (Extract)	

Table 4: Agar well diffusion of isolated compounds and extracts on *Enterobacter cloacae*

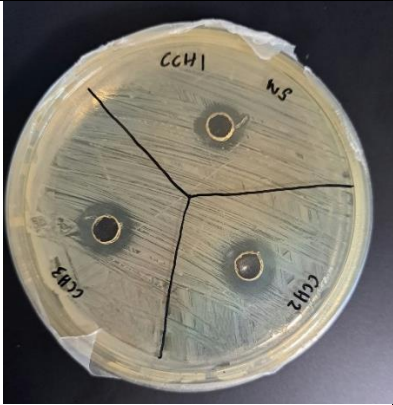
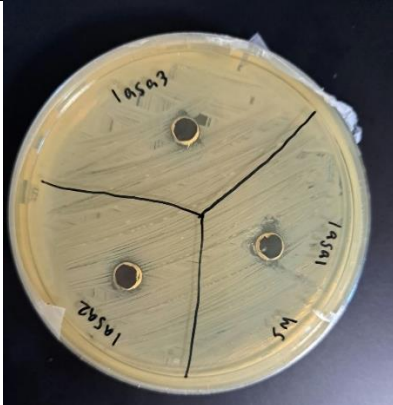
		
<i>C. soulattri</i> (Hexane extract)	<i>C. soulattri</i> (Ethyl acetate extract)	<i>C. soulattri</i> (Methanol extract)
		
<i>C. gracilentum</i> (Hexane extract)	<i>C. gracilentum</i> (Ethyl acetate extract)	<i>C. gracilentum</i> (Methanol extract)
		
Calanolide E	Calopolyanolide E	Euxanthone

Table 4: continued

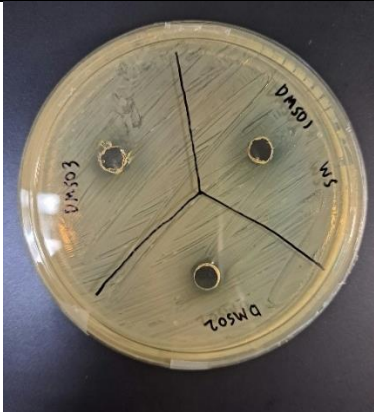
		
Souloxanthone	Friedelin	DMSO
		
Ampicillin (Compound)	Ampicillin (Extract)	

Table 5: Agar well diffusion of isolated compounds and extracts on *Serratia marcescens*

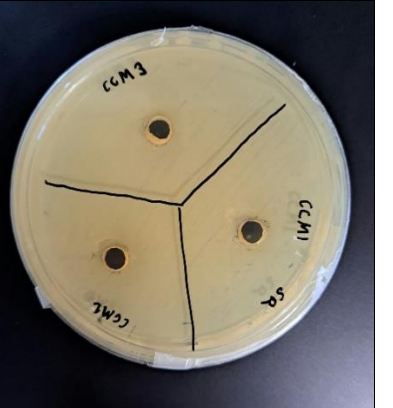
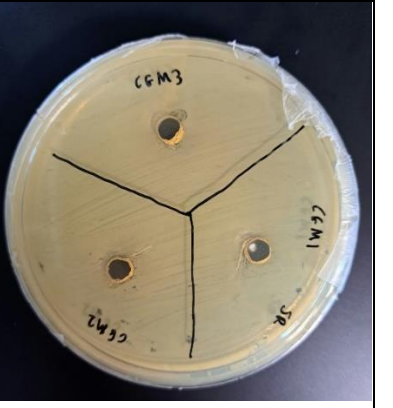
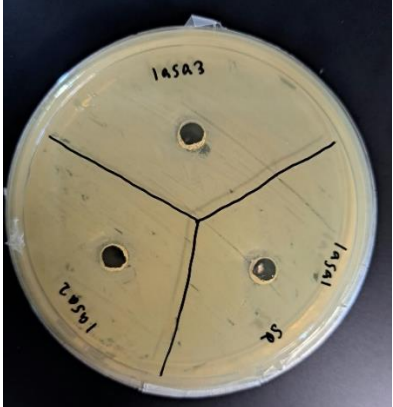
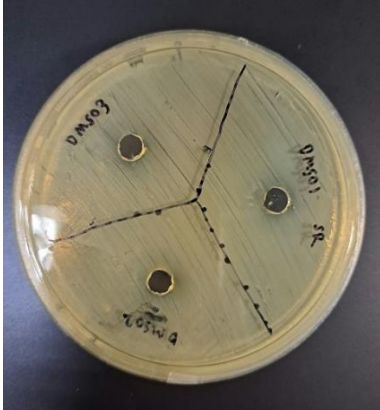
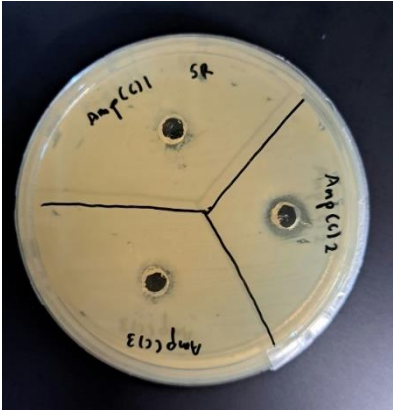
		
<i>C. soulattri</i> (Hexane extract)	<i>C. soulattri</i> (Ethyl acetate extract)	<i>C. soulattri</i> (Methanol extract)
		
<i>C. gracilentum</i> (Hexane extract)	<i>C. gracilentum</i> (Ethyl acetate extract)	<i>C. gracilentum</i> (Methanol extract)
		
Calanolide E	Calopolyanolide E	Euxanthone

Table 5: continued

		
Soulaxanthone	Friedelin	DMSO
		
Ampicillin (Compound)	Ampicillin (Extract)	