

**Bangladeshi Medicinal Plants: Ethnopharmacology,  
Phytochemistry and Anti-Staphylococcal Activity**

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## List of abbreviations

Abbreviations	Definition
ACOH	Acetic Acid
C	Cultivate
<sup>13</sup> C NMR	Carbon Nuclear Magnetic Resonance Spectroscopy
CDCl <sub>3</sub>	Detoriated Chloroform
CHCl <sub>3</sub>	Chloroform
COSY	Correlation Spectroscopy
d	Doublet
dd	Doublet of doublet
1D NMR	One dimensional Nuclear Magnetic Resonance
2D NMR	Two-dimensional Nuclear Magnetic Resonance
Da	Dalton
DEPT 135	Distortionless Enhancement through Polarization Transfer-135
EtOAc	Ethyl acetate
EUR	Euro
FIC	Informant consensus factor
FL	Fidelity level
GUAH	Govt. Unani and Ayurvedic Hospital
<sup>1</sup> H NMR	Proton Nuclear Magnetic Resonance
HMBC	Heteronuclear Multiple Bond Connectivity
HMQC	Heteronuclear Multiple Quantum Coherence
HRMS	High Resolution Mass Spectrometry
IR	Infrared spectroscopy
m	Multiplet
MBC	Minimum Bactericidal Concentration
MeOH	Methanol
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin Resistance <i>Staphylococcus aureus</i>
NBG	National Botanical Garden
NOESY	Nuclear Overhauser Effect Spectroscopy
t	Triplet
TLC	Thin Layer Chromatography

PTLC	Preparative Thin Layer Chromatography
q	Quadruplet
R <sub>f</sub> value	Retardation factor
RUBG	University of Rajshahi Botanical garden
s	Singlet
SPE	Solid Phase Extraction
SJ	Sunderban Jungle
USD	US dollar
VLC	Vaccum Liquid Chromatography
VS	Vanillin in Sulphuric acid
W	Wild
ZI	Zone of Inhibition

**Abstract:**

Antibacterial resistance is a major concern. Due to its new resistance mechanism, it is spreading and emerging globally and thereby threatening the treatment of common infectious diseases. There is no doubt to develop new antibiotic from natural sources to tackle antimicrobial resistance. This study involves, (1) ethnopharmacological survey in Bangladesh and (2) bioassay directed phytochemical investigation to explore antibacterial compounds against MRSA. In 2016, an ethnopharmacological survey conducted in Bangladesh led to the recommendation of 71 medicinal plants by 71 Ayurvedic/Unani practitioners, 21 Ayurvedic patients and 35 local inhabitants for the treatment of infectious diseases. Based on the review of literature, data analysis and ease of availability of the plants, 18 plants were initially selected and collected from Bangladesh. After initial antibacterial screening of 18 plants, five plants (MIC 32-512 µg/ml) were chosen based on potential antibacterial activity. These are (*Zingiber montanum*, *Uraria picta*, *Diospyros malabarica*, *Cynometra namiflora*, *Swertia chirayita*). Extensive phytochemical work using different chromatographic (VLC, Column chromatography, SPE, TLC, PTLC) and spectroscopic (NMR, Mass spectrometry, IR) techniques on five Bangladeshi medicinal plants led to the isolation and identification of 25 compounds. Eight terpenes (zerumbol, zerumbone, buddledone A, germacrone, furanodienone, (-) borneol, camphor and 8(17), 12-labdadiene-15, 16-dial) were isolated from *Zingiber montanum* with the MIC (32- >128 µg/ml). Eugenol and steriods were isolated from *Uraria picta* (MIC 64- >128 µg/ml). Lupane type triterpenoids (Lupeol, botulin, betulinaldehyde, betulone and messagenin) were isolated and identified from *Diospyros malabarica* with the MIC (64- >128 µg/ml) while pentacyclic triterpene (glutinol, glutinone), simple phenolic (ethyl 4-ethoxybenzoate) and steriods were isolated from *Cynometra ramiflora* with MIC (64- >128 µg/ml). A series of xanthones (swerchirin, swertiaperenin, belidifolin and decussatin) were identified from *Swertia chirayita* with MIC (>128 µg/ml). Among these compounds, 4-ethoxybenzoate, messagenin were identified as new natural compounds. In terms of activity, 8(17), 12-labdadiene-15, 16-dial (32 µg/ml against ATCC 5941) and zerumbol (32 µg/ml against EMRSA 15) showed potential antibacterial activity.

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### **Dedication**

This thesis is dedicated to the memories of my grandfather Mr Alam Siddique (1938-2011).

# **Chapter 1: Introduction**

# 1. Introduction

## 1.1 Antimicrobial resistance

The incidence of the number of bacterial pathogens bestowing antimicrobial and multidrug resistance to antibiotics has remarkably accelerated over the past few decades. The mishandling, misuse and abuse of antimicrobial agents are the main reasons for the emergence of the resistant gene in microorganisms. Apart from manifestation of the antimicrobial agent, antimicrobial resistance may occur due to the mutation in bacterial DNA or the acquisition of bacterial resistance gene through horizontal gene transfer (WHO, 2018). Antimicrobial resistance is now a serious and complex problem for global health requiring a multi-disciplinary approach involving partners from all health sectors including public health authorities and the scientific community.

According to the WHO report (2016), resistant infection has become the third leading cause of mortality worldwide. Current statistics show that around 25,000 people die each year in Europe due to AMR (antimicrobial resistance) and it is currently costing the health care system approximately EUR 1.5 billion per year (European Commission, 2017). AMR has made cancer chemotherapy, organ transplantation, diabetes management and major surgery like caesarean sections more challenging (WHO, 2018). Unless appropriate action is taken to tackle the threat, drug resistant infections will kill an extra 10 million people annually worldwide by 2050 which is more than the predicted death of cancer and it will cost 100 trillion USD of the world's economic output (Founou et al., 2017)

In February 2017 the WHO published a list of bacteria related to diseases in humans which urgently demanded new antibiotics for hard-to-treat infections. *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacteriaceae* (carbapenem- resistant) and *Staphylococcus aureus* (methicillin-resistant) that pose a particular threat in nursing homes and hospitals which have been categorised as critical in terms of AMR. To address the challenges of AMR, in July 2014, there have been disputation in the parliament of UK and the Prime Minster announced an exclusive review of AMR to search for techniques to discover new drugs. It has also been considered in the 'UK Five Year Antimicrobial Resistance Strategy 2013 to 2018' (Department of Health, 2013). It is important to discover alternative therapeutics for treating infectious diseases.

## 1.2 Medicinal plants in drug discovery

Plant-derived medicines have contributed largely to human health since antiquity; hence plants may be a source for the development of new novel antimicrobial compounds followed by subsequent pharmacological, chemical and clinical studies (Dias et al., 2012). For example, the most prominent anti-inflammatory agent acetylsalicylic acid commercially known as the drug aspirin isolated from the bark of the willow tree *Salix alba* L. (Der Marderosian and Beutler, 2003). Quinine isolated from bark of *Cinchona succirubra* has been used traditionally to treat malaria, indigestion, fever, mouth and throat disease for centuries (Der Marderosian and Beutler, 2003). **The nobel prize winner Youyou Tu discovered antimalarial compound artemisinin from *Artemisia annua* and saved millions of lives over the past 40 years (Guo, 2016).**

The roots of modern medicine lie in herbalism. It is estimated that plants have provided the natural product model for over 50% of drugs used in modern medicine. Every ancient civilisation has used plants for healing and in many cultures the use of herbal product has magical-religious significance with different points of view regarding the concepts of health and disease existing within the culture.

Today herbal medicine according to the WHO is still the primary form of healing employed by over 80% of the world's developing population (WHO, 2015). A report recently published in the Daily Sun (a national newspaper in Bangladesh) states that, 75% of the Bangladesh population are using plant originated medicine for fundamental health care (Jahan, 2016) because to local people they are more accessible, comparatively safer and more affordable compared to orthodox or conventional medicine. Bangladesh is enriched with vast plant diversity. About 450-500 plants with medicinal properties are indigenous to Bangladesh due to its favourable ecologic conditions such as temperate and humid weather with fertile alluvial soil (Banglapedia, 2015). Moreover, Bangladesh has hilly tracks, the largest mangrove forest (Sundorban, Khulna) and national botanical garden situated in the capital city where thousands of medicinal plants flourish. In addition, Bangladesh exports herbal and traditional products of about 30 million US dollars per annum.



### **1.3 Ayurveda**

Ayurvedic medicine, a traditional system of medicine that originated in India over 5000 years ago, is widely practiced in Bangladesh. The term Ayurveda is derived from two Sanskrit words 'Ayur' (Life) and 'Veda' (science or knowledge) which literally means "science of life" or "practices of longevity/ long life". The concept of Ayurvedic medicine in regard to health and disease is to promote the use of herbal remedies, special diet and other unique health practices. Local citizens of Bangladesh have a long history of using traditional medicine for primary healthcare. Ayurvedic and Unani practitioners (qualified practitioners) and local healers (using knowledge handed down through the generations) of Bangladesh prescribe such medicinal plants for the treatment of various diseases including different types of infections (Rahmat et al., 2012; Seikh et al., 2011). Bangladeshi medicinal plants that have been traditionally used for centuries by Ayurvedic and Unani practitioners to treat infectious diseases may contain phytochemicals with the potential for the development of new modern drugs. Ayurvedic/Unani practitioners are very popular among local inhabitants in the cities and are sought based on their skills in treating disease. In addition, local healers who are not qualified practitioners but recommend medicinal plants to village people in the rural areas of Bangladesh based on their experience and knowledge are well known in the Bengali community.

The knowledge and use of plants for medicinal purposes is an integral part of many ethnic cultures. This valuable indigenous knowledge associated with plants is now being recognised as a source of knowledge and the wisdom of using medicinal plants by traditional healers and communities which otherwise could be lost over time, needs to be conserved and documented to enable more research into the identification of the key chemical compounds and mechanism of action by which traditional medicine works (WHO, 2015).

### **1.4 Plant derived antimicrobial compounds:**

WHO has also emphasized the use of indigenous plants for the treatment of diseases when orthodox medicines are of little or no value. Plants are likely to produce antimicrobial metabolites as part of their defence strategy to counter bacteria and fungi in the environment. For example, the key compound, hyperforin (Fig.1.1), isolated from St John's Wort (*Hypericum perforatum*) is one

of the most interesting lead compounds in terms of antibacterial action (MIC values of 0.1  $\mu\text{g/ml}$  against MRSA and penicillin-resistant variants). Gibbons et al. (2002) reported significant activity of 33 out of 34 species and varieties of the genus *Hypericum* against a clinical isolate of methicillin-resistant *Staphylococcus aureus* with five extracts MICs of 64  $\mu\text{g/ml}$ . Ana and his colleagues (2011) isolated taxifolin-7-O- $\alpha$ -l-rhamnopyranoside (Figure 1.2) from *Hypericum japonicum* and reported its synergistic effect with conventional antibiotics (ampicillin, levofloxacin, ceftazidime and azithromycin) against MRSA clinical strains. The authors argued that taxifolin-7-O- $\alpha$ -l-rhamnopyranoside alone had moderate antibacterial activity whilst taxifolin in combination with ceftazidime showed good antibacterial activity with MIC of 2-16  $\mu\text{g/ml}$ .

For example, the chemical structures of isolated compounds (mentioned above) with potential antibacterial activity.

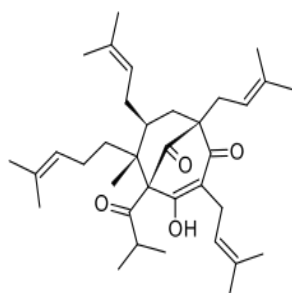


Figure 1.1: Hyperforin (Smelcerovic et al., 2006)

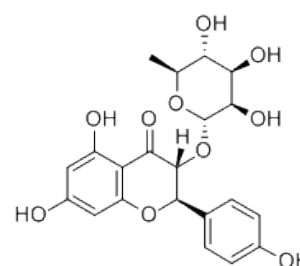


Figure 1.2: Taxifolin-7-O- $\alpha$ -l-rhamnopyranoside (Gabin et al., 2016)

Moreover, Tohidpour et al (2010) investigated the antibacterial activity of the traditional essential oil of *Thymus vulgaris* and *Eucalyptus globulus* against clinical isolates of Methicillin resistant *Staphylococcus aureus* (MRSA) and other standard bacterial strains. They claimed that *Thymus vulgaris* had shown lower MIC value (18  $\mu\text{g/ml}$ ) compared to *Eucalyptus globulus* (MIC value 37  $\mu\text{g/ml}$ ) the results showed that two of the essential oils had different antibacterial activity. From the above research findings, it is clear that medicinal plants are an effective resource of secondary metabolites. Thus, the development of novel antibiotics

from medicinal plants that have traditionally been used for decades for the treatment of infectious diseases would be a step forward to tackle this vital issue of antimicrobial resistance.

### **1.5 Ethnopharmacological survey in Bangladesh**

An ethnopharmacological survey is a strategy to select medicinal plants for scientific exploration of the biologically active agents. Several ethnopharmacological surveys have been conducted in different part of Bangladesh focussing on different aspects of health such as diabetes mellitus, diarrhoea or which have just simply catalogued the medicinal plants used by tribes of the hill tract areas (Kadir et al., 2012; Rahman, 2013; Rahman and Roy 2014; Uddin et al., 2015a; Uddin et al., 2015b; Nur et al., 2016,). These studies only featured the qualitative analysis of forest or hill tract areas of Bangladesh and lacked any data analysis using the various quantitative parameters usually associated with this type of study. Kadir et al published several studies on Bangladesh medicinal plants with data analysis however these focussed on the plants used to treat gastrointestinal disorders solely in the hill tracts of Bangladesh (Kadir et al., 2012, 2013 and 2014). According to the authors' knowledge, to date no studies have been carried out to investigate the use of Bangladeshi medicinal plants to treat infections. Thus, an ethnopharmacological survey was conducted in Bangladesh during autumn 2016 to document indigenous knowledge in regard to the treatment of infections with the ultimate aim of identifying plants that could be selected for phytochemical investigation to identify key secondary metabolites responsible for their anti-infective properties.

### **1.6 Phytochemical work:**

Based on the outcome of ethnopharmacological survey, the extensive phytochemical work was conducted on the selected plants to identify the specific compounds responsible for anti-infective biological activity. The phytochemical work has been discussed in the Method and the Results sections.

### **1.7 Antibacterial screening:**

In order to explore lead anti-infective compounds, a number of sensitivity tests including agar diffusion, disc diffusion, bioautography and microdilution titre assay are commonly used. In the current study, the microdilution titre assay was

applied to antibacterial screening of extracts, fractions and pure compounds in terms of minimum inhibitory concentrations (MICs). This technique offers several advantages over other methods. This is an easy method to perform offering quicker result within short period of time. Compared to other methods, it's less expensive.

### **1.8 Literature review:**

Based on the outcome of the ethnopharmacological survey and availability of plants, a total of 18 plants were initially selected for this study. These plants were extensively reviewed for data related to the isolation of secondary metabolites as well as for the biological activity. The key words used for the literature review were the scientific names of the plants. The results found were filtered based on the phytochemical reports as well as antibacterial, antifungal and related biological activity. The overall findings of the literature review of the phytochemical and biological/ pharmacological investigations are presented in table 1.1.

Table 1.1 Chemical and biological review on the selected plants.

Family	Scientific name	Local name	Literature review	
			Chemical	Biological
Acanthaceae	<i>Andrographis periculata</i>	Kalomegh	Andrographolide, Roseoside, 7,8-dimethoxy-2'-hydroxy-5-O-β-d-glucopyranosyloxyflavon, 14-deoxyandrographiside, and 5,4'-dihydroxyflavonoid-7-O-β-d-pyranglucuronatebutylester. (Zhang et al.,2015)	Antidengue efficacy of andrographolide with LC <sub>50</sub> 12ppm (Edwin et al., 2016)
Acanthaceae	<i>Justicia adhatoda</i>	Bashokpata	Alkaloid Vasicine. (Duraipandiyan et al., 2015) 2. 2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl (Dhankhar et al., 2014) 3. 2-acetyl benzylamine and vasicine acetate. (Ignacimuthu and Shanmugam, 2010) 4. Pyrroloquinazoline alkaloids- vasicinolone, vasicine acetate, vasicine, vasicinone, 2-acetyl benzyl amine (Singh and Sharma 2013)	Antibacterial activity of Vasicine acetate against <i>M. luteus</i> , <i>E. aerogenes</i> , <i>S. epidermidis</i> and <i>P. aeruginosa</i> (MIC125 µg/mL) (Duraipandiyan et al., 2015), against <i>E. coli</i> (MIC 20µg/ml), against <i>M. tuberculosis</i> and one multi-drug-resistant (MDR) strain and one sensitive strain (ZI 200 and 50 microg/ml, respectively) (Ignacimuthu and Shanmugam, 2010)  Antifungal activity against <i>C. albicans</i> at the dose of >55µg/ml (Singh and Sharma 2013)
Apocynaceae	<i>Tylophora indica</i>	Onontomul	No previous chemical work	No previous biological work
Combretaceae	<i>Terminalia arjuna</i>	Arjun	Arjunolic acid, Arjunic acid (Singh et al., 2002). Arjunoside I, II, Arjungenin (Honda et al., 1976), Terminoltin (Singh et al., 1995),  2α,19α-Dihydroxy-3Oxo-Olean-12-En28-Olic acid 28-O-β-d-glucopyranoside (Choubey and Srivastava, 2001)	Antibacterial activity against <i>Staphylococcus aureus</i> , <i>Bacillus subtilis</i> , <i>Klebsiella pneumoniae</i> , <i>Eschericia coli</i> , <i>Salmonella typhi</i> and <i>Pseudomonas aeruginosa</i> (ZI of bark extract 15.0 ± 0.7 mm, 15.5 ± 0.7 mm, 15.0 ± 1.5 mm, 15.5 ± 0.7 mm, 15.0 ± 0.7 mm, 15.0 ± 0.7

			<p>Arjunetosie (19<math>\alpha</math>-trihydroxyolean-12-en-28-oic acid 28-O-<math>\beta</math>-d-glucopyranoside, 3-O-<math>\beta</math>-d-glucopyranosyl-2<math>\alpha</math>, 3<math>\beta</math>) (Upadhyay et al., 2001).</p> <p>Terminic acid, <math>\beta</math>-Sitosterol, Terminic acid, Oleanolic acid (Anjaneyulu and Prasad, 1983) Arjunin, Arjunic acid, Arjunetin (Row et al., 1970).</p> <p>Kajiichigoside F1, 2<math>\alpha</math>,3<math>\beta</math>,23-trihydroxyurs-12,18-dien-28-oic acid 28-O-<math>\beta</math>-glucopyranosyl ester, Qudranoside VIII, 2<math>\alpha</math>,3<math>\beta</math>-dihydroxyurs-12,18-oic acid 28-O-<math>\beta</math>-d-glucopyranosyl ester, 3-O-methyl ellagic acid 4'-O-<math>\alpha</math>-l-rhamnophranoside (-)-epicatechin, 2<math>\alpha</math>,3<math>\beta</math>,23-trihydroxyurs-23-trihydroxyurs-12,19-dien-28-oic acid 28-O-<math>\beta</math>-d-glucopyranosyl ester (Wang et al., 2010).</p> <p>Arjunolone, Arjunone (Sharma et al, 1982) Arjunaphthanoloside (Ali et al, 2003)</p> <p>(-)-epigallocatechin, (+)-catechin, Quercetin, (+)-gallocatechin (Saha and Pawar, 2012)</p> <p>22<math>\beta</math>-diol-12-en-28 <math>\beta</math>-D-glucopyranosie-oic acid, Olean-3<math>\beta</math> (Patnaik et al., 2007) Terminarjunoside I and II (Alam et al., 2008) Termionic acid, Terminoside A (Ahmad et al., 1983)</p> <p>Arjunolitin (Tripathi et al, 1992) Galactopyranoside (Yadava and Rathore, 2000)</p> <p>Arjunasides A-E and Arjunglucoside IV and V (Wang et al., 2010)</p> <p>Luteolin, 14,16-dianhydrogitoxigenin 3-<math>\beta</math>-d-xylopyranosyl-(1 &gt; 2)-O-<math>\beta</math>-d- (Pettit et al., 1996)</p>	<p>mm; ZI of leave extract 13.5 <math>\pm</math> 0.7 mm, 16.5 <math>\pm</math> 0.7 mm, 14.0 <math>\pm</math> 0.5 mm, 15.0 <math>\pm</math> 0.5 mm, 13.5 <math>\pm</math> 0.7 mm, 14.0 <math>\pm</math> 0.7 mm) (Kumar et al., 2017), against <i>E. coli</i>, <i>P. aeruginosa</i> and <i>S. aureus</i> (MBC 25 mg/ml (Gupta and Kumar 2016)</p>
Combretaceae	<i>Terminalia chebula</i>	horitoki	Methyl gallate (Acharya et al.,2015)	Antibacterial activity of compounds against <i>Enterobacter aerogenes</i>

			Gallotannin 1,2,6-tri-O-galloyl-β-D-glucopyranose (Bag and Chattapadhyay 2017) Kaempferol-3-O-rutinoside, Pyrogallol (Singh and Kumar 2013)	(ZI- 31 mm; MIC 0.039 mg/ml (Singh and Kumar 2013), against <i>Helicobacter pylori</i> (MIC 125 mg/l; MBC 150 mg/l) (Malekzadeh et al., 2001)
Gentianaceae	<i>Swertia chirayita</i>	chirota	Secoiridoids and xanthones, i.e. mangiferin, amaroswerin, swertiamarin, amarogentin (Kumar et al., 2015) Swertiachiralatone A, swertiachoside A, swertiachirdiol A and swertiachoside B. (Zhou et al., 2015) 1-hydroxy-2,3,4,7-tetramethoxyxanthone and 1,5,8-trihydroxy-3-methoxy xanthone (Zhou et al., 2013) Swerchirin, decussatin , 1,8-dihydroxy-3,5,7-trimethoxyxanthone , 1-hydroxy-3,5,7,8-tetramethoxyxanthone , bellidifolin , 1-hydroxy-3, 7-dimethoxyxanthone , methylswertianin , 1-hydroxy-3,5-dimethoxyxanthone , erythrodiol , oleanolic acid , gnetiolactone , scopoletin , sinapaldehyde, syringaldehyde , and β-sitosterol (You et al., 2017) Amarogentin, gentiopicroside, amaroswerin, mangiferin and amaronitidin (Kumar et al., 2015) 1, 2, 8-trihydroxy-6-methoxy xanthone and 1, 2-dihydroxy-6-methoxyxanthone-8-O-β-D-xylopyranosyl (Mahendran et al., 2014)	Antibacterial activity against <i>Staphylococcus aureus</i> (ZI 19 mm (Alam et al., 2009)
Legumes	<i>Glycyrrhiza glabra</i>	Jeshthomodu	Licorice (Ota et al., 2018) Glabridin, formononetin, hispaglabridin B, hemileiocarpin, isoliquiritigenin, 4'-O-methylglabridin, and paratocarpin B (Chin et al., 2007) Macedonoside C-(Hayashi et al., 2000) Glucoliquiritin apioside, prenyllicoflavone A, shinflavone, shinpterocarpin, 1-methoxyphaseollin (Kitagawa et al., 1994) Glycyrrhizin, ergosterol, stigmasterol, glabridin and licochalcone (Chakotiya et al., 2017) Licochalcone C (Franceschelli et al., 2011) Licoagrodone (Li et al., 1998)	Antibacterial activity of glycyrrhizic acid against <i>Pseudomonas aeruginosa</i> (MIC 200 and 100 µg/ml) (Chakotiya et al., 2016), against <i>S. pyogenes</i> ATCC 19615 (MIC 39-156 µg/ml) (Siriwattanasatorn et al., 2016), against primary plaque colonizers and periodontal pathogens (mean ZI- 9.2 ± 1.09 mm and 10.6 ± 0.54 mm at 24 h (Sharma et al., 2016), against <i>Staphylococcus aureus</i> , <i>Bacillus subtilis</i> ,

				<i>Enterococcus faecalis</i> , <i>Candida albicans</i> (MIC of ethanolic extract of leave 0.312-5 mg/mL and root extract MIC 2.5-5 mg/mL) (Irani et al., 2010), against all the strains of <i>B. subtilis</i> (MIC of Licochalcone A 2-3 µg/ml) (Tsukiyama et al., 2002)
Legumes	<i>Cynometra ramiflora</i>	Shingra	No previous chemical work	No previous biological work
Malvaceae	<i>Abroma augusta</i>	Ulotkombol	Abromine with betain (Dasgupto and Basu, 1970) Taraxerol (Khanra et al., 2017)	No previous biological work
Meliaceae	<i>Tinospora cordata</i>	Goloncho	No previous chemical work	No previous biological work
Meliaceae	<i>Azadirachta indica</i>	Neem	Nimbin, desacetylnimbin, desacetylsalannin, azadirachtin and salanin. (Dwivedi et al., 2016) Azadirachtin R= (2H, 3H-cyclopent[b] furo [2',3':4,5] naphtho [2,4-d] heptalactone-[3,7] furan-6aceticacid, 3-(acetyloxy)-8-(3-furyl)-2a, 4a, 4b, 4c,5,5a, 6, 6a, 8, 9,9a, 10a,10b-13 hydrogen-2a,5a,6a,5-tetramethyl-3-[[[(2E)-2-methyl-1-oxo-2-butenyl]oxy]-methyl ester), Azadirachtin A, AZ-B, AZ-D, AZ-H, AZ-I, deacetylsalannin and azadiradione (Gao et al., 2017) Endophytic actinomycetes (Verma et al., 2009) Mahmoodin and Naheed (Siddiqui et al., 1992)	Antibacterial activity of compound against on <i>E. faecalis</i> (MBC 2.6%) (Shah et al., 2016) Antibacterial activity of extracts against <i>Lactobacillus curvatus</i> , <i>Campylobacter jejuni</i> , <i>Lactobacillus sakei</i> , <i>Carnobacterium spp.</i> , <i>Leuconostoc sp.</i> (ZI 11.33 ± 0.58 to 22.67 ± 0.58 mm) (Del et al., 2015), against <i>Salm. typhi</i> , <i>Escherichia coli</i> and <i>Vibrio cholera</i> (MIC 64 µg/ml and ZI 6.2-12.3 mm (Baritkar et al., 2014), against <i>L. monocytogenes</i> ATCC 43256 (MIC 4.0 mg/mL) and <i>L. monocytogenes</i> ATCC 49594 (MIC 5.0 mg/mL), <i>S. aureus</i> JCM 2151 (MIC 4.5 mg/mL) and <i>S. aureus</i> IFO 13276 (MIC 4.5 mg/mL) (Mahfuzul et al., 2017), against multi-drug-resistant <i>Vibrio cholerae</i> of serotypes O1, O139 and non-O1, non-O139 (MIC <sub>50</sub> 2.5



				mg/ml, MIC <sub>90</sub> > 5 mg/ml and MBC 10 mg/ml) (Thakurta et al., 2007)
Papilionaceae	<i>Uraria picta</i>	Sankarjata	4',5-dihydroxy-2',3'-dimethoxy-7-(5-hydroxyoxychromen-7yl)-isoflavanone , 5,7-Dihydroxy-2'-methoxy-3',4'-methylenedioxyisoflavanone (Rahman et al., 2007)	Antibacterial activity of isoflavones against gram positive, gram negative and fungi (MIC 12.5–200 µg/ml (Rahman et al., 2007)
Rutaceae	<i>Aegle marmelos</i>	Beal	Alkaloids, and phenols (Mujeeb et al.,2014) The structure of 5 coumarins are 6-(2-hydroxy-3-Hydroxymethyl-3-butenyl)-7-hydroxycoumarin, marmelonine and 8-hydroxysmyrindiol, 1-5, marmesiline, 6-(4-acetoxy-3-methyl-2-butenyl)-7-hydroxycoumarin (Kothari et al., 2011) Tannins, flavonoids, coumarins, saponins and triterpenoids (Chakthong et al., 2012)	Antibacterial activity against <i>S. epidermidis</i> (MIC 10-40mg/ml and 40-160mg/ml (Mujeeb et al.,2014), against <i>B. Cereus</i> , <i>B. Subtilis</i> and <i>S. aureus</i> , <i>E. Coli</i> , <i>P. Vulgaris</i> and <i>P. Aeruginosa</i> (ZI- 3.8cm) (Sudharameshwari and Radhika 2007), against <i>B. subtilis</i> , <i>S. aureus</i> , <i>E. coli</i> , <i>P. aeruginosa</i> (MIC 25-100 µg/ml) (Reijniemon et al., 2014)
Rutaceae	<i>Feronia limonia</i>	Kathbel	(-)-(2S)-5,3'-dihydroxy-4'-methoxy-6",6"-dimethylchromeno-(7,8,2",3")- flavanones (Rahman and Gray, 2002) Eudesma-4 , 11-dine, carvacrol and 1,5- cyclodecandine (Kumar et al., 2010)	No previous biological work
Rubiaceae	<i>Paedaria foetida</i>	Gondhobhadule	Iridoids, flavonoids, volatile oil (Wang et al., 2014)	Antibacterial activity of ethanolic leaf extract against <i>Enterobacter aerogenes</i> , <i>Klebsiella pneumoniae</i> , <i>Salmonella paratyphi</i> , <i>Escherichia coli</i> , <i>S. typhi</i> , <i>S. sonnei</i> , <i>Shigella dysenteriae</i> and <i>Vibrio cholera</i> (MIC 1.5-50 mg/ml) (Rath and Padhy,2015) Purging index was reduced at 500mg/kg doses in Castor oil and magnesium sulphate-induced diarrhoea models in mice (Afroz et al.,2006)
Scaridae	<i>Diospyros malabarica</i>	Gaab	No previous chemical work	No previous biological work

Zingiberaceae	<i>Zingiber montanum</i>	bunoda	γ-terpinene , sabinene, (E)-1-(3',4'-dimethoxyphenyl) buta-1,3-diene (DMPBD), β-phellandrene and terpinen-4-ol (Verma et al., 2018) Zerumbone (al-amin et al., 2012)	Antibacterial activity against MRSA strains (MIC 125-500 µg mL <sup>-1</sup> ) (Verma et al., 2018)
Zygophyllaceae	<i>Tribulus terrestris</i>	Gokhur kata	Flavonoids, alkaloids, saponins, lignin, amides and glycosides (Shahid et al.,2016)	Antimicrobial activity against <i>Streptococcus sanguis</i> , <i>Staphylococcus aureus</i> , <i>Actinomyces viscosus</i> <i>Escherichia coli</i> and <i>Enterococcus faecalis</i> (MIC 35.0- 20mg/ml. (Soleimanpour et al., 2015)

### 1.9 Aim and objectives:

The overall aim of the study was to focus on Bangladeshi medicinal plants for the isolation and identification of compounds with antibacterial activity against a number of bacteria including multi-drug resistant (MDR) and methicillin resistant *Staphylococcus aureus* (MRSA)

The objectives of the study are

**To interview** Ayurveda practitioners and local citizens of Bangladesh who frequently recommend or use medicinal plants for treating infective disease.

**To identify 17-18** medicinal plants based on ethno pharmacological survey used in the treatment of bacterial infections as well as literature review.

**To test** the antibacterial activity of the crude extracts of the selected medicinal plants against MRSA

**To isolate and purify** the bioactive compounds from crude extracts by using a range of chromatographic techniques.

**To identify the** anti-infective compounds by a series of spectroscopic methods such as Nuclear Magnetic Resonance (NMR) spectroscopy, Infrared Spectroscopy (IR) and High Resolution Mass Spectrometry (HRMS).

# **Chapter 2: Methods and Materials**

## **2. Methodology:**

### **2.1 Material and method: Ethnopharmacological survey.**

#### **2.1.1 Study area**

Bangladesh is a south Asian country located at the apex of the Bay of Bengal and boarded by India and Myanmar (latitudes 20° and 27°N and longitudes 88° and 93°E) with a population of over 162 million. It is well known for its rigorous greenery and numerous waterways. An ethnopharmacological survey was conducted to explore potential medicinal plants used for treating various infectious diseases by using structured and semi structured interviews according to the guidelines provided by Professor Michael Heinrich published later in the Journal of Ethnopharmacology (Heinrich et al., 2017). The interviews included Ayurvedic and Unani practitioners at their practice places, patients at Ayurvedic hospitals, local healers and inhabitants in common areas (National Botanical Fair and herb markets). As recommended by the practitioners of two recognised Ayurvedic and Unani colleges (Government Unani and Ayurvedic Degree College and Hospital at Mirpur and Tibbia Habibia Ayurvedic College and Hospital at Bokshi Bazar) in Bangladesh, the survey was carried out in greater Dhaka (Mirpur, New market, Chalk Bazar, Sutrapur, Jamalpur, Moulobhibazar, Shahabag), Tangail (near Modhupur forest), Gazipur (Telipara), Mymensingh and a northern district named Natore. The later district is well known for recommending medicinal plants by local healers. Various medicinal plants are also cultivated in this district

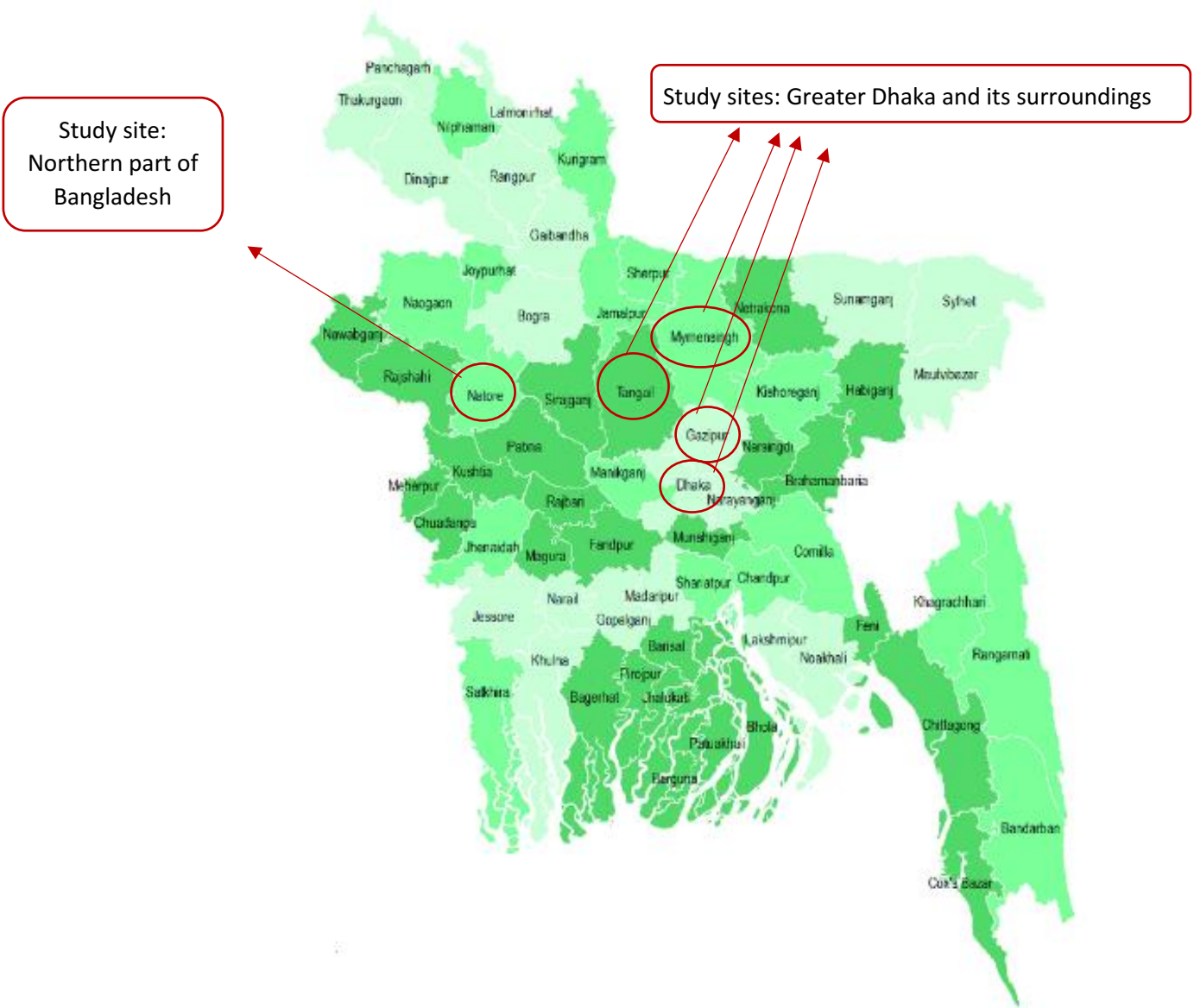


Figure 2.1: Map of the study area showing Dhaka divisions of Bangladesh.

### **2.1.2 Ethical approval for the study**

Ethical approval for conducting the ethnopharmacological survey was obtained from UREC (University Research Ethics Committee) of University of East London (Reference number: UREC- 1516 154). Relevant approval to carry out the survey was also sought from the University of Dhaka (Bangladesh). During the interview processes, all the participants were provided with a participant information sheet to explain the process and purpose of the study. Additionally, a participant consent form both in English and Bengali were also provided for participants to agree and sign prior to interview.

### **2.1.3 Ethnopharmacological survey**

The ethnopharmacological survey was carried out in Bangladesh during autumn 2016. Two different sets of questionnaires were compiled whereby 71 practitioners, 21 Ayurvedic hospital patients and 35 village inhabitants participated in the survey. The questionnaire for practitioners was structured to gather maximum information about medicinal plants. Some questions such as scientific name, dose and route of administration of suggested plant material or how many years they have been practicing as a practitioner were solely asked of practitioners. In addition, hospital patients and village inhabitants were asked about obvious signs and symptoms they experienced in relation to bacterial diseases, for example, a burning sensation while urinating was reported to be treated with *Kalanchoe pinnata*. Urinary tract infection, tuberculosis and malaria are common bacterial diseases in Bangladesh. The participants were asked about how long they had been taking the suggested plant(s) material to treat their symptoms, and whether they have ever been to an Ayurvedic and/or Unani practitioner. Information about medicinal plants to treat bacterial disease was collected from participants via face to face interview by using questionnaires

written in both English and Bengali (Questionnaires are available in [Appendix section; pp.182](#)).

The inclusion criteria for the selection of participants were as follows

- a. Adults above the age of 25
- b. The participants should be literate enough to read and sign the consent form written in either Bengali or English
- c. The Ayurvedic/ Unani practitioners interviewed should have a minimum of 5 years' experience in clinical practice.
- d. The local village people interviewed had to be familiar with the use of plants to treat themselves with herbal medicine

Information collected from experienced Ayurvedic /Unani practitioners and ayurvedic hospital patients or village inhabitants aged above 25 years were given higher priority as they tended to have more accurate knowledge on traditional medicinal plants. Two hospitals named Tibbia Habibia Unani College and Hospital and Government Unani and Ayurvedic Medical College and Hospital were visited. Ayurvedic/ Unani practitioners working in these hospitals were interviewed. A directory of Ayurvedic and Unani practitioners of Bangladesh published by Hamdard Laboratories Limited (renowned Ayurvedic manufacturing company in Indian subcontinent) also helped to communicate with some practitioners. Accordingly, most of the Ayurvedic and Unani practitioners who worked privately and were located in different areas of Dhaka city were contacted and interviewed at their practice place *via* appointments. With prior permission from the two mentioned hospitals, outpatients in the waiting area were also interviewed. Local inhabitants were interviewed at the National Tree Fair which was held in Agargaon, Dhaka from 1<sup>st</sup> July to 31<sup>st</sup> August, 2016 and also at a herb market located in Moulobhibazar, Dhaka. More interviews of inhabitants were



carried out in Dhaka (Mirpur, New market, Chalk bazar, Sutrapur, Jamalpur, Shahabag), Mymensingh, Gazipur (telipara), Tangail and Natore in social gathering places where people meet and greet in the evening.

Both sets of questionnaires ([Appendix section; pp. 182](#)) were comprised of two sections. The first section consisted of participants' personal information such as name, age, gender, profession, education level, how long they have been working as a practitioner (for Ayurvedic and Unani practitioners only), how long they had been admitted to the hospital (ayurvedic hospital patients only) and how they acquired their knowledge about traditional medicine (village inhabitants only).

The second set of questionnaires was comprised of open questions in order to gather information regarding medicinal plants used to treat infectious diseases. Respondents were asked to share their experiences of plants they use to treat bacterial disease. During the interview, a full description of the medicinal plants used for a bacterial disease (or symptoms indicative of bacterial infection) was requested in regard to its local name, scientific name (if known), use, therapeutic indication, parts of the plant used and growth form, route of administration, method of preparation, site of collection, form of usage (Fresh or dried), dosage, possible side effects, signs and symptoms of the disease (patients and inhabitants only), other therapeutic actions besides antibacterial activity (if any), rarity score. The above questionnaires were compiled following the guidelines for an ethnopharmacological survey as provided by Professor Michael Heinrich which was published subsequently Heinrich et al., 2017).

#### 2.1.4 Identification of plants:

Scientific names of those medicinal plants participants failed to identify were confirmed with the help of two books (Yusuf et al., 1994; Uddin, 2006) relevant to Bangladeshi medicinal plants. Moreover, selected plant materials collected following this survey were identified by the taxonomists of Bangladesh National Herbarium where the voucher specimens of all the selected plant materials were deposited.

#### 2.1.5 Data analysis:

The information gathered through this ethnopharmacological survey was analysed using various parameters including Use Value (UV), Informant Consensus Factor (FIC), Fidelity Level (FL) and Rank Order Priority (ROP).

**The Use Value** (UV) demonstrates the relative importance of the suggested plant species using the following formula-

$$UV = \frac{U \times 100}{N}$$

Where 'U' is the number of use reports cited by each participant for a given plant species and 'N' is the total number of participants (table 3.3) (Phillips et al., 1994).

The Informant Consensus Factor (FIC) was calculated to check the homogeneity of the information using the following formula (Heinrich et al., 1998). The medicinal plants that are effective in treatment of certain disease tend to have higher FIC values.

$$FIC = \frac{Nur - Nt}{Nur - 1}$$

Where 'Nur' is the number of use-reports for each disease category and 'Nt' is the number of plant species used. The diseases were classified into broad ailment categories in order to calculate the Fic (Informant consensus factor). FIC

is the suggestive value of how the participants uniformly agree about the use of a particular plant species for treating a particular ailment category. The value of FIC ranges from 0 to 1. A value close to 1 indicates that few plant species were used by a large group of participants. Alternatively, a low value indicates disagreement within the participants in regard to the use of a plant species for the treatment of a specific disease in an ailment category (Table 3.2).

The relative healing potential of a plant can be established by using the Fidelity Level index (FL; Friedman et al., 1986) determined by the following formula

$$FL = \frac{I_p}{I_u} \times 100$$

$I_p$  is the number of participants who independently cited the use of a plant species for the same disease and  $I_u$  is the total number of participants who mentioned the plant for any major disease. A high FL value means that the plant species was used frequently to treat a specific ailment category by the participants (table 3.3) (Friedman et al., 1986).

Chi Square ( $X^2$ ) test has also been calculated on demographic data using Microsoft excel (table 3.1).

Rank Order Priority (ROP) was determined by using the following formula (Eddouks et al., 2017).

$$ROP = FL \times RP$$

Where ROP is Rank order priority, FL is Fidelity level, RP is Relative popularity. ROP is the combination of fidelity level and relative popularity. Relative popularity was calculated in between the number of informants who suggested a particular plant species to the number of informants who cite the most frequently cited plant species (table 3.3).

## **2.2 Methods and Materials: Phytochemical investigation**

### **2.2.1. Collection of plant materials:**

Plants selected through the ethnopharmacological survey were collected from various parts of Bangladesh including Bangladesh National Botanical Garden (Mirpur, Dhaka) and Medicinal Plant Garden of Govt. Unani and Ayurvedic Hospital (Mirpur, Dhaka) and Sunderban Jungle (Khulna), Rajshahi University Botanical Garden (Rajshahi). The voucher specimens of these plant materials were submitted to the Bangladesh National Herbarium, Mirpur, Dhaka where the plants were identified by a qualified taxonomist.

### **2.2.2 Drying and grinding:**

All plant materials collected for this study were sun dried (35-40°C) for 2-3 days in Bangladesh. The plant materials included barks, stems, leaves or fruits. After bringing these plant materials to the UK, these were processed by cutting into small pieces with a plant pruner. Such plant materials were further dried in an oven at a temperature of 30-40°C for 30 minutes prior to grinding with a grinder. Subsequently, all plant materials were ground into fine powders to get them ready for extraction.

### **2.2.3. Preliminary extraction and bioassay:**

Extraction is a technique to separate a solute by dissolving in a solvent (Tissue, 1996). There are two types of extraction: hot extraction and cold extraction. In this study the hot extraction was carried out at 35-45°C using a Soxhlet apparatus (Adnan, 2015). Approximately 10-20 g of plant material was extracted sequentially with 80-100 ml solvents of increasing polarity (hexane, chloroform, and methanol) which offer efficient extractions with preliminary distribution of compounds into different extracts depending on their polarity. These extracts were evaporated to dried off using a rotary evaporator at 40°C.

Each of the different extracts was subjected to antibacterial screening by microtiter assay using 96 well plates (Rahman et al., 2008). Based on potential antibacterial activity (Table 3.31) against MRSA clinical strains (EMRSA-15, SA1199B, ATCC5941, XU212 and MRSA340702, MRSA24821), a total of 5 plants were selected for bioassay guided isolation and identification of potential antibacterial secondary metabolites.

#### **2.2.4. Large scale extraction:**

During large scale extraction, each of the plant materials (400-500 gm) was Soxhlet extracted using the identical protocol as described above with solvents (approximately 1 L) of increasing polarity. Similarly, each of the extracts was concentrated using a rotary evaporator under reduced pressure at a maximum temperature of 40°C to afford crude extracts as semi-solid mass.

**Table 2.1: List of the plants collected for the study**

Scientific name	Plant part(s) collected	Place of collection	Date of collection
<i>Paedaria foetida</i>	Leaf	NBG	7 <sup>th</sup> September 2016
<i>Aegle marmelos</i>	Fruit	NBG	8 <sup>th</sup> September 2016
<i>Justicia adhatoda</i>	Leaf	NBG	8 <sup>th</sup> September 2016
<i>Terminalia arjuna</i>	Bark	GUAH	9 <sup>th</sup> September 2016
<i>Diospyros malabarica</i>	Leaf	NBG	9 <sup>th</sup> September 2016
<i>Andrographis periculata</i>	Leaf	NBG	9 <sup>th</sup> September 2016
<i>Tinospora cordata</i>	Leaf	NBG	10 <sup>th</sup> September 2016
<i>Zingiber montanum</i>	Rhizome	NBG	10 <sup>th</sup> September 2016
<i>Azadirachta indica</i>	Bark and Leaf	NBG	11 <sup>th</sup> September 2016
<i>Tylophora indica</i>	Leaf	NBG	11 <sup>th</sup> September 2016
<i>Tribulus terrestris</i>	Thorn	NBG	12 <sup>th</sup> September 2016
<i>Uraria picta</i>	Leaf	RUBG	12 <sup>th</sup> September 2016
<i>Cynometra namiflora</i>	Leaf	SJ	13 <sup>th</sup> September 2016
<i>Swertia chirayita</i>	Leaf and thorn	NBG	13 <sup>th</sup> September 2016
<i>Terminalia chebula</i>	Fruit	NBG	13 <sup>th</sup> September 2016
<i>Glycyrrhiza glabra</i>	Fruit	NBG	13 <sup>th</sup> September 2016
<i>Abroma augusta</i>	Leaf with fruit	GUAH	13 <sup>th</sup> September 2016
<i>Feronia limonia</i>	Bark and Leaf	NBG	13 <sup>th</sup> September 2016

NBG= National Botanical Garden

GUAH= Govt. Unani and Ayurvedic Hospital

RUBG = Rajshahi University Botanical Garden

SJ= Sunderban Jungle

### **2.2.5 Antibacterial screening:**

Detailed methodology of antibacterial screening has been mentioned in section 2.2.9.

## **2.2.6. Chromatographic technique for the isolation of compounds:**

Various chromatographic techniques were used to isolate the compounds from the crude extracts which exhibited good antibacterial activity (MIC values equal to or below 256 µg/ml). Both traditional and modern chromatographic techniques have been applied during this study. The particular method was selected based on the type of compounds present in the crude extracts and/or fractions. Each of these techniques are discussed briefly.

### **2.2.6.1 Thin Layer Chromatography:**

This is a classical plane chromatographic technique which is based on the principle of moving the mobile phase (single solvent or combination of solvents) through the thin layer of stationary phase (usually silica gel) against the gravitational force. Both analytical and preparative TLC were used in the study.

#### **2.2.6.1.1 Analytical TLC:**

The initial screening of plant extracts or fractions was carried out by analytical TLC. Using a capillary tube, a very small amount of sample was applied to the TLC plate (pre coated silica gel 60 PF254) and placed into the TLC tank containing the mobile phase (Table 2.2). The TLC plate has allowed to run until it almost reached the top of the plate. Once the plate was developed, it was visualised under shortwave UV light (254nm) and longwave UV light (366 nm) followed by spraying with a reagent to identify the presence of compounds in the extracts and/ or fractions. In addition, the purity of isolated compounds was also determined by analytical TLC. The  $R_f$  value of pure compounds were calculated by measuring the distance travel by the compounds to the distance travel by solvents. During this study, various mobile phases (Table 2.2) with different ratios of solvents ranging from non-polar to polar were used.

#### **2.2.6.1.2 PTLC (preparative thin later chromatography):**

PTLC was applied for the separation and final purification of compounds from the fraction. The sample was applied uniformly as long streaks in the sample application zone (2 cm above from the bottom edge of TLC plate) on TLC

aluminium plates (pre coated silica gel 60 PF254). The TLC plates were developed up to the upper edge of the plates with suitable mobile phase. In addition, two or three development techniques were adapted for a better separation of compounds with similar polarity. After development, the plates were taken out of the TLC tank, dried off using hair dryer and observed under both short (254 nm) and long (366 nm) wavelength of UV light. If necessary, a small portion of TLC plates was sprayed by appropriate spray agent (section 2.2.7.2) in order to detect non UV active compounds. The specific compounds were recovered by scraping the sorbent layer from the region of interest with a spatula. The compounds were separated from the sorbent by eluting with chloroform or methanol depending on their solubility. Sometimes, materials recovered from the PTLC may require further purification by other chromatographic techniques. Pure compounds were analysed further for identification and structure elucidation by spectrometry (Sherma and Fried 1987).

**Table 2.2: List of mobile phases used during TLC and PTLC**

Code	Solvents	Ratio
A	Hexane : EtOAc	80: 20
B	Hexane : EtOAc	90:10
C	Hexane : EtOAc	95:5
D	Hexane : EtOAc: ACOH	85:14.5: 0.5
E	Hexane : EtOAc: ACOH	96: 3.5: 0.5
F	Hexane : EtOAc: ACOH	95: 4.5: 0.5
G	Hexane : EtOAc	98: 2
H	Hexane : EtOAc: ACOH	70: 29.5: 0.5
I	Hexane : EtOAc: ACOH	68: 31.5:0.5
J	Hexane : EtOAc: ACOH	64: 35.5: 0.5

### 2.2.6.2 VLC (Vacuum Liquid Chromatography):

VLC is a technique used extensively to fractionate crude extracts obtained with hexane, chloroform or methanol. This technique involves a preparative layer chromatographic separation run as a column, which activates by vacuum. The amount of extract to be fractionated was determined by the size of the column and height of the stationary phase (VLC grade silica; kieselgel 60 PF<sub>254</sub>). Firstly, the column was uniformly packed with VLC grade silica gel, then the sample material which was mixed in column grade silica gel was applied onto the



stationary phase. The column was then eluted with 200 ml of solvents of increasing polarity (Table 2.4 and 2.5). Initially, hexane and ethyl acetate with gradual increment in polarity were used followed by ethyl acetate and methanol of increasing polarity. The eluted fractions were collected in 250ml beaker monitored by TLC. Later the similar fractions were mixed together based on TLC results.

#### **2.2.6.3 Sephadex LH-20 column:**

Gel filtration chromatographic technique was applied followed by VLC fractionation of crude extracts. The glass column was packed with the slurry of sephadex LH-20 which was soaked in the selected solvent (100% hexane, 50% chloroform in hexane or 100% chloroform) half an hour prior to the packing of the column. The separation was achieved according to the molecular size of the compounds. The sample was dissolved in 50:50 hexane and chloroform solvent then applied onto the top of the adsorbent. The column was eluted with 70-100% hexane with 0-30% chloroform followed by 100% chloroform and chloroform and methanol mixture with increasing polarity (Table- 2.4). Finally, the column was washed with 100% methanol to remove all materials so that the sephadex LH20 recovered could be used further.

#### **2.2.6.4 Solid Phase Extraction: (SPE):**

SPE is a simple and rapid chromatographic technique to fractionate small amounts of extracts or VLC fractions prior to further chromatographic analysis such as PTLC. The basic principle of SPE is similar to VLC but SPE was used in smaller scale fractionation or further purification of compounds from the VLC fractions or pooled fractions from sephadex LH20 column chromatography.

The extract or fraction to be analysed by SPE was initially mixed with a small amount of silica and applied onto the top of the commercially available pre-packed column (usually silica as stationary phase). The column was eluted with solvents of increasing polarity using a mixture of hexane and ethyl acetate (Table 2.5). The similar fractions were mixed together and then concentrated with the help of a rotary evaporator.

### **2.2.7. Detection of compounds:**

The UV light and **spray** agent were used to detect the compounds from TLC and PTLC plates.

#### **2.2.7.1 UV light:**

The developed and dried TLC and PTLC plates were placed under UV light in both short (254 nm) and long (366nm) wavelength to detect the compounds which absorb UV light.

#### **2.2.7.2 Spray reagents:**

In this study, 1% vanillin in sulphuric acid was used to detect non-UV active compounds.

##### **1% Vanillin in H<sub>2</sub>SO<sub>4</sub> acid:**

This reagent was made by dissolving 1 g vanillin in 100ml concentrated H<sub>2</sub>SO<sub>4</sub>. This is a general reagent for the detection of various types of compounds. The plates were dried with the help of a dryer to develop a specific colour or range of colours which indicates the presence of the type of compounds. For example- terpene type compounds were detected as bright pink colour while flavonoids as yellow.

**Table 2.3: Extraction of *Zingiber montanum* (242 g), *Uraria picta* (300 g), *Cynometra ramiflora* (262 g), *Diospyros malabarica* (250 g), *Swertia chirayita* (231 g)**

Plant material	Extracts	Amount	% yield
<i>Zingiber montanum</i> (242 g)	Hexane	5.5 g	2.27
	Chloroform	10.2 g	4.21
	Methanol	23.0 g	9.50
<i>Uraria picta</i> (300 g)	Hexane	6.7 g	2.23
	Chloroform	7.7 g	2.56
	Methanol	9.87 g	3.29
<i>Cynometra ramiflora</i>	Hexane	1.33 g	0.51
	Chloroform	1.10 g	0.42
	Methanol	10 g	3.82
<i>Diospyros malabarica</i>	Hexane	6.99 g	2.80
	Chloroform	4 g	1.6
	Methanol	10 g	3.9
<i>Swertia chirayita</i>	Hexane	3.26 g	1.41
	Chloroform	4 g	1.73
	Methanol	10 g	4.32

**Table 2.4: Isolation of compounds from hexane extract of *Zingiber montanum***

Compound code	Initial fractionation	Further fractionation	Further purification procedure	Detection	Amount	R <sub>f</sub> value
MRS-2	CC (over Sephadex LH20) eluted with 50 % Chloroform in hexane	-	PTLC eluted with 15% EtOAc in hexane	Visible under short UV	3 mg	0.51
MRS-3	CC (over Sephadex LH20) eluted with 50 % Chloroform in hexane	-	PTLC eluted with 15% EtOAc in hexane	Bright Yellow with VS	10 mg	0.52
MRS-6	100% CHCl <sub>3</sub>	-	-	Bright pink with VS	39 mg	0.45
ZMH – 4	15% EtOAc in hexane	4% EtOAc in hexane	4% EtOAc in hexane with glacial acetic acid	UV active no colour with VS	7 mg	0.85
ZMH – 5	15% EtOAc in hexane	4% EtOAc in hexane	4% EtOAc in hexane with	Fade with VS	4 mg	0.61

			glacial acetic acid			
ZMH – 8	15% EtOAc in hexane	10% EtOAc in hexane	5% EtOAc in hexane with glacial acetic acid	Dark pink	5 mg	0.5
ZMH – 10	15% EtOAc in hexane	10% EtOAc in hexane	5% EtOAc in hexane with glacial acetic acid	Dark pink	7 mg	0.54

VS= 1% Vanillin in sulphuric acid

**Table 2.5: Isolation of compounds from chloroform extract of *Zingiber montanum* (The starting amount was 242 g)**

Compound code	VLC fraction	SPE/ Sephadex	PTLC	Detection	Amount	R <sub>f</sub> value
HZC 30/ZMH 14	25% EtOAc in hexane	10 % EtOAc in hexane	15 % EtOAc in hexane	Dark pink	4 mg	0.51
HZC 39/ZMH 4	10% EtOAc in hexane	4 % EtOAc in hexane (Sephadex LH-20 Column)	4 % EtOAc in hexane	Yellow spot turned blue with VS	6.5mg	0.55

**Table 2.6: Isolation of compounds from methanol extract of *Uraria picta***

Compound code	VLC fraction	SPE/ Sephadex	PTLC	Detection	Amount	R <sub>f</sub> value
UPM 3	10% EtOAc in Hexane	4% EtOAc in Hexane	5% EtOAc in Hexane	Dark pink with VS	5mg	0.75
UPM 46a	20-40% EtOAc in Hexane	50% chloroform in hexane	10% EtOAc in Hexane	Dark spot under short UV	4 mg	0.69

VS= 1% Vanillin in sulphuric acid

**Table 2.7: Isolation of compounds from Hexane extract of *Cynometra ramiflora***

Compound code	VLC fraction	SPE/ Sephadex	PTLC	Detection	Amount	R <sub>f</sub> value
CRH 9/12	33.3% EtOAc in hexane	-	15% EtOAc in Hexane	Visible in short UV, light violet after spray.	65 mg	0.69
CRH 16	33.3% EtOAc in hexane	-	15% EtOAc in Hexane	Visible after spray dark pink only	6 mg	0.37
CRH 34	60% EtOAc in hexane	17.5 % EtOAc in Hexane	20% EtOAc in Hexane with acetic acid	Dark spot under long UV	3 mg	0.82
CRC 10	-	25% CHCl <sub>3</sub> in hexane (Sephadex LH 20) 4% EtOAc in hexane (SPE fraction)	25% EtOAc in hexane	Visible in short UV	6 mg	0.49

**Table 2.8: Isolation of compounds from Hexane extract of *Diosphyros malabarica***

Compound code	VLC fraction	Sephadex LH 20	PTLC	Detection	Amount	R <sub>f</sub> value
DMH 2	10% EtOAc in Hexane	-	30% EtOAc in Hexane with few drops of Acetic acid	Dark green, Visible in short UV, green after spray.	3 mg	0.68
DMH 3/5	10% EtOAc in Hexane	-	30% EtOAc in Hexane with few drops of Acetic acid	Yellow, visible in short UV, turned dark yellow after spray	7 mg	0.47
DMH 10	60% EtOAc in Hexane	-	30% EtOAc in hexane with acetic acid	Yellow, turned green after spray	2 mg	0.65
DMH 15	6.6% Methanol in EtOAc	-	20% EtOAc in Hexane with Acetic acid	Visible in short UV	4 mg	0.70
DMC 22	10-20% EtOAc in Hexane	-	20% EtOAc in Hexane with Acetic acid	Visible in long UV	6mg	0.68

**Table 2.9: Isolation of compounds from Hexane extract of *Swertia chirayita***

Compound code	VLC fraction	PTLC	Detection	Amount	R <sub>f</sub> value
SCH 1	70% EtOAc in hexane	30% EtOAc in Hexane with few drops of acetic acid	Dark green, visible in short UV, turned green after spray	5 mg	0.75
SCH 2	70% EtOAc in hexane	30% EtOAc in Hexane with few drops of acetic acid	Yellow colour, visible in short UV, turned green after spray	2 mg	0.50
SCH 10	18.88% MeOH in EtOAc	32% EtOAc in hexane with few drops of acetic acid	Light yellow, visible under short UV	5 mg	0.69
SCH 11	18.88% MeOH in EtOAc	32% EtOAc in hexane with few drops of acetic acid	Visible in short UV	2 mg	0.48
SCH 15	0-33.3% EtOAc in methanol	36% EtOAc in hexane with few drops of acetic acid	Visible in short UV	1 mg	0.48

## 2.2.8. Spectroscopic methods:

### 2.2.8.1 Infrared spectroscopy:

Infrared spectrum is usually recorded on high resolution fourier transform IR spectrophotometer. The IR spectra of the isolated compounds was obtained using agilent FT-IR. IR spectra helps in elucidating chemical structure by providing information on the presence of certain functional groups such as – COOH, -OH, CO, -NH<sub>2</sub>, -CONH<sub>2</sub> in the compound.

### 2.2.8.2 Mass spectrometry:

Mass spectrometry is a useful analytical technique which reveals the mass of a molecule and its fragmentation pattern and thereby helps to generate the information about the structure of the molecule. Mass spectrometry complements the information provided by other spectroscopic method (UV, IR, and NMR). High resolution impact mass spectra allows the measuring of the molecular mass of small organic compounds within the accuracy of  $\leq 5$ ppm. Mass spectrometry data was obtained from Liverpool John Moores University (School of Pharmacy) using 6530 Accurate-Mass Q-TOF LC/MS from Agilent technologies.

### 2.2.8.3 NMR (Nuclear Magnetic Resonance) spectroscopy:

NMR is a non-destructive analytic method. **NMR is an analytical chemistry technique use to determine molecular structure, purity or content of the sample.** Samples are normally dissolved in deuterated solvents such as CDCl<sub>3</sub>, CD<sub>3</sub>OD etc. and placed into NMR spectrophotometer for both 1D and 2D NMR spectrum. Once all the experiments were finished, the samples were recovered (100% recovery), dried off with nitrogen gas and used for further analysis and bioassay. Proton magnetic resonance (<sup>1</sup>H NMR) spectra of pure compounds were obtained from Bruker AMX (500 MHz) NMR spectrometer in Queen Mary University of London. The <sup>13</sup>C and 2D NMR (COSY, NOESY, HMQC and HMBC) experiments were measured on the Bruker 500 MHz at UCL School of Pharmacy and Bruker 600 MHz and 700 MHz at UCL Department of Chemistry.



In this study NMR spectra was comprehensively used to characterize the isolated compounds and thus identify the chemical structures.  $^1\text{H}$  and  $^{13}\text{C}$  NMR helps to determine the presence of number of hydrogens and carbons respectively as well as their environment.

The 2D NMR spectroscopy facilitated unequivocal assignment of organic compounds.

#### **2.2.8.3.1 $^1\text{H}$ NMR spectroscopy:**

The presence of hydrogen was determined by proton NMR spectroscopy which is a first and fundamental experiment in elucidating the structure of isolated compounds. The number of NMR signals equals the number of different types of proton present in the compound. The position of signal denoted as chemical shift.

Protons in different environment give different signals. NMR spectrum provides the following basic information about the hydrogen present in a compound.

- Number of signal
- Chemical shift
- Intensity of signals ( how many protons in each signal)
- Spin- spin splitting of signal

The  $^1\text{H}$  NMR spectrum of MRSH-3 isolated from hexane extract of *Zingiber montanum* has shown in result section (figure 3.30). The sample was run in  $\text{CDCl}_3$  and solvent peak was calibrated at 7.26 ppm. The structure provides information about the hydrogen atom in the compound thus helps in its structure elucidation.

#### **2.2.8.3.2 $^{13}\text{C}$ NMR spectroscopy**

The  $^{13}\text{C}$  NMR identifies carbon atom in a different environment within the molecule. Each peak in  $^{13}\text{C}$  NMR spectra represent a carbon atom present in the compound. The  $^{13}\text{C}$  NMR spectra is usually single line spectra. The chemical shift is 0-230 ppm giving greater spectral dispersion. Unlike  $^1\text{H}$  NMR,  $^{13}\text{C}$  peak areas

are not related to number of carbons having the same shift. The carbon attached to the hydrogen in methyl group (CH<sub>3</sub>) gives the signal from 0-30 ppm. Carbon atoms are affected by electronegative atoms thus the carbon ranges from 160-180ppm gives the indication that carbon is attached to oxygen.

#### **2.2.8.3.3 Distortionless enhancement by polarisation transfer- 135 (DEPT-135)**

DEPT spectra separate fully carbon spectra differentiate into primary, secondary and tertiary carbon. The missing signal for quaternary carbon helps to separate C and CH<sub>2</sub> peaks in <sup>13</sup>C experiment. So it is useful NMR spectra for elucidating terpenoids in confirming CH<sub>2</sub> peaks.

#### **2.2.8.3.4 <sup>1</sup>H-<sup>1</sup>H correlation spectroscopy: (<sup>1</sup>H-<sup>1</sup>H COSY)**

The <sup>1</sup>H-<sup>1</sup>H COSY is two dimension homonuclear resonances which provides the information about protons that are coupled to each other. The COSY helps to identify the correlation in a complex structure that would be hidden in the clutter of peaks close to the diagonal (Derome, 1989)

#### **2.2.8.3.5 NOESY (Nuclear Overhauser Effect Spectroscopy):**

It is a very useful NMR spectrum for identifying dipolar interaction of spins for correlation of protons. It is similar to COSY spectrum but the cross peaks reveal interaction through space instead of scalar coupling through bonds.

#### **2.2.8.3.6 Heteronuclear correlation spectroscopy:**

**HMQC:** HMQC is the two dimension heteronuclear NMR spectroscopy which add some new feature in NMR to facilitate the structure elucidation of larger compounds. HMQC shows the direct correlation between hydrogen and carbon in the compound.

**HMBC:** The heteronuclear multiple bond coherence (HMBC) shows a correlation between

$^1\text{H}$  and  $^{13}\text{C}$  nuclei through 2, 3 or sometimes even 4. It confirms the structure by connecting various fragments through carbon-proton interactions through 2, 3 bonds in a molecule.

### **2.2.9. Antibacterial screening:**

Antibacterial screening can be accomplished in two ways; agar disc diffusion method and microtiter broth dilution assay (Peter, 1999). During this study, microtiter broth dilution method was followed for antimicrobial screening as adapted by leading antimicrobial groups (Appendino et al., 2008; Garvey et al., 2011; Rahman et al., 2007; Piddock et al., 2010; Rahman and Gibbons 2015; Shiu et al., 2012) which gave minimum inhibitory concentrations (MICs) of the extracts, fractions as well as pure compounds. Briefly, this method involves three days experiments- sub-subculture (day 1), assay (day 2) and observation of result (day 3).

#### **2.2.9.1 Bacterial strains subculture:**

A subculture is simply the transfer of a loopful of microorganism onto slope/ petri dish containing an appropriate medium followed by incubation at  $37^\circ\text{C}$  for approximately 18 hrs. Six clinical strains of MRSA (ATCC 5941, EMRSA 15, XU212, and MRSA340702, MRSA24821) were available. These bacterial strains were regularly monitored and sub cultured in order to keep the organism alive. A back up batch made up to maintain at  $-80^\circ\text{C}$ . Moreover, MRSA culture were regularly collected from UCL School of Pharmacy to avoid any unexpected problems with the clinical strains (Appendino et al., 2008; Shiu et al., 2012).

#### **2.2.9.2 MIC (Minimum Inhibitory Concentration) assay:**

Antimicrobial screening was conducted by MIC assay. 100  $\mu\text{l}$  of MHB was dispensed into 96 well micro titre plates using multi-channel pipette. Crude extracts, fractions, pure compounds and norfloxacin (a standard antibiotic) was diluted serially into every two rows of the first column. The content of the first column were mixed circumstantially by multi-channel pipette by switching 100  $\mu\text{l}$

of the contents to each column till 10<sup>th</sup> and 12<sup>th</sup> column and leaving 11<sup>th</sup> column for growth control. Afterwards, 100µl of diluted bacterial strains were pipetted to all wells of the plate upto 11<sup>th</sup> column, leaving 12<sup>th</sup> column for sterility control. The plates were incubated for 18 hours at 37°C.

### **2.2.9.3 Observation of results**

The MIC records the lowest concentration at which no growth was observed. On the third day of the experiment, 20µl of a 5 mg/ml methanolic solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-phenyltetrazolium bromide (MTT; Sigma) was added to each of the wells followed by incubation for 30 minutes at 37°C. A blue colouration indicates bacterial growth as MTT combines with bacterial mitochondria and forms a blue coloured complex whereas yellow colour (colour of MTT) was the indicator of antibacterial activity. These blue and yellow colour also observed in growth control (11<sup>th</sup> column in 96 well plate) and sterility control (last column in 96 well plate) (Shiu et al., 2012).

# Chapter 3: Results

### **3. Results**

#### **3.1 Ethnopharmacological survey:**

##### **3.1.1 Demographic data analysis:**

The data gathered through the ethnopharmacological survey carried out in Bangladesh was analysed based on the demographic variables such as gender, age, educational background, experience of the practitioners and occupation (Table 3.1). Among a total of 127 participants interviewed 55.91% of the major informants were male. A significant association ( $p < 0.05$ ) was observed in some of the demographic variables such as age, sex, educational background and profession. A particularly significant association ( $p < 0.01$ ) was recorded for the age group of male and female users compared to experience of the practitioners ( $p > 0.05$ ). However, no clear cut trend was observed in terms of age group and plant users. Most of the participants were young and fell within the age group of 25-35 yr (33.86%,  $n=127$ ) followed by the age range of 36-45 yr (23.62%,  $n=127$ ) and then 66-75 yr (21.26%  $n=127$ ). Surprisingly participants in the age range of 56-65 yr (10.24%) were the least plant users. Most of the participants were young because the majority of the interviewees were qualified Ayurvedic practitioners (71) between the ages of 25-35 compared to local people and Ayurvedic patients. Apart from qualified practitioners, local village people and patients from two different Ayurvedic hospitals claimed to have acquired ethnobotanical knowledge from their ancestors stating that botanical remedies were part of their culture. The literacy level of the participants was higher (40.94%) with 3 practitioners having higher degree qualifications (2 practitioners with PhD degree and 1 with MPhil degree). It is interesting to note that no interactions of herbal remedies with orthodox medicine were reported by any of the participants. In terms of empirical knowledge, 29.6% of practitioners had at

least 6-10 years' experience followed by 18.3% with 21-30 years of experience of treating patients with herbal medicine.

**Table 3.1: Classification of the informants, demographic data.**

Variable	Frequency	% Frequency	Chi-square value
<b>Informant category</b>			
Qualified practitioners	71	55.90%	
Ayurvedic patients	21	16.56%	
Local village people	35	27.56%	
<b>Gender</b>			
Female	56	44.09%	
Male	71	55.91%	
<b>Age-group (in years)</b>			
25-35	43	33.86%	0.000231 (< 0.01)
36-45	30	23.62%	
46-55	14	11.02%	
56-65	13	10.24%	
66-75	27	21.26%	
<b>Educational background</b>			
No education	22	17.32%	0.043081 (< 0.05)
Completed 8 years of education	12	9.45%	
Completed 10 years of education	9	7.09%	
Completed 12 years of education	11	8.66%	
Diploma	12	9.45%	
Bachelor or honours degree	52	40.94%	
Master's degree	4	3.15%	
Mphil	3	2.36%	
PhD degree	2	1.58%	
<b>Years of experience as practitioner</b>			
5 years	11	15.49%	0.169662 (> 0.05)
6-10 years	21	29.58%	
11-15 years	10	14.08%	
16-20 years	10	14.08%	
21-30 years	13	18.31%	
<b>Profession (practitioner only)</b>			
Hakim	5	3.94%	0.004628 (<0.05)
Unani	17	23.94%	
Ayurvedic	26	20.47%	
Medical officer of ayurvedic hospital	16	12.59%	
Other for inhabitants and patients (housewife, farmer, teacher or work in another field)	54	42.52%	

### 3.1.2 Graphical analysis of the frequency of the use of plant families

A total 71 medicinal plants were distributed in 54 families to manage five common infectious diseases namely chronic dysentery, urinary tract infection, and respiratory tract infection, wound healing and skin disease. Legumes, Asteraceae and Apocynaceae are the highest represented families followed by Lamiaceae, menispermaceae and Zingiberaceae in terms of families where the plants recommended belongs to.

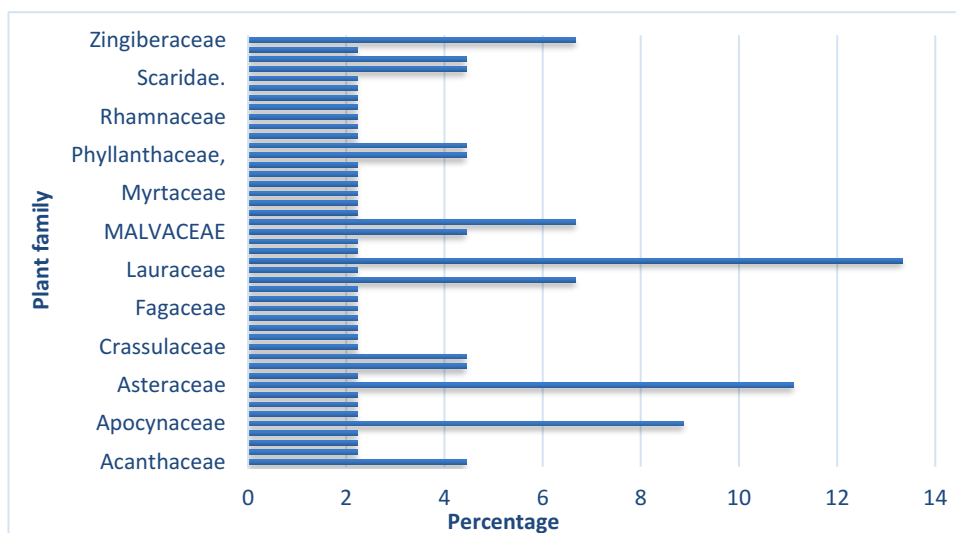


Figure 3.1: Frequency of the use of plant families in management of bacterial infection in Dhaka city and northern part of Bangladesh.

### 3.1.3 Informant consensus factor (FIC) for each category of ailments:

The agreement for the use of plants to treat particular ailment categories among the participants was determined by calculating the FIC value. Table 3.2 illustrates informants' consensus factor which varied from 0-0.94. Bacterial pneumonia recorded the highest FIC value 0.94 with 19 use reports for 2 plant species. *Ocimum tenuiflorum* was the species responsible for the highest consensus factor with 18 out of 19 reported events, followed by wound healing ( FIC - 0.85 ; 49 use reports, 8 species), gastrointestinal disorder (FIC - 0.83, 59 use reports, 11 species), rheumatic fever ( FIC - 0.83; 47 use reports , 9 species). A high FIC value indicated the efficiency of treating a particular ailment with medicinal plants. Bacterial pneumonia had the highest FIC value which confirmed that this ailment was common in the study area probably due to a weakened immune system caused by malnutrition. Moreover, there was good communication present among the informants for treating this ailment category.



**Table 3.2: Informant consensus factor (FIC) for each category of ailments:**

<b>Ailment category</b>	<b>Number of taxa (Nt)</b>	<b>Number of use reports (Nur)</b>	<b>Consensus factor</b>
<b>Bacterial pneumonia infection</b>	2	19	0.94
<b>Wound healing</b>	8	49	0.85
<b>Gastrointestinal disorder</b>	11	59	0.83
<b>Rheumatic fever</b>	9	47	0.83
<b>Gonorrhoea</b>	7	20	0.68
<b>Sinusitis</b>	3	7	0.67
<b>Liver disease</b>	3	7	0.67
<b>Dermatitis/ skin disease</b>	8	20	0.63
<b>Urinary tract infection</b>	13	31	0.60
<b>Respiratory tract infection</b>	4	7	0.50
<b>Tonsillitis</b>	3	5	0.50
<b>Chronic dysentery</b>	8	14	0.46

#### **3.1.4 Fidelity level:**

Fidelity level was calculated to determine the relative healing potential of a plant (section 2.1.5). **Fidelity level provides the information about the percentage of informants that use the certain plant for the same particular purpose.** Most of the plants in a particular disease category scored 100% but *Justicia adhatoda* scored the least (25%) compared to other plant species. Moreover, Rank Order Priority (ROP), number of citations, Used value (UV) and rarity score have been determined below.

**Table 3.3: Fidelity level (FL), rank order priority (ROP), number of citation, Use value (UV) and rarity score of the reported plants have been documented below**

Scientific name	Ailment category	Principle use	l <sub>p</sub>	l <sub>u</sub>	FL Value (%)	RP	ROP	No. of citation	UV value	Rarity score
<i>Andrographis periculata</i>	An injury to the surface of the skin	Wound	3	7	42.9	0.30	12.87	7	0.055	3
<i>Justicia adhatoda</i>	Disease of common cold	Respiratory tract infection	4	16	25	0.69	17.25	16	0.126	3
<i>Amaranthus spinosus</i>	Disease of the urinary tract	Urine infection	2	2	100	0.09	9	2	0.015	2
<i>Allium Sativum</i>	Disease of the arthritis	Gout	2	7	28.6	0.3	8.58	7	0.055	3
<i>Pistacia integerrima</i>	Disease of the respiratory system	Respiratory infection	2	2	100.0	0.09	9	2	0.015	2
<i>Tylophora indica</i>	An injury to the surface of the skin	Wound	3	7	42.9	0.3	12.87	7	0.055	3
<i>Holarrhena antidysenterica</i>	Disease of the digestive system	chronic dysentery	2	2	100.0	0.09	9	2	0.015	2
<i>Alstonia scholaris</i>	Disease of viral/bacterial infection	Fever	1	1	100.0	0.04	4	1	0.007	3
<i>Uraria picta</i>	Disease of the respiratory system	Respiratory tract infection	5	8	62.5	0.35	21.88	8	0.062	2
<i>Cuminum cyminum</i>	Disease of the digestive system	Diarrhoea	6	6	100.0	0.26	26	6	0.047	3
<i>Aloe vera</i>	An injury to the surface of the skin	Wound	2	11	18.18	0.48	8.73	11	0.086	3
<i>Acmella oleracea</i>	Disease of the digestive system	Dysentery, Malaria, Plague	2	2	100	0.09	9	2	0.016	1
<i>Tagetes erecta</i>	An injury to the surface of the skin	Wound	2	2	100.0	0.09	9	2	0.0157	3
<i>Eupatorium odoretum</i>	Disease of blood	Haemostatic,	1	1	100.0	0.04	4	1	0.007	3

<i>Artemisia absinthium</i>	Disease of liver	Hepatitis	1	1	100.0	0.04	4	1	0.007	1
<i>Sphaeranthus indicus</i>	Diseases of the genitourinary system	Skin disease. Gonorrhoea	1	1	100.0	0.04	4	1	0.007	2
<i>Musa acuminata</i>	Disease of the digestive system	Diarrhoea	6	6	100.0	0.26	26	6	0.047	3
<i>Ceiba speciosa</i>	Disease of the respiratory system	Tonsillitis,	1	1	100.0	0.04	4	1	0.007	3
<i>Bombex saiba</i>	Disease of the digestive system	Chronic dysentery	1	1	100.0	0.04	4	1	0.007	3
<i>Terminalia arjuna</i>	An injury to the surface of the skin	wound	3	9	33.3	0.39	12.99	9	0.071	3
<i>Terminalia chebula</i>	Disease of the digestive system	Diarrhoea	3	7	42.9	0.30	12.87	7	0.055	3
<i>Kalanchoe pinnata</i>	Disease of bladder	Urinary tract infection,	10	12	83.3	0.52	43.32	12	0.094	3
<i>Lagenaria siceraria</i>	Disease of digestive system	Cholera	7	9	77.7	0.39	30.30	9	0.070	3
<i>Vateria indica</i>	Disease of digestive system	Duodenal ulcer	1	1	100.0	0.04	4	1	0.007	3
<i>Chrozophora prostrata</i>	Disease of bladder	Urinary tract infection,	1	1	100.0	0.04	4	1	0.007	3
<i>Quercus infectoria</i>	Disease of digestive system	Chronic dysentery	2	3	66.6	0.13	8.66	3	0.023	3
<i>Fumaria officinalis/ indica</i>	Disease of bladder	Urinary tract Infection	2	2	100	0.09	9	2	0.015	1
<i>Swertia chirota</i>	An injury to the surface of the skin	Wound	3	7	42.9	0.3	12.87	7	0.055	1
<i>Ocimum tenuiflorum</i>	Inflammation in the lung	Bacterial Pneumonia,	8	18	44.4	0.78	34.63	18	0.141	3
<i>Menthe piperica</i>	Disease of the digestive system	Gastroenteritis	2	4	50	0.17	8.5	4	0.0314	3
<i>Vitex negunda</i>	Disease of the respiratory system	Respiratory tract infection	2	3	66.6	0.13	8.66	3	0.023	2

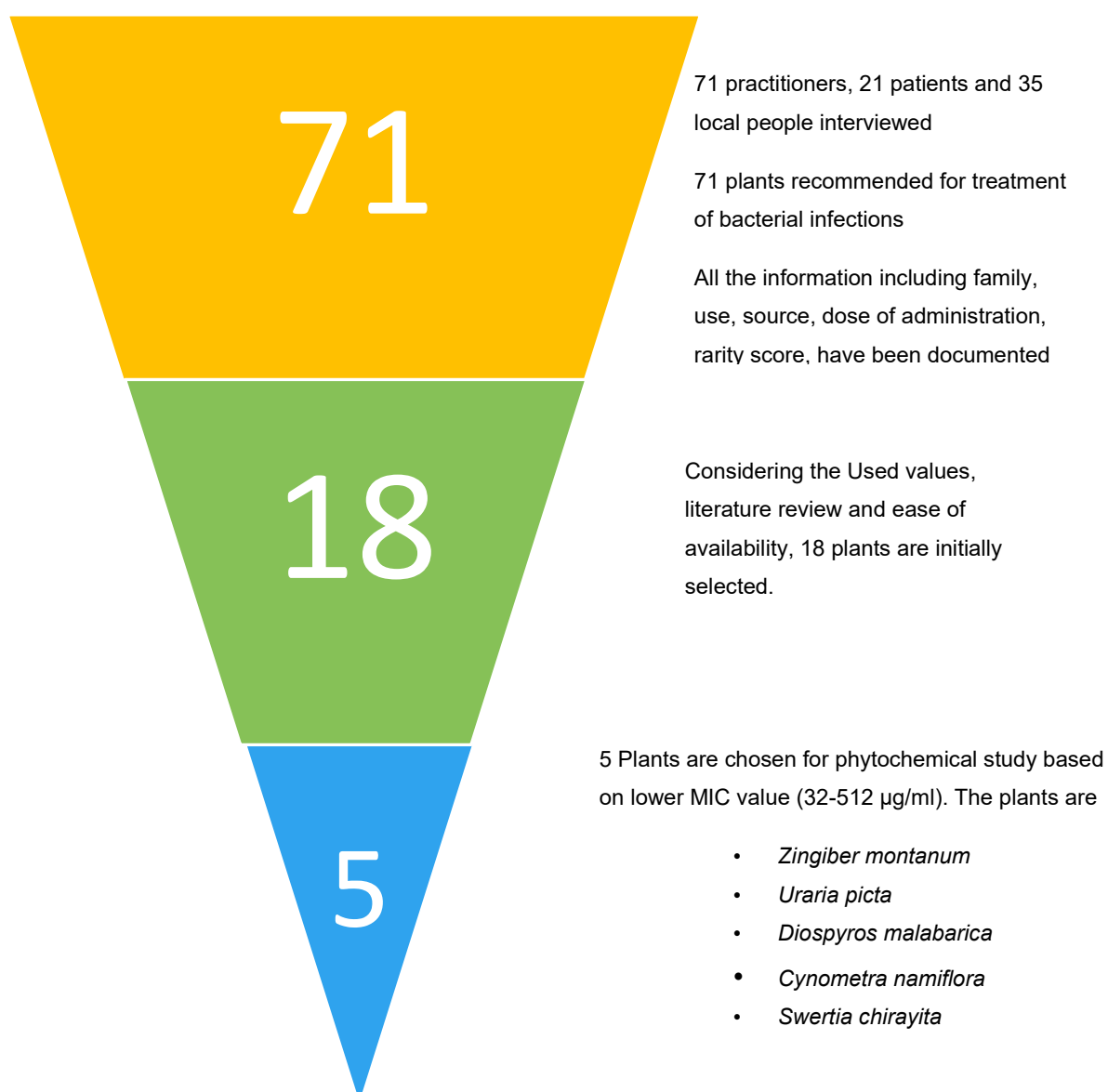
<i>Lavendula stoechas</i>	Disease of infectious sinus	Sinusitis	1	1	100.0	0.04	4	1	0.007	1
<i>Glycyrrhiza glabra</i>	Disease of the digestive system	Gastric Ulcer	3	7	42.9	0.3	12.87	7	0.055	3
<i>Cassia fistula</i>	Disease of liver	Hepatitis	1	1	100.0	0.04	4	1	0.007	3
<i>Cassia tora</i>	Disease of skin,	Eczema	2	2	100.0	0.09	9	2	0.0157	3
<i>Cassia angustifolia</i>	Disease of genitourinary system	Urinary tract infection, syphilis, gonorrhoea	1	1	100.0	0.04	4	1	0.007	3
<i>Saraca indica</i>	Disease of liver	Jaundice	1	1	100.0	0.04	4	1	0.007	3
<i>Cynometra ramiflora</i>	Disease of skin	Skin disease	3	7	42.9	0.3	12.87	7	0.055	2
<i>Lawsonia intermis</i>	Disease of bladder	Urinary tract infection,	1	3	33.3	0.13	4.33	3	0.023	3
<i>Abroma augusta</i>	Disease of bladder	Urinary tract infection	5	8	62.5	0.35	21.88	8	0.062	3
<i>Hibiscus rosa-sinensis</i>	Disease of digestive system	Dysentery	1	1	100.0	0.04	4	1	0.007	3
<i>Trinospora cordata</i>	Diseases of the genitourinary system	Gonorrhoea	3	6	50	0.26	13	6	0.047	3
<i>Cissampelos pareita</i>	An injury to the surface of the skin	Wound	1	1	100.0	0.04	4	1	0.007	3
<i>Tinospora cordifolia</i>	Disease of digestive system	Chronic dysentery	1	1	100.0	0.04	4	1	0.007	3
<i>Azadirachta indica</i>	An injury to the surface of the skin	wound	8	23	34.8	1	34.8	23	0.181	3
<i>Ficus heterophylla</i>	Disease of the skin	Boils	1	1	100.0	0.04	4	1	0.007	3
<i>Psidium guava</i>	Disease of respiratory disease	Tonsillitis, ear, throat, nose Infection	3	3	100.0	0.13	13	3	0.023	3

<i>Boerhaavia diffusa</i>	Disease of bladder	Urinary tract infection	1	1	100.0	0.04	4	1	0.007	3
<i>Nymphaea nouchali</i>	Disease of blood	Stops too much bleeding externally	1	1	100.0	0.04	4	1	0.007	3
<i>Nyctanthes arbor-tristis</i>	viral or bacterial infection	Fever	1	1	100.0	0.04	4	1	0.007	3
<i>Phyllanthus ninuni</i>	Disease of the cirrhosis of the liver	Jaundice	1	1	100.0	0.04	4	1	0.007	2
<i>Piper betle</i>	Disease of the skin	Skin disease	2	2	100.0	0.09	9	2	0.015	3
<i>Peperomia pellucida</i>	Disease of the bladder	Urinary tract infection, Gall bladder infection, Cystitis	1	1	100.0	0.04	4	1	0.007	2
<i>Cynodon dactylon</i>	An injury to the surface of the skin	Wound	6	12	50	0.52	26	12	0.094	3
<i>Poetuleca grantifolia</i>	Disease of metabolic disorder	Osteoporosis,	1	1	100.0	0.04	4	1	0.007	3
<i>Nigella sativa</i>	Bacterial infection	Rheumatic fever	1	1	100.0	0.04	4	1	0.007	3
<i>Ziziphus jujuba</i>	Disease of respiratory system	Respiratory tract infection	1	1	100.0	0.04	4	1	0.007	
<i>Neolamarckia cadamba</i>	Viral or bacterial infection	Dengue fever	1	1	100.0	0.04	4	1	0.007	3
<i>Paederia foetida</i>	Disease of digestive system	Chronic diarrhoea	3	6	50	0.26	13	6	0.047	3
<i>Aegle marmelos</i>	Disease of digestive system	Any stomach related disease	4	8	50.0	0.35	17.5	8	0.062	3
<i>Feronia limonia</i>	Disease of digestive system	Diarrhoea	2	9	22.2	0.39	8.66	9	0.071	3
<i>Santalum album</i>	Disease of the bladder	Urinary tract infection	3	3	100.0	0.13	13	3	0.023	3

<i>Diospyros malabarica</i>	Disease of the digestive system	Chronic Dysentery	3	6	50	0.26	13	6	0.047	3
<i>Solanum xanthocarpum</i>	Disease of the respiratory	Respiratory tract infection	3	3	100.0	0.13	13	3	0.023	3
<i>Solenanum nigrum</i>	Disease of bladder	Urinary tract infection,	1	1	100.0	0.04	4	1	0.007	1
<i>Centella asiatica</i>	Disease of digestive system	Diarrhoea	5	9	55.5	0.39	21.66	9	0.071	3
<i>Foeniculum vulgare</i>	Diseases of the genitourinary system	Gonorrhoea, Urinary tract infection,	1	2	50	0.09	4.5	2	0.015	3
<i>Viola odorata</i>	Disease of skin	Skin disease	1	1	100.0	0.09	9	1	0.007	3
<i>Curcuma Longa</i>	An injury to the surface of the skin	Wound	5	13	38.46	0.57	21.92	13	0.102	3
<i>Zinziber zerumbet</i>	Disease of the skin	Skin disease	1	1	100.0	0.09	9	1	0.007	2
<i>Zingiber montanum</i>	Disease of digestive system	gastroenteritis	3	6	50	0.26	13	6	0.047	2
<i>Tribulus terrestris</i>	Disease of the bladder	Urinary tract infection.	3	6	50	0.26	13	6	0.047	1

### 3.1.5 Selection of plants

Initially 71 plants recommended by practitioners, patients and local inhabitants, a total of 18 plants were preliminarily selected based on data analysis (used value  $\geq 0.05$ ), literature review (underresearched) and ease of availability to conduct initial bioassay against MRSA strains. Based on the best antibacterial screening results 5 plants were chosen for conducting extensive phytochemical work.



## 3.2 Phytochemical results

### 3.2.1 Simple phenolic compounds

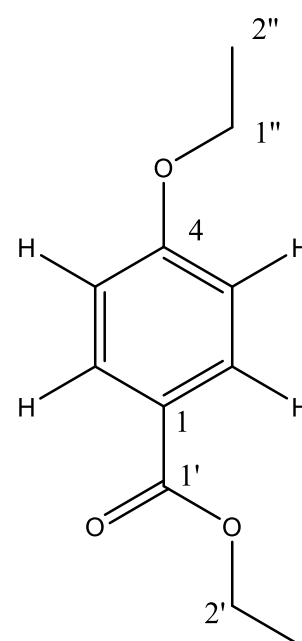
During this study, two simple phenolic compounds CRH 34 and UPM 3 were isolated. CRH 34 was isolated from the hexane extract of the leaf of *Diospyros malabarica* while UPM 3 was isolated from methanol extract of *Uraria picta*.

#### 3.2.1.1 Characterization of CRH 34 as ethyl 4-ethoxy benzoate (1):

The IR spectrum of CRH 34 revealed the presence of carbonyl at  $1714\text{ cm}^{-1}$ . The high resolution mass spectroscopy showed the  $[M+H]^+$  peak at  $m/z$  195.10213 (calculated for  $C_{11}H_{15}O_3$ , at 195.10212) which confirmed the molecular formula of CRH 34 as  $C_{11}H_{14}O_3$ .

The  $^1H$  NMR (700 MHz,  $CDCl_3$ , Table 3.4, Figure 3.2) spectrum of CRH 34 showed two sets of aromatic protons as AA'BB' pattern resonating at  $\delta_H$  7.99 (d,  $J=8.89$  Hz),  $\delta_H$  6.90 (d,  $J=8.89$  Hz), two sets of oxymethylene quadruplets at  $\delta_H$  4.34 ( $J=7.14$  Hz) and 4.09 ( $J=6.93$  Hz) and two sets of methyl protons  $\delta_H$  1.37 (t,  $J=7.12$  Hz) and  $\delta_H$  1.43 (t,  $J=6.93$  Hz). In the COSY experiment, AA'BB' system protons exhibited usual interactions among themselves whilst the methyl and oxymethylene protons showed usual interactions ( $\delta_H$  1.37 to 4.34;  $\delta_H$  1.43 to 4.09) as well.

The  $^{13}C$  NMR (175 MHz,  $CDCl_3$ , Table 3.4; Figure 3.2) spectrum of CRH 34 showed the presence of three quaternary carbons ( $\delta_C$  122.8, 162.8 and 166.5), two methyl carbons ( $\delta_C$  14.5 and 14.9), two oxymethylene carbons ( $\delta_C$  60.9 and 63.7) and two sets of aromatic methine carbons ( $\delta_C$  114.2 and 131.6; each representing two carbons). Assignments of all protons and carbons of the compound were confirmed by 2D NMR experiments, predominately by HMBC experiment. In the HMBC experiment, aromatic protons at  $\delta_H$  7.99 (H-2, 6;  $\delta_C$  131.6 from HSQC) and  $\delta_H$  6.90 (H-3, 5;  $\delta_C$  114.2 from HSQC) showed common interaction with quaternary carbon at  $\delta_C$  162.8 (C-4), H-2,6 and oxymethylene protons at  $\delta_H$  4.34 (H-2';  $\delta_C$  60.9) showed a common  $^3J$  HMBC correlation to an esteric carbonyl group at  $\delta_C$  166.5 ppm (C-1'). H-3,5 showed  $^3J$  HMBC correlation to another quaternary carbon at  $\delta_C$  122.8 (C-1). H-2' also revealed  $^2J$  HMBC connectivity to a methyl carbon at  $\delta_C$  14.5 (C-3';  $\delta_H$  1.37 from HSQC). The other



1



oxymethylene proton at  $\delta_H$  4.09 (H-1'');  $\delta_C$  63.7 from HSQC) showed HMBC connectivity to a methyl at  $\delta_C$  14.9 (C-2'');  $\delta_H$  1.43) by  $^2J$  and to a quaternary carbon at  $\delta_C$  162.8 (C-4) by  $^3J$ .

Therefore, based on these NMR and HRMS data, the compound CRH 34 was identified as ethyl 4-ethoxybenzoate (**1**). Although this compound was synthesized before, this is its first report as a natural product.

**Table 3.4:  $^1H$  (700 MHz) and  $^{13}C$  NMR (175 MHz) and HMBC spectroscopic data CRH 34 in  $CDCl_3$**

Position	$\delta_H$	$^{13}C$	HMBC	
			$^2J$	$^3J$
1	-	122.8		
2, 6	7.99, d, $J = 8.89$ Hz	131.6	114.2 (CH)	162.8 (C), 166.5 (C=O)
3, 5	6.90, d, $J = 8.89$ Hz	114.2	162.8 (C)	122.8 (C)
4	-	162.8	-	-
1'	-	166.5	-	-
2'	4.34, q, $J = 7.14$ Hz	60.9	14.5 (CH <sub>3</sub> )	166.5 (C=O)
3'	1.37, t, $J = 7.12$ Hz	14.5	-	60.9 (CH <sub>2</sub> -O)
1''	4.09, q, $J = 6.93$ Hz	63.7	14.9 (CH <sub>3</sub> )	162.8 (C)
2''	1.43, t, $J = 6.93$ Hz	14.9	63.7 (CH <sub>2</sub> -O)	-

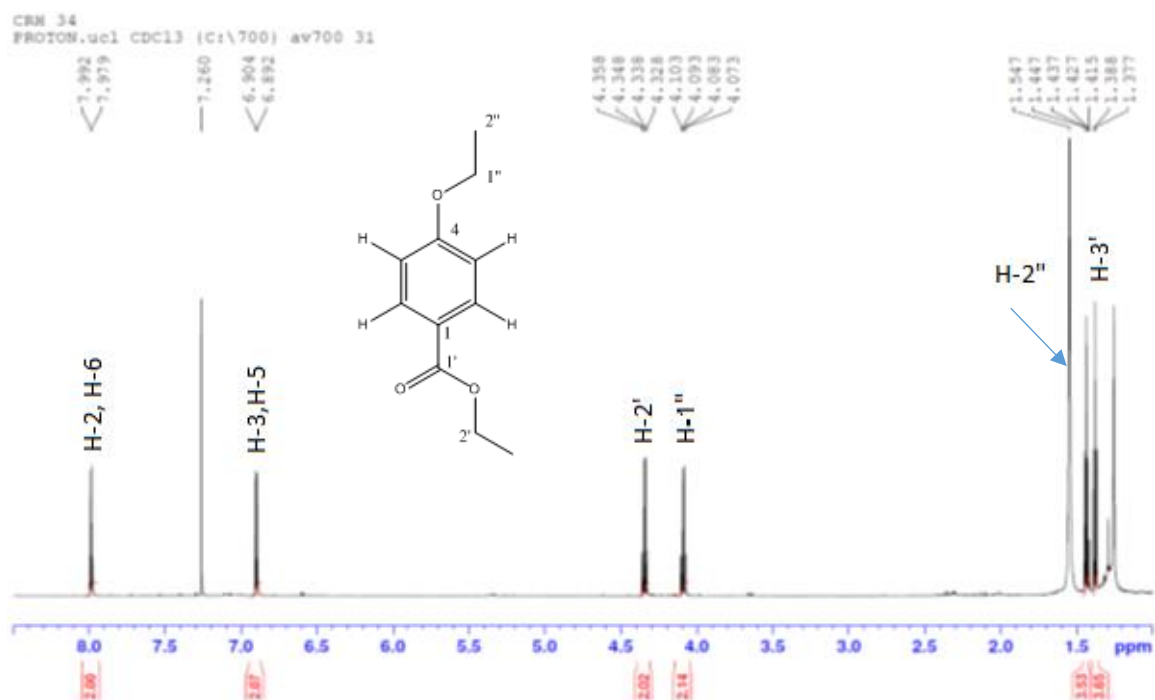


Figure 3.2:  $^1H$  NMR (700 MHz,  $CDCl_3$ ) spectrum of CRH 34

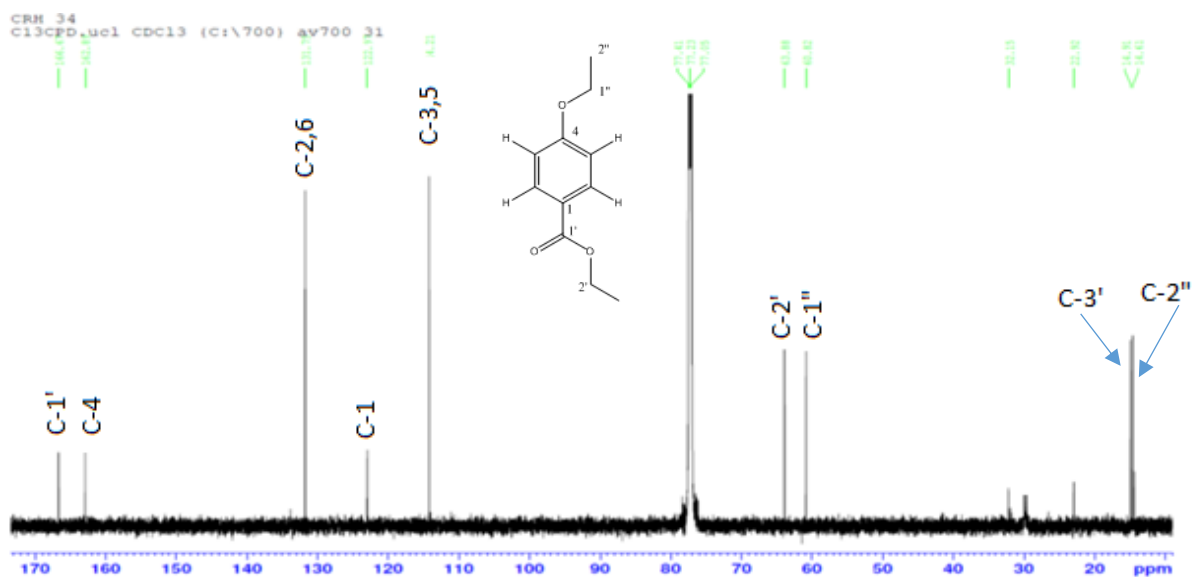


Figure 3.3:  $^{13}\text{C}$  NMR spectrum (175 MHz,  $\text{CDCl}_3$ ) of CRH 34

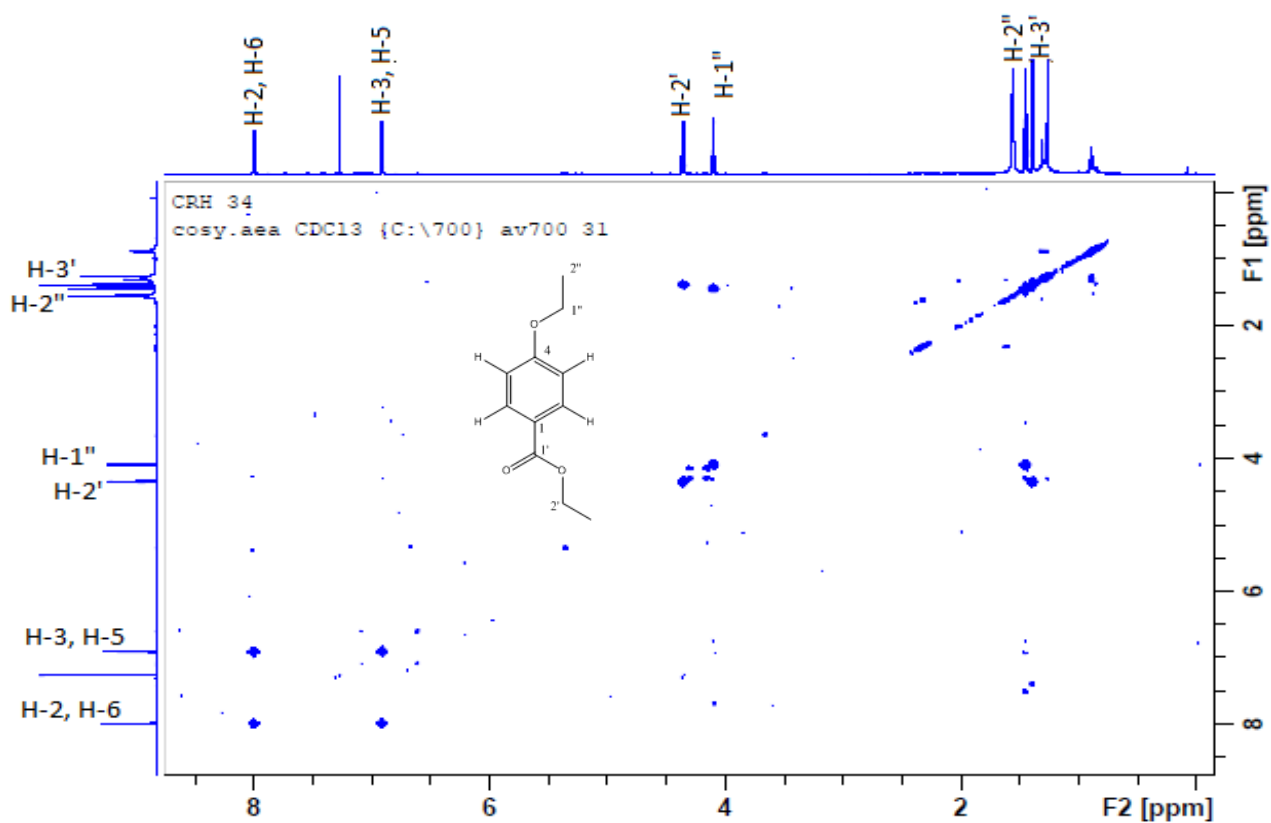


Figure 3.4: COSY NMR spectrum ( $\text{CDCl}_3$ ) of CRH 34

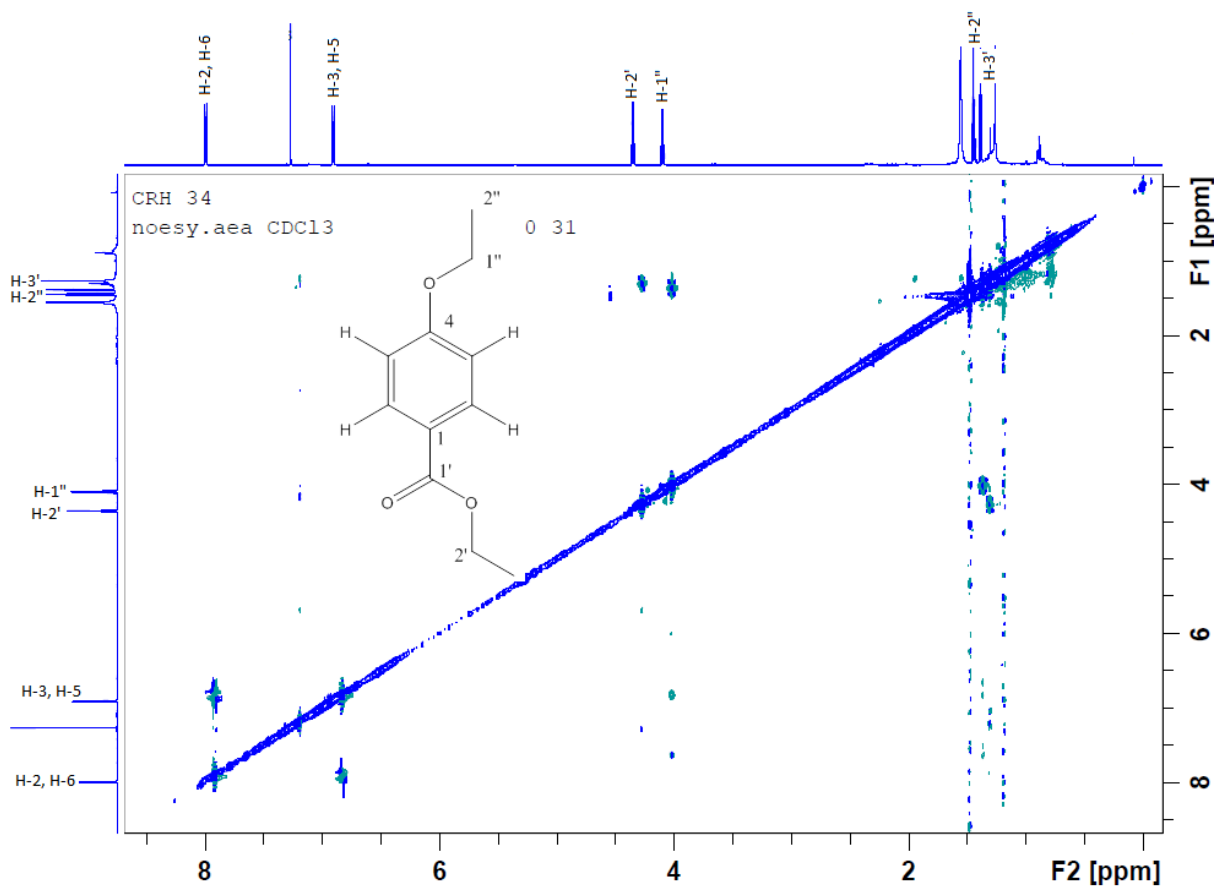


Figure 3.5: NOESY NMR spectrum (CDCl<sub>3</sub>) of CRH 34

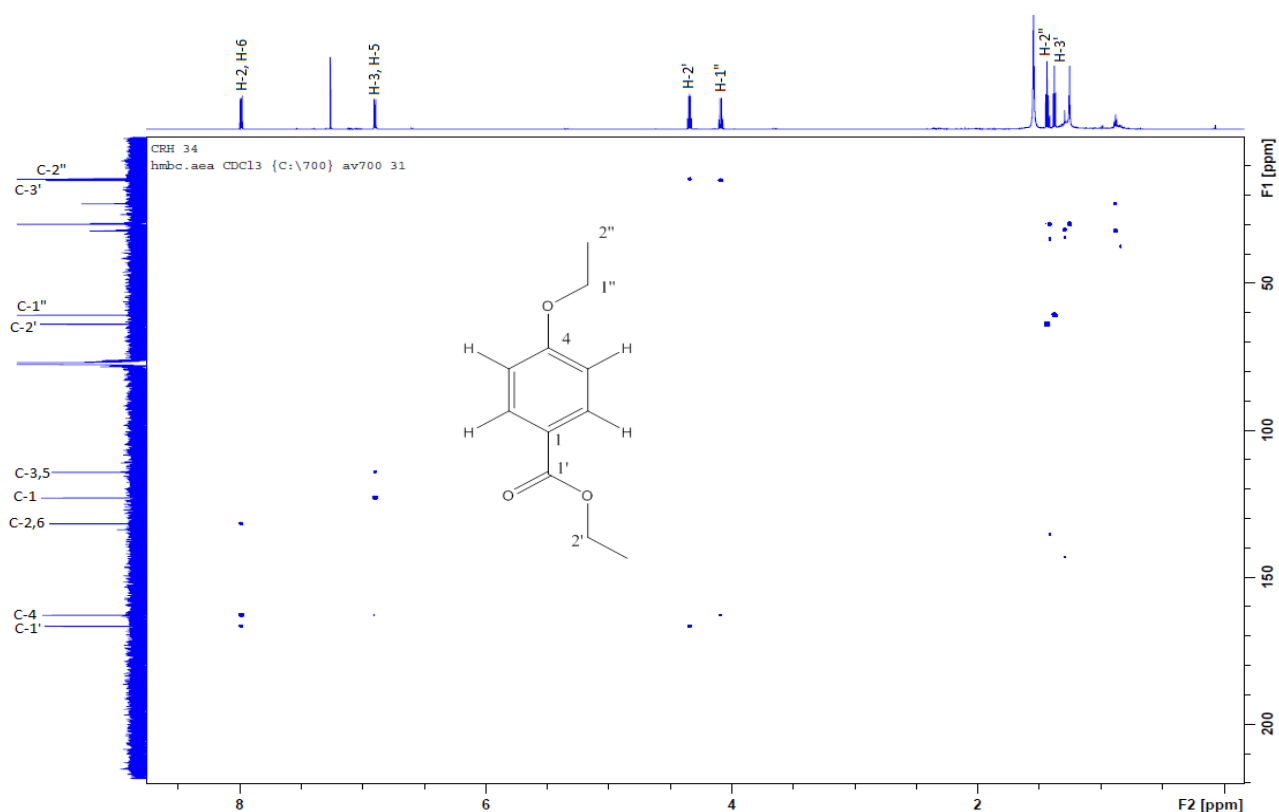
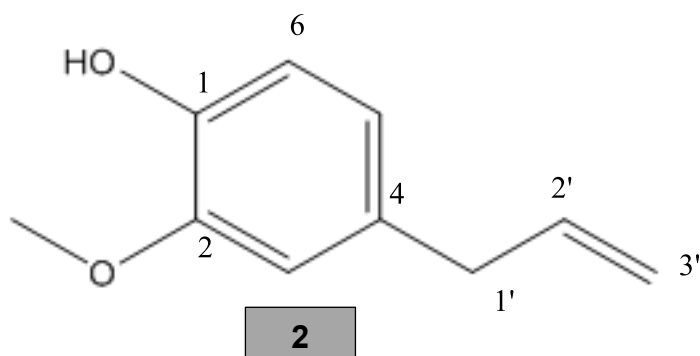


Figure 3.6: HMBC NMR spectrum ( $\text{CDCl}_3$ ) of CRH 34

### 3.2.1.2 Characterization of UPM 3 as Eugenol (4-allyl-2-methoxyphenol; **2**):

The IR spectrum of UPM-3 revealed the presence of hydroxyl group at  $3300\text{cm}^{-1}$  and the presence double bond at  $1677\text{ cm}^{-1}$ . The high resolution mass spectroscopy showed the  $[\text{M}+\text{H}]^+$  at  $m/z$  165.084120 (calculated for  $\text{C}_{10}\text{H}_{13}\text{O}_2$ , at 165.083730) which confirmed the molecular formula of UPM 3 as  $\text{C}_{10}\text{H}_{12}\text{O}_2$ .

The  $^1\text{H}$  NMR spectrum (500 MHz,  $\text{CDCl}_3$ , Table 3.5, Figure 3.7) of UPM 3 showed the presence of three aromatic protons, a methoxyl group, two sets of methylenes and one olefinic methine proton. The downfield singlet at  $\delta_{\text{H}}$  3.87 integrated as three protons was accounted as methoxyl group. The downfield protons at  $\delta_{\text{H}}$  6.69 (2H, *dd*,  $J=3.05$ ) and  $\delta_{\text{H}}$  6.85 (1H, *d*,  $J=7.05$ ) attributed to be ABX pattern aromatic protons which could be assigned as H-2, H-3 and H-5. The proton at  $\delta_{\text{H}}$  5.96 and  $\delta_{\text{H}}$  5.07 ( $J=1.89\text{ Hz}$ ) appeared to be olefinic protons which was part of side chain attached to the aromatic system. A total of 10 carbons were evident from  $^{13}\text{C}$  NMR spectrum (125 MHz,  $\text{CDCl}_3$ , Table 3.5; Figure 3.8)



including an exomethylene, three quaternary carbons two of which were oxygenated. The DEPT135 identified one methoxyl, 2 methylenes and 4 methine carbons.

In the HMBC, the methoxy protons at  $\delta_H$  3.87 ( $\delta_C$  56.0 from HMQC) and aromatic proton at  $\delta_H$  6.85 ( $\delta_C$  111.2 from HMQC) revealed  $^3J$  interaction to an oxygenated quaternary carbon at  $\delta_C$  146.5 (C-2). The protons at  $\delta_H$  6.69 (integrated for 2 protons,  $\delta_C$  121.3 and 113.3) showed  $^3J$  connectivity to methylene carbon at  $\delta_C$  40.0 ( $\delta_H$  3.33 from HMQC) and another quaternary carbon at  $\delta_C$  143.9 (C-1). The exomethylene protons at  $\delta_H$  5.07 ( $\delta_C$  115.7 from HMQC) was to connected methylene at  $\delta_C$  40.0 (C-1') by  $^3J$ . The  $^3J$  connectivity of olefinic proton ( $\delta_H$  5.96) to quaternary carbon at  $\delta_C$  132.0 (C-4) confirmed the connection of allyl side chain to the benzene ring through C-4. Accordingly, the compound was identified as 4-allyl-2methoxyphenol (**2**) commonly known as eugenol which is widely used for toothache and **antiseptic agent** (Sung et al., 2012). It has been isolated for the first time from *Uraria picta*. The NMR spectra were in agreement to those published in literature (Heinrich et al., 2012).

**Table 3.5:  $^1H$  (500 MHz) and  $^{13}C$  (125 MHz) and HMBC NMR spectroscopic data and  $^1H$ - $^{13}C$  correlation for UPM-3 in  $CDCl_3$**

Position	$\delta_H$	$^{13}C$	HMBC	
			$^2J$	$^3J$
1	-	143.9	-	-
2	-	146.5	-	-
3	6.69, dd, ( $J=3.75, 3.05$ Hz)	121.3	146.5 (C)	143.9 (C)
4	-	132.0	-	-
5	6.69, s	111.2	132.0 (C), 114.5 (CH)	143.9 (C), 121.3 (CH)
6	6.85, d, $J= 7.05$ Hz	114.5	143.9 (C), 111.2 (CH)	132.0 (C), 146.5 (C)
1'	3.33, d, $J= 7.02$ Hz	40.0	121.3 (CH), 132.0 (C), 137.9 (CH)	115.7 (CH <sub>2</sub> ), 111.2 (CH)
2'	5.96, m	137.9	132.0 (C)	-
3'	5.07, d, $J=1.89$ Hz	115.7	137.9 (CH)	-
2-OCH <sub>3</sub>	3.87, s	56.0	146.5 (C)	-



Figure 3.7: The <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) spectrum of UPM-3

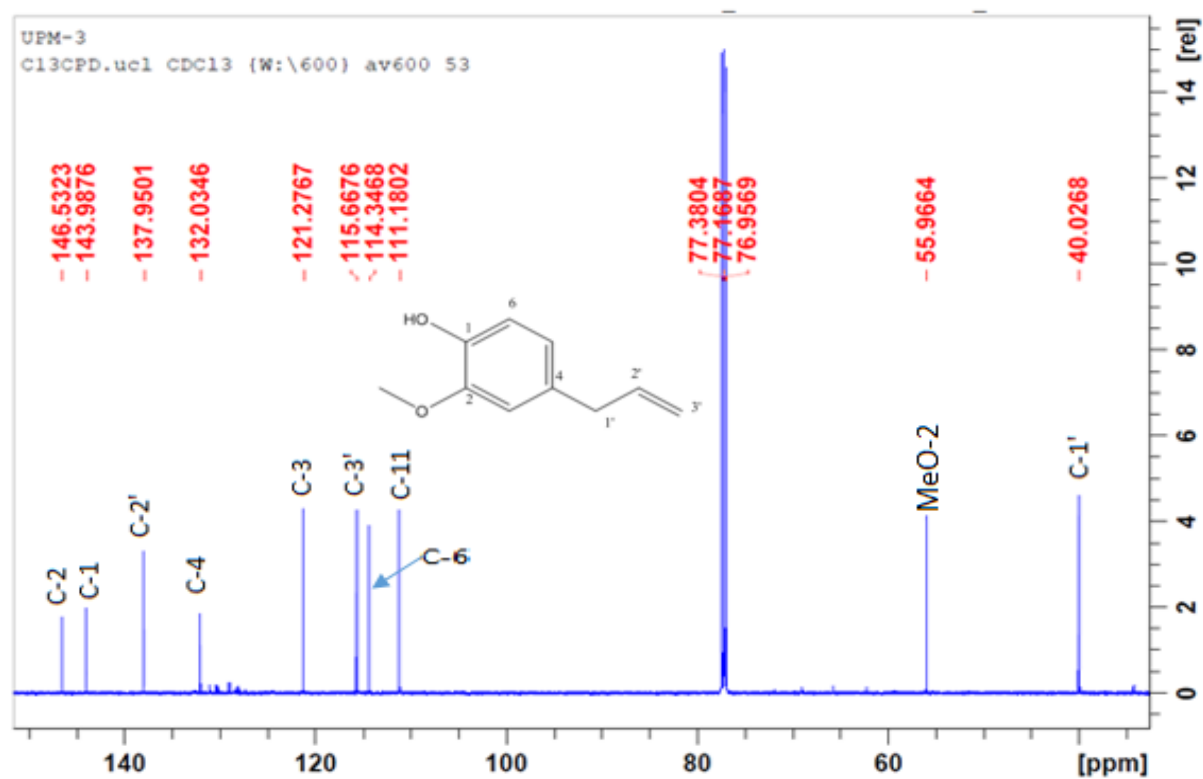


Figure 3.8: The <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>) spectrum of UPM-3

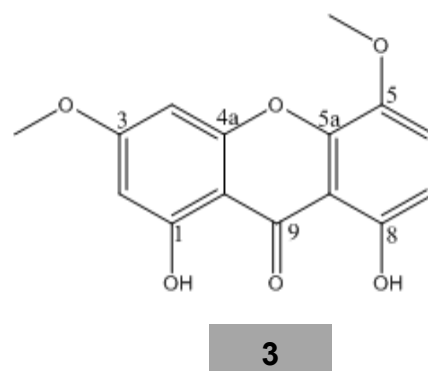
### 3.2.2 Xanthenes:

Xanthenes are known as phytonutrients and contain 3 ring cyclic compounds where two benzene rings are connected through tetrahydropyranone ring which is an important class in medicinal chemistry. Four xanthenes were isolated from the hexane extract of the whole plant of *Swertia chirayita*. These were coded as SCH 1, SCH 2, SCH 10 and SCH 15

#### 3.2.2.1 Characterization of SCH 10 as Swerchirin (3)

The IR spectrum of SCH 10 revealed the presence of carbonyl at  $1742\text{ cm}^{-1}$  and hydroxyl group at  $3013\text{ cm}^{-1}$ . The high resolution mass spectrometry showed the  $[M+H]^+$  at  $m/z$  289.07057 (calculated for  $C_{15}H_{13}O_6$ , at 289.071215) which confirmed the molecular formula of SCH 10 as  $C_{15}H_{12}O_6$ .

The  $^1\text{H}$  NMR spectrum (700 MHz,  $\text{CDCl}_3$ , Table 3.6, Figure 3.9) showed the presence of two methoxyl groups as singlets resonating at  $\delta_{\text{H}}$  3.89 and  $\delta_{\text{H}}$  3.96, two highly deshielded proton singlets at  $\delta_{\text{H}}$  11.99 and  $\delta_{\text{H}}$  11.40 attributed to the presence of two hydrogen bonded hydroxyl groups, two meta coupled ( $J= 2.24$  Hz) aromatic protons at  $\delta_{\text{H}}$  6.55 and  $\delta_{\text{H}}$  6.36 and two ortho coupled ( $J= 9.59$  Hz) protons at  $\delta_{\text{H}}$  7.23 and  $\delta_{\text{H}}$  6.72. The  $^{13}\text{C}$  NMR spectrum (175 MHz,  $\text{CDCl}_3$ , Table 3.7, Figure 3.10) revealed the presence of a total 15 carbons including carbonyl at  $\delta_{\text{C}}$  184.8, two methoxyl carbons at  $\delta_{\text{C}}$  56.2 and  $\delta_{\text{C}}$  57.7.



In the HMBC experiment (Table 3.8, Figure 3.12) the hydrogen bonded hydroxyl proton at  $\delta_{\text{H}}$  11.99 (HO-1) showed  $^2J$  connectivity at a quaternary carbon at  $\delta_{\text{C}}$  163.1 (C-1) and  $^3J$  and correlation with a quaternary carbon at  $\delta_{\text{C}}$  103.1 (C-1a). Methoxy protons at  $\delta_{\text{H}}$  3.89 ( $\delta_{\text{C}}$  56.2 from HSQC) showed  $^3J$  correlation with quaternary carbons at  $\delta_{\text{C}}$  167.7 (C-3) position which confirmed the position of methoxyl at C-3. Two aromatic protons at  $\delta_{\text{H}}$  6.36 (H-2;  $\delta_{\text{C}}$  98.1 from HSQC) and  $\delta_{\text{H}}$  6.55 (H-4;  $\delta_{\text{C}}$  93.3 from HSQC) exhibited  $^2J$  correlation with the quaternary carbon at 167.7 (C-3) and common  $^3J$  correlation with the quaternary carbon at 103.1 (C-1a). The protons at H-2 ( $\delta_{\text{H}}$  6.36;  $\delta_{\text{C}}$  98.1) and H-4 ( $\delta_{\text{H}}$  6.55;  $\delta_{\text{C}}$  93.3) showed  $^3J$  correlation to C-4 ( $\delta_{\text{C}}$  93.3;  $\delta_{\text{H}}$  6.55 from HSQC) and C-2 ( $\delta_{\text{C}}$  98.1;  $\delta_{\text{H}}$  6.36 from HSQC) respectively and thereby confirmed their *meta* position in the

aromatic ring. Another methoxyl proton at  $\delta_{\text{H}}$  3.96 ( $\delta_{\text{C}}$  57.5 from HSQC) showed  $^3J$  correlation with quaternary carbon at  $\delta_{\text{C}}$  140.1 (C-5) which confirmed the methoxyl group connected through C-5. The deshielded aromatic proton at  $\delta_{\text{H}}$  7.23 (H-6;  $\delta_{\text{C}}$  120.6 from HSQC) exhibited  $^2J$  correlation with quaternary carbons at  $\delta_{\text{C}}$  140.1 (C-5) and  $^3J$  correlation with  $\delta_{\text{C}}$  154.4 (C-8) while another aromatic proton at  $\delta_{\text{H}}$  6.72 (H-7;  $\delta_{\text{C}}$  109.6 from HSQC) showed correlation with same quaternary carbons at  $\delta_{\text{C}}$  140.1 by  $^3J$  and  $\delta_{\text{C}}$  154.4 by  $^2J$ . In addition, another highly downfield proton at 11.40 (HO-8) showed  $^2J$  connection with quaternary carbon at  $\delta_{\text{C}}$  154.1 (C-8) and  $^3J$  showed connection with aromatic methine carbon at  $\delta_{\text{C}}$  109.4 (C-7). According to IR, Mass spectrometry and NMR spectral data, the compound SCH 10 was identified as 1,8-dihydroxy-3,5-dimethoxy-9H-xanthen-9-one commonly known as swerchirin (**3**; Bajpai et al., 1991; Saxena et al., 1996). The spectral data were identical to those reported previously (Jia et al., 2011).

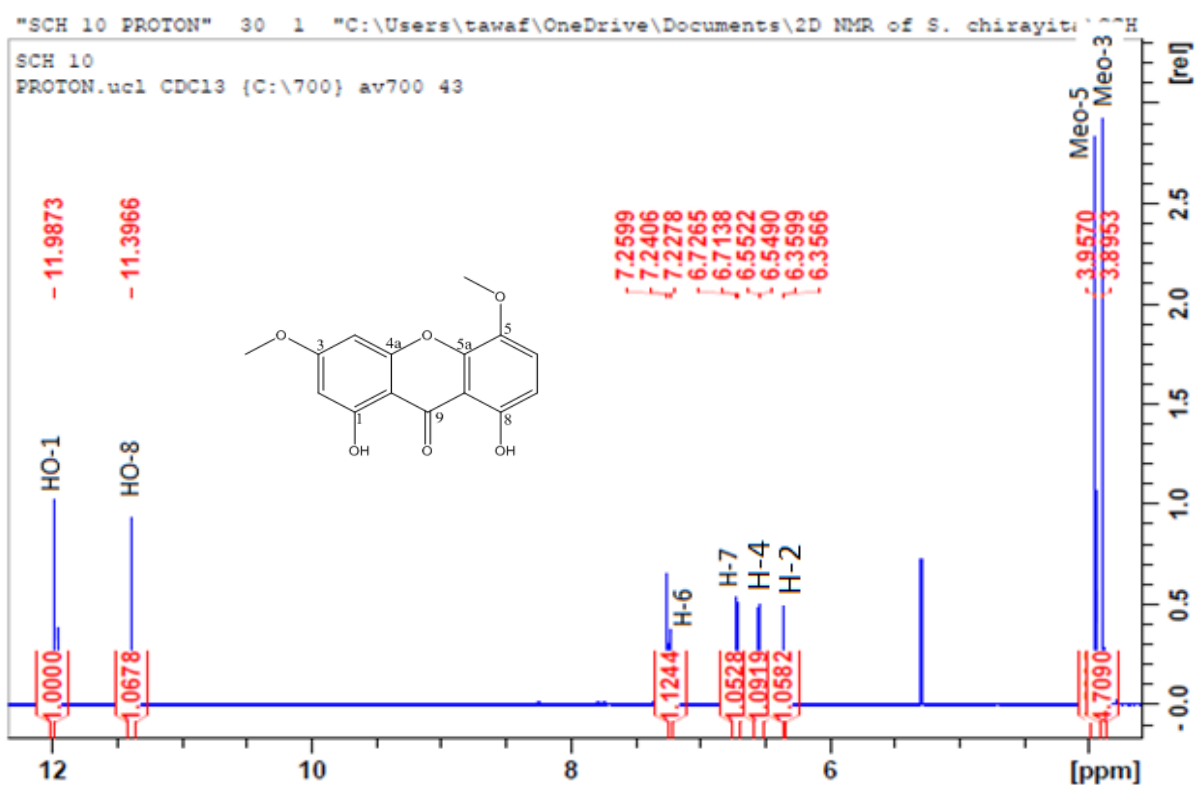


Figure 3.9:  $^1\text{H}$  NMR (700 MHz) spectrum of SCH 10 in  $\text{CDCl}_3$



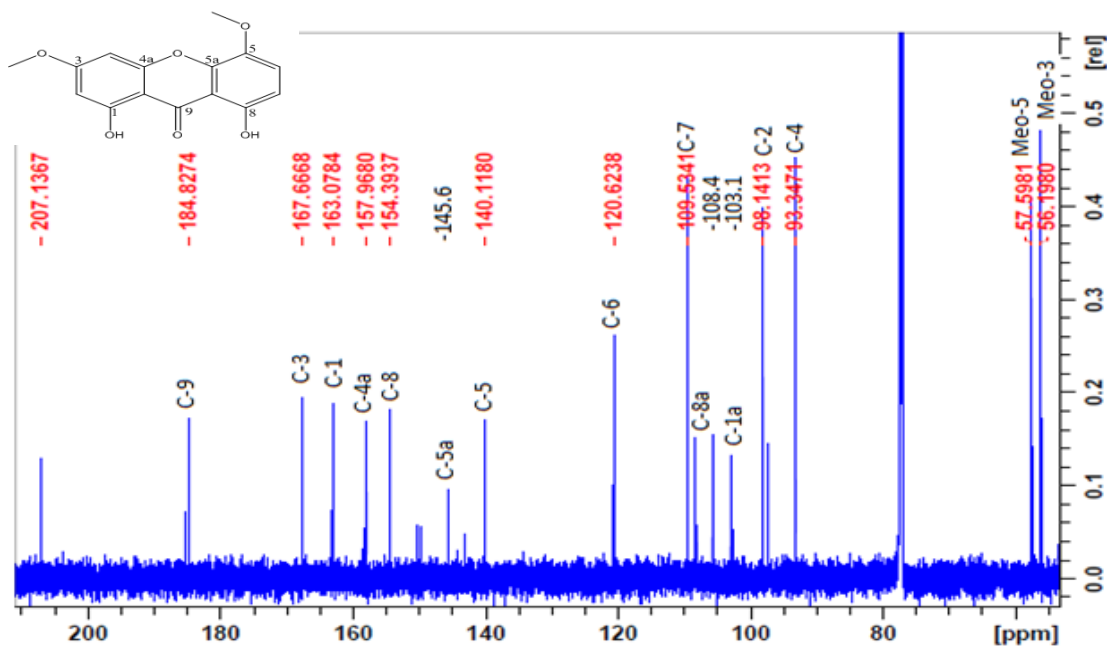


Figure 3.10:  $^{13}\text{C}$  NMR (175MHz) spectrum of SCH 10 in  $\text{CDCl}_3$

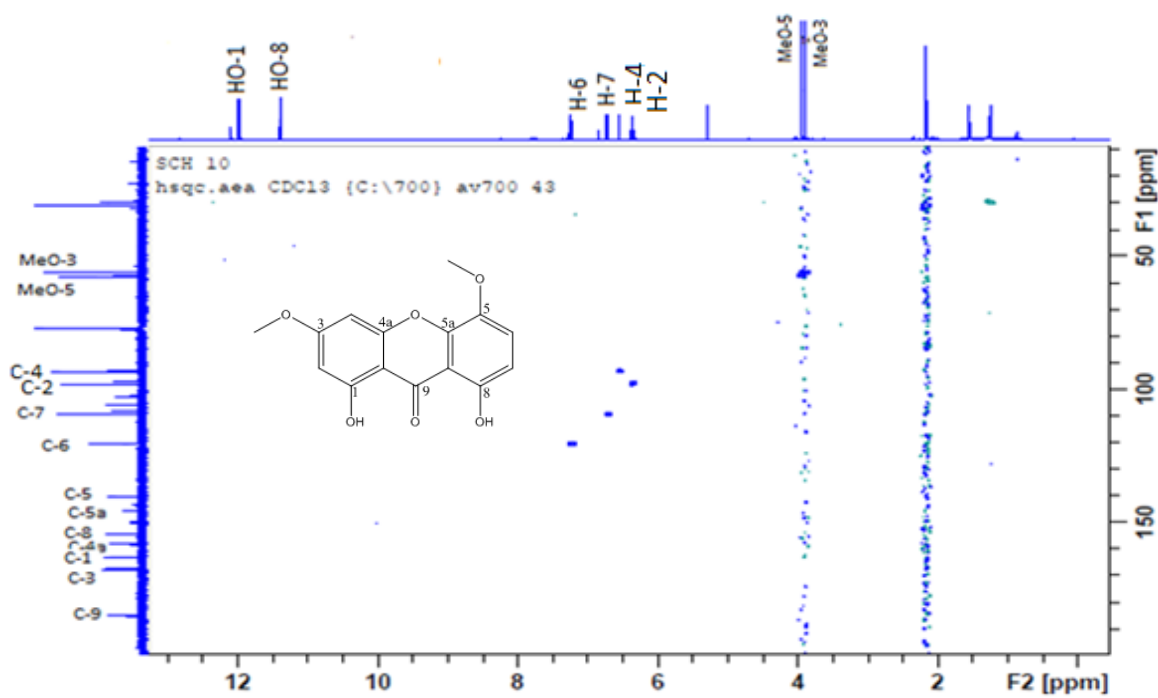


Figure 3.11: HSQC NMR spectrum of SCH 10 in  $\text{CDCl}_3$

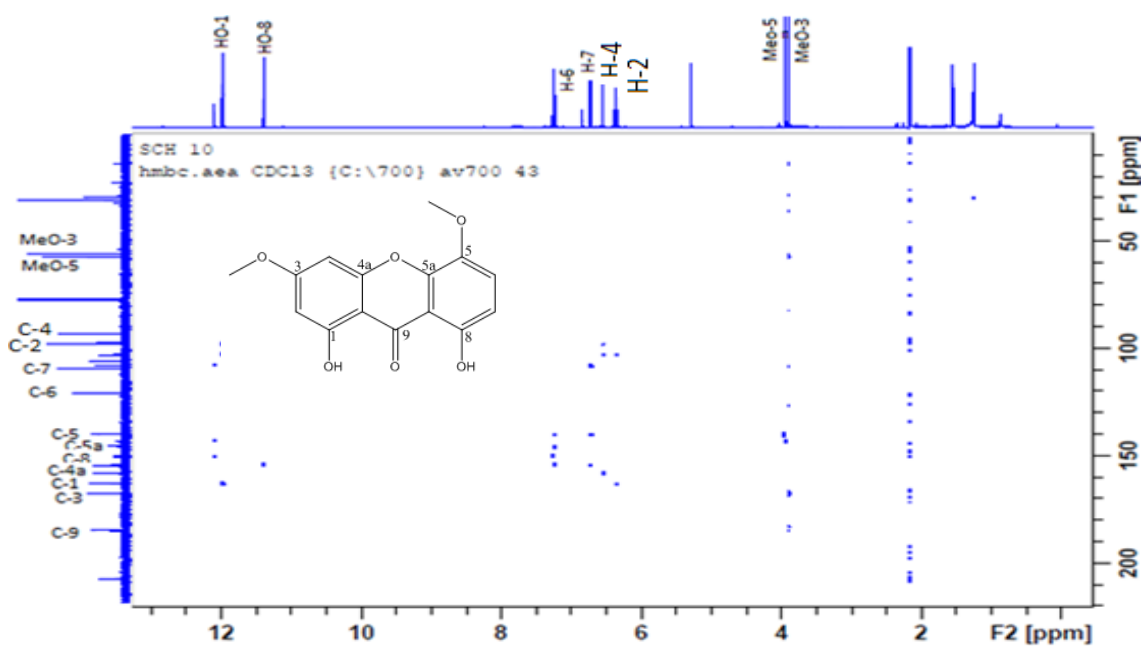


Figure 3.12: HMBC NMR spectrum of SCH 10 in CDCl<sub>3</sub>

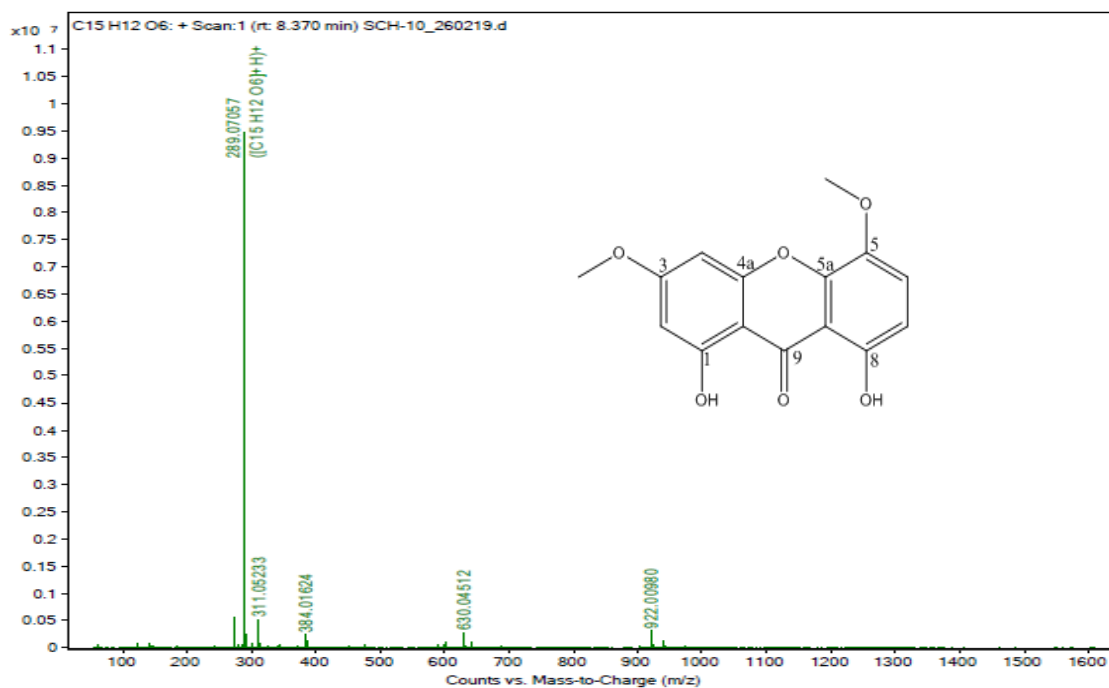
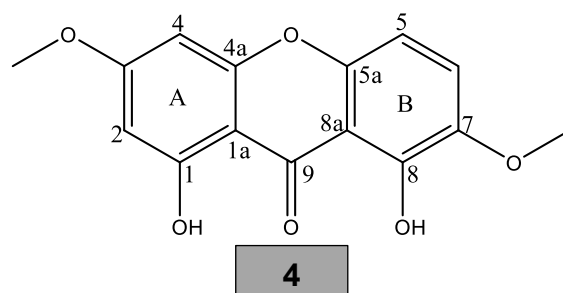


Figure 3.13: HRMS data of SCH 10

### 3.2.2.2 Characterization of SCH 1 as a mixture (1:1) of Swerchirin (3) and Swertiaperenin (4):

The high resolution mass spectroscopy showed the  $[M+H]^+$  at  $m/z$  289.07086 (calculated for  $C_{15}H_{13}O_6$ , at 289.071215) which confirmed the molecular formula of SCH 1 as  $C_{15}H_{12}O_6$  which indicates the mixture of two isomers.

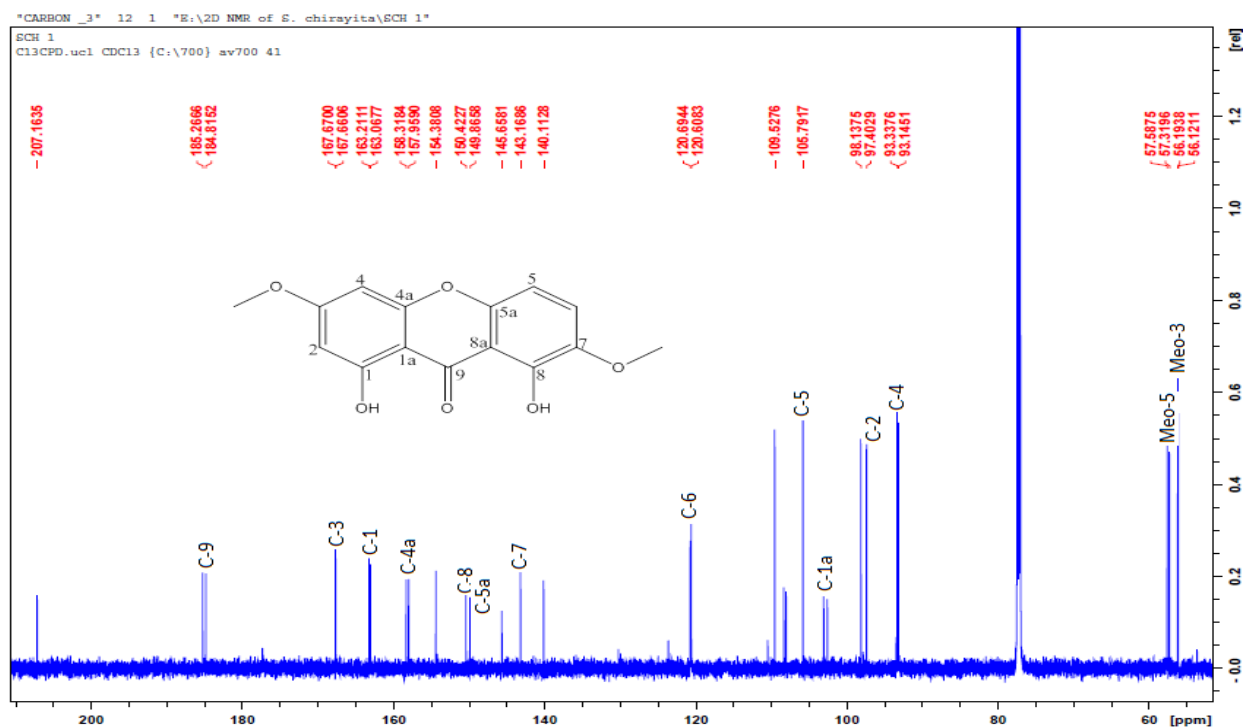
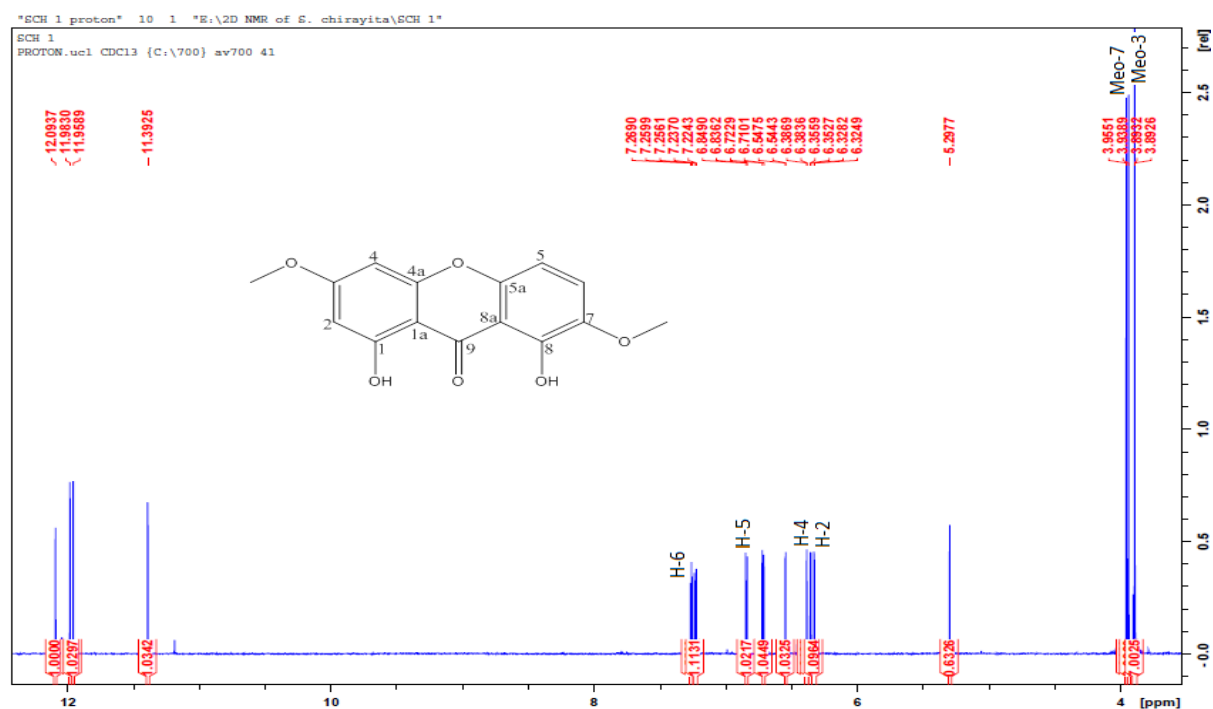
The  $^1H$  NMR spectrum (700 MHz,  $CDCl_3$ , Table 3.6, Figure 3.13) of SCH 1 revealed the presence of four hydrogen bonded hydroxyl groups resonated at  $\delta_H$  11.39, 11.96, 11.98, 12.09, two sets of *meta* coupled aromatic protons at  $\delta_H$  6.36 ( $J=2.24$  Hz), 6.55 ( $J=2.24$  Hz), 6.33 ( $J=2.31$ Hz), 6.39 ( $J=2.31$ Hz), two sets of *ortho* coupled aromatic protons resonated at  $\delta_H$  6.72 ( $J=9.59$  Hz), 7.23 ( $J=9.59$  Hz), 6.84 ( $J=8.96$  Hz) and 7.27 ( $J=9.40$  Hz) and a total of 4 methoxyl groups at  $\delta_H$  3.89 (x2), 3.94, 3.96.



The  $^{13}C$  NMR (175 MHz,  $CDCl_3$ , Table 3.7, Figure 3.14) spectrum data also showed the presence of carbonyl at  $\delta_C$  184.9 including oxygenated quaternary carbons at  $\delta_C$  163.1,  $\delta_C$  167.7,  $\delta_C$  143.2,  $\delta_C$  150.4,  $\delta_C$  109.4 and  $\delta_C$  154.4 and methoxyl carbons at  $\delta_C$  56.1,  $\delta_C$  56.2,  $\delta_C$  57.7,  $\delta_C$  57.3. The presence of 2 sets of hydroxyl protons at  $\delta_H$  11.39,  $\delta_H$  11.98, *meta* coupled protons at  $\delta_H$  6.36 (98.1 from HSQC),  $\delta_H$  6.55 (93.4 from HSQC), *ortho* coupled protons at  $\delta_H$  7.23 (H-6;  $\delta_C$  120.6 from HSQC),  $\delta_H$  6.72 (H-7;  $\delta_C$  140.1 from HSQC), methoxyl protons at  $\delta_H$  3.89 ( $\delta_C$  56.1),  $\delta_H$  3.94 ( $\delta_C$  57.3) were identical to those of SCH 10. Thus, this part of the molecule is identical to SCH 10.

In the HMBC experiment, (Table 3.8), ring A of the other compound were identical to ring A of SCH 1. In ring B, the methoxyl proton at  $\delta_H$  3.94 (H-7;  $\delta_C$  57.3 from HSQC) showed  $^3J$  correlation with quaternary carbon at  $\delta_C$  143.2. In addition, the hydrogen bonded hydroxyl proton at  $\delta_H$  12.09 (H-8) exhibited  $^2J$  correlation with quaternary carbon at  $\delta_C$  150.4 and  $^3J$  correlation with another quaternary carbon at  $\delta_C$  108.1 and oxygenated quaternary carbon at  $\delta_C$  143.2. Ortho coupled aromatic proton at  $\delta_H$  6.84 (H-5;  $\delta_C$  105.8 from HSQC) exhibited  $^2J$  correlation with quaternary carbon at  $\delta_C$  149.9 and  $^3J$  correlation with another quaternary carbon at  $\delta_C$  143.2 and  $\delta_C$  108.1. In addition, other ortho coupled

aromatic proton at  $\delta_H$  7.27 (H-6;  $\delta_C$  120.6 from HSQC) showed  $^2J$  correlation with quaternary carbon at  $\delta_C$  143.2. So these spectral data and their correlation supported the identification of the later part as swertiaperenin. Accordingly, SCH-1 was identified as the mixture of swerchirin (**3**) and swertiaperenin (**4**) in a ratio of 1:1. The spectra data were in agreement with the published literature (Jia et al., 2011)



**Table 3.6: <sup>1</sup>H (700 MHz, CDCl<sub>3</sub>) NMR data of SCH 10 and part of SCH 1**

Position	$\delta_{\text{H}}$ of SCH 10	$\delta_{\text{H}}$ of part of SCH 1
2	6.36, d, $J= 2.24$ Hz	6.33, d, $J=2.31$ Hz
4	6.55, d, $J=2.24$ Hz	6.39, d, $J=2.31$ Hz
5	-	6.84, d, $J=8.96$ Hz
6	7.23, d, $J=9.59$ Hz	7.27, d, $J=9.40$ Hz
7	6.72, d, $J=9.59$ Hz	-
HO-1	11.99, s	11.96, s
HO-8	11.40, s,	12.09, s
MeO-3	3.89, s	3.89, s
MeO-5	3.96, s	-
MeO-7		3.94, d, $J= 11.34$ Hz

**Table 3.7: <sup>13</sup>C (175MHz, CDCl<sub>3</sub>) NMR data of SCH 10 and SCH 1**

Position	<sup>13</sup> C of SCH 10	<sup>13</sup> C of SCH 1
1	163.1	163.1
2	98.1	97.4
3	167.7	167.7
4	93.3	93.2
5	140.1	105.8
6	120.6	120.6
7	109.4	143.2
8	154.4	150.4
9	184.8	184.9
1a	103.1	102.6
4a	157.9	158.4
5a	145.6	149.9
8a	108.4	108.1
MeO-3	56.2	56.1
MeO-5	57.7	
MeO-7	-	57.3

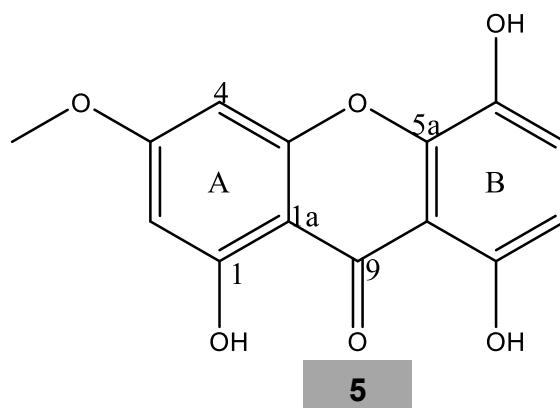
**Table 3.8: HMBC data (700 MHz, CDCl<sub>3</sub>) of SCH 10 and SCH 1**

Protons	HMBC of SCH 10		HMBC of SCH 1	
	<sup>2</sup> J	<sup>3</sup> J	<sup>2</sup> J	<sup>3</sup> J
H-2	167.7 (C)	93.3 (CH), 103.1 (C)	167.7 (C)	102.6 (C), 93.2 (CH)
H-4	167.7 (C)	103.1 (C), 98.1 (CH)	167.7 (C), 158.4 (C)	102.6 (C), 97.4 (CH)
H-5	-	-	149.9 (C)	143.2 (C), 108.1 (C)
H-6	140.1 (C)	145.6 (C), 154.4 (C)	143.2 (C)	-
H-7	154.4 (C)	108.4 (C), 140.1 (C),	-	-
H-8	-	-	-	-
HO-1	163.1 (C)	103.1 (C)	163.1 (C)	102.6 (C), 97.4 (CH)
HO-8	154.4 (C)	109.4 (CH)	150.4 (C)	143.2 (C), 108.1 (C)
MeO-3	-	167.7 (C)	-	167.7 (C)
MeO-5	-	140.1 (C)	-	-
Meo-7			-	143.2 (C)

### 3.2.2.3 Characterization of SCH 15 as belidifolin (1,5,8-tri-hydroxy-3-methoxyxanthone; **5**)

The high resolution mass spectroscopy showed the  $[M+H]^+$  at  $m/z$  275.05519 (calculated for  $C_{14}H_{11}O_6$ , at 275.055565) which confirmed the molecular formula of SCH 15 as  $C_{14}H_{10}O_6$ .

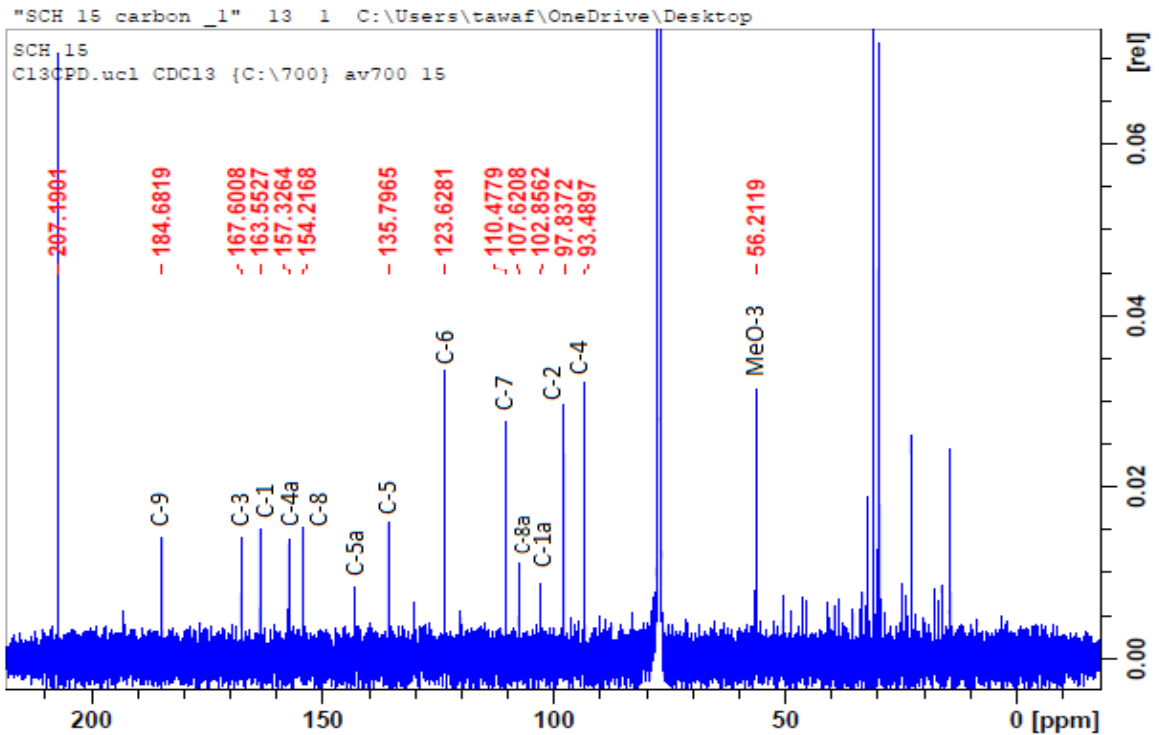
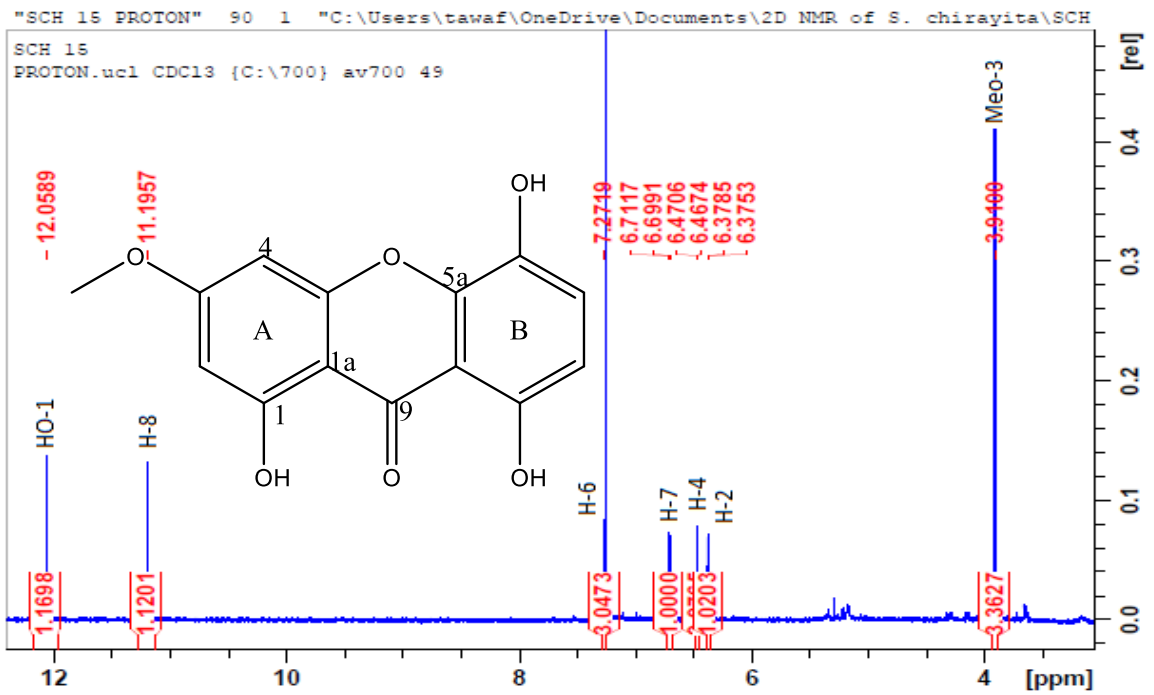
The  $^1H$  NMR spectrum (700 MHz,  $CDCl_3$ , Table 3.9, Figure 3.15) is very similar to those in SCH 10. Like SCH 10, the  $^1H$  NMR spectrum (700MHz,  $CDCl_3$ ) of SCH 15 revealed two highly downfield protons singlets at  $\delta_H$  11.19 and  $\delta_H$  12.06 attributed to hydrogen bonded hydroxyl groups, two meta coupled ( $J=2.24$  Hz) aromatic protons resonating at  $\delta_H$  6.37 and 6.45 and two ortho coupled  $J= 8.89$  Hz and  $J= 8.40$  Hz protons resonating at  $\delta_H$  6.70 and 7.27, respectively.



However, there was only one methoxyl group proton as singlet at  $\delta_H$  3.91. The  $^{13}C$  NMR spectrum (175MHz,  $CDCl_3$ , Table 3.9, Figure 3.16) showed the presence of 14 carbons including carbonyl carbon at  $\delta_C$  184.7 and methoxyl carbon at  $\delta_C$  56.2.

In the HMBC experiment (Table 3.10), the key HMBC correlations of ring A was identical to those of SCH 10. In ring B, the ortho coupled aromatic methine protons at  $\delta_H$  7.27 (H-6;  $\delta_C$  123.6 from HSQC) and  $\delta_H$  6.70 (H-7;  $\delta_C$  110.5 from HSQC) revealed correlation with oxygenated quaternary carbon at  $\delta_C$  135.8 by  $^2J$  and  $^3J$  respectively, therefore, 135.8 was identified as C-5 oxygenated quaternary carbon.

Considering  $^1H$ ,  $^{13}C$  NMR data and their correlations in the HSQC and HMBC, the compound SCH 15 was identified as 1, 5, 8-tri-hydroxy-3-methoxyxanthone also known as belidifollin (**5**). (Timsina et al., 2018). Belidifollin was previously isolated from another species of genus *Swertia* (*Swertia multicaulis*). (Timsina et al., 2018). The NMR spectral data were identical to previously reported data. (Xue-qing et al., 2017)





### 3.2.2.4 Characterization of SCH 2 as decussatin (6) (1-hydroxy-3, 7, 8-trimethoxyxanthone):

The high resolution mass spectroscopy showed the  $[M+H]^+$  at  $m/z$  303.08689 (calculated for  $C_{16}H_{15}O_6$ , at 303.086865) which confirmed the molecular formula of SCH 2 as  $C_{16}H_{14}O_6$ .

The  $^1H$  NMR spectral data (700 MHz,  $CDCl_3$ ) (Table 3.9; Figure 3.17) of SCH 2 were very similar to SCH 10. Like SCH 10,  $^1H$  NMR spectrum revealed the presence of four aromatic doublet protons resonating at  $\delta_H$  6.32 ( $J=2.24Hz$ ),  $\delta_H$  6.34 ( $J=2.24Hz$ ),  $\delta_H$  7.16 ( $J=9.17 Hz$ ),  $\delta_H$  7.33 ( $J=9.17 Hz$ ), one eminent downfield proton singlet at  $\delta_H$  13.25 and three methoxyl singlets resonating at  $\delta_H$  3.88, 4.00 and 3.93.

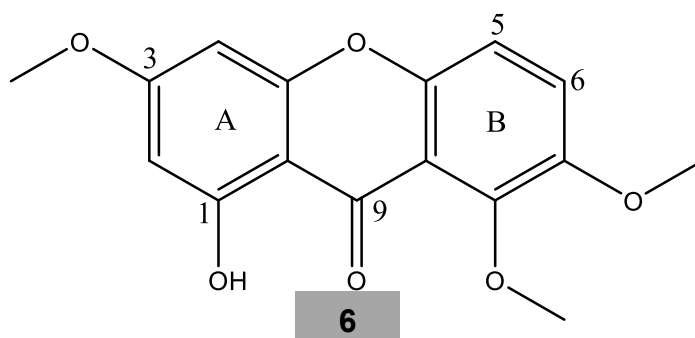
The  $^{13}C$  NMR spectrum (175 MHz,  $CDCl_3$ , Table 3.9, Figure 3.18) revealed the presence of a total 16 carbons including carbonyl  $\delta_C$  181.4, methoxyl carbons at  $\delta_C$  56.0,  $\delta_C$  62.0,  $\delta_C$  57.4 respectively.

The key HMBC corrections in ring A was identical to those of SCH 10. In ring B,

aromatic proton at  $\delta_H$  7.33

(H-5,  $\delta_C$  120.7 from HSQC) revealed  $^2J$  correlation with quaternary carbons at C-5a ( $\delta_C$  151.2) and  $^3J$  connection with C-7 ( $\delta_C$  149.2). Another aromatic proton at  $\delta_H$  7.16 (H-6,  $\delta_C$  112.9 from HSQC) showed  $^3J$  correlation to a quaternary carbon at  $\delta_C$  149.5 (C-8).

On the other hand, methoxyl protons at  $\delta_H$  4.00 ( $\delta_C$  62.0 from HSQC) and  $\delta_H$  3.93 ( $\delta_C$  57.4 from HSQC) showed  $^3J$  connection with oxygenated quaternary carbons at  $\delta_C$  149.2 and  $\delta_C$  149.5 confirmed its positions at C-5 and C-6, respectively. According to NMR spectrum, the compound of SCH 2 was identified as 1-hydroxy-3, 7, 8-trimethoxy-9H-xanthen-9-one (**6**; He et al., 2015; Timsina et al., 2018). It has been previously isolated from *Swertia chirayita* (You et al., 2017) as well. These spectral data complied with the data published in the literature (Alaribe et al., 2011).



**Table 3.9:  $^1\text{H}$  (700 MHz,  $\text{CDCl}_3$ ) and  $^{13}\text{C}$  (175MHz,  $\text{CDCl}_3$ ) data of SCH 15, SCH 2**

Position	$\delta_{\text{H}}$ of SCH 15	$^{13}\text{C}$ of SCH 15	$\delta_{\text{H}}$ of SCH 2	$^{13}\text{C}$ of SCH 2
1	-	163.6	-	164.1
2	6.37, d, $J= 2.24$ Hz	97.8	6.32, d, $J= 2.24$ Hz	97.0
3	-	167.6	-	166.6
4	6.45, d, $J= 2.24$ Hz	93.5	6.34, d, $J=2.24$ Hz	92.2
5	-	135.8	7.33, d, $J= 9.17$ Hz	120.7
6	7.27, $J=8.40$ Hz	123.6	7.16, d, $J=9.17$ Hz	112.9
7	6.70, d, $J=8.89$ Hz	110.5	-	149.2
8	-	154.3	-	149.5
9	-	184.7	-	181.4
1a	-	102.9	-	104.3
4a	-	157.3	-	157.4
5a	-	143.1	-	151.2
8a	-	107.4	-	116.0
HO-1	12.06, s	-	13.25, s	-
HO-8	11.19, s	-	-	-
MeO-3	3.91, s	56.2	3.88, s	56.0
MeO-5	-	-	4.00, s	62.0
MeO-8	-	-	3.93, s	57.4

**Table 3.10: HMBC data (700 MHz,  $\text{CDCl}_3$ ) of SCH 15, and SCH 2**

Protons	HMBC of SCH 15		HMBC of SCH 2	
	$^2J$	$^3J$	$^2J$	$^3J$
H-2	167.6 (C), 163.6 (C)	93.5 (CH)	166.6 (C), 164.1 (C)	104.3 (C), 92.2 (CH)
H-4	157.3 (C), 167.6(C)	97.8 (CH)	166.6 (C), 157.4 (C)	104.3 (C), 97.0 (CH)
H-5	-	-	151.2	149.2
H-6	135.8 (C)	154.3 (C)	-	149.5
H-7	154.3 (C)	135.8 (C)	-	-
H-8	-	-	-	-
HO-1	-	-	164.1 (C)	104.3 (C), 97.0 (CH)
MeO-3	-	167.6 (C)	-	166.6 (C)
MeO-5	-	-	-	149.2 (C)
MeO-8	-	-	-	149.5 (C)

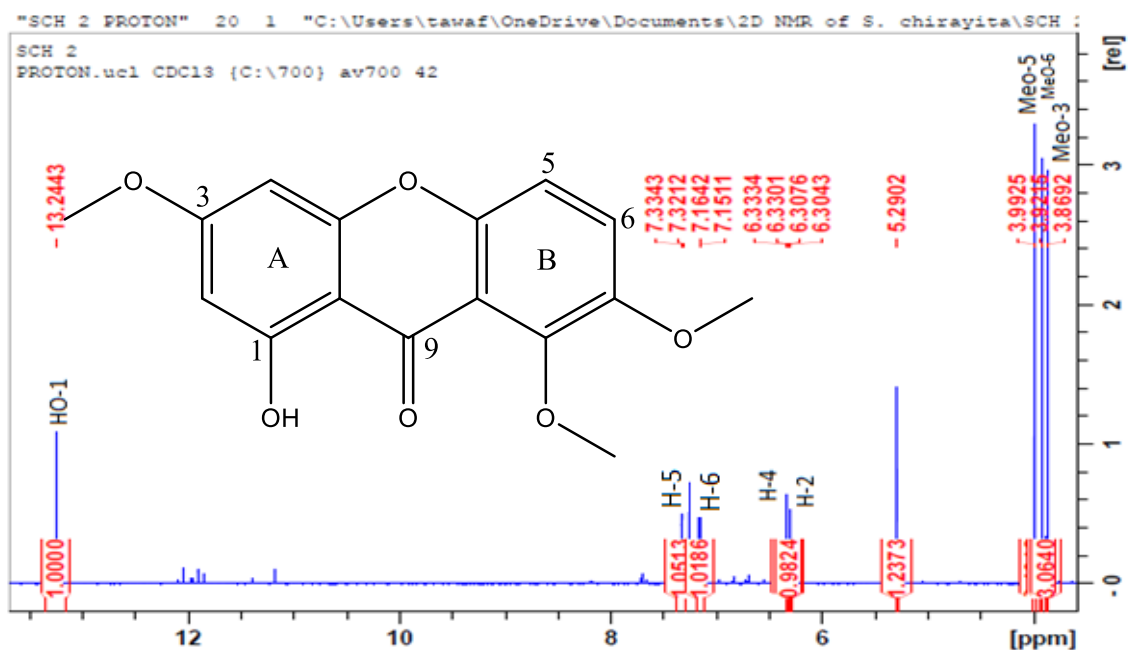


Figure 3.18:  $^1\text{H}$  NMR (700 MHz) spectrum of SCH 2 in  $\text{CDCl}_3$

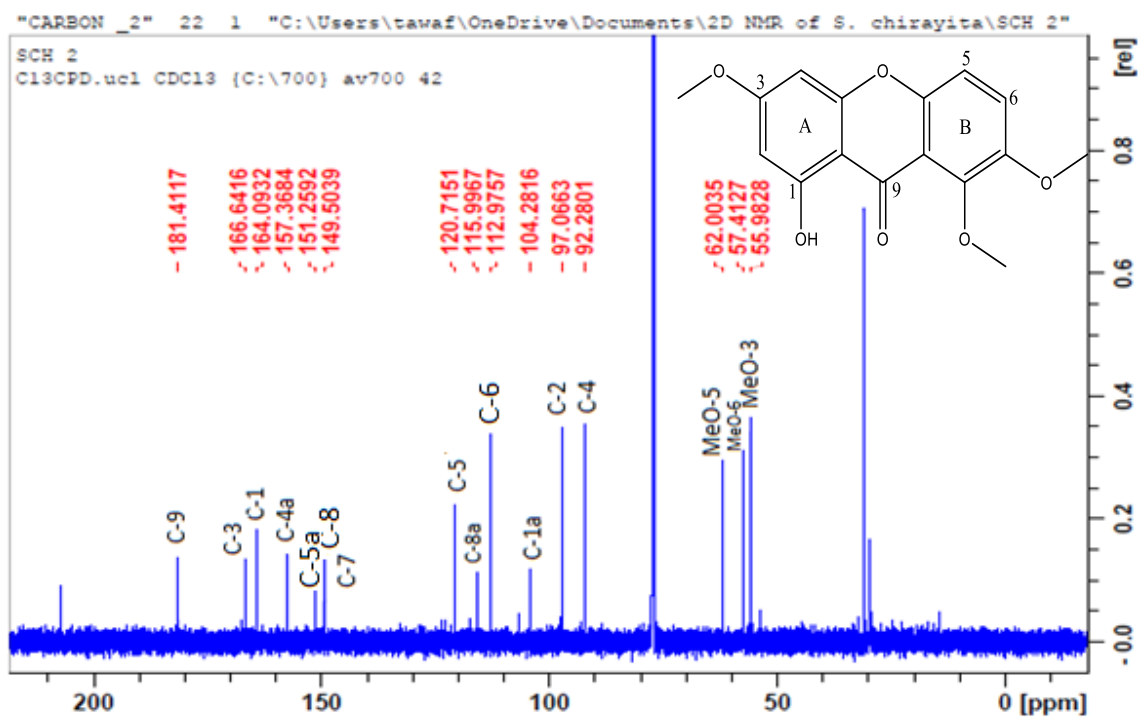


Figure 3.19:  $^{13}\text{C}$  NMR (175MHz) spectrum of SCH 2 in  $\text{CDCl}_3$

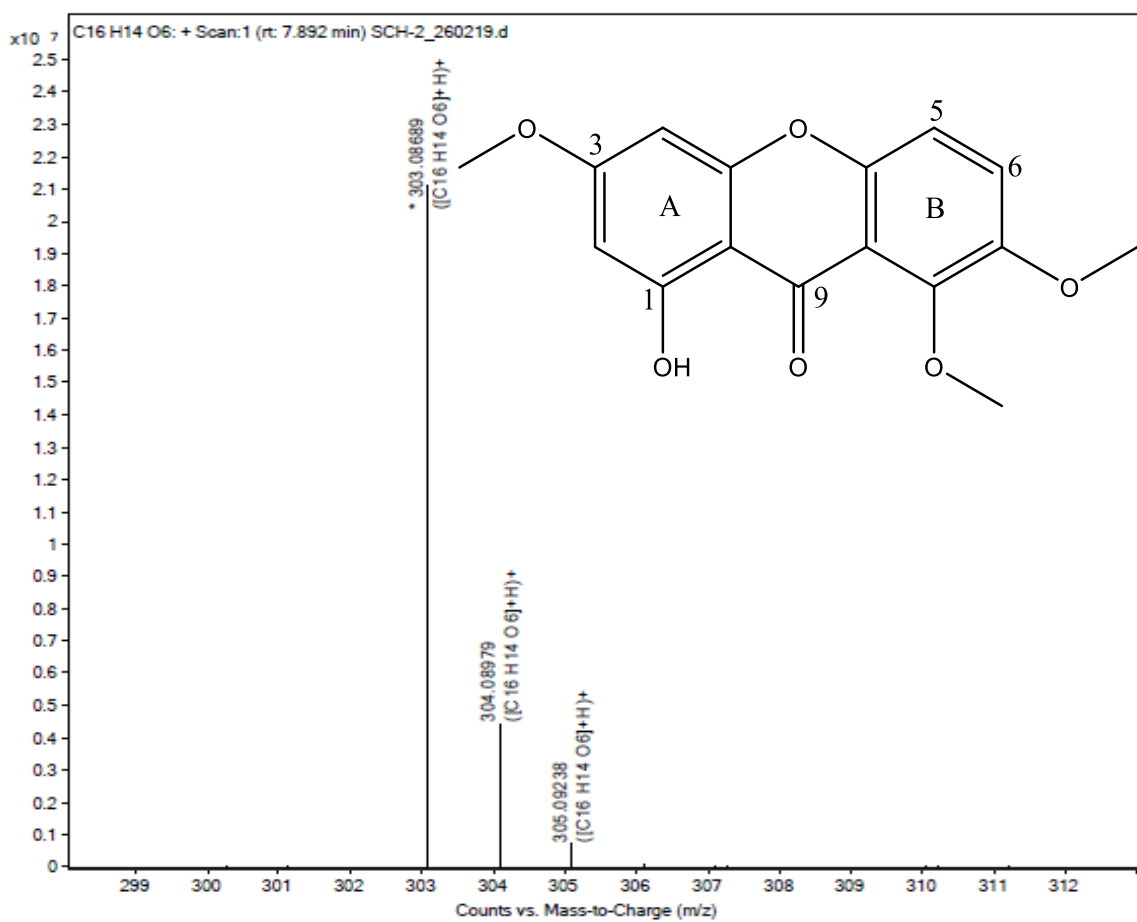


Figure 3.20: HRMS data of SCH 2

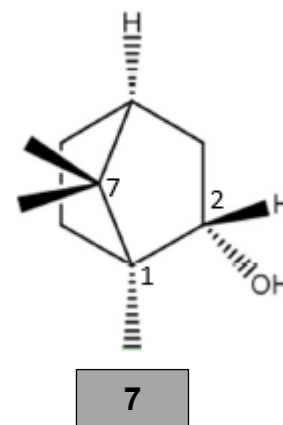
### 3.2.3 Monoterpenes:

Monoterpenes consist of 2 isoprene units. In this study, two monoterpenes were isolated from hexane extract of the rhizome of *Zingiber montanum*. These are coded as ZMH 10 and ZMH 8

#### 3.2.3.1 Characterization of ZMH 10 as (-) borneol (7):

The IR spectrum of ZMH 10 revealed the presence of hydroxyl group at 3019  $\text{cm}^{-1}$ . The high resolution of mass spectroscopy showed the  $[\text{M}+\text{H}]^+$  at  $m/z$  155.13628 (calculated for  $\text{C}_{10}\text{H}_{19}\text{O}$ , at 155.135765) which confirmed the molecular formula of ZMH 10 as  $\text{C}_{10}\text{H}_{18}\text{O}$ .

The  $^1\text{H}$  NMR spectrum (600MHz,  $\text{CDCl}_3$ , Table 3.11, Figure 3.19) revealed the presence of 3 methyl singlets at  $\delta_{\text{H}}$  0.82, 0.90 and 1.01, an oxymethine at  $\delta_{\text{H}}$  3.62 (dd,  $J= 7.50$  Hz, 3.96 Hz) and a number of signals resonated between  $\delta_{\text{H}}$  0.96-1.75 due to the presence of methine and methylene protons.

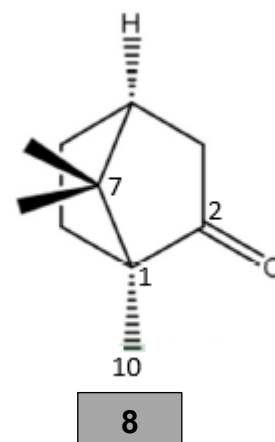


The  $^{13}\text{C}$  NMR spectrum (150 MHz,  $\text{CDCl}_3$ ) (Table 3.11, Figure 3.20) revealed the presence of total 10 carbons in the structure including oxymethine carbon at  $\delta_{\text{C}}$  80.1 and three methyl carbons at  $\delta_{\text{C}}$  20.6, 11.4, 20.4. The DEPT NMR spectrum confirmed the presence of 3 methyls, 3 methylenes, 2 methine and remaining 2 quaternary carbons.

In HMBC experiment (Table 3.12), the methyl protons at  $\delta_{\text{H}}$  0.82 (H-8,  $\delta_{\text{C}}$  20.6 from HSQC) and  $\delta_{\text{H}}$  1.01 (H-9,  $\delta_{\text{C}}$  20.4) showed common  $^3J$  correlation with quaternary carbon at  $\delta_{\text{C}}$  49.1 (C-1) and methine carbon at  $\delta_{\text{C}}$  45.2 (C-4). Moreover, the methyl proton at  $\delta_{\text{H}}$  0.82 ( $\delta_{\text{C}}$  20.6 from HSQC) showed  $^3J$  connection with methyl carbon at  $\delta_{\text{C}}$  20.4 ( $\delta_{\text{H}}$  1.01) and *vice versa*. On the other hand, methyl proton at  $\delta_{\text{H}}$  1.01 (H-9,  $\delta_{\text{C}}$  20.4 from HSQC) showed common  $^2J$  connectivity with the quaternary carbon at  $\delta_{\text{C}}$  46.6 (C-7). The methine proton at (H-4)  $\delta_{\text{H}}$  1.71 ( $\delta_{\text{C}}$  45.2 from HSQC) and methylene proton at (H-6)  $\delta_{\text{H}}$  1.50 and 1.01 ( $\delta_{\text{C}}$  27.4 from HSQC) showed correlation with oxymethine carbon at (C-2)  $\delta_{\text{C}}$  80.1 ( $\delta_{\text{H}}$  3.62, dd,  $J= 7.50$ , 3.96Hz). Based on the NMR and IR data, the structure of ZMH 10 was confirmed as (-) borneol (7). Previously, Verma and his colleagues detected (-) borneol from the essential oil of *Zingiber montanum* (Verma et al., 2018).

### 3.2.3.2 Characterization of ZMH 8 as Camphor (8):

The IR spectrum of ZMH 8 revealed the presence of carbonyl at  $1750\text{ cm}^{-1}$ . The high resolution of mass spectroscopy showed the  $[M+H]^+$  at  $m/z$  153.12628 (calculated for  $C_{10}H_{17}O$ , at 153.120115) which confirmed the molecular formula of ZMH 8 as  $C_{10}H_{16}O$ . The  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR data of ZMH-8 are very similar to those of ZMH-10. The  $^1\text{H}$  NMR spectrum (600MHz,  $\text{CDCl}_3$ , Table 3.11, Figure 3.21) revealed the presence of 3 methyl singlets at  $\delta_{\text{H}}$  0.94,  $\delta_{\text{H}}$  0.82 and  $\delta_{\text{H}}$  0.89 and a number of methine and methylene protons resonated between  $\delta_{\text{H}}$  1.31-2.34, however it did not show any peaks around 3.62 indicating the absence of oxymethine proton.



The  $^{13}\text{C}$  NMR spectrum (150 MHz,  $\text{CDCl}_3$ , Table 3.11) revealed the presence of similar type of carbons like ZMH 10 in the molecule including carbonyl at  $\delta_{\text{C}}$  218.0 (C-2) instead of oxymethine carbon at 80.1 as evident in ZMH 10. The DEPT NMR spectrum confirmed the presence of 3 methyls, 3 methylenes, and 1 methine.

In HMBC experiment, the key  $^1\text{H}$  and  $^{13}\text{C}$  correlation of ZMH 8 is very much similar to ZMH 10. The methyl proton at 0.89 (H-10; 9.4 from HSQC) and methine proton at 2.07 (H-4; 43.1 from HSQC) revealed common  $^3J$  correlation with the carbon of carbonyl at 218.0 (C-2) which was missing at the carbon spectrum. Based on the NMR, IR, and mass spectral data, the compound ZMH 8 has been identified as camphor (**8**). Previously it was isolated from the essential oil of the rhizome of *Zingiber montanum* (Verma et al., 2018).

**Table 3.11:  $^1\text{H}$  (600 MHz) and  $^{13}\text{C}$  NMR spectroscopic data of ZMH 10 and ZMH 8 in  $\text{CDCl}_3$**

Position	ZMH 10		ZMH 8	
	$^1\text{H}$	$^{13}\text{C}$	$^1\text{H}$	$^{13}\text{C}$
1	-	49.1	-	57.9
2	3.62, dd, $J=7.50$ Hz, $j=3.96$ Hz	80.1	-	218.0
3	1.65-1.75, m	40.5	1.83, 2.34, m	43.3
4	1.71, m	45.2	2.07, m	43.1
5	1.65, m 1.01, m	27.4	1.92, m 1.31, m	27.2
6	1.50, m 0.96, m	34.1	1.66, m 1.38, m	30.1
7	-	46.6	-	47.0
8	0.82, s	20.6	0.94, s	19.2
9	1.01, s	20.4	0.82, s	19.9
10	0.90, s	11.4	0.89, s	9.4

**Table 3.12:  $^1\text{H}$ - $^{13}\text{C}$  correlation for ZMH 10 and ZMH 8 in  $\text{CDCl}_3$**

Protons	HMBC of ZMH 10		HMBC of ZMH 8	
	$^2J$	$^3J$	$^2J$	$^3J$
H-2	49.1 (C)	-	-	-
H-3	45.2 (CH)	27.4 ( $\text{CH}_2$ )	-	27.2 ( $\text{CH}_2$ )
H-4	27.4 ( $\text{CH}_2$ )	80.1 (CH-O)	47.0 (C)	30.1 ( $\text{CH}_2$ ), 19.2 ( $\text{CH}_3$ ), 218 (C=O)
H-5	-	40.5 ( $\text{CH}_2$ )	-	57.9 (C)
H-6	49.1 (C), 27.4 ( $\text{CH}_2$ )	80.1 (CH-O),	57.9 (C), 27.2 ( $\text{CH}_2$ )	9.4 ( $\text{CH}_3$ )
H-8	-	20.4 ( $\text{CH}_3$ ), 45.2 (CH), 49.1 (C)	47.0 (C)	19.2 ( $\text{CH}_3$ ), 43.1 (CH), 57.9 (C)
H-9	46.6 (C)	20.6 ( $\text{CH}_3$ ), 45.2 (CH), 49.1 (C)	47.0 (C)	57.9 (C), 43.1 (CH), 19.9 ( $\text{CH}_3$ )
H-10	49.1 (C)	34.1 ( $\text{CH}_2$ ), 46.6 (C), 80.1 (CH-O)	57.9 (C)	47.0 (C), 30.1 ( $\text{CH}_2$ ), 218.0 (C=O)

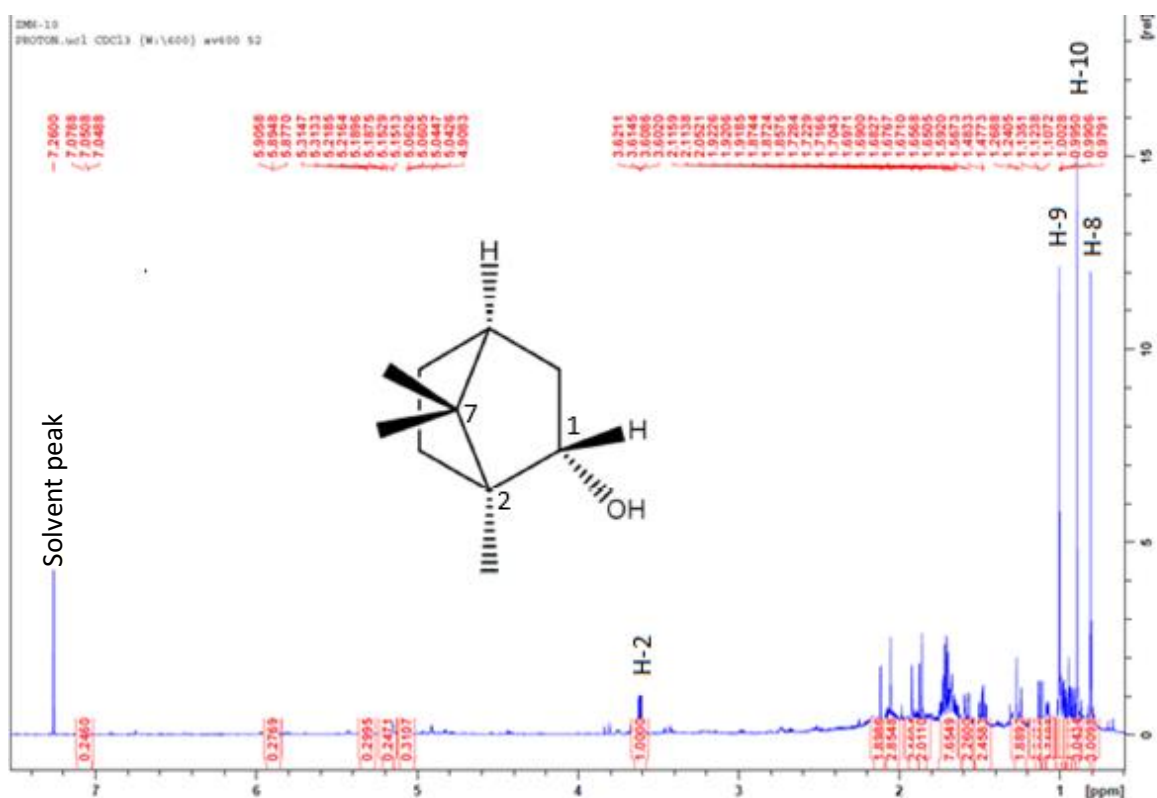


Figure 3.21:  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ ) spectrum of ZMH 10

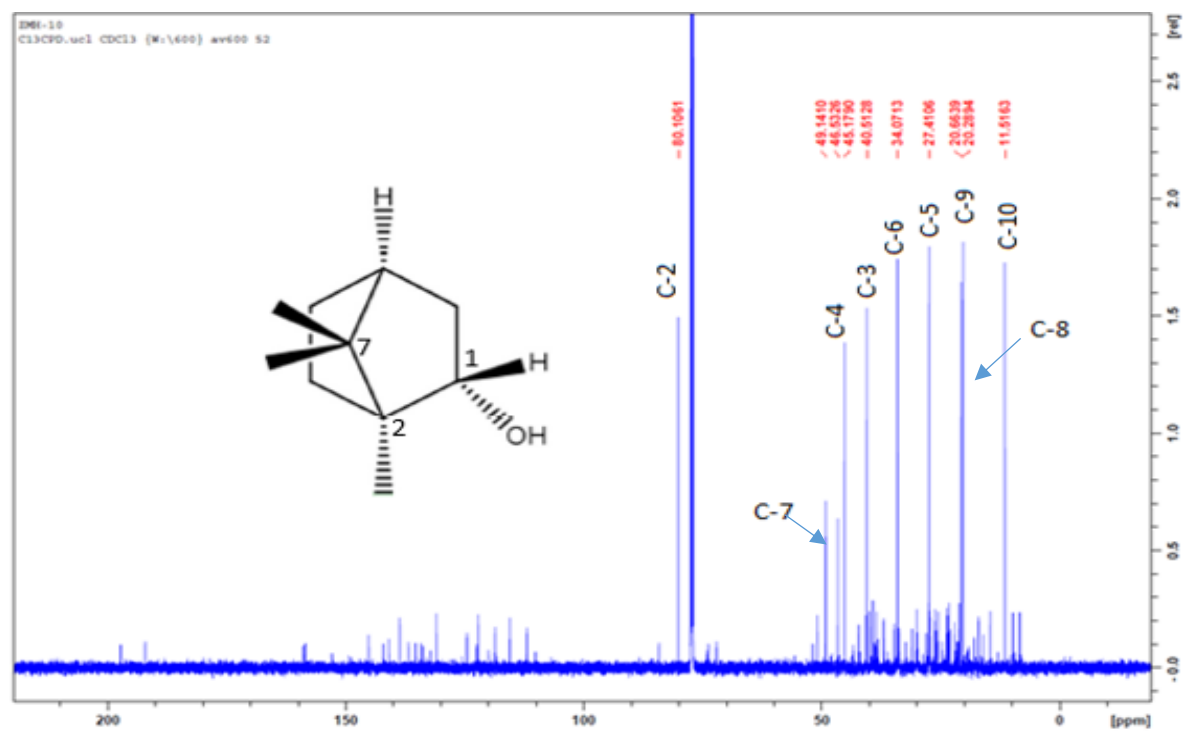


Figure 3.22:  $^{13}\text{C}$  NMR (150 MHz,  $\text{CDCl}_3$ ) spectrum of ZMH 10



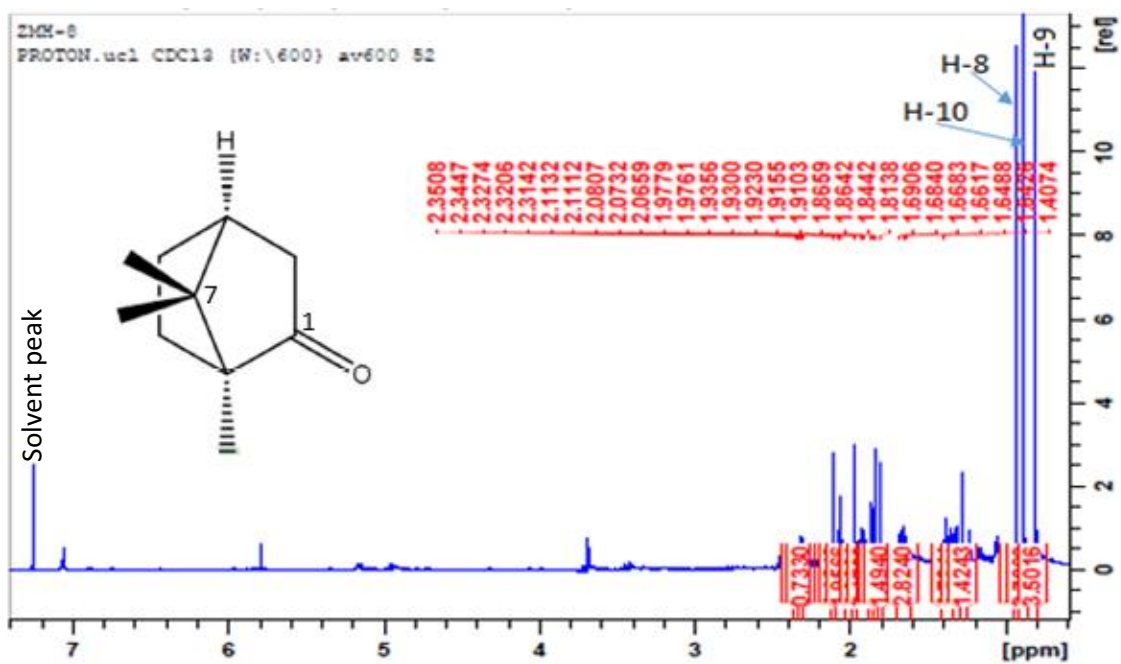


Figure 3.23:  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ ) spectrum of ZMH 8

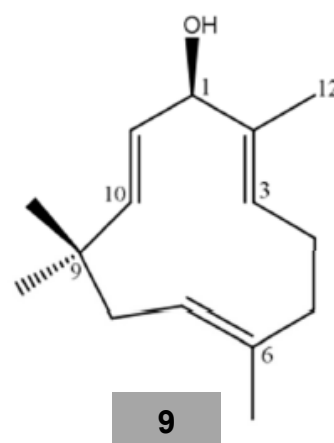
### 3.2.4 Sesquiterpenes:

Sesquiterpenes are comprised of 3 isoprene units. In this study, a total of five sesquiterpenes were isolated and identified from hexane and chloroform extract of rhizome of *Zingiber montanum*.

#### 3.2.4.1 Characterization of MRSH-6 as (2Z, 6Z, 10E) -2,6,9,9-tetramethylcycloundeca-2,6,10-trien-1-ol (Zerumbol; 9):

MRSH 6 was isolated as colourless oil from the n-hexane extract of *Z. montanum*. The IR spectrum revealed the presence of a hydroxyl group ( $3300\text{ cm}^{-1}$ ) and alkene group ( $1600\text{ cm}^{-1}$ ). The high resolution of mass spectroscopy showed the  $[M+H]^+$  at  $m/z$  221.18956 (calculated for  $C_{15}H_{25}O$ , at 221.19054) which confirmed the molecular formula of MRSH 6 as  $C_{15}H_{24}O$ . The  $^1H$  NMR spectrum ( $CDCl_3$ , 500 MHz, Table 3.13, Figure 3.22) of MRSH 6 showed the presence of four methyl singlets resonating at  $\delta_H$  1.04, 1.06, 1.43, 1.65, four sets of olefinic protons at 4.82 (dd,  $J= 10.2, 4.4$  Hz), 5.20, d,  $J= 7.5$  Hz), 5.23 (d,  $J= 16.2$  Hz) and 5.56 (dd,  $J= 16.2, 7.5$  Hz), an oxymethine proton at  $\delta_H$  4.63 as doublet ( $J= 7.5$  Hz) and also couple of methylene protons peaks between  $\delta_H$  1.87-2.35 Hz.

The  $^{13}C$  NMR spectrum (125 MHz,  $CDCl_3$ ; Table 3.13; Figure 3.23) revealed the presence of a total of 15 carbons including an oxymethine carbon at  $\delta_C$  78.8. The DEPT135 identified four methyl, three methylene, one oxymethine, four olefinic methines and the remaining three as quaternary carbons. Among the later three quaternary carbons, one at  $\delta_C$  37.3 was aliphatic and remaining two were connected double bonds. The complete structure of this compound was established by 2D NMR spectra, predominantly by HSQC and HMBC. In the  $^1H$ - $^1H$  COSY spectrum (Figure 3.24), the *trans* double bonded protons showed expected interaction between them. In the HMBC experiment (Table 3.14; Figure 3.25) the *trans* double bonded proton at  $\delta_H$  5.23 ( $\delta_C$  139.5 from HSQC) and methyl protons at 1.65 (H-12;  $\delta_C$  12.8 from HSQC) revealed a common  $^3J$  interaction to an oxymethine carbon at 78.8 (C-1). Olefinic protons at 5.20 (H-3;  $\delta_C$  124.8 ppm from HSQC) and  $\delta_H$  4.82 (H-7;  $\delta_C$  125.0 ppm from HSQC) and methyl protons at 1.43 (H-13;  $\delta_C$  15.3 ppm from HSQC) showed  $^3J$  correlation to a methylene carbon at 39.5 (C-5;  $\delta_H$  2.35 ppm from HSQC).



Protons at  $\delta_{\text{H}}$  2.20 and  $\delta_{\text{H}}$  2.24 (H-4;  $\delta_{\text{C}}$  24.4 from HSQC) exhibited  $^3J$  correlations to quaternary carbons at 142.2 (C-2) and 133.2 (C-6). Two sets of methyl protons at 1.04 (H-14;  $\delta_{\text{C}}$  24.9) and 1.06 (H-15;  $\delta_{\text{C}}$  29.9) revealed common  $^2J$  correlation to a quaternary carbon at  $\delta_{\text{C}}$  37.3 (C-9) and  $^3J$  interaction to olefinic methine carbon at 139.5 (C-10,  $\delta_{\text{H}}$  5.23) and methylene at 42.4 (C-8,  $\delta_{\text{H}}$  1.87, 2.32). H-10 also revealed  $^3J$  interaction to both methyl group carbons at 24.9 (C-14) and 29.9 (C-15). The COSY experiment exhibited usual interaction (H-10 to H-11; H-4 to both H-3 and H-5; H-7 to H-8). Accordingly, MRS 6 was identified as (2Z, 6Z, 10E)-2, 6, 9, 9- tetramethylcycloundeca-2,6 , 10-trien-1-ol, commonly known as Zerumbol (**9**). Previously it was synthesized from Zerumbone (Takashi et al., 2002). The NMR spectra of MRS 6 are in agreement with published literature (Songsiang et al., 2010)

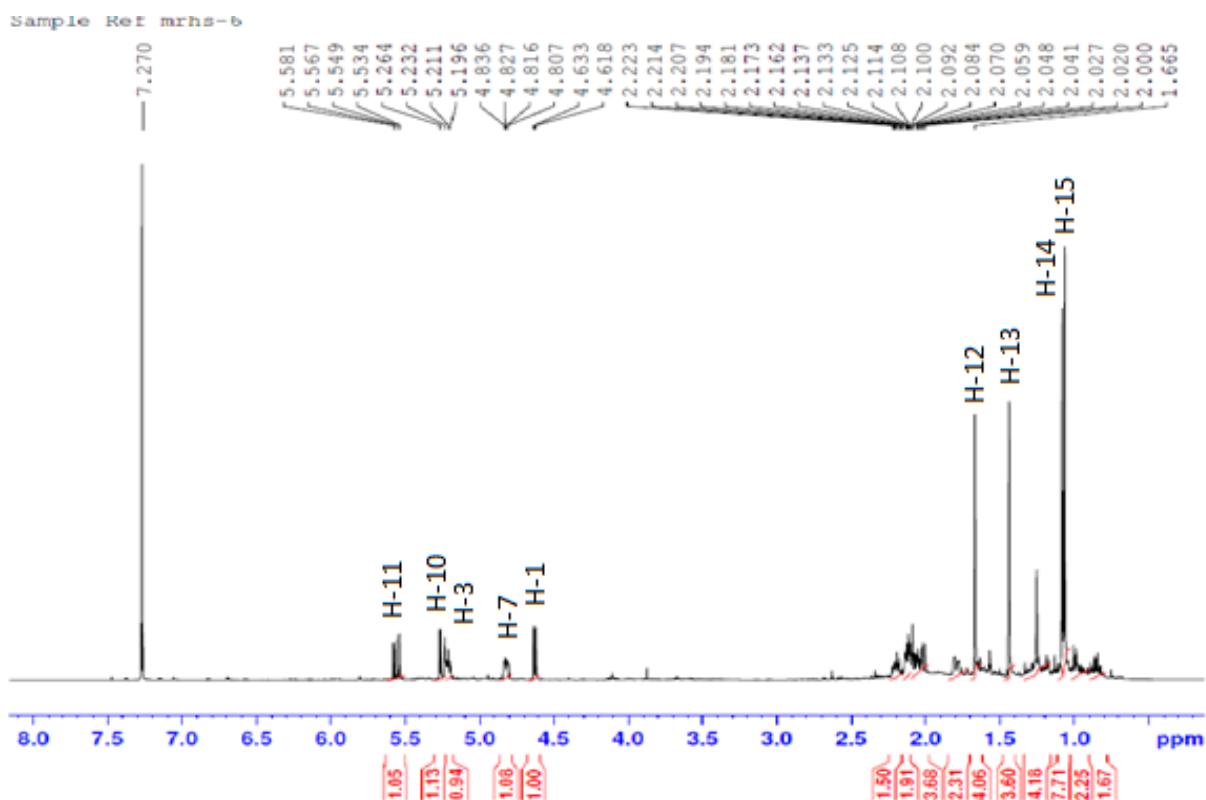


Figure 3.24:  $^1\text{H}$  NMR (500MHz) spectrum of MRS 6 in  $\text{CDCl}_3$

Sample Ref MRHS\_6

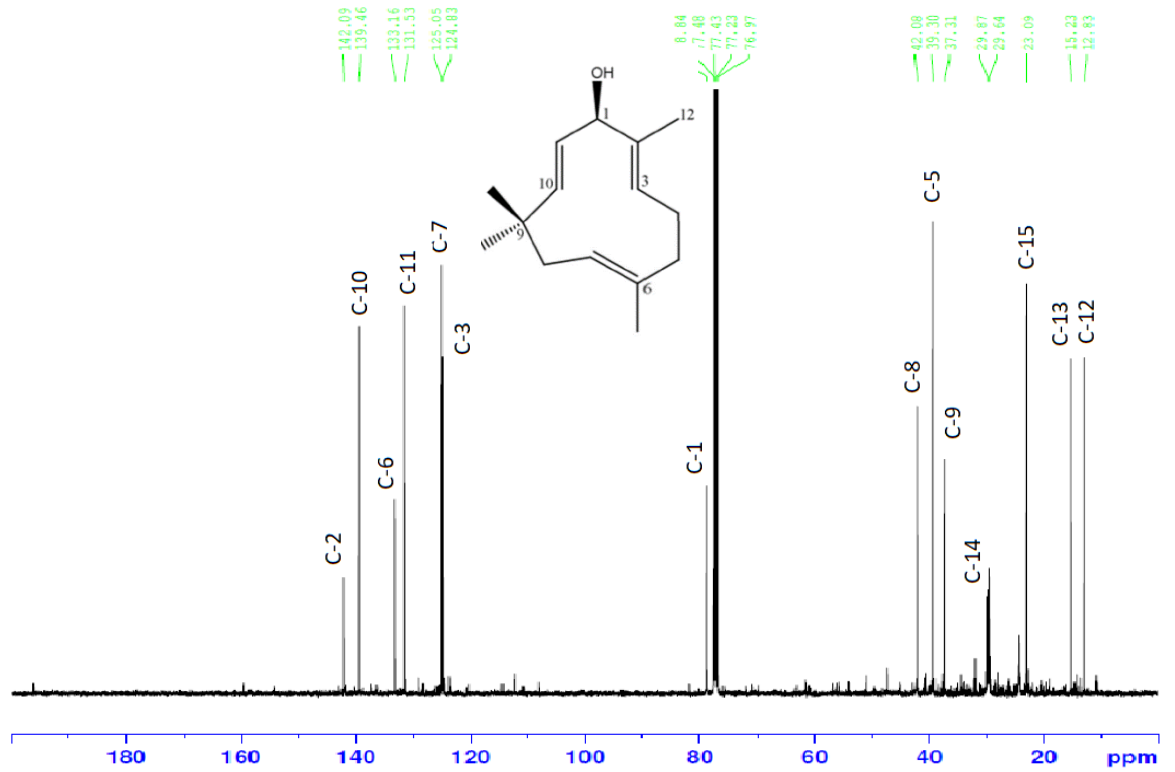


Figure 3.25:  $^{13}\text{C}$  NMR (125MHz) spectrum of MRSH 6 in  $\text{CDCl}_3$

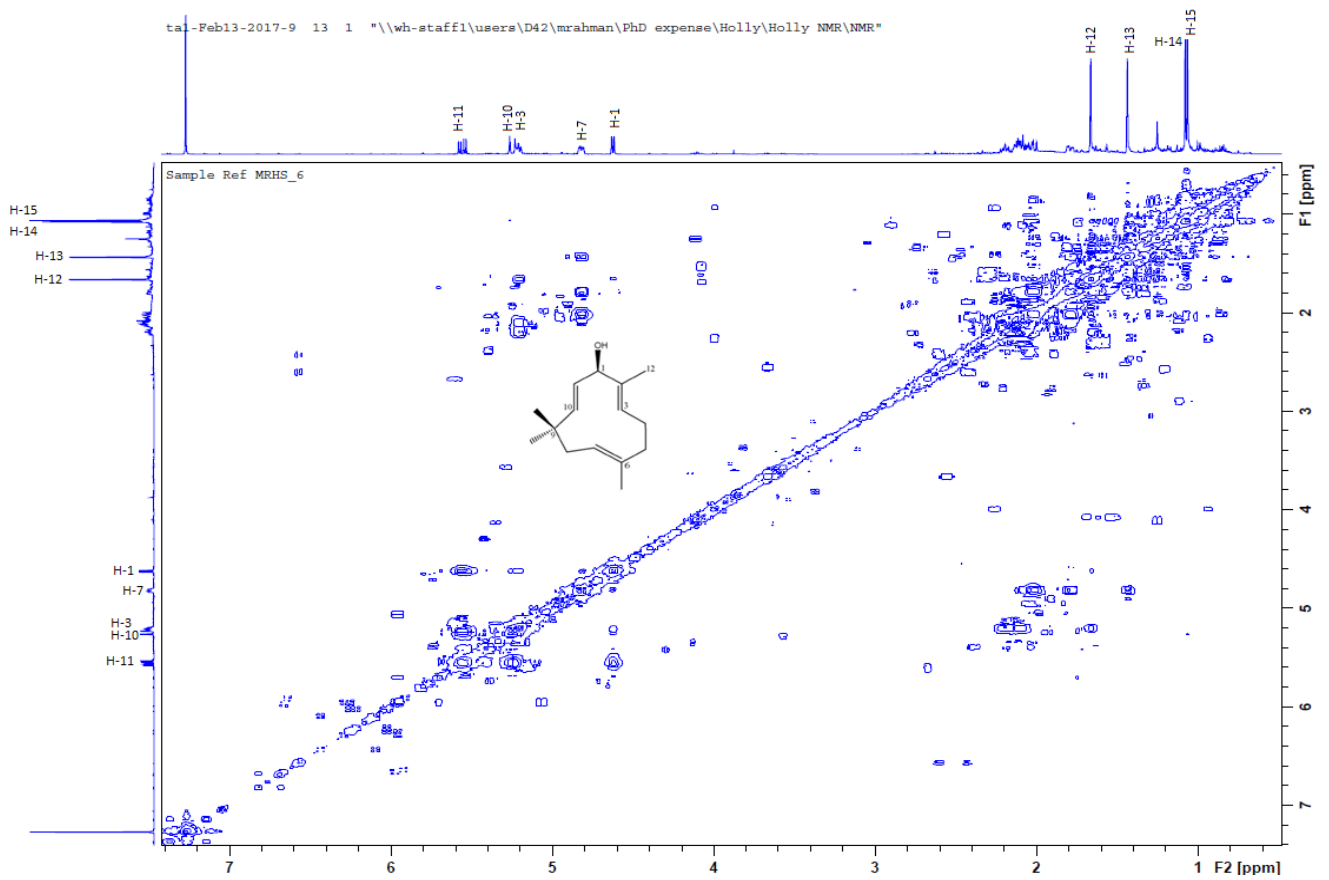
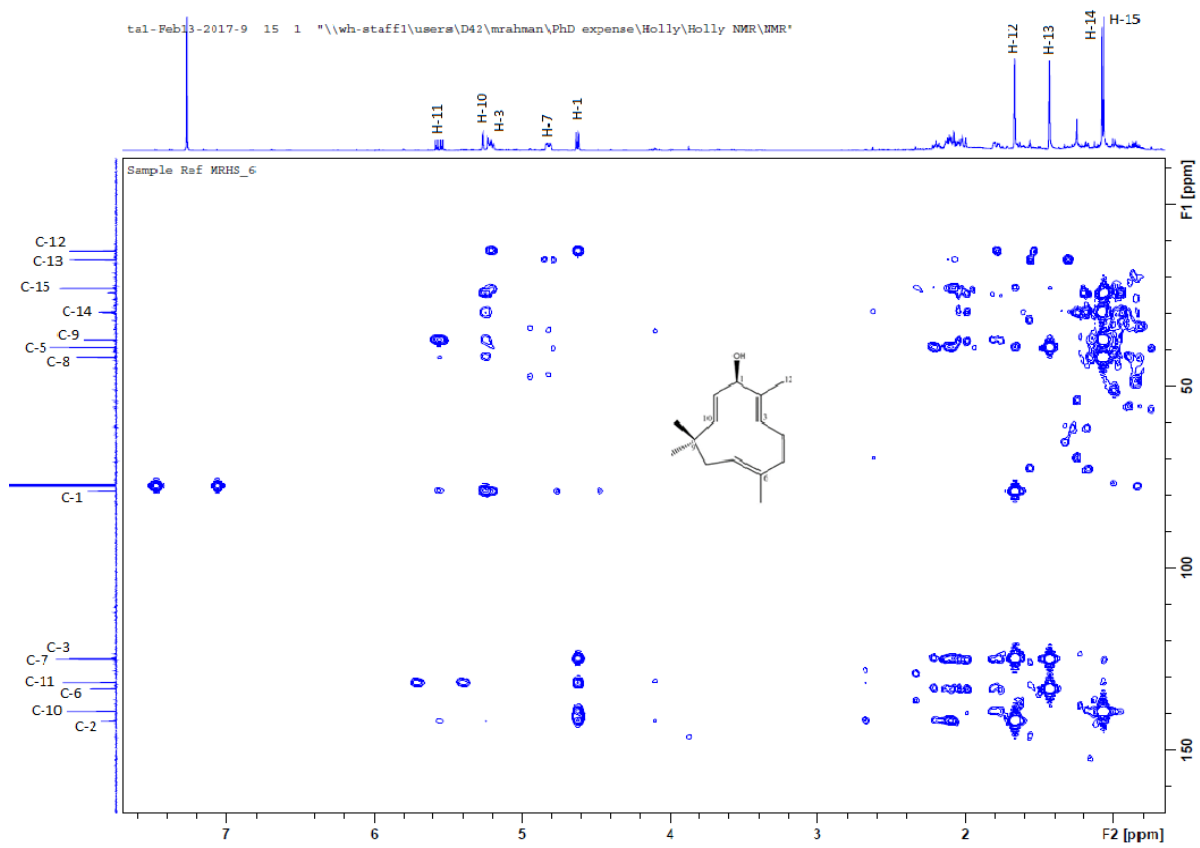


Figure 3.26: COSY NMR spectrum of MRSH 6 in  $\text{CDCl}_3$

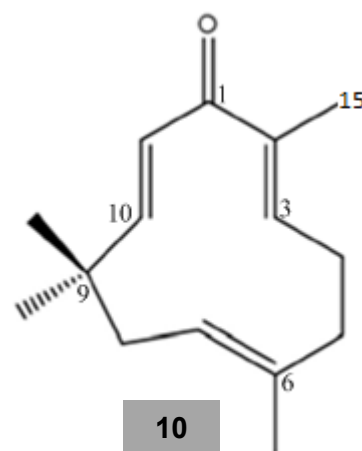


### 3.2.4.2 Characterization of MRSH 3 as (2Z, 6Z, 10E) -2,6,9,9-tetramethylcycloundeca-2, 6, 10- trien-1-one (Zerumbone; 10):

The IR spectrum of MRSH 3 showed the presence of carbonyl group at  $1658\text{ cm}^{-1}$  (Figure 3.6). The high resolution of mass spectrometry showed the  $[M+H]^+$  at  $m/z$  219.1746 (calculated for  $C_{15}H_{23}O$  at 219.174890) which confirmed the molecular formula of MRHS-3 as  $C_{15}H_{22}O$ .

The  $^1H$  NMR spectrum of MRSH 3 ( $CDCl_3$ , 500 MHz; Table 3.13; Figure 3.26) showed the presence of four methyl singlets resonating at  $\delta_H$  1.04, 1.07, 1.51, 1.77, four sets of olefinic protons at  $\delta_H$  5.22 (d,  $J=11.5\text{ Hz}$ ), 5.83 (d,  $J=16.5\text{ Hz}$ ), 5.94 (d,  $J=16\text{ Hz}$ ) and 5.99 (d,  $J=11\text{ Hz}$ ), and also couple of methine and methylene protons peaks between  $\delta_H$  1.85-2.44 Hz. The  $^{13}C$  NMR spectrum (125 MHz,  $CDCl_3$ ; Table 3.13; Figure 3.27) revealed the presence of a total of 15 carbons including a carbonyl at  $\delta_C$  204.4. The DEPT135 (Figure 3.28) identified four methyl, three methylene, four olefinic carbons. So the remaining three carbons were quaternary. Among the later three quaternary carbons, one at  $\delta_C$  37.9 was aliphatic and remaining two were connected double bonds. The complete structure of this compound was established by 2D NMR spectra, predominantly by HMQC and HMBC. In the  $^1H$ - $^1H$  COSY spectrum (Figure 3.29), the *trans* double bonded protons showed expected interaction between them.

In the HMBC experiment (Table 3.14; Figure 3.31), the *trans* double bonded proton at  $\delta_H$  5.83 ( $\delta_C$  160.8 from HMQC) and methyl protons at  $\delta_H$  1.77 ( $\delta_C$  11.9 from HMQC) revealed common  $^3J$  interaction to a carbonyl at  $\delta_C$  204.4 (C-1). Olefinic proton at  $\delta_H$  5.12 (H-7;  $\delta_C$  125.0 ppm from HMQC) showed  $^3J$  correlation to quaternary carbon at  $\delta_C$  136.5 ( $^2J$  connectivity to methyl carbon at  $\delta_C$  15.3 (H-13;  $\delta_H$  1.51). The later methyl protons were also exhibited  $^3J$  correlation at carbon  $\delta_C$  125 (C-7) and methylene carbon at  $\delta_C$  39.5 (C-5;  $\delta_H$  2.35 from HMQC). Two methyl groups at  $\delta_H$  1.17 ( $\delta_C$  24.3) and 1.04 ( $\delta_C$  29.5) revealed common  $^3J$  interaction to methine carbon at  $\delta_C$  168.8 (C-10,  $\delta_H$  5.82) and methylene at  $\delta_C$  42.5 (C-8,  $\delta_H$  1.87). H-10 also revealed  $^3J$  interaction to both methyl's at  $\delta_C$  24.3 (C-14) and  $\delta_C$  29.5 (C-15). The cosy experiment exhibited usual interaction (H-



10 to H-11); H-3 to H-4; H-7 to H-6 and H-8). According to the compound spectra, it was identified as (2Z, 6Z, 10E)-2,6,9,9- tetramethylcycloundeca-2,6,10-trien-1-one (**10**), known as zerumbone (Riyanto, 2007), this compound was previously reported from *Zingiber zerumbet* and also known as ginger sesquiterpene. (Takashi et al., 2002)

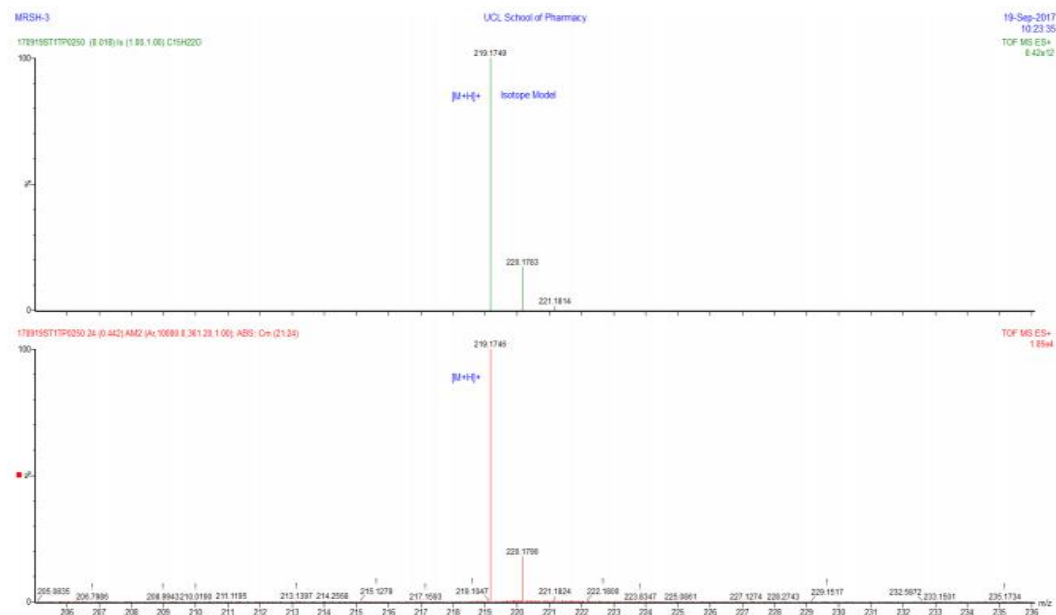


Figure 3.28 HRMS data of MRSH 3

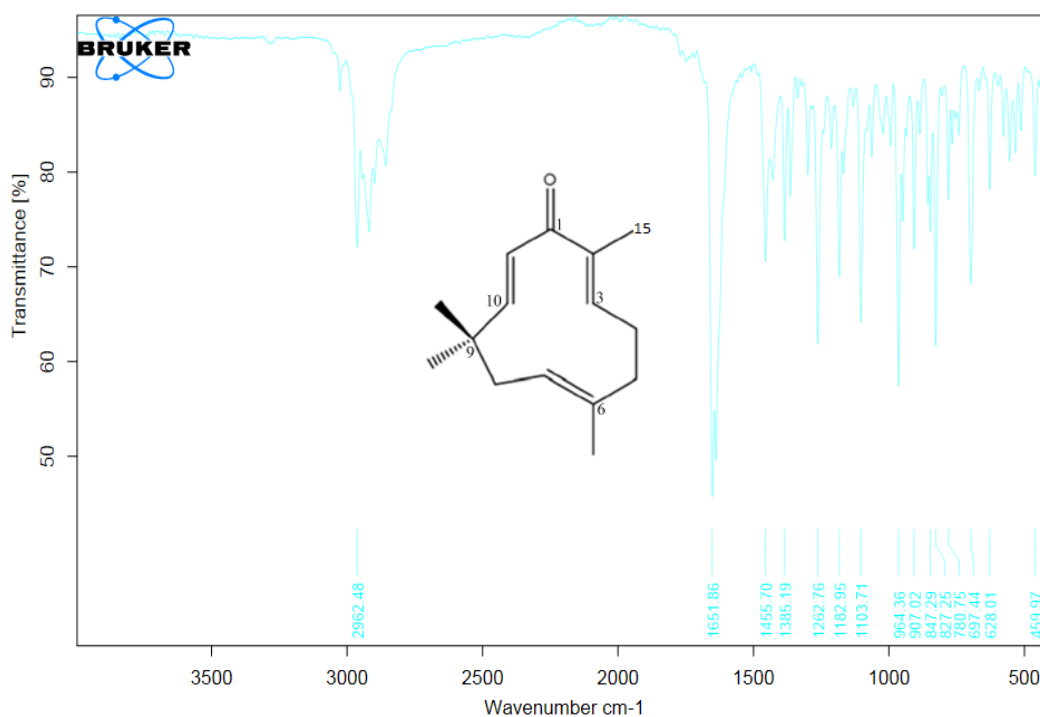


Figure 3.29: IR data of MRSH 3

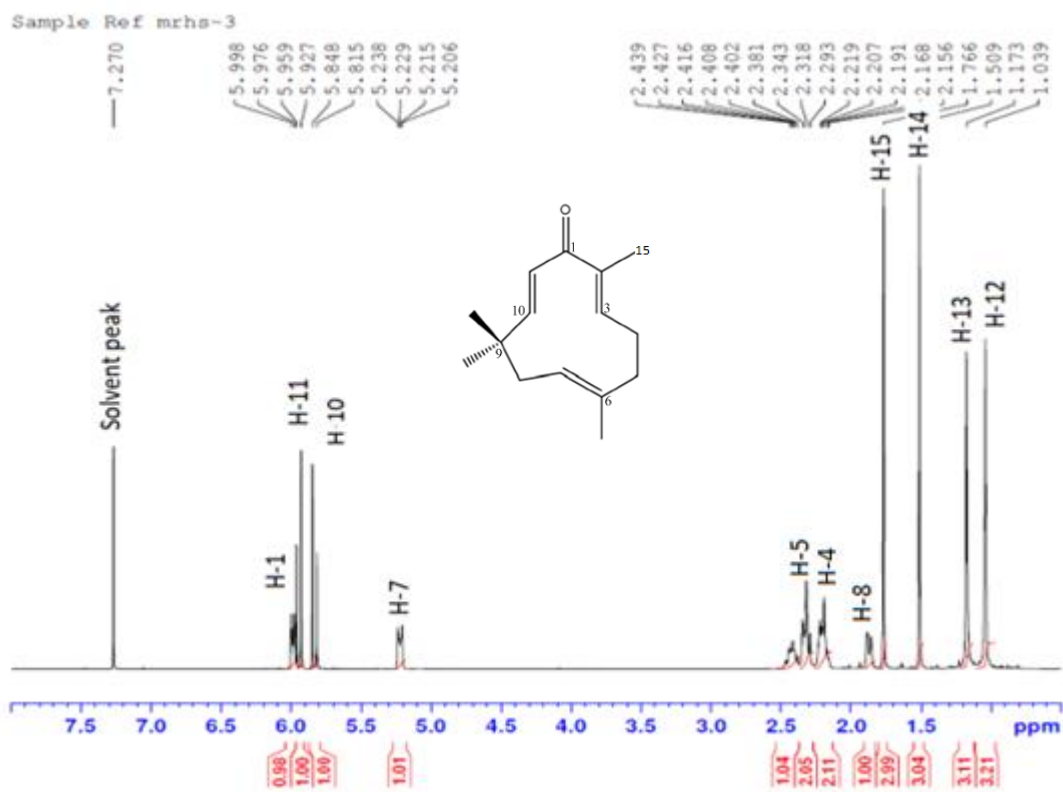


Figure 3.30: <sup>1</sup>H NMR spectrum (500MHz) of MRSH 3 in CDCl<sub>3</sub>

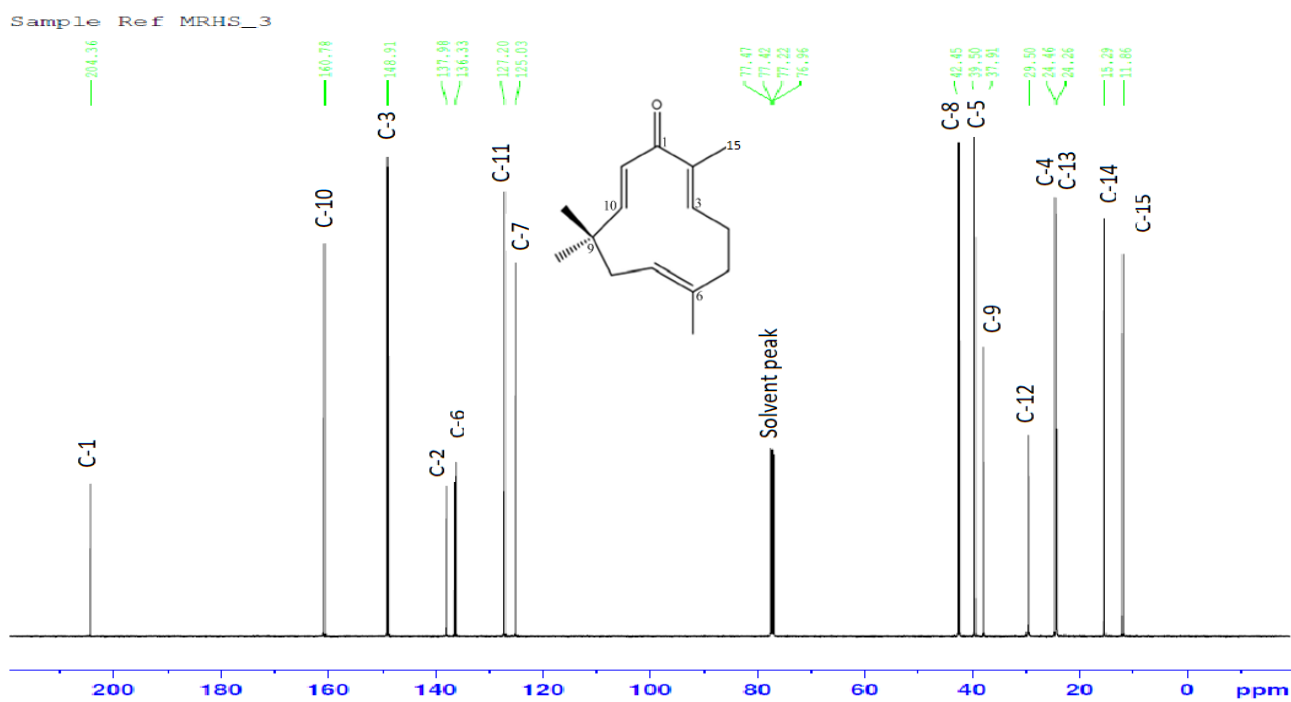


Figure 3.31: <sup>13</sup>C NMR spectrum (125MHz) of MRSH 3 in CDCl<sub>3</sub>



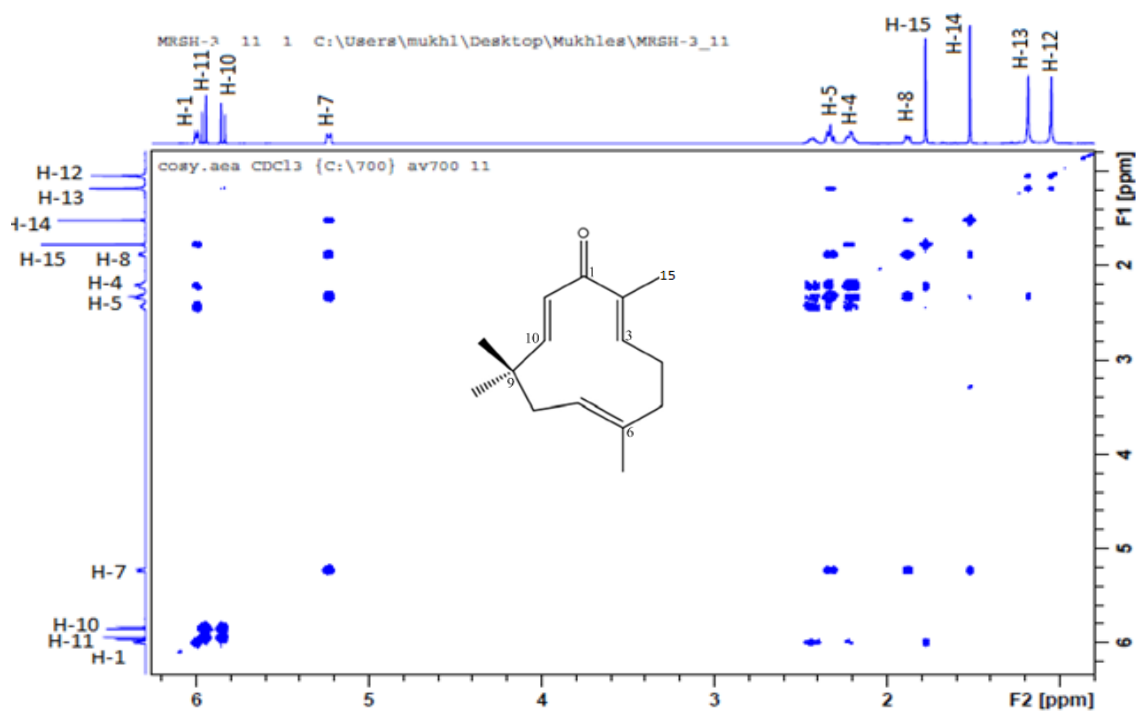
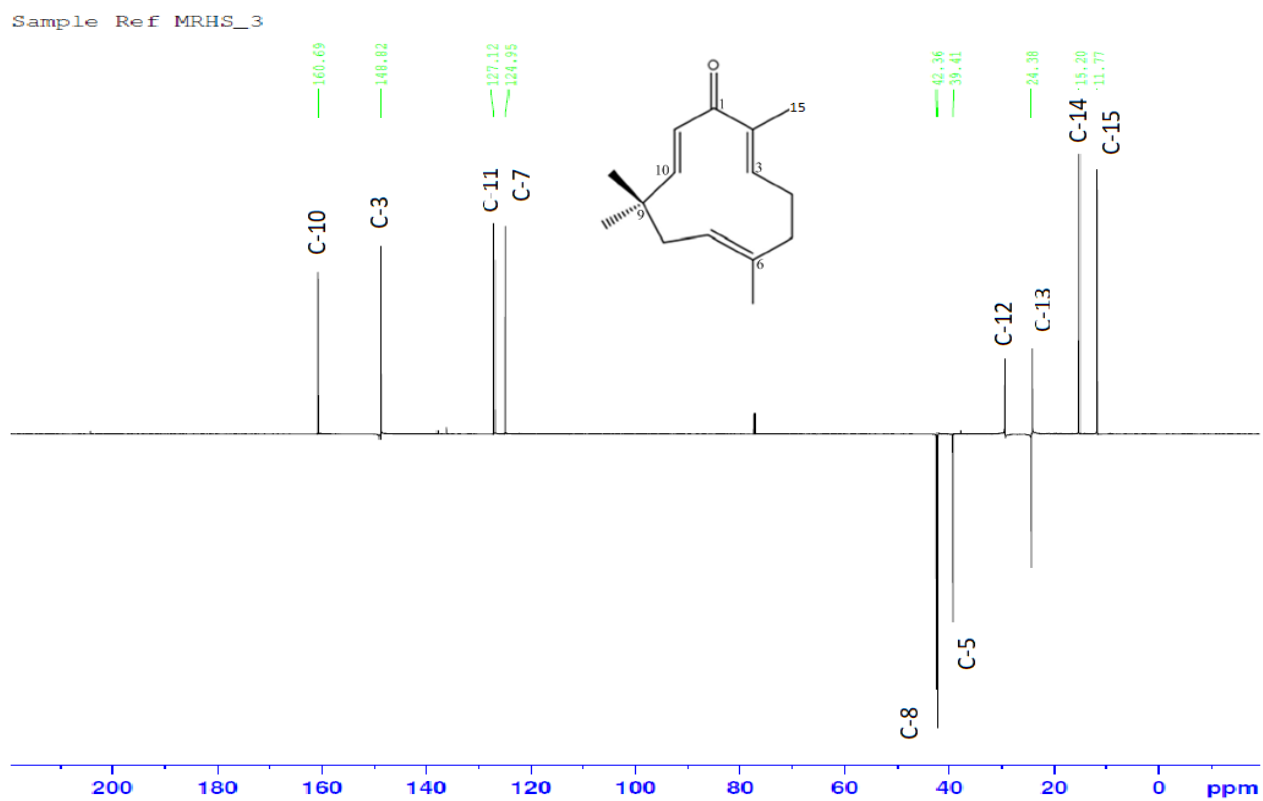


Figure 3.33: COSY NMR spectrum of MRSH 3 in  $\text{CDCl}_3$

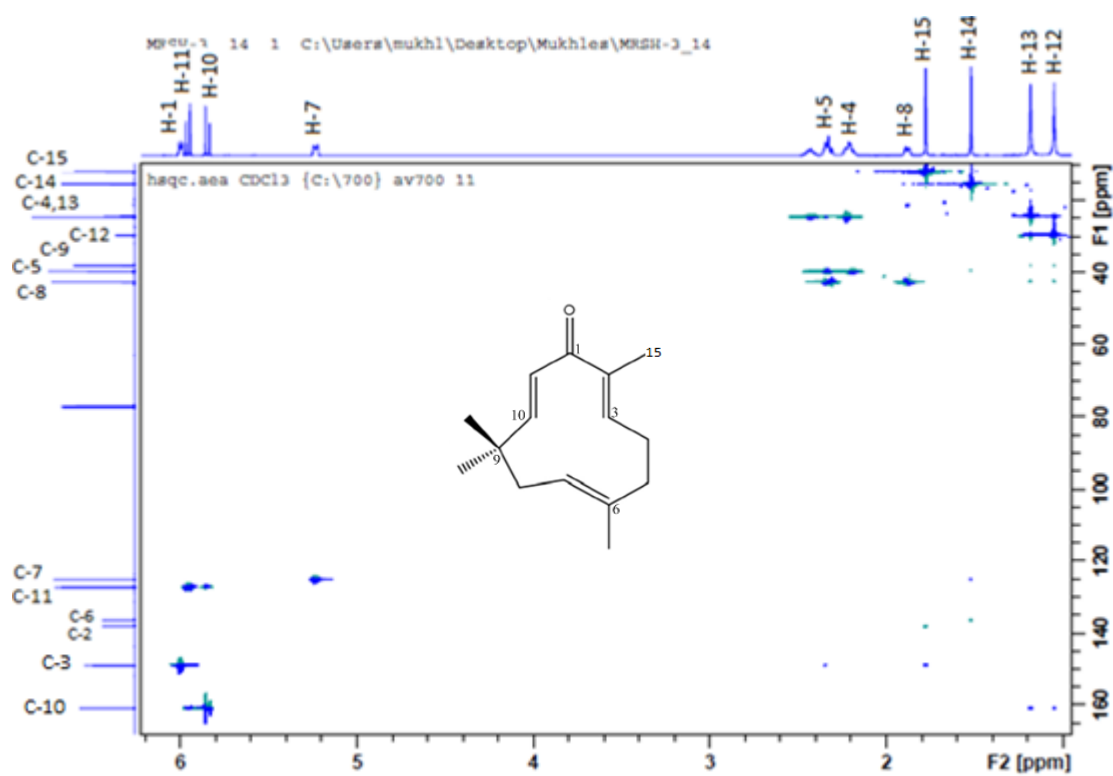


Figure 3.34: HSQC NMR spectrum of MRSH 3 in CDCl<sub>3</sub>

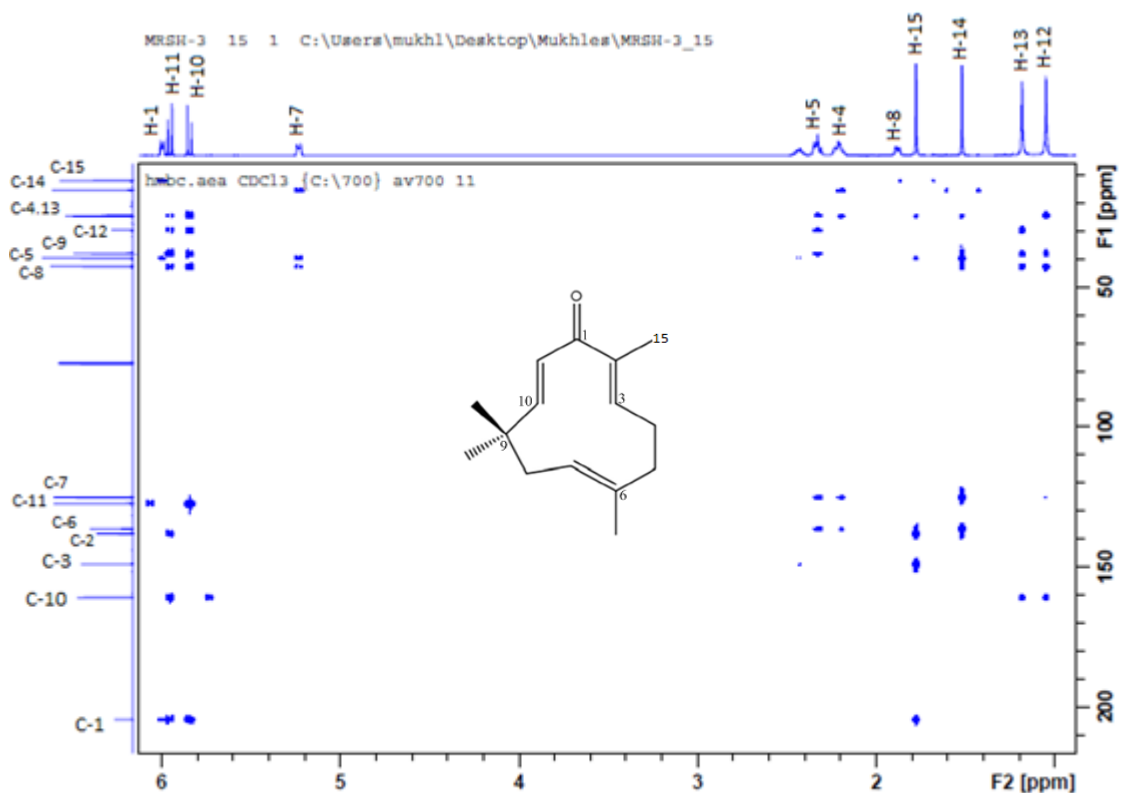
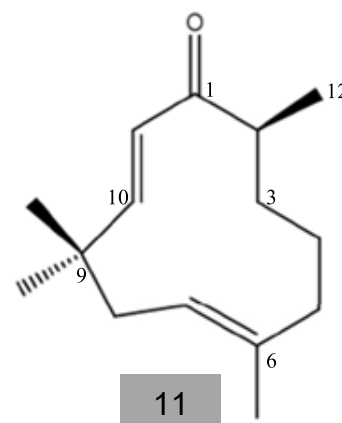


Figure 3.35: HMBC NMR spectrum of MRSH 3 in CDCl<sub>3</sub>

### 3.2.4.3 Characterization of MRSH-2 as (6E, 10E)-2, 6, 9, 9-tetramethylcycloundeca-6, 10-dien-1-one (**11**; Buddledone A):

The IR spectrum showed the presence of carbonyl group at  $1692\text{ cm}^{-1}$ . The high resolution of mass spectroscopy showed the  $[M+H]^+$  at  $m/z$  221.19103 (calculated for  $C_{15}H_{25}O$  at 221.19054) which confirmed the molecular formula of MRHS-2 as  $C_{15}H_{24}O$ .

The  $^1H$  and  $^{13}C$  NMR data of MRSH 2 were very similar to those of MRSH 3. Like MRSH 3, the  $^1H$  NMR (500 MHz,  $CDCl_3$ ) (Table 3.13; Figure 3.32) spectrum of MRSH 2 showed the presence of four methyl singlets at  $\delta_H$  1.02, 1.15, 1.16, 1.42, trans double bonded protons at  $\delta_H$  6.02 (H-10) and  $\delta_H$  6.20 (H-11) and one methine proton as doublet at  $\delta_H$



5.06 ( $J= 8.5\text{Hz}$ ). The only difference was that there was no olefinic proton at  $\delta_H$  5.99 at position H-3, however the presence of methine was observed at  $\delta_H$  2.52, t,  $J= 8\text{ Hz}$  at position H-2. Like MRSH 3, the  $^{13}C$  NMR spectra (125 MHz,  $CDCl_3$ ) (Table 3.13; Figure 3.33) showed the presence of total 15 carbons including carbonyl. DEPT NMR spectra showed the presence of 3 methyl, 3 olefinic methine carbons remaining methylene and 2 quaternary carbons. In comparison with  $^1H,^{13}C$  NMR of MRSH 3, MRSH 2 was identified as (6E, 10E)-2, 6, 9, 9-tetramethylcycloundeca-6, 10-dien-1-one (**11**), also known as Buddledone A, previously reported from Chinese traditional medicinal plant *Buddleja globosa*.(Cai et al., 2012).

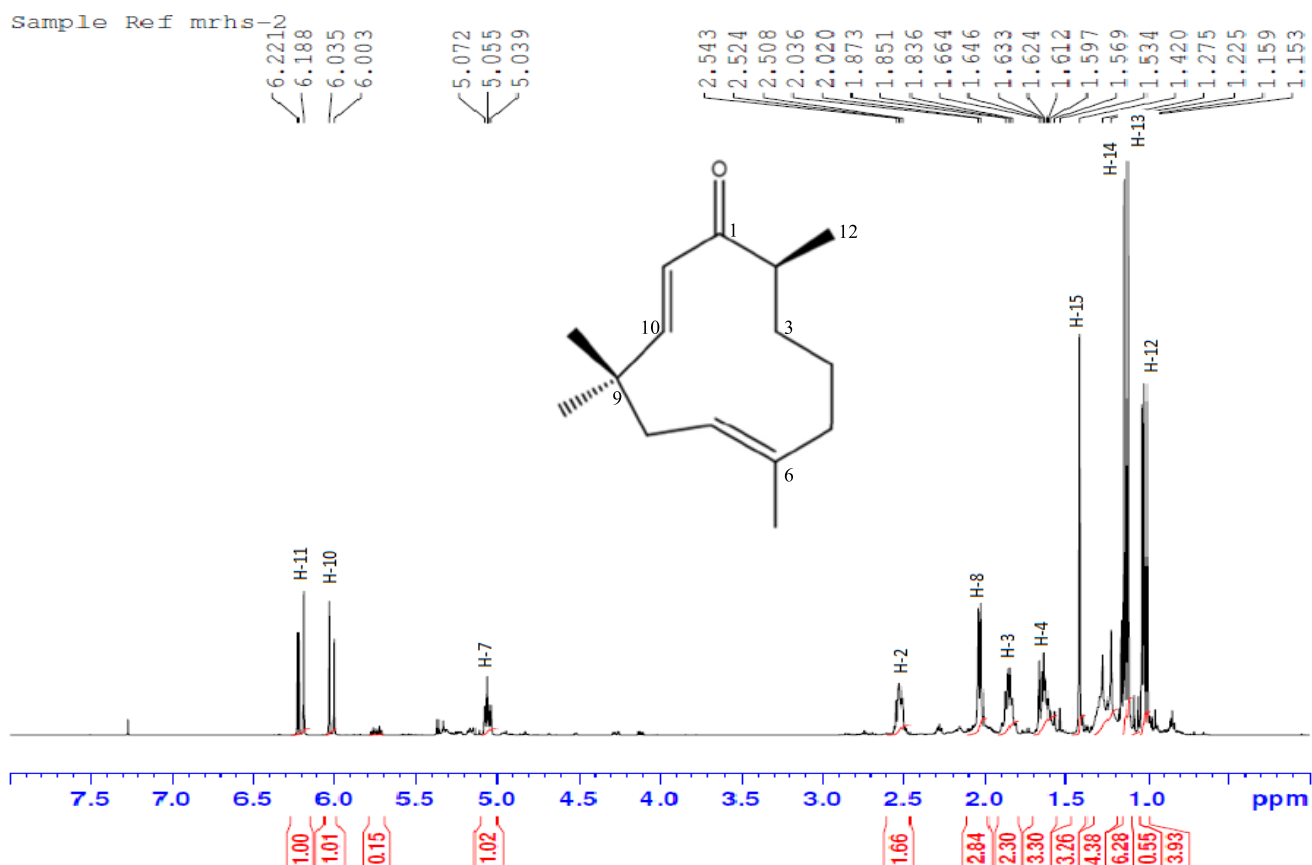


Figure 3.36:  $^1\text{H}$  NMR spectrum (500MHz) of MRSH 2 in  $\text{CDCl}_3$

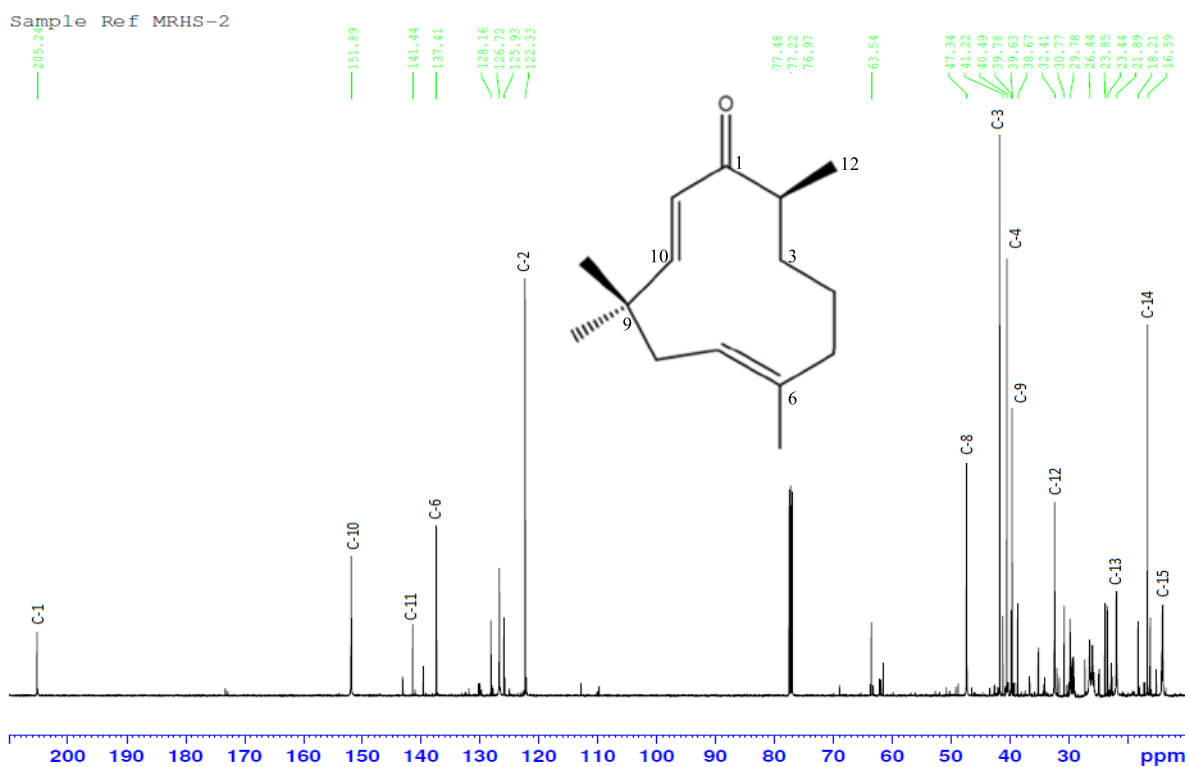


Figure 3.37:  $^{13}\text{C}$  NMR spectrum (125MHz) of MRSH 2 in  $\text{CDCl}_3$

**Table 3.13:  $^1\text{H}$  (500 MHz) and  $^{13}\text{C}$  NMR (125 MHz) spectroscopic data for MRSH-6, MRSH-3 and MRSH-2 in  $\text{CDCl}_3$**

MRHS- 6			MRSH -3		MRSH -2	
Position	$\delta_{\text{H}}$	$^{13}\text{C}$	$\delta_{\text{H}}$	$^{13}\text{C}$	$\delta_{\text{H}}$	$^{13}\text{C}$
1	4.63, d, $J= 7.5$ Hz	78.8	-	204.4	-	205.2
2	-	142.2	-	138.0	2.52, t, $J= 8$ Hz	122.3
3	5.20, d, $J= 7.5$	124.8	5.99, d, $J=11$ Hz	148.9	1.85, t, $J= 7.5$ Hz	42.0
4	2.20, m 2.24, m	24.4	2.20, m; 2.24,m	24.4	1.65, m	40.5
5	2.35, m	39.5	2.35, m	39.5	1.26, m	38.6
6	-	133.2	-	136.3	-	137.4
7	4.82, dd, $J= 10$ Hz	125.0	5.22, dd, $J= 11.5$ Hz	125.0	5.06, d, $J= 8.5$ Hz	123.3
8	1.87, m 2.32, m	42.4	1.87, m; 2.32, m	42.4	2.03, d, $J=8$ Hz	47.3
9	-	37.3	-	37.9	-	39.6
10	5.23, d, $J= 16.5$ Hz	139.5	5.83, d, $J=16.5$ Hz	160.8	6.02, d, $J=16.5$ Hz	151.9
11	5.56, dd, $J= 16$ Hz	131.2	5.94, d, $J=16$ Hz	127.2	6.20, d, $J= 16$ Hz	141.4
12	1.65, s	12.8	1.04, s	29.5	1.02, s	32.4
13	1.43, s	15.3	1.17, s	24.3	1.15, s	21.2
14	1.06, s	29.9	1.51, s	15.3	1.16, s	16.6
15	1.04, s	24.9	1.77, s	11.9	1.42, s	14.1

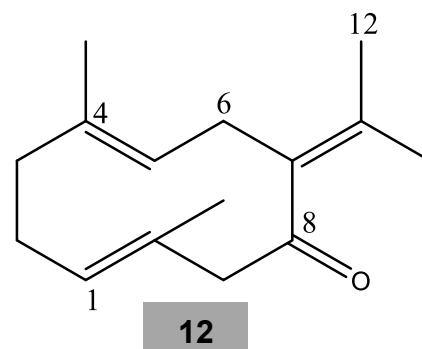
**Table 3.14: HMBC correlation data for MRSH-6, MRSH-3 in CDCl<sub>3</sub>**

Protons	HMBC of MRHS - 6		HMBC of MRSH - 3	
	<sup>2</sup> J	<sup>3</sup> J	<sup>2</sup> J	<sup>3</sup> J
H-3	-	78.8 (CH), 12.8 (CH <sub>3</sub> )	-	204.4 (C), 11.9 (CH <sub>3</sub> )
H-4	124.8 (CH)	133.2 (C)	148.9(CH)	136.5 (C)
H-7	-	39.5 (CH <sub>2</sub> )	42.5 (CH <sub>2</sub> )	39.5 (CH <sub>2</sub> ), 15.3 (CH <sub>3</sub> )
H-8	125.0 (CH)	133.2 (C)	125.0(CH)	136.3 (C)
H-10	-	78.8 (CH)	-	204.4 (C), 42.5 (CH <sub>2</sub> )
H-11	78.8 (CH), 139.5 (CH)	-	204.4 (C), 160.8 (CH)	37.9 (C)
H-12	142.2 (C)	78.8 (CH),124.8 (CH)	138.0 (C)	148.9 (CH), 204.4(C)
H-13	133.2 (C)	39.5 (CH <sub>2</sub> )	136.3 (C)	125.0 (CH)
H-14	37.3 (C)	139.5 (CH)	-	160.8 (CH)
H-15	37.3 (C)	139.5 (CH)	-	160.8 (CH)

#### 3.2.4.4 Characterization of ZMH 4 as 1(10), 4, 7(11)-Germacatrien-8-one (12):

The IR spectrum of ZMH 4 showed the presence of carbonyl group at 1676 cm<sup>-1</sup>. The high resolution of mass spectrometry showed the [M+H]<sup>+</sup> at m/z 219.17423 (calculated for C<sub>15</sub>H<sub>23</sub>O, at 219.17489) which confirmed the molecular formula of ZMH 4 as C<sub>15</sub>H<sub>22</sub>O.

The <sup>1</sup>H NMR spectrum (600MHz, CDCl<sub>3</sub>; Table 3.15; Figure 3.34) showed the presence of 4 methyl singlets resonating at δ<sub>H</sub> 1.44, 1.63, 1.72 and 1.77, two olefinic protons at δ<sub>H</sub> 4.98 and 4.70 and couple of methine and methylene peaks between 2.0-3.5 ppm. The <sup>13</sup>C NMR spectrum (150 MHz, CDCl<sub>3</sub>; Table 3.15; Figure 3.35)



revealed the presence of a total of 15 carbons including carbonyl at δ<sub>C</sub> 208.2. The DEPT135 spectrum confirmed the presence of 4 methylene, 4 methyl and two olefinic methine carbons and remainders as quaternary carbons.

The structure of this compound was confirmed by 2D NMR, predominantly the HMBC experiment. The two methyl groups (δ<sub>H</sub> 1.72 and 1.77) showed common <sup>2</sup>J connection to a quaternary carbon at δ<sub>C</sub> 137.4 (C-11) and <sup>3</sup>J correlation to another quaternary carbons at δ<sub>C</sub> 129.8 (C-7). So, this two methyl are connected to each other. The olefinic proton at 4.98 (H-1; δ<sub>C</sub> 132.9 from HMQC) was connected to methylene carbon at δ<sub>C</sub> 56.1 (C-2; δ<sub>H</sub> 3.41, 2.93 from HMQC) and a methyl carbon at δ<sub>C</sub> 16.9 (C-15; δ<sub>H</sub> 1.63 from HSQC). The other olefinic proton at δ<sub>H</sub> 4.71 (H-5) showed HMBC correlations to methyl carbon at δ<sub>C</sub> 15.8 (C-14; δ<sub>H</sub> 1.44) and methylene carbon at δ<sub>C</sub> 38.3 (C-3; δ<sub>H</sub> 2.09, 2.16, m). The methyl proton at δ<sub>H</sub> 1.63 (H-15, δ<sub>C</sub> 16.9 from HSQC) showed <sup>2</sup>J connection with olefinic quaternary carbon at δ<sub>C</sub> 127.0 (C-10) and <sup>3</sup>J connection with methylene carbon at δ<sub>C</sub> 56.1 (C-9; δ<sub>H</sub> 2.93, 3.41, m) and olefinic methine carbon at δ<sub>C</sub> 132.9 (C-1; δ<sub>H</sub> 4.98, d, J= 11.3 Hz). According to the <sup>1</sup>H, <sup>13</sup>C NMR spectra and HSQC and HMBC experiment, the compound ZMH 4 was identified as 1(10),4,7(11)-Germacatrien-8-one (12), commonly known as Germacrone (Yan et al., 2005) which was previously isolated and identified from *Curcuma heyneana* (one of the zingiberaceous plant) (Firman et al., 1988)

**Table 3.15:  $^1\text{H}$  (600 MHz) and  $^{13}\text{C}$  (150 MHz) NMR spectroscopic data of ZMH 4 in  $\text{CDCl}_3$**

Position	$\delta_{\text{H}}$	$^{13}\text{C}$
1	4.98, d, $J=10.74$ Hz	132.9
2	2.07, m 2.37, m	24.3
3	2.16, m 2.09, m	38.3
4	-	135.3
5	4.71, d, $J=9.54$ Hz	125.6
6	2.85, m 2.95, m	29.5
7	-	129.8
8	-	208.2
9	2.93, m 3.41, d, $J=10.38$ Hz	56.1
10	-	127.0
11	-	137.4
12	1.77, s	20.1
13	1.72, s	22.6
14	1.44, s	15.8
15	1.63, s	16.9

**Table 3.16:  $^1\text{H}$ - $^{13}\text{C}$  correlation of ZMH 4 in  $\text{CDCl}_3$**

Protons	HMBC of ZMH 4	
	$^2J$	$^3J$
H-1	-	56.1 ( $\text{CH}_2$ ), 16.9 ( $\text{CH}_3$ )
H-2	38.3 ( $\text{CH}_2$ )	127.0 (C)
H-3	24.3 ( $\text{CH}_2$ ), 135.3 (C)	125.2 (CH), 15.8 ( $\text{CH}_3$ )
H-5	-	38.3 ( $\text{CH}_2$ ), 15.8 ( $\text{CH}_3$ )
H-6	125.2 (CH), 129.8 (C)	135.3 (C), 137.4 (C)
H-9	127.0 (C)	132.9 (CH), 16.9 ( $\text{CH}_3$ )
H-12	137.4 (C)	22.6 ( $\text{CH}_3$ ), 129.8 (C)
H-13	137.4 (C)	20.1 ( $\text{CH}_3$ )
H-14	135.3 (C)	125.0 (CH)
H-15	127.0 (C)	56.1 ( $\text{CH}_2$ ), 132.9 (CH)



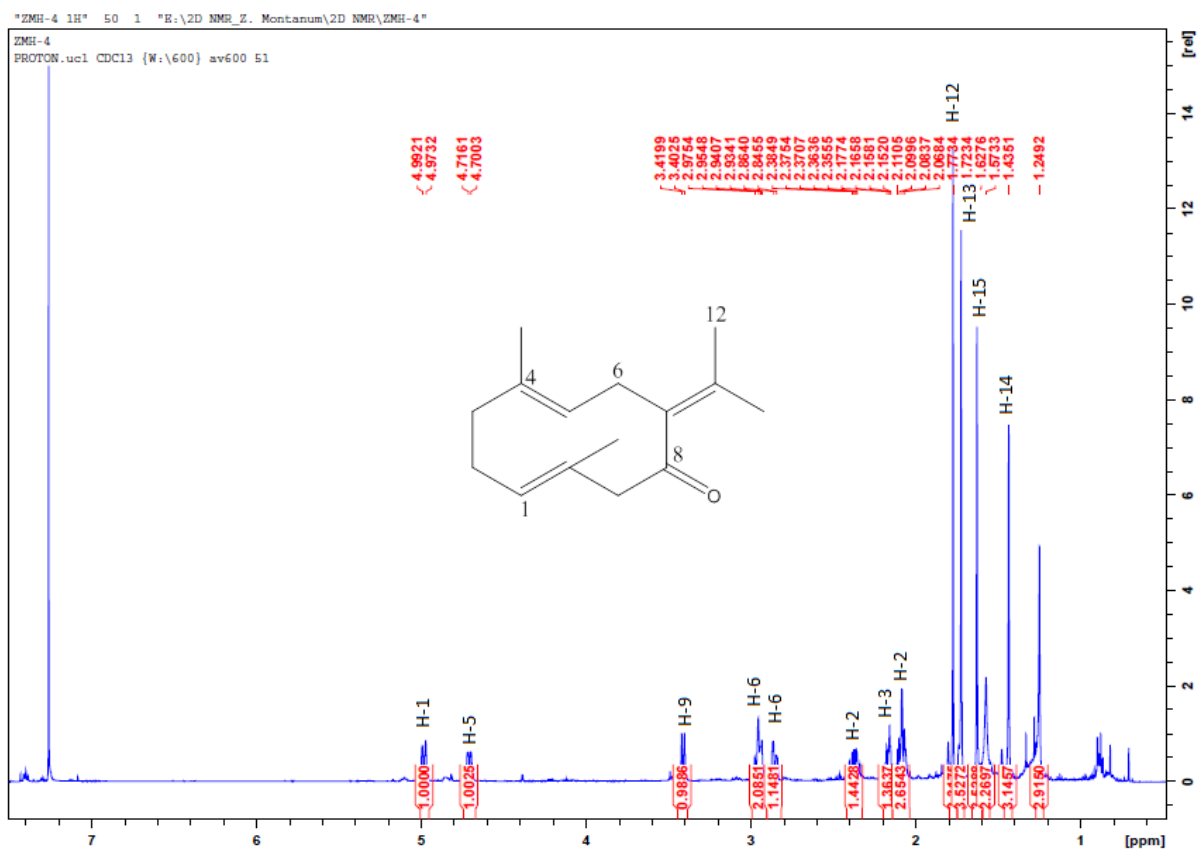


Figure 3.38:  $^1\text{H}$  NMR spectrum (600MHz) of ZMH 4 in  $\text{CDCl}_3$

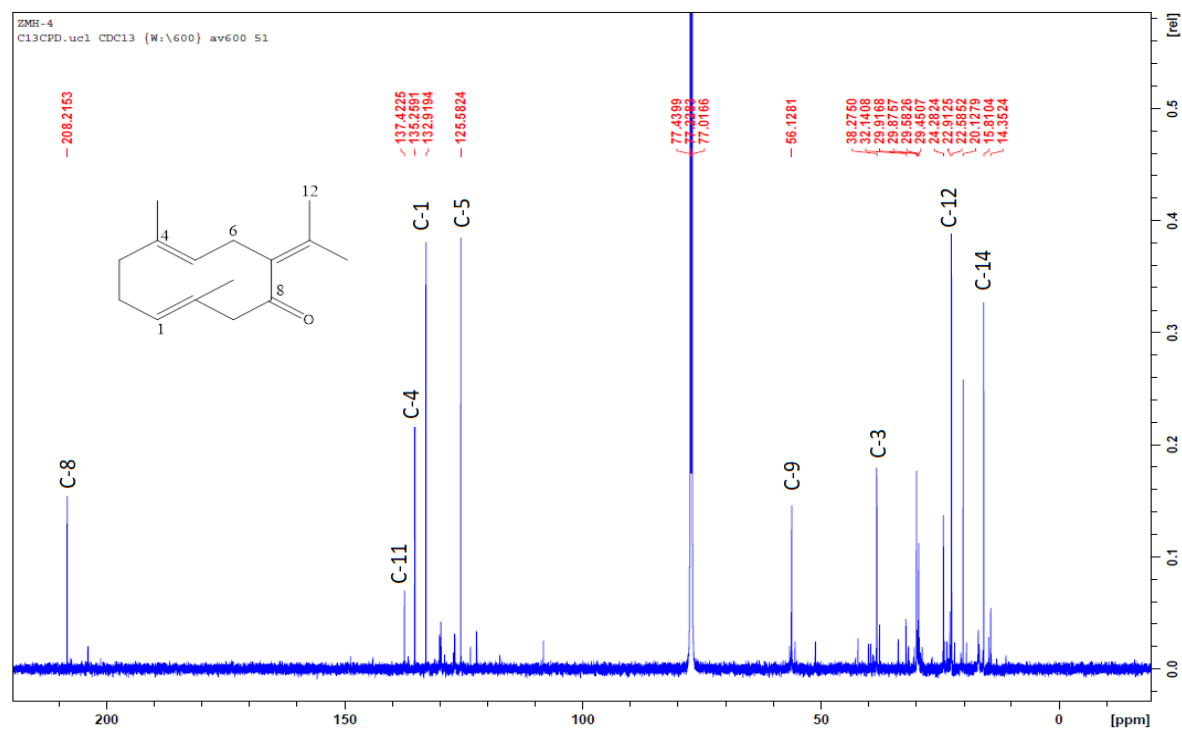
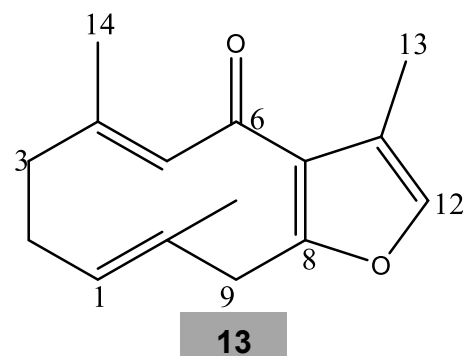


Figure 3.39:  $^{13}\text{C}$  NMR spectrum (150MHz) of ZMH 4 in  $\text{CDCl}_3$

### 3.2.4.5 Characterization of ZMH 5 as 8, 12-Epoxy-1(10), 4, 7, 11-germacratetraen-6-one (13; Furanodienone):

The IR spectrum revealed the presence of carbonyl group at  $1760\text{ cm}^{-1}$ . The high resolution mass spectrometry showed the  $[M+H]^+$  at  $m/z$  231.13720 (calculated for  $C_{15}H_{19}O_2$ , at 231.13851) which confirmed the molecular formula of ZMH 5 as  $C_{15}H_{18}O_2$ .

$^1H$  NMR (600 MHz,  $CDCl_3$ , Table 3.17, Figure 3.36) showed the presence of three sets of methyl signals at  $\delta_H$  1.30,  $\delta_H$  1.99 and  $\delta_H$  2.13, three sets of olefinic protons at  $\delta_H$  7.08 (s),  $\delta_H$  5.81 (s), and  $\delta_H$  5.18 (dd,  $J= 11.52, 4.44$  Hz). There were also a number of methine and methylene peaks resonated at  $\delta_H$  2.13- 3.70.



$^{13}C$  NMR spectrum (150MHz,  $CDCl_3$ ; Table 3.17; Figure 3.37) revealed the presence of a total 15 carbons including carbonyl at 190.4. The DEPT 135 NMR spectra identified 3 methyl, 3 methylene, 4 methine and 6 quaternary carbons. Among the quaternary carbons, 5 were connected with double bonds and the remainder was the carbon of carbonyl at C-6 ( $\delta_C$  190.4)

In HMBC experiment (Table 3.18), the olefinic methine at  $\delta_H$  5.18 (H-1;  $\delta_C$  130.8 from HSQC) revealed  $^2J$  connection with methylene carbon at  $\delta_C$  41.9 (C-2; 3.70 from HSQC) and  $^3J$  correlation with methyl carbon at  $\delta_C$  16.2 (C-15; 2.13 from HSQC) and methylene carbon at  $\delta_C$  26.5 (C-9). The olefinic proton at  $\delta_H$  5.81 (H-5;  $\delta_C$  132.5) and methyl proton at  $\delta_H$  1.99 (H-14;  $\delta_C$  19.3 from HSQC) exhibit common  $^2J$  interaction with the double bonded quaternary carbon at  $\delta_C$  146.0 (C-6). However, olefinic proton at  $\delta_H$  5.81 (H-5) showed  $^2J$  connection with carbonyl carbon at  $\delta_C$  190.4 (C-6) while methyl proton at  $\delta_H$  1.99 (H-14;  $\delta_C$  19.3 from HSQC) showed  $^3J$  connection with olefinic methine carbon at  $\delta_C$  132.6 C-5;  $\delta_H$  5.81 from HSQC) and methylene carbon at  $\delta_C$  40.8 (C-3;  $\delta_H$  2.46 and 1.88 from HSQC). However, the downfield olefinic proton at  $\delta_H$  7.08 (H-12;  $\delta_C$  138.3 from HSQC) showed connection with quaternary carbon 122.4 (C-11) by  $^2J$  and oxygen bearing quaternary carbon at  $\delta_C$  156.7 (C-8) by  $^3J$ . Methyl protons at  $\delta_H$  1.30 (H-15;  $\delta_C$  16.2 from HSQC) showed  $^2J$  connection with quaternary carbon at  $\delta_C$  135.2 (C-10) and  $^3J$  interaction with the olefinic carbon at  $\delta_C$  130.7 (C-1;  $\delta_H$  5.18 from HSQC). Another the methyl proton at  $\delta_H$  2.13 (H-13;  $\delta_C$  9.7 from HSQC)

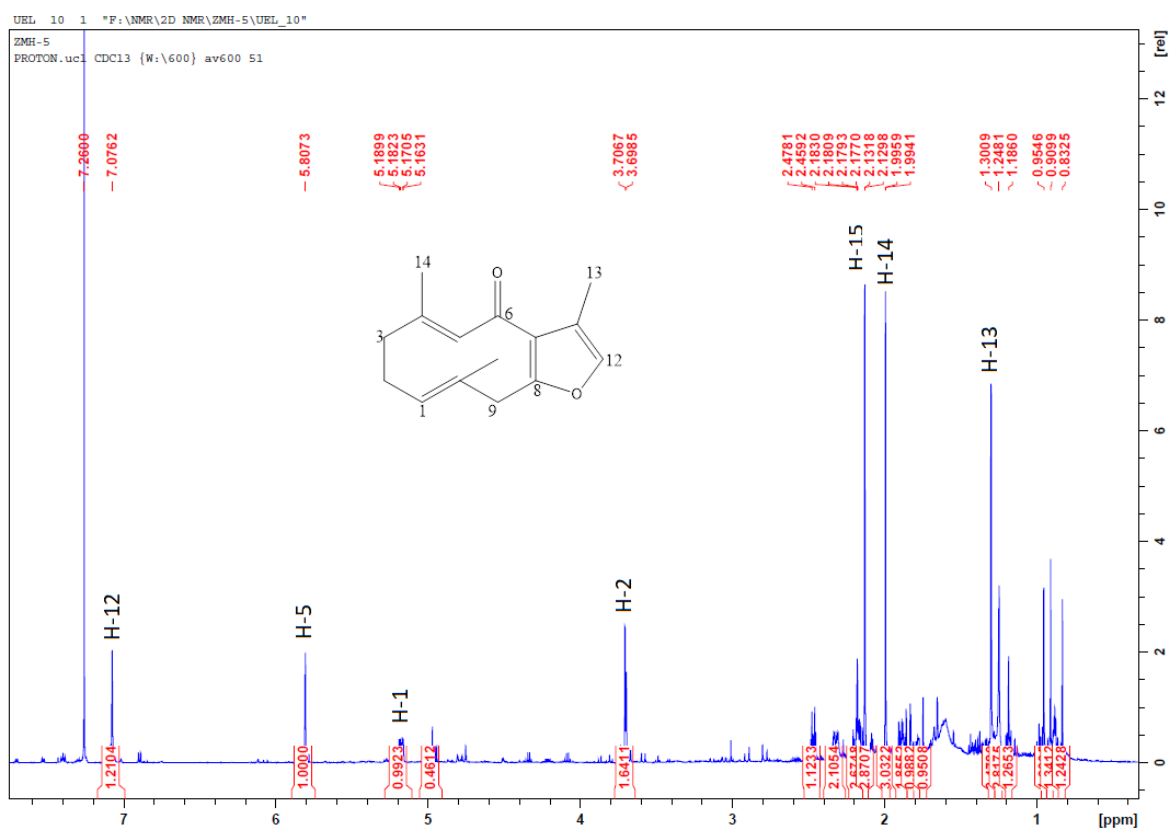
revealed 2 bond away correlation with quaternary carbon at  $\delta_C$  122.4 (C-11) and  $^3J$  connection with downfield olefinic carbon resonating at  $\delta_C$  138.3 ( $\delta_H$  7.08). According to NMR spectrum, the compound was identified as 8, 12-epoxy-1(10), 4, 7, 11-germacratetraen-6-one (**13**) also known as furanodienone (Joshi, 2014). Previously it was isolated from wild ginger *Siphonochilus aethiopicus*. (Igoli et al., 2012).

**Table 3.17:  $^1H$  (600 MHz) and  $^{13}C$  (150 MHz) NMR spectroscopic data of ZMH 5 in  $CDCl_3$**

Position	$^1H$	$^{13}C$
1	5.18, dd, $J= 11.52, 4.44$ Hz	130.7
2	3.70, d, $J=4.92$ Hz	41.9
3	2.46, m 1.88, m	40.8
4	-	146.1
5	5.81, s	132.6
6	-	190.4
7	-	123.9
8	-	156.7
9	2.32, m 2.18, m	26.5
10	-	135.2
11	-	122.4
12	7.08, s	138.3
13	2.13, d, $J= 1.20$ Hz	9.7
14	1.99, d, $J=2.88$ Hz	19.3
15	1.30, s	16.2

**Table 3.18:  $^1\text{H}$ - $^{13}\text{C}$  correlation for ZMH 5 in  $\text{CDCl}_3$**

Protons	HMBC	
	$^2J$	$^3J$
H-1	41.9 ( $\text{CH}_2$ )	16.2 ( $\text{CH}_3$ ), 26.5 ( $\text{CH}_2$ )
H-2	130.7 ( $\text{CH}$ )	135.2 ( $\text{C}$ )
H-3	146.1 ( $\text{C}$ )	19.3 ( $\text{CH}_3$ ), 130.7 ( $\text{CH}$ ), 132.6 ( $\text{CH}$ )
H-5	146.1 ( $\text{C}$ ), 190.4 ( $\text{C}=\text{O}$ )	19.3 ( $\text{CH}_3$ ), 40.8 ( $\text{CH}_2$ ),
H-9	-	130.7 ( $\text{CH}$ )
H-12	122.4 ( $\text{C}$ )	156.7 ( $\text{C}$ )
H-13	122.4 ( $\text{C}$ )	138.3 ( $\text{CH}$ )
H-14	146.1 ( $\text{C}$ )	40.8 ( $\text{CH}_2$ ), 132.6 ( $\text{CH}$ )
H-15	135.2 ( $\text{C}$ )	130.7 ( $\text{CH}$ )



**Figure 3.40:  $^1\text{H}$  NMR spectrum (600MHz) of ZMH 5 in  $\text{CDCl}_3$**

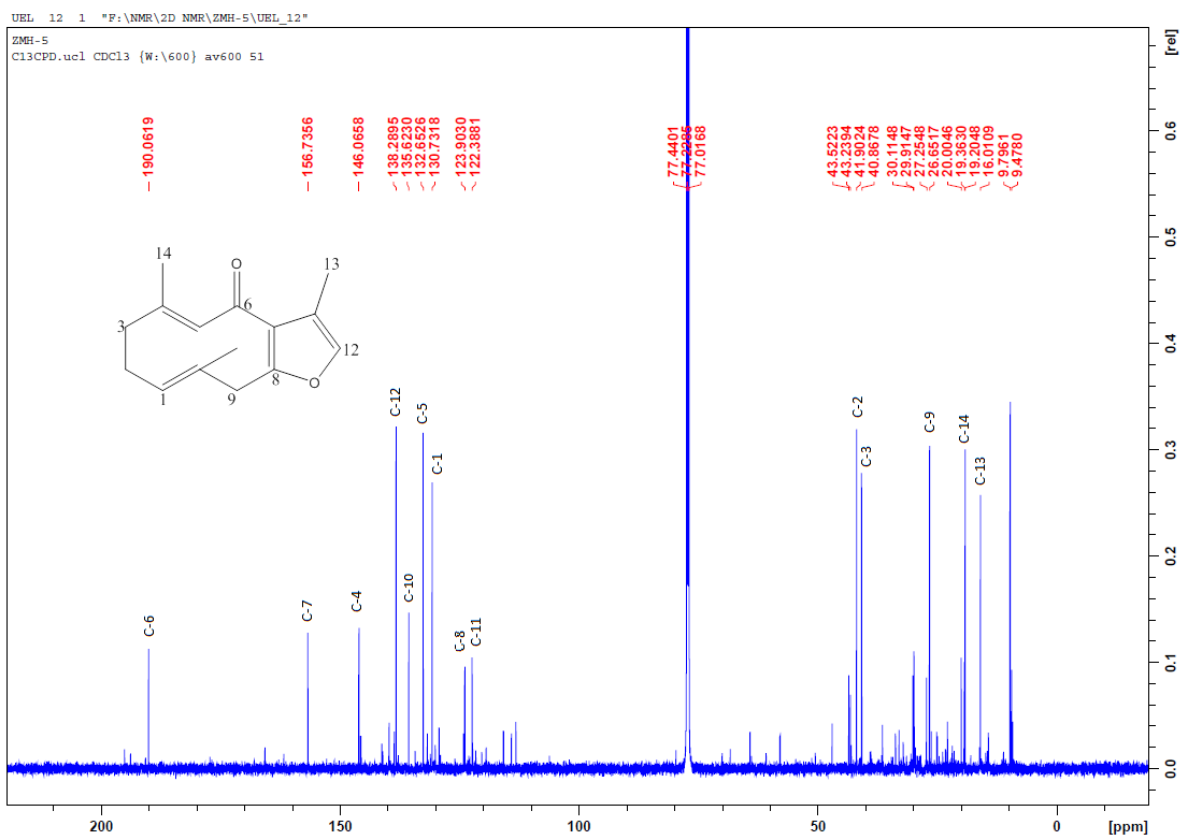


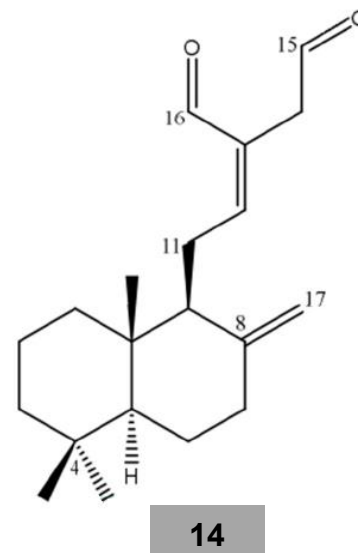
Figure 3.41: <sup>13</sup>C NMR spectrum (150MHz) of ZMH 5 in CDCl<sub>3</sub>

### 3.2.5 Diterpene:

#### 3.2.5.1 Characterization of ZMH 14 as 8(17), 12-Labdadiene-15, 16-dial (14):

ZMH 14 was isolated as a colourless amorphous powder from the  $\text{CHCl}_3$  extract of *Z. montanum*. The molecular formula was established as  $\text{C}_{20}\text{H}_{30}\text{O}_2$  from the  $[\text{M}+\text{H}]^+$  at  $m/z$  303.23122 (calculated for  $\text{C}_{20}\text{H}_{31}\text{O}_2$ , at 302.22458) in the high resolution mass spectroscopy which confirmed the molecular formula of ZMH 14 as  $\text{C}_{20}\text{H}_{30}\text{O}_2$ . The  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ , Table 3.19, Figure 3.38) NMR spectrum showed the presence of two sets of aldehyde protons ( $\delta_{\text{H}}$  9.40 and 9.63), one olefinic proton resonating at  $\delta_{\text{H}}$  6.76 ( $J= 6.6\text{Hz}$ ), exomethylene protons at 4.36 and 4.86, three sets of methyl protons and a number of peaks for methine and methylene protons.

The  $^{13}\text{C}$  NMR (150 MHz,  $\text{CDCl}_3$ ; Table 3.19; Figure 3.39) spectrum showed the presence of a total of 20 carbons including two aldehyde carbons ( $\delta_{\text{C}}$  193.8 and 197.5), an exomethylene (108.1), an olefinic methine (160.4), two aliphatic methines, four quaternary carbons, three methyl carbons and seven methylene carbons. In HMBC (Figure 3.41), two sets of methyl protons at  $\delta_{\text{H}}$  0.82 ( $\delta_{\text{C}}$  22.1 from HSQC) and  $\delta_{\text{H}}$  0.89 ( $\delta_{\text{C}}$  33.7 from HSQC) showed a common  $^2J$  connectivity to a carbon at 33.7 (C-4) and  $^3J$  connection with methylene carbon at  $\delta_{\text{C}}$  42.0 (C-3;  $\delta_{\text{H}}$  1.41 and 1.18 from HSQC) and methine carbon at  $\delta_{\text{C}}$  55.8 (C-5;  $\delta_{\text{H}}$  1.13 from HSQC). H-5 revealed  $^3J$  HMBC correlation to methylene carbons at 42.0 (C-3), 38.1 (C-7;  $\delta_{\text{H}}$  2.02 and 2.44 from HSQC), methine carbon at 56.6 (C-9;  $\delta_{\text{H}}$  1.90 from HMQC) and methyl carbon at 14.6 (C-20;  $\delta_{\text{H}}$  0.72 from HSQC). H-9 exhibited HMBC interactions to C-10 (by  $^2J$ ), C-11 (by  $^2J$ ), C-12 (160.4 by  $^3J$ ;  $\delta_{\text{H}}$  6.76 from HSQC), C-17 (108.4 by  $^3J$ ;  $\delta_{\text{H}}$  4.36 and 4.86 from HSQC) and C-20 (by  $^3J$ ). H-12 showed  $^3J$  HMBC connectivity to C-9, methylene carbon at 39.6 (C-14;  $\delta_{\text{H}}$  3.41 and 3.46 from HSQC) and aldehydic carbon at 193.8 (C-16;  $\delta_{\text{H}}$  9.40 from HSQC). The other aldehydic proton at H 9.63 (H-15; C 197.5 from HSQC) showed  $^3J$  HMBC correlation to a quaternary carbon at 135.0 (C-13). Accordingly, structure of 1 was confirmed as 8(17), 12-Labdadiene-15, 16-dial (**14**). This compound has previously reported only from *Alpinia chinensis*. This is the first report of its



isolation from the genus, *Zingiber*. The NMR data of ZMH 14 are in agreement with published literature (Lai-King and Brown 1997).

**Table 3.19:  $^1\text{H}$  (500 MHz) and  $^{13}\text{C}$  (125) NMR spectroscopic data of ZMH 14 in  $\text{CDCl}_3$**

Position	$^1\text{H}$	$^{13}\text{C}$
1	1.68, m 1.06, m	39.4
2	1.50, m 1.57, m	19.5
3	1.41, m 1.18, m	42.0
4	-	33.6
5	1.13, m	55.8
6	1.75, m 1.34, m	24.4
7	2.02, m 2.41, m	38.1
8	-	148.4
9	1.90, m	56.6
10	-	39.8
11	2.49, m 2.31, m	24.8
12	6.76, t, $J=6.55$ Hz	160.4
13	-	135.0
14	3.42, dd, $J=16.7$ Hz	39.6
15	9.63, t, $J=1.44$ Hz	197.5
16	9.40, s	193.8
17	4.86, s 4.46, s	108.1
18	0.88, s	33.7
19	0.82, s	22.1
20	0.72, s	14.6

Table 3.20:  $^1\text{H}$ - $^{13}\text{C}$  correlation for ZMH 14 in  $\text{CDCl}_3$

Protons	HMBC	
	$^2J$	$^3J$
H-2	42.0 ( $\text{CH}_2$ )	56.6 ( $\text{CH}$ )
H-3	-	22.1 ( $\text{CH}_3$ ), 33.7 ( $\text{CH}_3$ )
H-5	24.4 ( $\text{CH}_2$ )	14.60 ( $\text{CH}_3$ ), 22.1 ( $\text{CH}_3$ ), 24.3 ( $\text{CH}_2$ ), 38.1 ( $\text{CH}_2$ ), 56.6 ( $\text{CH}$ )
H-6	38.3 ( $\text{CH}_2$ ), 55.6 ( $\text{CH}$ )	-
H-7	148.4 ( $\text{C}$ )	55.6 ( $\text{CH}$ ), 108.1 ( $\text{CH}_2=\text{}$ )
H-9	39.8 ( $\text{C}$ )	14.6 ( $\text{CH}_3$ ), 24.8 ( $\text{CH}_2$ ),
H-11	-	56.6 ( $\text{CH}_2$ )
H-12	-	39.6 ( $\text{CH}_2$ ), 56.6 ( $\text{CH}$ ), 197.5 ( $\text{CH}$ ),
H-14	135.0 ( $\text{C}$ )	160.4 ( $\text{CH}$ ), 24.8 ( $\text{CH}_2$ ),
H-15	135.0 ( $\text{C}$ )	39.5 ( $\text{CH}_2$ )
H-16	39.6 ( $\text{CH}_2$ )	135.0 ( $\text{C}$ )
H-17	38.1 ( $\text{CH}_2$ )	56.6 ( $\text{CH}$ )
H-18	-	22.1 ( $\text{CH}_3$ ), 42.0 ( $\text{CH}_2$ ), 55.6 ( $\text{CH}$ )
H-19	-	33.7 ( $\text{CH}_3$ ), 42.0 ( $\text{CH}_2$ ), 55.6 ( $\text{CH}$ )
H-20	39.8 ( $\text{C}$ )	55.6 ( $\text{CH}$ )

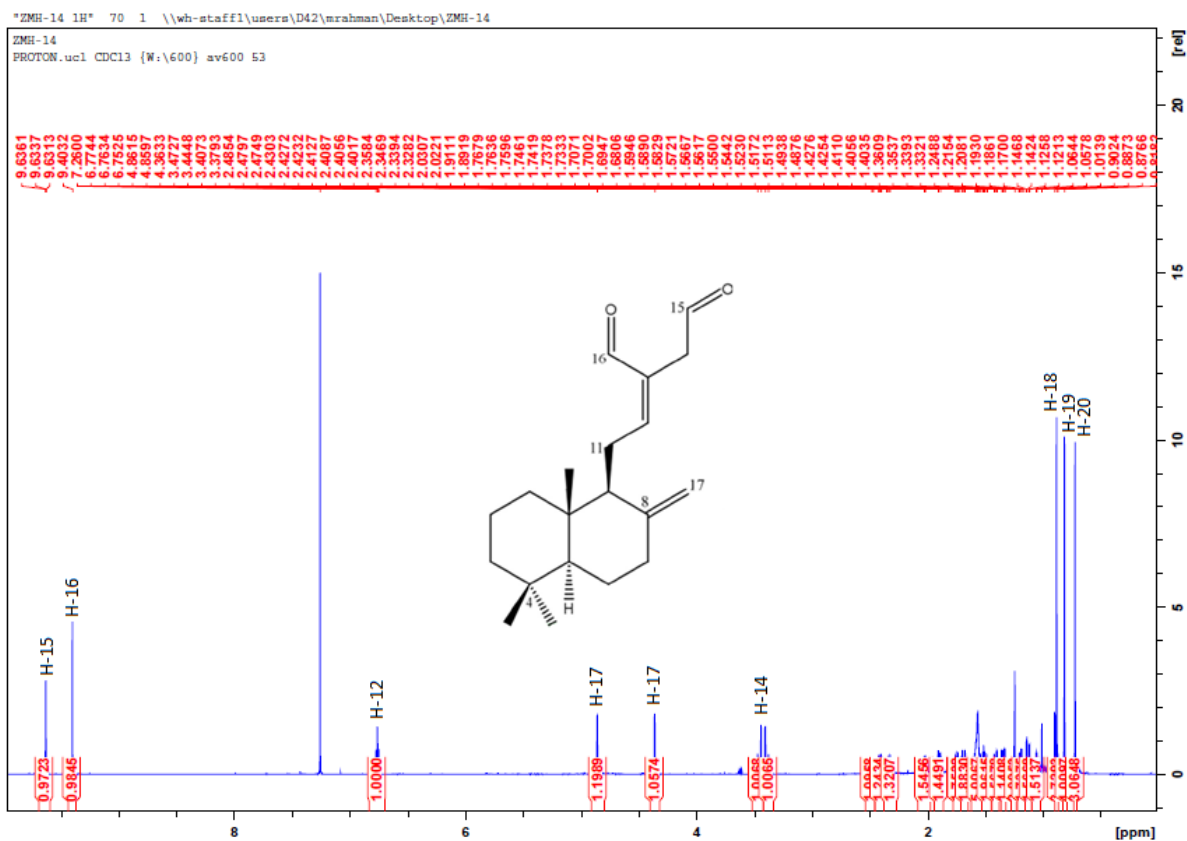
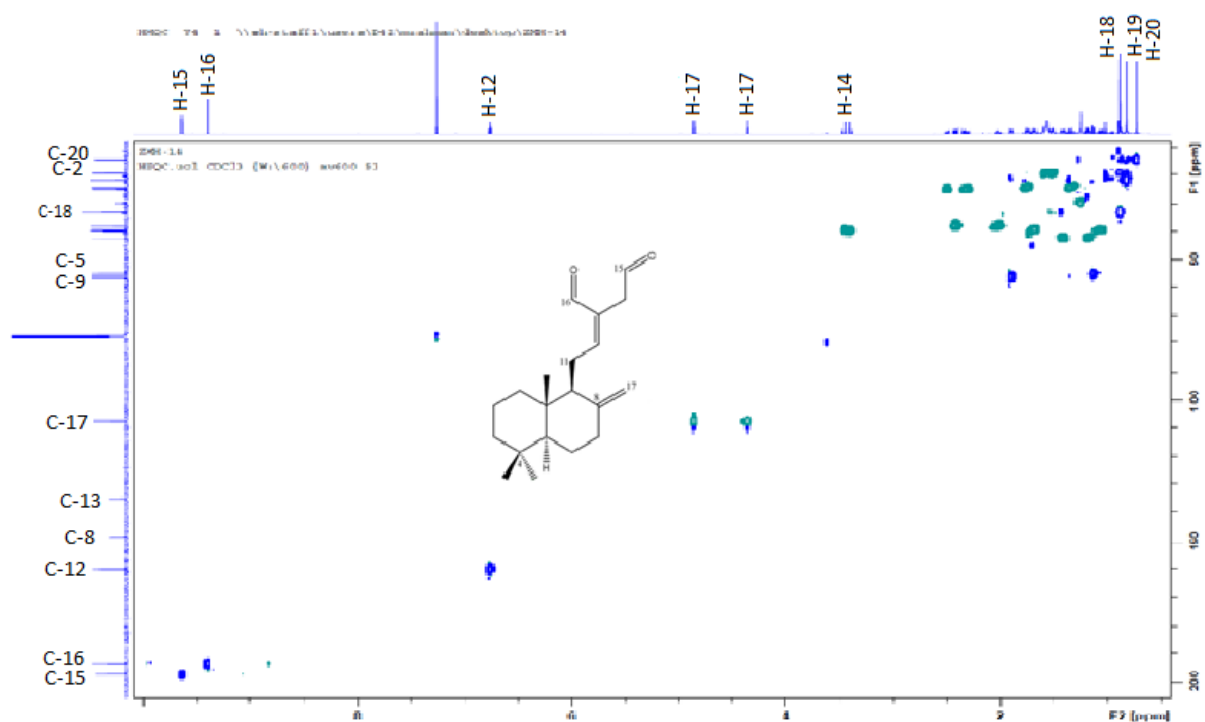
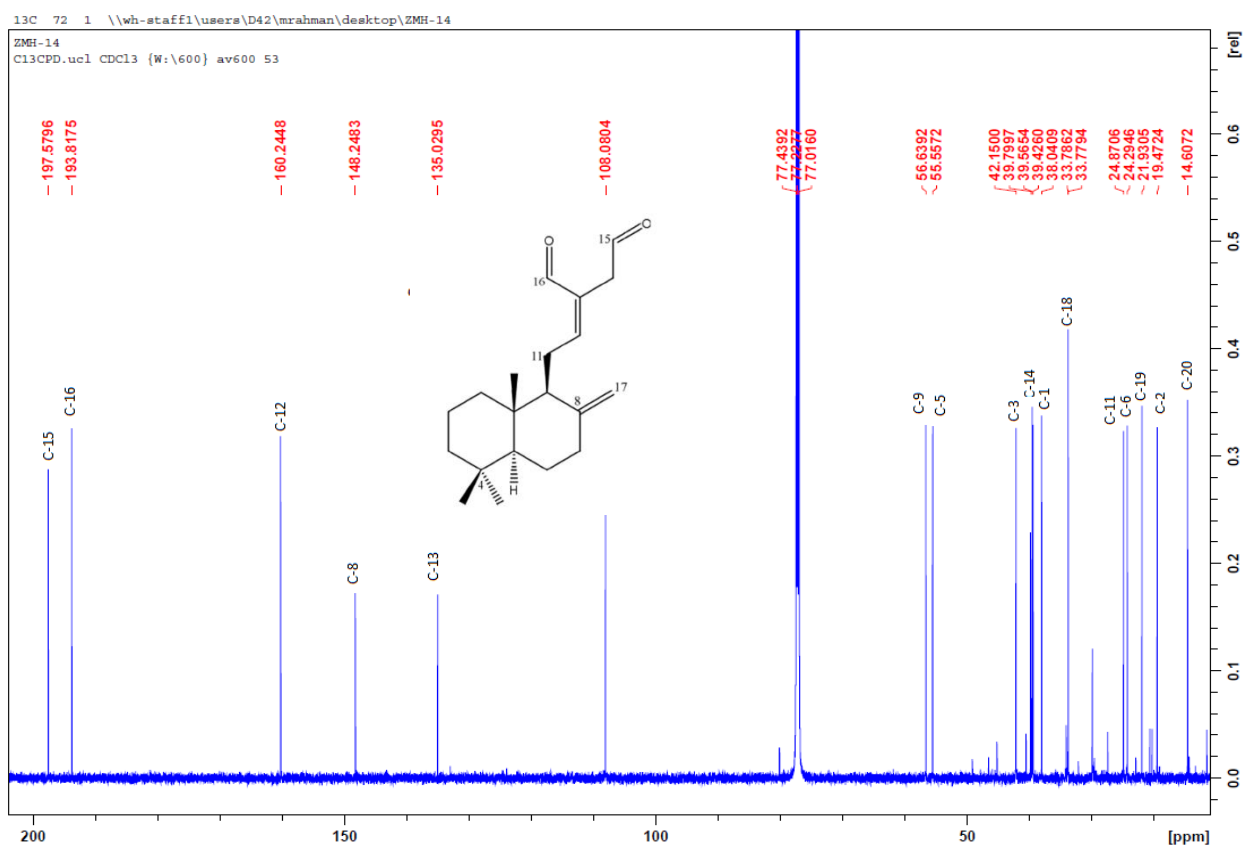


Figure 3.42:  $^1\text{H}$  NMR spectrum (600 MHz) of ZMH 14 in  $\text{CDCl}_3$





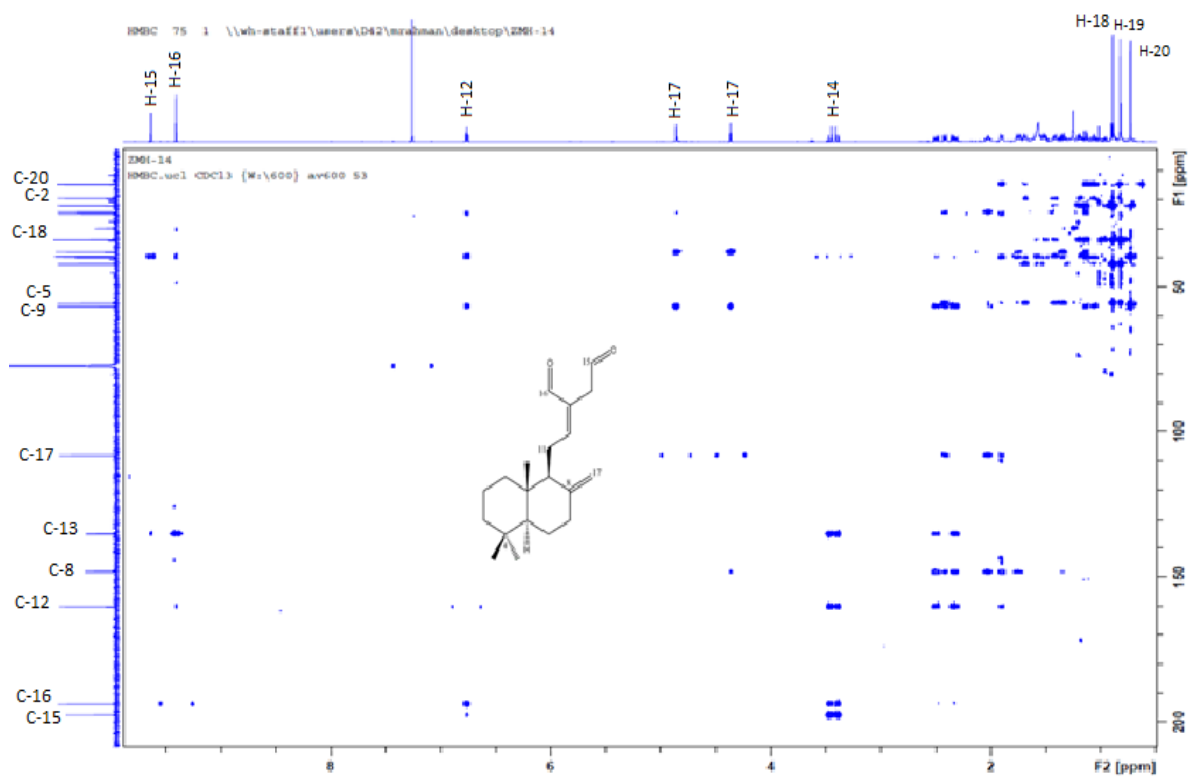


Figure 3.45: HMBC NMR spectrum of ZMH 14 in CDCl<sub>3</sub>

### 3.2.6 Triterpenes

Triterpenes are the most abundant and diverse group of chemical compounds widely distributed in the plants. They contain 6 isoprene units. This study has led to the isolation of 5 triterpenes lupine series from the hexane and chloroform leaf extract of *Diosphyros malabarica* and two pentacyclic triterpenes from hexane and chloroform extract of *Cynometra ramiflora*. The triterpenes isolated from *Diosphyros malabarica* were codes as DMH 2, DMH 3, DMH 10, DMH 15 and DMC 22.

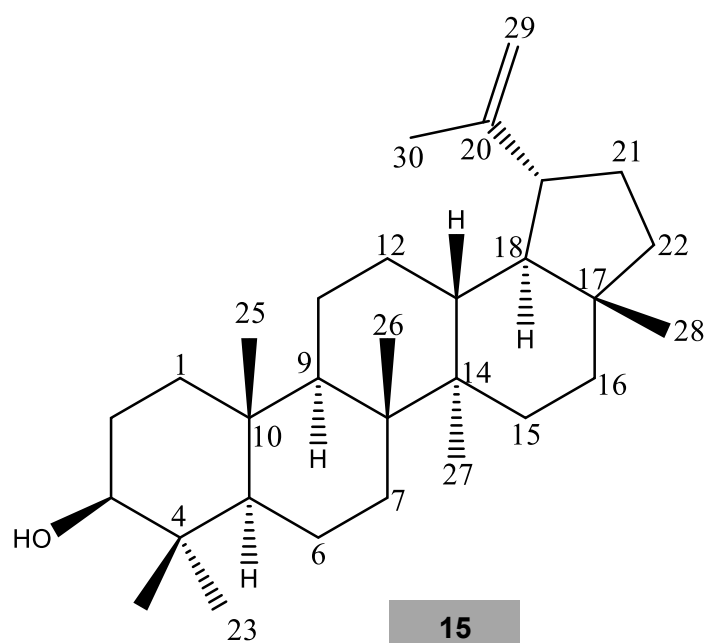
#### 3.2.6.1 Characterization of DMC 22 as Lupeol (15):

The IR revealed the presence of exomethylene and hydroxyl group by revealing the characteristic bands at  $884\text{ cm}^{-1}$  and  $3361\text{ cm}^{-1}$  respectively. The high resolution mass spectrometry showed the  $[M+H]^+$  at  $m/z\ 427.39478$  (calculated for  $C_{30}H_{51}O$  at  $427.393990$ ) which confirmed the molecular formula of DMC 22 as  $C_{30}H_{50}O$ .

The  $^1\text{H}$  NMR spectrum (700 MHz,  $\text{CDCl}_3$ , Table 3.21, Figure 3.42) showed the presence of an exomethylene protons resonating at  $\delta_{\text{H}}\ 4.69$  ( $J=2.24\text{ Hz}$ ) and  $4.57$  ( $J=2.31\text{ Hz}$ ), oxymethine signal  $\delta_{\text{H}}\ 3.19$  (dd,  $J= 11.55\text{ Hz}, 4.69\text{ Hz}$ ), a vinylic methyl at 1.68, five methyls between 0.76-1.03, and numerous signals between 0.92-2.38 due to the presence of methine and methylene protons.

The  $^{13}\text{C}$  NMR (175 MHz,  $\text{CDCl}_3$ ; Table 3.22; Figure 3.43) revealed a total of 30 carbons including an oxymethine at  $\delta_{\text{C}}\ 79.1$ , exomethylene carbon  $\delta_{\text{C}}\ 109.5$ , six methyl signals at  $\delta_{\text{C}}\ 14.7, 15.6, 16.2, 16.3, 18.2, 19.5,$  and  $28.2$ . The DEPT 135 NMR spectrum confirmed the presence of 7 methyls, 11 methylenes, 6 methines and remaining six as quaternary carbons.

The 2D NMR spectra including HSQC and HMBC played key role in assigning the protons and carbons of the compound. In HMBC experiment (Table 3.23), two sets of methyl protons at  $\delta_{\text{H}}\ 0.76$  (H-24;  $\delta_{\text{C}}\ 15.6$  from HSQC) and  $\delta_{\text{H}}\ 0.97$  (H-



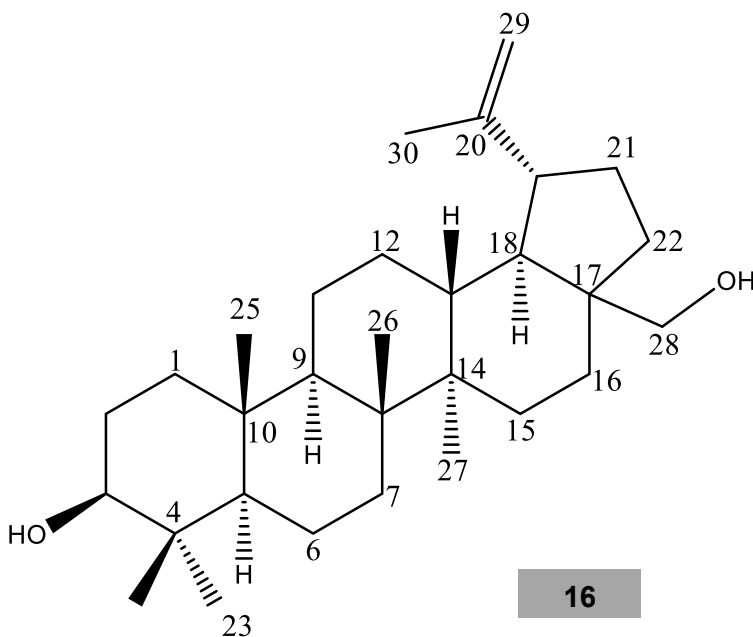
23;  $\delta_C$  28.2 from HSQC) showed a common  $^2J$  correlation with a quaternary carbon at  $\delta_C$  39.1 (C-4) and  $^3J$  connectivity with an oxymethine carbon at  $\delta_C$  79.2 (C-3;  $\delta_H$  3.19) and methine carbon at 55.6 (C-5;  $\delta_H$  0.68 from HSQC). In addition, the oxymethine proton at  $\delta_H$  3.19 (H-3;  $\delta_C$  79.2 from HSQC) also showed  $^3J$  correlation with methyl carbons at  $\delta_C$  15.6 (C-24;  $\delta_H$  0.76) and  $\delta_C$  28.2 (C-23;  $\delta_H$  0.97). The methyl protons at  $\delta_H$  0.83 (H-25;  $\delta_C$  16.3 from HSQC) revealed  $^2J$  correlation with quaternary carbon at  $\delta_C$  37.3 and  $^3J$  connection with methine carbons at  $\delta_C$  55.6 (C-5;  $\delta_H$  0.68 from HSQC) and methylene carbon at  $\delta_C$  38.9 (C-1;  $\delta_H$  0.92). Another set of methyl protons at  $\delta_H$  1.03 (H-26;  $\delta_C$  16.2 from HSQC) showed connectivity with a quaternary carbon at  $\delta_C$  41.1 by  $^2J$  and connection with methylene carbon at  $\delta_C$  34.5 (C-7;  $\delta_H$  1.41 from HSQC) and quaternary carbon at  $\delta_C$  43.0 by  $^3J$ . Both methyl protons  $\delta_H$  0.83 (H-25;  $\delta_C$  16.3 from HSQC) and  $\delta_H$  1.03 (H-26;  $\delta_C$  16.2 from HSQC) showed common  $^3J$  correlation with methine carbon at  $\delta_C$  50.6 (C-9;  $\delta_H$  1.28 from HSQC). Moreover, another methyl proton at  $\delta_H$  0.94 (H-27;  $\delta_C$  14.2 from HSQC) exhibited  $^2J$  correlation with a quaternary carbon at  $\delta_C$  43.0 (C-14) and  $^3J$  correlation with methylene carbon at  $\delta_C$  27.7 (C-15;  $\delta_H$  1.36 and 1.67 from HSQC), methine carbon at  $\delta_C$  38.3 (C-13;  $\delta_H$  1.67) and quaternary carbon at  $\delta_C$  41.0. The methyl proton at  $\delta_H$  0.79 (H-28;  $\delta_C$  18.2 from HSQC) revealed connectivity with quaternary carbon at  $\delta_C$  43.2 (C-17) by  $^2J$  and connection with methylene carbon at  $\delta_C$  35.8 (C-16;  $\delta_H$  1.48, 1.38 from HSQC) and  $\delta_C$  40.2 (C-22;  $\delta_H$  1.19,  $\delta_H$  1.38 from HSQC) and methine carbon at  $\delta_C$  48.5 (C-18;  $\delta_H$  1.36 from HSQC) by  $^3J$ . On the other hand, the methyl proton at  $\delta_H$  1.68 (H-29;  $\delta_C$  19.5 from HSQC) had  $^2J$  interaction with quaternary carbon  $\delta_C$  151.2 at C-20 and  $^3J$  correlation with exomethylene carbon at  $\delta_C$  109.5 (C-30;  $\delta_H$  4.69,  $\delta_H$  4.57 from HSQC) and methine carbon at  $\delta_C$  48.2 (C-19;  $\delta_H$  2.38 from HSQC) and vice versa. Based on the spectral data and HMBC connectivities, the compound DMC 22 was identified as 20(29)-Lupen-3beta-ol commonly known as Lupeol (**15**). The spectral data were identical to those reported previously (Silva et al., 2017). It was isolated previously from most of the *Diospyros* species including *Diospyros abyssinica*, *Diospyros canaliculata*, *Diospyros chevalieri* (Zhong et al., 1984).

### 3.2.6.2 Characterization of DMH 3 as Betulin (16):

The IR revealed the presence of exomethylene and hydroxyl group by revealing the characteristic band at  $883\text{ cm}^{-1}$  and  $3365\text{ cm}^{-1}$  respectively.

The high resolution mass spectrometry showed the  $[M+Na]^+$  at  $465.37056\text{ m/z}$  (calculated for  $C_{30}H_{50}O_2Na$ , at  $465.37085$ ) which confirmed the molecular formula of DMH 3 as  $C_{30}H_{50}O_2$ .

The  $^1\text{H}$  NMR spectrum (700 MHz,  $\text{CDCl}_3$ , Table 3.21, Figure 3.44) and  $^{13}\text{C}$  NMR (175 MHz,  $\text{CDCl}_3$ , Table 3.22, Figure 3.45) of DMH 3 showed very similar signals compared to those of DMC 22.  $^1\text{H}$  NMR spectrum (700 MHz,  $\text{CDCl}_3$ , Table 3.21, Figure 3.44 ) revealed the presence of five methyl peaks from  $\delta_{\text{H}}$  0.78-0.98 including a vinylic proton peak at  $\delta_{\text{H}}$  1.68, an exomethylene singlets at  $\delta_{\text{H}}$  4.68 and  $\delta_{\text{H}}$  4.58 and oxymethine peaks at  $\delta_{\text{H}}$  3.18 (dd,  $J=11.55\text{ Hz}$ ,  $j=4.55\text{ Hz}$ ). However, the presence of extra peaks at  $\delta_{\text{H}}$  3.33 (t,  $J=9.38\text{ Hz}$ ) and  $\delta_{\text{H}}$  3.80 (d,  $10.78\text{ Hz}$ ) were accounted due



to the presence of hydroxymethyl group in the compound, instead of methyl as evident in DMC 22.

$^{13}\text{C}$  NMR (175 MHz,  $\text{CDCl}_3$ ; Table 3.22; Figure 3.45) exhibited the presence of 30 carbons including the signal of oxymethine carbon at  $\delta_{\text{C}}$  79.5, exomethylene carbon at  $\delta_{\text{C}}$  109.9 and hydroxymethyl carbon at  $\delta_{\text{C}}$  60.5. In HMBC experiment, the key HMBC correlations of DMH 3 were very much similar to DMC 22. The hydroxymethyl protons at  $\delta_{\text{H}}$  3.33 and  $\delta_{\text{H}}$  3.80 showed  $^3J$  correlation with methylene carbons at 34.2 (C-22;  $\delta_{\text{H}}$  1.86 from HSQC) and 29.4 (C-16;  $\delta_{\text{H}}$  1.21 and 1.86 from HSQC). On the other hand, methylene protons  $\delta_{\text{H}}$  1.21 and 1.86 (C-16; 29.4 from HSQC) and methine proton at 1.58 (C-18; 48.8 from HSQC) revealed the  $^3J$  connection with hydroxymethyl carbon at  $\delta_{\text{C}}$  60.5 (C-28;  $\delta_{\text{H}}$  3.33 and  $\delta_{\text{H}}$  3.80 from HSQC).

Thus, based on the IR, Mass spectrometry,  $^1\text{H}$ ,  $^{13}\text{C}$  NMR spectral data and their HMBC connectivity the compound of DMH 3 was identified as 20(29)-Lupene-3, 28-diol; commonly known as Betulin, the spectral data were identical to those reported previously (Siddiqui et al., 1988). This compound had been previously isolated from most of the *Diospyros* species including *Diospyros abyssinica*, *Diospyros canaliculata*, *Diospyros chevalieri*) of genus *Diospyros* (Zhong et al., 1984).

### 3.2.6.3 Characterization of DMH 10 as 30- norlupane- $3\beta$ , 28- diol-20-one (Messagenin; 17):

The IR of DMH 10 revealed the presence of hydroxyl group at  $3019\text{ cm}^{-1}$  and carbonyl group at  $1700\text{ cm}^{-1}$ . The high resolution mass spectrometry showed the  $[\text{M}+\text{H}]^+$  at  $m/z$  445.36684 (calculated for  $\text{C}_{29}\text{H}_{49}\text{O}_3$  at 445.36817) which confirmed the molecular formula of DMH 10 as  $\text{C}_{29}\text{H}_{48}\text{O}_3$ .

DMH 10 showed very similar signals as DMH 3 in both the  $^1\text{H}$  (700MHz,  $\text{CDCl}_3$ ) and  $^{13}\text{C}$  (175MHz,  $\text{CDCl}_3$ ) NMR spectra. The  $^1\text{H}$  NMR (700MHz,  $\text{CDCl}_3$ ,

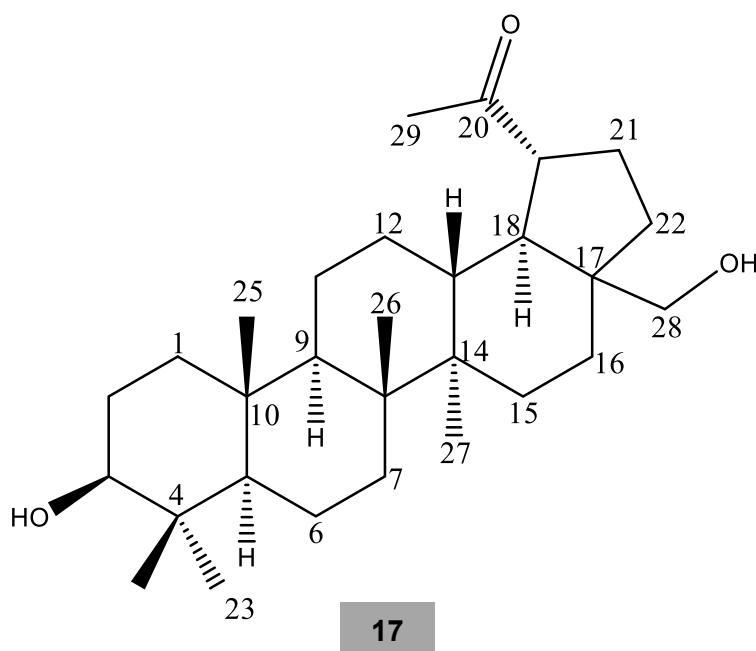


Table 3.21, Figure 3.46) revealed the presence of 6 methyl peaks from  $\delta_{\text{H}}$  0.76-2.17 and hydroxymethyl signals at  $\delta_{\text{H}}$  3.25 (d,  $J= 10.71$  Hz) and  $\delta_{\text{H}}$  3.79 (d,  $J=10.71$  Hz) and oxymethine multiplet signal at  $\delta_{\text{H}}$  3.20 but missing the exomethylene protons. The chemical shift of one methyl proton was 2.17 was assumed to be due to the presence of acetyl group in the molecule.

The  $^{13}\text{C}$  (175MHz,  $\text{CDCl}_3$ ; Table 3.22; Figure 3.47) NMR spectrum of DMH 10 showed total 29 carbons present in the compound including acetyl carbonyl at  $\delta_{\text{C}}$  212.4, oxymethine at  $\delta_{\text{C}}$  79.1 and hydroxymethyl at  $\delta_{\text{C}}$  60.8. The key HMBC correlations were very much similar to those of DMH 3. In the HMBC, the methine proton at  $\delta_{\text{H}}$  2.63 (C-19;  $\delta_{\text{C}}$  52.2 from HSQC) and methyl protons at  $\delta_{\text{H}}$  2.17 ( $\delta_{\text{C}}$  29.6 from HSQC) exhibited a common  $^2J$  correlation with the carbonyl carbon of acetyl group at  $\delta_{\text{C}}$  212.4 (C-20).

Therefore, based on the IR, Mass Spectrometry and NMR data and HMBC correlations, the compound DMH 10 was confirmed as 30- norlupane- 3 $\beta$ , 28-diol-20-one (**17**) also known as Messagenin. Messagenin was synthesized from betulinic acid previously (Macias, et al., 1994). However, this is the first reported NMR data of DMH 10.

**Table 3.21: <sup>1</sup>H NMR data (700 MHz, CDCl<sub>3</sub>) of DMC 22, DMH 3, DMH 10**

Position	$\delta_H$ of DMC 22	$\delta_H$ of DMH 3	$\delta_H$ of DMH 10
1	0.92, m	0.90, m 1.66, m	1.65, m
2	1.69, m 1.67, m	1.57, m	1.08, m 1.01, m
3	3.19, dd, $J= 11.55, 4.69$ Hz	3.18, dd, $J= 11.55, 4.55$ Hz	3.20, m
5	0.68, d, $J= 9.31$ Hz	0.68, d, $J=10.01$ Hz	0.69, d, $J=10.01$ Hz
6	1.38, m 1.51, m	1.40, m 1.53, m	1.42, m 1.53, m
7	1.41, m	1.39, s 1.04, m	1.40, m
9	1.28, m	1.27, s	1.29, m
11	1.24, m 1.42, m	1.21, m	1.27, m
12	1.07, m 1.67, m	1.05, m 1.63, m	1.55, m
13	1.67, m	1.64, m	1.57, m
15	1.36, m 1.67, m	1.69, m 1.04, m	1.69, m
16	1.38, m 1.19, m	1.21, m 1.86, m	1.94, m
18	1.36, m	1.58, t, $J= 11.76$ Hz	2.07, d, $J= 11.83$ Hz
19	2.38, m	2.39, m	2.63, m
21	1.92, m 1.29, m	1.41, m 1.25, m	2.09, m
22	1.48, m 1.38, m	1.86, m	2.43, m 2.47, m
23	0.97, s	0.96, s	0.76, s
24	0.76, s	0.78, s	0.98, s
25	0.83, s	0.82, s	0.82, s
26	1.03, s	1.02, s	1.01, s
27	0.94, s	0.98, t, $J=8.12$ Hz	1.01, s
28	0.79, s	3.80, d, $J=10.78$ Hz 3.33, t, $J= 9.38$ Hz	3.79, d, $J= 10.71$ Hz 3.25, d, $J= 10.71$ Hz
29	4.69, d, $J= 2.24$ Hz 4.57, q, $J= 2.31$ Hz	4.68, s 4.58, s	2.17, s
30	1.68, s	1.68, s	-

**Table 3.22:  $^{13}\text{C}$  NMR data (175 MHz,  $\text{CDCl}_3$ ) of DMC 22, DMH 3, DMH 10**

<b>Carbon</b>	<b>DMC 22</b>	<b>DMH 3</b>	<b>DMH 10</b>
C-1	38.9	38.9	38.7
C-2	27.6	27.6	27.4
C-3	79.1	79.5	79.1
C-4	39.1	39.1	39.1
C-5	55.6	55.6	55.4
C-6	18.6	18.5	18.6
C-7	34.5	34.4	34.4
C-8	41.1	41.1	41.1
C-9	50.6	50.5	50.4
C-10	37.3	37.3	37.4
C-11	21.5	21.0	29.9
C-12	25.4	25.5	27.6
C-13	38.3	37.5	36.4
C-14	43.0	42.9	42.8
C-15	27.7	27.3	27.2
C-16	35.8	29.4	29.1
C-17	43.2	47.9	48.1
C-18	48.5	48.8	49.8
C-19	48.2	48.1	52.2
C-20	151.2	150.7	212.4
C-21	29.9	29.9	27.8
C-22	40.2	34.2	34.2
C-23	28.2	28.3	15.6
C-24	15.6	15.6	14.9
C-25	16.3	16.3	16.3
C-26	16.2	16.2	16.2
C-27	14.7	15.0	15.0
C-28	18.2	60.5	60.8
C-29	109.6	109.9	29.6
C-30	19.5	19.3	-



**Table 3.23: HMBC correlation (700 MHz, CDCl<sub>3</sub>) of DMC 22, DMH 3 and DMH 10**

Proton	HMBC of DMC 22		HMBC of DMH 3		HMBC of DMH 10	
	<sup>2</sup> J	<sup>3</sup> J	<sup>2</sup> J	<sup>3</sup> J	<sup>2</sup> J	<sup>3</sup> J
H-1	ns	ns	37.3 (C)	79.5 (CH-O)	27.4 (CH <sub>2</sub> )	55.4 (CH)
H-2	ns	ns	79.5 (CH-O)	37.3 (C)	-	55.4 (CH)
H-3	-	15.6 (CH <sub>3</sub> ), 28.2 (CH <sub>3</sub> )	-	15.6 (CH <sub>3</sub> ), 28.3 (CH <sub>3</sub> )	-	39.1 (C), 15.6 (CH <sub>3</sub> ), (14.9 (CH <sub>3</sub> ))
H-5	-	28.2 (CH <sub>3</sub> )	18.5 (CH <sub>2</sub> ), 39.1 (C)	16.3 (CH <sub>3</sub> )	18.6 (CH <sub>2</sub> ), 39.1 (C)	15.6 (CH <sub>3</sub> ), 34.4 (CH <sub>2</sub> )
H-6	-	37.3 (C), 41.1 (C)	-	41.1 (C), 50.5 (CH)	55.4 (CH)	41.1 (C)
H-7	ns	ns	41.1 (C)	50.5 (CH), 55.6 (CH)	41.1 (C)	16.2 (CH <sub>3</sub> ), 55.4 (CH), 50.4 (CH)
H-9	21.5 (CH <sub>2</sub> ), 37.3 (C), 41.1 (C)	16.3 (CH <sub>3</sub> )	41.1 (C), 37.3 (C)	55.6 (CH)	37.4 (C), 41.1 (C)	16.3 (CH <sub>3</sub> )
H-11	ns	ns	25.5 (CH <sub>2</sub> )	-	-	-
H-12	ns	ns	37.5 (CH)	48.1 (CH)	-	49.8 (CH), 42.8 (C)
H-13	43.0 (C)	-	48.8 (CH)	27.3 (CH <sub>2</sub> )	42.8 (C), 49.8 (CH)	48.1 (CH), 27.2 (CH <sub>2</sub> ), 15.0 (CH <sup>3</sup> )
H-15	ns	ns	42.9 (C)	41.1 (C)	42.8 (C), 29.1 (CH <sub>2</sub> )	41.1 (C)
H-16	ns	18.2 (CH <sub>3</sub> ), 16.2 (CH <sub>2</sub> ), 48.6 (CH)	27.3 (CH <sub>2</sub> )	60.5 (CH <sub>2</sub> -O)	48.1 (C)	60.8 (CH <sub>2</sub> -O), 49.8 (CH)
H-18	-	151.1 (C)	48.1 (CH), 37.5 (CH)	60.5 (CH <sub>2</sub> -O)	52.2 (CH), 48.1 (C), 36.4 (CH)	60.8 (CH <sub>2</sub> -O), 42.8 (C), 34.2 (CH <sub>2</sub> )
H-19	48.5 (CH), 151.2 (C)	19.5 (CH <sub>3</sub> ), 29.9 (CH <sub>2</sub> ), 109.5 (CH <sub>2</sub> )	48.8 (CH), 29.9 (CH <sub>2</sub> ), 150.7 (C)	19.3 (CH <sub>3</sub> ), 109.9 (CH <sub>2</sub> )	27.8 (CH <sub>2</sub> ), 49.8 (CH), 212.4 (C)	36.4 (CH)

H-21	ns	ns	-	-	52.2 (CH), 34.2 (CH <sub>2</sub> )	49.8 (CH), 48.1 (C)
H-22	ns	18.2 (CH <sub>3</sub> )	29.9 (CH <sub>2</sub> )	60.5 (CH <sub>2</sub> -O), 48.8 (CH),	-	36.4 (CH)
H-23	39.1 (C)	15.6 (CH <sub>3</sub> ), 55.6 (CH), 79.2 (CH)	39.1 (C)	79.5(CH-O), 15.6 (CH <sub>3</sub> ), 55.6 (CH)	39.1 (C)	14.9(CH <sub>3</sub> ), 55.4 (CH), 79.1 (CH-O),
H-24	39.1 (C)	28.2 (CH <sub>3</sub> ), 55.6 (CH), 79.2 (CH)	39.1 (C)	79.5 (CH-O), 28.3 (CH <sub>3</sub> ), 55.6 (CH)	39.1 (C)	15.6 (CH <sub>3</sub> ), 55.4 (CH), 79.1(CH-O)
H-25	37.3 (C)	38.9 (CH <sub>2</sub> ), 50.6 (CH), 55.6 (CH)	37.3 (C)	38.9 (CH <sub>2</sub> ), 50.5 (CH), 55.6 (CH)	37.4 (C)	50.4 (CH), 55.4 (CH), 38.9 (CH <sub>2</sub> )
H-26	41.1 (C)	34.5 (CH <sub>2</sub> ), 43.0 (C), 50.6 (CH)	41.1 (C)	42.9 (C), 50.5 (CH),	41.1 (C)	42.8 (C), 50.4 (CH), 34.4 (CH <sub>2</sub> )
H-27	43.0 (C)	27.7 (CH <sub>2</sub> ), 38.3 (CH), 41.0 (C)	42.9 (C)	41.1 (C)	42.8 (C)	41.1 (C) , 36.4 (CH), 27.2 (CH <sub>2</sub> )
H-28	43.2 (C)	35.8 (CH <sub>2</sub> ), 40.2 (CH <sub>2</sub> ), 48.5 (CH)	-	34.2 (CH <sub>2</sub> ), 29.4 (CH <sub>2</sub> )	48.1 (C)	29.1( CH <sub>2</sub> ), 34.2 (CH <sub>2</sub> )
H-29	-	19.5 (CH <sub>3</sub> ), 48.2 (CH)	150 (C)	19.3 (CH <sub>3</sub> ), 48.1 (CH)	212.4 (C)	52.2 (CH)
H-30	151.2 (C)	109.5 (CH <sub>2</sub> =), 48.2 (CH)	150 (C)	109.9 (CH <sub>2</sub> =), 48.1 (CH)	-	-

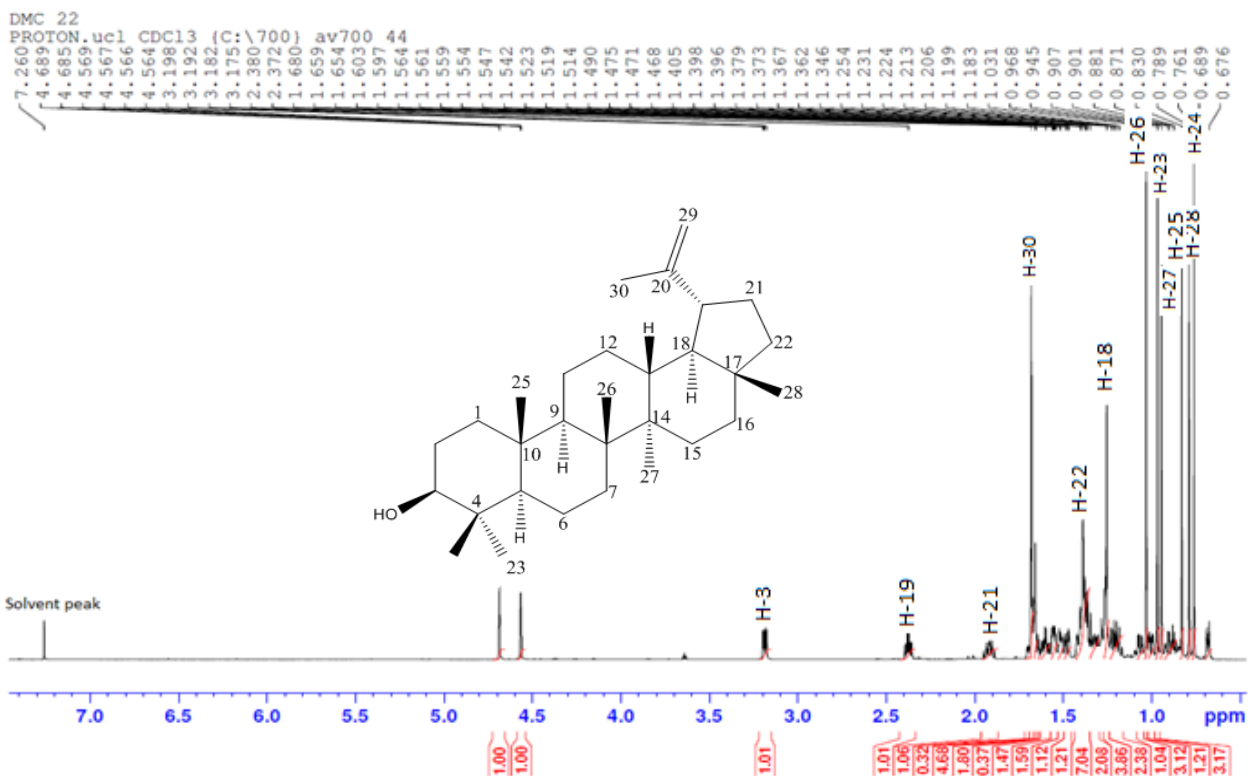


Figure 3.46:  $^1\text{H}$  NMR spectrum (700 MHz) of DMC 22 in  $\text{CDCl}_3$

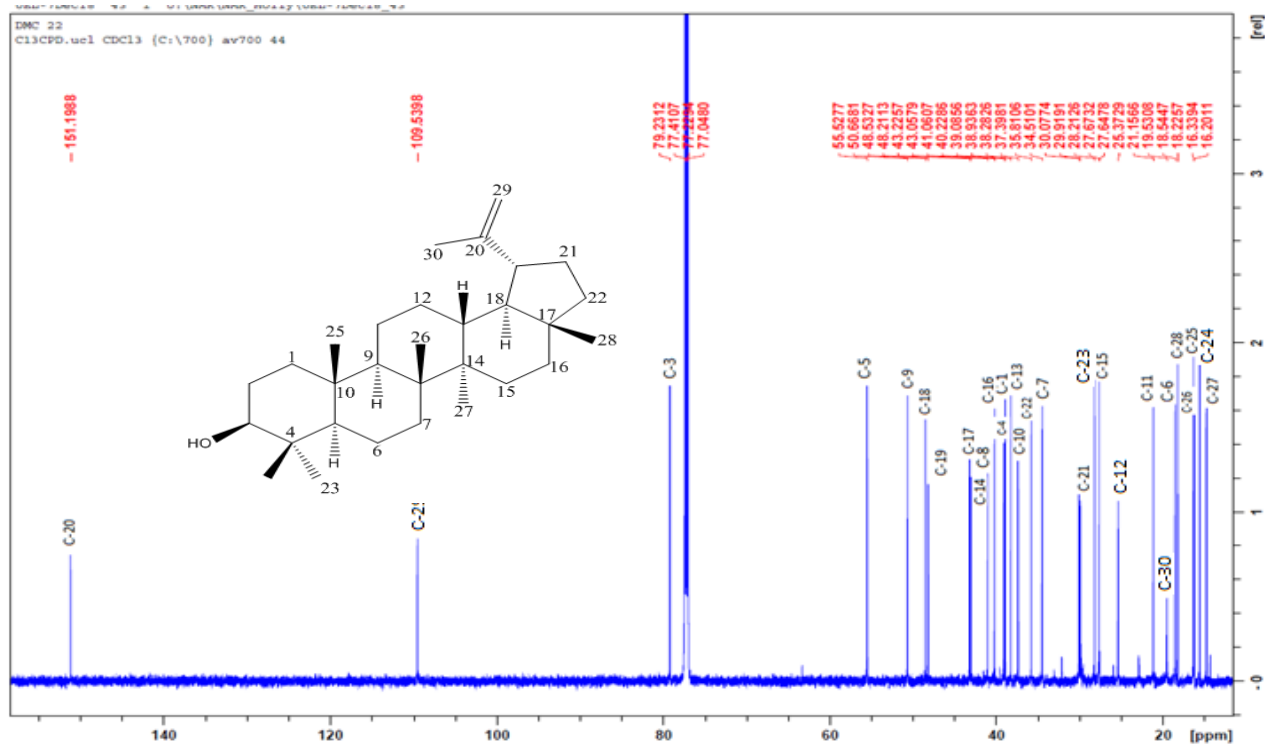


Figure 3.47:  $^{13}\text{C}$  NMR spectrum (175MHz) of DMC 22 in  $\text{CDCl}_3$

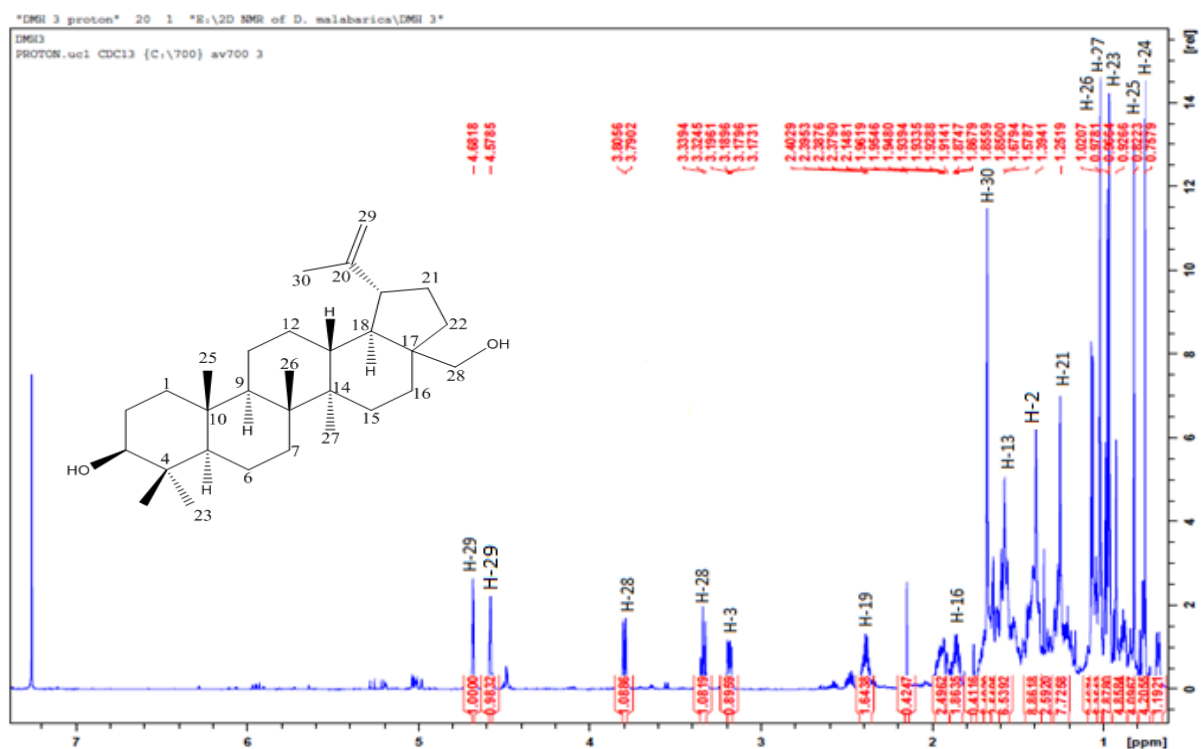


Figure 3.48:  $^1\text{H}$  NMR spectrum (700MHz) of DMH 3 in  $\text{CDCl}_3$

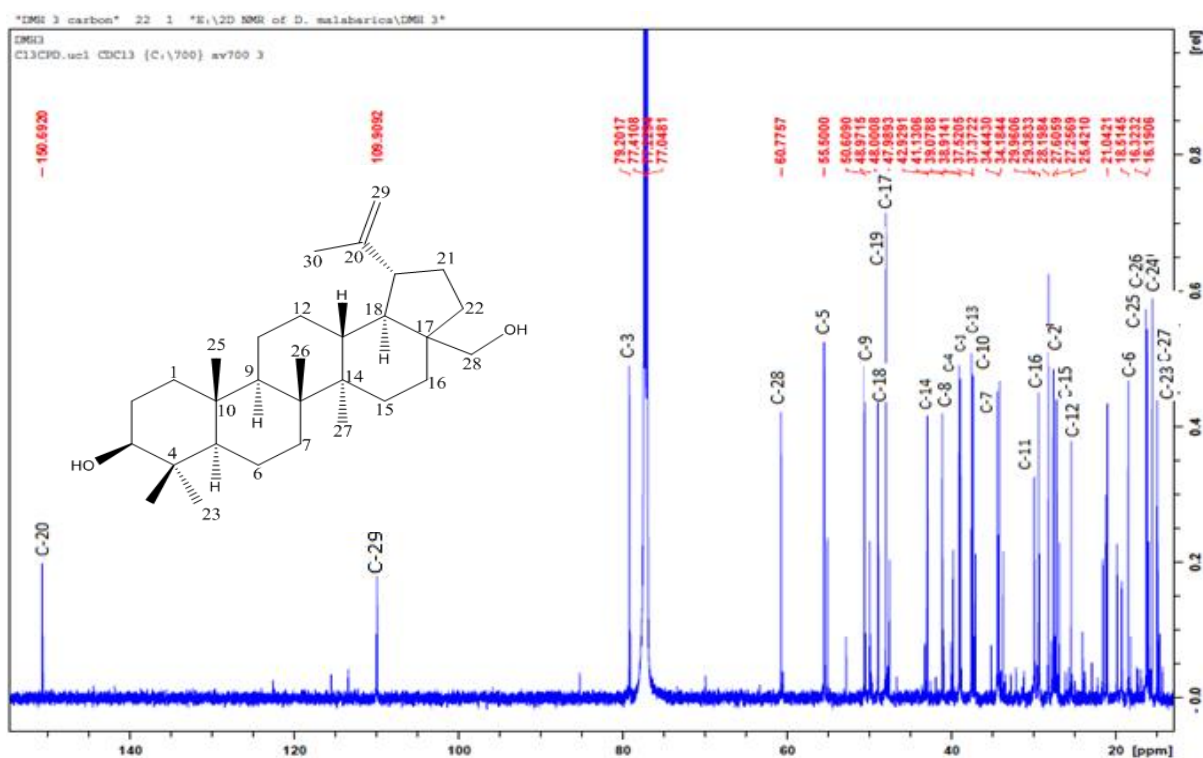


Figure 3.49:  $^{13}\text{C}$  NMR spectrum (175 MHz) of DMH 3 in  $\text{CDCl}_3$

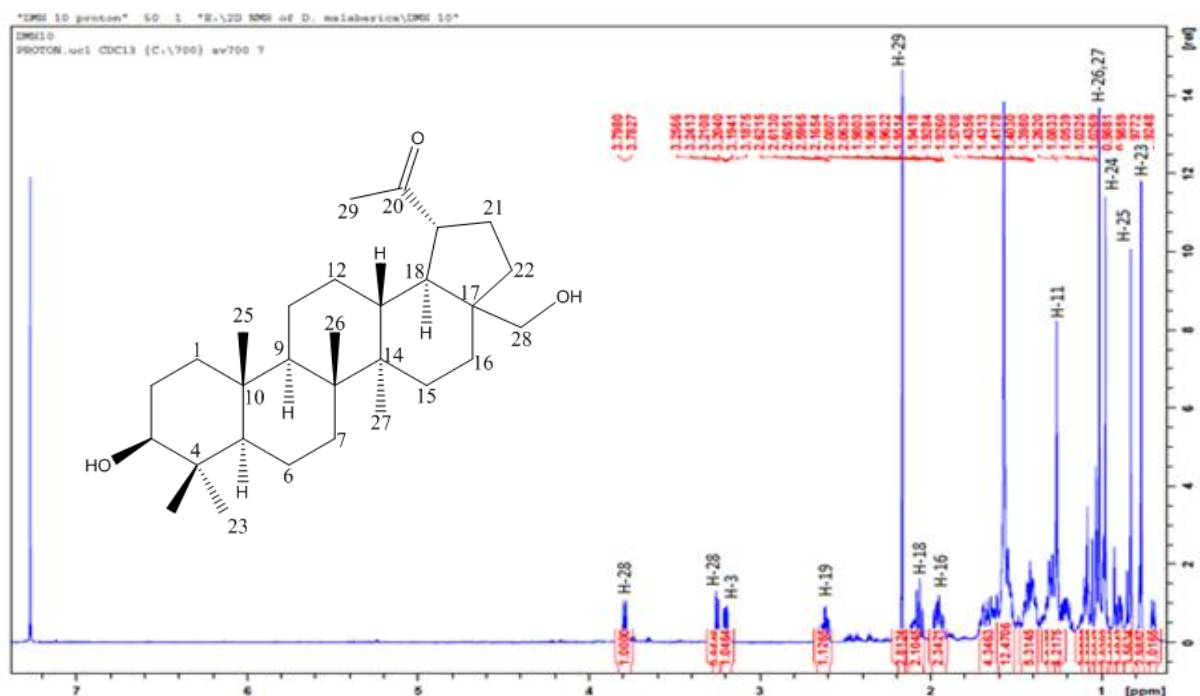


Figure 3.50:  $^1\text{H}$  NMR spectrum (700MHz) of DMH 10 in  $\text{CDCl}_3$

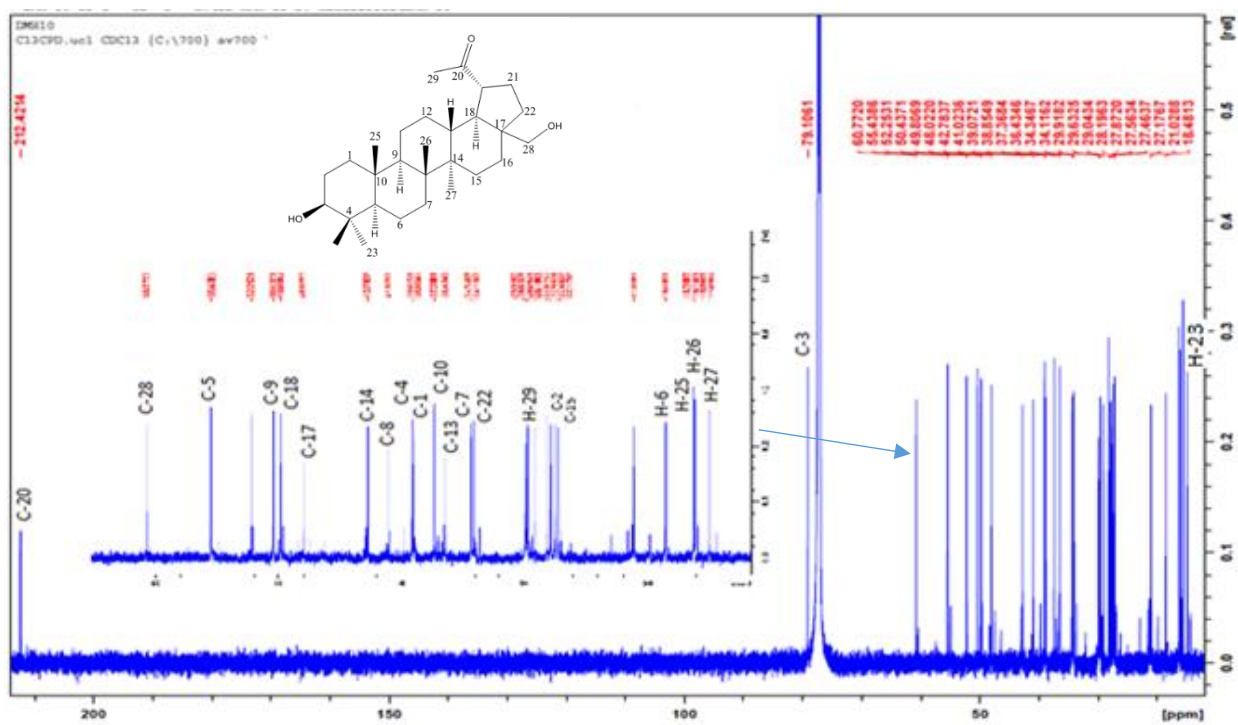


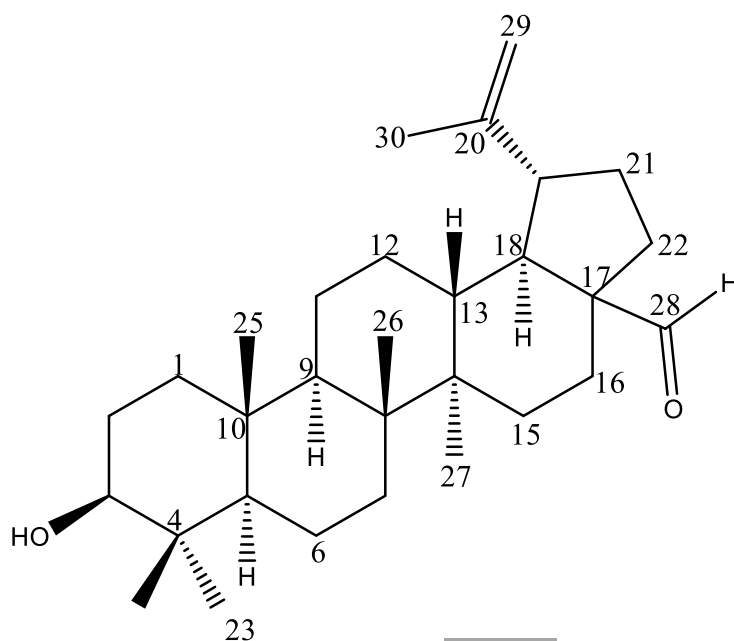
Figure 3.51:  $^{13}\text{C}$  NMR spectrum (175MHz) of DMH 10 in  $\text{CDCl}_3$

### 3.2.6.4 Characterization of DMH 15 as betulinaldehyde (18)

The high-resolution mass spectrometry showed the  $[M+H]^+$  at  $m/z$  441.37430 (calculated for  $C_{30}H_{49}O_2$  at 441.373255) which confirmed the molecular formula of DMH 15 as  $C_{30}H_{48}O_2$ .

The  $^1H$  and  $^{13}C$  NMR spectrum of DMH 15 showed very similar signals to those of DMH 3. Like DMH 3,  $^1H$  NMR spectrum (700 MHz,  $CDCl_3$ , Table 3.24, Figure 3.48) of DMH 15 showed the presence of five methyl peaks from  $\delta_H$  0.76-0.98 including a vinylic methyl singlet at  $\delta_H$  1.69, an exomethylene singlets at  $\delta_H$  4.76 and  $\delta_H$  4.63, however it also exhibited the presence of aldehyde proton singlet peak at  $\delta_H$  9.68 instead of hydroxymethyl.

The  $^{13}C$  NMR spectrum (175 MHz,  $CDCl_3$ ; Table 3.24; Figure 3.49) showed the presence of 30 carbons in the molecule including an aldehyde at  $\delta_C$  206.9, oxymethine carbon at  $\delta_C$  79.2 and exomethylene carbon at  $\delta_C$  110.4.

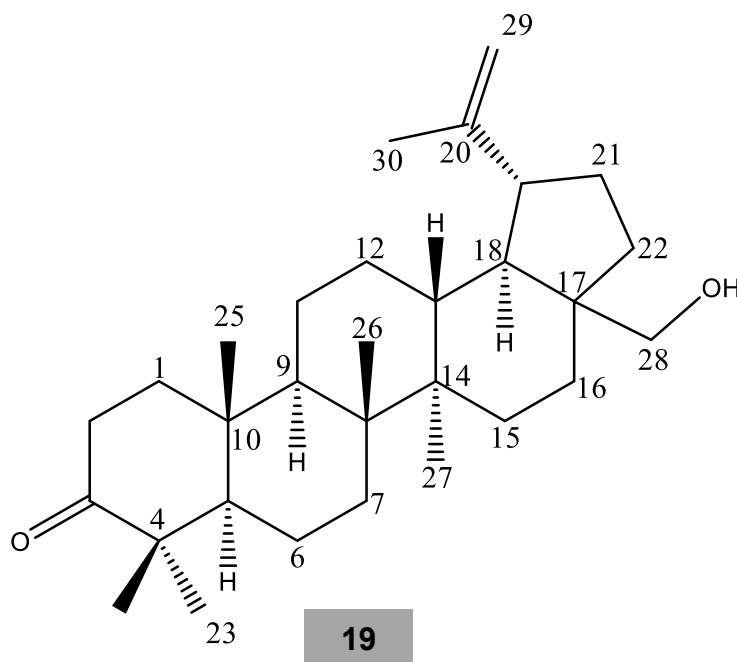


The DEPT experiment confirmed that the carbonyl at 206.9 as an aldehyde group and also the presence of methyl, methylene and methines like DMH 3. The HMBC correlations were also very much similar to those of DMH 3. In HMBC experiment (Table 3.25), the proton of aldehyde group at  $\delta_H$  9.68 (C-28;  $\delta_C$  206.9 from HSQC) exhibited  $^2J$  connection with the quaternary carbon at  $\delta_C$  59.6 (C-17). Thus based on NMR and Mass spectrometry data, the compound DMH 15 was identified as 3-hydroxy-20(29)-Lupen-28-al (**18**) commonly known as betulinaldehyde. The spectral data of DMH 15 were in agreement with literature data (Haque et al., 2006). This is a common plant derived compound which was also previously reported from the same genus *Diospyros* but different species of the bark of *Diospyros canaliculata* (Zhong et al., 1984).

### 3.2.6.5 Characterization of DMH 2 as Betulone (19):

The IR spectrum of DMH 2 revealed the presence of exomethylene at  $882\text{ cm}^{-1}$  and carbonyl at  $1702\text{ cm}^{-1}$ . The high resolution of mass spectroscopy showed (Figure 3.50) the  $[M+H]^+$  at  $m/z$  441.37232 (calculated for  $C_{30}H_{49}O_2$ , at 441.373255) which confirmed the molecular formula of DMH 2 as  $C_{30}H_{48}O_2$ .

The  $^1\text{H}$  NMR spectrum (700 MHz,  $\text{CDCl}_3$ ; Table 3.24; Figure 3.51) showed the presence of exomethylene protons as singlets at  $\delta_{\text{H}}$  4.69 and 4.59, the hydroxymethyl signals resonating at  $\delta_{\text{H}}$  3.80 (d,  $J= 10.78\text{ Hz}$ ) and  $\delta_{\text{H}}$  3.35 (d,  $J= 10.78\text{ Hz}$ ), five methyl proton signals between  $\delta_{\text{H}}$  0.99-1.02 including a vinylic methyl at  $\delta_{\text{H}}$  1.68 and numerous signals between  $\delta_{\text{H}}$  1.06-2.49 due to the presence of methine and methylene protons.



The  $^{13}\text{C}$  NMR (175 MHz,  $\text{CDCl}_3$ ; Table 3.24; Figure 3.52) revealed a total of 30 carbons including a carbonyl at  $\delta_{\text{C}}$  218.2, exomethylene carbon at  $\delta_{\text{C}}$  109.9 and hydroxymethyl carbon at  $\delta_{\text{C}}$  60.7. The DEPT 135 identified 6 methyl, 11 methylene including exomethylene and hydroxymethyl, 5 methines and 8 quaternary carbons.

The Key HMBC correlations were very much similar to DMH 3. The only difference was the presence of carbonyl  $\delta_{\text{C}}$  218.2 at C-3 position instead of oxymethine of DMH 3. In HMBC experiment (Table 3.25), the methyl protons at  $\delta_{\text{H}}$  1.07 (H-23; 26.6) and  $\delta_{\text{H}}$  1.02 (H-24;  $\delta_{\text{C}}$  21.3) showed  $^3J$  connectivity with the carbon of carbonyl  $\delta_{\text{C}}$  218.2 at (C-3) position. Moreover, the methylene protons at  $\delta_{\text{H}}$  2.41, 2.49 (H-2;  $\delta_{\text{C}}$  34.4 from HSQC) and  $\delta_{\text{H}}$  1.39 and 1.89 (H-1;  $\delta_{\text{C}}$  39.8 from HSQC) exhibited  $^2J$  and  $^3J$  correlation respectively, with the carbonyl at  $\delta_{\text{C}}$  218.2 (C-3).

Therefore, based on the NMR spectra, IR and Mass spectrometry data, and its direct comparison with published data, the compound was identified as 20 (29) - lupine-3, 28-diol (**19**) commonly known as Betulone (Satiraphan et al., 2012). Previously it was isolated from the bark of *Betula lenta* (Cole et al., 1991).

**Table 3.24:  $^1\text{H}$  NMR data (700 MHz,  $\text{CDCl}_3$ ) and  $^{13}\text{C}$  NMR data (175 MHz,  $\text{CDCl}_3$ ) of DMH 15 and DMH 2**

Position	$\delta^1\text{H}$ of DMH 15	$^{13}\text{C}$ of DMH 15	$\delta^1\text{H}$ of DMH 2	$^{13}\text{C}$ of DMH 2
1	1.66, m 0.90, m	38.9	1.39, m 1.89, m	39.8
2	2.02, m	27.6	2.41, m 2.49, m	34.4
3	3.64, t, $J=6.65$ Hz	79.2	-	218.2
4	-	39.1	-	47.6
5	0.68, d, $J= 10.01$ Hz	55.5	1.33, m	55.1
6	1.38, m 1.51, m	18.5	1.45, m 1.55, m	19.8
7	2.34, m 2.43, m	34.4	1.45, m	33.7
8	-	41.1	-	41.0
9	1.25, s	50.7	1.39, m	49.9
10	-	37.3	-	37.0
11	0.85, m	20.9	1.42, m 1.26, m	21.5
12	1.75, m 1.05, m	25.8	1.05, m 1.66, m	25.4
13	2.02, m	38.9	1.69, m	37.6
14	-	42.9	-	42.9
15	1.56, m	27.6	1.08, m 1.72, m	27.2
16	1.45, m 1.25, m	29.5	1.23, m 1.95, m	29.3
17	-	59.6	-	34.3
18	1.71, m	48.3	2.08, m	48.9
19	2.86, m	47.6	2.40, m	47.9
20	-	149.6	-	150
21	1.45, m 1.25, m	29.8	1.44, m 1.97, m	29.9
22	2.35, m 2.44, m	33.9	1.06, m 1.86, m	34.1
23	0.97, d, $J=8.33$ Hz	28.2	1.07, d, $J= 7.28$	26.8
24	0.76, s	15.6	1.02, s	21.3
25	0.82, s	16.4	0.93, s	16.2
26	0.91, s	16.2	1.06, s	16.0
27	0.98, d, $J= 8.33$ Hz	14.3	0.99, s	14.9
28	9.68, s	206.9	3.80, d, $J= 10.78$ 3.35, d, $J= 10.78$	60.7
29	4.76, s 4.63, s	110.4	4.69, s 4.59, s	109.9
30	1.69, s	19.2	1.68, d, $J= 6.79$	19.2



**Table 3.25: HMBC correlation (700 MHz, CDCl<sub>3</sub>) of DMH 15 and DMH 2**

Protons	HMBC of DMH 15		HMBC of DMH 2	
	<sup>2</sup> J	<sup>3</sup> J	<sup>2</sup> J	<sup>3</sup> J
H-1	-	55.5 (CH)	36.9 (C)	218.2 (C=O), 16.1 (CH <sub>3</sub> ),
H-2	-	39.1 (C)	218.2 (C=O), 39.8 (CH <sub>2</sub> )	47.5 (C)
H-3	27.6 (CH <sub>2</sub> )		-	-
H-5	18.5 (CH <sub>2</sub> )	34.4 (CH <sub>2</sub> )	36.9 (C), 47.5 (C)	16.1 (CH <sub>3</sub> ), 21.3 (CH <sub>3</sub> )
H-6	55.5 (CH)	41.1 (C)	33.7 (CH <sub>2</sub> ), 55.1 (CH)	16.1 (CH <sub>3</sub> )
H-7	-	-	19.8 (CH <sub>3</sub> )	15.9 (CH <sub>3</sub> ) 55.1 (CH), 49.9 (CH)
H-9	37.3 (C), 41.1 (C), 29.8 (CH <sub>2</sub> )	38.9 (CH)	41.0 (C)	15.9 (CH <sub>3</sub> )
H-11	-	37.3 (C), 16.4 (CH <sub>3</sub> ), 38.9 (CH)	49.9 (CH)	41.0 (C)
H-12	20.9 (CH <sub>2</sub> )	48.3 (CH), 59.6 (C)	-	-
H-13	48.3 (CH), 42.9 (C)	14.3 (CH <sub>3</sub> ), 29.5 (CH <sub>2</sub> )	48.8 (CH), 42.7 (CH)	14.9 (CH <sub>3</sub> )
H-15	29.5 (CH <sub>2</sub> ), 42.9 (C)	14.3 (CH <sub>3</sub> )	42.7 (C)	14.9 (CH <sub>3</sub> )
H-16	-	59.6 (C)	34.3 (C)	42.7 (C), 48.8 (CH)
H-18	38.9 (CH), 47.6 (CH)	-	34.3 (C)	29.9 (CH <sub>2</sub> )
H-19	29.8 (CH <sub>2</sub> ), 149.6 (C), 48.3 (CH)	19.2 (CH <sub>3</sub> ), 38.9 (CH), 110.4 (CH <sub>2</sub> =)	29.9 (CH <sub>2</sub> ), 48.8 (CH)	19.2 (CH <sub>3</sub> )
H-21	47.6 (CH)	59.6 (C)	-	48.8 (CH), 150 (C)
H-22	-	-	29.9 (CH <sub>2</sub> )	48.8 (CH), 60.7 (CH <sub>2</sub> OH)
H-23	39.1 (C)	55.5 (CH), 79.2 (CH-O)	47.5 (C)	26.8 (CH <sub>3</sub> ), 55.1 (CH)
H-24	39.1 (C)	55.5 (CH), 79.2 (CH-O)	47.5 (C)	21.3 (CH <sub>3</sub> ), 218.2 (C), 55.1 (CH)
H-25	37.3 (C)	50.7 (CH), 55.5 (CH)	36.9 (C)	39.8 (CH <sub>2</sub> ), 55.1 (CH), 49.9 (CH)
H-26	41.1 (C)	34.4 (CH <sub>2</sub> ), 42.9 (C), 50.5 (CH)	41.0 (C)	33.7 (CH <sub>2</sub> ), 49.9 (CH), 42.7 (CH)
H-27	42.9 (C)	41.1 (C)	42.7 (C)	37.6 (CH), 41.0 (C), 27.2 (CH <sub>2</sub> )
H-28	59.6 (C)	-	-	47.9 (CH), 29.3 (CH <sub>2</sub> ), 34.1 (CH <sub>2</sub> )
H-29	-	19.2 (CH <sub>3</sub> ), 47.6 (CH)	150.5 (C)	19.2 (CH <sub>3</sub> ), 47.9 (CH)
H-30	-	47.6 (CH)	150.5 (C)	109.9 (CH <sub>2</sub> =)

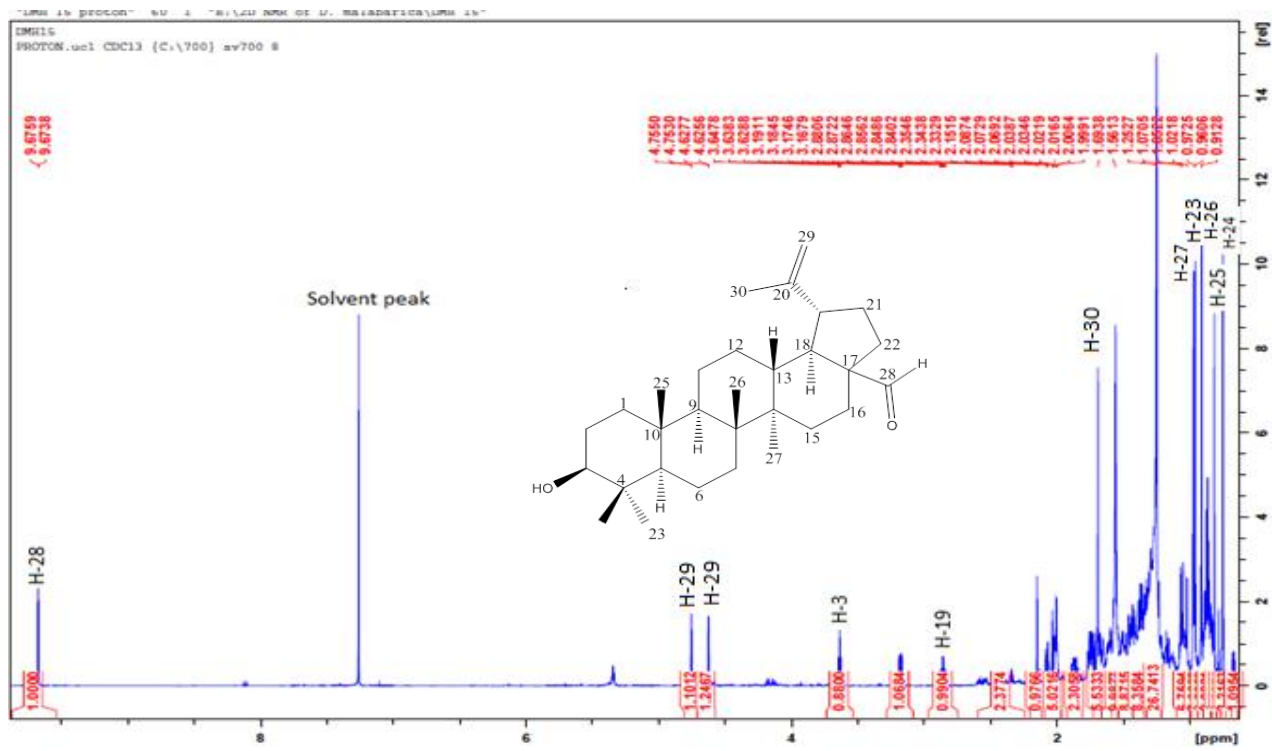
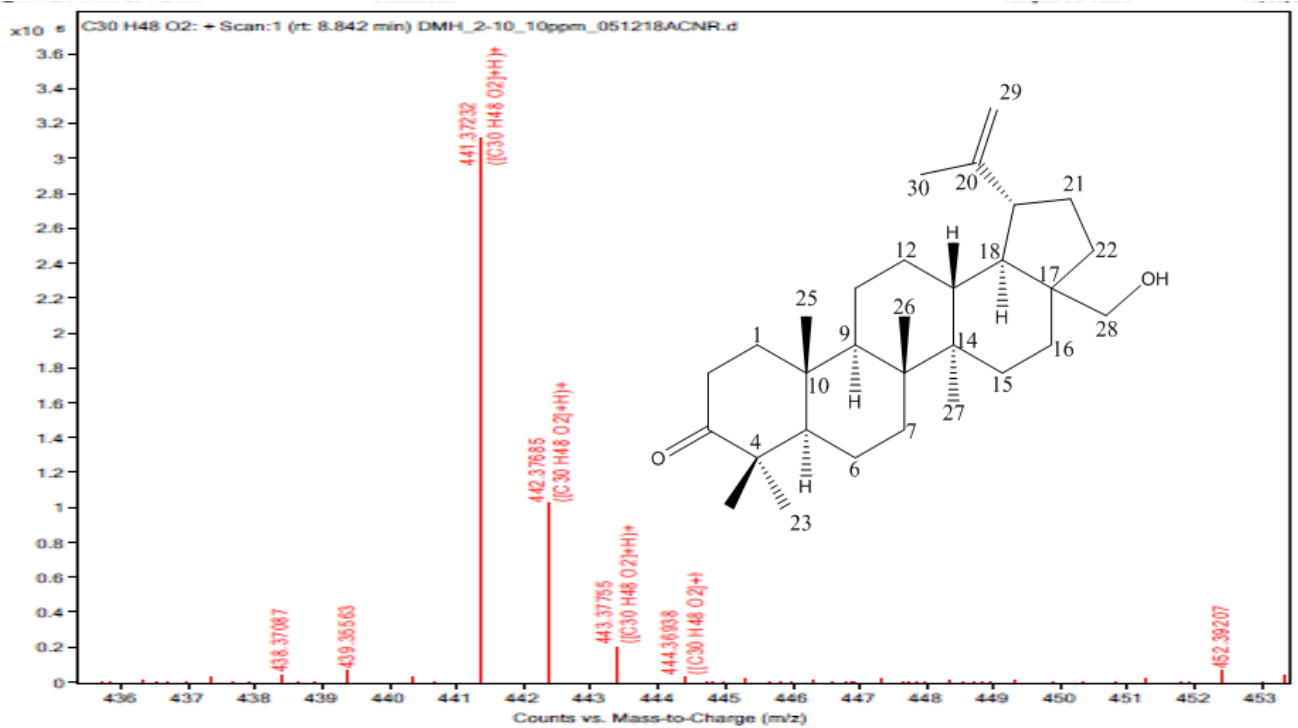
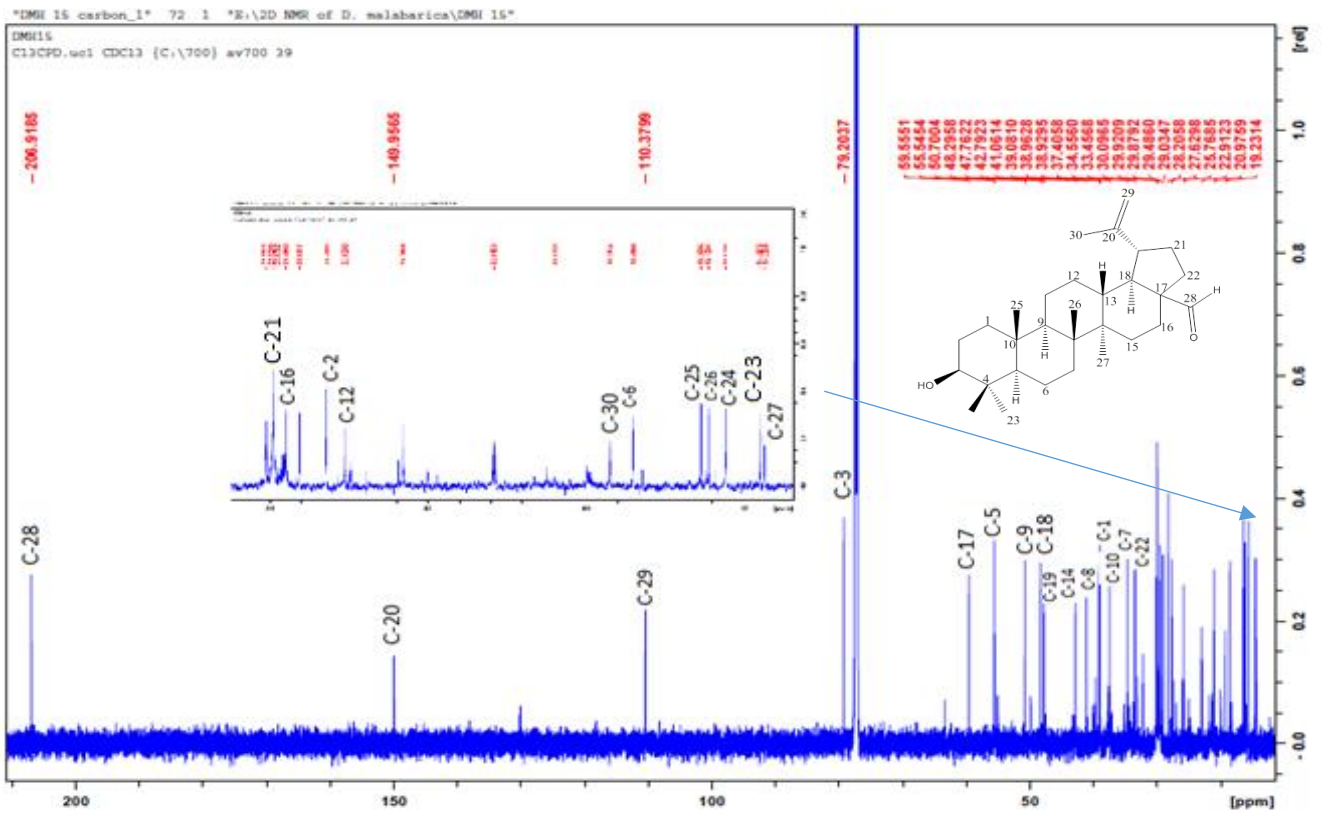


Figure 3.52: <sup>1</sup>H NMR spectrum (700MHz) of DMH 15 in CDCl<sub>3</sub>



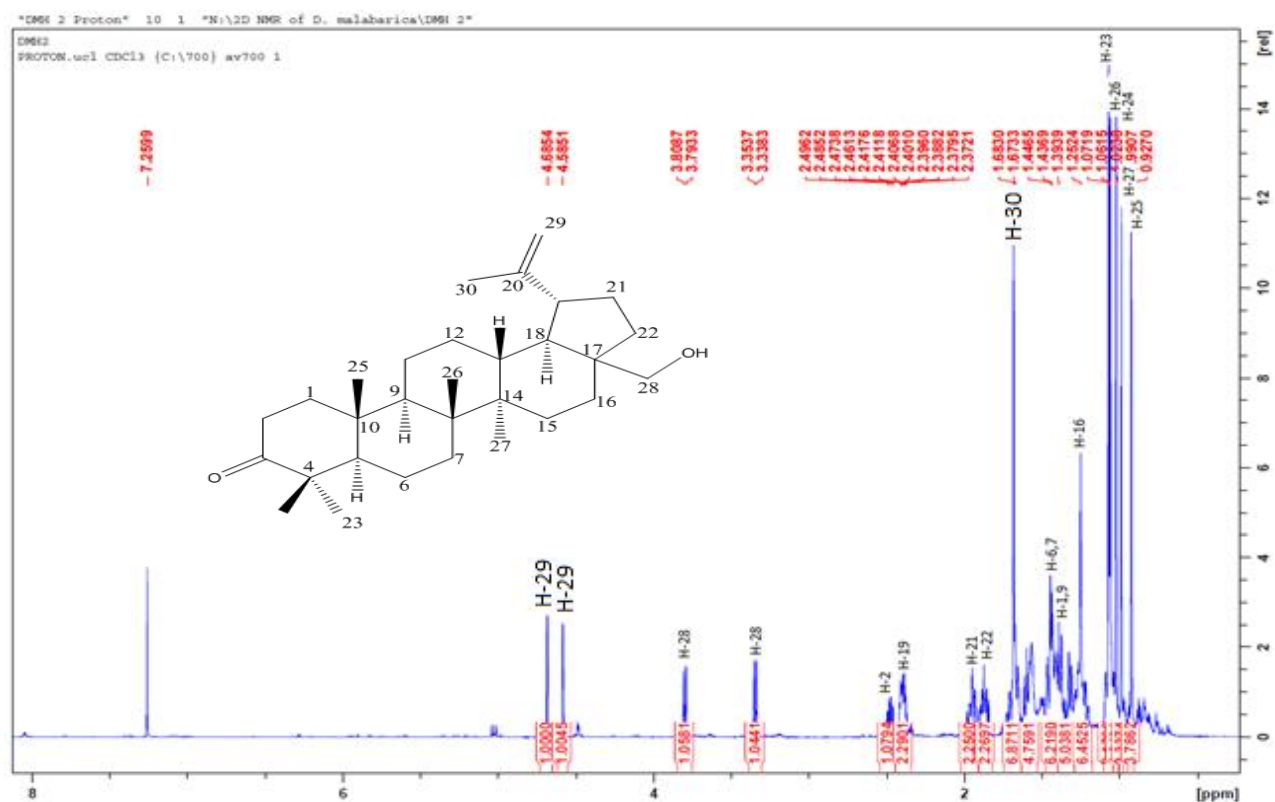


Figure 3.55:  $^1\text{H}$  NMR spectrum (700MHz) of DMH 2 in  $\text{CDCl}_3$

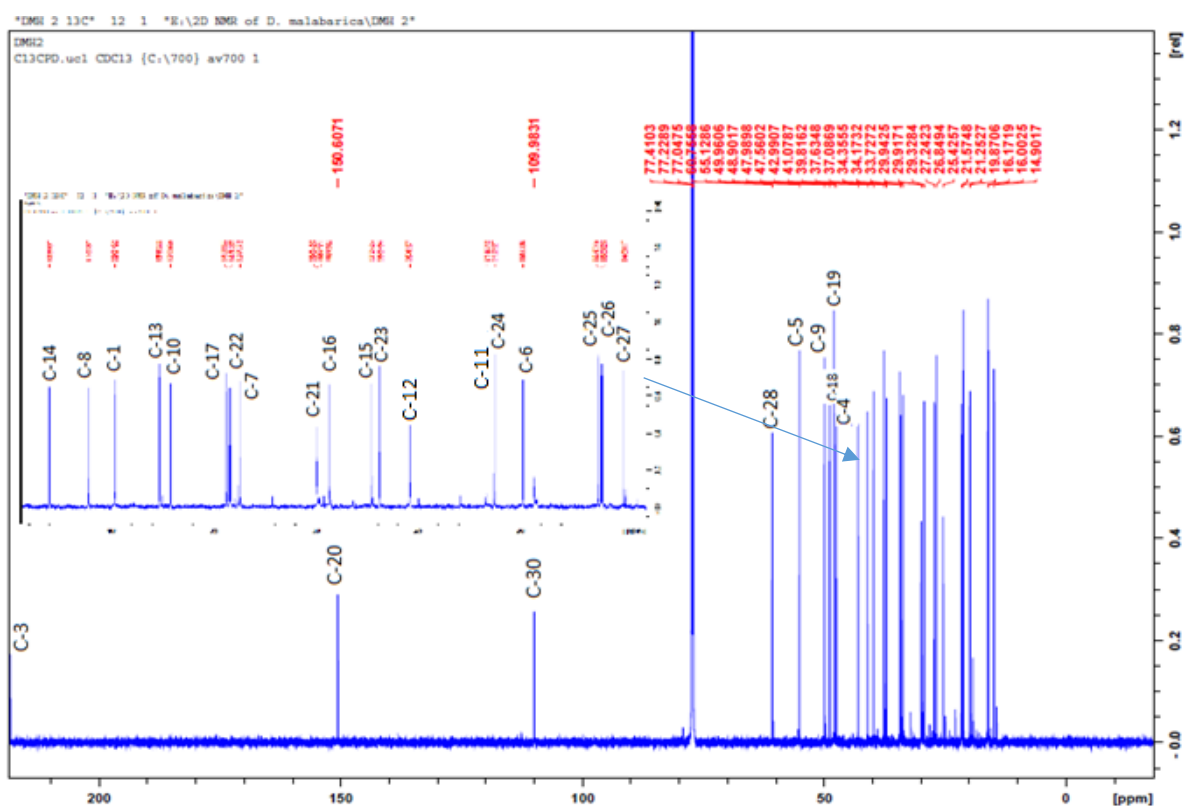


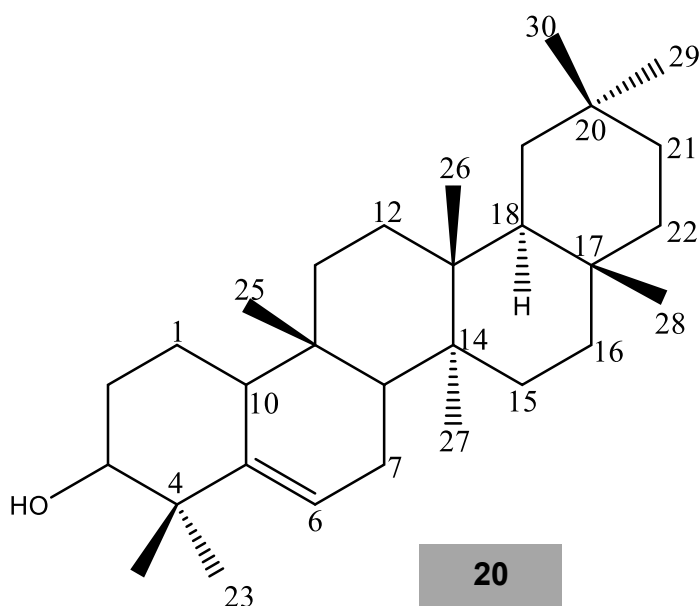
Figure 3.56:  $^{13}\text{C}$  NMR spectrum (175MHz) of DMH 2 in  $\text{CDCl}_3$

### 3.2.6.6 Characterization of CRH 9 as glutinol (20)

The  $^1\text{H}$  NMR spectra (700MHz,  $\text{CDCl}_3$ , Table 3.26, Figure 3.53) of CRH 12 exhibited the presence of eight methyl groups resonated between  $\delta_{\text{H}}$  0.85-1.16, an olefinic proton at  $\delta_{\text{H}}$  5.63 (d,  $J=6.16$  Hz), an oxymethine at  $\delta_{\text{H}}$  3.46 and a number of signals between  $\delta_{\text{H}}$  0.91-1.69 due to the presence of numerous methine and methylene protons in the molecule.

The  $^{13}\text{C}$  NMR spectrum (175 MHz,  $\text{CDCl}_3$ , Table 3.26, Figure 3.54) showed a total of 30 carbons including an oxymethine at  $\delta_{\text{C}}$  76.6, an olefinic methine at  $\delta_{\text{C}}$  122.3 and a quaternary carbon at  $\delta_{\text{C}}$  141.8. The DEPT experiment also confirmed a total of 8 methyl, 10 methylene, 5 methine and 7 quaternary carbons in the molecule.

The complete structure of this compound was established by 2D NMR spectra, predominantly by HMQC and HMBC. In HMBC experiment (Table 3.27),



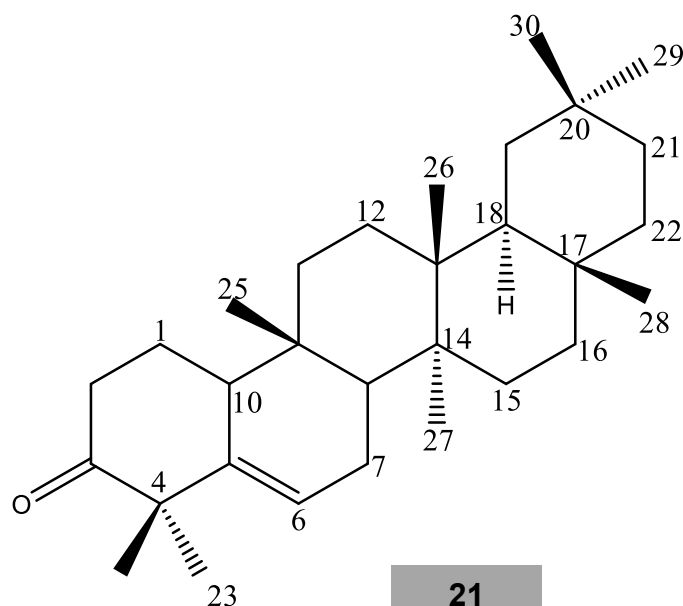
methylene protons at  $\delta_{\text{H}}$  1.69,  $\delta_{\text{H}}$  1.88 (H-2;  $\delta_{\text{C}}$  28.0 from HSQC) showed  $^2J$  correlation with oxymethine carbon at  $\delta_{\text{C}}$  76.6 (C-3;  $\delta_{\text{H}}$  3.46). Moreover, methylene protons at  $\delta_{\text{H}}$  1.46,  $\delta_{\text{H}}$  1.53 (H-1;  $\delta_{\text{C}}$  18.3 from HSQC) and two methyl protons at  $\delta_{\text{H}}$  1.14 (H-23;  $\delta_{\text{C}}$  25.6 from HSQC) and 1.05 (H-24;  $\delta_{\text{C}}$  29.2 from HSQC) exerted  $^3J$  connectivity with oxymethine carbon at  $\delta_{\text{C}}$  76.6 (C-3;  $\delta_{\text{H}}$  3.46). The downfield olefinic methine proton at  $\delta_{\text{H}}$  5.62 (H-6;  $\delta_{\text{C}}$  122.3 from HSQC) revealed  $^2J$  connection with the quaternary carbon at  $\delta_{\text{C}}$  141.6 (C-5) and  $^3J$  connection with quaternary carbon at  $\delta_{\text{C}}$  41.0 (C-4) and methine carbons at  $\delta_{\text{C}}$  47.6 (C-8;  $\delta_{\text{H}}$  1.51 from HSQC) and  $\delta_{\text{C}}$  49.9 (C-10;  $\delta_{\text{H}}$  1.99). In addition, the angular methyl proton at  $\delta_{\text{H}}$  0.85 (H-25;  $\delta_{\text{C}}$  16.4) also showed  $^3J$  connection with methine carbons at  $\delta_{\text{C}}$  47.6 (C-8;  $\delta_{\text{H}}$  1.51 from HSQC) and  $\delta_{\text{C}}$  49.9 (C-10;  $\delta_{\text{H}}$  1.99). Moreover, the methine protons at  $\delta_{\text{H}}$  1.51 (H-8;  $\delta_{\text{C}}$  47.6) also showed  $^3J$  connection with two methyl carbons at  $\delta_{\text{C}}$  16.4 (C-25;  $\delta_{\text{H}}$  0.85) and  $\delta_{\text{C}}$  19.8 (C-27;  $\delta_{\text{H}}$  1.09) and methine carbon at  $\delta_{\text{C}}$  49.9 (C-10;  $\delta_{\text{H}}$  1.99).

The methyl protons at  $\delta_{\text{H}}$  1.00 (H-26;  $\delta_{\text{C}}$  18.6), H-27 ( $\delta_{\text{H}}$  1.09,  $\delta_{\text{C}}$  19.8) and H-28 ( $\delta_{\text{H}}$  1.16,  $\delta_{\text{C}}$  32.1) were shown  $^2J$  connection with the quaternary carbon attached next to them at C-13 ( $\delta_{\text{C}}$  38.0), C-14 ( $\delta_{\text{C}}$  47.6) and C-17 ( $\delta_{\text{C}}$  30.3). However, methyl protons notably H-26 and H-28 showed common  $^3J$  correlation with methine carbon at  $\delta_{\text{C}}$  43.3 (C-18;  $\delta_{\text{H}}$  1.57 from HSQC). Methyl protons at H-26 ( $\delta_{\text{H}}$  1.00;  $\delta_{\text{C}}$  18.6) also exhibit  $^3J$  correlation with methylene carbon at  $\delta_{\text{C}}$  30.6 (C-12;  $\delta_{\text{H}}$  1.34 from HSQC) and quaternary carbon at 38.0 (C-14). However, methyl proton at H-27 ( $\delta_{\text{H}}$  1.09,  $\delta_{\text{C}}$  19.8) showed connection with methine carbon at 47.6 (C-8;  $\delta_{\text{H}}$  1.51) and quaternary carbon at 39.6 (C-13) by  $^3J$ . Another angular methyl proton at H-28 ( $\delta_{\text{H}}$  1.16,  $\delta_{\text{C}}$  32.1) showed  $^3J$  connection with methylene carbons at  $\delta_{\text{C}}$  36.2 (C-16;  $\delta_{\text{H}}$  1.38, 1.53) and 39.2 (C-22;  $\delta_{\text{H}}$  0.91, 1.54 from HSQC).

On the other hand, Methyl protons at  $\delta_{\text{H}}$  0.95 (H-29;  $\delta_{\text{C}}$  34.8) and  $\delta_{\text{H}}$  0.98 (H-30;  $\delta_{\text{C}}$  32.6) showed  $^2J$  connection with quaternary carbon at C-20 ( $\delta_{\text{C}}$  28.5) and  $^3J$  connection with methylene carbon at  $\delta_{\text{C}}$  33.4 (C-21;  $\delta_{\text{H}}$  1.46, 1.24 from HSQC). Based on NMR, IR data, and its direct comparison with published data, the compound CRH 9 has been identified as glutinol (**20**; Adebayo et al., 2017).

### 3.2.6.7 Characterization of CRC 10 as glutinone (**21**)

The  $^1\text{H}$  NMR spectrum (700MHz,  $\text{CDCl}_3$ , Table 3.26, Figure 3.55) and  $^{13}\text{C}$  NMR (175MHz,  $\text{CDCl}_3$ ) of CRC 10 are almost similar to those in CRH 9. Like CRH 9, the  $^1\text{H}$  NMR of CRC 10 showed there is the presence of 8 methyl signals between  $\delta_{\text{H}}$  0.83-1.25, an olefinic methine proton at  $\delta_{\text{H}}$  5.69 and a number of signals between  $\delta_{\text{H}}$  0.94-2.26 representing numerous methine and



methylene protons in the molecule but however, there was no signal at 3.45 confirming the absence of oxymethine peak at C-3. The  $^{13}\text{C}$  NMR (175MHz,  $\text{CDCl}_3$ , Table 3.26, Figure 3.56) spectrum revealed the presence of a total of 30

carbons including carbonyl at  $\delta_C$  215.0 instead of oxymethine carbon, olefinic methine carbon at  $\delta_C$  122.5 and olefinic quaternary carbon at  $\delta_C$  142.3.

The key HMBC correlations were almost identical to CRH 9. The only difference was the presence of carbonyl carbon 215.0 instead of oxymethine carbon at the same position. In HMBC experiment, the methylene proton at  $\delta_H$  2.34, 2.45 (H-2;  $\delta_C$  34.4) showed  $^2J$  correlation with the carbonyl carbon at  $\delta_C$  215.0 (C-3). The methylene protons at  $\delta_H$  1.63, 1.88 (H-1;  $\delta_C$  21.8) and methyl proton at  $\delta_H$  1.24 (H-24;  $\delta_C$  24.6) showed  $^3J$  connection with the carbon of carbonyl at  $\delta_C$  215.0 (C-3). The rest of the HMBC correlations were identical those in CRH 9. Therefore, based on the NMR data, the compound was identified as alnusenone (**21**; glutinone). However, this is the first time report NMR data of alnusenone.

**Table 3.26:  $^1H$  (500 MHz) and  $^{13}C$  (125 MHz) NMR spectroscopic data for CRH 9 and CRC 10 in  $CDCl_3$**

Position	CRH 9		CRC 10	
	$\delta_H$	$^{13}C$	$\delta_H$	$^{13}C$
1	1.46, m 1.52, m	18.3	1.88, m 1.63, m	21.8
2	1.69, m 1.88, m	28.0	2.45, m 2.34, m	34.4
3	3.46, s	76.6	-	215.0
4	-	41.0	2.25, m	50.3
5	-	141.8	-	142.3
6	5.63, d, $J= 6.16$ Hz	122.3	5.69, m	122.5
7	1.97, m 1.84, m	23.8	2.01, m	23.8
8	1.51, m	47.6	1.68, m	47.3
9	-	35.1	-	29.4
10	1.99, m	49.9	2.26, m	50.7
11	1.52, m 1.39, m	34.8	0.96, m	34.8
12	1.34, m	30.6	1.40, m	30.6
13	-	39.6	-	39.5
14	-	38.0	-	38.1
15	1.46, m 1.29, m	32.2	0.99, m	32.5
16	1.53, m 1.38, m	36.2	1.94, m	39.2
17	-	30.3	-	30.3
18	1.57	43.3	1.59, m	43.3
19	1.37, m 1.25, m	35.2	1.41, m	35.3
20	-	28.5	-	28.5
21	1.46, m 1.24, m	33.4	1.49, m 1.29, m	33.3
22	1.54, m 0.91, m	39.2	ns	36.2

23	1.14, s	25.6	1.25, s	28.8
24	1.05, s	29.2	1.24, s	24.6
25	0.85, s	16.4	0.83, s	15.8
26	1.00, s	18.6	1.10, s	19.6
27	1.09, s	19.8	1.04, s	18.6
28	1.16, s	32.1	1.18, s	32.2
29	0.95, s	34.8	0.97, s	34.7
30	0.98, s	32.6	1.00, s	32.6

**Table 3.27: HMBC spectroscopic data for CRH 9 and CRC 10 in CDCl<sub>3</sub>**

Protons	HMBC of CRH 9		HMBC of CRC 10	
	<sup>2</sup> J	<sup>3</sup> J	<sup>2</sup> J	<sup>3</sup> J
H-1	28.0 (CH <sub>2</sub> ), 49.9 (CH)	76.6 (CH-O)	50.7 (CH)	142.3 (C), 215.0 (C=O)
H-2	18.3 (CH <sub>2</sub> ), 76.6 (CH-O)	49.9 (CH)	21.8 (CH <sub>2</sub> ), 215.0 (C=O)	50.7 (CH)
H-3	18.3 (CH <sub>2</sub> )	141.8 (C)	-	-
H-6	141.8 (C)	41.0 (C), 47.6 (CH), 49.9 (CH)	23.8 (CH <sub>2</sub> )	47.3 (CH), 50.7 (CH)
H-7	47.6 (CH)	35.1 (C)	-	29.4 (C)
H-8	35.1 (C), 23.8 (CH <sub>2</sub> )	49.9 (CH), 38.0 (C), 19.8 (CH <sub>3</sub> ), 16.4 (CH <sub>3</sub> )	23.8 (CH <sub>2</sub> )	15.8 (CH <sub>3</sub> ), 19.6 (CH <sub>3</sub> )
H-10	35.1 (C)	18.4 (CH <sub>2</sub> ), 16.4 (CH <sub>2</sub> ), 47.6 (CH), 41.0 (C)	29.4 (C)	-
H-11	35.1 (C)	16.4 (CH <sub>3</sub> ), 38.0 (C)	30.6 (CH <sub>2</sub> )	-
H-12	34.8 (CH <sub>2</sub> )	18.6 (CH <sub>3</sub> ), 39.6 (C)	-	-
H-15	39.6 (C), 36.2 (CH <sub>2</sub> )	38.0 (C), 19.8 (CH <sub>3</sub> )	-	-
H-16	30.3 (C)	43.3 (CH), 32.1 (CH <sub>3</sub> )	-	32.2 (CH <sub>3</sub> )
H-18	30.3 (C)	39.2 (CH <sub>2</sub> ), 32.1 (CH <sub>3</sub> ), 18.6 (CH <sub>3</sub> ), 28.5(C)	39.5 (C)	-
H-19	43.3 (CH), 28.5 (C)	38.0 (C), 34.8 (CH <sub>3</sub> ), 32.6 (CH <sub>3</sub> ), 30.3 (C)	-	39.5 (C)
H-21	39.2 (CH <sub>2</sub> ), 28.5 (C)	32.6 (CH <sub>3</sub> ), 30.3 (C)	-	34.7 (CH <sub>3</sub> )
H-22	33.4 (CH <sub>2</sub> )	43.3 (CH)	-	-
H-23	41.0 (C)	29.2 (CH <sub>3</sub> ), 76.6 (CH-O), 141.8 (C)	-	24.6 (CH <sub>3</sub> )
H-24	41.0 (C)	25.6 (CH <sub>3</sub> ), 76.6 (CH-O), 141.8 (C)	50.3 (C)	28.8 (CH <sub>3</sub> ), 215.0 (C=O), 142.3 (C)
H-25	35.1 (C)	49.9 (CH), 47.6 (CH)	29.4 (C)	47.3 (CH), 50.7 (CH)
H-26	39.6 (C)	38.0 (C), 43.3 (CH), 30.6 (CH <sub>2</sub> )	38.1 (C)	39.5 (C)
H-27	38.0 (C)	47.6 (CH), 39.6 (C)	39.5 (C)	30.6 (CH <sub>2</sub> ), 43.3 (CH)



H-28	30.3 (C)	36.2 (CH <sub>2</sub> ), 39.2 (CH <sub>2</sub> ), 43.3 (CH)	30.3 (C)	36.2 (CH <sub>2</sub> ), 43.3 (CH)
H-29	28.5 (C)	33.4 (CH <sub>2</sub> )	28.5 (C)	33.3 (CH <sub>2</sub> ), 35.3 (CH <sub>2</sub> )
H-30	28.5 (C)	33.4 (CH <sub>2</sub> )	28.5 (C)	33.3 (CH <sub>2</sub> )

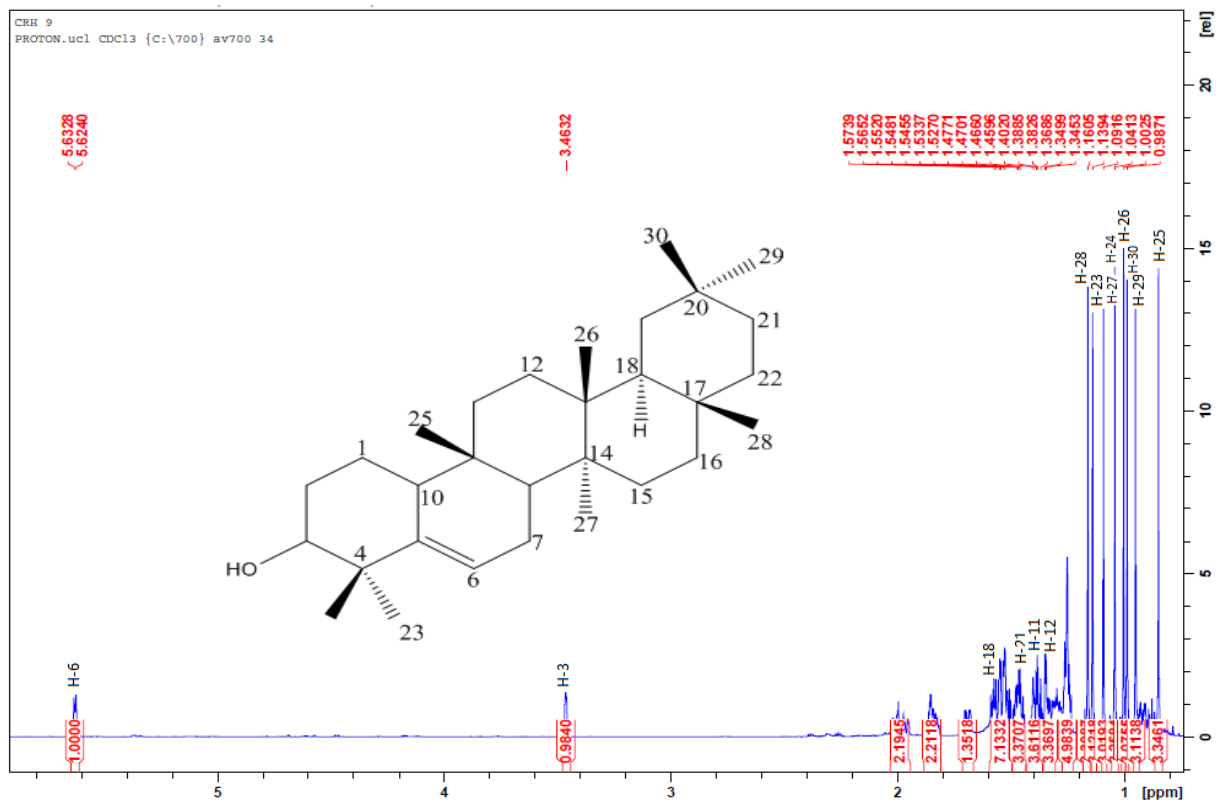


Figure 3.57: <sup>1</sup>H NMR spectrum (700MHz) of CRH 9 in CDCl<sub>3</sub>

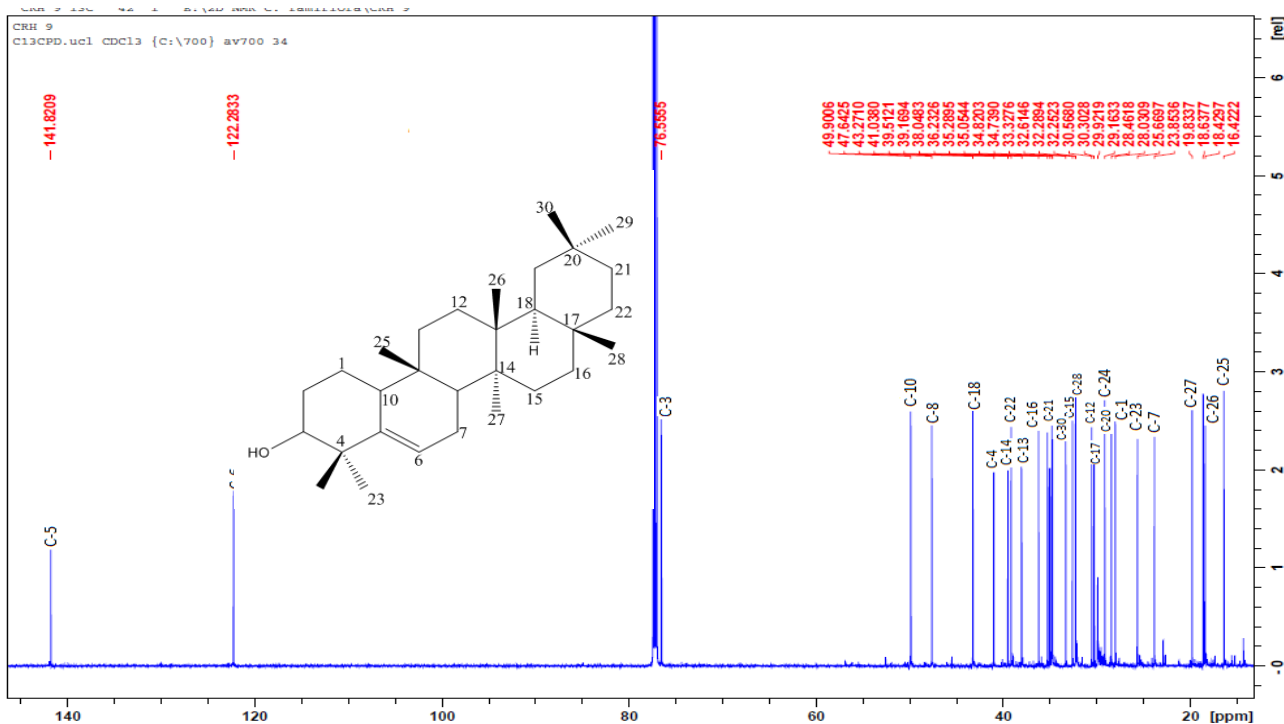


Figure 3.58: <sup>13</sup>C NMR spectrum (175MHz) of CRH 9 in CDCl<sub>3</sub>

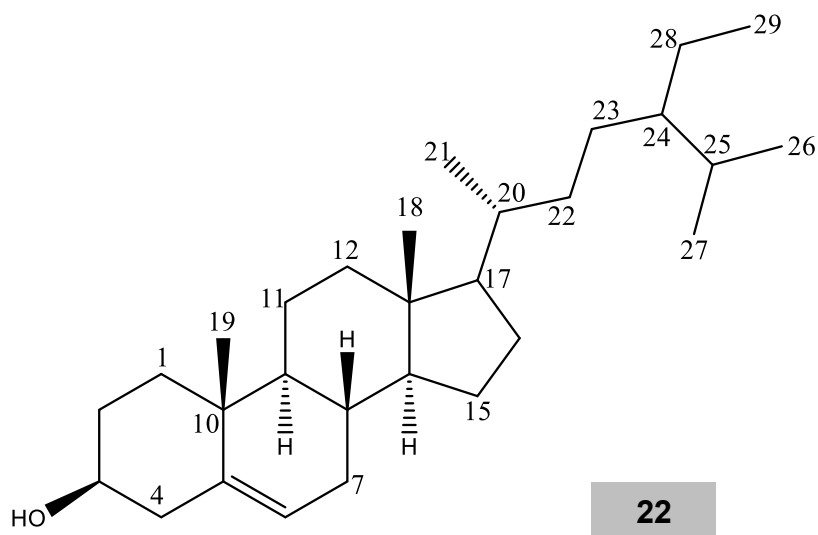


### 3.2.7 Steroids:

Steroids are the most common and abundant secondary metabolites widely distributed in the plants. In this study, the steroids with different functional groups have been isolated from the hexane extracts of the leave of *Cynometra ramiflora* and *Uraria picta*. These are CRH 16 and UPH 46a.

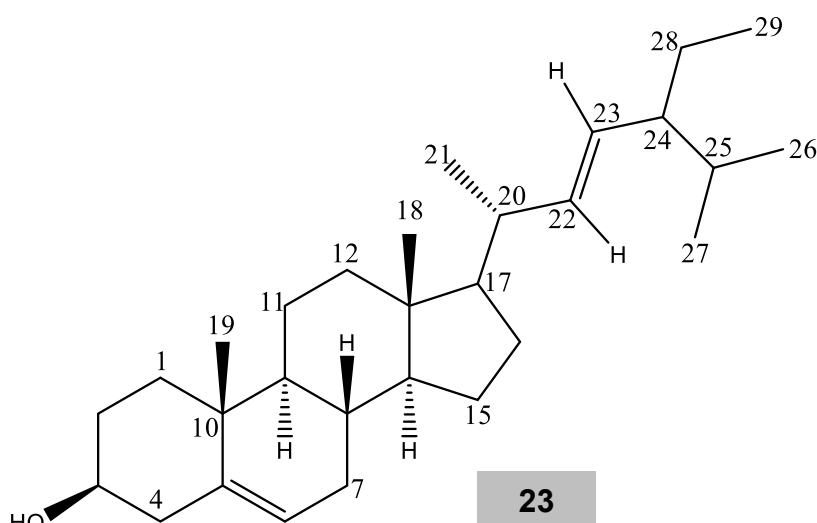
#### 3.2.7.1 Characterization of CRH 16 as a mixture 4:1 of $\beta$ sitosterol (22) and stigmasterol (23):

The  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra of CRH 16 revealed signals which have been interpreted as a mixture of two compounds in a ratio of 4:1. The  $^1\text{H}$  NMR spectrum (700MHz,  $\text{CDCl}_3$ , Table 3.28) showed the presence of olefinic proton as doublet at  $\delta_{\text{H}}$  5.36



( $J = 2.31$  Hz), an oxymethine signal at  $\delta_{\text{H}}$  3.52, signals between  $\delta_{\text{H}}$  0.68-1.01 indicating the presence of six sets of methyl protons and numerous multiplets between  $\delta_{\text{H}}$  1.05-3.55 for methine and methylene protons. Besides the above signal in the  $^1\text{H}$  NMR, the minor compound also revealed the presence of two olefinic protons at  $\delta_{\text{H}}$  5.15 (dd,  $J = 15.12$  Hz, 8.75 Hz) and  $\delta_{\text{H}}$  5.02 (dd,  $J = 15.61$  Hz, 8.65 Hz) which appeared to be *trans* double bonded to each other.

The  $^{13}\text{C}$  NMR spectrum (175 MHz,  $\text{CDCl}_3$ ; Table 3.28; Figure 3.57) exhibited a total of 29 carbons including an oxygenated methine ( $\delta_{\text{C}}$  72.0; typical C-3 of steroid/terpene), an



olefinic methine at  $\delta_{\text{C}}$  121.9 and a quaternary carbon at  $\delta_{\text{C}}$  140.9. The minor

compound revealed the presence of two olefinic methines at  $\delta_C$  138.6 and  $\delta_C$  129.5 which replaced two methylene carbons in the major compound. In HMBC (Table 3.30), oxygenated methine at  $\delta_H$  3.52 (H-3;  $\delta_C$  72.0) and methyl protons at  $\delta_H$  1.02 (H-19;  $\delta_C$  19.6 from HSQC) showed  $^3J$  correlation to a quaternary carbon at  $\delta_C$  140.9 (C-4). The olefinic proton at  $\delta_H$  5.36 (H-6;  $\delta_C$  121.9 from HSQC) showed  $^2J$  correlation with C-4 at  $\delta_C$  140.9 and  $^3J$  connectivity to a quaternary carbon at  $\delta_C$  37.1 (C-10) and a methylene carbon at  $\delta_C$  42.4 (C-4;  $\delta_H$  2.24; 2.29 from HSQC) and a methine carbon at  $\delta_C$  32.2 (C-8;  $\delta_H$  1.46 from HSQC). Both methyl groups at  $\delta_H$  0.68 (H-18;  $\delta_C$  12.0 from HSQC) and  $\delta_H$  0.92 (H-21;  $\delta_C$  18.9) were connected to a methine carbon at  $\delta_C$  56.7 (C-17;  $\delta_H$  1.10). Three sets of methyl protons at  $\delta_H$  0.81 (H-26;  $\delta_C$  19.2 from HSQC),  $\delta_H$  0.84 (H-27;  $\delta_C$  20.0 from HSQC) and  $\delta_H$  0.85 (H-29;  $\delta_C$  12.1 from HSQC) showed a common  $^3J$  correlation to a methine carbon at  $\delta_C$  46.1 (C-24;  $\delta_H$  0.93 from HSQC). Methyl protons H-26 and H-27 were connected to C-25 ( $\delta_C$  29.3;  $\delta_H$  1.66) by  $^2J$  whereas H-29 was connected to C-28 ( $\delta_C$  23.2) by  $^2J$ . In the major compound, H-24 ( $\delta_H$  0.93;  $\delta_C$  46.1) was connected to a methylene carbon at  $\delta_C$  34.4 (C-22;  $\delta_H$  1.02, 1.32) by  $^3J$ . Because of the presence of another double bond between C-22 and C-23, the protons and carbons chemical shift from C-17 to C-24 were changed which were confirmed from HMBC. In HMBC, the proton at  $\delta_H$  5.16 (H-22;  $\delta_C$  138.9 from HSQC) showed HMBC correlation to carbons at  $\delta_C$  129.5 (C-23),  $\delta_C$  40.8 (C-20),  $\delta_C$  21.4 (C-21),  $\delta_C$  51.1 (C-24) and  $\delta_C$  56.4 (C-17). Similarly, another olefinic proton at  $\delta_H$  5.03 (H-23;  $\delta_C$  129.5 from HSQC) showed HMBC correlation with the carbons at  $\delta_C$  138.6 (C-22),  $\delta_C$  40.8 (C-20),  $\delta_C$  51.1 (C-24). Accordingly, CRH 16 was characterized as a mixture (4:1) of  $\beta$ -sitosterol (**22**) and stigmasterol (**23**), two common phytosterols. The spectral data comply with those reported before. (Rahman, 2002)

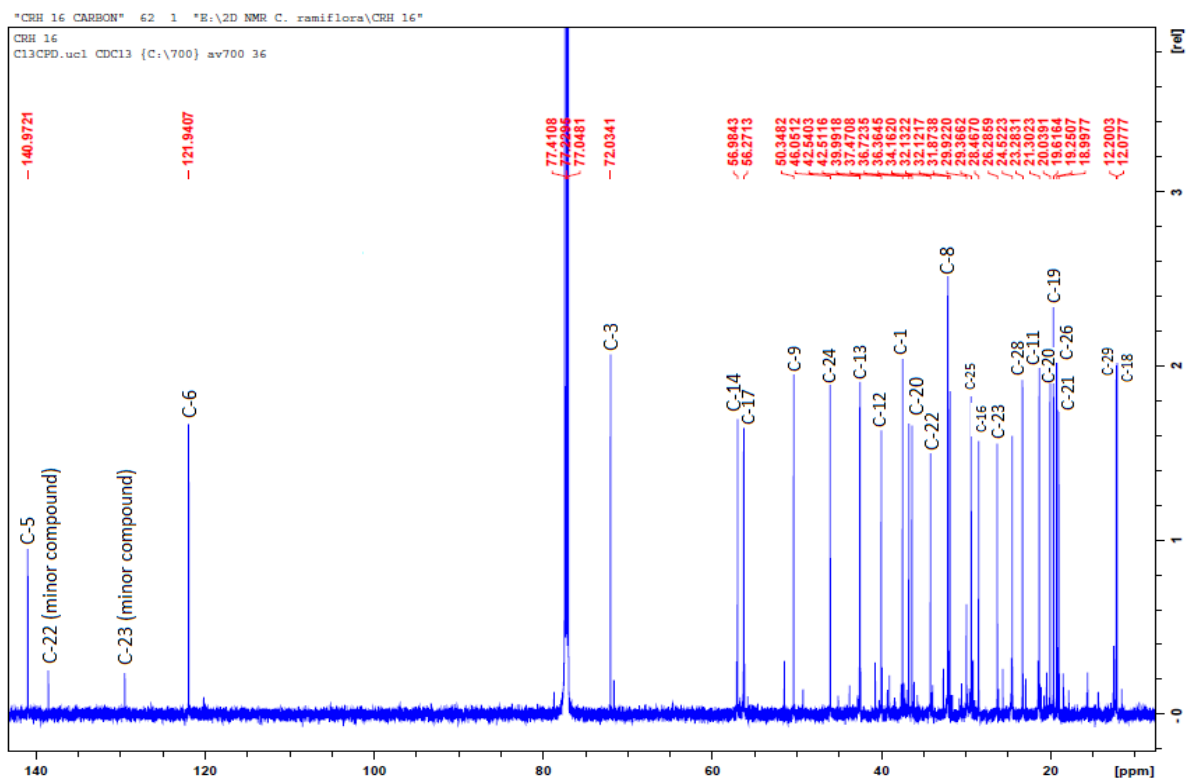


Figure 3.61:  $^{13}\text{C}$  NMR spectrum (175MHz) of CRH 16 in  $\text{CDCl}_3$

**Table 3.28: <sup>1</sup>H (500 MHz) and <sup>13</sup>C NMR (125 MHz) data for CRH 16 and UPH**

CRH 16			UPH 46a	
Position	$\delta^1\text{H}$	$\delta^{13}\text{C}$	$\delta^1\text{H}$	$\delta^{13}\text{C}$
1	1.85, m 1.09, m	37.5	1.69, m 2.02, m	35.9
2	1.83, m 1.51, m	31.8	2.34, m 2.43, m	34.2
3	3.53, m	72.0	-	199.9
4	2.29, m 2.24, m	42.4	ns	ns
5	-	140.9	-	171.9
6	5.36, d, $J=2.31$ Hz	121.9	5.73, s	123.9
7	1.53, m 1.97, m	31.8	1.10, m 1.60, m	24.4
8	1.46, m	32.2	ns	32.1
9	0.95, m	50.4	1.02, q, $J=11.62$ Hz	56.2
10	-	37.1	-	38.8
11	1.50, m 1.47, m	21.3	1.42, m 1.51, m	21.2
12	2.01, m 1.16, m	39.9	1.16, m 2.03, m	39.8
13	-	42.5	-	42.6
14	1.00, m	56.9	0.92, m	54.0
15	ns	ns	ns	ns
16	1.27, m	28.6	ns	ns
17	1.11, m	56.3	1.11, m	56.1
18	0.68, s	12.1	0.70, d, $J=12.88$ Hz	12.1
19	1.02, s	19.6	0.81, q, $J=10.25$ Hz	19.2
20	1.36, m	36.3	1.36, m	36.3
21	0.92, d, $J=6.58$ Hz	18.9	0.91, d, $J=6.65$ Hz	18.9
22	1.32, m 1.02, m	34.4	1.02, m 1.32, m	34.2
23	1.17, m	26.3	1.16, m	26.3
24	0.93, m	46.1	0.93, m	46.0
25	1.67, m	29.3	1.66, m	29.4
26	0.82, d, $J=6.86$ Hz	19.3	0.80, q, $J=10.5$ Hz	12.4
27	0.84, m	20.2	0.81, q, $J=10.25$ Hz	19.2
28	1.23, m 1.28, m	23.2	0.93, m	23.3
29	0.85, t, $J=6.86$ Hz	12.2	ns	ns

**Table 3.29: <sup>13</sup>C NMR (175 MHz) data of Stigmasterol and stigmasterone**

Position	Stigmasterol (CRH 16)	Stigmasterone (UPH 46a)
C-1	37.5	35.9
C-2	31.8	34.2
C-3	72.0	199.9
C-4	42.4	ns
C-5	140.9	171.9
C-6	121.9	123.9
C-7	31.8	24.4
C-8	32.2	32.1
C-9	50.4	56.2
C-10	37.1	38.8
C-11	21.3	21.2
C-12	39.9	39.8
C-13	42.5	42.6
C-14	56.9	54.0
C-15	ns	ns
C-16	28.6	ns
C-17	56.4	56.1
C-18	12.1	12.1
C-19	19.6	19.2
C-20	40.8	40.6
C-21	21.4	21.3
C-22	138.6	138.3
C-23	129.5	129.6
C-24	51.5	51.3
C-25	29.3	29.4
C-26	19.3	12.4
C-27	20.2	19.2
C-28	23.2	23.3
C-29	12.2	ns

**Table 3.30: HMBC NMR spectroscopic data for CRH 16 in CDCl<sub>3</sub>**

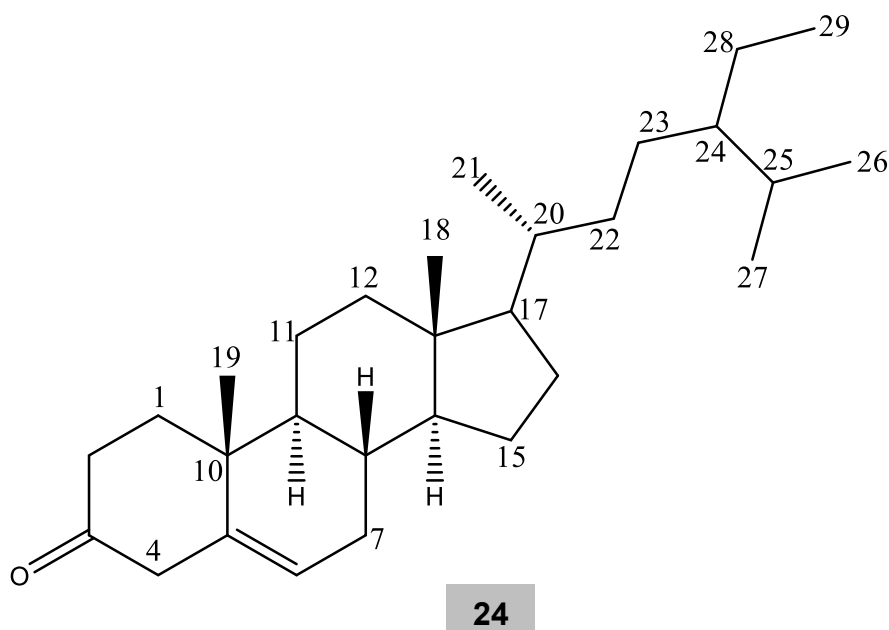
Protons	HMBC	
	<sup>2</sup> J	<sup>3</sup> J
H-1	72.0 (CH-O)	19.6 (CH <sub>3</sub> ), 50.3
H-2	37.5 (CH <sub>2</sub> )	140.9 (C)
H-3	31.9 (CH <sub>2</sub> )	140.9 (C), 37.5 (CH <sub>2</sub> )
H-4	72.0 (CH)	31.8 (CH <sub>2</sub> ), 37.1 (C)
H-6	140.9 (C)	42.5 (CH <sub>2</sub> ), 37.1 (C), 32.2 (CH)
H-7	121.9 (CH)	140.9 (C)
H-8	50.3 (CH)	37.1 (C)
H-9	32.3 (CH <sub>2</sub> )	-
H-11	50.4 (CH), 39.9 (CH <sub>2</sub> )	-
H-12	21.3 (CH <sub>2</sub> ), 42.6 (C)	-
H-14	42.7 (C), 32.2 (CH)	-
H-15	-	56.9 (CH), 39.9 (CH <sub>2</sub> )
H-16	-	46.0 (CH), 42.6 (C)
H-17	42.6 (C)	39.9 (CH <sub>2</sub> ), 12.2 (CH <sub>3</sub> )
H-18	42.6 (C)	56.3 (CH), 39.9 (CH <sub>2</sub> )
H-19	-	37.2 (CH <sub>2</sub> ), 50.4 (CH), 140.9 (C)
H-20	18.9 (CH <sub>3</sub> )	-
H-21	36.3 (CH)	34.4 (CH <sub>2</sub> )
H-22	36.3 (CH)	12.2 (CH <sub>3</sub> )
H-23	46.1 (CH)	29.3 (CH)
H-24	29.3 (CH), 26.3 (CH <sub>2</sub> ), 23.2 (CH <sub>2</sub> )	34.4 (CH <sub>2</sub> ), 19.2 (CH <sub>3</sub> )
H-25	46.1 (CH)	23.2 (CH <sub>2</sub> )
H-26	29.3 (CH)	46.1 (CH)
H-27	29.3 (CH)	46.1 (CH)
H-28	12.1 (CH <sub>3</sub> ), 46.1 (CH)	29.3 (CH)
H-29	23.2 (CH <sub>2</sub> )	46.1 (CH)



### 3.2.7.2 Characterization of UPH 46a as a mixture of (3:1) $\beta$ -sitosterone (24) and stigmasterone (25)

Both  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra of UPH 46a showed presence of signals for two compounds with intensity ratio of 3:1.

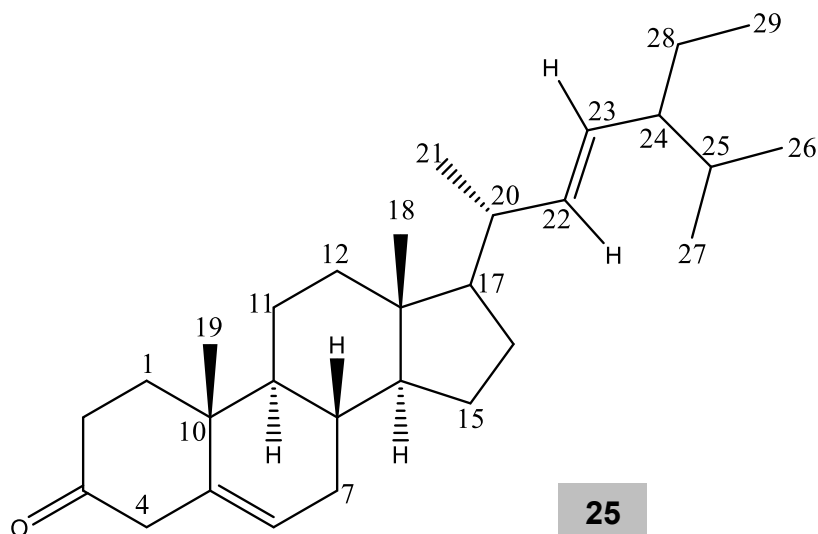
The  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra of UPH 46a were very similar to CRH 16. Like CRH 16, the  $^1\text{H}$  NMR spectrum (700 MHz,  $\text{CDCl}_3$ ) and  $^{13}\text{C}$  NMR



(175 MHz,  $\text{CDCl}_3$ ) spectra revealed signals as a mixture of two compounds in a ratio of 3:1. The  $^1\text{H}$  NMR spectrum showed the presence of olefinic methine at  $\delta_{\text{H}}$  5.73, six methyls resonating between  $\delta_{\text{H}}$  0.70-0.93 and numerous multiplet signals between  $\delta_{\text{H}}$  1.02-2.38 resonating for methine and methylene protons. However, no oxymethine peak was evident in the spectrum. Besides the above signal in the  $^1\text{H}$  NMR, the minor compound also revealed the presence of two olefinic protons at  $\delta_{\text{H}}$  5.14 ( $J=15.12$  Hz) and  $\delta_{\text{H}}$  5.02 ( $J=14.98$  Hz) which appeared as *trans* double bonded to each other.

The  $^{13}\text{C}$  NMR spectrum 175 MHz,  $\text{CDCl}_3$ ) revealed the presence of 29 carbons including a carbonyl at  $\delta_{\text{C}}$  199.9, a downfield quaternary at  $\delta_{\text{C}}$  171.9 and an olefinic methine at  $\delta_{\text{C}}$  123.9 in addition

with two other olefinic methine carbons at  $\delta_{\text{C}}$  138.3 and  $\delta_{\text{C}}$  129.7 (indicates the mixture compound). The key HMBC correlation showed almost similar with the CRH 16. The



only difference was the presence of carbonyl  $\delta_{\text{C}}$  199.9 (C-3) in the molecule instead of oxygen-bearing methine. The methylene protons at  $\delta_{\text{H}}$  2.34, 2.43 ( $\delta_{\text{C}}$  34.2 from HSQC) and  $\delta_{\text{H}}$  1.69, 2.02 ( $\delta_{\text{C}}$  35.9 from HSQC) showed  $^2J$  and  $^3J$  connectivity with the carbon of carbonyl at  $\delta_{\text{C}}$  199.9 (C-3). In the minor compound, the chemical shift from C-17-C-24 were changed due to the presence of double bond between C-22 and C-23 similarly like the minor compound of CRH 16. In HMBC, the proton at  $\delta_{\text{H}}$  5.15 (H-22;  $\delta_{\text{C}}$  138.3 from HSQC) showed HMBC correlation to carbons at  $\delta_{\text{C}}$  21.3 (C-21),  $\delta_{\text{C}}$  40.6 (C-20) and  $\delta_{\text{C}}$  51.2 (C-24). Another olefinic proton at  $\delta_{\text{H}}$  5.02 (H-23;  $\delta_{\text{C}}$  129.6 from HSQC) showed HMBC correlation to the carbons at  $\delta_{\text{C}}$  40.6 (C-20) and  $\delta_{\text{C}}$  51.2 (C-24).

Thus, based on the spectral data and correlations in the HMBC, the compound UPH 46a was identified as a mixture (3:1) of  $\beta$ -sitosterone (**24**) and stigmasterone (**25**) (Rahman, 2002).

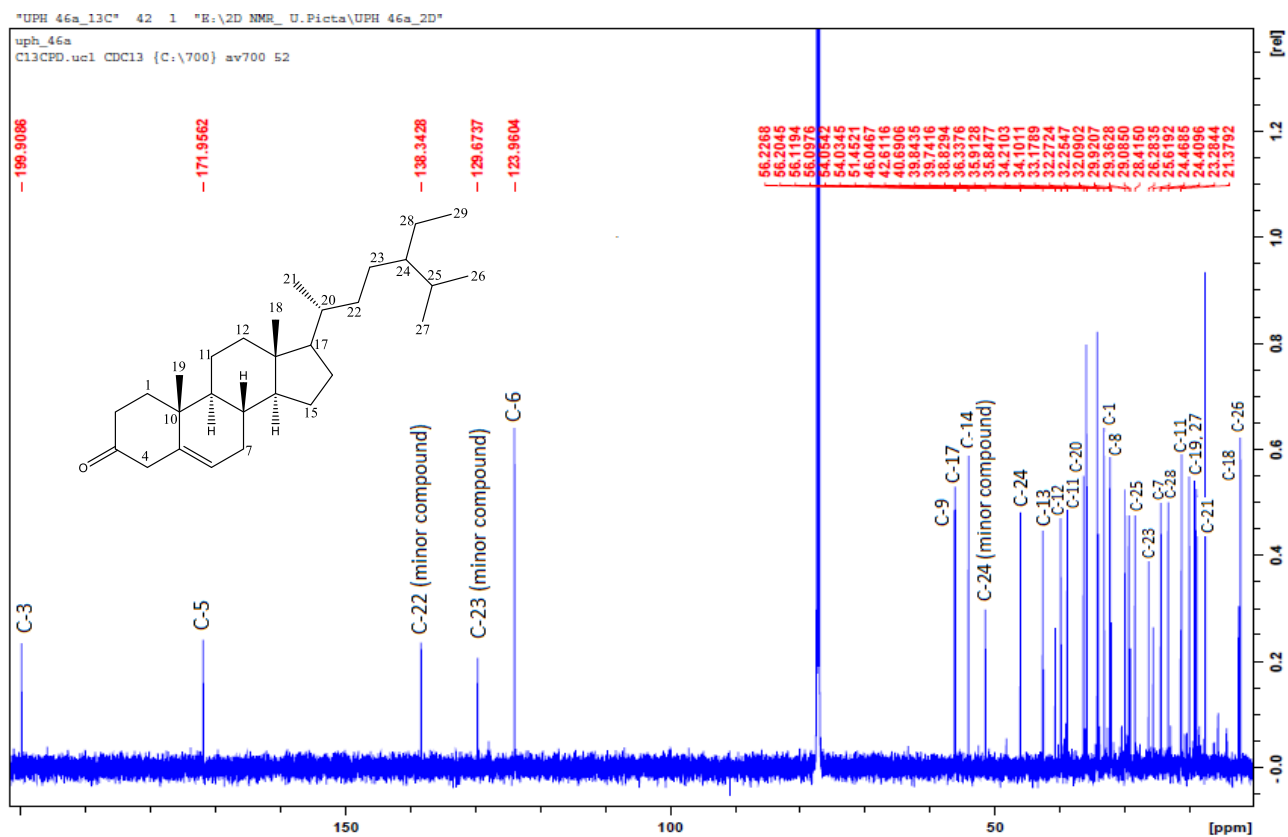


Figure 3.62:  $^{13}\text{C}$  NMR spectrum (175MHz) of UPH 46a in  $\text{CDCl}_3$

### 3.3 Antibacterial screening

**3.3.1 Antibacterial screening of 18 selected plants:** Based on the results of data analysis of ethnopharmacological survey and review of literature 18 out of 71 plants were initially selected and collected from Bangladesh in September 2016. These 18 plants were initially extracted sequentially with hexane, chloroform and methanol in small scale, these extracts were then screened for antibacterial activity against clinical strains of MRSA by MIC assay. The MRSA strains were EMRSA 15, SA1199B and XU212. ATCC 25941 is a standard bacterial strain only. The starting concentration was 512  $\mu\text{g/ml}$  (4 times more diluted than stock concentration). Mostly the plant extracts with the MIC concentration  $\leq 256 \mu\text{g/ml}$  were chosen for further phytochemical investigation.

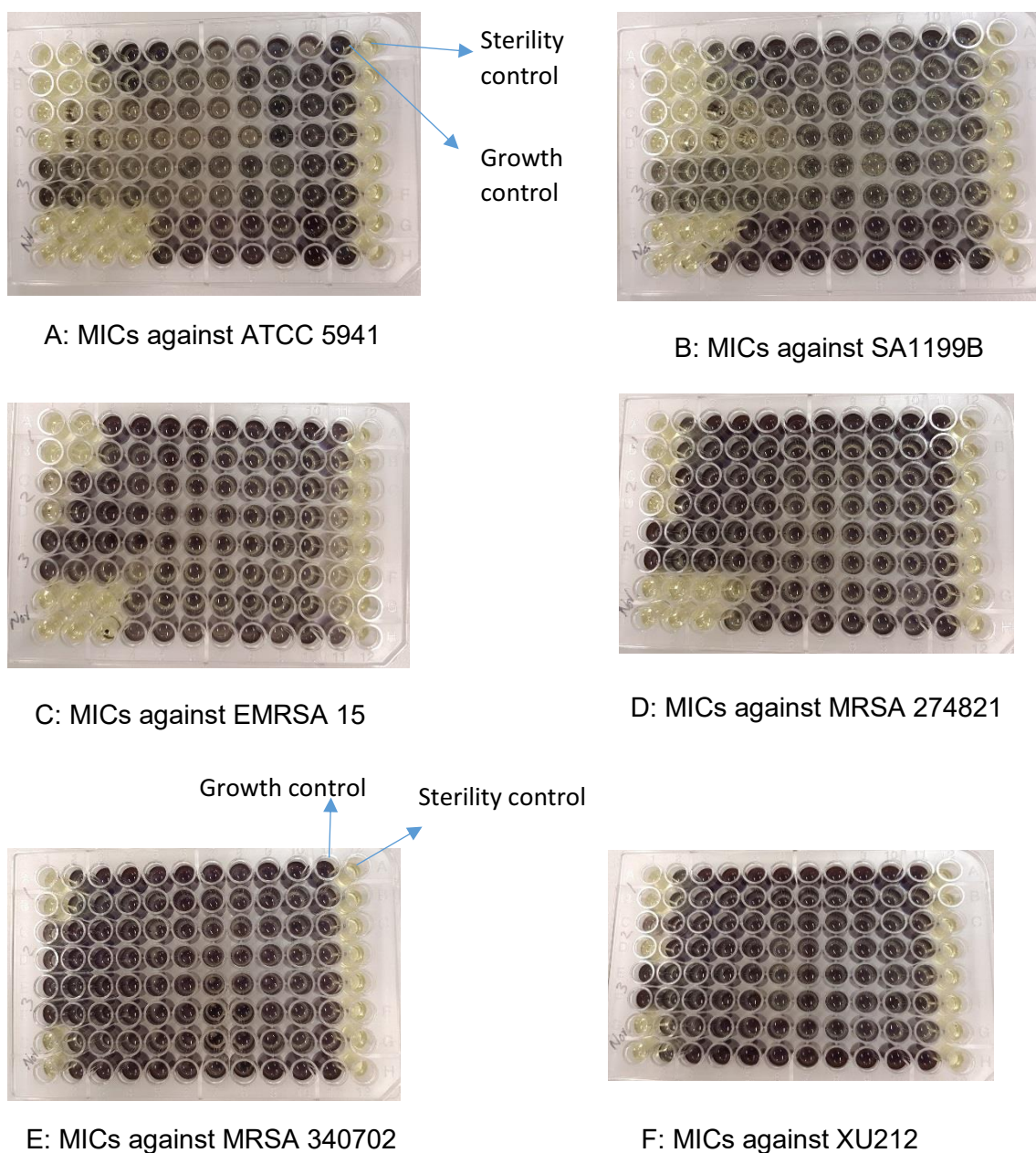
**Table 3.31: The MIC result of 18 selected plants against MRSA clinical strains in  $\mu\text{g/ml}$**

Plant extract/ standard	MRSA strains in $\mu\text{g/ml}$			
	EMRSA 15	ATCC 25941	SA1199B	XU212
<i>Paedaria foetida</i>				
Hexane	512	512	512	512
Chloroform	512	512	512	512
Methanol	>512	>512	>512	>512
<i>Justicia adhatoda</i>				
Hexane	128	512	128	512
Chloroform	128	512	128	512
Methanol	256	512	512	512
<i>Aegle marmelos</i>				
Hexane	128	64	128	512
Chloroform	128	64	128	512
Methanol	256	512	512	512
<i>Abroma augusta</i>				
Hexane	>512	>512	>512	>512
Chloroform	>512	>512	>512	>512
Methanol	>512	>512	>512	>512
<i>Terminalia arjuna</i>				
Hexane	>512	>512	>512	>512
Chloroform	>512	>512	>512	>512
Methanol	>512	>512	512	128
<i>Diospyros malabarica</i>				
Hexane	64	128	>512	512
Chloroform	64	128	>512	512
Methanol	>512	>512	>512	>512
<i>Andrographis periculata</i>				
Hexane	256	512	512	512
Chloroform	256	512	512	512
Methanol	256	512	512	>512
<i>Tinospora cordata</i>				
Hexane	>512	>512	>512	>512
Chloroform	>512	>512	>512	>512
Methanol	>512	>512	>512	>512
<i>Azadirachta indica</i>				
Hexane	>512	>512	>512	>512
Chloroform	>512	>512	>512	>512
Methanol	>512	>512	>512	>512
<i>Tylophora indica</i>				
Hexane	512	512	>512	>512
Chloroform	512	512	>512	>512
Methanol	>512	>512	>512	>512
<i>Tribulus terrestris</i>				
Hexane	64	>512	32	64
Chloroform	64	>512	32	64
Methanol	>512	>512	512	>512

<i>Uraria picta</i>				
Hexane	128	64	32	64
Chloroform	128	64	32	64
Methanol	64	64	128	128
<i>Cynometra namiflora</i>				
Hexane	128	64	128	64
Chloroform	128	64	128	64
Methanol	256	>512	512	256
<i>Swertia chirayita</i>				
Hexane	>512	512	>512	>512
Chloroform	>512	512	>512	>512
Methanol	256	512	512	>512
<i>Terminalia chebula</i>				
Hexane	128	256	512	512
Chloroform	128	256	512	512
Methanol	>512	>512	>512	>512
<i>Glycrrhiza glabra</i>				
Hexane	512	512	128	256
Chloroform	512	512	128	256
Methanol	>512	>512	>512	>512
<i>Feronia limonia</i>				
Hexane	512	>512	>512	>512
Chloroform	512	>512	>512	>512
Methanol	128	>512	512	>512
<i>Zingiber montanum</i>				
Hexane	256	128	128	128
Chloroform	128	256	64	128
Methanol	>512	>512	>512	>512
Norfloxacin	16	16	32	64

**Table 3.32: Antibacterial activity (MIC assay in µg/ml) of pure compounds against MRSA strains**

Compounds	MRSA strains (MIC values in µg/ml)					
	XU212	ATCC 5941	EMRSA 15	MRSA 340702	MRSA 24821	SA1199B
<i>Zingiber montanum</i>						
ZMH 4	>128	64	>128	>128	>128	>128
MRSH 3	>128	128	64	>128	NS	64
ZMH 14	64	32	64	64	128	64
MRSH 6	128	64	32	128	128	128
ZMH 8	128	128	>128	>128	>128	>128
ZMH 5	>128	>128	>128	>128	>128	>128
MRSH 2	128	128	128	128	128	128
ZMH 10	>128	128	>128	>128	>128	>128
<i>Cynometra ramiflora</i>						
CRH 34	>128	64	128	>128	>128	64
CRH 9	128	>128	128	>128	>128	64
CRC 10	>128	64	>128	>128	>128	128
<i>Diospyros malabarica</i>						
DMC 22	64	128	>128	>128	>128	128
DMH 2	>128	128	>128	>128	>128	>128
DMH 10	64	>128	64	>128	>128	>128
DMH 5	>128	64	>128	>128	>128	>128
DMH 15	>128	64	>128	>128	>128	64
<i>Swertia chirayita</i>						
SCH 1	>128	>128	>128	>128	>128	>128
SCH 10	>128	>128	>128	>128	>128	>128
SCH 15	>128	>128	>128	>128	>128	>128
SCH 2	>128	>128	>128	>128	>128	>128
<i>Uraria picta</i>						
UPM 5	64	128	128	128	>128	>128
Control (Norfloxacin)	64	32	16	64	64	32



**Figure 3.63: 96 well plate for the determination of MICs of compounds**

The plate represented MICs of 1= MRSH 6, 2= ZMH 14, 3= MRSH 2, Nor= Norfloxacin (Control) against A: ATCC 5941, B: SA1199B, C: EMRSA 15, D: MRSA274821, E: MRSA340702, F: XU212

This figure represents the MIC assay of pure samples against MRSA clinical strains. The starting concentration of the pure samples in well plates were 128  $\mu\text{g/ml}$ . Here 11<sup>th</sup> column represents growth control meaning no samples were added on that column whilst the 12<sup>th</sup> column as sterility control because no MRSA strains were added on that column. Column 1 and 2 of A and B showed yellow colour identical to the sterility control which indicated that the pure sample inhibited the bacterial growth in column 1 and 2. The samples were duplicated so three samples were added in each well plates followed by norfloxacin (positive control) at the end of the plate. Blue colour in the plates indicated the growth of the bacteria as identical to growth control (column 11). In presence of bacterial strain, its mitochondria react with MTT reagent forming a blue colour complex.



# Chapter 4

## Discussion and Conclusion

## **4 Discussion:**

### **4.1 Ethnopharmacological survey:**

An ethnopharmacological survey was carried out in 10 different locations of the capital city Dhaka and its surrounding areas and also in a northern district, Natore. Dhaka is one of the world's largest cities and the 4th most densely populated city in the world with the population of 18.89 million (Population and housing census, 2011). Despite the improved economic system in Bangladesh, the country is still experiencing remarkable public health issues (Shahabuddin et al., 2015; Edmunds et al., 2015; Asadullah et al., 2011). There is a strong inclination to improve complementary medicine system in Dhaka city due to limited medical sources. 62.0% of medical doctors in Dhaka treat patients with alternative or Ayurvedic medicine and 39.7% recommended Ayurvedic medicine to be a part of the orthodox medicine (Yoshida et al., 2017). At Natore district of Bangladesh, around 200 farmers shifted their livelihood from conventional crops and vegetables to medicinal plants among which *Aloe vera* was the main medicinal plant to be cultivated since 2000. The market in Bangladesh for herbal medicine is huge both locally and internationally. The market size for importing herbal medicine is more than 100 millions dollar annually. An ethnopharmacological survey was conducted in Bangladesh to accurately document information in regard to the herbal remedies used traditionally for the management of infective diseases such as syphilis, gonorrhoea, chronic dysentery and bacterial pneumonia in order to identify the anti-infective lead compounds from selected plant materials and to preserve valuable ethno medical information.

55.91% males and 44.09% females participated in the survey. The majority of the participants were Ayurvedic or Unani doctors (71 practitioners out of 127 participants). Yoshida and her colleagues (2015) interviewed 86 male medical doctors and 73 female doctors to determine the attitude and perception of the doctors to Ayurvedic medicine. A significant gender difference was observed in terms of education because it is a common practice not to educate daughters in rural households of Bangladesh as it is believed that females are only born to manage the households (Amin and Nuzhat, 2016)

Some inhabitants were interviewed at the national tree fair in 2016. The national tree fair, held in Dhaka comprises foliage and plants. Around 100 stalls

displayed thousands of varieties of saplings including flowers, fruit trees and medicinal plants. Local inhabitants get the opportunity to buy the saplings for their garden. Most of the selected plants were collected from the National Botanical Garden of Bangladesh with prior permission from the authority. The National Botanical Garden of Bangladesh is a well-known recreation place and the largest plant preservation centre (210 acres) as it is the accumulation of 56,125 trees, shrubs, herbs and aquatic plants (Botanic Gardens Conservation International, 2018). It is known as a knowledge centre for botanists and nature lovers because exotic and rare medicinal plant species are well adapted. The samples of all selected plants were deposited in Bangladesh National Herbarium. Bangladesh National Herbarium (established in 1970) is a scientific centre where the collected dried plant specimens were recorded and preserved for reference purposes (Banglapedia, 2015).

The patients of two Ayurvedic and Unani hospitals "Tibbia Habibia Unani College and Hospital" and "Government Unani and Ayurvedic Medical College" were interviewed. Two hospitals were non profit medical colleges and hospitals where people get the opportunity to get the bachelor degree in Unani or Ayurvedic medicine and also patients get treatment based on herbal medicine. The suggestion of plants from practitioners was different from local inhabitants and patients. Inhabitants and patients suggested commonly used medicinal plants mostly for cough and cold such as aloe vera, turmeric or tulsi basil however, the practitioners suggested plants used for herbal formulations which were either manufactured by an herbal company or practitioners themselves prepared the formula with proper instructions and then handed this over to the patients the next day.

Out of 71 reported plants (distributed over 54 families), legumes, asteraceae and apocynaceae were the highest represented families with the highest number of species closely followed by lamiaceae, menispermaceae and zingiberaceae. The remaining 48 families were represented by only one species each. The possible reason for the use of plants from specific families is that the flora of these families has a tendency to dominate the pharmacopoeia due to the monsoon climate of Bangladesh (Sharma et al., 2012). Gurib-karim, (2006) reported that asteraceae, rutaceae and laminaceae were shown to be important families to contribute to drug discovery and development. Legumes were

reported to be the highest family used. One factor that may explain the extensive use of this taxonomic family is that legumes are the third largest family with 20,000 species. Moreover, Legumes are an economically important family due to its agricultural relevance. Most of the plants of the legume family provide edible oils, food for both human and animals, fibres and edible seeds that contain high protein and essential amino acids. (Gepts et al., 2005)

Results from the survey indicated that various plant parts (stem, bark, leaf, root, flower, and rhizome) were used for the herbal formulations. Among these plant parts, leaves (26%) were reported to be used most frequently for making herbal formulations. A similar result was found from the previous ethnopharmacological surveys conducted in different tropical regions (Khulna, Rangamati and Chittagong hill tracts) of Bangladesh for identifying the important species used in traditional medicine (Kadir et al., 2012, 2014; Dulla and Jahan, 2017). The predominance of using the leaves is their easy availability to collect and use compared to other plant parts. In addition, there is generally a higher concentration of active compounds present in the leaves due to the presence of photosynthates (Ghorbani, 2005). This can be explained by the fact that the leaves act as reservoirs for photosynthesis or exudates that are thought to contain toxins for plant protection and survival which consequently find medical values in treating diseases. (Wambugu *et al.*, 2011, Vitalini *et al.* 2009 and Namsaa *et al.*, 2009). The second most frequently used plant parts reported were fruit (16%) and bark (15%) whereas the root was not commonly used because scraping away the soil to reach the plant's root may lead to cessation of the plant. Previously Alzweiri *et al.*, 2011 suggested that the selection of the correct plant parts was important to achieve a therapeutic response to an herbal formulation as different plant parts contain different chemical constituents. The current study showed that village people of Bangladesh used plant parts based on cultural heritage not on scientific grounds. Most of the plant parts mentioned in the present study were collected by the informants from the wild and the method of preparation was mostly powder form followed by mash and decoction. The possible reason behind this was most of the participants were Ayurvedic practitioners (71 out of 127) who prescribed their formulations as a powder form. Moreover, local people also tend to pick medicinal plants and dry them to store as a powder form for future use. Therefore, fresh plant parts were collected and

sun dried during the spring season prior to making into powder (Alzweiri et al., 2011).

The current study found that informants commonly mixed the herbal powder remedy with water followed by honey and sugar to improve the compliance when taken orally. Previously Chintamunnea and Mahomodally, (2012) reported that there was a direct association between the taste of a preparation and the need to use additives to improve compliance.

In this study, three main ethnopharmacological quantitative indexes were calculated to figure out the main medicinal plants used by the participants. The first parameter used was UV (use value) which was applied to identify relative importance of each plant species mentioned by the participants; the FIC (Informants consensus factor) was calculated to determine the main categories of anti-bacterial diseases for which plants were used. Finally, FL (fidelity level) was applied to determine the most important plant species from the selected disease category.

According to the high UV value (Table 3.3), *Azadirachta indica* (23 citations) and *Ocimum tenuiflorum* (18 citations) followed by *Justicia adhatoda* (16 citations) and *Curcuma longa* (13 citations) were the most cited plants by the informants. The current study showed that the fresh leaf of *Azadirachta indica* is used for wound healing. This property has been endorsed by several studies (Shrivastav et al., 2018; Babu et al., 2016). For example, Narendhirakannan and his colleagues (2012) also conducted an in vitro study on *A. indica* and other traditional medicinal plants originating from the neighbouring country India and claimed that the presence of flavonoids (polyphenols), terpenes, alkaloids, saponins in *A.indica* were responsible for exhibiting strong antibacterial and antioxidant activity which contributing to significantly quicker wound healing (83%) compared to a commercial ointment (76%). Notably, *A. indica* has been widely mentioned in the literature in regard to its medicinal value. In Swahili, this plant is referred to as “Muarubaini” (meaning treat 40 different diseases and frequently used as an insecticide, antimicrobial, antimalarial and antiviral (Baritkar et al., 2014; Mahfuzul et al., 2007; Thakurta et al., 2007).

*Ocimum tenuiflorum* is widely known as tulsi or holy basil. In Ayurveda, tulsi is recognised as “Mother Medicine of Nature” and “the Queen of Herbs”. The fresh

leaves of *O. tenuiflorum* were used for treating common cough and cold and bacterial pneumonia by the participants. Previous studies reported antibacterial, antioxidant, wound healing, antifertility, antidiabetic, anticancer, immunomodulatory, anti-inflammatory, radiation protective and anti-stress activity of *Ocimum tenuiflorum*. (Godhwani et al., 1988; Prashar et al., 1998, Prakash and Gupta, 2000, Kelm et al., 2000, Ahmed et al., 2002, Gholap and Kar, 2004, Udupa et al., 2006, Trevisan et al., 2006, Vats et al., 2004, Gupta et al., 2006, Subramanian et al., 2005, Yanpallewar et al., 2004, Mukherjee et al., 2005, Karthikeyan et al., 1999, Geetha and Vasudevan, 2004)

The leaf of *Justicia adhatoda* was used fresh or as a powder for treating respiratory tract infection by the participants. A double-blind randomized placebo-controlled study confirmed that the leaf of *Justicia adhatoda* relieved common cough and cold in combination with the root of *Echinacea purpurea* and *Eleutherococcus senticosus* (Barth et al., 2015). Several investigators reported antibacterial activity of the isolated compounds from *Justicia adhatoda*. For instance, Durairandiyar and his colleagues (2015) reported that *Justicia adhatoda* contained vasicine acetate which possessed antibacterial activity against *M. luteus* (125 µg/mL), *E. aerogenes* (125 µg/mL), *S. epidermidis* (125 µg/mL), and *P. aeruginosa* (125 µg/mL). 2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl isolated from *Justicia adhatoda* showed promising antioxidant activity (Dhankhar et al., 2014). Plants with a low UV value (Table 3.3) are not necessarily less important but this may be an indication that the traditional knowledge on these plants has been lost or may be attributed to scarcity of the plant species (Sharma et al., 2012).

For the calculation of FIC value (Table 3.2), the reported diseases were grouped into 12 different ailment categories. Diseases mentioned such as tuberculosis, cholera, scalp infection, dengue fever, syphilis and gall bladder infection, cystitis and jaundice were not included in this category because only one plant was suggested for this ailment category. Bacterial pneumonia was recorded to have the highest FIC value 0.94 which was generally treated with the second highest cited plant *Ocimum tenuiflorum* followed by wound healing (0.85), Gastrointestinal infection (0.83) and rheumatic fever (0.83). The high FIC values of these ailments indicated that the information in regard to the plants are homogenous and used by a large number of people. Several authors have

reported that medicinal plants that scored a high FIC value were assumed to be efficient in treating a certain ailment category and that information was passed between informants. (Boulogne et al., 2011), (Uddin and Hassan, 2014). Therefore, it can be concluded that most of the plants mentioned in the current study may be effective in treating infective diseases.

The fidelity level value (Table 3.3) was calculated to identify the most important plant in each disease category. Most of the plant species showed 100% FL value which indicated that most of the plant species might have potential for good healing to treat a particular disease (Ayyanar and Ignacimuthu, 2011). In respiratory tract infection, *Justicia adhatoda* scored the lowest FL value i.e. (25%) which indicated that culturally it was probably a less important plant for treating that particular disease.

Rank order priority (ROP) (Table 3.3) index were calculated to determine the knowledge of the species with regard to the abundance of the resources mentioned in the studied use category (Eddouks et al., 2017). *Kalanchoe pinnata* (43.32%), *Azadirachta indica* (34.80%), *Ocimum tenuiflorum* (34.63%) had the highest ROP value, which indicated that these species were the most familiar to informants. while few plants such as *Alstonia scholaris* (0.04), *Paederia foetida* (0.04) showed a lower priority among the reported medicinal plants by the informants.

However, the mode of action of the 71 suggested plants was beyond the aim of this study. Some of the listed plants in our investigation were used to treat other diseases besides infectious disease. For example, Alzweiri et al., 2011 and Chintamunnea and Mahomodally, (2012) reported that the bulb of *Allium sativum* L. was used for snake bites, muscle relaxation, blood circulation problem, asthma, gastrointestinal disorder or hypertension but in the current study participants suggested *A. sativum* solely for treating metabolic disorder such as gout. Similarly Chintamunnea and Mahomodally, (2012) mentioned *A. indica* was used in the tropical region of Mauritius as an antidiabetic agent while in Bangladesh most of the people used it for treating common cold and cough.

## 4.2 Phytochemistry:

Based on the results of the data analysis of the ethnopharmacological survey and subsequent literature review of all the suggested plants and ease of availability, 18 plants out of 71 plants were initially selected and collected from Bangladesh in September 2016. These plants were initially screened for antibacterial activity against clinical strains of MRSA. The number of plants were narrowed down to five (*Zingiber montanum*, *Uraria picta*, *Cynometra namiflora*, *Diospyros malabarica* and *Swertia chirayita*) based on their potential antibacterial activity.

Bioassay directed phytochemical investigations on the 5 selected plants led to the isolation of a total of 25 compounds including simple phenolics, xanthenes, mono, di, sesqui, triterpenes and steroids. Structure elucidation of the compounds was achieved by spectroscopic techniques (1D, 2D NMR, mass spectrometry, IR).

*Z. montanum* (Fam. Zingiberaceae) is an herbaceous plant that produces a clump of leaves from large rhizomes. It is indigenous to Bangladesh where it is widely used in the northern part of Bangladesh. The ethnopharmacological survey showed that *Z. montanum* was being used by traditional practitioners and local village people for the treatment of gastrointestinal infection.

A total of eight terpenes were isolated from both hexane and chloroform extracts of *Zingiber montanum*. These compounds were characterized as zerumbone, zerumbol, buddledone A, camphor, borneol, furanodienone, germacrone, 8(17), 12-labdadiene-15, 16-dial by spectral data, mostly 1D and 2D NMR spectroscopy and mass spectrometry. Verma and his colleagues (2018) reported the essential oils from *Zingiber montanum* originating from India. The authors found major constituents of the oil were  $\gamma$ -terpinene (1.1–4.8%), sabinene (13.5–38.0%), terpinen-4-ol (9.0–31.3%), (E)-1-(3', 4'-dimethoxyphenyl) buta-1, 3-diene (DMPBD) (20.6–35.3%) and  $\beta$ -phellandrene (1.0–4.4%) including minor components camphor and borneol. As *Zingiber montanum* is native to India, Malaysia, Thailand, Indonesia, Bangladesh and Sri Lanka, previous studies of the essential oil of leaf and rhizome exhibited tangible variation in the chemical constituents. For example, the rhizome oil from *Zingiber montanum* of Thailand were dominated by sabinene, (E)-1-(3',4'-Dimethoxyphenyl) buta-1,3-diene (DMPBD) and terpinen-4-ol (Bua-in and



Paisooksantivatana, 2009) (Sukatta et al., 2009). However, the rhizome oil from Malaysian *Zingiber montanum* possessed higher amounts of sesquiterpenes (Kamazeri et al., 2012). Previous studies on Bangladeshi *Zingiber montanum* showed that rhizome oil characterized by triquinacene-1,4-bis (methoxy), (Z)-ocimene and terpinen-4-ol (Bhuiyan et al., 2008). Moreover, the leaf oil of north-east Indian *Zingiber montanum* was characterized by furanodien-6-one, curzerenone and  $\beta$ -sesquiphellandrene (Bordoloi et al., 1999). Similarly furanodien-6-one was also isolated from the rhizome of *Zingiber montanum* in the current study.

The presence of camphor and borneol in the rhizomes essential oil was in agreement with the study conducted by Verma and his colleagues (2018) on Indian *Zingiber montanum* but the presence of zerumbone, zerumbol, buddledone A, furanodiene, germacrone, and labdadiene in the rhizome of *Zingiber montanum* from Bangladesh were reported for the first time. Zerumbone is a sesquiterpene isolated previously from another species of wild ginger (*Zingiber zerumbet*) (Takashi et al., 2002). Takashi and his colleagues (2002) synthesised optically active zebumbol from zerumbone as the starting material for conversion to useful chiral product such as paclitaxel. However, this compound has not been isolated naturally before from any plant species. Therefore, zerumbol appeared to be a new natural product. Buddledone A was previously isolated from the Chinese traditional medicinal plant, *Buddleja globosa*. (Cai et al., 2012). Germacrone was previously isolated from *Geranium macrorrhizum*, *Curcuma wenyujin* and *Rhododendron adamsii* (Yan et al., 2005) while 8(17),12-Labdadiene-15,16-dial was previously isolated and identified from the perennial herb from Hong Kong *Alpinia chinensis* that belongs to the Zingiberaceae family (Lai-King and Brown 1997).

Rahman and co-workers (2007) reported the isolation of two new isoflavones, along with triterpenes and steroids from *Uraria picta*. During this study, eugenol and a mixture of (3:1)  $\beta$  sitosterone and stigmasterone were isolated from *Uraria picta*. Eugenol, the major compound present in *Syzygium aromaticum* has been reported from other plants however this was the first time it was reported from *Uraria picta*.

*Cynometra ramiflora* is a medicinal plant indigenous to India and Bangladesh predominantly in the mangrove forest sundarban, Sri Lanka and tropical areas of Africa and Australia. This plant has been investigated for anti-

ulcer activity (Paguigan et al., 2014; Uddin et al., 2011). However, according to the literature review, no chemical constituents have been isolated or previously identified. Therefore,  $\beta$  sitosterol, glutinol, glutinone and ethyl 4-ethoxy benzoate have been isolated for the first time from *Cynometra ramiflora*. Among these compounds, ethyl 4-ethoxybenzoate appeared to be a new natural product.

Glutinol and glutinone are triterpenoids isolated from hexane and ethyl acetate extracts of the root barks of *Uvaria narum* Wall and *Uvaria hookeri* King and tested for antibacterial, antifungal and anthelmintic activities (Padmaja et al., 1993). Glutinol that coats the outer layer of leaf, works as a natural defense compound against pathogens and herbivores. Glutinol and glutinone have been isolated and identified for the first time from the hexane extract of *Cynometra ramiflora* leaf in this study.

*Diospyros malabarica*, a 30 m long tree indigenous to India, Pakistan and Bangladesh, produces edible seasonal fruit. Two bioactive flavonoid glycoside, namely luteoline-4'-methyl-ether-7-O-glucoside and quercetin-3-O-(glucosyl)-glucoside were isolated from the aqueous extract of *D. malabarica* fruit with potent antioxidant activity (Sahu et al., 2012). Apart from the isolation of bioactive flavonoid glycosides, no other phytochemical work was reported from this species. However, the genus *Diospyros* was widely researched in terms of phytochemistry. The genus *Diospyros* is dominated by the production of lupane series triterpenoids and naphthoquinones. (Zhong et al., 1984). In this current study, the lupane series triterpenoids- lupeol, betulone, betulin, betulinaldehyde and messagenin were isolated and reported for the first time from the hexane leaf extract of *Diospyros malabarica* species. The lupane series are common natural compounds mostly abundant in fruit and vegetables like green pepper, mangoes, grapes, white cabbage and in medicinal plants such as *Celastrus paniculatus*, *Tamarindus indica*, *Himatanthus sucuuba*, *Zanthoxylum riedelianum*, *Leptadenia hastata*, *Allanblackia monticola*, *Crataeva nurvala*, *Sebastiania adenophora* and *Bombax ceiba* (Wal et al., 2015). In 1984, Zhong and his colleagues revealed the presence of triterpenoids of the lupane series such lupeol, betulin and betulinaldehyde from the genus *Diospyros*, which is in agreement with the current findings of the *Diospyros malabarica* species. Messagenin had been synthesised before (Marcias et al; 1994) but there is no literature found on isolating this

compound from natural sources. Therefore, the natural compound messagenine was reported for the first time in this study.

*Swertia chirayita*, the popular ethnomedicinal herb indigenous to the Himalayas has been well-documented in Ayurveda, Unani, Siddha, and other conventional medical systems for its wide spectrum of pharmacological properties. The chemical review revealed a considerable amount of research that explored the different chemical constituents of *Swertia chirayita* (Kumar et al., 2015, Zhou et al., 2015, Zhou et al., 2013, You et al., 2017). A series of xanthenes had previously been isolated from other species of the genus *Swertia* (*S. kouitchensis*, *S. corymbosa*, *S. mussoitii* Franch and *S. bimaculata*) (Phoboo et al., 2012). 1-hydroxy-3, 5, 6-trimethoxyxanthone (commonly known as decussatin) was identified from the ethyl acetate fraction of the whole plant of *Swertia kouitchensis*. (He et al., 2015)

The current study has led to the isolation of xanthenes including swerchirin, swertiaperenin, decussatin and bellidifolin from the hexane extract of *Swertia chirayita*. Swerchirin (isolated from hexane extract of whole plant) is known to be hypoglycemic, antimalarial (Bajpai et al., 1991; Saxena et al., 1996). However, this series of xanthenes had already been identified previously from the whole plant of hexane extract of *Swertia chirayita* (You et al., 2017).

#### **4.3 Antibacterial screening:**

Antibacterial screening was expressed in terms of minimum inhibitory concentration (MIC) value in  $\mu\text{g/ml}$ . The antibacterial activities of the pure compounds were evaluated against a panel of clinical strains of methicillin resistance *Staphylococcus aureus* (MRSA), including XU212, EMRSA15, SA1199B, MRSA 340702 and MRSA 24821 along with MRSA standard strain ATCC 5941. These efflux strains are resistant to common antibiotics such as SA1199B that is resistant to fluoroquinolones and XU212 resistant to tetracycline. The antimicrobial activities of the active compounds were compared to the standard antibiotics norfloxacin. The MIC of norfloxacin was found to be in the range of 16-128  $\mu\text{g/ml}$ .

ZMH 14 (8(17), 12-Labdadiene-15, 16-dial) (table 3.32) exhibited the most potent antibacterial activity with the MIC value 32-128  $\mu\text{g/ml}$ . ZMH 14 (MIC value 64  $\mu\text{g/ml}$ ) showed the same activity as the control (Norfloxacin, MIC 128  $\mu\text{g/ml}$ )

against XU212 and MRSA 340702 bacterial strains. ZMH 14 is a labdadiene diterpene with exomethylene, olefin and two aldehyde groups. The presence of these groups and unsaturations could account for the significant antibacterial activity against MRSA strains.

Moreover, sesquiterpene MRSH-6 (Zerumbol) showed moderate antibacterial activity with an MIC result of 32-128 µg/ml. Although MRSH 6 and MRSH 3 are structurally very similar, they differ in activity. The author suggests that the presence of a hydroxyl group in MRSH 6 instead of carbonyl group might make MRSH 6 more active than MRSH 3 (Thosar et al., 2013). The antibacterial activity of MRSH-6 against SA1199B was the same as the crude hexane extract of *Z. montanum* however it showed 3 fold potent antibacterial activity against XU212 and 2 fold potent antibacterial activity against ATCC clinical strain of MRSA compared to the crude extract.

It is evident from table 3.32 (result section) that the MIC of MRSH-3 (Zerumbone) was 64-128 µg/ml. The presence of a carbonyl group instead of hydroxyl group of MRSH 6 showed a 1 fold reduction in MIC activity. Its reduced antibacterial activity compared to crude extract indicated that the possible synergistic effect of the crude extract was better than the isolated compound. This finding is in agreement with the previous finding by Kamazeri and his colleagues (2012) who isolated 2, 6, 9, 9- tetramethyl- 2,6,10- cycloundecatrien-1-one and  $\beta$  caryophyllene from the essential oil of *Z. montanum* but found very low antibacterial activity against both gram positive and gram negative bacteria. Other isolated compounds ZMH 4 and ZMH-5, ZMH 8 and ZMH 10 exhibited moderate activity against MRSA clinical strains. This needs to be investigated further.

The lupane series was isolated from *Diospyros malabarica* were tested against MRSA strains. DMH 2 were inactive against all the MRSA stains while lupeol (DMC 22) exerted moderate antimicrobial activity against XU212 (64 µg/ml) and ATCC 5941 (128 µg/ml). Lupeol is a triterpene with an exomethylene and hydroxyl group which confer better antimicrobial activity compared to DMH 2. Other compound such as DMH 10 displayed activity against XU212 and EMRSA 15 with MIC 64 µg/ml, DMH 15 exerted activity against ATCC 5941 and SA1199B with the MIC of 64-128 µg/ml while DMH 5 exerted activity against ATCC 5941 (64 µg/ml) only.

*Swertia chirayita* contains a number of xanthone derivatives which are documented as exhibiting antimicrobial activity, however, *Swertia* and its xanthone derivatives did not exert good antibacterial activity.

In 2007, Rahman and co-workers isolated two new isoflavones, along with triterpenes and steroids from *Uraria picta*. The MIC results of two isoflavones 5,7-dihydroxy-2'-methoxy-3',4'-methylenedioxyisoflavanone and 4',5-dihydroxy-2',3'-dimethoxy-7-(5-hydroxyoxochromen-7yl)-isoflavanone were found to be in the range of 12.5-200 µg/ml against both gram positive and gram negative bacteria including *Proteus vulgaris*, *Bacillus subtilis*, *Aspergillus niger*, *Staphylococcus aureus* and *Candida albicans*. However, these were not tested against clinical isolates of MRSA. During this study, eugenol was isolated from the methanol extract of whole plant of *Uraria picta*. UPM 5 (eugneol) exerted moderate antimicrobial activity with an MIC of 64-128 µg/ml against XU212, ATCC 5941, EMRSA 15 and MRSA 340702. Eugenol is a common natural compound mostly found in the essential oil of several plants. It is a potent bioactive compound with broad spectrum antimicrobial activity against food or human pathogen (Marchese et al., 2017).

## 5 Conclusion

The traditional knowledge on medicinal plants still provides the foundation for pharmacological research in many aspects. This study involved conducting an ethnopharmacological survey on Bangladeshi medicinal plants by interviewing qualified Ayurvedic/Unani practitioners, ayurvedic hospital patients and local village people. This survey founded the selection of medicinal plants for the isolation and identification of compounds with possible antibacterial activity against MRSA clinical strains. There had been a handful of previous research conducted on Bangladeshi medicinal plants with the purpose of focusing on searching for plants with antidiabetic, antidiarrhoeal or antidysentery activity. Some authors just simply catalogued the medicinal plants used by tribes of the hill tract areas (Kadir et al., 2012; Rahman, 2013; Rahman and Roy 2014; Uddin et al., 2015a; Uddin et al., 2015b; Nur et al., 2016,). However, there was a lack of scientific data regarding the use of medicinal plants for the management of infective diseases in Bangladesh. To date, this work can be described as the first comprehensive study to establish the existence of accurate knowledge from qualified/ experienced Ayurvedic/ Unani practitioners as well as local village people on medicinal plants in the treatment of infectious diseases. In addition, the ethnopharmacological knowledge on Bangladeshi medicinal plants and demonstration of its antiinfective activity can assist the preservation of medicinal plants in decline. This survey contributed to the selection of medicinal plants for extensive phytochemical research. Plants contain numerous compounds whilst their individual metabolites can be shown to have antibacterial activity. For instance, the root of *P. reptans* exhibited potent antibacterial activity against *E. coli* (MIC 3.9 µg/ml) compared to the broad-spectrum antibiotic control chloramphenicol (Rahman et al., 2008) which gives a clear indication of the pharmaceutical interest in isolated compounds.

In this study, the combination of traditional knowledge and extensive scientific work using a wide range of chromatographic and spectroscopic techniques led to the identification of a total of 25 compounds including terpenes and simple phenolic compounds with potential antibacterial activity against clinical isolates of a panel of methicillin resistant *Staphylococcus aureus* (MRSA). One of the key elements of this project was to select medicinal plants from Bangladesh to conduct phytochemical research. Based on the used value and

other scientific analysis, 30 plants were initially selected for phytochemical study. Following a literature review the number of plants was narrowed down to 18. Five of the 18 plants were finally selected for phytochemical research based on the antibacterial screening report. A total of 8 terpenes were isolated and identified from *Zingiber montanum* of which 8(17), 12-labdadiene-15, 16-dial (32 µg/ml against ATCC 5941) and zerumbol (32 µg/ml against EMRSA 15) isolated first time from the rhizome of *Zingiber montanum* and that showed potential antibacterial activity. *D. malabarica* and *C. ramiflora* medicinal plants were under researched in terms of identifying chemical constituents and their antibacterial activity. The, lupane series, triterpenes and simple phenolic compounds were reported for the first time from the current study from *D. malabarica* and *C. ramiflora* respectively. Among the lupane series phenolic compounds, messagenin isolated from *D. malabarica* and ethyl 4-ethoxybenzoate isolated from *C. ramiflora* were identified as new natural compounds.

Such approach used in this study for the identification of new antibacterial compounds showed benefit to the inhabitants of neighbouring countries who are using ayurvedic medicine for primary health care; also the inhabitants of other countries who prefer herbal medicine as an alternative therapy. Qualified/ experienced Ayurvedic/ Unani practitioners around the world will be encouraged to enhance their knowledge with scientific investigation of the chemical constituents of selected medicinal plants reported for their possible antibacterial activity. Such novel drug discovery approach might contribute in tackling the problem of AMR in near future.

This ethnopharmacological research survey identified medicinal plants that have been used in traditional medicine for the treatment of infectious diseases. Among the plants recommended by the qualified practitioners, patients and local village people, a total of five plants were investigated phytochemically for the isolation and identification of compounds with antibacterial activity. This such research has reasonably justified the traditional use of medicinal plants for the treatment of infections including urinary tract, respiratory, gastrointestinal infection. The project has led to the isolation and identification of 25 compounds. Among the compounds terpenes such as 8(17), 12-labdadiene-15, 16-dial (32 µg/ml against ATCC 5941), zerumbol (32 µg/ml against EMRSA 15), germacrone (64 µg/ml against ATCC 5941), zerumbone (64 µg/ml against EMRSA 15),

triterpenes such as lupeol (64 µg/ml against XU212), messagenin (64 µg/ml against XU212 and EMRSA 15) and glutinone (64 µg/ml against ATCC 5941) and simple phenolic compound ethyl 4-ethoxybenzoate (64 µg/ml against ATCC 5941 and SA1199B) showed moderate activity against MRSA strains. These types of terpenes are known in the literature to produce antibacterial activity against bacteria but not clinical isolates of MRSA. Such moderate activity is not enough to take into the development stage. However, the findings from the ethnopharmacological survey could be the basis for further extensive phytochemical research to explore anti infective compounds from other plants recommended through the survey.

## **6 Fututre Work**

Compounds with potential antibacterial activity against the clinical isolates of MRSA strains, and are available in sufficient quantities will be tested for anti-mycobacterial activity against multi-drug resistant Mycobacterium tuberculosis and also for cytotoxicity against HeLA and other cell-lines..

Methanol extracts could be investigated further using preparative HPLC to explore the presence of bioactive compounds.

Literature review will be continued in order to keep track of the current studies regarding these selected plants. Other plants can be selected from 71 suggested plants and test antibacterial, antifungal activity for isolating secondary active compounds.



# **Chapter 5**

## **References**

## 7. References

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Chapter 6

Appendix



## 8. Appendix

### Publications

1. Siddique, H., Pendry, B. and Rahman, M.M. (2019). Terpenes from *Zingiber montanum* and Their Screening against Multi-Drug Resistant and Methicillin Resistant *Staphylococcus aureus*. *Molecules*. 24(3).
2. Siddique, H., Pendry, B. and Rahman, M.M. Medicinal plants used to treat infectious diseases in central part of Bangladesh and in a northern district, Natore- an ethnopharmacological perception (Manuscript in preparation)
3. Siddique, H., Pendry, B. and Rahman, M.M. Anti-infective medicinal plants- an ethnopharmacological survey in Bangladesh. An abstract was published and oral and poster were presented at APSGB conferences 2017. The poster was awarded 2<sup>nd</sup> prize in poster competition.
4. Siddique, H., Pendry, B. and Rahman, M.M. Antibacterial mono- and sesquiterpenes from *Zingiber montanum*. An abstract was published and a poster was presented at the International Symposium of the Phytochemical Society of Europe (PSE) in Liverpool, John Moores University, 2018. The travel grant of £250 was awarded.



5 August 2016

Dear Holly,

<b>Project Title:</b>	<b>Identification of anti-infective lead compounds from Bangladeshi medicinal plants to tackle antimicrobial resistance</b>
<b>Principal Investigator:</b>	<b>Dr Mukhlesur Rahman</b>
<b>Researcher:</b>	<b>Holly Siddique</b>
<b>Reference Number:</b>	<b>UREC 1516 154</b>

I am writing to confirm the outcome of your application to the University Research Ethics Committee (UREC), which was considered by UREC **on Wednesday 20 July 2016**.

The decision made by members of the Committee is **Approved**. The Committee's response is based on the protocol described in the application form and supporting documentation. Your study has received ethical approval from the date of this letter.

Should you wish to make any changes in connection with your research project, this must be reported immediately to UREC. A Notification of Amendment form should be submitted for approval, accompanied by any additional or amended documents:

<http://www.uel.ac.uk/wwwmedia/schools/graduate/documents/Notification-of-Amendment-to-Approved-Ethics-App-150115.doc>

Any adverse events that occur in connection with this research project must be reported immediately to UREC.

#### Approved Research Site

I am pleased to confirm that the approval of the proposed research applies to the following research site.

<b>Research Site</b>	<b>Principal Investigator / Local Collaborator</b>
Bangladesh	Dr Mukhlesur Rahman



### Approved Documents

The final list of documents reviewed and approved by the Committee is as follows:

<b>Document</b>	<b>Version</b>	<b>Date</b>
UREC application form	2.0	4 August 2016
Participant Information Sheet	2.0	4 August 2016
Consent Form	1.0	5 July 2016
Questionnaires for Ethno pharmacological survey of Ayurvedic medicinal plants of Bangladesh	1.0	5 July 2016
Bengali translated Participant Information Sheet	1.0	4 August 2016
Bengali translated Consent Form	1.0	4 August 2016
Bengali translated Questionnaire	1.0	4 August 2016
Letter from Professor Abdur Rashid confirming consent to collaborate on Holly Siddique's research	1.0	5 July 2016
Letter from Professor Saiful Islam confirming that Professor Abdur Rashid is collaborating on Holly Siddique's research study and gatekeeper permission is not required.	1.0	4 August 2016

Approval is given on the understanding that the UEL Code of Practice in Research is adhered to.

The University will periodically audit a random sample of applications for ethical approval, to ensure that the research study is conducted in compliance with the consent given by the ethics Committee and to the highest standards of rigour and integrity.

**Please note, it is your responsibility to retain this letter for your records.**

With the Committee's best wishes for the success of this project.

EXTERNAL AND STRATEGIC DEVELOPMENT SERVICES

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Quality Assurance and Enhancement



Yours sincerely,

Catherine Fieulleteau  
Research Integrity and Ethics Manager  
University Research Ethics Committee (UREC)  
Email: [researchethics@uel.ac.uk](mailto:researchethics@uel.ac.uk)

Uxbridge Campus, Uxbridge Way, London E16 9FD



A copy of consent form, and questionnaire which was used during the survey provided below



**Annexe 2**

**UNIVERSITY OF EAST LONDON**

**Consent to Participate in a Programme Involving the Use of Human Participants.**

**Project Title**

IDENTIFICATION OF ANTI-INFECTIVE LEAD COMPOUNDS FROM BANGLADESHI MEDICINAL PLANTS TO TACKLE ANTIMICROBIAL RESISTANCE

**Student Researcher: Holly Siddique**

**Supervisors:**

**Dr. Mukhlesur Rahman**  
**Dr. Barbara Pendry**  
**Dr. Joanne Tocher**

Please tick as appropriate:

	YES	NO
I have read the information leaflet relating to the above programme of research in which I have been asked to participate and have been given a copy to keep. The nature and purposes of the research have been explained to me, and I have had the opportunity to discuss the details and ask questions about this information. I understand what is being proposed and the procedures in which I will be involved have been explained to me.		
I understand that my involvement in this study, and particular data from this research, will remain strictly confidential as far as possible. Only the researchers involved in the study will have access to the data. It has been explained to me what will happen once the programme has been completed		
I understand that my participation in this study is entirely voluntary, and I am free to withdraw at any time during the research without disadvantage to myself and without being obliged to give any reason. I understand that my data can be withdrawn up to the point of data analysis and that after this point it may not be possible.		
I hereby freely and fully consent to participate in the study which has been fully explained to me and for the information obtained to be used in relevant research publications.		

Participant's Name (BLOCK CAPITALS) .....

Participant's Signature .....

Investigator's Name (BLOCK CAPITALS) ...HOLLY SIDDIQUE.....

Investigator's Signature .....

Questionnaires for practitioners

Ethnopharmacological survey of Ayurvedic medicinal plants of Bangladesh

**a. Informants' details:**

Name of the interviewer	
Name of the interviewee	
Date of interview:	
Gender	
Age	
Occupation	
Education (for example- apprenticeship, diploma or university degree)?	
Number of years you have been practising as an Ayurvedic/ Unani practitioner? (if applicable)	
Location/Residence	

**b. Questions for Ayurvedic/ Unani practitioners:**

Information about plants:

What specific plants do you prescribe to patients for treating infectious diseases such as boils, skin diseases, minor cut, cholera, dengue fever, diarrhoea, gonorrhoea, syphilis, pneumonia, urinary tract infection?					
	1	2	3	4	5
Name of the disease					
Plants used (Local name)					
Scientific name of plant/s					
Which plant part do you use? (stem, bark or root)					
What is the habit of the plant? (tree, shrub, herb or climber)					
What is the perfect time of plant collection?					
In what condition do the plants used? (Fresh or dried form of plant)					
How the plants are prepared? Do you prepare it by yourself or you give instruction to patients to prepare it by themselves?					
Did the patients experience any side effect if the plant extract take with allopathic medicine?					
Route of administration, i.e. how is the plant taken? (oral/ ointment/ etc)					
What is the dosage of prescribe plant?					
Do these plants have other uses besides antimicrobial activity? (If any)					

c. Indigenous information about plants:

Where does the plant grow? Does it grow in wild or cultivated as food?					
Is the plant common, less common or rare?					
What is the location of collecting the plant? Farm land, home garden, wild habitat or roadside?					
What are the conservative needs?					
Conservation efforts made by Government and local residence					

Plants identified with ranking.....(Scientific name)

Researcher signature:.....

Date:.....