Strathclyde Institute of Pharmacy and Biomedical Science

Libyan Propolis: A Comprehensive Chemical, *in vitro* Biological Investigation and Metabolomic profiling of Antiprotozoal Activity

A thesis presented

For

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By

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DECLARATION

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This thesis is dedicated to my parents,

My husband and my lovely kids

Over 1400 years ago the **Quran** refers to bees that generate the honey as females (the Arabic grammar is in the female mode): [Quran 16.68-69] and your Lord (Allah) revealed to the bees: Build your hives in mountains, trees and in what they build. The **Quran** used "Kuli" (females).

In the name of of Allah the Merciful

﴿ وَأَوْحَىٰ رَبُّكَ إِلَى النَّحْلِ أَنِ اتَّخِذِي مِنَ الْجِبَالِ بُيُوتًا وَمِنَ الشَّجَرِ وَمِمَّا يَعْرِشُونَ (68) ثُمَّ كُلِي مِنْ كُلِّ الثَّمَرَاتِ فَاسْلُكِي سُبُلَ رَبِّكِ ذُلُلًا يَخْرُجُ مِنْ بُطُونِهَا شَرَابٌ مُخْتَلِفٌ أَلْوَانُهُ فِيهِ شِفَاءٌ لِلنَّاسِ إِنَّ فِي ذَٰلِكَ لَآيَةً لِقَوْمٍ يَتَفَكَّرُونَ (69) ﴾

[القران الكريم سورة النحل اية 68-69]

(68) And your Lord inspired the bee: "Set up hives in the mountains, and in the trees, and in what they construct"

(69) "Then eat of all the fruits, and go along the pathways of your Lord, with precision. From their bellies emerges a fluid of diverse colors, containing healing for the people. Surely in this is a sign for people who reflect".

*[**Quran**, surah al-nahl 16.verse 68-69]*

ABSTRACT

Propolis (bee-glue) is collected by bees from plants as a defensive substance in response to environmental pressures which include a range of microorganisms and parasites. These parasites are known to include the protozoal species *Crithidia*. Since it is collected by bees for the specific purpose of providing chemotherapeutic protection this increases the likelihood of finding active compounds in propolis compared with random screening of plants. Twelve samples of Libyan propolis (P1-P12) were collected from different geographic zones of Libya. Ethanolic extracts of the twelve propolis samples were prepared and these were profiled initially by NMR which gave some general indication of the type of compounds which might be found in them providing signals typical of diterpene aldehydes and cycloartane triterpenoids depending on the origin of the sample. There were limited signals in the aromatic region between 6 and 8 ppm in contrast to Northern European samples where many signals from flavonoid compounds would be expected. The extracts were profiled by high resolution LC-MS and the LC-MS data was extracted and modelled by SIMCA-P software using PCA with HCA, which separated the samples into five main groups based on their chemical composition. The groups were according to Geographic origin which the samples from North East, North West, South East and Southwest Libya grouping together. The sample extracts were tested against a wide range of microorganisms including *T. brucei*, *L. donovani*, *P. falciparum*, *C. fasiculata*, *M. marinum*, *S. aureus*, *K. pneumoniae* and *T. spiralis*. In addition, cell based assays for cytotoxicity and anti-inflammatory activities were carried out.

Eighteen isolated compounds were isolated including: eight diterpenes **(1)** 13-epi-torulosal, **(2)** 13-O- acetyl epi-cupressic acid**, (3)** 13-epicupressic acid, **(4)** 13-epitorulosol, **(15)** acetylisocuppressic acid, **(16)** Agathadiol, **(17)** Isocupressic acid and, **(18)** isoagatholal, three lignans; **(5)** sesamin**, (6)** Demethylpiperitol, **(7)** 5', methoxy piperitol, **(8)** the flavonoid flavanone taxifolin-3-acetate-4'-methyl ether and five triterpenes of the cyclo artane type; **(9)** cycloartanol, **(10)** mangiferolic acid, **(11)** mangiferonic acid, **(12)** ambolic acid, **(13)** 27-hydroxymangeferonic acid and the resorcinol **(14)** cardol. Both the crude extracts and isolated compounds exhibited activity against the range of microorganisms were tested such as *T. brucei*, *L. donovani*, *P. falciparum*, *C. fasiculata*, *M. marinum*, *S. aureus*, *K. pneumoniae* and *T. spiralis.*

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ABBREVIATIONS

Abbreviations	*Definition*
^{1}D ^{1}H NMR	One dimensional proton NMR spectra
2D NMR	Two-dimensional nuclear magnetic resonance spectroscopy
ANOVA	Analysis of variance
APCI-MS	Atmospheric pressure chemical ionization, ion trap–mass spectromry
B.C.	Before Christ
C. fasciculate	*Crithidia fasciculate*
CAPE	Caffeic acid phenethyl ester
CC	Column chromatography
CCl_4	Carbon tetrachloride
CHI	Chalcone isomerase
COSY	Correlation spectroscopy
$CDCL_3$	Chloroform-d
DCM	Dichloromethane
DME	Dulbecco' s modified eagle medium
DMSO	Dimethylsulfoxide
DMSO-d_6	Deuterated dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNP	Dictionary of Natural Products
DPPH	2,2-diphenylpicrylhydrazyl
E. coli	*Escherichia coli*
EEP	Ethanol extracted propolis
EI-MS	Electron impact mass spectrometry
ELISA	Enzyme-linked Immunosorbent Assay
ELSD	Evaporative light scattering detector
ESI-MS	Electrospray ionisation mass spectrometry
EtOAc	Ethyl acetate
FC	Flash chromatography
FCS	Fetal Calf Serum
FDA	Food and Drug Administration

FDR	False discovery rate
FNS	Flavone Synthase
GC	Gas liquid chromatography
GC-MS	Gas chromatography mass spectrometry
HCA	Hierarchical cluster analysis
HCV	Hepatitis C virus
HILIC	Hydrophilic interaction liquid chromatography
HIV	Human immunodeficiency virus
HMBC	Heteronuclear Multiple-Bond Correlation Spectroscopy
HMDB	The human metabolome database
HMGB1	High-mobility group protein B1
HMQC	Heteronuclear multiple-quantum correlation
HMQC	Heteronuclear Multiple Quantum Coherence
HPLC	High performance liquid chromatography
HRESI-MS	High resolution electrospray ionisation mass spectrometry
HRLC-MS	High Resolution Liquid Chromatography mass spectrometry
HRMS	High resolution mass spectrometry
HSV-1	Herpes simplex type 1
IC_{50}	Half of maximum concentration
IPmac	Intraperitoneal macrophages
L. donovani	*Leishmania donovani*
LPS	Lipopolysaccharide
LC	Liquid chromatography
LC-MS	Liquid chromatography mass spectrometry
M. marinum	*Mycobacterium marinum*
m/z	Mass to Charge Ratio
MBC	Minimal bactericidal concentration
MeCN	Acetonitrile
MeOH	Methanol
MHz	Megahertz
MIC	Minimum inhibition concentration
ml	Millilitre
PPBs	polycyclic polyisoprenylated benzophenones
µl	Microlitre

MPLC	Medium pressure liquid chromatography
MRSA	Methicillin-resistant staphylococcus aureus
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MVA	Multivariate analysis
NaCl	Sodium chloride
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NP	Normal phase chromatography
№	Number
^{o}C	Celsius
OPLS	Orthogonal partial least squares
P. falciparum	*Plasmodium falciparum*
PAHs	Polycyclic aromatic hydrocarbons
PCA	Principal component analysis
PLS	Partial least squares
PPB	Polycyclic polyisoprenylated benzophenones
ROS	Reactive oxygen species
RP	Reverse phase
RT	Retention time
S. aureus	*Staphylococcus aureus*
SIDR	Strathclyde Institute for Drug research
SIMCA-P	Soft-Independent Modelling of Class Analogy
SIPBS	Strathclyde Institute of Pharmacy and Biomedical Sciences
TMS	Trimethylsilyl
T. cruzi	*Trypanosoma cruzi*
T. evansi	*Trypanosoma evansi*
T. brucei	*Trypanosoma brucei*
T. brucei clone (s427)	Drug-sensitive *T.brucei* clone, Lister strain 427
TIC	Total ion current
TLC	Thin layer chromatography
TMS	Tetramethylsilane
TNF-α	Tumour necrosis factor alpha

UK	United kingdom
UV	Ultraviolet
v/v	Volume per volume
VIP	Variable Importance in the Projection
w/v	Weight per volume
WHO	World health organization

LIST OF PUPLICATIONS

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Book Chapter

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Posters

Weam Siheri, Tong Zhang, Ruan Edrada-Ebel, Carol Clements, John Igoli, David G. Watson Strathclyde Institute of Pharmacy and Biomedical Sciences, Glasgow G4 0RE. *The Use of LC-MS Profiling and Bioassay In The Isolation And Identification of Antitrypanosomal Diterpenes From Libyan Propolis*. (9th International Metabolomics Conference 1-4 July SECC Glasgow 2013.

Ruwida Omar, **Weam Siheri**, Sultan Al Mutairi, Carol Clements, James Fearnley, Ruangelie Edrada-Ebel, David Watson And Tong Zhang,

*Chemical profiling of African propolis by LC-UV-ELSD, high resolution MS and MS/MS and correlation to biological activity***.** Poster presented in (9th International Metabolomics Conference 1-4 July SECC Glasgow 2013.

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Weam Siheri, Tong Zhang, Godwin Unekwuojo Ebiloma, Marco Biddau, Nicola Woods[1], Muattaz Hussain, Carol Clements, James Fearnley, Ruangelie Edrada Ebe, Tim Paget, Sylke Muller, Katharine C. Carter, Val Ferro, Harry P. De Koning And David G. Watson. *Metabolomic profiling of Anti-protozoal Activity of Libyan propolis.* Scottish Metabolomics Network Meeting. December 2015. Awarded a prize as the people's choice for best poster.

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1 Chapter 1

Introduction

1.1 Introduction

1.2 Propolis background

Bees have been in existence for more than 125 million years and their evolutionary success has allowed them to become perennial species that can exploit virtually all habitats on Earth. This success is attributed to chemistry and application of the specific products that bees manufacture such as honey, propolis beeswax, pollen and royal jelly.

The first reports on the use of propolis in folk medicine were around 300 B.C, as pointed out by a number of research studies propolis used from ancient times, as medicine, food products and cosmetics. In addition to the fact that propolis is one of the few natural remedies that has maintained its popularity over such a long period of time (Lotfy, 2006).

Propolis (bee glue), is a resinous natural substance composed mainly of plant resins and beeswax, that honey bees collect from different plant exudates and utilize to fill the gaps and to seal parts of the hive. The word propolis originates from the Greek *pro* meaning in front of and *polis* (the city) which relates to the defence of the hive. The bees mix the plant exudates with bees wax and β-glycosidase which removes sugars from the flavonoids in order to produce a water insoluble resin to seal the hive, which is called propolis (Petrova et al., 2010, Laskar et al., 2010, Gavanji et al., 2012).

Propolis has been used as a remedy for several centuries for various diseases and its antimicrobial properties which are present in propolis from many different origins have been extensively studied in recent decade. In addition, recent studies have focused on the other pharmacological properties of

propolis in relation to the chemical composition. Different samples of propolis possess antiparaisitic, antiviral, immuno- stimulating, healing, antitumor, anti-inflammatory and analgesic activities (Chattopadhyay and Kumar, 2006, Alsiheri, 2010).

Currently, there are many commercial applications of propolis where it is included in over the counter medications to cure different symptoms such as: colds, as well as for dermatological treatment in wound healing and in the treatment of burns. In addition, propolis is used in mouth washes, tooth paste to prevent caries, gingivitis and stomatitis (Sarkez, 2014, ZHOU et al., 2004, Pietta et al., 2002).

1.3 Geographical variations of propolis.

The chemical compositions and biological activities of propolis in various countries have been studied extensively by various scientific research groups and significant variations have been observed in the chemical composition of propolis. This is greatly affected by variations in geographical origins (Popova et al., 2005, Woo et al., 2011, Bankova et al., 2000, Hegazi et al., 2000, Kartal et al., 2003, Trusheva et al., 2006, Banskota et al., 2001b, Nagaoka et al., 2003, Petrova et al., 2010, Watson et al., 2006). The composition of propolis samples depends on the collecting location, time and plant sources used by bees (Lotfy, 2006).

One of the greatest differences affecting propolis composition is variation of geographical origin. This point attracted researchers to analyse different samples from different geographical locations and compare the biological activities of the samples (Woo et al., 2011).

Watson, *et al.* (2006), chemically and biologically analysed 43 propolis samples which were collected in different parts of the world (Africa, Asia, Brazil,

Europe and Solomon Islands). The results showed that chemical variation parallel to different origins of propolis. Also the biochemical profiling of the propolis components could be attributed to the various biological activity (Watson et al., 2006).

Sawaya, *et al*. (2009), found that the ESI-MS fingerprinting showed that the propolis samples varied according to the species of bee, month of collection and geographic origin (Sawaya et al., 2009).

Trusheva, *et al* (2004), conducted a study into the variation in the chemical composition of propolis in relation to geographic and plant origins. This clearly showed that in regions of temperate climate such as Europe and North America *Apis mellifera* bees obtain resins mainly from the buds of species of *Populus* and the main active components are flavonoids while in the equatorial, tropical and subtropical climate the chemical composition and plant origin are much more variable (Trusheva et al., 2004).

Indeed, more recent studies suggest that propolis from tropical regions, such as Cuba and Venezuela, have polyisoprenylated benzophenones as major constituents. On the other hand the major sources of propolis have been shown to be *Populus* buds in Europe, while apical buds of *Baccharis dracunculifolia* is the source of propolis in southeast Brazil and propolis from the secretions of flowers of *Clusia major* and *Clusia minor* in Venezuela, and *Clusia nemorosa* in Cuba (de Castro Ishida et al., 2011). This is evidence that *Clusia* species seem to be important as sources of propolis in tropical regions. Numerous species of *Clusia* resins are utilized by pollinating bees (de Castro Ishida et al., 2011, Simone-Finstrom and Spivak, 2010).

Many different studies have concluded that the propolis samples from Europe contain predominantly flavonoids and esters of phenolic acids, while the best known propolis from Brazil (green propolis from the Southeast) and Cuba

contains predominantly prenylated benzophenones, derivatives of p-coumaric acids and terpenes (Trusheva et al., 2004, Popova et al., 2005, Woo et al., 2011). However, isoflavonoids, prenylated benzophenones, and a naphthoquinone, were also isolated from a red propolis sample from northeast Brazil (Trusheva et al., 2006).

On the other hand, a research study done by Kalogeropoulos, *et al*. (2009) investigated propolis extracts from Greece and Cyprus. The results showed that the composition presented differences from European propolis and similarities with East Mediterranean propolis since the Mediterranean propolis contained mainly diterpenes. An interesting finding was the discovery of the diterpene totarol, which was identified by means of the mass spectrum of its TMS derivative. This was the first record of totarol in a European propolis which showed antimicrobial action against Gram-positive bacteria; *Staphylococcus aureus* and *Staphylococcus epidermidi*s (Kalogeropoulos et al., 2009).

1.4 Chemical composition of propolis

In general, the main components of propolis are fatty, aliphatic and aromatic acids, flavonoids, alcohols, terpenes, sugars and esters. Several studies have confirmed the differences in percentages of individual components of propolis, depending on the origin of the plants from which the resin is collected. The variety of propolis chemical composition is mentioned in (Table 1-1) (Markiewicz-Żukowska et al., 2012).

Recently a systematic database literature search for the chemical composition of propolis was carried out by Shuai Huang, *et al* (2014) and stated that 241 compounds were identified in propolis for the first time between 2000 and

2012; and from numerous chemical classes such as flavonoids, phenylpropanoids, terpenenes, stilbenes, lignans, coumarins, and their prenylated derivatives, showing a pattern consistent with around 300 previously reported compounds. The chemical characteristics of propolis are attributed to the variety of geographical locations, botanical sources and bee species (Huang et al., 2014).

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Table 1-1 Summary review of propolis chemical composition based on previous studies[a]

Compounds (percentage of contents)				
Fatty and Aliphatic acids (24–26%)	**Flavonoids (18–20%)**	**Microelements (0.5–2.0%)**	**Sugars (15–18%)**	**Others (21–27%)**
Butanedioic acid (Succinic acid)	Astaxanthin	Aluminum (Al)	Sorbopyranose	Cyclohexanone
Propanoic acid (Propionic acid)	Apigenin	Copper (Cu)	D-Erythrotetrofuranose	3-methyl,antitricyclo undec-3-en 10-one
Decanoic acid (Capric acid)	Chrysin	Magnesium (Mg)	D-Altrose	Cyclohexane
Undecanoic acid	Tectochrysin	Zinc (Zn)	D-Glucose	Cyclopentene
Malic acid	Pinobanksin	Silicon (Si)	Arabinopyranose	5-n-propyl-1,3 dihydroxybenzene
D-Arabinoic acid	Squalene	Iron (Fe)	d-Arabinose	Butane
Tartaric acid	Pinostrobin chalcone	Manganese (Mn)	α-D-Galactopyranose	2(3H)-Furanone
Gluconic acid	Pinocembrin	Tin (Sn)	Maltose	L-Proline
α-D-Glucopyranuronic acid	Genkwanin	Nickel (Ni)	α-D-Glucopyranoside	2-Furanacetaldehyde
Octadecanoic acid (Stearic acid)	Galangin	Chrome (Cr)	D-Fructose	2,5-is-3-phenyl-7-pyrazolopyrimidine
Hexadecanoic acid	Pilloin	**Aromatic acids (5–10%)**	**Esters (2–6%)**	Cliogoinol methyl derivative
β-D-Glucopyranuronic acid	Acacetin	Benzoic acid	Caffeic acid phenethyl ester	Fluphenazine
9,12-Octadecadienoic acid	Kaemferide	Caffeic acid	4,3-Acetyloxycaffeate	4,8-Propanoborepinoxadiborole
Tetradecanoic acid	Rhamnocitrin	Ferulic acid	Cinnamic acid,	1,3,8-Trihydroxy-6-methylanthraquinone

Pentanedioic acid	7,4′-dimethoxyflavone	Cinnamic acid	3,4 dimethoxy-trimethylsilyl ester	1-5-oxo-4,4-diphenyl-2-imidazolin-2-yl guanidine
Glutamic acid	5-hydroxy-4′7-dimethoxyflavone		3-Methoxy-4-cinnamate	3,1,2-Azaazoniaboratine/Piperonal
2,3,4-trihydroxy butyric acid	5,7-dihydroxy-3,4′dihydroxyflavone	**Alcohols and Terpenes (2–3.3%)**	Cinnamic acid 4 methoxy 3 TMS ester	3-Cyclohexene
Phosphoric acid	3,5-dihydroxy-7,4′-dimethoxyflavone	Glycerol	2-propenoic acid methyl ester	1H-Indole
Isoferulic acid		Erythritol		1H-Indole-3-one
		α-Cedrol		2-Furanacetaldehyde
Vitamins (2–4%)		Xylitol		Guanidine
A, B1 , B2 , E, C, PP				2(3H) Furanone
				1,3,8-trihydroxy-6-meyhylanthraquinone

N.B; [a]; This table has been prepared based on the following papers (Ozkul et al., 2005, Machado et al., 2007, Eremia and Dabija, 2007, Vardar-Ünlü et al., 2008, Maciejewicz et al., 2001, Wang et al., 2010, Kumazawa et al., 2004, Burdock, 1998).

In addition, propolis is characterized by mean contents of 50% balsams and resins, 30% waxes, 10% essential oils, 5% pollen and 5% of various other substances and organic debris. This matrix usually contains a variety of compounds such as phenolic compounds, terpenes, sesquiterpenes and stilbenes, β-steroids, aromatic aldehydes and alcohols (Chattopadhyay and Kumar, 2006, Papotti et al., 2012). Propolis is broadly characterised into propolis from temperate regions mainly originating from poplar tree exudates and rich in phenolics such as flavonoids, aromatic acids and esters (Bankova et al., 2002).

On the other hand propolis from tropical areas, contains traces of poplar constituents but is rich in other substances including prenylated derivatives of p-coumaric acids, diterpenes and lignans (Bankova et al., 1999, Marcucci, 1995, Marcucci and Bankova, 1999), prenylated benzophenones (Cuesta-Rubio et al., 2002) and prenylated flavonoids (Raghukumar et al., 2010).

1.4.1 Flavonoids and phenolics occurring in propolis

Flavonoids are a group of heterocyclic organic compounds which occur in plants and their components such as fruits, vegetables, nuts, seeds, stems and flowers and are commonly found among the secondary metabolites (Abdel-Fattah and Nada, 2007).

Propolis has variable composition and can contain up to 200 constituents. Polyphenolic compounds such as flavonoids, phenolic acids and their esters and phenyl proponoids such as caffeic acid, cinnamic acid and their esters occur widely in propolis. The main of compounds which are usually present as major components are flavonoids which include flavones, flavonols (Amoros et al., 1992b, Cui-ping and Fu-liang, 2009), flavanones, dihydroflavonols and chalcones (Yang et al., 2013).

1.4.2 Terpenoids in propolis

Terpenoids are compounds with an extensive hydrocarbon skeleton which may be oxygenated to produce compounds such as aldehydes, ketones, alcohols, phenols and oxides. They include mono, sesqui-, di- and triterpenes (Aharoni et al., 2005). Terpenes are a unique group of hydrocarbon-based natural products and are volatile constituents of plant essential oils (Marquez Hernandez et al., 2010, Banskota et al., 2001b, Salatino et al., 2005, Campos et al., 2010).

Since they are the only mono sesquiterpens ''volatile'' terpenes cause the aroma of the plant. Monoterpenes (Patricio et al., 2002) and sesquiterpenes are composed of two and three isoprene units (Huang et al., 2014, Simionatto et al., 2012). Monoterpene alcohols such as terpineol (2) have antiseptic properties. Some representative terpenoids are shown in (Figure 1-1) (Bankova et al., 2000, Kalogeropoulos et al., 2009, Watanabe et al., 2011, Melliou and Chinou, 2004, Bankova et al., 2014, Falcão et al., 2016).

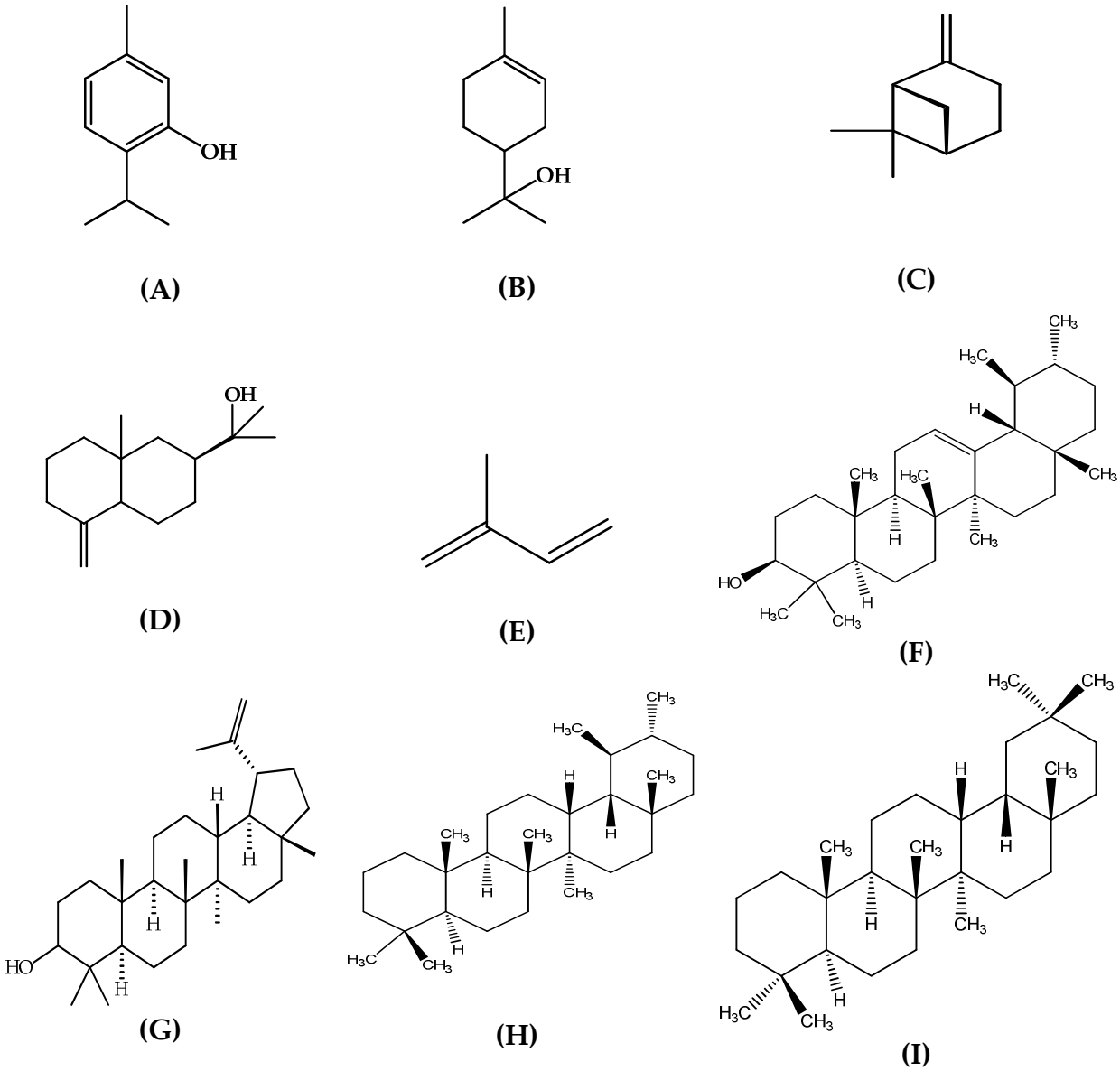


Figure 1-1 Chemical structures of some representative terpenes, (A); Thymol, (B). Terpineol, (C); β-Pinene, (D); Eudesmol, (E); Isoprenyl unit, (F); β-amyrins, (G); Lupeol, (H); Ursane, (I); Oleanane.

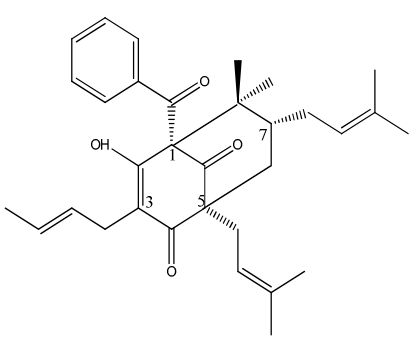
It was observed that volatile oils in propolis have moderate anti-microbial activity and it was found that in tropical samples the volatile oil fraction is more important for antimicrobial activity than in samples from Europe (Saloma˜o et al., 2004, Trusheva et al., 2006, Trusheva et al., 2010). Several studies have investigated the terpenoid composition of ethanolic extracts of propolis through different analytical means, especially gas chromatography–

mass spectrometry (GC–MS) and high performance liquid chromatography (HPLC) and Nuclear Magnetic Resonance (NMR) spectroscopy (El Sayed and Ahmad, 2012, Ashry et al., 2012, Sforcin and Bankova, 2011, Kardar et al., 2014, Valencia et al., 2012, Li et al., 2009a, Li et al., 2009b, Zhou et al., 2009).

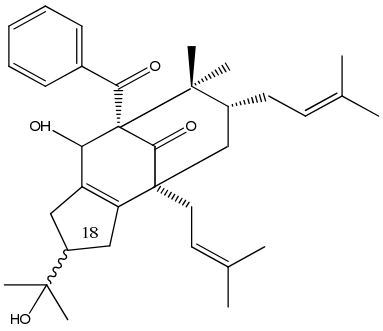
On the other hand, polycyclic polyisoprenylated benzophenones (PPBs) form a class of acylphloroglucinols that is confined to the *Guttiferae*, a plant family almost exclusively tropical, which is a rich source of biologically active metabolites. Many PPBs have an oxygenated and substituted bicyclo-[3.3.1]-nonane-2, 4, 9-trione core to which a benzoyl group and prenyl or geranyl side chains are attached. Secondary cyclizations involve the β-diketone and pendant olefinic groups affording adamantanes, homoadamantanes and dihydrofuran- or pyran-fused structures. PPBs exhibit a wide variety of biological activities, the most interesting of which are cytoprotection against HIV, antibacterial activity against methicillin-resistant *S. aureus*, antioxidant and cytotoxic activity. More recent studies have confirmed that acylphloroglucinols are labile compounds sensitive to light, oxygen, and heat (Hernández et al., 2005). Nemorosone, a PPB with a bicyclo-[3.3.1]-nonane-2,4,9-trione system of type A (benzoyl group at C1), is found in resins and latex of *Clusia* species and it is the major constituent of *C. rosea* floral resin and brown Cuban propolis (Figure 1-2).

Nemorosone is responsible for the antimicrobial activity of *Clusia spp*. resin and propolis (Mangas Marín et al., 2008, Cuesta-Rubio et al., 2002) Nemorosone exhibits activity as a free radical scavenger of the same order of magnitude as α-tocopherol and has moderate anti-human immunodeficiency virus (HIV) activity. It has also been demonstrated that it exerts a significant cytotoxic activity against a panel of tumour cell lines (Piccinelli et al., 2009).

The instability of PPBs carrying an enolizable 1, 3-diketone system has been suggested, and the elucidation of this aspect is very basic for the evaluation of their biologic activity (Piccinelli et al., 2011, Matsuhisa et al., 2002). In order to define the stability of nemorosone, (ESI-MS^n) was employed to shed light on the origin of the degradants of nemorosone. Initially MS^n experiments were performed on seven related PPBs (Figure 1-2) to obtain useful information for structural characterization of this class of compounds and to identify the degradation products of nemorosone. The proposed fragmentation pathways, supported by exact mass measurements, allowed the nature of side chains on the bicyclo core and the type and position of their modifications to be established. Several studies have revealed that nemorosone undergoes rapid degradation in *n*-hexane and chloroform solutions (Table 1-2). The degradation products have been identified by HPLC-ESI/MS^n and NMR (Cuesta-Rubio et al., 2001).



(A)



(B)

(C)

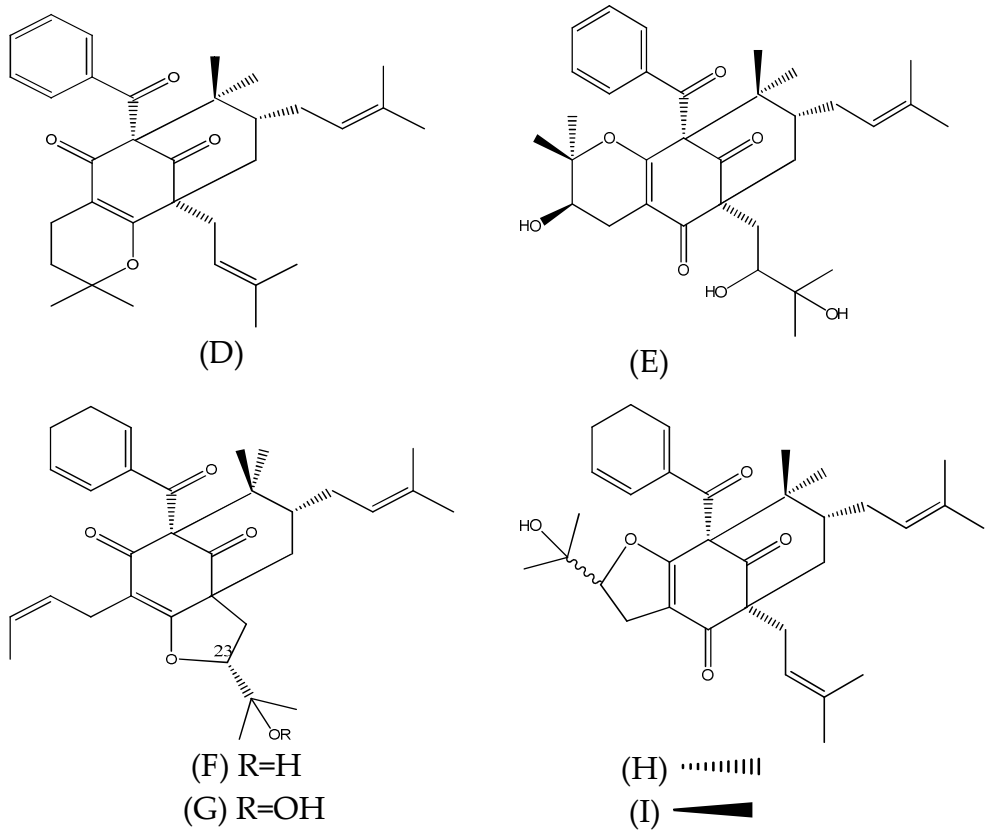


Figure 1-2 The structures of the polycyclic polyisoprenylated benzophenones included in previous study: (A); Nemorosone, (B); Garcinielliptone I, (C); Hyperibone B, (D); Propolone A, (E); Propolone B, (F); Propolone C, (G); Propolone D, (H); Propolone D hydroperoxide and (I); 18-epi-propolone C.
Adapted from (Piccinelli et al., 2009).

Table 1-2 Accurate masses for product ions of the PPBs and their elemental compositions [a]

Compounds [b][M +H]$^+$ion	***[m/z]*** **Measured**	**Elemental composition**	***[m/z]*** **Calculated**	***[m/z]*** **(ppm)**
Nemorosone **(A)**	503.317	$C_{33}H_{43}O_4^+$	503.316	1.5
Garcinielliptone I **(B)**	519.311	$C_{33}H_{43}O_5^+$	519.311	–0.5
Hyperibone B **(C)**	519.311	$C_{33}H_{43}O_5^+$	519.311	0.3
Propolone A **(D)**	503.315	$C_{33}H_{43}O_4^+$	503.316	–1.7
Propolone B **(E)**	553.317	$C_{33}H_{45}O_7^+$	553.317	0.1
Propolone C **(F)**	519.311	$C_{33}H_{43}O_5^+$	519.311	0.1
Propolone D **(G)**	519.310	$C_{33}H_{43}O_5^+$	519.311	–1.3
Propolone D hydroperoxide **(H)**	535.308	$C_{33}H_{43}O_6^+$	535.306	3.2
18-*epi*-propoloneC **(I)**	519.311	$C_{33}H_{43}O_5^+$	519.311	–0.3

N.B; [a]; Chemical structure of compounds **(A-I),** shows in figure 1-2 based on (Piccinelli et al., 2009), [b] M; moleclular weight and H; proton $^+$ion.

1.4.3 Environmental contamination in propolis

Bees and beehive products have been widely used as bio-indicators of environmental contamination (Porrini et al., 2003). The most important contaminants in propolis are the substances used for the control of bee pests. Chemical protection of bee hives is commonly carried out by treatment with different kinds of pesticide (Guo et al., 2011).

Additionally, bees collect nectar, honeydew, resinous substances, pollen and water from the surrounding environment. Such products may be contaminated by different xenobiotics such as pesticides, insecticides, radionuclides, heavy metals and polycyclic aromatic hydrocarbons (PAHs),

may be considered as contaminants of propolis. PAHs may be expected in the analysis of some propolis samples (Porrini et al., 2003, Bogdanov, 2006).

A research study was carried out on raw propolis and propolis-based dietary supplements (North-East Italy) obtained from beekeepers and from the local market. The samples were analysed using a Varian model 9010 HPLC gradient pump and the results showed the presence of 13 polycyclic aromatic hydrocarbons (PAHs), including eight high molecular weight compounds (Moret et al., 2010). The contaminants that were detected included phenanthrene, anthracene, fluoranthene, pyrene and benz[a]anthracene (Moret et al., 2010).

1.4.4 Propolis extraction and isolation

The extraction of propolis is a process, which aims to remove waxy materials and to preserve the bioactive fractions. Raw propolis is considered to be difficult to use in cosmetics, foods or medicine unless it passes through a range of purification steps which extract materials such as wax, consequently the remaining extract is enriched with the bioactive components which are responsible for the biological activity of propolis (Sforcin and Bankova, 2011) Ethanol and methanol are the most widely used solvents in extraction of propolis. The most often utilized solvent is ethanol containing different percentages of water, 70% ethanol was found to extract most of the active components of propolis but not waxes. As propolis might contain up to 20–30% wax, this solvent has been applied in many studies. It was found that water has also been used on many occasions; however, it is important to note that in general, water dissolves only a small part of propolis constituents, about 10% of its weight, whereas 70% ethanol may dissolve 50–70% of active

ingredient, depending on the amount of wax in propolis extracts (Bankova et al., 1995).

1.4.4.1 Solubility of propolis.

Propolis solubility is attributable to the complex structure of propolis compositions, it cannot be used directly. Propolis is extracted commercially with a suitable solvent. The most common solvents used for extraction are water, methanol, ethanol, chloroform, dichloromethane, ether, and acetone. Many of the bactericidal components are soluble in water or alcohol (Kumar et al., 2008), which should remove the inert material and preserve the desired compounds.

Propolis composition depends upon the geographical region and also the method of extraction (Marcucci, 1995); the solvent should be carefully chosen (Cowan, 1999). The main solvents used for extraction of bioactive compounds and other chemical compounds extracted are outlined in Table 1-3.

Table 1-3 Summary of different solvents used for the extraction of propolis and major components extracted.

Water	Methanol	Ethanol	Chloroform	Dichloromethane	Ether	Acetone
Anthocyanins, starches, tannins, saponins, terpenoids, polypeptides, and lectins	Anthocyanins, terpenoids, saponins, tannins, xanthoxyline, totarol, quassinoids, lactones, flavones, phenones polyphenols, polypeptides and lectins	Tannins, polyphenol, polyacetylenes, terpenoids, sterols, and alkaloids	Terpenoids, flavonoids	Terpenoids, tannins, polyphenols, polyacetylenes, sterols, and alkaloids	Alkaloids, terpenoids, coumarins, and fatty acids	Flavonols

Adapted from (Wagh, 2013, Fokt et al., 2010).

1.4.5 Chemical analysis methods of propolis

Several analytical techniques have been used for chemical profiling of propolis compositions using different instrumental techniques (Table 1-4). These techniques include high performance liquid chromatography (HPLC) (Kumazawa et al., 2003, Zhou et al., 2009, Pietta et al., 2002), mass spectrometry (MS) (Zhang et al., 2014) and liquid chromatography mass spectrometry (LC-MS) (Catchpole et al., 2015, Kumazawa et al., 2003, Falcão et al., 2013b, Siheri et al., 2016, Pellati et al., 2011, Castro et al., 2014), Nuclear magnetic resonance spectroscopy (NMR) (Aga et al., 1994, Cuesta-Rubio et al., 2007, Bertelli et al., 2012, Watson et al., 2006), Gas liquid chromatography (GC), Gas Chromatography Mass Spectrometry (GC/MS) (Kartal et al., 2002, Popova et al., 2010, Maciejewicz et al., 2001, Cheng et al., 2013, Popova et al., 2005). Due to variations in the chemical composition of propolis, it is difficult to evaluate propolis by a single instrumental technique (Zhang et al., 2014, Cuesta-Rubio et al., 2007, Aliboni et al., 2010, Kasote et al., 2014, Siheri et al., 2016, Nina et al., 2015). Hence, different chemical techniques have been used. For instance LC-MS is useful in the determination of the different components of propolis (Saleh et al., 2015, Falcão et al., 2013b). It is considered the most versatile technique for the quality control of different propolis samples. While GC/MS has been used for the analysis of volatile components in some propolis samples which have no compounds that can be detected with UV spectrophotometry in combination with HPLC such as terpenoids (Hernández et al., 2005, Popova et al., 2010). There has been also extensive work on the analysis of propolis with nuclear magnetic resonance spectroscopy (NMR) which is a technique useful for the analysis of all groups of chemical constituents which have H and C in their structure (Bertelli et al., 2012, Watson et al., 2006). In addition, atmospheric pressure chemical ionization, ion trap–mass spectrometry (APCI-

MS) has been used, which allows characteristic fingerprints of complex natural materials (Cuesta-Rubio et al., 2007, Campo Fernández et al., 2008, Chattopadhyay and Kumar, 2007). According to research study by Zhang *et al*, (2014) on the different propolis samples from Africa, ultra violet (UV), evaporative light scattering (ELSD), and MS were used for identification, by using two detectors in LC, which led to more universal component detection. Some propolis samples show ELSD signals with few instances of UV indicating the presence of non-phenolic compounds. HRMS and NMR carried out extra elucidation. The use of different types of detectors helps in structure identification; some detectors are more universal and less specific than others and chosen depending on the expected nature of the isolated components.

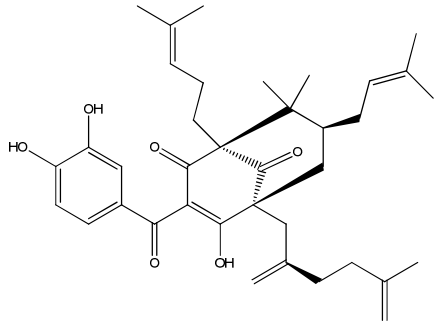
Moreover, dereplication of already investigated compounds using different analytical techniques likes ^{1}H and ^{13}C NMR, LC-MS, and GC-MS in both CID and EI-MS. Then comparing their structures with the already published compounds helps in making the screening process robust for the investigation of novel compounds (Zhang et al., 2014).

A study explored the composition of 65 different Cuban propolis samples, which were collected from different regions of Cuba and extracted with methanol. A classification method for the samples was developed by using a combination of NMR, HPLC-PDA, and HPLC-ESI/MS techniques. The analysis of ^{1}H and ^{13}C NMR spectra and chromatographic profiles of all propolis extracts allowed the definition of three main types of Cuban propolis directly related to their secondary metabolite classes which are: brown Cuba propolis (BCP), rich in polyisoprenylated benzophenones, red Cuban propolis (RCP), containing isoflavonoids as the main constituents, and yellow Cuban propolis (YCP) which probably contains aliphatic compounds. Subsequently, the principal compounds of the brown and red types were characterized by HPLC-

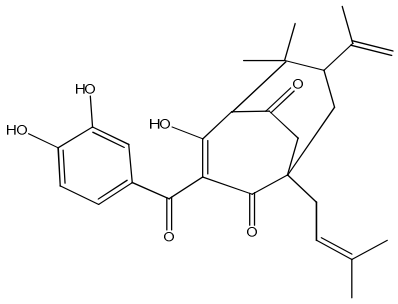
ESI/MS analysis. Indeed the Instrumental techniques used were complementary LC-PDA and LC-MS techniques, which were useful tools for qualitative and quantitative analysis of marker compounds of Cuban propolis. On the other hand NMR was shown to be a quick and informative tool for the rapid analysis of crude propolis polar extracts and allowed the identification of the main class of secondary metabolites (Cuesta-Rubio et al., 2007). Research has also been carried out on Mexican propolis and the occurrence of isoflavonoids in Mexican propolis has been reported for the first time. In addition, the presence of compounds with a 1, 3-diarylpropane and a 1, 3-diarylpropene carbon skeleton were found for the first time in compounds isolated from propolis (Lotti et al., 2010). Isolation of three new compounds, 1-(3', 4'-dihydroxy-2'-methoxyphenyl)-3-(phenyl) propane, (Z)-1-(2'-methoxy-4', 5'dihydroxyphenyl)-2-(3-phenyl) propene and 3-hydroxy-5, 6-dimethoxyflavan, together with seven known flavanones, isoflavans, and pterocarpans was carried out. Profiling of the samples was carried out by ESI-MS/MS techniques combined with 2D NMR. The characteristic compounds of red Mexican propolis have a very restricted distribution in the plant kingdom and occur almost exclusively in the *Leguminosae* family; the chemical study supported that the botanical origin of the reddish propolis as *Dalbergia* genus (Lotti et al., 2010).

Furthermore, a study carried out by de Castro Ishida, *et al*. (2011), prepared ethanolic extracts of four propolis samples collected from Manaus (North Brazilian Amazon) produced by *Apis mellifera* from different hives. The extracts from the 4 samples were analysed by HPLC/DAD/ESI–MS/MS and GC/EIMS. The major constituents of E2 and E4 were analysed by NMR (^{1}H and ^{13}C) and ESI/MS/MS. The main constituents of E2 and E4 are polyprenylated benzophenones: 7-epi-nemorosone, 7-epi-clusianone (major E4 constituents),

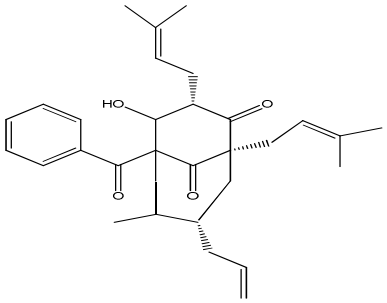
xanthochymol and gambogenone major E2 constituents, as illustrated in (Figure 1-3) making up a chemical profile so far unreported for Brazilian propolis. Aristophenone, methyl insigninone, 18-ethyloxy-17-hydroxy-17, 18-dihydroscrobiculatone B and derivatives of dimethyl weddellianone A and B, propolones, and a scrobiculatone derivative, were detected as minor constituents. While Triterpenoids (β-amyrins, β-amyrenone, lupeol and lupenone) were ubiquitous and predominant in E1 and E3. The extracts E2 and E4 were highly active against the cariogenic bacteria; E2 was more active than E4, probably due to a higher content of 2-epi-nemorosone. E4 was richer in di-hydroxylated compounds. Minor benzophenones present in E2 and E4 could not be isolated, due to the low contents and chemical complexity of samples, and tendency to rapid decomposition (de Castro Ishida et al., 2011). It was clear from this study these substances were highly liable to oxidation and decomposition during the processes of isolation.



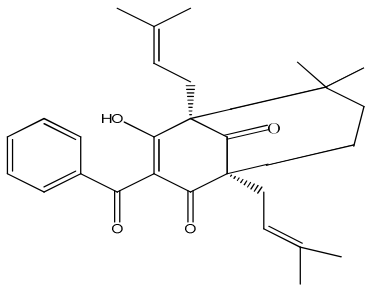
Xanthochymol



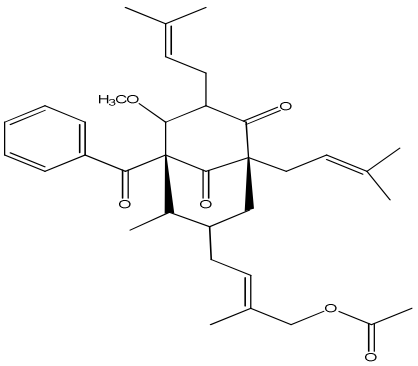
Gambogenon



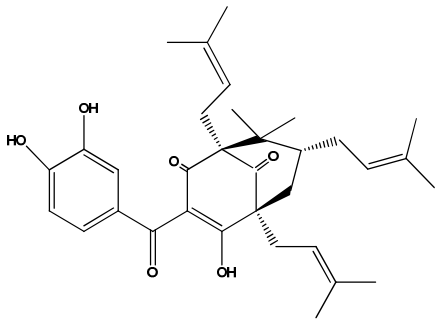
7-epi-Nemorosone



7-epi-Clusianone



Methyl insigninone



Aristophenone

Figure 1-3 Structures of polyprenylated benzophenones detected in the ethanol extracts of two samples of propolis (E2 and E4) from Manaus (Brazilian Amazon region).
Adapted from (de Castro Ishida et al., 2011).

The triterpenoids α- and β-amyrins, lupeol, β lupenone and amyrenone were detected in E1–E4 by using GC-MS. They predominated in E1 and E3, especially in the latter. Polyprenylated benzophenones have been reported as constituents of red propolis. In Brazil, the red propolis has so far been known only from the northeastern region. Outside Brazil, red propolis has been reported as typical for Cuba and Venezuela, where it is produced with a resin collected from *Clusia scrobiculata.* The study showed that both GC/MS and ESI–MS analysis could be used to explain the differences observed in antimicrobial activity. E3 and E1 exhibited lower antimicrobial activity, probably due to the higher amounts of triterpenoids, and virtually undetected amounts of polyisoprenylated benzophenones and polyprenylated di-hydroxylated benzophenones, such as xanthochymol and gambogenone as in (Figure 1-3).

Propolis from Europe and South America especially from Brazil has been intensively studied in the last decades and propolis from Asia such as, China (Bankova et al., 2000, Ahn et al., 2007, Yang et al., 2011, Cheng et al., 2013), Japan (Kumazawa et al., 2007, Mishima et al., 2005), Taiwan (Chang et al., 2002, Lu et al., 2003, Liu et al., 2004, Chen et al., 2008), Nepal (Shrestha et al., 2007b, Shrestha et al., 2007a, Huang et al., 2014)and Myanmar (Li et al., 2009b, Li et al., 2009a) etc., has recently become the subject of detailed studies.

However, in the previous research studies of propolis there are numerous reports on the isolation and structural elucidation of phytochemical compounds from propolis collected in Europe (Hegazi et al., 2000, Popova et al., 2010), South America (Trusheva et al., 2004), Asia and the Pacific region (Chen et al., 2008, Cheng et al., 2013). There is a lack in information on African propolis and its the exact chemical constituents although there is great diversity in vegetation of Africa

There were a few studies carried out on propolis from some areas of Africa which has antibacterial and antiradical activity (Khadem and Marles, 2010, Kardar et al., 2014).

In 2014, Kardar, *et al*, reported on the phytochemical analysis of propolis samples from the West of the Cameroon and the study characterised nine triterpenes, thirteen alk(en)ylphenols and nine alk(en)ylresorcinols (Kardar et al., 2014)

However, research into African propolis is scarce and limited to North African regions, such as Tunisia (Kouidhi et al., 2010, Martos et al., 1997), Algeria (Lahouel et al., 2010, Piccinelli et al., 2013) and Egypt (El Hady and Hegazi, 2002, El-Bassuony, 2009, Noori et al., 2012). There is almost no data on chemical composition of propolis from Sub-Saharan Africa (Martos et al., 1997, Petrova et al., 2010, Velikova et al., 2000).

In 2010, Petrova, *et al.* conducted a study to two ethanolic extracts of propolis obtained from two different places of Mwingi and Voi in Kenya which were subjected to preliminary chemical screening by TLC and GC-MS. Each extract demonstrated an individual chemical profile. Their TLC and GC-MS fingerprints were obviously different from European poplar type propolis, as well as from other tropical propolis types, such as Brazilian green, Brazilian red and Pacific propolis. Consequently, detailed chemical studies of the Kenyan propolis samples were performed. Separation was carried out by column chromatography and then fractions were further purified by preparative TLC using GC-MS and NMR pure compounds were characterised and two new components were isolated from propolis samples from Kenya which were arylnaphtalene lignans, tetrahydrojusticidin B **(A)** and 6-methoxydiphyllin (**B)**,

along with four known phenolic compounds (**E–H**), found for the first time in propolis. The structures of the compounds were elucidated based on their spectral properties. The geranylstilbenes **(G** and **H)** demonstrated antibacterial activity against *S. aureus*, and the geranylflavone macarangin **(F)** possessed antiradical activity against DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) free-radicalas illustrated in the (Figure 1-4) (Petrova et al., 2010) .

Additional constituents of the Mwingi sample, recognized by GC-MS were triterpenes (mainly alcohols of amyrine type) and a number of sugars (mono and disaccharides). Both compound types are common propolis constituents in different geographic locations. The plant source of this sample, especially the source of the arylnaphtalene lignans, is yet unknown. Moreover, the results of the study were consistent with the idea that tropical propolis is highly variable and this is a promising for further studies on African propolis.

The previous studies indicated that the chemical composition of propolis product is highly variable although of different chemical composition; propolis always demonstrates considerable a biological activity, especially antimicrobial activity. For this reason, the chemical diversity of propolis has the potential to provide valuable leads to active components and new types of propolis from unexplored regions continue to attract growing attention among scientists searching for new bioactive molecules (Petrova et al., 2010).

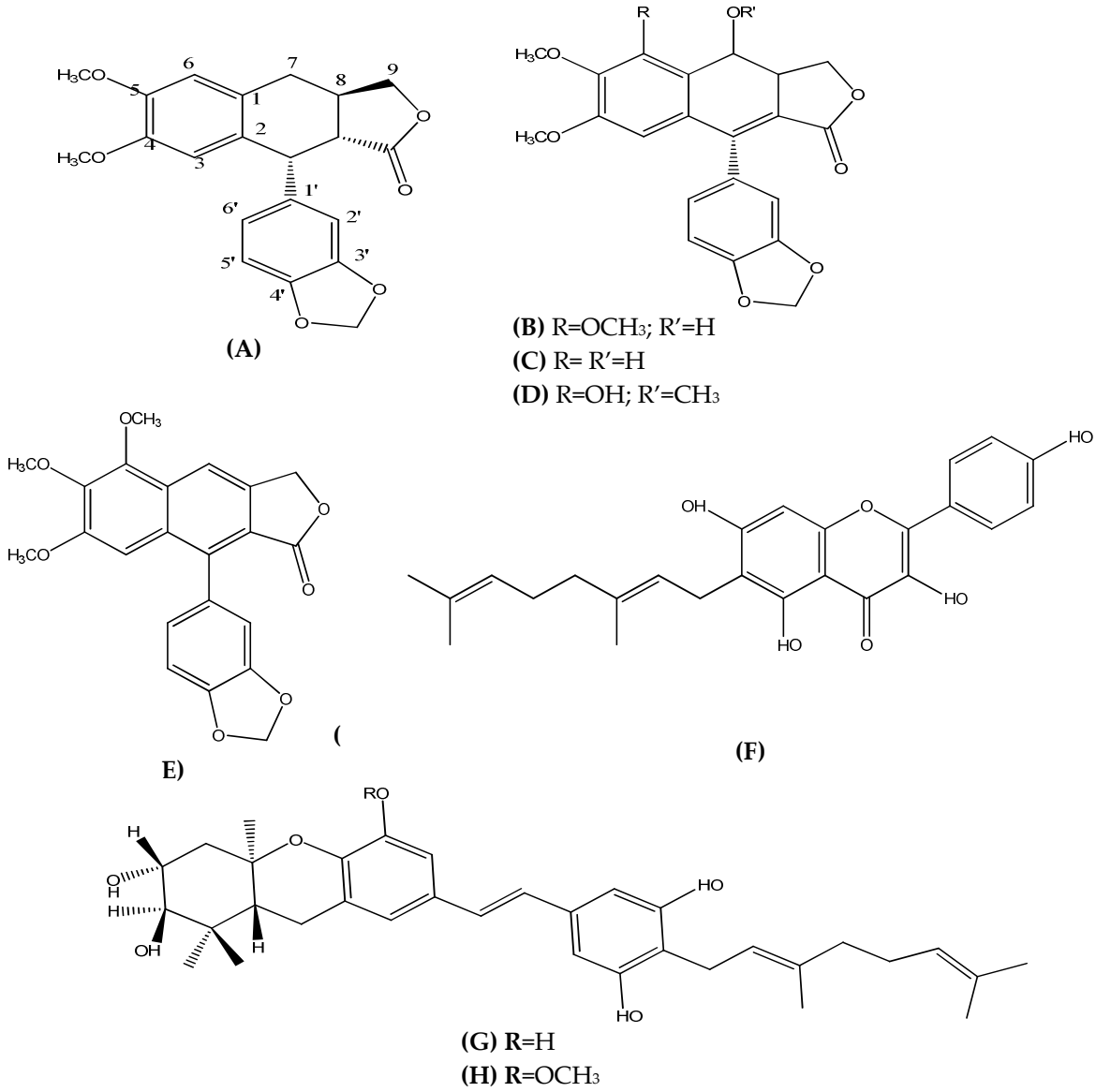


Figure 1-4 Chemical new components isolated from Kenyan propolis (A) tetrahydrojusticidin B, (B) 6-methoxydiphyllin, (C) diphyllin; (D) 6 hydroxyjusticidin A, (E) phyllamyricin C, (F) macarangin (G); geranylstilbenes, (H) schweinfurthin B.
Adapted from (Petrova et al., 2010).

Table 1-4 Summary review on some studies of propolis.

Propolis Sample	Region of collection	Chemical Analysis				References
		Extraction Methods		Results		
		Identification Techniques used	Material and solvent system	Chemical profile	Biological Activity profile	
Four propolis samples from Manaus	Brazilian Amazon	HPLC/DAD/ESI–MS/MS, GC/EIMS, NMR (^{1}H and ^{13}C) and ESI/MS/MS.	Ethanolic extracts	Polyprenylated benzophenones: 7- epi-nemorosone, 7-epi-clusianone xanthochymol and gambogenone Aristophenone, methyl insigninone, 18-ethyloxy-17-hydroxy-17, 18-dihydroscrobiculatone B, and derivatives of dimethyl weddellianone A and B, propolones, and a scrobiculatone derivative, were detected as minor constituents. Triterpenoids (β-amyrins, β-amyrenone, lupeol and lupenone.	Antimicrobial activity	(de Castro Ishida et al., 2011)
Six propolis samples from different regions of Turky	Turkey	TLC and GC-MS	Ethanolic extracts	phenolic and flavonoid content diterpenic acids and high percent of cinnamyl cinnamate, hydroxy fatty acids and triterpenic alcohols, phenolic glycerides	Antibacterial activity	(Popova et al., 2005)
Two locations with two different dominant floras (TypeI; Pine trees and Type II; Oak trees).	Jordanian	Column chromatograph, NMR and LCMS	Chloroform extract and twice with methanol	Three pure phenolic compounds (three flavonoids) namely, pinobanksin-3-O-acetate, pinocemberin and chrysin.	Antibacterial activity against MRSA	(Darwish et al., 2010)

Three propolis samples	Khanpur, U.S. Nagar, Uttarakhand		Aqueous and ethanol extracts of Indian propolis	High polyphenols content.		(Laskar et al., 2010)
43 propolis samples	From different parts of the world Africa, Europe Brazil, Solomon Islands, Pollen and Asia	HPLC and NMR (^{1}H and ^{13}C)	Ethanolic extract	Caffeic acid, Pinocembrin, Cinnamic acid, Naringenin, Chrysin and Galangin	DPPH free radical assay Antioxidant	(Watson et al., 2006)
40 propolis samples collected from countries varies from Tropical, subtropical, temperate	From Africa North and South America,/Asia Oceania, and from Mediterranean and Baltic Zones		Ethanolic Extracts		Antibacterial	(Seidel et al., 2008)
17 Propolis samples from apiaries at 17 different places	Malta and the island of Gozo in Mediterranean	GC–MS analysis Analytical TLC	Ethanol ethyl acetate pyridine, Silica gel Alufolien F254	Diterpene compounds. A total of 32 individual diterpenes was identified, 22 of them were present in each one of the 17 samples. Most abundant were the diterpene acids. Diterpenes and sugars were the most abundant groups of constituents. Aliphatic hydroxy acids, aromatic and fatty acids, triterpenes (mainly alcohols of the amyrin type) only one sample minor amounts of typical poplar flavonoids (chrysin, pinocembrin chalcone) pentenyl caffeates) were detected.	Antibacterial (*S. aureus*, *E. coli)* Antifungal (*C. albicans)*	(Popova et al., 2011)

3 different propolis samples from *A. mellifera* hives located in different zones	Portuguese Bragança Coimbra Beja	Hydro – alcoholic methanolic Aques				(Silva et al., 2012)
Red propolis sample	Macei ´ o, state of Alagoas, in northeastern Brazil	Chloroform, ethyl acetate and methanol extracts		Narigenin-8-*C*-hexoside, in propolis. The main constituent found was characterized as 3, 4, 2_, 3_-tetrahydroxychalcone. Other important constituents. were the chalcone isoliquiritigenin, the isoflavans (3*S*)-vestitol, (3*S*)-7-*O*-methylvestitol, the pterocarpan medicarpin, the phenylpropenes *trans*-anethol,methyl eugenol, elimicin, methoxyeugenol and *cis*-asarone, and the triterpenic alcohols lupeol and α- and β- amyrins.	Antimicrobial activity toward Gram-positive and Gram-negative bacteria.	(Righi et al., 2011)
Twenty propolis samples	Bologna, Italy		HPLC-MS/MS	pinobanksin p-coumaric acid ferulic acid caffeic acid, quercetin , naringenin , pinocembrin ,pinostrobin, kaempferol, galangin, chrysin and apigenin		(Papotti et al., 2012)
Propolis Samples	Iran Isfahan Isfahan province	(GC-MS) flash chromatography	light petroleum (ethyl acetate) EtOAc	Terpene esters of substituted benzoic acids. prenylated coumarine suberosin pinocembrin, pinobanksin acetate, pentenyl caffeates, caffeic acid phenethyl ester (CAPE Tschimgin (bornyl p-hydroxybenzoate), tschimganin (bornyl vanillate), ferutinin (ferutinolp-hydroxybenzoate), tefernin (ferutinol vanillate)	Antibacterial (*S. aureus*, *E. coli)*. Antifungal (*C. albicans)* *And* DPPH	(Trusheva et al., 2010)

Five propolis samples	Greek	GC-MS	94 components were identified from the oils	Junipene, α-pinene, manoyl oxide, *trans*-β-terpineol α-eudesmol, *n*-decanal, cedrol, *n*-nonanal, manool the predominance of terpenoids, especially of α-pinene in all samples	Antibacterial (*S.aureus, S. epidermis, P aeruginosa, Enterobacter loacae, Klebsiella pneumonia* and *Escherichia).* Antifungal (*C. albicans, C. tropicalis, C. glabrata)*	(Melliou et al., 2007)
Two samples of propolis	Tunisia and Spain	HPLC NMR		Chrysin, galangin, tectochrysin pinocembrin, pinobanksin, dimethylallyl caffeate, phenylethyl caffeate.pinobanksin, pinocembrin, pinobanksin 3-acetate, chrysin, galangin, galangin 3-methyl ether, pinocembrin 7-methyl ether, and tectochrysin		(Martos et al., 1997)
Twenty two propolis samples	African propolis	HPLC/UV-ELSD LC-MS/GC-MS	Ethanolic extracts	The diversity of the composition of these African propolis samples could be observed by heat mapping the LC–UV and ELSD data. The characteristic chemical components were uncovered by applying PCA Analysis to the LC–HRMS data and a preliminary dereplication was carried out by searching their accurate masses in the (DNP).		(Zhang et al., 2014)
12 climatically diverse regions across the US	compare the metabolite profiles among those samples using LC–MS based metabolomic methods	LC–MS-based metabolomic methods		Characterize the bee pathogens *P. larvae* and *Ascosphaeraapis*. Metabolomic analysis of regional propolis samples revealed that each sample was compositionally distinct, and LC–FTMS profiles from each sample contained a unique number of shared and exclusive peaks		(Wilson et al., 2015)

1.5 The Biological properties of propolis

1.5.1 Introduction

The antimicrobial activity of propolis until now is the most widely investigated property of propolis and hundreds of publications on this topic have appeared in the last 40 years (Bogdanov, 2012). These findings explain why propolis plays such an important role in bee hives since it can be considered as a chemical weapon against pathogenic microorganisms (Fokt et al., 2010, Bankova, 2005a). Different propolis types contain many chemical constituents responsible for their antimicrobial properties (Bankova, 2005a) and it seems that the sum of the propolis antimicrobial components, rather than individual substances, are responsible for the observed antimicrobial actions (Bogdanov, 2012, Kujumgiev et al., 1999). Propolis has: antibacterial (Silici and Kutluca, 2005, Kujumgiev et al., 1999, Grange and Davey, 1990, Sforcin et al., 2000), antifungal (Kartal et al., 2003, Kujumgiev et al., 1999, Ota et al., 2001), antiviral (Amoros et al., 1992b, Amoros et al., 1992a), antiprotozoal (Freitas et al., 2006, Dantas et al., 2006a, Dantas et al., 2006b), anti-tumour (Callejo et al., 2001, Komericki and Kränke, 2009, Banskota et al., 2000), anti-inflammatory (Khayyal et al., 1992, Dobrowolski et al., 1991, Fokt et al., 2010), local-anaesthetic (Marcucci, 1995), antioxidant (Russo et al., 2002, Fokt et al., 2010, Kumazawa et al., 2007), immunostimulating (Dimov et al., 1992, Oršolić et al., 2004), cytostatic (Christov et al., 1998, Banskota et al., 1998) and hepatoprotective (Banskota et al., 2001a, Won Seo et al., 2003) activities.

There are many components that are responsible for the biological activity of propolis and these vary with propolis sample type and the solvents

used in the extraction of the propolis (Ugur and Arslan, 2004). Flavonoids and esters of phenolic acids are generally regarded as bioactive compounds which are responsible for antimicrobial activity (Fokt et al., 2010).

However, there are many other components with activity and these are summarised in (Tables 1-5 and Table1-6) for different types of propolis and for two of the main types.

Table- 1-5 Biological effects of propolis components[a]

Propolis active ingredient and propolis type	**Biological activity**
Polyphenols and flavonoids Mostly poplar and all propolis types	Antibacterial, antiviral, antifungal
Caffeic acid phenethyl ester (CAPE) and other caffeates. Poplar and Bacharis	Antibacterial, antiviral, fungicidal
Caffeic acid (CA) Poplar and Baccharis	Antiviral
Terpenes Greece, Crete, Croatia, Brazil	Antibacterial, antifungal
Essential oils Brazil, Poland	Antibacterial
Furfuran lignans Canary islands	Antibacterial

N.B; [a]*; Adapted from* (Bogdanov, 2012).

Table 1-6 Biologically active ingredients in Poplar and Baccharis propolis[a]

Biological activity	Propolis type, active ingredient
Antibacterial	**Poplar**: different flavonones, flavones, phenolic acids and their esters **Bacharis**: prenylated *p*-coumaric acids, labdane diterpenes
Antifungal	**Poplar**: pinocembrin, galangin, benzoic acid, salicylic acid, vanillin **Baccharis**: mono and sesquiterpenes, Artipellin C
Antiviral	**Poplar**: Polyphenols, phenyl carboxylic acids, and esters of substituted cinnamic acids, caffeic acid, quercetin, luteolin, fisetin, and quertecagetin **Baccharis**: activity detected but no substances identified

N.B;[a]; *Adapted from* (Bogdanov, 2012).

1.5.2 Antibacterial activity of propolis

Antimicrobial activity is recognised as the most important property of propolis, particularly activity against bacteria. Several studies have been performed to evaluate this property against a large group of Gram-positive and Gram-negative bacteria; both aerobic and anaerobic types. The bacteria studied are summarised in (Table 1-7). These bacteria were either from laboratory collections or were isolated from clinical samples. The studies used propolis of different geographical origins and chemical composition, and employed different experimental approaches such as disc diffusion and disc dilution to investigate antibacterial activity. In the disc diffusion method, antibacterial activity is determined by measuring the diameter of the bacterial growth inhibition zone in the agar layer surrounding a disc containing propolis extracts (Kujumgiev et al., 1999). On the other hand, the dilution method is used to determine the minimal inhibitory concentration (MIC) and the minimal

bactericidal concentration (MBC) which are, respectively, the lowest concentrations that inhibit visible bacterial growth and the lowest concentration that kills bacteria (Grange and Davey, 1990, Stepanović et al., 2003). The vast majority of the antibacterial activity studies were carried out by using *in vitro* bioassays as mentioned above. Although the composition of propolis differs considerably depending on its botanical origin, all examined types of propolis have revealed a strong antibacterial activity (Kujumgiev et al., 1999, Bankova, 2005b, Bankova et al., 2007). Also the activity of propolis may depend of the type of bee collecting it since it was found that poplar propolis collected by *Apis mellifera caucasica* had a higher antibacterial activity than that collected by *Apis mellifera anatolica* and *Apis mellifera carnica* (Silici and Kutluca, 2005).

Tests for the antibacterial activity of propolis were carried out against a range of different pathogenic bacteria in several studies as summarised in (Table 1-7) (Banskota et al., 2001b, Ghisalberti, 1979, Grange and Davey, 1990). It has been reported that propolis is more active against Gram-positive pathogens but many Gram-negative bacteria are also inhibited (see Table 1-7) (Fokt et al., 2010, Wagh, 2013).

Table 1-7 Bacteria used in the determination of the antibacterial activity of propolis [a]

Type	**Gram-positive**	**Gram-negative**
Aerobic	*Bacillus spp.* • *B. cereus* • *B. subtilis* *Enterococcus spp.* • *E. faecalis* *Micrococcus luteus* *Nocardia asteroids* *Rhodococcus equi* *Staphylococcus spp.* • *S. aureus* • *S. auricularis* • *S. capitis* • *S. epidermidis* • *S. haemolyticus* • *S. hominis* • *S. mutans* • *S. warnerii* *Streptococcus spp.* • *S. cricetus* • *S. faecalis* • *S pneumioniae* • *S. pyogenes* • *S. β- haemolyticus* • *S. mutans* • *S. sobrinus* • *S. viridians*	*Aeromonas hydrophila* *Brucella abortus* *Corynebacterium spp.* • *C. pseudotuberculosis* *Escherichia coli* *Helicobacter pylori* *Klebsiella pneumoniae* *Salmonella sp* • *S. enteritidis,* • *S. typhi* • *S. typhimurium)* *Pseudomonas aeruginosa* *Proteus spp.* • *P. mirabilis* • *P. vulgaris* *Shigella dysenteriae*
Anaerobic	*Actinomyces naeslundii* *Lactobacillus acidophilus* *Peptostreptococcus micros*	*Actinobacillus actinomycetemcomitans* *Capnocytophaga gingivalis* *Porphyromonas spp.* • *P. anaerobius* • *P. gingivalis* *Fusobacterium nucleatum* *Prevotella spp.* • *P. intermedia* • *P. melaninogenica* • *P. oralis* *Veillonella parvula*

N.B;[a]; Adapted from (Fokt et al., 2010).

The data collected from a range of studies of the antibacterial properties of propolis support the fact that propolis is active mainly against Gram-positive bacteria and either displays much lower activity against the Gram-negative ones or is not active at all (Marcucci, 1995, Silici and Kutluca, 2005, Kujumgiev et al., 1999, Drago et al., 2007, Sforcin et al., 2000, Grange and Davey, 1990, Kartal et al., 2003, Dobrowolski et al., 1991, Fadaly and EEY, 2001). Kujumgiev et al. (1999), who evaluated propolis samples, can see such results in the study from different geographic regions (tropical and temperate zones) against *Staphylococcus aureus* and *Escherichia coli*. All the extracts exhibited significant antibacterial activity against *S. aureus* but none were active against *E.coli*. (Kujumgiev et al., 1999).

However, it was reported that ethanolic extracts from propolis (EEP) completely inhibited the growth of *S. aureus*, *Enterococcus spp*. and *Bacillus cereus,* and moderately inhibited the Gram-negative organisms *Pseudomonas aeruginosa* and *E. coli*. (Grange and Davey, 1990). The antibacterial activity of EEP from Brazilian propolis, collected during four seasons, was found to inhibit the growth of Gram-positive bacteria and higher concentrations of EEP were needed to inhibit Gram-negative bacterial growth but the extracts had no effect on *Klebsiella pneumoniae* (Sforcin et al., 2000).

More recent research has revealed antibacterial activity of propolis against *Micrococcus luteus, Salmonella typhimurium* (Uzel et al., 2005) and *K. pneumonae* (Victorino et al., 2007), and in earlier studies (Grange and Davey, 1990) it was stated that *Listeria monocytogenes* is not sensitive to propolis but more recent studies revealed significant activity against this organism (Ozcan et al., 2004, Yang et al., 2006). It was also found that propolis had a strong antibacterial activity against 13 different bacterial plant pathogens (Basim et al., 2006).

The antibacterial effect of propolis is bactericidal (Grange and Davey, 1990) and it was proposed that it works by inhibiting bacterial mobility. In addition, it has been shown that the antibacterial activity of poplar propolis is based on inhibition of quorum sensing (QSI), the flavonoid pinocembrin being an important QSI agent (Savka et al., 2015)

The flavonoids, galangin, pinocembrin and pinostrobin, have been most associated with the antibacterial properties of propolis shown in (Table1-6) (Dimov et al., 1992), but also it has been reported that propolis samples containing only traces of flavonoids demonstrate an antibacterial action (Tomás-Barberán et al., 1993). In addition, ferulic and caffeic acids, prenylated coumaric acid and benzophenone derivatives or diterpenic acids have also been reported as antibacterial compounds (Ghisalberti, 1979, Castaldo and Capasso, 2002, Kujumgiev et al., 1999, Popova et al., 2007, Mirzoeva et al., 1997, Burdock, 1998).

In recent years, there has been a considerable interest in using propolis in hospitals as an antibacterial agent due to the increase of antibiotic resistance (Bogdanov, 2012). It has been shown that the components in propolis act synergistically against bacteria

(Onlen et al., 2007, Orsi et al., 2006, Scazzocchio et al., 2006, Speciale et al., 2006, Stepanović et al., 2003). Several authors point out that the antimicrobial activity of propolis is related to its highly complex and variable constituents and their synergistic action (Bonvehí and Coll, 1994, Freitas et al., 2006, Scazzocchio et al., 2006, Mirzoeva et al., 1997, Takaisi-Kikuni and Schilcher, 1994, Burdock, 1998).

Compounds which were active against *Mycobacterium marinum*, the closest genetic relative to *Mycobacterium tuberculosis* were isolated from Saudi Arabian propolis. The strongest activity was for the flavonoid psiadiarabin which had activity only 5 times less than the gentamycin control (Almutairi et al., 2014a, Almutairi et al., 2014b). Twelve ethanolic extracts of propolis from different areas within Libya were tested against *M. marinum* in order to determine whether or not observed activity was associated with specific components in the samples. The extracts had moderate to strong activity against *M. marinum* (Siheri et al., 2016).

Commonly, the biological activity of a natural product medicine decreases with increasing storage time, but Meresta (1997) stated that ethanolic solutions of propolis stored for 10-15 years gave increased antibacterial activity (Meresta, 1997).

1.6 Antiprotozoal and Antihelminthic activity of propolis

Recently, attention has been focused on the antiparasitic activity of propolis since an improvement from the existing drugs against several tropical diseases caused by different protozoa is required. Numerous assessments have been performed using different *in vivo* and *in vitro* experiments to investigate the activity of raw propolis and active compounds isolated from propolis. Accordingly, the literature has reported significant effects against different parasitic species including: *Cholomonas paramecium*, *Eimeria magna, Media perforans*, *Giardia lambia, Giardia duodenalis, Trichomonas vaginalis, Trypanosoma cruzi* and *Trypanosoma evansi* (Freitas et al., 2006, Falcão et al., 2013a, Bogdanov, 2012, Parreira et al., 2010). Several research studies have been performed that show the activity of propolis and its components against a range of protozoan

parasites which cause various human diseases including *Trypanosoma brucei* which causes sleeping sickness and *Trypanosoma cruzi* which causes Chagas disease (Higashi and De Castro, 1994, De Castro and Higashi, 1995, Marcucci et al., 2001, Dantas et al., 2006a, Salomão et al., 2010, Falcão et al., 2013a, Almutairi et al., 2014b, Siheri et al., 2014, Omar et al., 2015, Siheri et al., 2016). Antiprotozoal effects of different propolis samples was reported against *Leishmania donovani*, which causes visceral leishmaniasis, and for other strains of leishmania (Siheri et al., 2016, Da Silva et al., 2013, Duran et al., 2008, Pontin et al., 2008, Ozbilge et al., 2010, Monzote et al., 2011, Amarante et al., 2012). Recent studies have observed antiprotozoal effects of propolis extracts against *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium vivax* and *Plasmodium ovale*, all of which cause malaria (Olayemi, 2014, Siheri et al., 2016). Propolis is also effective against *Entamoeba histolytica* and *Giardia lamblia*, which cause intestinal infections (dysentery and diarrhoea), and also against the multicellular organisms including the intestinal worms including helminths such as *Schistosoma spp*., *cestodes* such as tapeworms, nematodes such as roundworms, and trematodes such as flukes (Freitas et al., 2006, Issa, 2007, Hegazi et al., 2007, Abdel-Fattah and Nada, 2007, Noweer and Dawood, 2008, Alday-Provencio et al., 2015, Hassan et al., 2016). Some of the studies are described in more detail below.

Extracts of Portuguese propolis and of its potential sources such as poplar buds were screened against different protozoa including: *Plasmodium falciparum; Leishmania infantum; Trypanosoma brucei* and *Trypanosoma cruzi* (Falcão et al., 2013a). The toxicity of the extracts against MRC-5 fibroblast cells was also evaluated to assess toxic selectivity. The propolis extracts had moderate

activity against these parasites, with the highest inhibitory effect being observed against *Trypanosoma brucei* (Falcão et al., 2013a).

Recently, extracts from twelve samples of propolis collected from different regions of Libya were tested for their activity against *Trypanosoma brucei*, *Leishmania donovani*, *Plasmodium falciparum*, and *Crithidia fasciculate,* while the cytotoxicity of the extracts was also tested against mammalian cells. All the extracts were active to some degree against all of the protozoa, exhibiting a range of EC_{50} values between 1.65 and 53.6 μg/ml (Siheri et al., 2016), while only exhibiting moderate to negligible cytotoxicity.

The activity of propolis against Chagas disease (caused by *Trypanosoma cruzi*) was assessed in comparison with crystal violet, a standard drug recommended to prevent the transmission of Chagas disease via blood (De Castro and Higashi, 1995). The relationship between trypanocidal activity and the chemical composition of propolis has been widely investigated by several authors and these studies confirmed that Brazilian green propolis had high activity against *T. cruzi* transmission (Dantas et al., 2006a, De Castro and Higashi, 1995, Higashi and De Castro, 1994).

The activity of ethanol extracts from a Brazilian (Et-Bra) and a Bulgarian (Et-Blg) propolis against *T. cruzi* were tested and it was found that, although there were differences in the chemical composition between both extracts, they were both active against *T. cruzi*. The study also confirmed that in European samples biological activity was associated with the presence of flavonoids and aromatic acids and their esters. In Brazilian propolis, amyrins occur as components that might contribute to the anti-trypanosomal activity (Higashi and De Castro, 1994, Saloma˜o et al., 2004).

The activity of acetone and ethanol extracts of two Bulgarian propolis samples (Bur and Lov) against *T. cruzi* was evaluated. Both extracts showed similar chemical composition with a high content of flavonoids and strong inhibitory activity against *T. cruzi* proliferative epimastigotes which were more susceptible than trypomastigotes. While in the presence of blood, the activity of Et-Bur or Et-Lov against trypomastigotes was similar to that of the standard drug, crystal violet (Prytzyk et al., 2003). It was also found that two different samples from Bulgarian propolis had significant activity against *T. cruzi in vitro* (Saloma˜o et al., 2004, Salomão et al., 2009, Dantas et al., 2006a).

The current therapy of *T. evansi* infections is not effective for the vast majority of animals with relapsing parasitemia and clinical signs. The susceptibility of *T. evansi* to a propolis extract *in vitro* and *in vivo* was evaluated. A dose-dependent trypanocidal activity of the propolis extract was observed *in vitro*. All trypomastigotes were killed within 1 h after incubation with 10μg/ml of the extract. However, *in vivo* assessment of the concentrations of 100, 200, 300 and 400 mg kg^{-1} administered orally for 10 consecutive days presented no curative effect, and the rats died from the disease. However, rats treated with the two highest concentrations of propolis extract showed higher longevity than the other groups. Based on these data the study concluded that despite the lack of curative efficacy observed *in vivo* at the concentrations tested, the propolis extract can prolong life in rats infected with the protozoan (Gressler et al., 2012).

A comprehensive chemical profiling study was carried out on 22 African propolis samples collected from the sub-Saharan region. Results revealed that triterpenoids were the major chemical components in more than half of the propolis samples analysed in this study and some others were classified as

temperate and Eastern Mediterranean type of propolis. Based on the comparative chemical profiling, one propolis sample from southern Nigeria stood out from others by having prenylated isoflavonoids which indicated that it was more like Brazilian red propolis (Zhang et al., 2014). This propolis was further investigated and ten phenolic compounds were isolated including a new dihydrobenzofuran. All the isolated compounds were tested against *T. brucei and* displayed moderate to high activity. Some of the compounds tested had similar activity against wild type *T. brucei* and two strains displaying pentamidine resistance. The Nigerian propolis from Rivers State had some similarities with Brazilian red propolis and exhibited antitrypanosomal activity at a potentially useful level (Omar et al., 2015).

The chemical profile and antitypanosomal activity of Ghanian propolis against *T. brucei* was also investigated. Two compounds were isolated, a prenylated tetrahydroxy stilbene and a geranylated tetrahydroxy stilbene. These compounds exhibited moderate activity against *T. brucei*. In the same paper, isolation of a new phloroglucinone analogue from Cameroon propolis was reported. The compound was found to possess a high potency which was comparable to that of suramin (Almutairi et al., 2014b).

The EEP of Libyan propolis was tested activity against *T. brucei*. One of the samples was fractionated and yielded a number of active fractions. Three of the active fractions contained single compounds which were found to be 13-epitorulosal, acetyl-13-epi-cupressic acid and 13-epi-cupressic acid which had been identified previously in Mediterranean propolis. Two of the compounds had a MIC value of 1.56 μg/mL against *T. brucei* (Siheri et al., 2014).

The chemical composition and biological activity of a propolis sample collected from Saudi Arabia were investigated. A new diterpene, propsiadin, was isolated along with two flavonoids and a known diterpene, psiadin. The compounds had MICs in the range 30.9-78.1 μM against *T. brucei*. The propolis was thought to originate from *Psiadia arabica* and *Psiadia punctulata* and it represented a new type of propolis (Almutairi et al., 2014a).

According to WHO (2004), Leishmaniasis has been reported as an endemic disease in 88 countries in tropical and sub-tropical regions across the world, affecting more than 12 million people. There are no vaccines available for any form of the disease and the chemotherapy of this disease is still inadequate and expensive (Kayser et al., 2003, Croft et al., 2006). An intense search for potential natural products isolated from plants or propolis for the treatment of Leishmaniasis has been carried out during the last decades. In previous literature, there are several reports on the activity of a variety of crude natural extracts, especially from plants collected in tropical zones, against Leishmania (Croft et al., 2006).

Previous studies have reported that propolis samples from various origins possess activity as anti-leishmanial agents due to the presence of flavonoids and amyrins (Machado et al., 2007).

A study of propolis from Turkey investigated the effects of propolis against *Leishmania tropica* and it was observed with microscopic examination that propolis inhibited parasite growth at ≥ 32 μg/ml concentration. Also it was found that the antileishmanial effects of propolis increased with increasing concentrations and incubation periods (Ozbilge et al., 2010).

The activity of *Baccharis dracunculifolia*, which is the most important plant source of the Brazilian green propolis, against promastigote forms of *L. donovani*, was investigated and IC_{50} values of 42μg/ml were obtained. The extract also displayed high activity in a schistosomicidal assay (Parreira et al., 2010).

The activity of eighteen Cuban propolis extracts collected in different geographic areas were screened against *Leishmania amazonensis* and *Trichomonas vaginalis*. The study observed that all propolis extracts produced an inhibitory effect on intracellular amastigotes of *L. amazonensis*. Only five samples decreased the viability of *T. vaginalis* trophozoites at concentrations lower than 10 μg/ml (Monzote et al., 2011). Brazilian green propolis was tested against *L. braziliensis* by experimental infection of mice. The results showed an IC_{50} value of 18.1 μg/ml against promastigote forms of *L. brasiliensis*. IC_{50} values were in the range 78–148 μg/ml against the M2904 strain of *L. brasiliensis* and the extract also had antiproliferative activity on *L. brazilensis* promastigotes at 100 μg/ml (Da Silva et al., 2013).

The EEP of Libyan propolis collected from North East Libya was found to be active against *L. donovani* and four compounds, three diterpenes and a lignan were isolated. These compounds exhibited moderate to strong activity against *L .donovani,* the compounds having IC_{50} values in the range 5.1–21.9 μg/ml (Siheri et al., 2014). These results were replicated in subsequent assays on *L. donovani* involving twelve extracts of Libyan propolis where IC_{50} values ranged from 2.67 μg/ml to 16.2 μg/ml (Siheri et al., 2016).

The activity of methanolic extracts of ten Bolivian propolis samples was studied against *L. amazonensis* and *L. braziliensis*. The most active samples

towards *Leishmania* species had IC_{50} values in the range 78-121 μg/ml against *L. amazonensis* and *L brasiliensis* (Nina et al., 2016).

It was reported that an ethanolic extract of European propolis had activity against *Toxoplasma gonodi* (De Castro, 2001).

The activity of Nigerian propolis was tested against *Plasmodium berghei* using mice experimentally infected with *P. berghei*, with chloroquine as a positive control. The propolis significantly reduced the level of parasitemia in treated mice, and there was no significant difference from mice treated with chloroquine (Olayemi, 2014).

Propolis extract inhibited the growth of the intestinal parasites *Giardia lamblia*, *Giardia intestinalis* and *Giardia duodenalis*. The extract decreased the growth of trophozoites and the level of inhibition varied according to the extract concentration and incubation times. Significant decreases of parasite growth were detected in cultures exposed to 125, 250 and 500 μg/ml of propolis respectively, in all incubation periods (24, 48, 72 and 96 h). Growth reduction by 50% was observed in cultures treated with 125 μg/ml of the extract, and concentrations of 250 and 500 μg/ml were able to inhibit growth by more than 60% (Freitas et al., 2006).

Mice were orally infected with axenically cultivated *Giardia lamblia* trophozoites. The trophozoite count in the intestines, measurements of interferon-gamma serum levels, and histopathological examination of duodenal and jejunal sections were carried. The results showed that propolis as a prophylaxis produced a significant decrease in the intensity of infection. While as a treatment, propolis provided a more significant decrease in trophozoite count than that obtained by metronidazole. However, mice treated

with propolis alone showed a reversed $CD4^+$: $CD8^+$ T-lymphocyte ratio resulting in a strong immune enhancing effect which resulted in an adverse increase in inflammatory response at the intestinal level. The combined therapy of metronidazole and propolis showed a stronger efficacy in reducing the parasite count than that gained by each drug alone (Abdel-Fattah and Nada, 2007).

Propolis was used as a foliar application or soil drench on fava bean plants. Propolis treatment increased total chlorophyll and carotenoids and the magnitude of increase was more noticeable after applying a higher concentration (1000 mg/l). It was found that fava bean plants treated with propolis extract, either as a foliar application or soil drench, were able to overcome the inhibitory influence of nematode infection on chlorophyll formation (Noweer and Dawood, 2008).

A study was carried out in BALB/c mice to investigate the synergistic effect of the EEP of Egyptian propolis and immunization with *Taenia saginata* crude antigen in the prevention of bovine cysticercosis. After 24 weeks of challenge the mice in G2 (given both EEP and immunisation) showed the highest level of protection (100%) with no cyst being detected rather than mice in G1 (which received only immunisation). The latter got just 33.3% protection. Additionally, the ELISA results in this study showed higher antibody titres in G2, with reduction in the alteration of liver and kidney functions, compared to mice in G1 (Kandil et al., 2015).

There are several papers on the antihelmintic effects of the propolis extracts. Propolis inhibited the growth of the helminth parasite *Fasciola gigantica* (Hegazi et al., 2007). In tests against schistosomiasis in mice, a

significant reduction in the number of schistosomules of 59.2% was obtained in the group treated with propolis compared to a reduction of 98.9% in the praziquantel treated group (Issa, 2007). A study was carried out to evaluate the effect of Egyptian propolis against *Toxocara vitulorum*. Adult worms were incubated for 24 h in several concentrations of EEP (100, 50, 25, 12 and 6 μg/ml) and assessed by light and scanning electron microscopy following 24 h incubation. It was observed that the extract possessed anthelmintic efficacy and the mortality rate was concentration dependent: 6.9 μg/ml was the LC_{25}, 12.5 μg/ml was LC_{50}, and LC_{90} was 53.4 μg/ml. The authors thus confirmed the nematodicidal effect of Egyptian propolis (Hassan et al., 2016).

1.7 Anti-inflammatory activity of propolis

Inflammation is a complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, irritants, and free radicals. Anti-inflammatory activity means the primary effect of the host defence system.

De Almeida and Menezes, (2002), has been reviewed the anti-inflammatory activity of propolis and it has been reported that propolis has inhibitory effects on myeloperoxidase activity, NADPH-oxidase ornithine decarboxylase, tyrosine-protein kinase and hyaluronidase from guinea pig mast cells (De Almeida and Menezes, 2002). This anti-inflammatory activity can be explained by the presence of flavonoids and cinnamic acid derivatives. The former includes acacetin, quercetin, and naringenin; the latter includes caffeic acid phenyl ester (CAPE) and caffeic acid (CA) (Almutairi et al., 2014b), CAPE and galangin, both being typical poplar propolis constituents, exhibited anti-inflammatory activity and significantly inhibited carrageenan oedema,

carrageenan pleurisy, and adjuvant arthritis inflammations in rats (Franzblau et al., 1998, Fidock et al., 1998). An ethanol extract of propolis suppressed prostaglandin and leukotriene generation by mouse peritoneal macrophages *in vitro* and during zymosan-induced acute peritoneal inflammation *in vivo*. Dietary propolis significantly suppressed the lipoxygenase pathway of arachidonic acid metabolism during inflammation *in vivo.* CAPE was a more potent modulator of arachidonic acid metabolism than caffeic acid, quercetin, and naringenin (Laine et al., 2015, Wagh, 2013).

1.8 Other pharmacological activities and therapeutic uses of propolis

Several studies have reported that propolis exhibits a range of pharmacological activities such as; antidiabetic (Alcolea et al., 2014, Bacchi et al., 1974), antioxidant (Cowan, 1999, Oliver et al., 1998), immunomodulatory (Vincent et al., 2012), antitumour (Fokt et al., 2010, Wagh, 2013, Kumar et al., 2008), antioxidant activities (Mamas et al., 2011, Goodwin et al., 2000) and has been clinically use against the vaginal yeast *Candida albicans* (Mamas et al., 2011, Fukusaki and Kobayashi, 2005).

Also it has been reported that propolis has an action on oral cavity (Harrigan and Goodacre, 2012, Bates et al., 2012)

Zhao, *et al.* (2009), have stated that propolis exhibits a hepatoprotective effect in animal model studies and concluded that propolis has potential as a hepatoprotective agent (Zhao et al., 2009). Another group of authors highlighted the wound healing properties of the propolis. The anti-

inflammatory activity of ethanolic extract of green Brazilian propolis was evaluated by determination of wound healing parameters (Moura et al., 2011).

Also it has been reported that propolis provides relief of the different symptoms of allergy, rhinitis, and asthma. Shinmei, *et al*. (2009), studied the effect of Brazilian propolis on sneezing and nasal rubbing in experimental allergic rhinitis of mice, concluding that propolis may be effective in the relief of symptoms of allergic rhinitis through inhibition of histamine release (Shinmei et al., 2009).

Khayyal, *et al*. (2003), studied the effect of the administration of an aqueous extract of propolis 13% daily for 2 months to asthmatic patients with mild to moderate symptoms (Khayyal et al., 2003). Propolis-treated patients showed a reduced incidence and severity of nocturnal attacks which was associated with decreased prostaglandins, leukotriene and proinflammtory cytokines (TNF-α, IL-6, and IL-8) and increased IL-10 (Mamas et al., 2011, Alsaadi et al., 2012, Wagh, 2013).

Table 1-8 summarises the chemical constituents that responsible for the biological properties of propolis.

Table 1-8 Summary of the compounds responsible for the biological activities of propolis [a].

Propolis type	Antibacterial activity	Anti-inflammatory activity	Antitumour activity	Hepatoprotective activity	Antioxidant activity	Anti-allergic action
European poplar type	Flavonoids, flavones, phenolic acid And their esters	Flavanones, flavones, Phenolic acids and their ester	Caffeic acid Phenethylester	Caffeic acid, ferulic acid, caffeic acid Phenethylester	Flavonoids, phenolic and their ester	3,3 Dimethylayl caffeate
Brazilian (Baccharis type)	Prenylated p-coumaric acid labdanediterpenes	Unidentified	Prenylated p-coumaric acid, Clerodane Diterpens, benzofuranes.	Prenylated p-coumaric acid, flavonod Lignans, caffeoyl quinic acid	Prenylated p-coumaric acid, flavonoids	No tested
Cuban	Prenylated benzophenones	No tested	Prenylated benzophenoe	Unidentified	Prenylated benzophenones	not tested
Taiwanese	Not tested	Not tested	Prenylated flavanones	Not tested	Flavanones	not tested

N.B; [a]; *Adapted from* (Bankova, 2005b)

1.9 Libyan propolis

Based on the information in the literature Libya covers an area of over 1,759,540 km^2 and the Libyan Desert, which constitutes approximately 90% of Libya, is one of the most arid places on earth. Oases can be found scattered throughout Libya, the most important of which are Ghadames and El-Kufra. The northern regions enjoy a milder Mediterranean climate. Most of the commercial beekeepers are located in an agricultural belt that extends to about 30 km from the coast (Shaibi et al., 2009a).

Libya is bordered by the Mediterranean Sea, Egypt, Sudan, Chad, Niger, and Tunisia. The country position is between 18°33°N and 9°25°E and consisting mainly of desert and the Mediterranean coast. Libya coastline (1,770 km) is one the longest of any African country bordering the Mediterranean. There are 2103 species of plant in Libya belonging to 856 genera and 155 families. The floristic composition in Libyan reflects a plant's strategy to resist extreme weather (Yang et al., 2013, Keshlaf, 2014).

From the extensive literature on propolis it is clear that propolis differs from area to area according to the geographical region and the type of vegetation present can affect propolis composition from different areas. Because there is no previous work on Libyan propolis and the different areas of Libyan may show significant differences in Libyan propolis composition the study in this thesis carried out a comprehensive study of Libyan propolis.

There was a previous study, which investigated the honeybee populations of *A. mellifera* in Saharan and coastal locations in Libya to fill out a North African gap in biogeography and the distribution of honeybees. The study used morphology and mtDNA analysis. It was found that Libyan honeybees are different, morphologically and genetically, from adjacent subspecies; and

majority of Libyan bees (92%) belong to an oriental evolutionary lineage (O). In addition, there was local impact of imported European honeybees. Further studies named the Libyan bees as a separate subspecies (Shaibi et al., 2009a, Shaibi et al., 2009b).

However, Libyan propolis did not become a subject of research by biologists and chemists although there are many wild-bee hives and Libya is famous for its good quality honey used in treating many ailments. The actual ingredients in individual propolis products may differ significantly, according to a number of variables including the type of bees that produced the propolis, time of the year and the geographic location of the hives (Scott Schneider et al., 2004).

The main honey plants in Libya including; *Eucalyptus spp*., *Acacia spp*., *Citrus spp*., *Pinus spp*., *Cupressus spp*., ***T**hymus **v**ulgaris*, ***L**antana **c**amara*, ***H**isbiscus **r**osa-**s**inensis*, ***M**edicago **s**ativa* and many wild plants as displayed in (Table 1-9).

However, Eucalyptus honey, from *Eucalyptus spp*., is one of the main honeys produced and consumed in Libya especially in the north where there are extensive areas of trees, which flower in November and December. Because of the consecutive blooming of the different *Eucalyptus* species, it is regarded as the most important source of nectar and pollen to colonies in drought periods (Keshlaf, 2014, Shaibi et al., 2009a). In the western region of Libya, there are three main honey flows, the heaviest from spring flowering plants in late March and April. Many beekeepers move their colonies to hilly country located east of Tripoli for the second flow from wild flowers of (Sider), ***Z**izaphus **S**pina,* from May to June, then for the third flow from thyme, *T. vulgaris*, in June to July. In desert areas, tamarisk, ***T**amarix **n**ilotica*, of provides an exceptional honey flow in the eastern region there are other bee plants such as schamiry, ***A**rbtus **p**avarii*, carob and ***C**eratonia **s**iliquea* (Abd El-Rahman, 2010).

Table 1-9 Main bee plants in Libya and its flowering period [a]

Common name of plants	Scientific name of plants	Flowering season
Orange tree	*Citrus spp.*	March – April
African rue (harmal)	*Peganum harmala*	April – May
Sedr Zizaphus S	*Pina Christi*	May – June
Thyme (Za'atar)	*Thymus capitatus L.*	June – July
Tamarix	*Tamarix Africana*	July –August
Carob tree	*Ceratonia siliqua*	August-October
Schamiry	*Arbtus pavarii*	December – January

N.B; [a]*; Adopted from* (Keshlaf, 2014).

There are few studies focus on the effect of seasonal collection time of propolis and corresponding findings on the chemical composition and biological activities of propolis. There was research study which stated that seasonality does not significantly change the chemical composition of propolis qualitative chromatographic profile, but it can influence the quantitative chemical profile of propolis (Valencia et al., 2012). A study was carried out on Sonoran propolis ad stated that that season had no significant effects on the relative abundance of the main chemical constituents and the biological activity (Sforcin et al., 2000).

1.9.1 Research studies review of Libyan propolis.

Until the date of this project there was no chemical profiling or profiling of the biological activity of ethanolic extracts of Libyan propolis, apart from the study by Abd El-Rahman, (2010). The study examined ethanolic extracts of crude

propolis from west Libya and examined the effects of the topical application of propolis extract, rosemary extract and a mixture of both extracts together on 12-O-tetradecanoylphorbol 13-acetate (TPA) –induced tumour promotion in mice previously initiated with 7,12 dimethylbenz[a]anthracene. The propolis extract, rosemary extract and a mixture of both extracts together were applied topically 15 min prior to the application of 5 nmol TPA for 20 weeks. A decrease in the number of skin tumours per mouse by 27, 39 and 71% was seen for the respective treatments, as well as a decrease in the number of mice with tumours by 22, 31, and 75%. The treatment inhibited the tumour size per mouse by 46, 62 and 72% for propolis, rosemary and both together respectively. It is likely that the combination of their activities such as antioxidant and cytotoxic ones as well as the combination of several components in both are responsible for their inhibitory effect on carcinogenesis. Therefore, further investigations were recommended in order to establish the conditions under which topical application of propolis had either protective or deleterious effects (Abd El-Rahman, 2010).

Sarkez *et al* (2014), investigated the antimicrobial properties of an ethanolic extract of Libyan propolis from Zawia in West Libya against *S. aureus* under different incubation temperatures (6, 20 and 37 °C), salt concentrations (5 and 10%), and pH values (3.5, 6.3). Several fractions were obtained from the partial purification of propolis: crude ethanolic extract, resinous material ethanolic solution, and alkaline hydrolysis of water soluble compounds solution. Tests conducted included measurement of inhibition zone by the disk diffusion method, minimal inhibitory concentration by the tube dilution method, and minimal bactericidal concentration by agar plating. Only the crude ethanolic extract of propolis exhibited effective inhibition zone at different seed concentrations: a diameter of 19.62 mm at 30 mg/150 μl and 19.73 mm at 40 mg/

200 µl. There was no antibacterial effect of the extract at 10 mg/50 µl concentration. The minimal inhibitory concentration and minimal bactericidal concentration were found to be 26.04 mg/ml, and 34.72 mg/ml, respectively. Higher temperature (37^{o}C) at pH of 3.5 enhanced the antimicrobial activity of ethanolic extract of propolis, while high salt concentration, 10%, showed no added antibacterial effect. Possible use of propolis extract as food or pharmaceutical preservative, or topical treatment for skin diseases caused by *S. aureus* is encouraging is promising (Sarkez, 2014).

A study by Azab, *et al*, (2015a), investigated an aqueous extract of the Libyan propolis from West (Surman) with regard to it hypolipidemic and antiatherogenic effects in lead acetate intoxicated male albino mice. The study was carried out on thirty-two adult male albino mice which were divided into four groups as follows. The 1st group was control group, the 2nd was the propolis group which orally received propolis (200 mg/kg body wt), the 3rd was the experimental and received lead acetate (500 mg /kg diet), and the 4th one co-administered lead acetate (500 mg/kg diet) with propolis (200 mg/kg body wt) daily for 30 days. Blood samples were obtained for assessment of serum cholesterol, triglycerides, HDL, LDL, parameters. It was found that the aqueous extract of Libyan propolis showed hypolipidemic and antiatherogenic effects in lead acetate intoxicated male albino mice (Azab et al., 2015a).

A study by Asab *et al* (2015b) was carried out to evaluate the effectiveness of aqueous extract of Libyan propolis (Surman) West Libya as a natural source of antioxidants to decrease the harmful effects of sodium nitrite induced haematotoxicity and hyperlipidemia in Guinea pig. In this study, twenty-four adult male Guinea pigs were used and divided into four groups. The 1st group

was a control group, the 2nd was the propolis group which orally received propolis (200 mg/kg body wt), the 3rd was the experimental and received sodium nitrite orally at a dose of 80 mg/kg body weight, the 4th one co-administered sodium nitrite orally at a dose of 80 mg/kg body weight with propolis (200 mg/kg body wt) daily for 35 days. Blood samples were obtained for the assessment of haematological parameters and serum lipid profile. In sodium nitrite treated animals, there were severe haematological changes and dyslipidemia. Haematologically, Guinea pigs that received sodium nitrite orally at a dose of 80 mg/kg body weight daily for 35 days had significantly ($p<0.05$) lower red blood cell count, hemoglobin content, haematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell count, and platelets count than those in the control animals, because of sodium nitrite adverse effects,there fore Propolis supplement showed a remarkable decrese of these side effects on sodium nitrite treated male Guinea pigs (Azab et al., 2015b).

Azab *et al*., (2015b). A brief of summary of the research work on Libyan propolis has been is shown in (Table 1-10) (Azab et al., 2015b)

Table 1-10 Summary review of previous research on Libya propolis

Propolis Samples Origin and Number	Propolis Extracts	Chemical Analysis	Biological Activity Finding	References
Surman city, West Libya one sample	Ethanolic extract	TLC chemical investigation/CAPE	Cytotoxicity and Antioxidant activity	(Abd El-Rahman, 2010)
Zawia city, West-Libya One sample propolis	Ethanolic extract	Partial purification	Propolis has effective inhibitory effect against *S. aureus*.	(Sarkez, 2014)
Alaquria and Tokra, North East Libya Two samples of propolis	Ethanolic extracts and purified compounds	LC-MS-GC-MS HPLC-UVELSD NMR Diterpenes, lignin compounds	High activity against *T. brucei*., *L. donovani*	(Siheri et al., 2014)
Surman city, West Libya One sample of propolis	Aqueous extract	No chemical analysis	*In vivo* hypolipidemic and antiatherogenic effects in lead acetate intoxicated male albino	(Azab et al., 2015a)
Surman city, West Libya propolis sample	Aqueous extract	No chemical analysis	Hepatoprotective and Hypolipidemic Effects of Aqueous Against Sodium Nitrite Induced Haematotoxicity and Hyperlipidemia in Guinea Pigs	(Azab et al., 2015b)
Different Geographic areas West, East, south east and south west of Libya 12 propolis samples	Ethanolic extract	LC-MS, PCA, combined analysis with HCA and divide samples into five groups. The outlying groups had different sets of dominant	Activity against *T. brucei*, *L. donovani*, *P. falciparum*, *C. fasciculata* and *M. marinum* and the cytotoxicity of the extracts was tested against mammalian cell	(Siheri et al., 2016)

1.10 Aims and objectives

1. Biological properties and chemical profile of Libyan propolis have not been investigated before. This research work aims to shed light on the chemical and biological profile of a twelve propolis samples collected from different locations in Libya.
2. Chemical profiling will be carried out by using HPLC, LC-MS, HPLC-ELSD, GC-MS and NMR.
3. Principal component analysis (PCA) will be used to classify propolis samples according to their chemical profile. Orthogonal partial least squares (OPLS) analysis will be used to link the activity of each extract against the different microorganisms to particular components in the extracts.
4. Biological profiling will be carried out *in vitro* to assess antiparasitic activity against *T. brucei*, *L. donovani*, *P. falciparum*, *C. fasciculate* and *M. marinum*, *S. aureus* and *E. coli*. Anti-helminthic activity will be tested against *Trichinella spiralis* and *Caenorhabditis elegans*. Cytotoxicity of the crude extracts will be tested against mammalian cell line (U937 cells).

Active extracts will be fractionated by open column chromatography and MPLC. Then profiled using liquid chromatography with UV and evaporative light scattering detection (ELSD), LC-MS and GC-MS and NMR. Pure components will be identified by NMR. The biological activity of purified components will be tested against a range of bacteria and protozoa.

2 Chapter 2

Materials and Methods

2.1 MATERIALS AND METHODS

2.2 Materials and equipments

0.22μm filter (Millipore, UK)

4ml glass vials 45×14.75 mm (Kinesis Ltd, UK)

5 ml glass vials (Kinesis Ltd, UK)

Absolute ethanol (Fisher Scientific, UK)

ACE C_{18} column (3 mm x 150mm, 3μm) (Hichrom, UK).

Automatic pipettes (Gilson, Anachem, UK)

Balance weight (Adventure, UK)

Blue tip pipette (Star Lab, UK)

Chloroform-d (Sigma Aldrich, UK)

Columns glass prepared by the glassblower at Glasgow University

Conical flasks (VWR International Lutterworth, UK)

Dimethylsulfoxide (DMSO HPLC Grade, Sigma Aldrich, UK)

E. coli (American Type Culture Collection (ATCC 8739) (Fisher Scientific, UK)

Ethanol (Sigma -Aldrich, UK)

Ethyl acetate (Analytical grade) (Fisher Scientific, UK)

Ethyl acetate (Sigma Aldrich, UK)

Formic acid (90%) BDH-Merck (Leicestershire UK)

GC-MS vails (Thermo,Germany)

Hexane (HPLC grade) (Fisher Scientific, UK)

HPLC vials (Kinesis Ltd,UK)

LC-MS vails (Thermo, Germany)

Mycobacterium marinum a (ATCC BAA535)(Fisher Scientific, UK)

Methanol (HPLC grade) (Fisher Scientific, UK)

Nylon filter membrane disc (Nylasorb™, USA)

Pasteur pipette (VWR International Lutterworth, UK)

Rotary evaporator (Rotavapor RII) (Büchi, Switzerland)

S. aureus (ATCC 29213) (Fisher Scientific, UK)

Sample Concentrator (TECHNE)®

Silica gel (coarse for dry loading) (Sigma -Aldrich, UK)

Silica gel 60, 0.04 -0.06mm mesh size (Sigma -Aldrich, UK)

Silica-Amorphous, precipitated (Sigma -Aldrich, UK)

Sonicator (Ultrasonic bath) (Lanson 2510, USA)

Syringe filters (Acrodisc, Fisher Scientific,UK)

T. brucei (ATCC (s427) blood stream form)(Fisher Scientific, UK)

Thin walled NMR sample tubes (VWR® International, USA)

Water (HPLC grade) (produced in house by Milli Q system, Millipore, UK)

Wilmad® NMR tubes, 5mm, 300MHz, 7inL, 507-PP (Sigma-Aldrich, USA)

2.3 Collection of propolis samples

Twelve raw propolis samples (Figure 2-2) were collected from different geographical localities in Libya; (see map in Figure 2-1); Tukra (Al`Aquriyah) a small village located about 70km East of Benghazi city Libya) (P1), Qaminis (53km South of Benghazi while) (P2), Bayda (East of Benghazi city Libya)(P3), Quba (East of Benghazi city Libya) (P4), Kufra A (of south east of Libya) (P5), Kufra B (of south east of Libya) (P6), Kufra C (of south east of Libya) (P7), Ghadames (south west) (P8), Tripoli (North west of Libya) (P9), Kasser khiar (located 80 km east Tripoli) (P10), Khumas (located 120km from east of Tripoli)

(P11), and Khumas (located 120km from east of Tripoli) (P12). The raw samples were used in this study (P1-P12).

Time of the collection and areas of collection of the raw propolis samples were described in (Table 2-1) and Libyan map in (Figure 2-1) illustrates the localities of the Libyan Propolis samples used in this study.

The samples (P1 and P2) were collected December 2012 and (P2-P7) samples collected in July 2013 and other samples (P8-P12) from March 2014 (Figure 2-2). The beekeeper scraped the propolis sample off the top of the hive using a spatula and collected it in a clean tray

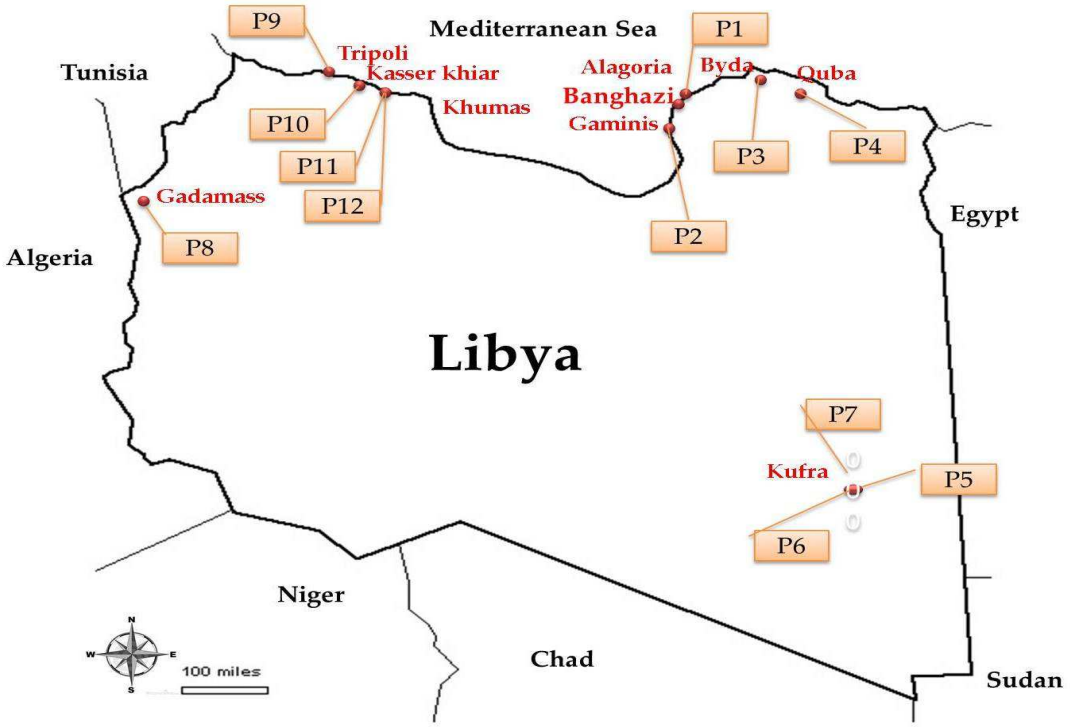


Figure 2-1 Libyan map including the localities of the analysed Libyan Propolis samples. P1 (Alagoria), P2 (Gaminis), P3 (Byda), P4 (Quba), P5 (Kufra (A), P6 (Kufra (B), P7 (Kufra (C), P8 (Gadamass), P9 (Tripoli), P10 (Kasser khiar), P11 (Khumas (A), P12 (Khumas (B).

Table 2-1 Different propolis samples collected from different geographical regions in Libya.

Propolis samples codes	Area of Collection of libyia	Name of the Cities	Time of the collection
P1	North East	Alagoria	December 2012
P2	North East	Gaminis	December 2012
P3	North East	Byda	July 2013
P4	North East	Quba	July 2013
P5	South East	Kufra (A)[a]	July 2013
P6	South East	Kufra (B)[b]	July 2013
P7	South East	Kufra (C)[c]	July 2013
P8	South West	Gadamass	October 2013
P9	North West	Tripoli	November2013
P10	North West	Kasser khiar	December 2013
P11	North West	Khumas (A)[d]	March 2014
P12	North West	Khumas (B)[e]	March 2014

N.B; [a, b, c]; Kufra (A, B, C); are different propolis samples collecting from different areas in Kufra city. [d, e] ; Khumas (A) and (B); are different propolis samples collecting from different areas in Khumas city

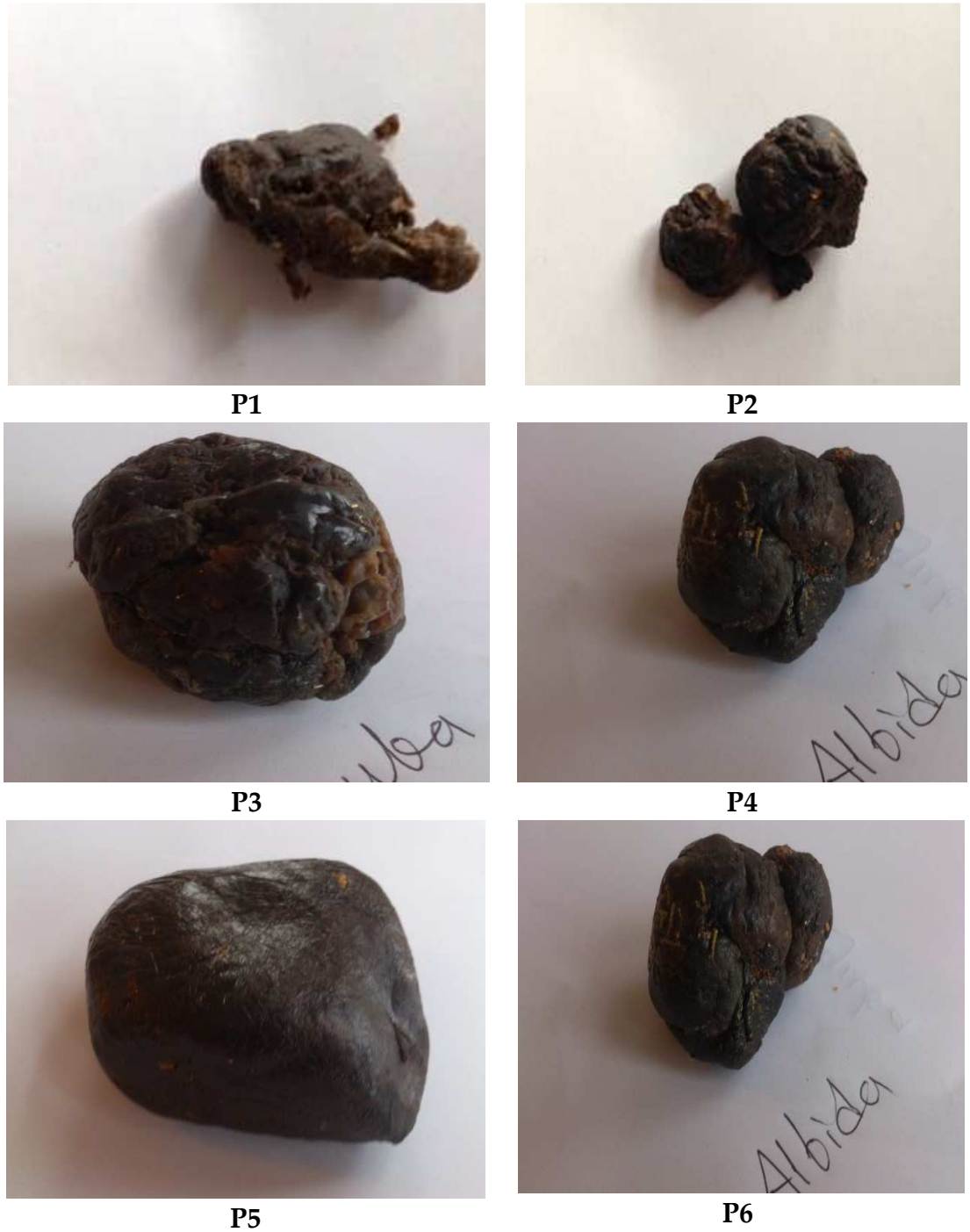


Figure 2-2 Raw Libyan propolis samples (P1-P12) collected from different geographical regions in Libya.

P7

P8

P9

P10

P11

P12

(Figure 2-2 Continued)

2.4 Extraction of Libyan raw propolis

A sample (20g) of each raw propolis sample (P1-P12) as in (Figure 2-2) and (Table 2-1), was extracted with 100 ml of absolute ethanol by sonication for 60min, and then the extract was filtered and re-extracted twice with 100ml of ethanol, filtering each time after that. The extracts were combined and the solvent was evaporated then dried using a rotary evaporator and then weighed. The residue was re-dissolved in the flask by using 5ml of ethyl acetate and by sonicating it to help the residue dissolve. The extracted solution was transferred to labelled empty weighed vials (Table 2-1). All labelled vials containing extracts P1-P12 were kept in the fridge.

Ethanolic extracts of each crude propolis EEP (P1-P12), were subjected to further comprehensive chemical and biological profiling investigations.

2.5 Propolis purification procedures

The crude propolis samples subjected to the further purification and isolation of the compounds using chromatographic procedures as the follows:

2.5.1 Open column chromatography for crude propolis

The column was prepared by putting some cotton wool at the base of the column the silica gel was prepared in slurry and poured into the column. The elution was carried out under gravity using a gradient elution sequence with mobile phase gradient as described in (Table 2-2 and 2-3.).

EEP of P1 propolis samples were chosen initially for further fractionation and purification by different chromatographic techniques. The fractionation of the crude P1 extract was carried out by open column chromatography on silica gel. 2 g of EEP of P1 of propolis was added to 6 g of silica gel in beaker and the

extract was mixed with silica gel and by drying the mixture and blow of the solvent by use sample concentrator then the sample was dried on the surface of the silica gel.

Then (50g) of silica was weighed and poured to clean beaker and hexane (200ml) was added to make slurry and which was then poured into a dry glass column then drain the hexane from the column just leaving 2 cm of hexane on the top of the column to avoid the cracking of silica in the column. Then the 2g of dried crude EEP of P1 sample on silica gel was added by dry loading. After that elution was carried out using mobile phase 200ml of hexane/ethyl acetate, F1 (90:10), then 200ml of hexane/ethyl acetate F2 (60:40), 200ml of hexane /ethyl acetate F3 (40:60), 200ml of ethyl acetate F4 and 200 ml of methanol F5 and finally 200ml of methanol/water (60:40 The eluted fractions were collected in 50 ml conical flasks labelled (1-4) in each elution), as shown (Table 2-2).

Subsequently, all fractions pooled from open column chromatography were concentrated by rotary evaporation and re-dissolved and transferred to labelled weighed empty vials and the final concentration and dryness was carried out by using sample concentrator and reweighing the vials to find the final weights of each fraction.

Based on the weights collected from open column fractions, high weights of more than 100 mg were further fractionated by medium pressure liquid chromatography (MPLC) on silica gel using a Grace Revelris® system with ELSD detection. Isolated fractions were profiled by reversed phase HPLC with ELSD and by GC-MS (Thermo DSQ). NMR was carried out by using a JEOL 400 MHz instrument.

The following (Table 2-2) shows the fractions collected with the gradient of mobile phase used for separation and amount of hexane, ethyl acetate and methanol used for the chromatographic condition of low pressure open column chromatography of P1.

Later, the next sample of propolis chosen for further fractionation was EEP P2 propolis sample was chosen subsequently for further purification and fractionation and purification by different chromatographic techniques. Started with open column chromatography and the fractionation of the crude P2 extract was carried out by open column chromatography on silica gel 2.5g of ethanolic extract of P2 of propolis was added to 6 g of silica gel in beaker and the extract was mixed with silica gel and by drying the mixture and blow of the solvent by use sample concentrator then the sample was dried on the surface of the silica gel. Then 50 g of silica weighed and poured to clean beaker and hexane (200 ml) was added to make slurry and which was then poured into a dry glass column then drain the hexane from the column just left 2 cm of hexane on the top of the column to avoid the cracking of silica in the column. Then the 2.5 g of dried crude EEP P2 sample on silica gel was added by dry loading. After that elution was carried out using mobile phase 200ml of hexane/ethyl acetate, F1 (90:10), after the elute the first fraction there strong band on the column very dark colour bands these act to added more gradient then 200ml of hexane/ethyl acetate F2 (80:20), 200ml of hexane /ethyl acetate F3 (70:30), 200ml of hexane/ethyl acetate F4 (60:40), 200ml of hexane/ethyl acetate ethyl acetate F5 (50:50) and 200 ml hexane/ethyl acetate of F6 (40:60) and 200 ml hexane/ethyl acetate of F7 (70:30) and 200 ml hexane/ethyl acetate F8 (80:20) and 200 ml hexane/ethyl acetate F9 (90:10)and 200 ml ethyl acetate of h methanol F5 and finally 200ml of methanol/water (60:40). The eluted fractions were collected in 50 ml conical flasks labelled (1-4) in each elution), as shown (Table 2-3).

Subsequently, all fractions obtained from open column chromatography were concentrated by rotary evaporation, re-dissolved, and transferred to labelled weighed empty vials and the final concentration and dryness was carried out

by using sample concentrator and reweighing the vials to find final weights of each fraction.

These collected fractions, were further fractionated by medium pressure liquid chromatography (MPLC) on silica gel using a Grace Revelris® system with ELSD detection. Isolated fractions were profiled by reversed phase HPLC with ELSD and characterized / analysed by GC-MS (Thermo DSQ). NMR was carried out by using a JEOL 400 MHz instrument.

The following (Table 2-3) shows the fractions collected with the gradient of mobile phase used for separation and amount of solvent system of hexane, ethylacetate and methanol used for the chromatographic condition of low pressure open column chromatography of P2.

Table 2-2 Collected fractions from open column fractionation of P1 sample

P1 Fractions	Solvent system [a](all volumes were 200ml)		Volume (ml) collected
	Hexane (%)	**Ethyl acetate (%)**	
P1-1	90	10	50
P1-2	90	10	50
P1-3	90	10	50
P1-4	90	10	50
P1-5	60	40	50
P1-6	60	40	50
P1-7	60	40	50
P1-8	60	40	50
P1-9	40	60	50
P1-10	40	60	50
P1-11	40	60	50
P1-12	40	60	50
P1-13	0	100	50
P1-14	0	100	50
P1-15	0	100	50
P1-16	0	100	50
	Methanol (%)	**H_2O (%)**	**Volume (ml)**
P1-17	100	0	50
P1-18	100	0	50
P1-19	100	0	50
P1-20	100	0	50
P1-21	100	0	50
P1-22	60	40	50
P1-23	60	40	50
P1-24	60	40	50
P1-25	60	40	

N.B. [a]; Each eluted solvent gradient was 200ml were collected every 50ml in separately.

The investigations of all fractions were carried out by using the LC-HRMS and in the same condition used to analyse the crude P1 and P2 samples. In addition all these fractions were investigated by HPLC-ELSD.

Table 2-3 Collected fractions from open column fractionation of P2 sample.

P2 Fractions	Solvent system [a](all volumes were 200ml)		Volume (ml) collected
	Hexane (%)	Ethyl acetate (%)	
P2-1	90	10	50
P2-2	90	10	50
P2-3	90	10	50
P2-4	90	10	50
P2-5	90	10	50
P2-6	90	10	50
P2-7	80	20	50
P2-8	80	20	50
P2-9	80	20	50
P2-10	80	20	50
P2-11	60	60	50
P2-12	60	60	50
P2-13	60	40	50
P2-14	60	40	50
P2-15	40	60	50
P2-16	40	60	50
P2-17	40	60	50
P2-18	40	60	50
P2-19	50	50	50
P2-20	50	50	50
P2-21	50	50	50
P2-22	50	50	50
P2-23	30	70	50
P2-24	30	70	50
P2-25	30	70	50
P2-26	30	70	50
P2-27	20	80	50
P2-28	20	80	50
P2-29	20	80	50

(Table 2-3 Continued)

P2-30	20	80	50
P2-31	0	100	50
P2-32	0	100	0
P2-33	0	100	0
P2-34	0	100	0
P2-35	0	100	0
P2-36	0	100	0
P2-37	0	100	0
P2-38	0	100	0
	Methanol (%)	**Ethyl acetate (%)**	
P2-39	50	50	50
P2-40	50	50	50
P2-41	50	50	50
P2-42	50	50	50

N.B; [a]; Each eluted solvent gradient was 200ml were collected every 50ml in separately.

2.5.2 High pressure liquid chromatography-evaporative light scattering detectors (HPLC-UV-ELSD).

The EEP of each of the crude propolis samples (P1-P12), were prepared at 0.5mg/ml by reconstituted with the mobile phase at the ratio of the initial composition of the liquid chromatography (LC) gradient program dissolving in acetonitrile and then adding water to give a solution in water/acetonitrile (70:30) of each EEP then were profiled using an Agilent 1100 HPLC linked to a Shodex ELSD and an Agilent UV detector. The preliminary profiling for all EEPs from P1-P12 was carried out

Additionally, the open column fractions from P1 such as P1-3 were profiled by reversed phase HPLC with ELSD and P1-3 was analysed started using the general conditions and the column was used a C_{18} column and mobile phase (water: acetonitrile) and detection was with ELSD and UV with volume of injection 10 μl.

The following programme was used: 0.5 ml/min start with 70% acetonitrile and ramp to 100 % acetonitrile over 70 min. The samples were prepared at 0.5

mg/ml by dissolving in acetonitrile and then adding water to give 30:70 (water to acetonitrile).and then hold for 6min with 100% acetonitrile. Detector 1 was ELSD Range 1: Bipolar, 1250 mV, 12.5 Samp. Per Sec., Detector 2: DA D Signal A Range 2: Bipolar, 100000 mA U, 10 Samp per Sec., and Detector 3: DA D: Signal B Range 3: Bipolar, 100000 mA U, 10 Samp. per sec. Then isolated fractions and pure isolated compounds from the Grace Revelris® of P1-3 and other samples fractions from P2, P7 and P9, were also analysed under the same conditions.

2.5.3 Medium pressure liquid chromatography using the Grace (Revelris®).

2.5.3.1 Purification of open column fractions from P1

Further purification of open column fraction P1 (P1-3) as shown in (Table 2-2), the yield weight was (1g) as displayed later on in (section 3.7, Table 3-26), was carried out using the Revelris® MPLC. One gram of P1-3 was dissolved in hex: EtOAc (50:50) in a beaker with addition 1.9 g of Celite® and the sample was dried by using the sample concentrator to dryness so that the celite was coated and could then be transferred to the dry loader for the Revelris® MPLC.

The Revelris® MPLC was set up with a 24 g silica column to run a stepwise gradient at 12 ml/min flow rate and start 100% hexane by use programme 100% hexane to hexane ethyl acetate (80:20) in 30 minutes and then to 100% ethyl acetate at 50 minutes. The fractions associated with the same peak according to the mass spectrometry detection and chromatogram were combined, evaporation of the fractions was carried out by using a rotary evaporator and then hex: EtOAc (50:50) was used to re-dissolve and transfer to weighed labelled vials and the sample was dried then the vials were reweighed. The fractions were labelled as P1-3-1, P1-3-2 and P1-3-3 consequently P1-3-28, as

displayed later on the (section 3.8, Table 3-28), all belong to the original P1-3 open column fraction.

Additionally, the open column fraction of P1-2 as showed in (Table 2-3), the yield weight was (251.9mg) as displayed in later on (section 3.7, Table 3-26), was dissolved in 5 ml of ethyl acetate and mixed with 503mg celite® and blown dry. The sample was packed into an empty "dryloader" cartridge loaded onto the Revelris® MPLC system, fitted with a 12 g silica gel cartridge and then eluted a linear gradient from 0% to 100% ethyl acetate which were started with 100% hexane for 5 min, then hexane ethyl acetate (60:40) for 20 min, followed by hexane ethyl acetate (50:50) for 20 min. then hexane ethyl acetate (40:60) for 20 min and then hexane ethyl acetate (20:80) for 10 min and finally ethyl acetate 100% for 25 min with a flow rate of 12mL/min. Fractions were collected automatically when triggered by the ELSD response. The fractions associated with the same peak according to the ELSD chromatogram were combined, and the solvent was removed and weighed sample was dried by using the sample concentrator to dryness, different fractions were collected and weight included P1-2-16, as described later on (section 3.8, Table 3-29)

In addation, open column fraction P1 (P1-9) as shown in (Table 2-2), the yield weight was (255.2mg) as displayed later on in (section 3.7, Table 3-26), was loaded onto celite® (800mg) and packed into a dry loading cartridge. The Revelris® MPLC was set up with a 12 g silica gel column to run a stepwise gradient at 12 mL/min flow rate using linear gradients as follows: 100% hexane 0 min, hexane ethyl acetate (40:60) 30 min and 100% ethyl acetate 50 min. Fractions were collected automatically when triggered by the ELSD response. The fractions associated with the same peak according to the ELSD chromatogram were combined, and the solvent was removed and weighed

sample was dried by using the sample concentrator to dryness and weighed as described in (section 3.8, Table 3-30).

2.5.3.2 Purification of open column fractions from P2

Open column fraction P2 (P2-12) as shown in (Table 2-3), the yield weight was (354.2mg) as displayed in (section 3.7, Table 3-27), was reconstituted in ethyl acetate (5 mL) and added to (708.4mg) celite® mixed and blown dry and the sample was packed into an empty "dryloader" cartridge allowing it to be eluted onto the Revelris® MPLC system. The system was fitted with a 12 g silica gel cartridge and was eluted with 100% hexane 0 min to 5min, hexane ethyl acetate (60:40) for 20 min then (40:60) for 20 min then (20:80) for 10 min then 100% ethyl acetate for 20 min in liner gradient with a flow rate of 12mL/min. Fractions were collected automatically when triggered by the ELSD response. The fractions associated with the same peak according to the ELSD chromatogram were combined, and the solvent was removed and weighed sample was dried by using the sample concentrator to dryness and weight as described in (section 3.8, Table 3-31).

Also, the open column fraction P2 (P2-24) as shown in (Table 2-3), the yield weight was (207.5mg) as displayed in (section 3.7, Table 3-27), was dissolved in 5 ml of ethyl acetate and mixed with celite®, (621mg), and loaded onto the Grace Revelris® system fitted with a Grace 12 g C18 cartridge. The sample was eluted isocratically with acetonitrile: water (40:60) at 12mL/min over 30min followed by linearly increasing acetonitrile to 100% over 30min. The fractions associated with the same peak according to the ELSD chromatogram were combined, and the solvent was removed and weighed sample was dried using the sample concentrator to dryness and weight as different fraction include P2-24-7, (20mg).

2.5.3.3 Direct fractionation of P7 by MPLC

One gram of EEP of sample P7, was dissolved in ethyl acetate (5ml) and was mixed with celite® (2 g), dried in a fume cupboard and the sample was packed into an empty "dryloader" cartridge (Alltech, Carnforth, UK) allowing it to be transferred onto to a Grace Davison Reveleris® flash chromatography system. A linear gradient with 100 % hexane and 100% ethyl acetate was used with a flow rate of 24 mL/min and silica gel (24g) cartridge (Alltech). Fractions were collected automatically when triggered by the ELSD response. The fractions associated with the same peak according to the ELSD chromatogram were combined, and the solvent was removed and weighed sample was dried by using the sample concentrator to dryness weight as described later in (section 3.8.1, Table 3-32)

2.5.3.4 Direct fractionation of P9 by MPLC

Then EEP of P9 (1gm) was dissolved in ethyl acetate (5ml) and mixed with celite® (2g), blown dry and the sample was packed into an empty "dryloader" cartridge allowing it to be eluted onto the Revelris® MPLC system which was fitted with a prepacked 24 g silica column (Alltech, Carnforth, UK). The detection threshold was set at medium and by using hexane: ethyl acetate ranging from 0% to 100% over a 60 min linear gradient, varying volumes was collected according to the threshold setting monitored using HPLC-UV-ELSD.

2.5.4 Liquid chromatography high resolution-mass spectrometry (LC-HRMS).

The EEP of crude propolis samples (P1-P12) and fractions obtained from open column chromatography and the purified factions and compounds obtained from MPLC chromatography were dissolved in methanol (HPLC grade) to get a concentration of 1mg/ml and sample solution (20µl) was injected into the LC-

MS. The mobile phase was 0.1% formic acid in acetonitrile: 0.1% formic acid in water at a flow rate of 300µl/min. The high resolution mass spectra were obtained by using an LTQ Orbitrap MS (Thermo Orbitrap mass spectrometer) in negative ion mode with a needle voltage of -4.0 kV.

The separation of ethanolic extracts was performed on an ACE-C18 column (150×3 mm, 3 µm) from HiChrom UK with 0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile as B. The detectors used in this and HPLC gradient used in athe study are shown in Table 2-4 and Table 2-5, respectively.

Table 2-4 LC/UV/MS system specifications.

HPLC system	**Surveyor pump, detector and auto sampler**
Mass spectrometer	LTQ Orbitrap
Column	ACE C18 -column (150×3 mm, 3 µm)
Mobile phase	**A;** 0.1% formic acid in H_2O
	B; 0.1% formic acid in ACN

Table 2-5 Chromatography conditions of LC-MS for propolis extraction.

Time (min)	Phase A	Phase B	Flow Rate (µl/min)
0	70	30	300
15	70	30	300
25	50	50	300
40	50	50	300
50	40	80	300
51			300
59	0	100	500
60	70	30	300
70	70	30	300
0	70	30	300

N.B; **A**; aqueous phase and **B**; organic phase (**A**% (0.1% v/v formic acid in H_2O) and B% (0.1 % v/v formic acid in Acetonitrile).

The investigation of all fractions weighing more than 10 mg collected from open column chromatography was carried out by using the LC-MS as described above.

2.5.5 Gas chromatography–mass spectrometry (GC-MS).

A trace GC system was fitted with a Rtx-1 ms column (30 m×0.25 µm film × 0.2mm i.d., Thames Restek UK). The oven was programmed 100 °C (1min), 20 °C per min to 320 °C and held for 5 min. Injector temperature was 250 °C and the detector temperature was 250 °C. The GC was interfaced to a DSQ II MS operated in electron impact mode at 70eV. EEP of crude P1 and P2 propolis samples initially were investigated by GC-MS after analysis by LC-MS. Consequently P1-3 open column fraction was chosen for further fractionation was tested by GC-MS and then the fractions collected by Grace Revelris® flash chromatography from P1-3 were analysed by GC-MS. A portion of each fraction (3mg) was dissolved in 3 of ml hexane/ethyl acetate (2:1) by sonicating

for 30 min. Then the sample was transferred into a vial for the GC and a run was carried out. Xcalibur software was used to manipulate data by library searching the NIST library to find the spectra of compounds with high similarity (correlation value >800).

2.5.6 Nuclear magnetic resonance (NMR).

Proton NMR (^{1}H NMR), was used to determine some information about the crude EEP and to elucidate the structure of the isolated compounds from the propolis samples in this study.

A portion (100mg) of EEP of all the crude samples (P1-P12) was dissolved in Deuterated DMSO (DMSO-d_6, 0.75 ml) and transferred to an NMR tube. Then proton NMR carried out for all crude samples. The observed chemical shift δ values were obtained in (ppm) and the coupling constant (J) in Hz. ^{1}H NMR spectra were measured at a magnetic field strength of 400.13 MHz using a JEOL Delta GX 400 MHz FT nuclear magnetic resonance (NMR) Spectrometer MNOVA software was used for processing the samples. ChemBioDraw Ultra, Version 11, was used to draw compound structures, and also to predict the chemical name for the pure compounds ^{1}H NMR data. For the pure compounds J-modulated ^{13}C and 2D NMR COSY, HMBC, HSQC, were measured using same NMR spectrometer. Observed chemical shift δ values were obtained in ppm and the coupling constant (J) in Hz. Spectra were referenced to the residual proton in deuterated chloroform or other solvent such as DMSO-d_6…etc. Broad band decoupled ^{13}C NMR was used to determine the number of carbons, their type and where necessary DEPT experiments were obtained to distinguish the carbons according to the extent of their proton attachments. DEPT spectrum is a pulse sequenced experiment that transforms the information of the CH signal multiplicity and spin-spin coupling into phase relationship. In the DEPT 135 spectrum, CH_3 and CH are

directed towards the positive phase of the spectrum while CH_2 is facing the negative phase. The advantage of the DEPT spectrum over carbon spectrum is that it is 4 times more sensitive as it uses ^{1}H-^{13}C polarisation transfer.

Correlation spectroscopy (COSY) was carried out for pure compounds. The proton shifts are plotted on both axes with the contour plot along the diagonal of the square. Results called as cross peaks are arranged in the square symmetrically about the diagonal. Thus the cross peaks refer to the spin-spin coupled protons. The correlations observed are due to geminal (2J) and vicinal (3J) couplings. Heteronuclear Multiple-Bond Correlation Spectroscopy (HMBC) is one of 2D ^{1}H-^{13}C experiments and provides considerable structural information through carbon-proton coupling via two, three or four bonds. HMBC experiments were carried out for compounds **(1)** to **(18)** in this study for highly-substituted compounds which lack sufficient protons to 'track' the carbons. Heteronuclear Multiple Quantum Coherence (HMQC) is 2D 1H-^{13}C-experiments were used to identify one-bond (1J) connections In a HMQC spectrum, the ^{1}H and ^{13}C (or DEPT) spectrum is plotted along the abscissa and ordinate, respectively (or vice versa). Cross-peaks show protons and carbons that are directly connected to each other; the pure compounds were subjected in the HMQC in order to elucidate their chemical structures. Mnova NMR software was used to processing the NMR spectra.

2.5.7 Optical rotation measurement

The optical rotation for some pure compounds obtained was using a Perkin–Elmer 241 polarimeter with a sodium lamp at 20 °C (PerkinElmer Inc., USA). In order to measure their optical rotation 1mg of each of the compounds was dissolved in solvent (chloroform) to get 1mg/ml. The average of ten

readings was taken and then optical rotation was calculated using the equation below:

Equation: $[\propto] = \mathbf{100} \times \boldsymbol{\alpha} / \boldsymbol{l} \times \boldsymbol{c}$

> Where $[\alpha]$ is the specific rotation at wavelength λ, α is the average of the measured rotation, T is the temperature at 20 °C, $\boldsymbol{l}$ is the path length in decimetres, and $\boldsymbol{c}$ is the concentration of the solution in g/mL.

2.5.8 Software and data processing for LC-HR-MS

Xcalibur 2.2 from Thermo Fisher Scientific was used to check the raw LC-HRMS and GC-MS data and generate the MS based chromatograms shown in the manuscript. Clarity from DataApex was used to handle the LC-UV-ELSD data. Also MZMine 2.10 was used for LC-HR-MS data processing. The generated peak lists from both ESI positive and negative modes were combined and imported to SIMCA-P 13 (Umetrics, Sweden) for Principal Component Analysis (PCA). Using an in-house macro coded by Visual Basic Application in Excel (Microsoft office 2010) the first 100 LC-HRMS features from each sample were selected based on the peak area and putatively identified by searching for the accurate mass in Dictionary of Natural Products (version 2013).

2.6 Biological assays of propolis samples (P1-P12)

The EEP from twelve crude propolis samples (P1-P12) were subjected to the various screening tests to assess the biological activity against a range of pathogenic protozoa e.g.; *Trypanosoma brucei, Leishmania donovani, Plasmodium falciparum, Crithidia fasciculate* and *Mycobacterium marinum*. Also activity against a range of Game-positive such as *S. epidermidi*s (ATCC 12228) *S. aureus* (ATCC 29213), and Gram-negative bacteria such as *E. coli* (ATCC 8739) and *K.*

pneumoniae (ATCC13883) were also assessed. The cytotoxicity of the extracts was tested against mammalian cells. Moreover, the purified fractions collected from flash chromatography (MPLC) were tested as well.

2.6.1 Antitrypanosomal assay of propolis samples (P1-P12) against *T. brucei*

An AlamarBlue® assay was used to determine drug sensitivity against African trypanosomes *in vitro*. The tests were carried out by Mrs. Carol Clements from the Strathclyde Institute for Drug research (SIDR).

Preliminary *in vitro* antitrypanosomal testing crude propolis samples (P1-P12), was carried out against a standard drug-sensitive *T. brucei* clone, Lister strain 427 (s427) (Omar et al., 2015, de Koning et al., 2000), and the results were expressed as EC_{50} values based on three replicates at each concentration. The assay is based on viable cells metabolizing the blue non-fluorescent dye resazurin to resorufin, which is pink and fluorescent. The assays were performed using serial dilutions in white opaque plastic 96-well plates (F Cell Star, Greiner Bio-one GmbH, Frickenhausen, Germany), with each compound or mixture double diluted over 2 rows of the plate (i.e. double dilutions and a no-drug control well), facilitating an optimally-defined EC_{50} value after plotting of the reading to a sigmoid curve with variable slope (GraphPad Prism 5.0). The seeding density at the start of the assay was 2×10^4 cells/well, and the cells were exposed for 48 h to the test compounds, at 37°C/5% CO_2, before the addition of the resazurin dye and a further incubation of 24 h under the same conditions. Fluorescence was determined in a FLUOstar Optima (BMG Labtech, Ortenberg, Germany) at wavelengths of 544 nm and 620 nm for excitation and emission, respectively.

2.6.2 Antileishmanial assay of propolis samples (P1-P12) against *L. donovani*

The antileishmanial assay of EEP of (P1-P12) was carried out by Mutazz Hussain at Strathclyde University and Dr. Chris Carter at Strathclyde University as follows; intraperitoneal macrophages were recovered from the peritoneal cavity of BALB/c mice 3 days after intraperitoneal injection with 1mL 3% w/v aqueous sterile starch solution. The mice were then euthanized, and 3mL of incomplete medium (RPMI-1640, 100 μg/mL penicillin–streptomycin and 200mML-glutamine) was injected into the peritoneal cavity. The macrophage-containing medium was then removed and collected, and the resulting cell suspension centrifuged at 3000 × g for 5 min and then re-suspended in 10 mL of complete medium (in complete RPMI-1640 supplemented with 10% heat inactivated fetal calf serum (FCS) [v/v]). The cells were then used in antileishmanial assays. Bone marrow was then harvested from the femurs of each mouse by flushing out the removed bone with 5ml of bone marrow medium (Dulbecco's modified Eagle's medium, 20% heat-inactivated fetal calf serum (FCS) [v/v], 30% L-Cell solution [v/v], 100μg/mL penicillin–streptomycin and 200mML-glutamine). The cell suspension was added to sterile petri dishes (one petri dish/mouse) and incubated for 7 days at 37°C in an atmosphere of 5% CO_2:95% air. The medium was removed from the plate, and 7mL TrypLE Express was added to detach the bone marrow-derived macrophages. The resulting suspension of bone marrow-derived macrophages was collected, pelleted by centrifugation and re-suspended in 10mL of incomplete medium and then used in antileishmanial assays. The number of live macrophages per millilitre was determined microscopically using a haemocytometer, by mixing a cell sample with 1:1 trypan blue (20μL) and viewing at ×10 magnification. In all cases, cell viability was >95%. Cells (0.5 × 10^5 in 200 μL complete medium) were added to the appropriate wells of a 96-

well tissue culture plate and incubated for 24 h at 37°C in an atmosphere of 5% CO_2: 95% air. Cells were then infected with *L. donovani* luciferase-expressing promastigotes, produced at the University of Strathclyde using strain MHOM/ET/67:LV82, using a 20:1 parasite/host cell ratio. The plate was incubated as before for 24 h. The medium was removed from each well and replaced with 200μL complete medium (Control, *n=6*) or various concentrations of the one of the extracts (diluted in 4% DSMO v/v in complete medium, *n=3*) or Amphotericin B solution (4–0.02 μg/mL). The plate was incubated as before for 72 h, the medium was then removed, and 150μL of luciferin solution (150 μg/mL luciferin in complete RPMI-1640) was added to each well. The bioluminescence intensity (BLI) emitted per well was determined using the IVIS® imaging system (Caliper Life Sciences, Runcorn, UK) (Siheri et al., 2014, Alsaadi et al., 2012). The suppression in bioluminescent signal for each test sample was compared with the mean control value. The mean IC_{50} value was then calculated for each sample by probit analysis. Data were analysed using MINITAB® software version 16.1.1 supplied by Minitab Ltd. Coventry, UK, and an Anderson–Darling test was used to establish if the data were normally distributed. Parametric data were analysed using a Student's unpaired t-test or by one-way analysis of variance dependent on the number of treatments/experiments, and significance was confirmed by a Fisher test. A Mann–Whitney or Kruskal– Wallis test was used to analyse data that did not have a normal distribution. Results were considered statistically significant at a *p-value* of <0.05.

2.6.3 Antimalarial assay of propolis samples (P1-P12) against *Plasmodium falciparum*

Antimalarial activity assessment was carried out against *P. falciparum* (3D7, The Netherlands) and was determined as described previously (Fidock et al., 1998,

Laine et al., 2015), the assay was carried out by Dr Marco Biddau in Institute of Infection, Immunity and Inflammation, College of Medical, Veterinary and Life Sciences University of Glasgow. Synchronous ring stage parasites were seeded and incubated in triplicate into 96 well plates at 0.5% parasitemia and 2.5% haematocrit, using hypoxanthine free RPMI 1640 (Sigma Aldrich, Dorset, UK) medium, containing 0.5% [v/v] AlbuMAX II (Life technologies, Paisley, UK), 2 mml-glutamine (Sigma Aldrich, Dorset, UK) and increasing concentrations of each compound (0.1 to 200 µg/mL and no drug control; final DMSO concentration < 0.5% v/v). Increasing concentrations of Chloroquine (Sigma Aldrich, Dorset, UK) were used as a positive control (0.05 to 100 nM and no drug control). Parasites were cultured for 48 h before 5 µCi/mL [^{3}H]-hypoxanthine (American Radiolabeled Chemicals, Saint Louis MO, USA) was added to each well to be then incubated for an additional 24 h before being frozen at -20°C. After thawing, plates were harvested onto filter mats with a Harvester 96™ Mach III (TomTec, Hamden CT, USA) and [^{3}H]-hypoxanthine incorporation determined by scintillation counting using a Wallac 1450 MicroBeta Trilux counter (Perkin Elmer, Waltham MA, USA).

2.6.4 Anti-Crithidia assays of propolis samples (P1-P12) against *C. fasciculata*

The anti-Crithidia activity test was carried out against *Crithidia fasciculata* for all crude samples EEP (P1-P12), the test was carried out by Timothy Paget Dept. of Pharmacy, Health and Well-being, University of Sunderland, *C. fasciculata* (ATCC50083) was grown in RPMI 1640 medium supplemented with L-glutamine and 10% v/v heat inactivated foetal bovine serum for 24 h with shaking prior to use (Alcolea et al., 2014). These cells were then used to inoculate wells of a 96 well plate with 1 x 10^5 cells per well in 100µl of medium. Stock extracts were prepared in DMSO for each concentration so that there was

a constant percentage of DMSO per well (2.5% v/v). The absorbance of plates was determined at 620nm (T_0) using a Bio Rad xMark Microplate Spectrophotometer (Hemel Hempstead, UK) and plates and these were then incubated for 48 h at 25°C. The absorbance of the wells was then determined again at 620nm (T_{48}). For compounds showing no change in absorbance (T_{48}-T_0) terminal subculture was performed and growth determined by absorbance at 620nm and by microscopy. Pentamidine was included as a control drug in all assays but it shows variable activity against *C. fasciculata* (Bacchi et al., 1974) and thus Menadione was used as an additional control drug.

2.6.5 Anti-Mycobacterium marinum assay of propolis samples (P1-P12) against *M. marinum*.

The EEP from twelve crude propolis samples (P1-P12) were subjected to the Antimycobacterial activity screening using an AlamarBlue® Assay (resazurin–reduction test) was carried out against *Mycobacterium marinum*. The assay was carried out by Mrs. Carol Clements from the SIDR, Strathclyde University.

The antibacterial bioassays against *Mycobacterium marinum* (ATCC.BAA535) were performed in 96-well micro titre plates using a modification of the well-established AlamarBlue® method (Almutairi et al., 2014b, Franzblau et al., 1998). *M. marinum* was inoculated on to a Columbia agar with chocolated horse blood slope (Fisher Scientific, UK) and incubated at 31°C for 5 days. A loopful of the 5 day old *M. marinum* culture was transferred to a sterile universal container containing 10 ml saline plus (425–600µm) glass beads (Sigma Aldrich, Dorset, UK). The bacterial suspension was mixed vigorously and allowed to settle, an aliquot of the bacterial suspension was transferred to a tube containing saline, and the turbidity was matched to that of a 0.5 McFarland standard (~$1.5x10^8$ CFUs/ml) and then diluted with MHB (Cation Adjusted Mueller Hinton Broth, TREK Diagnostic Systems Ltd. UK) to $1.5x\ 10^7$

CFUs/ml and then 1:1 in the assay microplate to give a final concentration of 0.75 x 10^7 CFUs/ml. The assay microplate was incubated at 31°C for 6 days, after which 10% AlamarBlue® was added and the incubation continued for a further 24 h. Fluorescence was determined using a Wallac Victor 2 microplate reader (Excitation 560nm Emission 590nm) (Perkin Elmer, Waltham MA, USA). The samples were tested in duplicate over a concentration range of 100–0.19μg/ml and negative and positive controls were included containing 1–0.0019% DMSO and 100–0.78 μg/ml gentamycin respectively.

2.6.6 Antibacterial assay of propolis samples (P1-P12)

The EEP of twelve crude propolis samples (P1-P12) were tested for antibacterial activity against Gram-positive and Gram-negative bacteria.

Antimycobacterial activity against some pathogenic gram-positve and gram-negative bacteria screening. The assay was carried out by Mrs. Carol Clements from the SIDR, Strathclyde University.

Microorganisms.

All Culti-Loops® of the following bacterial were purchased from Fisher Scientific (UK). Two Gram-positive bacteria, namely, *Staphylococcus aureus* (ATTCC 29213), *Staphylococcus epidermidis* (ATCC 12228), and two Gram-negative bacteria, *Escherichia coli* (ATCC 8739) and *Klebsiella. pneumoniae* (ATCC 13883).

Inoculum preparation.

Bacterial suspensions were prepared from loops primarily in nutrient broth (Sigma Aldrich, UK) by incubating at 37 °C overnight. Cultures were then transferred into nutrient agar plates (Sigma Aldrich, UK) and incubated at 37

°C overnight. For the assay, organisms were subcultured once onto fresh nutrient agar in two days (1st and 2nd Day), and inocula were prepared by transferring colonies to Tryptone Soya broth (4–5 mL) (Sigma Aldrich, UK). Following incubation for 2–4 h at 37 °C, bacterial suspensions were diluted to match a McFarland 0.5 standard by transferring colonies to saline (0.9% w/v NaCl). Aliquots (100 μL) were then transferred to cation-adjusted Mueller–Hinton broth (10 mL) (Sensititre®, Trek-Diagnostic System, East Grinstead, UK).

The assay was carried out in two conservative days as the follows:

On day one, a loopful of each bacterial strain required was streaked on to individual Columbia with chocolated horse blood agar slopes gar slopes (These were incubated at 37°C ~ 20 hours).

Then on the second day, the same method was followed for all of the above bacterial strains. A loopful of bacterial culture a loopful of bacteria from the agar slope culture transferred to a sterile universal container containing (~10mls) of sterile 0.9% NaCl and glass beads. The bacterial suspension was mixed vigorously and allowed to settle, (~ 1ml), of the supernatant was added to a fresh tube containing (~10mls) saline (sterile MHB type) this tube had previously been used to Zero the turbidity meter. The turbidity was compared to that of a 0.5 McFarland standard (~1.5 x 10^8 CFUs/ml) and adjusted to have the same optical density (OD). A few drops of Tween 80 0.02% (filter sterilised) was added to homogenise the suspension. The suspension was shaken and the inoculum was diluted 1 in 1000 with Mueller Hinton Broth (MHB). i.e. 1μl in 1ml (10μl in 10ml of diluted suspension per plate plus some excess).

N.B; All the steps in this assay was done in open manipulations/transfer of bacteria within a class II cabinet

2.6.7 Cytotoxicity assay of propolis samples (P1-P12)

The assay was carried out by Nicola Woods University of Strathclyde, Strathclyde Institute of Pharmacy and Biomedical Science, Cell culture U937 (human malignant monocytic cells) cells were grown in a humidified incubator at 37°C with 5% CO_2. They were cultured in RPMI 1640 cell culture media (Lonza) supplemented with 10% foetal bovine serum (FBS) (Biosera), 2mM L-Glutamine (Life Technologies), and penicillin/streptomycin (500u/500µg, respectively, Life Technologies).

Cytotoxicity testing is a necessary step to determine the toxicity and effect each sample has on various cell lines. The results of cytotoxicity testing may determine the nature of the compounds within the extract and their bioactivity, and thus could indicate possible future uses. In this study, cytotoxicity tests were carried out using resazurin sodium salt (Sigma Aldrich) in solution using a modified version of the manufactures protocol for the *in vitro* toxicology assay kit (TOX8). The resazurin sodium salt was purchased on its own and prepared as a solution as required. The resazurin solution was prepared by adding 5mg of resazurin salt to 50ml deionised water. This was then sterilised filter using a 0.2µl filter (Sigma Aldrich).

The U937 cells were grown until approximately 70-80% confluence and were counted directly from the cell culture flask (75cm^2, Sigma Aldrich) and centrifuged before plating at 1x10^5cells/ml in a 96 well plate. The cell plates were then incubated overnight (20-24 hours) at 37°C, 5% CO_2, before the samples were added.

Following the overnight incubations, samples were prepared on a dilution plate in normal cell culture media respective to the cell line used. For initial testing, samples were added to the cells at a range of different concentrations to

determine the IC_{50} value for each sample. Samples were prepared at 2x the final concentration (200µg/ml) before being diluted 1 in 2 which resulted in a serial dilution from 200µg/ml to 1.56µg/ml. 100µl of each sample was then added to 100µl of the pre-plated cells which consequently halved the concentration of the samples. The final serial dilution was from 100µg/ml to 0.78µg/ml. Four controls were included on each plate: a final concentration of 10% DMSO was added to cells as a cell death control; normal cell culture media was added to cells as a growth control; 0.5-1% DMSO was added to cells as a solvent control; and media was added to some well without cells as a background control.

Following the addition of the extracts, the cell plates were incubated for 24 hours at 37°C. The plates were then removed from the incubator and resazurin solution was added to a final concentration of 10% (v/v). The cell plates were incubated at 37°C in the dark for 4 hours and 24 hours before the fluorescence reading (560nm excitation, 590nm emission) was recorded on a Spectramax Plate Reader using Softmax Pro software. These results were transferred to Microsoft Excel for analysis. Readings after 24 hours were deemed as optimal and thus only these results are shown.

Each sample was tested in triplicate and the results are expressed as cell viability as a percentage of the cell only control. The equation (see Equation 1) used to determine the cell viability is shown below:

$$\%\ \text{cell viability} = \frac{\text{Mean of sample (OD560} - \text{OD590)}}{\text{Mean of control (OD560} - \text{OD590)}} \text{ x } 100$$

Equation 1 Equation used to determine the cell viability.

Extracts were considered to be toxic if they caused a reduction in cell viability by at least 50% or more. Statistical analysis was carried out using ANOVA with a Dunnet's post-test using MiniTab 16 and graphs were plotted using Origin Pro 9.0.

2.6.8 Anti-inflammatory TNF-α ELISA assay of propolis samples (P1-P12)

Evaluation of anti-inflammatory activity for all crude propolis P1-P12 was carried out using TNF-α ELISA as per the protocol (DY201, R&D systems). The assay was carried out by Nicola Woods, University of Strathclyde, Strathclyde Institute of Pharmacy and Biomedical Sciences. Samples were tested in triplicate and data is represented as mean ± SEM.

TNF-α was used to prepare a standard curve to determine the level of production of TNF-α in the supernant after treatment. Samples were diluted before they were added to the ELISA plate to ensure the readings were within the range of the reference standard. Therefore, all results were then multiplied by the dilution factor. Statistical analysis was carried out using ANOVA with a Dunnet's post-test using Minitab 16 and graphs were plotted using Origin Pro 9.0.

2.6.8.1 Stimulation of THP-1 cells with Lipopolysaccharide (LPS)

Tested propolis extract (1mg) was dissolved in 1ml complete media (CM) in sterile 20 ml universal centrifuge tubes. Vigorous shaking using a vortex was employed to ensure dissolving of the tested samples. They were filtered through a 0.22µm filter into sterile Bijoux. Lipopolysaccharide (10mg) was dissolved in 1ml CM and aliquoted. The LPS was vigorously mixed by vortex before use. THP-1 cells (1×10^6 cells/ml, 1ml) were added to each well of a 24-well plate, together with 100µl/well of 1mg/ml propolis tested samples with and without LPS (10µl/well final well concentration 0.05mg/ml). Wells were made up to a final volume of 1110µl with CM. Each treatment was performed in triplicate. Plates were incubated for 24h at 37°C, 5% CO_2 and 100% humidity. Afterwards, the supernatants were transferred to 1.5ml sterile microfuge tubes and centrifuged in a micro centrifuge at 13,000rpm for 5 min at

4°C. The supernatants were transferred to fresh microfuge tubes and stored at -20°C until assayed for cytokine levels by ELISA.

2.6.8.2 Enzyme linked immunosorbent assay (ELISA)

A sandwich ELISA was carried out with supernatants (section 2.7), from the LPS stimulation assay to evaluate the production of tumour necrosis factor alpha (TNF-α) cytokine, using an ELISA Kit. All procedures were carried out according to the manufacturer's instructions, while wash steps were carried out using phosphate buffered saline (PBS), pH 7.4 containing (v/v) Tween®. F16 Maxisorp Loose Nunc-immuno modules were coated with 100 µl/well capture antibody (1:250 dilution) in coating buffer and incubated overnight at 4°C. Wells were washed three times with wash buffer and blocked with 200 µl/well assay diluent. The modules were incubated at room temperature for 1h and then washed. The supplied TNF-α standard (5µl) was added to 10ml assay diluent to make a top standard concentration of 500pg/ml. 100µl/well was added in duplicate wells at the top of the modules and a 1:2 serial dilution was carried out down the modules. The bottom two wells contained assay diluent only (blank). For the samples, 100µl/well of cell supernatant was added in triplicate and modules incubated at room temperature for 2h. The modules were then washed five times and 100 µl/well detection antibody in diluent (1:250 dilution) added. The modules were incubated at room temperature for 1h, followed by another five washes. Then, 100 µl/well of Avidin-HRP in diluent (1:250 dilutions) was added and incubated for 30min at room temperature and the modules washed seven times. The wells were developed with 100µl/well substrate solution, and incubated for 15min at room temperature. The enzyme reaction was stopped by adding 50µl/well 10% (v/v) sulphuric acid (H_2SO_4) and read at 450nm. The concentration of TNF-α

produced in each sample was calculated from the slope equation of the standard curve.

2.6.9 Anthelmintic assay of propolis samples (P1-P12)

The anthelmintic assay was carried out for all crude propolis samples EEP (P1-P12), the assay was carried out by Pilaslak Akrachalanont and Dr Catherine Lawrence at Strathclyde University, which Female C57BL/6 and BALB/c mice, aged 8-12 weeks, were bred and maintained under conventional animal house conditions and in accordance with Home Office regulations in the animal unit of the University of Strathclyde. Experimental groups consisted of a minimum of five mice. Animals were euthanized by CO_2 inhalation in all cases. These animals were used to maintain parasites.

2.6.9.1 *In vitro* bioassy of (P1-P12) against *Trichinella spiralis*.

Parasites were maintained by serial passage through wild type BALB/c mice for *Trichinella spiralis* nfective *T. spiralis* muscle larvae were obtained from digestion of infected BALB/c mice (> 30 days post infection). Mice were killed by CO_2 inhalation and skins, snouts, extremities and abdominal organs were removed. The carcasses were then cut into pieces and then homogenised in a Kenwood blender. The material was then digested in at least 200ml 0.9% NaCl/ 0.5% Pepsin/ 0.5% HCl solution per mouse at 37^{o}C under agitation for 1 hour 30 minutes. Digests were then filtered through a coarse sieve (mesh size 1mm) to remove undigested tissue and bone fragments. The larvae were collected by a series of three successive washings and sedimentations in 0.9% NaCl solution. The larvae were finally suspended in 50ml 0.9% NaCl solution and the total number determined by counting under a Leica light microscope. Experimental mice were infected orally with 400 larvae in 200µl volume of 0.1% agarose.

2.6.9.2 *In vitro* bioassy of propolis (P1-P12) against *Caenorhabditis elegans*.

The wild-type N2 strain (strain CB4856) of *C. elegans* and *Escherichia coli* OP50 were kindly provided 2 culture plates by Dr Nick Tucker (SIPBS, University of Strathclyde) was cultured on nematode growth medium (NGM) under standard conditions (Brenner, 1974). The starter culture of *E. coli* OP50 was used to isolate single colonies on a streak plate of a LB Broth. Inoculated cultures were allowed to grow overnight at 37°C. The *E. coli* OP50 solution was then used to seed NGM plates. The *E. coli* OP50 liquid culture was stored at 4°C and remained usable for several months. *C. elegans* was maintained in the laboratory on NGM. 0.05 ml of *E. coli* OP50 liquid culture was added to NGM. *C. elegans* could be visualized using a dissecting microscope equipped with a transmitted light source. The transferring frequency depended on the temperature at growing condition, and the plan to work with them. This project *C. elegans* stocks was maintained at 4°C and grow at 25°C.

3 Chapter 3

Results

3.1 Results

3.2 Chemical profiling

The twelve crude propolis samples (P1-P12), used in this study were subjected to investigation using a variety of different analytical and biological techniques were used to determination the active ingredients in the crude samples and these were related to the biological activities and their metabolomic profiles. The following diagram describes all the research work steps that have been carried out in this study (Diagram 1).

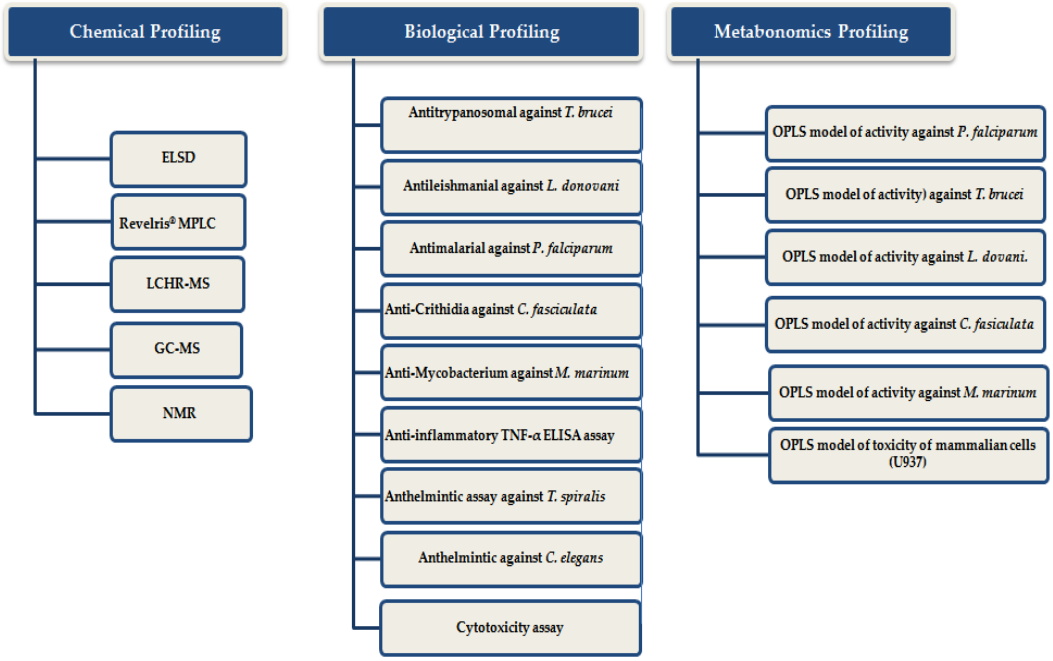


Diagram 1 Research study scheme for Libyan propolis.

3.3 Extraction of Libyan crude propolis (P1-P12).

A sample (20g) of each propolis samples (P1-P12) (Figure 3-1) as (Table 3-2), was extracted with 100ml of absolute ethanol followed by sonication for 60min, and then the extract was filtered and re-extracted twice with 100ml of ethanol,

filtering each time after that. The extracts were combined and the solvent was evaporated then ethanolic extract of each crude propolis extract was subjected to chemical and biological profiling.

Each sample (Table 3-1) showed a different physical appearance and properties, some of examples can be seen in Figure 3-1.

Some differences in odour, texture were observed and the colour varied, where for instance P1 possessed an intense orange-like odour, was light brown and had a very sticky texture, whereas P2 was darker brown and less sticky and had a less intense odour. P3 and P4 had a very intense colour and were less sticky than P7 and P6, which were light brown. P10 and P11 were light brown extracts and non-sticky. P12 was darker brown and P10 and P11 were dark yellow. P5, P6 and P7 were very dark brown but did not have a strong odour (see Table 3-1).

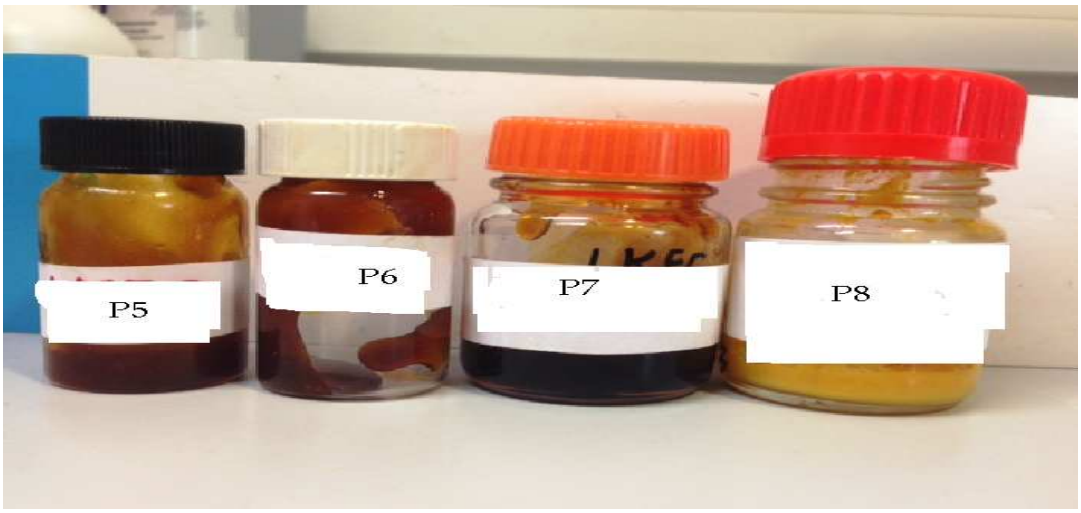


Figure 3-1 Examples of different appearance for different EEP of some Libyan propolis samples (P5, P6, P7 and P8).

Table 3-1 Physical properties and yields percentage of propolis samples EEP of (P1-P12)

Propolis Samples	Total weight (g)	Extracted weight (g)	Yields (%)	Colour	Odour	Consistency
P1	23.0	10.3	44.9	Dark brown	Orange odour	Sticky
P2	20.2	8.3	41.2	Dark red brown	Intense odour	Semi sticky
P3	19.8	6.5	32.7	Light brown	Olive leave odour	non sticky
P4	20.2	6.9	34.4	Dark yellow	Mild	Sticky
P5	25.0	7.5	29.8	Dark brown	No odour	Very sticky
P6	25.1	6.9	27.7	Dark brown	No odour	Very sticky
P7	41.9	20.0	47.7	Dark brow	Mild	Very sticky
P8	5.80	2.4	40.5	Yellow	Mild flower	Non-sticky
P9	13.9	5.9	43.1	Dark yellow light honey	Intense orange – Flower	Semi sticky
P10	15.4	4.36	28.2	Light brown	Flowery odour	Non sticky
P11	20.5	5.4	26.4	Light brown	Mild odour	Sticky
P12	20.5	6.4	31.2	Honey – brown	Mild odour	Sticky

Table 3-1 shows the extraction yields for each sample.

The crude EEPs of P1-P12 were prepared at 1mg/ml and were profiled by using an LTQ Orbitrap in negative ion mode using an ACE-C18 column (150×3 mm, 3 μm) from HiChrom UK with 0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B.

Then further purification and fractionation was carried out for samples P1, P2, P7 and P9 using open column chromatography and flash chromatography for profiling and isolation and separation for active fractions, and these were further chemically investigated by LC-MS and GC-MS and NMR.

3.4 Nuclear magnetic resonance (NMR) profiling of the propolis samples (P1-P12)

3.4.1 ^{1}H NMR spectra of propolis samples (P1-P12)

In order to gain some immediate information about the compounds present in extracts of P1-12 ^{1}H-spectra were obtained for the extracts. The ^{1}H NMR spectra of crude P1-P12 samples are shown Figures 3-2 to Figures 3-13 respectively, and demonstrated significant differences in the spectra. P5, P6 and P7 partly appear different from the other spectra. P1 and P9 show similarities in their spectra as can be seen in Figures 3-2 and 3-10 and both P1 and P9 have the same pattern in chemical shifts especially a peak at 9.7 ppm which is more abundant in P9. Both of the extracts appear to be rich in diterpenes and display characteristic exomethelene signals plus other alkene proton signals suggesting diterpenes with two double bond systems with an absence of aromatic signals.

Extract P2 looked different from P1 and P9 as shown in Figure 3-14.

P2 has an aliphatic region similar to the P1 and P9 but has no exomethylene system. In addition, there was sharp signal at 6.00ppm representing the presence of lignan compounds in P1 sample.

The NMR spectra for P10, P11 and P12 as shown in Figure 3-11 to 3-13, were different from P1, P9 and P2, and there is signal at 9.8 ppm which in P11 is more intense than in P10 and P12 and also different from P5, P6 and P7.

Interestingly, P5, P6, and P7 were quite similar in their chemical shift patterns (Figures 3-6 to 3-8 respectively) and they are different from the other P1-P12 samples. NMR spectra indicated that the all crude samples contained abundant aliphatic protons with there being only low responses for aromatic and double bond protons and there were significant difference representing cycloaratane indicated by two singlets at 0.56ppm and 0.76ppm which represent a finger print of cycolartane triterpenes as mentioned earlier (Figure 2-1 and Table 2-1) the samples P5, P6 and P7 were collected from South East libya where the region is more tropical and different from the Mediterranean sample in the North West.

According, to the Figure 3-14, there is similarity, between P5, P6 and P7 but it can be seen P5 slightly is different from P6, P7 in the aromatic region.

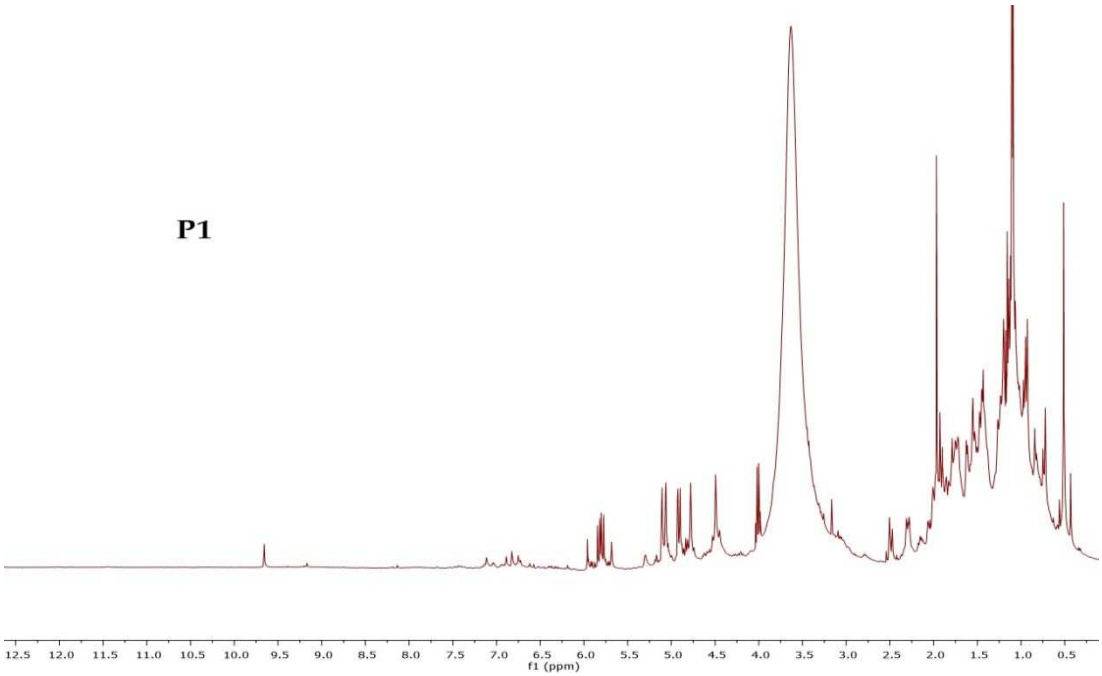


Figure 3-2 Full ^{1}H NMR spectrum (400 MHz, DMSO-d_6) (0-12.5ppm) of P1.

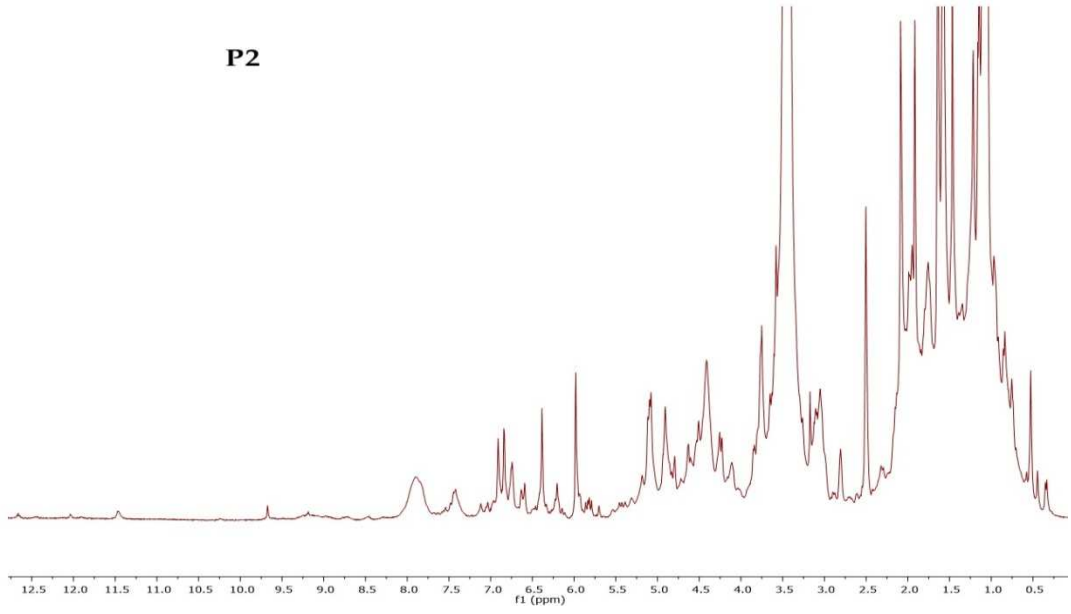


Figure 3-3 Full ^{1}H NMR spectrum (400 MHz, DMSO-d_6) (0-12.5ppm) of P2.

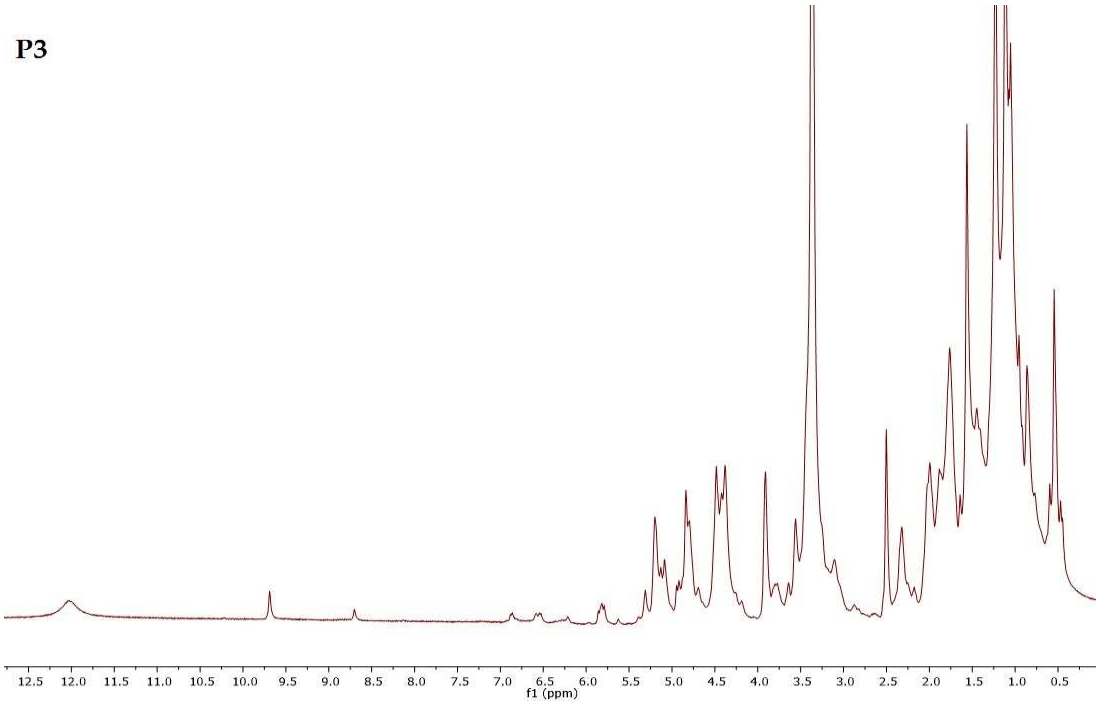


Figure 3-4 Full ^{1}H NMR spectrum (400 MHz, DMSO-d_6) (0-12.5ppm) of P3.

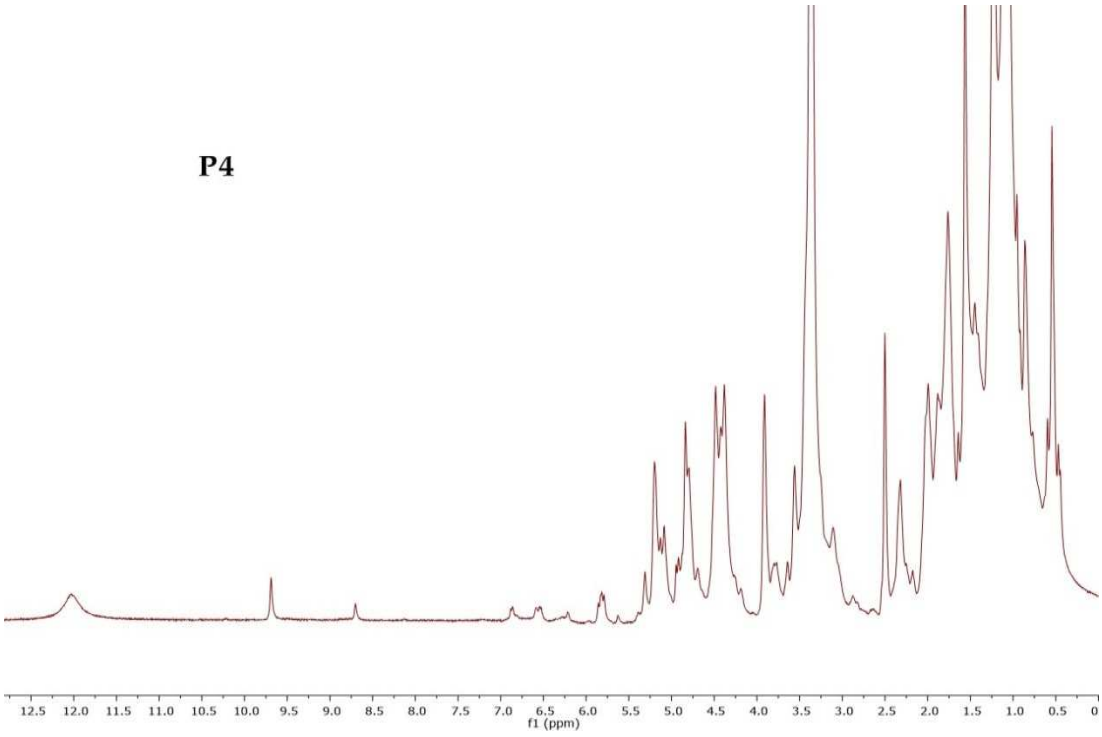


Figure 3-5 Full ^{1}H NMR spectrum (400 MHz, DMSO-d_6) (0-12.5ppm) of P4.

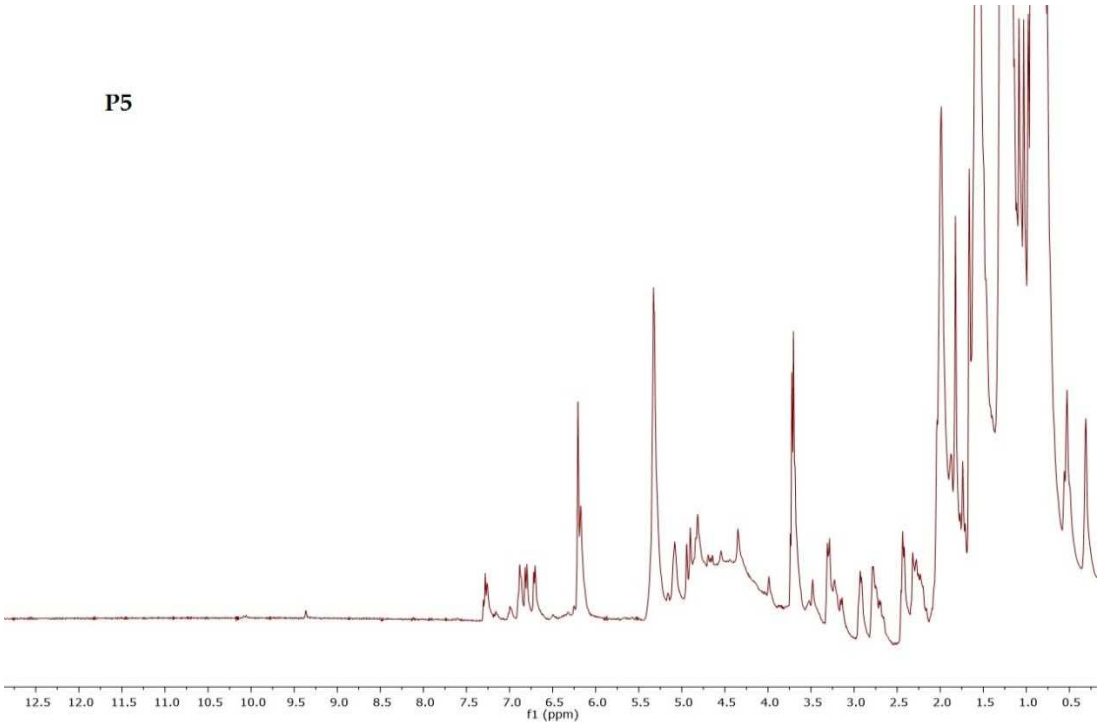


Figure 3-6 Full ^{1}H NMR spectrum (400 MHz, DMSO-d_6) (0-12.5ppm) of P5.

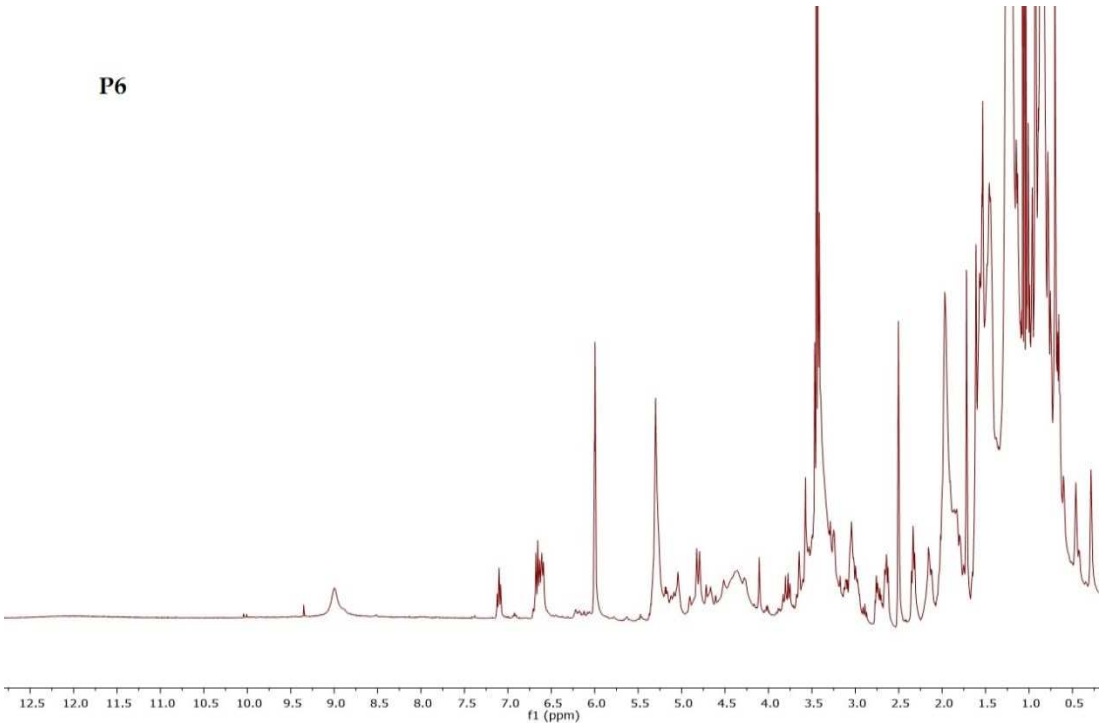


Figure 3-7 Full ^{1}H NMR spectrum (400 MHz, DMSO-d_6) (0-12.5ppm) of P6.

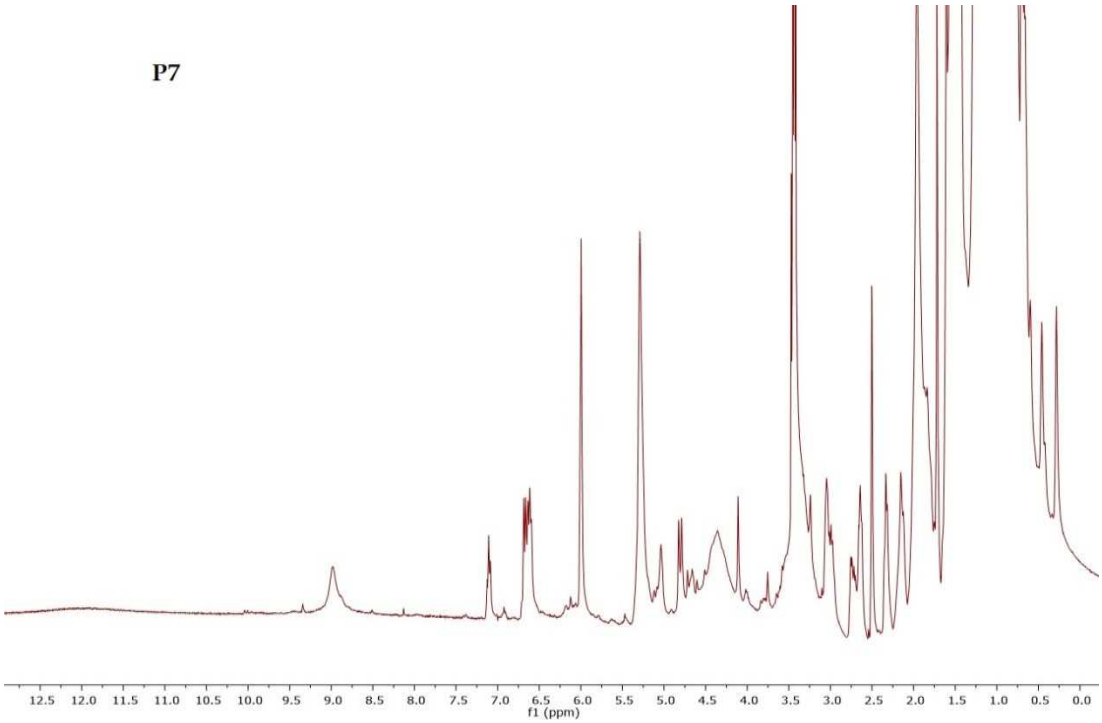


Figure 3-8 Full ^{1}H NMR spectrum (400 MHz, DMSO-d_6) (0-12.5ppm) of P7.

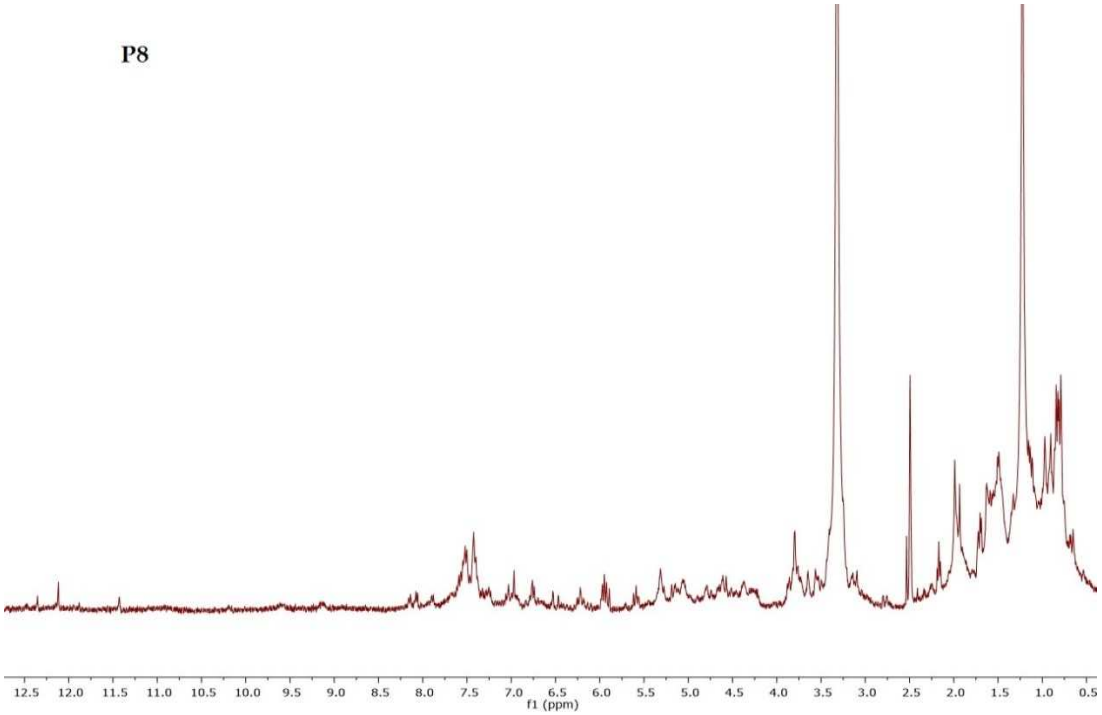


Figure 3-9 Full ^{1}H NMR spectrum (400 MHz, DMSO-d_6) (0-12.5ppm) of P8.

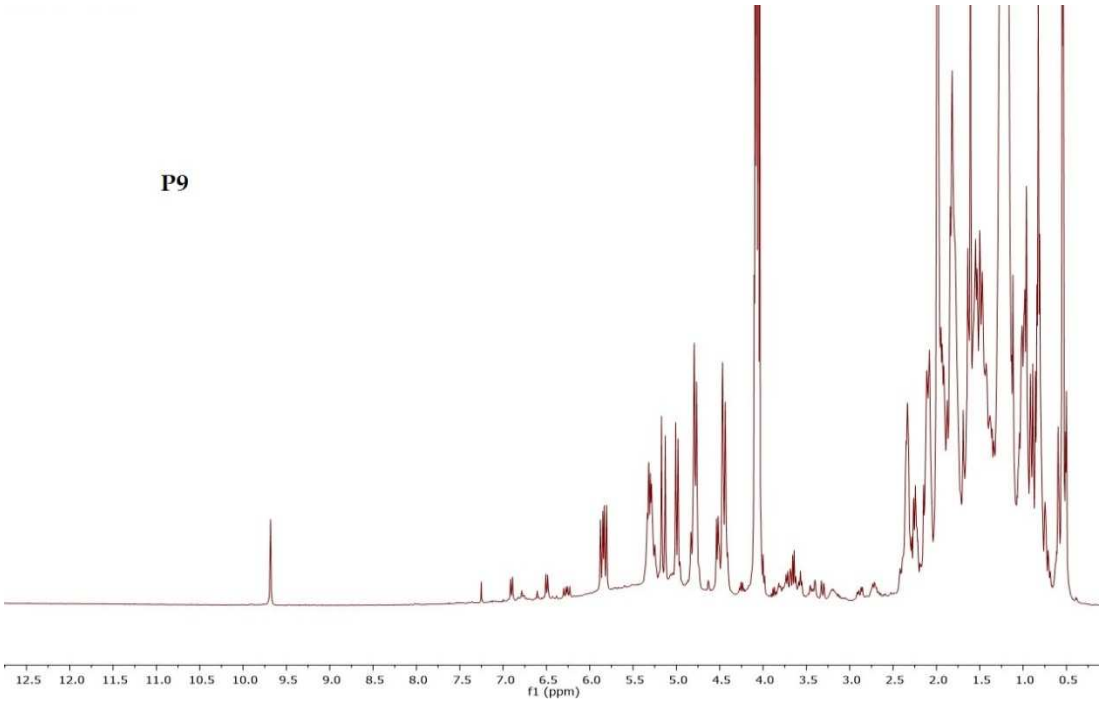


Figure 3-10 Full ^{1}H NMR spectrum (400 MHz, DMSO-d_6) (0-12.5ppm) of P9.

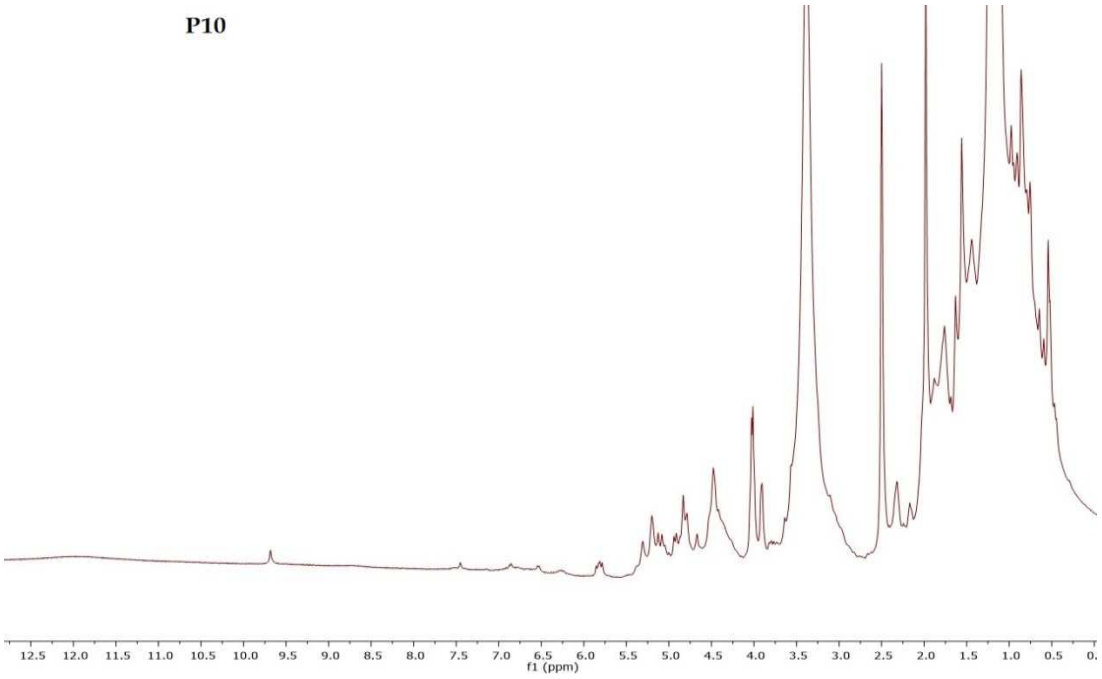


Figure 3-11 Full ^{1}H NMR spectrum (400 MHz, DMSO-d_6) (0-12.5ppm) of P10.

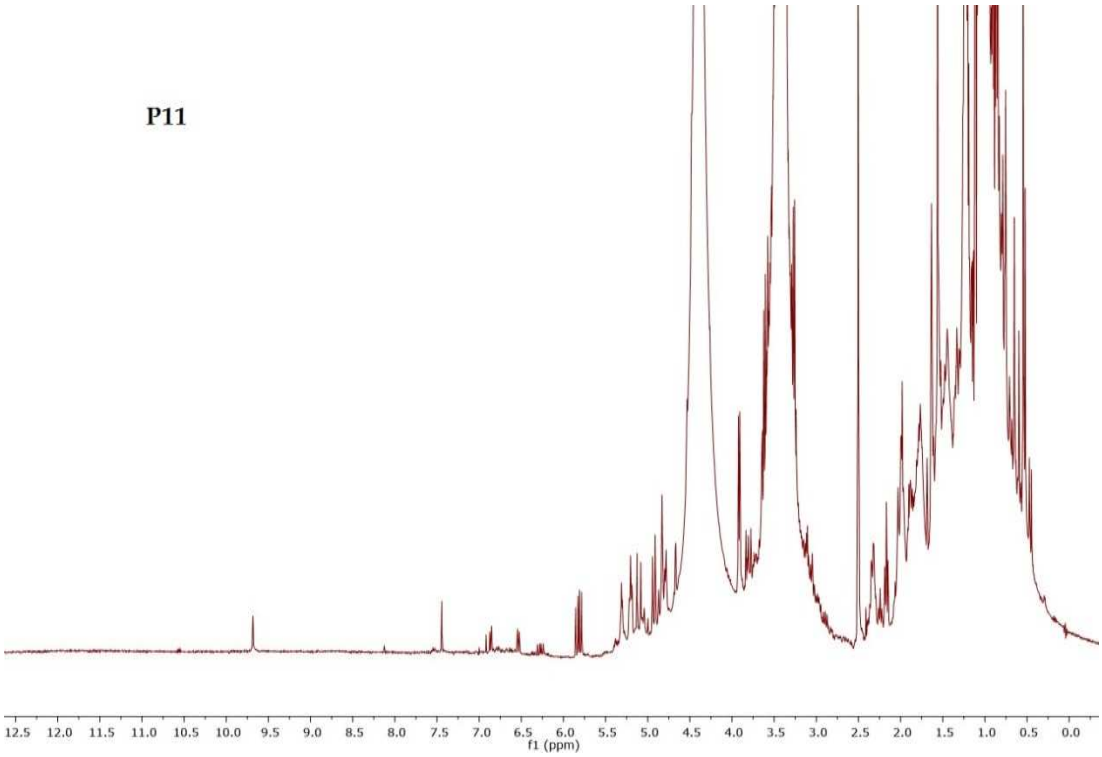


Figure 3-12 Full ^{1}H NMR spectrum (400 MHz, DMSO-d_6) (0-12.5ppm) of P11.

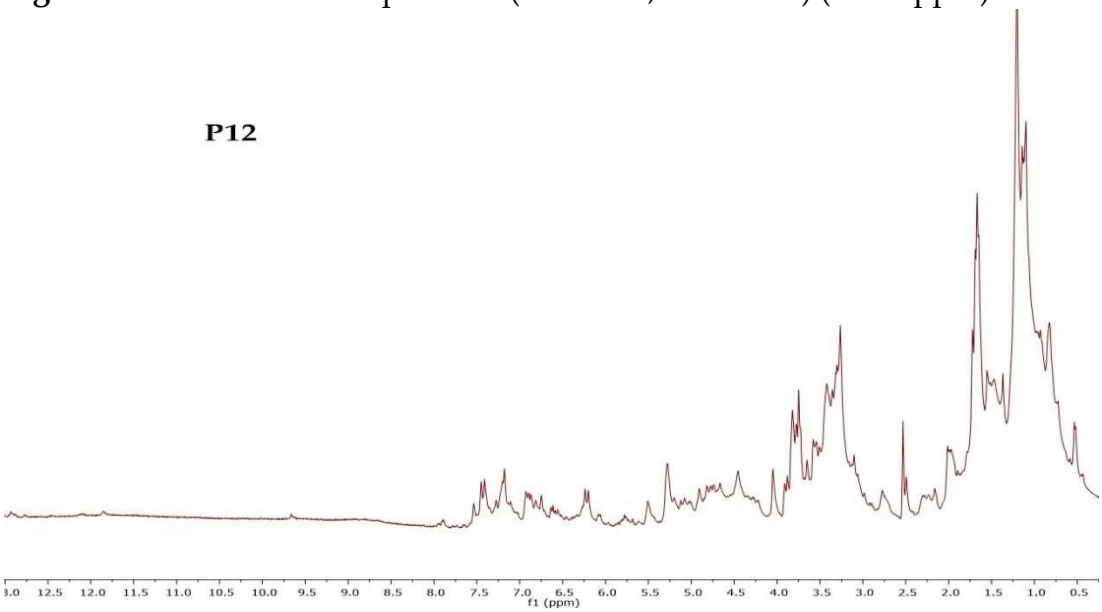


Figure 3-13 Full ^{1}H NMR spectrum (400 MHz, DMSO-d_6) (0-12.5ppm) of P12.

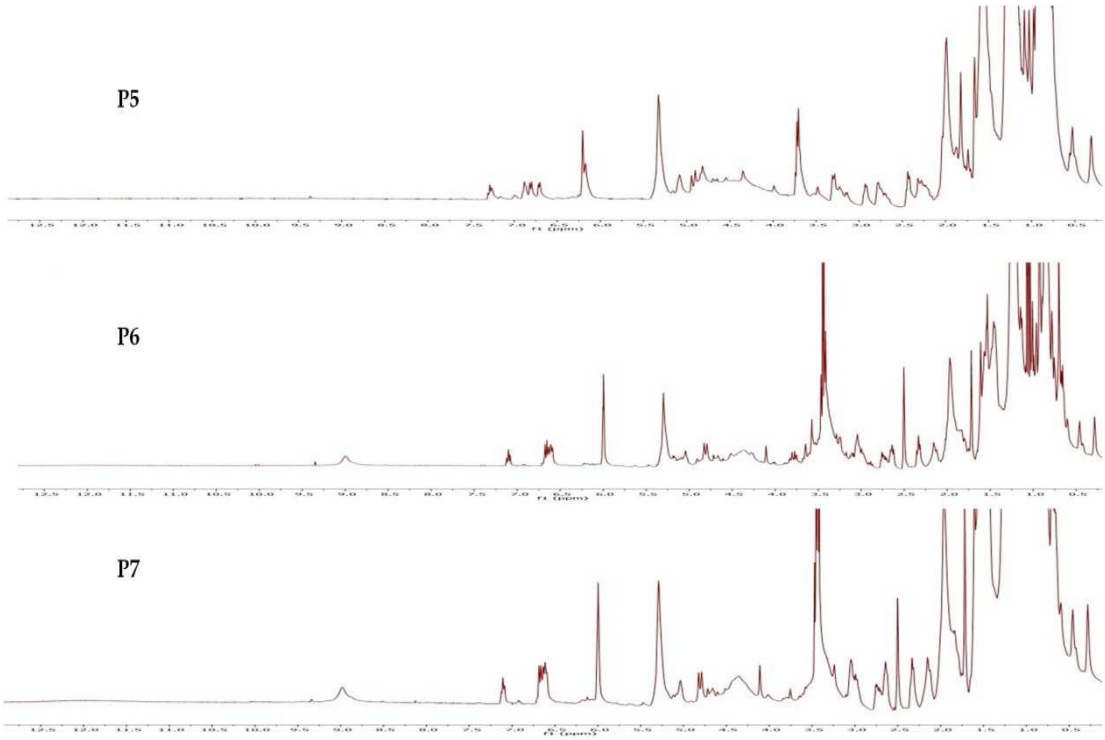


Figure 3-14 Comparisons of ^{1}H NMR spectra of P5, P6 and P7 in (0.5-12.5ppm) (400 MHz, DMSO-d_6).

3.5 Liquid chromatography high resolution mass spectroscopy (LC-HRMS)

The EEPs of samples P1-P12 were subjected to a comprehensive chemical profiling using high resolution mass spectrometry LC-HRMS under the conditions previously mentioned (section 2.5.4) and the fractions collected from open column of both (P1 and P2) and the fractions collected from MPLC as were also profiled.

The results of the analysis of LC-HRMS of P1-P12 samples can be seen in the LC-MS chromatograms in negative ion mode 0-70 min also can be seen in (Tables 3-2 to 3-13).

The components in the samples were putatively identified from accurate masses which are within 5 ppm deviation of the exact mass of the proposed elemental composition obtained by using Xcalibur. Stronger signals are

obtained in the negative ion mode in comparison with the positive mode for propolis samples and the negative ion mode gives clear signals as shown in

(Figures 3-15 to 3-26).

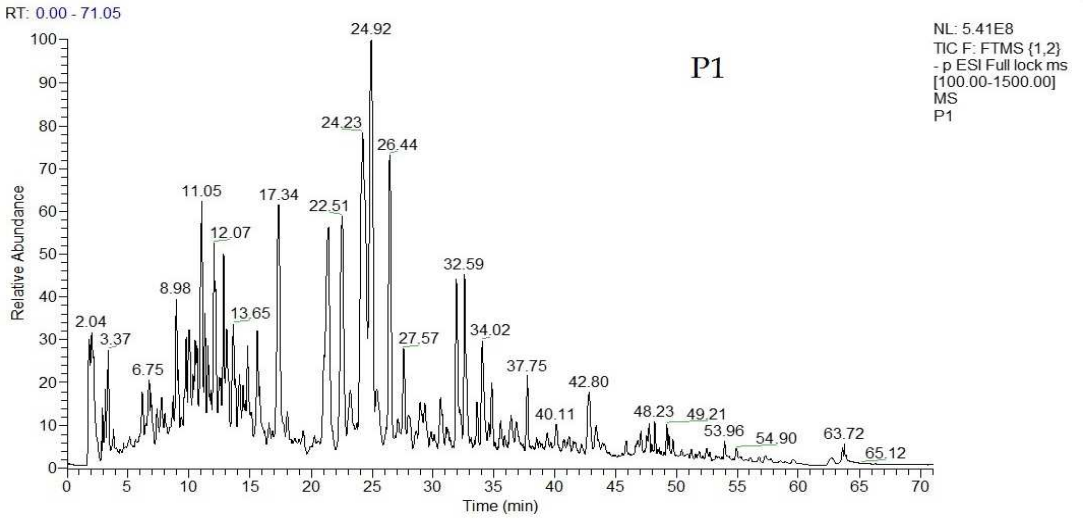


Figure 3-15 LC-MS chromatogram of P1 sample in negative ion mode 0-70 min

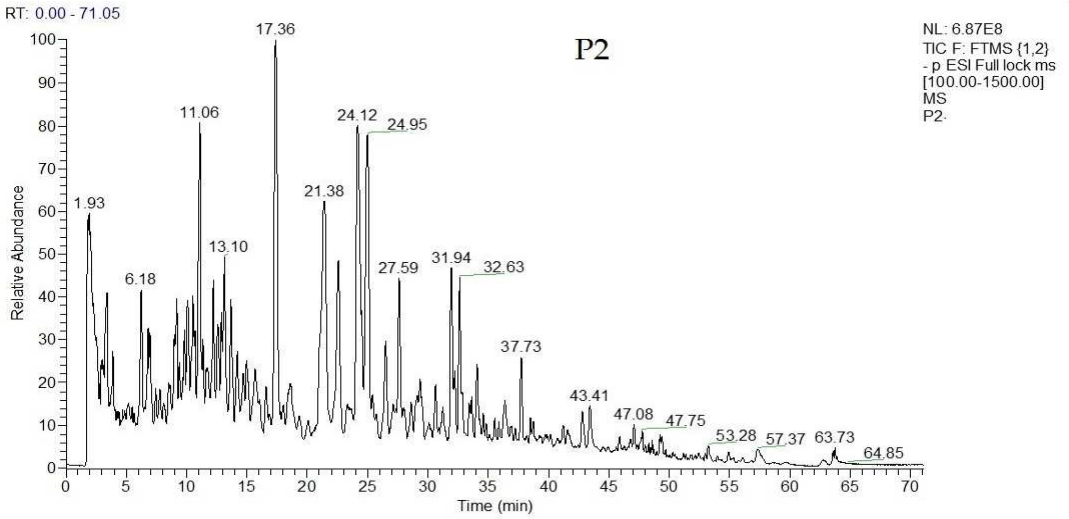


Figure 3-16 LC-MS chromatogram of P2 sample in negative ion mode 0-70 min.

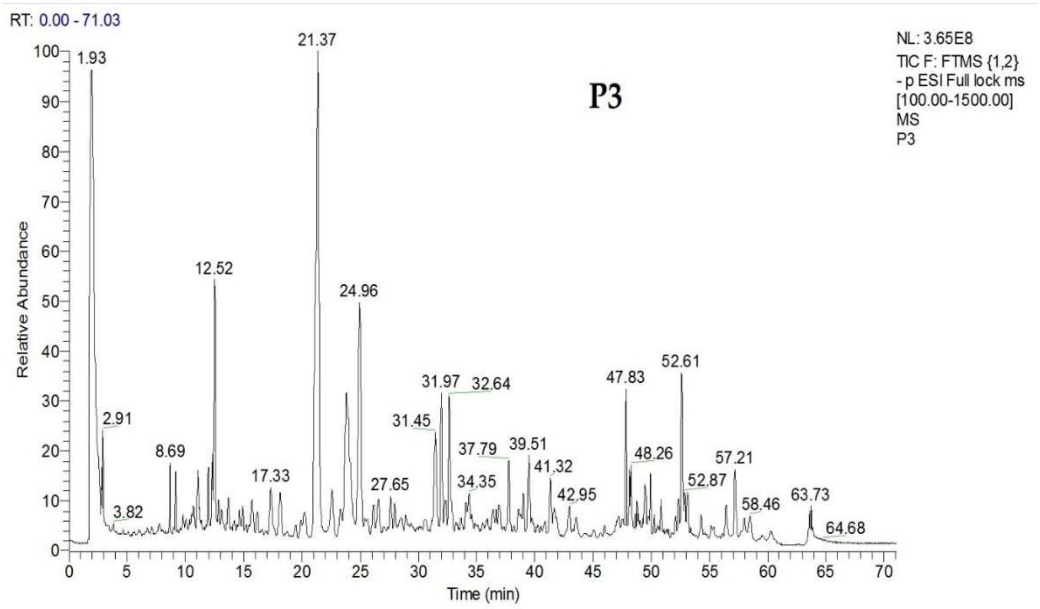


Figure 3-17 LC-MS chromatogram of P3 sample in negative ion mode 0-70 min.

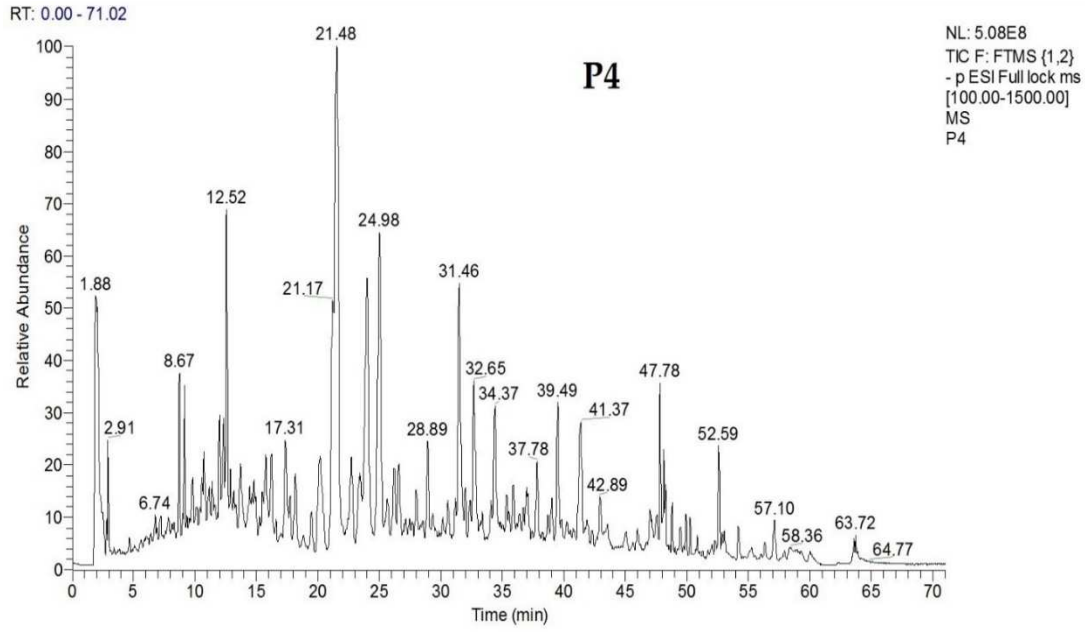


Figure 3-18 LC-MS chromatogram of P4 sample in negative ion mode 0-70 min.

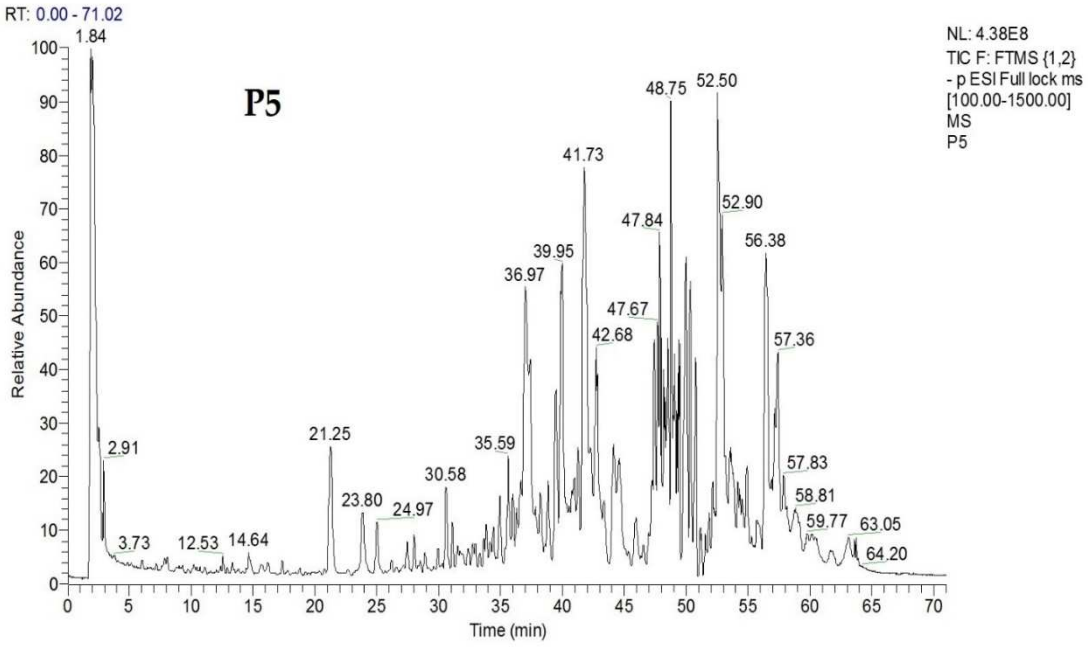


Figure 3-19 LC-MS chromatogram of P5 sample in negative ion mode 0-70 min.

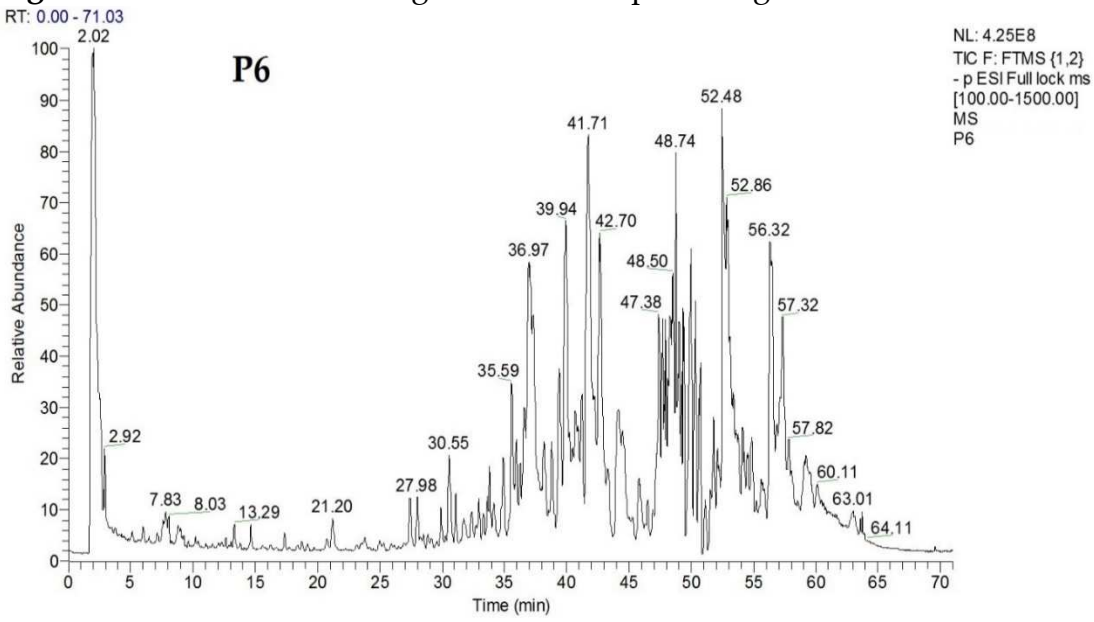


Figure 3-20 LC-MS chromatogram of P6 sample in negative ion mode 0-70 min.

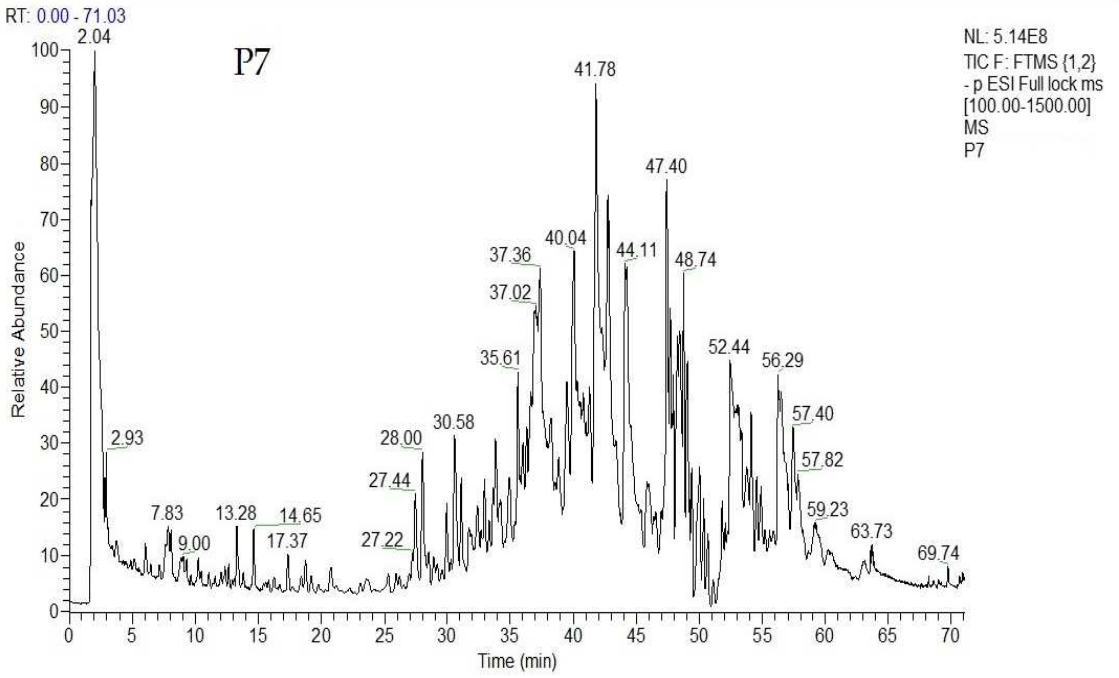


Figure 3-21 LC-MS chromatogram of P7 sample in negative ion mode 0-70 min.

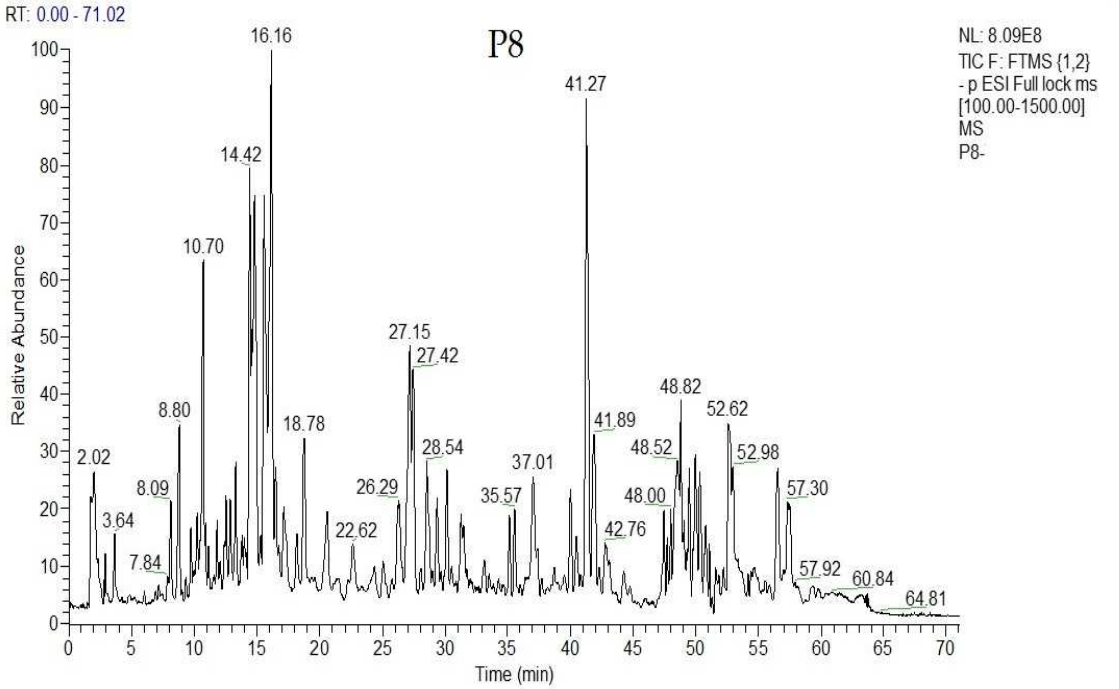


Figure 3-22 LC-MS chromatogram of P8 sample in negative ion mode 0-70 min.

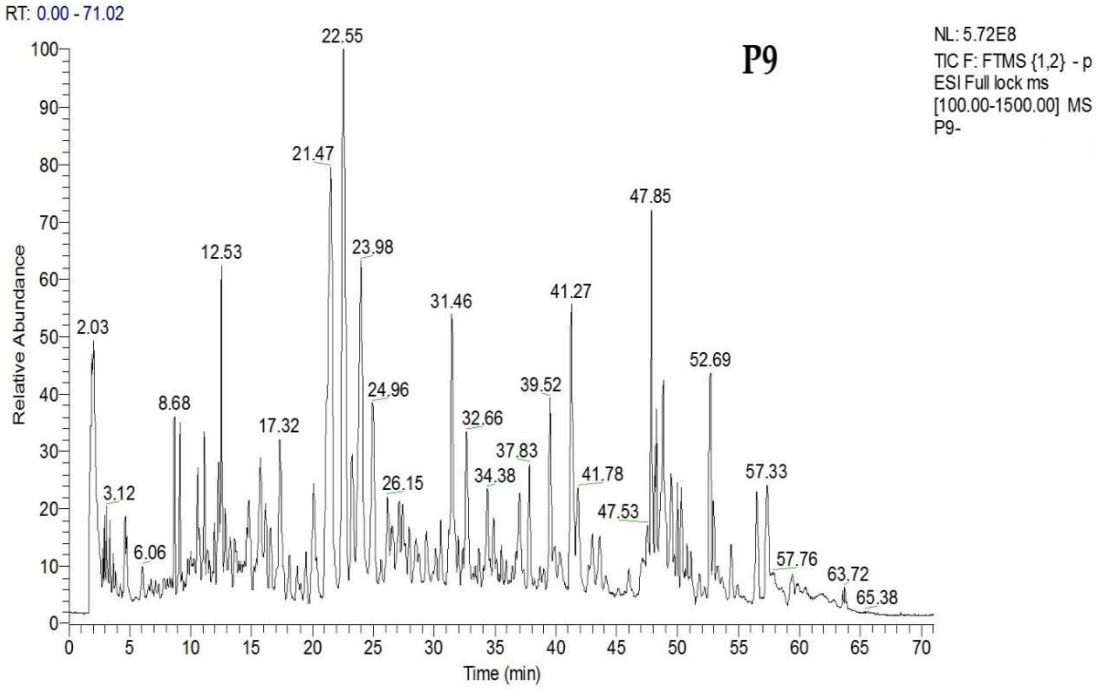


Figure 3-23 LC-MS chromatogram of P9 sample in negative ion mode 0-70 min.

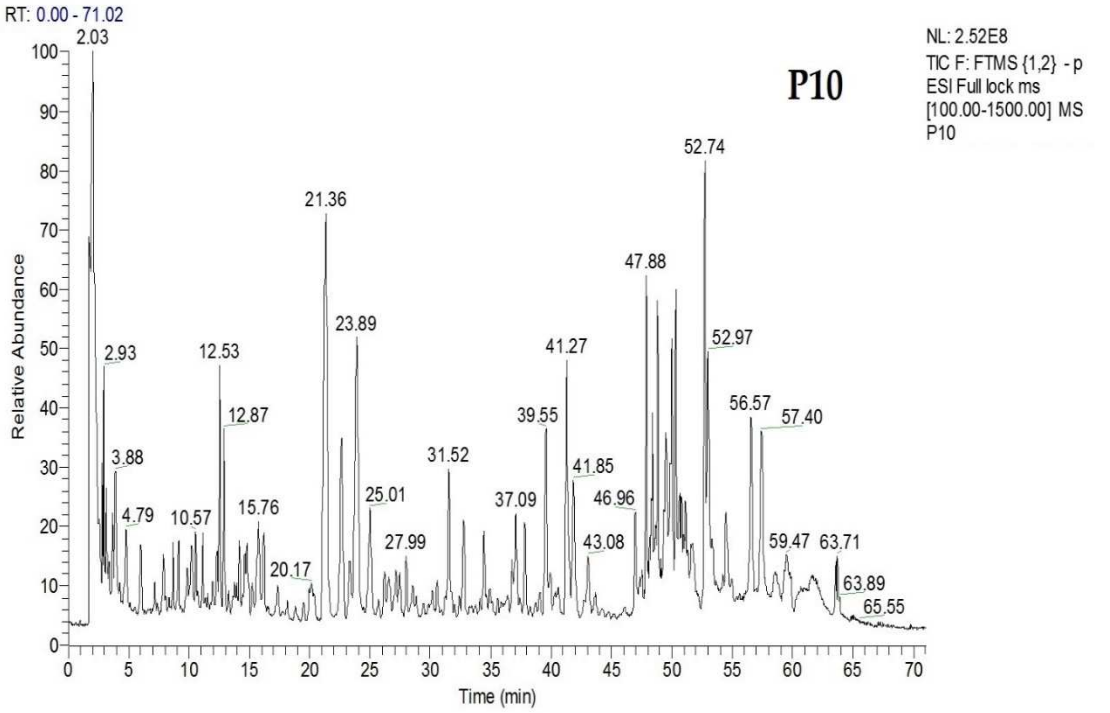


Figure 3-24 LC-MS chromatogram of P10 sample in negative ion mode 0-70 min.

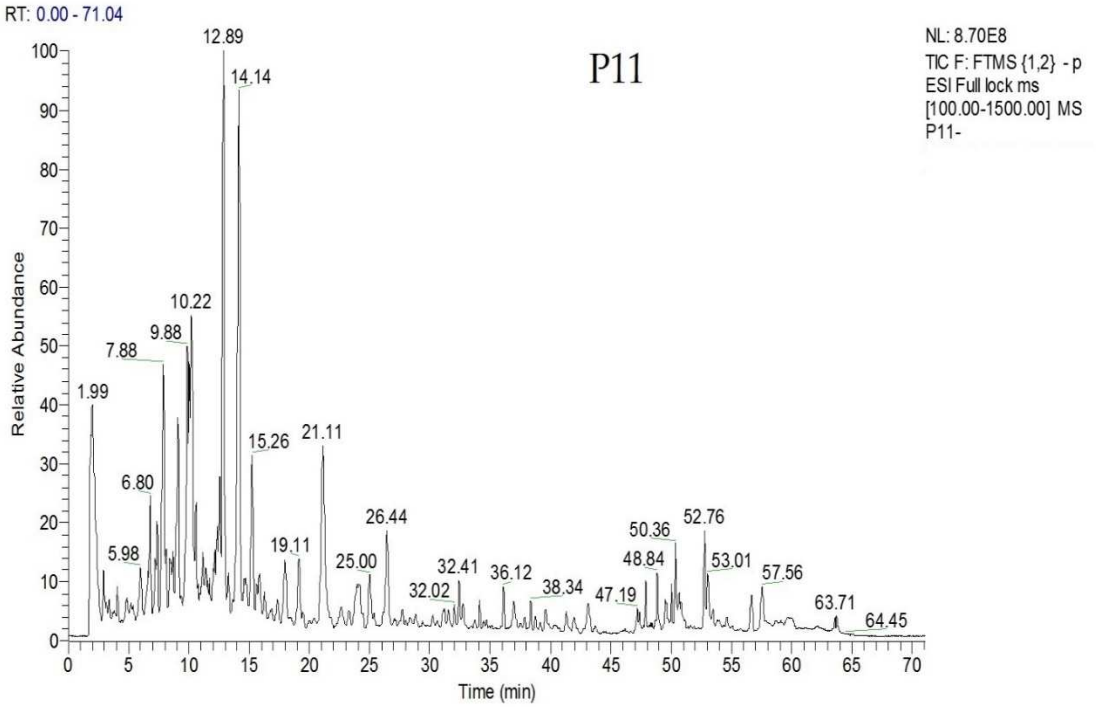


Figure 3-25 LC-MS chromatogram of P11 sample in negative ion mode 0-70 min.

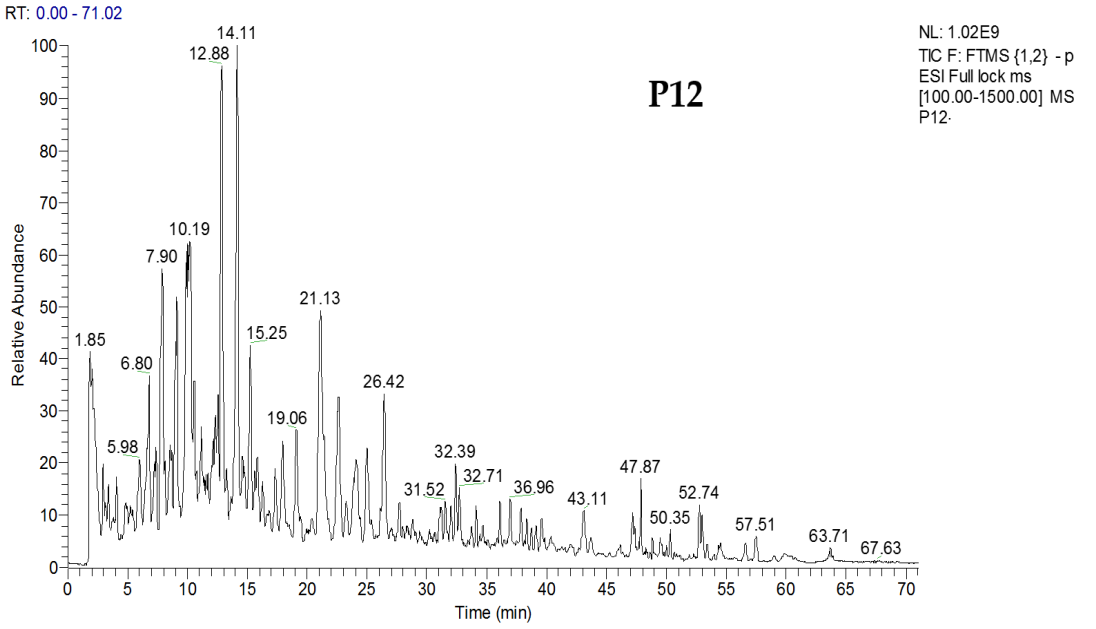


Figure 3-26 LC-MS chromatogram of P12 sample in negative ion mode 0-70 min.

Tables 3-2 to 3-13 show the retention times and elemental compositions for the most abundant negative ion peaks and obtained from the LC-MS analysis of the EEPs of P1-P12.

Table 3-2 LC-MS profile of P1 sample in negative ion masses $[M-H]^-$

RT (min)	[M-1]	Formula	RDB	Delta (ppm)	Intensity
1.9	341.11	$C_{12}H_{21}O_{11}$	2.5	-0.512	1.42E+08
6.8	359.15	$C_{20}H_{23}O_6$	9.5	1.304	1.43E+08
6.2	351.22	$C_{20}H_{31}O_5$	5.5	1.517	1.28E+08
3.4	255.11	$C_7H_{13}O_8$	1.5	0.442	1.93E+07
9.8	341.13	$C_{20}H_{21}O_5$	10.5	0.771	1.16E+08
8.9	377.17	$C_{18}H_{25}O_6$	6.5	1.24	2.61E+07
10.2	341.13	$C_{20}H_{21}O_5$	10.5	-0.05	1.15E+08
10.9	359.07	$C_{18}H_{15}O_8$	11.5	0.416	1.33E+08
11.6	331.19	$C_{20}H_{27}O4$	7.5	1.804	2.58E+00
12.1	349.21	$C_{20}H_{29}O_5$	6.5	0.896	1.06E+08
12.8	329.07	$C_{17}H_{13}O_7$	11.5	1.957	1.28E+08
12.1	349.20	$C_{20}H_{29}O_5$	6.5	-0.422	1.98E+06
15.5	255.06	$C_{15}H_{11}O_4$	10.5	-0.283	1.06E+07
15.5	319.19	$C_{19}H_{27}O_4$	6.5	-0.227	5.57E+07
15.8	395.20	$C_{21}H_{31}O_7$	6.5	0.439	1.55E+07
13.7	357.13	$C_{20}H_{21}O_6$	10.5	-0.397	5.40E+08
14.8	335.22	$C_{20}H_{31}O_4$	5.5	1.633	2.11E+07
14.1	421.09	$C_{23}H_{17}O_8$	15.5	1.091	1.09E+07
14.8	335.21	$C_{20}H_{31}O_4$	5.5	1.185	1.78E+08
15.6	333.20	$C_{20}H_{29}O_4$	6.5	1.252	7.55E+07
15.7	333.20	$C_{20}H_{29}O_4$	6.5	0.892	1.30E+08
16.6	315.197	$C_{20}H_{27}O_3$	7.5	1.244	1.06E+08
17.3	333.20	$C_{20}H_{29}O_4$	6.5	1.433	7.57E+07
19.3	345.17	$C_{20}H_{25}O_5$	8.5	0.269	5.46E+07
21.4	341.13	$C_{20}H_{21}O_5$	10.5	0.067	1.79E+08
22.6	317.21	$C_{20}H_{29}O_3$	6.5	0.857	1.70E+08
24.3	317.21	$C_{20}H_{29}O_3$	6.5	0.952	1.80E+08
24.5	319.21	$C_{20}H_{31}O_3$	5.5	0.883	5.90E+07
24.9	325.14	$C_{20}H_{21}O_4$	10.5	0.085	1.90E+08
25.2	325.14	$C_{20}H_{21}O_4$	10.5	0.761	1.80E+08
25.4	343.15	$C_{20}H_{23}O_5$	9.5	1.145	1.47E+08
26.3	315.19	$C_{20}H_{27}O_3$	7.5	1.339	1.67E+08
27.6	345.17	$C_{20}H_{25}O_5$	8.5	0.964	1.28E+08
28.9	317.21	$C_{20}H_{29}O_3$	6.5	0.763	1.30E+08
30.7	313.14	$C_{19}H_{21}O_4$	9.5	1.461	1.08E+08

(Table 3-2 Continued)

31.9	479.24	$C_{29}H_{35}O_6$	12.5	-0.359	1.28E+08
37.7	315.19	$C_{20}H_{27}O_3$	7.5	1.72	1.06E+08
31.2	341.13	$C_{20}H_{21}O_5$	10.5	0.331	1.40E+07
31.9	361.23	$C_{22}H_{33}O_4$	6.5	2.207	2.20E+07
31.1	477.22	$C_{29}H_{33}O_6$	13.5	-0.675	4.02E+06
33.6	345.20	$C_{21}H_{29}O_4$	7.5	0.774	5.40E+07
32.2	381.14	$C_{26}H_{21}O_3$	16.5	-4.376	9.16E+07
32.6	329.17	$C_{20}H_{25}O_4$	8.5	-0.16	1.80E+08
34.0	361.23	$C_{22}H_{33}O_4$	6.5	1.266	2.28E+07
34.2	345.20	$C_{21}H_{29}O_4$	7.5	1.122	2.40E+07
34.8	359.22	$C_{22}H_{31}O_4$	7.5	0.995	2.69E+07
36.8	373.20	$C_{22}H_{29}O_5$	8.50	1.08	1.29E+06
37.7	315.19	$C_{20}H_{27}O_3$	7.5	1.72	1.77E+08
37.6	351.17	$C_{26}H_{23}O$	15.5	-4.581	4.50E+06
46.7	343.28	$C_{20}H_{39}O_4$	1.5	0.953	6.67E+07
43.9	339.23	$C_{23}H_{31}O_2$	8.5	0.962	9.31E+07
43.4	301.21	$C_{20}H_{29}O_2$	6.5	0.951	1.32E+07
42.8	347.22	$C_{21}H_{31}O_4$	6.5	0.165	7.60E+06
56.8	375.29	$C_{24}H_{39}O_3$	5.5	0.244	6.71E+07

Table 3-3 LC-MS profile of P2 sample in negative ion masses $[M-H]^-$

RT (min)	[M-1]	Formula	RDB	Delta (ppm)	Intensity
2.2	215.03	$C_{12}H_7O_4$	9.5	-0.925	4.45E+07
3.4	623.19	$C_{29}H_{35}O_{15}$	12.5	0.893	1.44E+08
3.3	595.16	$C_{27}H_{31}O_{15}$	12.5	1.372	9.11E+07
6.2	351.21	$C_{20}H_{31}O_5$	5.5	0.833	4.02E+07
6.2	397.22	$C_{21}H_{33}O_7$	5.5	1.141	3.40E+07
6.2	703.44	$C_{40}H_{63}O_{10}$	9.5	-0.244	8.70E+07
6.7	359.15	$C_{20}H_{23}O_6$	9.5	0.636	2.24E+08
7.4	331.19	$C_{20}H_{27}O_4$	7.5	-0.219	1.28E+08
8.1	381.19	$C_{20}H_{29}O_7$	6.5	1.085	1.54E+07
8.4	361.16	$C_{20}H_{25}O_6$	8.5	1.74	2.23E+08
8.9	357.13	$C_{20}H_{21}O_6$	10.5	1.227	2.38E+08
9.4	365.23	$C_{21}H_{33}O_5$	5.5	1.321	2.24E+08
10.1	357.13	$C_{21}H_{33}O_5$	5.5	1.321	2.23E+08
11.1	359.07	$C_{18}H_{15}O_8$	11.5	0.249	2.29E+08
11.3	357.13	$C_{20}H_{21}O_6$	10.5	1.563	2.36E+08
11.6	343.11	$C_{19}H_{19}O_6$	10.5	1.511	2.28E+08
12.1	349.20	$C_{20}H_{29}O_5$	6.5	0.896	2.26E+07
12.1	333.20	$C_{20}H_{29}O_4$	6.5	1.523	1.55E+08
12.7	357.13	$C_{20}H_{21}O_6$	10.5	0.527	7.62E+08
13.1	359.07	$C_{18}H_{15}O_8$	11.5	1.697	9.40E+07
13.8	357.13	$C_{20}H_{21}O_6$	10.5	0.863	2.63E+08
14.1	421.09	$C_{23}H_{17}O_8$	15.5	0.165	2.55E+07
15.1	347.18	$C_{20}H_{27}O_5$	7.5	1.333	2.54E+08
15.7	349.20	$C_{20}H_{29}O_5$	6.5	0.81	2.37E+08
15.7	343.08	$C_{18}H_{15}O_7$	11.5	0.39	2.28E+08
16.6	315.19	$C_{20}H_{27}O_3$	7.5	1.339	2.26E+08
17.4	333.20	$C_{20}H_{29}O_4$	6.5	0.802	2.23E+08
21.4	341.13	$C_{20}H_{21}O_5$	10.5	1.035	2.19E+08
18.6	329.13	$C_{19}H_{21}O_5$	9.5	1.346	2.23E+08
22.5	317.21	$C_{20}H_{29}O_3$	6.5	0.952	2.31E+08
22.6	363.21	$C_{21}H_{31}O_5$	6.5	0.641	3.31E+08
24.2	317.21	$C_{20}H_{29}O_3$	6.5	1.046	2.36E+08
24.8	325.14	$C_{20}H_{21}O_4$	10.5	1.038	2.30E+08
26.7	315.19	$C_{20}H_{27}O_3$	7.5	1.434	6.68E+08
27.7	345.17	$C_{20}H_{25}O_5$	8.5	-0.281	3.40E+08
30.6	313.14	$C_{19}H_{21}O_4$	9.5	-0.008	2.28E+08

(Table 3-3 Continued)

32.4	329.17	$C_{20}H_{25}O_4$	8.5	-0.069	3.13E+08
34.2	345.20	$C_{21}H_{29}O_4$	7.5	0.514	9.73E+08
37.7	361.20	$C_{21}H_{29}O_5$	7.5	0.284	1.27E+07
43.2	437.22	$C_{21}H_{31}O_4$	6.5	0.856	3.38E+08
43.4	301.21	$C_{20}H_{29}O_2$	6.5	1.051	1.16E+08

Table 3-4 LC-MS profile of P3 sample in negative ion masses $[M-H]^-$

RT (min)	[M-1]	Formula	RDB	Delta (ppm)	Intensity
2.0	387.11	$C_{13}H_{23}O_{13}$	2.5	1.178	6.67E+07
8.1	399.39	$C_{21}H_{35}O_7$	4.5	0.534	3.38E+07
12.3	351.21	$C_{20}H_{31}O_5$	5.5	1.175	2.31E+08
17.3	333.26	$C_{20}H_{29}O_4$	6.5	1.433	7.76E+08
17.2	289.18	$C_{18}H_{25}O_3$	6.5	0.699	7.79E+08
21.1	341.13	$C_{20}H_{21}O_5$	10.5	0.419	1.30E+08
22.2	317.21	$C_{20}H_{29}O_3$	6.5	0.952	6.64E+07
23.9	317.25	$C_{20}H_{29}O_3$	6.5	1.172	1.28 E+08
24.9	325.14	$C_{20}H_{21}O_4$	10.5	1.315	2.13E+08
27.0	345.17	$C_{20}H_{25}O_5$	8.5	-0.455	1.68E+08
31.6	297.24	$C_{18}H_{33}O_3$	2.5	1.083	7.19E+06
31.9	341.13	$C_{20}H_{21}O_5$	10.5	-0.402	2.58E+06
32.6	329.17	$C_{20}H_{25}O_4$	8.5	0.964	2.13E+08
31.4	343.19	$C_{21}H_{27}O_4$	8.5	-0.037	1.70E+08
37.9	655.45	$C_{40}H_{63}O_7$	9.5	-0.713	5.45E+07
39.5	301.21	$C_{20}H_{29}O_2$	6.5	0.752	1.15E+07
39.3	661.37	$C_{40}H_{53}O_8$	14.5	1.915	5.36E+07
47.9	369.30	$C_{22}H_{41}O_4$	2.5	2.452	6.17E+07
48.1	495.31	$C_{31}H_{43}O_5$	10.5	2.061	2.93E+06
49.9	345.24	$C_{22}H_{33}O_3$	6.5	2.521	1.30E+08
50.9	579.45	$C_{38}H_{61}O_5$	8.5	1.138	1.10E+07
57.2	375.29	$C_{24}H_{39}O_3$	5.5	1.386	6.17E+07
52.2	573.45	$C_{36}H_{61}O_5$	6.5	0.436	1.81E+07
57.9	667.40	$C_{43}H_{55}O_6$	16.5	0.788	
58.5	411.38	$C_{26}H_{51}O_3$	1.5	1.263	6.03E+07

Table 3-5 LC-MS profile of P4 sample in negative ion masses $[M-H]^-$

RT (min)	[M-1]	Formula	RDB	Delta (ppm)	Intensity
1.6	225.06	$C_7 H_{13} O_8$	1.5	-0.047	3.46E+00
1.9	387.11	$C_{13} H_{23} O_{13}$	2.5	1.178	5.49E+07
5.9	187.09	$C_9 H_{15} O_4$	2.5	0.255	9.11E+06
5.9	397.22	$C_{21} H_{33} O_7$	5.5	0.839	3.60E+07
8.7	353.23	$C_{20} H_{33} O_5$	4.5	0.839	1.82E+07
8.7	399.23	$C_{21} H_{35} O_7$	4.5	0.785	2.77E+07
9.1	399.23	$C_{21} H_{35} O_7$	4.5	-0.743	3.34E+07
9.17	353.23	$C_{20} H_{33} O_5$	4.5	-0.021	1.82E+07
9.8	341.13	$C_{20} H_{21} O_5$	10.5	-0.05	9.47E+07
10.6	395.20	$C_{21} H_{31} O_7$	6.5	0.743	4.90E+06
11.9	337.23	$C_{20} H_{33} O_4$	4.5	0.288	4.91E+07
11.9	383.24	$C_{21} H_{35} O_6$	4.5	0.282	1.17E+07
11.1	359.07	$C_{18} H_{15} O_8$	11.5	1.53	8.92E+07
11.1	527.28	$C_{27} H_{43} O_{10}$	6.5	0.738	9.65E+07
12.5	351.21	$C_{20} H_{31} O_5$	5.5	0.662	3.74E+08
12.6	397.22	$C_{21} H_{33} O_7$	5.5	0.537	2.56E+07
13.9	357.13	$C_{20} H_{21} O_6$	10.5	0.023	2.28E+07
14.3	325.14	$C_{20} H_{21} O_4$	10.5	0.177	1.17E+08
14.8	247.09	$C_{14} H_{15} O_4$	7.5	0.962	3.45E+07
15.7	269.04	$C_{15} H_9 O_5$	11.5	0.161	2.79E+07
15.7	313.18	$C_{20} H_{25} O_3$	8.5	1.124	1.99E+07
16.1	283.09	$C_{17} H_{15} O_4$	10.5	-0.185	2.52E+07
16.3	345.17	$C_{20} H_{25} O_5$	8.5	1.051	3.37E+07
17.4	333.20	$C_{20} H_{29} O_4$	6.5	0.052	2.23E+05
18.0	335.22	$C_{20} H_{31} O_4$	5.5	0.439	4.57E+07
21.4	341.13	$C_{20} H_{21} O_5$	10.5	-0.49	1.58E+08
23.2	319.22	$C_{20} H_{31} O_3$	5.5	0.789	1.31E+08
24.9	325.14	$C_{20} H_{21} O_4$	10.5	1.407	1.31E+08
28.9	317.21	$C_{20} H_{29} O_3$	6.5	1.172	1.31E+08
31.5	317.21	$C_{20} H_{29} O_3$	6.5	1.046	5.22E+07
31.4	363.28	$C_{21} H_{31} O_5$	6.5	1.301	1.57E+07
32.7	329.17	$C_{20} H_{25} O_4$	8.5	0.964	1.20E+08
34.3	361.23	$C_{22} H_{33} O_4$	6.5	0.158	5.51E+08
34.3	407.24	$C_{23} H_{35} O_6$	6.5	-0.03	2.02E+07
34.3	507.16	$C_{28} H_{27} O_9$	15.5	4.425	1.05E+06
37.8	365.23	$C_{21} H_{33} O_5$	5.5	0.801	8.88E+07

(Table 3-5 Continued)

37.8	501.32	$C_{30}H_{45}O_6$	8.5	0.693	9.23E+06
38.9	383.24	$C_{21}H_{35}O_6$	4.5	1.325	3.19E+07
39.5	347.22	$C_{21}H_{31}O_4$	6.5	0.424	2.37E+07
39.5	301.21	$C_{20}H_{29}O_2$	6.5	0.354	5.25E+07
41.3	365.21	$C_{24}H_{29}O_3$	10.5	0.662	7.20E+07
41.8	633.41	$C_{40}H_{57}O_6$	12.5	0.99	2.46E+06
42.9	603.44	$C_{40}H_{59}O_4$	11.5	1.138	3.57E+06
44.9	583.23	$C_{35}H_{35}O_8$	18.5	1.404	2.58E+06
45.9	567.36	$C_{35}H_{51}O_6$	10.5	1.335	8.76E+06
45.1	583.23	$C_{35}H_{51}O_6$	10.5	1.335	2.14E+06
47.8	517.39	$C_{32}H_{53}O_5$	6.5	0.796	1.70E+07
48.1	495.31	$C_{31}H_{43}O_5$	10.5	0.954	2.23E+07
48.2	625.35	$C_{40}H_{49}O_6$	16.5	1.835	1.27E+07
48.2	635.43	$C_{40}H_{59}O_6$	11.5	1.711	2.23E+06
48.8	369.24	$C_{24}H_{33}O_3$	8.5	1.034	5.41E+07
49.9	345.24	$C_{22}H_{33}O_3$	6.5	0.642	2.06E+07
50.3	371.25	$C_{24}H_{35}O_3$	7.5	0.624	2.88E+07
50.8	397.27	$C_{26}H_{37}O_3$	8.5	1.137	2.29E+06
52.5	373.27	$C_{24}H_{37}O_3$	6.5	-0.022	5.66E+07
55.1	627.36	$C_{40}H_{51}O_6$	15.5	1.016	2.00E+07
56.3	401.32	$C_{26}H_{41}O_3$	6.5	0.851	1.81E+08
57.2	375.29	$C_{24}H_{39}O_3$	5.5	1.283	6.15E+07
63.6	505.33	$C_{33}H_{45}O_4$	11.5	2.903	2.75E+06
58.6	583.41	$C_{40}H_{55}O_3$	13.5	0.396	1.46E+06
54.2	517.38	$C_{32}H_{53}O_5$	6.5	-0.731	1.70E+07
58.1	609.35	$C_{40}H_{49}O_5$	16.5	-0.046	6.06E+06
62.3	585.43	$C_{40}H_{57}O_3$	12.5	1.18	1.18E+07

Table 3-6 LC-MS profile of P5 sample in negative ion masses $[M-H]^-$

RT (min)	[M-1]	Formula	RDB	Delta (ppm)	Intensity
2.2	215.03	$C_{12}H_7O_4$	9.5	-0.925	4.45E+07
3.4	623.19	$C_{29}H_{35}O_{15}$	12.5	0.893	1.44E+08
3.3	595.16	$C_{27}H_{31}O_{15}$	12.5	1.372	9.11E+07
6.2	351.21	$C_{20}H_{31}O_5$	5.5	0.833	4.02E+07
6.2	397.22	$C_{21}H_{33}O_7$	5.5	1.141	3.40E+07
6.2	703.44	$C_{40}H_{63}O_{10}$	9.5	-0.244	8.70E+07
6.7	359.15	$C_{20}H_{23}O_6$	9.5	0.636	2.24E+08
7.4	331.19	$C_{20}H_{27}O_4$	7.5	-0.219	1.28E+08
8.1	381.19	$C_{20}H_{29}O_7$	6.5	1.085	1.54E+07
8.4	361.16	$C_{20}H_{25}O_6$	8.5	1.74	2.23E+08
8.9	357.13	$C_{20}H_{21}O_6$	10.5	1.227	2.38E+08
9.4	365.23	$C_{21}H_{33}O_5$	5.5	1.321	2.24E+08
10.1	357.13	$C_{21}H_{33}O_5$	5.5	1.321	2.23E+08
11.1	359.07	$C_{18}H_{15}O_8$	11.5	0.249	2.29E+08
11.3	357.13	$C_{20}H_{21}O_6$	10.5	1.563	2.36E+08
11.6	343.11	$C_{19}H_{19}O_6$	10.5	1.511	2.28E+08
12.1	349.20	$C_{20}H_{29}O_5$	6.5	0.896	2.26E+07
12.1	333.20	$C_{20}H_{29}O_4$	6.5	1.523	1.55E+08
12.7	357.13	$C_{20}H_{21}O_6$	10.5	0.527	7.62E+08
13.1	359.07	$C_{18}H_{15}O_8$	11.5	1.697	9.40E+07
13.8	357.13	$C_{20}H_{21}O_6$	10.5	0.863	2.63E+08
14.1	421.09	$C_{23}H_{17}O_8$	15.5	0.165	2.55E+07
15.0	347.18	$C_{20}H_{27}O_5$	7.5	1.333	2.54E+08
15.7	349.20	$C_{20}H_{29}O_5$	6.5	0.81	2.37E+08
15.7	343.08	$C_{18}H_{15}O_7$	11.5	0.39	2.28E+08
16.6	315.19	$C_{20}H_{27}O_3$	7.5	1.339	2.26E+08
17.4	333.20	$C_{20}H_{29}O_4$	6.5	0.802	2.23E+08
21.4	341.13	$C_{20}H_{21}O_5$	10.5	1.035	2.19E+08
18.6	329.13	$C_{19}H_{21}O_5$	9.5	1.346	2.23E+08
22.5	317.21	$C_{20}H_{29}O_3$	6.5	0.952	2.31E+08
22.6	363.21	$C_{21}H_{31}O_5$	6.5	0.641	3.31E+08
24.2	317.21	$C_{20}H_{29}O_3$	6.5	1.046	2.36E+08
24.8	325.14	$C_{20}H_{21}O_4$	10.5	1.038	2.30E+08
26.7	315.19	$C_{20}H_{27}O_3$	7.5	1.434	6.68E+08
27.7	345.17	$C_{20}H_{25}O_5$	8.5	-0.281	3.40E+08
30.6	313.14	$C_{19}H_{21}O_4$	9.5	-0.008	2.28E+08
32.4	329.17	$C_{20}H_{25}O_4$	8.5	-0.069	3.13E+08

(Table 3-6 Continued)

34.2	345.20	$C_{21}H_{29}O_4$	7.5	0.514	9.73E+08
37.7	361.20	$C_{21}H_{29}O_5$	7.5	0.284	1.27E+07
43.2	437.22	$C_{21}H_{31}O_4$	6.5	0.856	3.38E+08
43.4	301.21	$C_{20}H_{29}O_2$	6.5	1.051	1.16E+08

Table 3-7 LC-MS profile of P6 sample in negative ion masses $[M-H]^-$

RT(min)	[M-1]	Formula	RDB	Delta (ppm)	Intensity
1.9	179.06	$C_6H_{11}O_6$	1.5	1.333	5.91E+07
2.1	225.06	$C_7H_{13}O_8$	1.5	0.531	1.22E+08
27.4	485.33	$C_{30}H_{45}O_5$	8.5	0.685	8.70E+07
27.4	531.33	$C_{31}H_{47}O_7$	8.5	0.758	1.39E+07
27.9	335.19	$C_{19}H_{27}O_5$	6.5	-0.171	8.59E+07
29.3	379.25	$C_{22}H_{35}O_5$	5.5	0.956	1.90E+08
30.6	403.25	$C_{24}H_{35}O_5$	7.5	0.056	4.27E+07
32.3	297.24	$C_{18}H_{33}O_3$	2.5	0.847	4.26E+06
33.8	405.26	$C_{24}H_{37}O_5$	6.5	0.598	5.54E+07
34.9	517.35	$C_{31}H_{49}O_6$	7.5	-0.372	2.36E+07
35.6	407.28	$C_{24}H_{39}O_5$	5.5	1.062	1.61E+08
37.3	385.24	$C_{24}H_{33}O_4$	8.5	0.408	1.79E+08
38.8	515.34	$C_{31}H_{47}O_6$	8.5	-0.917	3.34E+07
39.9	517.35	$C_{31}H_{4}9O_6$	7.5	0.691	3.38E+73
41.6	469.33	$C_{30}H_{45}O_4$	8.5	-0.582	8.86E+07
42.6	389.27	$C_{24}H_{37}O_4$	6.5	0.583	2.01E+08
45.9	471.35	$C_{30}H_{47}O_4$	7.5	0.672	8.60E+07
47.9	511.34	$C_{32}H_{47}O_5$	9.5	0.063	3.40E+07
47.7	317.21	$C_{20}H_{29}O_3$	6.5	-0.467	8.90E+07
48.5	439.29	$C_{28}H_{39}O_4$	9.5	1.018	1.24E+08
49.9	345.24	$C_{22}H_{33}O_3$	6.5	1.453	1.22E+08
50.3	371.26	$C_{24}H_{35}O_3$	7.5	0.059	1.82E+08
51.9	443.32	$C_{28}H_{43}O_4$	7.5	-0.413	1.35E+08
51.1	415.19	$C_{26}H_{39}O_4$	7.5	1.293	1.34E+08
52.8	373.27	$C_{24}H_{37}O_3$	6.5	0.138	2.01E+08
54.8	361.27	$C_{23}H_{37}O_3$	5.5	-0.023	1.35E+08
56.4	401.31	$C_{26}H_{41}O_3$	6.5	0.153	1.38E+08
57.3	375..29	$C_{24}H_{39}O_3$	5.5	1.63	2.30E+08
59.4	431.35	$C_{28}H_{47}O_3$	5.5	-0.368	1.39E+08
60.1	389.31	$C_{25}H_{41}O_3$	5.5	1.032	1.95E+08

Table 3-8 LC-MS profile of P7 sample in negative ion masses $[M-H]^-$

RT(min)	[M-1]	Formula	RDB	Delta (ppm)	Intensity
6.0	301.03	$C_{15}H_9O_7$	11.5	0.778	9.61E+06
7.0	225.06	$C_7H_{13}O_8$	1.5	0.042	1.26E+06
7.1	669.21	$C_{34}H_{37}O_{14}$	16.5	-0.013	5.32E+06
7.1	598.25	$C_{36}H_{38}O_8$	18	-1.047	1.81E+06
7.8	582.26	$C_{36}H_{38}O_7$	18	-1.428	2.55E+07
8.0	628.26	$C_{37}H_{40}O_9$	18	-2.851	6.68E+06
8.9	629.22	$C_{32}H_{37}O_{13}$	14.5	2.282	2.46E+06
8.9	577.26	$C_{30}H_{41}O_{11}$	10.5	-0.2	3.45E+06
8.9	575.26	$C_{30}H_{39}O_{11}$	11.5	-0.27	3.65E+06
10.3	285.04	$C_{15}H_9O_6$	11.5	0.557	2.20E+08
12.1	251.12	$C_{14}H_{19}O_4$	5.5	-0.129	2.03E+07
13.3	279.16	$C_{16}H_{23}O_4$	5.5	-0.761	3.94E+07
14.6	287.22	$C_{16}H_{31}O_4$	1.5	-0.532	3.88E+07
16.2	311.22	$C_{18}H_{31}O_4$	3.5	1.373	3.27E+07
17.4	307.19	$C_{18}H_{27}O_4$	5.5	0.643	2.24E+07
18.8	291.16	$C_{17}H_{23}O_4$	6.5	-0.18	3.89E+07
19.3	503.33	$C_{30}H_{47}O_6$	7.5	1.008	4.03E+07
20.8	533.34	$C_{31}H_{49}O_7$	7.5	-0.557	3.07E+07
25.3	519.36	$C_{31}H_{51}O_6$	6.5	-0.775	3.28E+07
26.2	503.33	$C_{30}H_{47}O_7$	7.5	1.365	4.22E+07
32.5	533.34	$C_{31}H_{49}O_7$	7.5	-0.0859	9.55E+07
34.6	531.33	$C_{31}H_{47}O_7$	8.5	-1.085	4.22E+07
34.4	485.32	$C_{30}H_{45}O_5$	8.5	-0.0778	8.06E+07
34.5	549.37	$C_{32}H_{53}O_7$	6.5	-1.178	1.19E+07
35.1	549.32	$C_{34}H_{45}O_6$	12.5	1.416	2.85E+07
36.7	549.30	$C_{30}H_{45}O_9$	8.5	2.456	5.51E+07
37.8	357.24	$C_{23}H_{33}O_3$	7.5	1.591	5.51E+07
37.7	403.24	$C_{24}H_{35}O_5$	7.5	2.573	1.43E+08
37.9	485.32	$C_{30}H_{45}O_5$	8.5	2.839	9.61E+06
37.8	531.33	$C_{31}H_{47}O_7$	8.5	-2.573	1.26E+06
37.8	521.38	$C_{31}H_{53}O_6$	5.5	1.75	5.32E+06
37.1	471.23	$C_{27}H_{35}O_7$	10.5	4.1312	1.81E+06
37.9	575.35	$C_{33}H_{51}O_8$	8.5	-2.21	2.55E+07
40.5	519.33	$C_{31}H_{51}O_6$	7.5	2.285	6.68E+06
42.6	517.35	$C_{31}H_{45}O_6$	7.5	0.575	2.46E+06
44.3	517.35	$C_{31}H_{45}O_6$	7.5	0.111	3.45E+06
46.9	517.35	$C_{31}H_{49}O_6$	7.5	-1.667	3.65E+06

(Table 3-8 Continued)

46.7	517.35	$C_{31}H_{45}O_6$	7.5	1.406	2.20E+08
50.4	553.35	$C_{34}H_{49}O_6$	10.5	3.3	2.03E+07
48.9	469.33	$C_{30}H_{45}O_4$	8.5	3.316	3.94E+07
48.8	515.33	$C_{31}H_{47}O_6$	8.5	3.469	3.88E+07
46.7	551.33	$C_{34}H_{47}O_6$	11.5	-3.958	3.27E+07
46.7	553.31	$C_{34}H_{49}O_6$	10.5	-3.98	2.24E+07
46.6	555.30	$C_{36}H_{49}O_6$	15.5	-3.567	3.89E+07
52.4	555.33	$C_{33}H_{47}O_7$	10.5	-1.687	4.03E+07
48.2	553.29	$C_{36}H_{41}O_6$	16.5	2.895	3.07E+07
51	551.33	$C_{34}H_{47}O_6$	11.5	-4.503	3.28E+07
45.8	549.32	$C_{34}H_{45}O_6$	12.5	-0.696	4.22E+07
39.1	487.34	$C_{30}H_{47}O_5$	7.5	0.632	9.55E+07
38.0	545.34	$C_{32}H_{49}O_7$	8.5	1.032	4.22E+07
38.0	543.33	$C_{32}H_{47}O_7$	9.5	2.542	8.06E+07
38.0	553.31	$C_{33}H_{45}O_7$	11.5	-0.88	1.19E+07
38.2	403.24	$C_{24}H_{35}O_5$	7.5	-2.0628	2.85E+07
38.2	357.24	$C_{23}H_{33}O_3$	7.5	1.171	5.51E+07
38.01	471.23	$C_{27}H_{35}O_7$	10.5	4.131	5.51E+07
38.03	473.23	$C_{33}H_{49}O_3$	19.5	2.265	1.43E+08
38.8	517.35	$C_{31}H_{49}O_6$	7.5	2.382	9.61E+06
39.4	478.34	$C_{30}H_{47}O_5$	7.5	0.312	1.28E+08
39.4	355.22	$C_{23}H_{31}O_3$	8.5	1.3	3.90E+07
40.3	487.34	$C_{30}H_{47}O_5$	6.5	0.2989	2.37E+08
40.6	335.18	$C_{19}H_{27}O_5$	6.5	1.7423	2.16E+08
41.0	517.35	$C_{31}H_{49}O_6$	7.5	-0.146	2.39E+08
42.7	501.35	$C_{31}H_{49}O_5$	7.5	1.839	1.20E+08
48.8	469.32	$C_{30}H_{45}O_4$	8.5	0.334	4.01E+07
45.2	467.31	$C_{30}H_{43}O_4$	9.5	0.593	2.53E+07
51.2	387.25	$C_{24}H_{35}O_4$	7.5	0.705	1.44E+08
37	485.32	$C_{30}H_{45}O_5$	8.5	0.046	2.14E+08
50.4	371.25	$C_{24}H_{35}O_3$	7.5	1.702	2.37E+08
51.8	443.31	$C_{28}H_{43}O_4$	7.5	-0.481	1.49E+08
56.5	401.30	$C_{26}H_{41}O_3$	6.5	0.079	2.11E+08
57.7	375.29	$C_{24}H_{39}O_3$	5.5	1.63	1.57E+08
60.2	389.30	$C_{25}H_{41}O_3$	5.5	1.417	2.44E+08

Table 3-9 LC-MS profile of P8 sample in negative ion masses $[M-H]^-$

RT (min)	[M-1]	Formula	RDB	Delta (ppm)	Intensity
1.9	225.06	$C_7 H_{13} O_8$	1.5	-0.758	2.28E+07
2.9	387.17	$C_{18} H_{27} O_9$	5.5	1.406	7.81E+08
3.7	359.08	$C_{18} H_{15} O_8$	11.5	1.53	9.90E+07
3.6	179.04	$C_9 H_7 O_4$	6.5	0.212	1.15E+07
8.8	285.08	$C_{16} H_{13} O_5$	10.5	0.432	4.01 +E8
10.2	285.04	$C_{15} H_9 O_6$	11.5	0.452	2.94E+08
10.7	271.06	$C_{15} H_{11} O_5$	10.5	0.75	4.03E+08
9.8	269.05	$C_{15} H_9 O_5$	11.5	-0.508	4.08E+08
9.3	267.07	$C_{16} H_{11} O_4$	11.5	-0.008	4.08E+08
10.2	285.04	$C_{15} H_9 O_6$	11.5	0.452	3.43E+08
10.8	299.09	$C_{16} H_{11} O_6$	11.5	0.23	5.31E+08
11.8	283.06	$C_{16} H_{11} O_5$	11.5	0.188	3.28E+08
12.5	315.05	$C_{16} H_{11} O_7$	11.5	0.679	4.92E+08
12.9	397.23	$C_{24} H_{29} O_5$	10.5	1.165	5.47E+07
13.3	329.07	$C_{17} H_{13} O_7$	11.5	1.045	5.21E+08
13.8	397.21	$C_{24} H_{29} O_5$	10.5	0.938	7.74E+07
14.4	247.11	$C_{14} H_{15} O_4$	7.5	-0.131	3.86E+08
14.7	269.08	$C_{16} H_{13} O_4$	10.5	0.475	4.05E+08
15.6	255.07	$C_{15} H_{11} O_4$	10.5	0.541	3.78.+E8
16.6	313.07	$C_{17} H_{13} O_6$	11.5	0.059	5.46E+08
16.5	285.08	$C_{16} H_{13} O_5$	10.5	0.748	3.87E+08
17.3	283.06	$C_{16} H_{11} O_5$	11.5	0.294	3.63E+08
18.2	311.22	$C_{18} H_{31} O_4$	3.5	1.148	4.86E+08
18.8	295.11	$C_{18} H_{15} O_4$	11.5	0.365	2.74E+08
20.2	327.09	$C_{18} H_{15} O_6$	11.5	0.24	4.47E+08
20.6	327.09	$C_{18} H_{15} O_6$	11.5	0.24	5.43E+08
22.3	317.21	$C_{18} H_{15} O_6$	11.5	0.24	4.34E+08
24.4	417.13	$C_{25} H_{21} O_6$	15.5	0.739	2.50E+08
25.1	475.14	$C_{27} H_{23} O_8$	16.5	-0.149	6.53E+07
25.0	325.09	$C_{20} H_{21} O_4$	10.5	1.407	5.10E+08
27.3	381.21	$C_{24} H_{29} O_4$	10.5	1.41	2.43E+08
28.5	381.21	$C_{24} H_{29} O_4$	10.5	1.095	4.43E+08
30.1	355.12	$C_{20} H_{19} O_6$	11.5	-0.089	1.38E+08
30.5	431.15	$C_{24} H_{27} O_5$	11.5	-0.145	7.90E+07
35.1	423.12	$C_{26} H_{31} O_5$	11.5	1.259	8.31E+08
35.2	423.22	$C_{26} H_{31} O_5$	11.5	1.046	8.30E+07
37.1	471.35	$C_{30} H_{47} O_4$	7.5	-0.686	6.50E+07

(Table 3-9 Continued)

39.4	417.35	$C_{30}H_{47}O_4$	7.5	-0.749	6.50E+07
41.3	365.21	$C_{24}H_{29}O_3$	10.5	-0.241	4.85E+08
41.8	469.33	$C_{30}H_{45}O_4$	8.5	0.718	6.53E+07
42.9	389.27	$C_{24}H_{37}O_4$	6.5	1.2	2.49E+08
43.4	387.25	$C_{24}H_{35}O_4$	7.5	1.206	2.45E+08
44.9	387.25	$C_{24}H_{35}O_4$	7.5	0.974	3.31E+08
44.8	599.36	$C_{33}H_{51}O_7$	8.5	0.738	1.14E+00
44.1	501.23	$C_{31}H_{33}O_6$	15.5	1.173	2.25E+06
45.3	373.27	$C_{24}H_{37}O_3$	6.5	0.058	5.08E+08
47.5	365.21	$C_{24}H_{29}O_3$	10.5	0.827	4.20E+08
48.8	369.24	$C_{24}H_{33}O_3$	8.5	1.684	1.48E+08
49.9	345.12	$C_{22}H_{33}O_3$	6.5	0.932	1.08E+08
48.5	403.32	$C_{24}H_{39}O_3$	5.5	0.884	2.04E+08
49.5	415.51	$C_{26}H_{39}O_4$	7.5	1.365	1.01E+08
50.4	371.38	$C_{26}H_{39}O_4$	7.5	1.365	1.55E+08
50.8	379.27	$C_{26}H_{37}O_3$	8.5	0.055	4.48E+08
51.1	415.29	$C_{26}H_{39}O_4$	7.5	1.149	1.01E+08
52.8	373.27	$C_{24}H_{37}O_4$	6.5	0.018	3.28E+08
52.9	347.26	$C_{24}H_{37}O_3$	6.5	1.049	1.11E+08
56.6	401.31	$C_{26}H_{41}O_3$	6.5	0.851	4.83E+08
54.2	571.44	$C_{36}H_{59}O_5$	7.5	0.913	2.26E+06
55.6	375.25	$C_{24}H_{39}O_3$	5.5	0.884	2.34E+07
56.6	401.31	$C_{33}H_{51}O_7$	8.5	0.631	2.41E+08
57.4	375.29	$C_{24}H_{39}O_3$	5.5	1.39	4.20E+08

Table 3-10 LC-MS profile of P9 sample in negative ion masses $[M-H]^-$

RT (min)	[M-1]	Formula	RDB	Delta (ppm)	Intensity
1.9	225.21	$C_7 H_{13} O_8$	1.5	0.664	6.65E+07
4.7	503.18	$C_{22} H_{31} O_{13}$	7.5	-0.803	4.46E+07
9.1	399.24	$C_{21} H_{35} O_7$	4.5	0.459	4.17E+07
10.6	527.29	$C_{27} H_{43} O_{10}$	6.5	-0.532	8.31E+07
11.2	527.29	$C_{27} H_{43} O_{10}$	6.5	0.852	8.34E+07
12.5	351.22	$C_{20} H_{31} O_5$	5.5	1.175	9.31E+07
13.3	329.07	$C_{17} H_{13} O_7$	11.5	1.319	1.38E+07
13.6	357.13	$C_{20} H_{21} O_6$	10.5	1.059	1.45E+07
14.8	253.05	$C_{15} H_9 O_4$	11.5	0.229	6.67E+07
14.7	433.09	$C_{24} H_{17} O_8$	16.5	-0.556	1.56E+07
15.8	269.05	$C_{15} H_9 O_5$	11.5	0.273	2.62E+08
16.2	313.07	$C_{17} H_{13} O_6$	11.5	0.73	4.59E+07
17.2	333.21	$C_{20} H_{29} O_4$	6.5	0.142	2.48E+08
18.8	295.12	$C_{18} H_{15} O_4$	11.5	-0.482	2.37E+00
20.1	347.19	$C_{20} H_{27} O_5$	7.5	1.074	3.28E+08
21.5	341.14	$C_{20} H_{21} O_5$	10.5	0.771	3.25E+08
22.6	317.18	$C_{20} H_{29} O_3$	6.5	1.046	2.23E+08
23.9	319.23	$C_{20} H_{31} O_3$	5.5	0.225	2.23E+08
24.9	325.14	$C_{20} H_{21} O_4$	10.5	-0.192	2.58E+08
26.1	413.21	$C_{24} H_{29} O_6$	10.5	-0.368	7.55E+07
27.6	381.21	$C_{24} H_{29} O_4$	10.5	0.124	1.71E+08
29.3	381.21	$C_{24} H_{29} O_4$	10.5	0.124	1.82E+08
31.4	343.19	$C_{21} H_{27} O_4$	8.5	-0.124	1.58E+08
32.7	329.18	$C_{20} H_{25} O_4$	8.5	0.782	2.50E+08
35.6	423.22	$C_{26} H_{31} O_5$	11.5	0.101	3.14E+07
37.0	471.35	$C_{30} H_{47} O_4$	7.5	0.672	3.21E+07
39.6	301.02	$C_{20} H_{29} O_2$	6.5	-0.377	7.78E+07
40.4	381.21	$C_{24} H_{29} O_4$	10.5	1.567	1.65E+08
41.3	365.21	$C_{24} H_{29} O_3$	10.5	1.018	2.12E+08
43.6	301.22	$C_{20} H_{29} O_2$	6.5	0.852	1.28E+08
46.0	567.37	$C_{35} H_{51} O_6$	10.5	0.912	2.26E+06
47.3	619.44	$C_{40} H_{59} O_5$	11.5	0.939	9.47E+07
48.6	403.32	$C_{26} H_{43} O_3$	5.5	1.243	7.67E+07
49.9	345.24	$C_{22} H_{33} O_3$	6.5	-0.053	1.35E+08
50.4	371.26	$C_{24} H_{35} O_3$	7.5	1.432	1.34E+08
50.8	397.28	$C_{26} H_{37} O_3$	8.5	0.91	1.34E+08
51.1	415.19	$C_{26} H_{39} O_4$	7.5	1.149	7.57E+07

(Table 3-10 Continued)

52.6	373.28	$C_{24}H_{37}O_3$	6.5	0.808	1.05E+08
52.9	347.26	$C_{22}H_{35}O_3$	5.5	-0.225	6.61E+07
54.4	417.31	$C_{26}H_{41}O_4$	6.5	-0.175	7.23E+07
56.5	401.23	$C_{26}H_{41}O_3$	6.5	0.851	7.64E+07
57.3	375.29	$C_{24}H_{39}O_3$	5.5	1.044	1.33E+08

Table 3-11 LC-MS profile of P10 sample in negative ion masses $[M-H]^-$

RT (min)	[M-1]	Formula	RDB	Delta (ppm)	Intensity
2.01	225.06	$C_7H_{13}O_8$	1.5	-0.625	6.07E+07
2.9	353.09	$C_{16}H_{17}O_9$	8.5	1.458	3.73E+07
3.2	335.08	$C_{16}H_{15}O_8$	9.5	0.177	2.92E+06
3.9	301.01	$C_{16}H_{15}O_8$	9.5	0.177	2.96E+07
4.7	503.18	$C_{22}H_{31}O_{13}$	7.5	-0.684	2.47E+07
6.1	371.14	$C_{17}H_{23}O_9$	6.5	0.982	4.03E+07
7.9	315.05	$C_{16}H_{11}O_7$	11.5	1.346	3.25E+07
8.7	353.23	$C_{20}H_{33}O_5$	4.5	1.366	3.71E+07
9.1	399.24	$C_{21}H_{35}O_7$	4.5	-0.217	2.21E+07
10.2	329.07	$C1_7H_{13}O_7$	11.5	1.501	3.90E+07
11.1	527.29	$C_{20}H_{33}O_5$	4.5	1.366	2.06E+07
12.5	351.22	$C_{20}H_{31}O_5$	5.5	1.004	3.85E+07
12.9	315.16	$C_{19}H_{23}O_4$	8.5	1.356	4.14E+07
14.5	315.16	$C_{19}H_{23}O_4$	8.5	1.166	5.45E+07
14.7	253.05	$C_{19}H_{23}O_4$	8.5	1.166	2.34E+07
15.8	269.05	$C_{15}H_9O_5$	11.5	-0.396	1.67E+07
16.2	313.07	$C_{17}H_{13}O_6$	11.5	1.114	9.73E+06
16.2	345.17	$C_{20}H_{25}O_5$	8.5	1.312	1.02E+07
21.3	319.23	$C_{20}H_{31}O_3$	5.5	0.006	5.25E+00

Table 3-12 LC-MS profile of P11 sample in negative ion masses $[M-H]^-$

RT (min)	[M-1]	Formula	RDB	Delta (ppm)	Intensity
1.9	225.06	$C_7H_{13}O_8$	1.5	-0.491	1.09E+08
5.9	247.09	$C_{14}H_{15}O_4$	7.5	-0.495	3.00E+07
6.7	331.15	$C_{19}H_{23}O_5$	8.5	-0.021	6.20E+08
7.9	315.05	$C_{16}H_{11}O_7$	11.5	0.394	6.13E+08
9.1	317.06	$C_{16}H_{13}O_7$	10.5	-0.177	8.87E+08
9.9	299.05	$C_{16}H_{11}O_6$	11.5	-0.272	2.34E+08
10.3	329.06	$C_{17}H_{13}O_7$	11.5	0.286	5.93E+08
12.7	315.16	$C_{19}H_{23}O_4$	8.5	1.579	6.07E+08
14.9	315.16	$C_{19}H_{23}O_4$	8.5	-0.071	6.15E+08
15.3	315.16	$C_{19}H_{23}O_4$	8.5	0.785	6.00E+08
18.0	287.16	$C_{18}H_{23}O_3$	7.5	0.704	6.06E+08
19.1	357.17	$C_{21}H_{25}O_5$	9.5	1.352	2.79E+08
21.2	357.17	$C_{21}H_{25}O_5$	9.5	0.932	5.90E+08
25.1	325.14	$C_{20}H_{21}O_4$	10.5	0.638	6.12E+08
25	439.14	$C_{24}H_{23}O_8$	13.5	-4.875	2.26E+06
29.1	299.16	$C_{19}H_{23}O_3$	8.5	0.776	6.21E+08
30.2	355.11	$C_{20}H_{19}O_6$	11.5	1.291	3.84E+06
32.4	587.33	$C_{37}H_{47}O_6$	14.5	0.864	3.99E+07
36.9	345.20	$C_{21}H_{29}O_4$	7.5	1.035	2.09E+07
38.3	441.26	$C_{27}H_{37}O_5$	9.5	-0.334	1.23E+07
41.6	469.33	$C_{30}H_{45}O_4$	8.5	-1.029	1.51E+07
41.2	365.21	$C_{24}H_{29}O_3$	10.5	-0.159	1.09E+08
43.1	347.22	$C_{21}H_{31}O_4$	6.5	1.144	2.27E+07
43.1	569.38	$C_{35}H_{53}O_6$	9.5	-0.321	1.85E+07
53.4	419.32	$C_{30}H_{43}O$	9.5	-4.863	2.33E+07
56.7	401.30	$C_{26}H_{41}O_3$	6.5	0.926	3.27E+07
57.5	375.29	$C_{24}H_{39}O_3$	5.5	1.044	7.82E+07

Table 3-13 LC-MS profile of P12 sample in negative ion masses $[M-H]^-$

RT (min)	[M-1]	Formula	RDB	Delta (ppm)	Intensity
2.1	377.08	$C_{18}H_{17}O_9$	10.5	-3.992	8.23E+07
2.1	225.06	$C_7H_{13}O_8$	1.5	-0.18	9.43E+07
7.7	315.05	$C_{16}H_{11}O_7$	11.5	-0.209	7.E6E8
6.7	331.15	$C_{19}H_{23}O_5$	8.5	1.458	7.73E+07
8.9	317.06	$C_{16}H_{13}O_7$	10.5	0.896	7.36E+08
9.9	299.05	$C_{16}H_{11}O_6$	11.5	0.965	2.84E+08
10.1	331.15	$C_{19}H_{23}O_5$	8.5	1.549	7.54E+08
12.5	315.16	$C_{19}H_{23}O_4$	8.5	0.119	7.81E+08
14.1	315.16	$C_{19}H_{23}O_4$	8.5	1.356	7.73E+08
15.7	329.06	$C_{17}H_{13}O_7$	11.5	1.866	7.66E+08
17.9	287.16	$C_{18}H_{23}O_3$	7.5	0.356	1.50E+08
19.1	357.11	$C_{21}H_{25}O_5$	9.5	0.932	3.23E+08
21.2	357.17	$C_{21}H_{25}O_5$	9.5	1.184	7.44E+08
22.5	317.21	$C_{20}H_{29}O_3$	6.5	0.952	7.70E+08
23.2	509.27	$C_{27}H_{41}O_9$	7.5	-0.424	2.05E+07
25.1	325.14	$C_{20}H_{21}O_4$	10.5	1.13	3.36E+08
32.4	587.33	$C_{37}H_{47}O_6$	14.5	-0.277	8.70E+07
33.7	345.20	$C_{21}H_{29}O_4$	7.5	-0.297	4.80E+07
34.7	381.22	$C_{21}H_{33}O_6$	5.5	1.464	3.08E+07
38.8	587.33	$C_{37}H_{47}O_6$	14.5	-0.277	8.74E+07
42.9	559.34	$C_{36}H_{47}O_5$	13.5	1.094	2.56E+07
43.3	569.38	$C_{35}H_{53}O_6$	9.5	1.295	9.16E+07
47.8	341.27	$C_{20}H_{37}O_4$	2.5	0.841	6.95E+07
57.5	375.29	$C_{24}H_{39}O_3$	5.5	0.884	6.00E+07

3.5.1 Metabolomic chemical profiling

Tables 3-2 to 3-13 present the results obtained from the general profiling of the EEPs of P1-P12 by LC-MS in negative mode. The prevalence on C_{20} compounds indicates the presence of both diterpenes or isoprenylated flavonoids and lignans and C_{15} to C_{18} compounds with a high degree of unsaturation indicates the presence of flavonoids. The general profiles of twelve Libyan propolis samples as demonstrated in the above tables contain many chemical compounds. In order to make a fingerprint comparison the mass spectrometry data were extracted by using m/z Mine to produce an Excel spreadsheet of masses, which could be matched to metabolites in the Dictionary of Natural Products database. In order to get an overview of the differences in the chemical composition of the different propolis samples PCA was used. This method reduces the hundreds of variables (chemical compounds) in the samples to a few principle components using the covariance within the data, essentially mapping the samples according to how close they are in composition.

Figure 3-27 shows a PCA based on the 300 features with the highest mean intensity across the samples selected by m/z mine from the negative ion data which included 30020 features. A principal component analysis (PCA) model was constructed which, in combination with hierarchical cluster analysis (HCA), divided the samples into five groups the data was Pareto scaled and log transformed. HCA was used to divide the samples into 5 groups. Only samples P5, P6 and P7 from the Southeast of the country gave a distinct group and they were grouped close to the sample from the Southwest P8. The samples from the coast did not divide according to longitude and the two groups P3, P4, P9, P10 and P11, P12 are composed of samples from the East and

West of the country and although P10 was collected from a site close to P11 and P12 it seems to be quite different in composition.

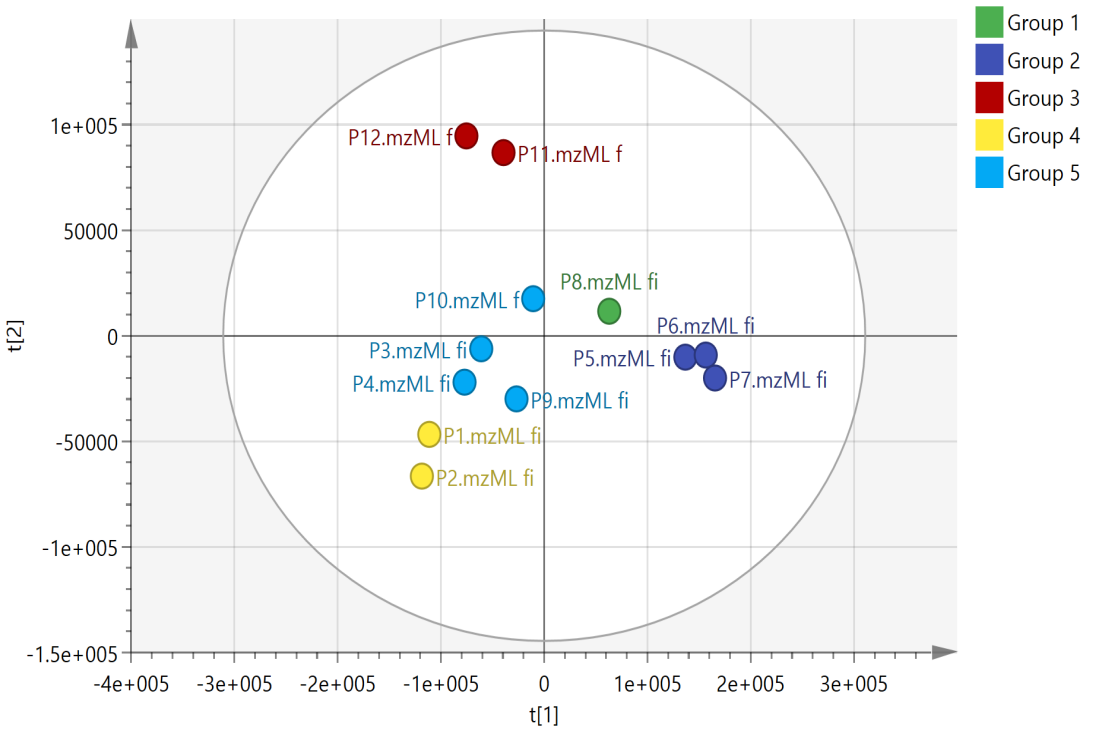


Figure 3-27 PCA with HCA based on the 300 most intense features obtained in negative ion mode for the 12 propolis samples R^2X 0.689, Q^2 0.48.

In general, most of the compounds of interest are detected in negative ion mode since. Data extraction of the positive ion data yielded 6363 features of which the top 500 by mean intensity were selected for PCA. The groupings obtained were similar to those obtained with the negative ion data as shown in Figure 3-28.

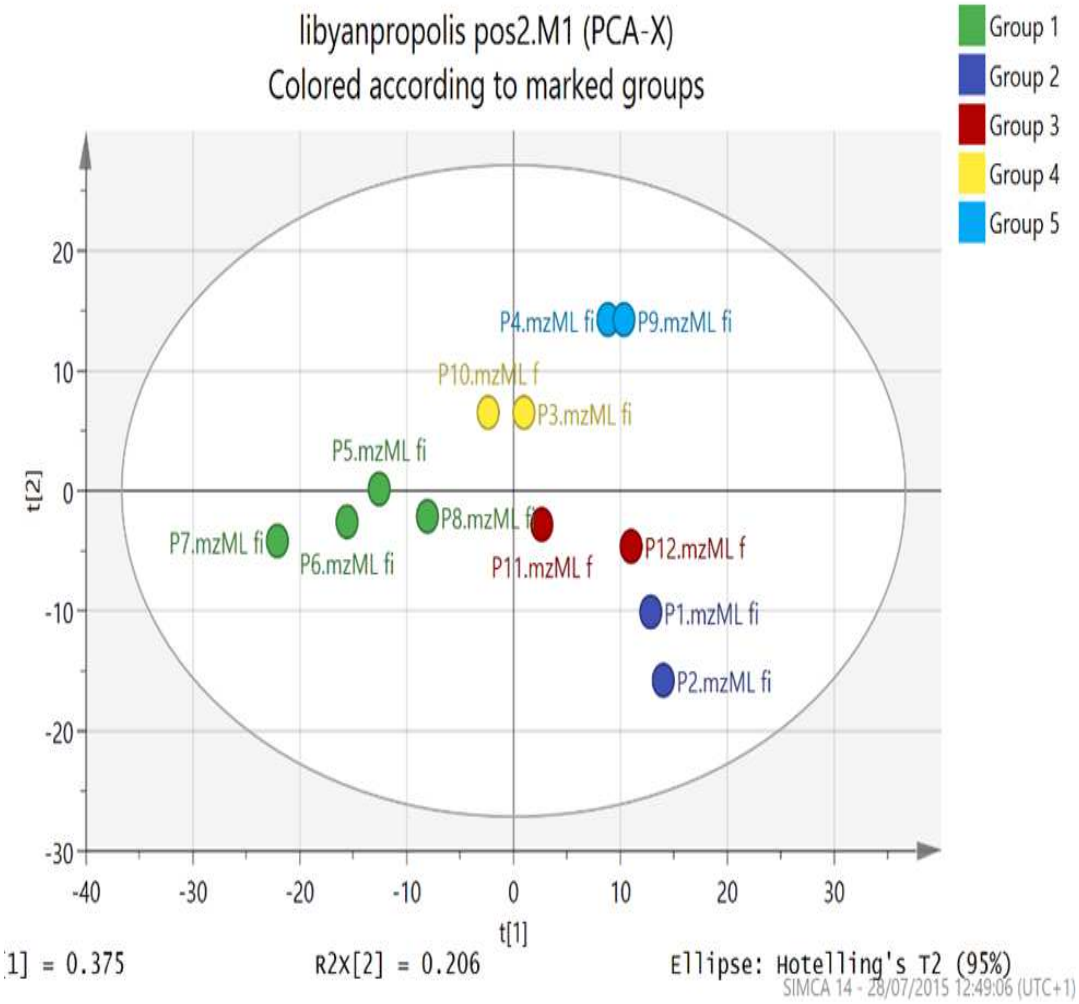


Figure 3-28 PCA with HCA based on the 500 most intense features obtained in positive mode for the 12 propolis samples

Table 3-14 lists the ten most important variables (VIPs) used in the PCA classification of the samples for each group (Galindo-Prieto et al., 2014). However, in the PCA model shown in Figure 3-14 the most important variables for the classification of the samples were compounds with m/z values in negative ion mode at m/z 325.145 and m/z 341.140. All masses deviated by < 2 ppm from the proposed elemental composition. As can be seen in Table 3-14 the DNP often has many isomeric possibilities matching the elemental compositions of the VIPs. A compound with m/z 373.27 in negative ion mode has the highest importance for locating P5, P6 and P7 and is present in smaller amounts in the other samples. Samples P11 and P12 from the West of the

country also have clear marker compounds whereas the weightings of the VIPs in samples P3, P4, P9 and P10 are weak indicating that these samples have an average composition.

Table 3-14 The top ten VIPs composed of negative ion masses measured to within 2 ppm of that of the proposed elemental compositions responsible locating the groups shown in (Figure 3-27) .

[m/z]	Rt (min)	Molecular formula	Isomers in DNP	VIP
		P1/P2		
325.145	25	$C_{20}H_{22}O_4$	109	10.1
341.14	21.4	$C_{20}H_{22}O_5$	188	8.2
595.168	3.3	$C_{27}H_{32}O_{15}$	52	3.5
329.067	11.1	$C_{17}H_{14}O_7$	163	3.5
325.145	10.1	$C_{20}H_{22}O_4$	109	2.8
331.155	17.7	$C_{19}H_{24}O_5$	106	2.7
341.14	13.6	$C_{20}H_{22}O_5$	188	2.6
341.103	10.5	$C_{19}H_{18}O_6$	127	2.5
421.093	14.2	$C_{23}H_{18}O_8$	16	2.4
357.135	29	$C_{20}H_{22}O_6$	236	2.2
301.217	43.6	$C_{20}H_{30}O_2$	598	2
381.192	8.2	$C_{20}H_{30}O_7$	184	2
		P5/P6/P7		
373.275	52.6	$C_{24}H_{38}O_3$	45	13
401.306	56.4	$C_{26}H_{42}O_3$	27	10.1
375.291	57.4	$C_{24}H_{40}O_3$	27	9.3
369.244	48.8	$C_{24}H_{34}O_3$	11	7.1
385.239	36.8	$C_{24}H_{34}O_4$	45	5.7
345.244	50	$C_{22}H_{34}O_3$	127	5
387.254	49.1	$C_{24}H_{36}O_4$	51	4.8
347.259	52.9	$C_{22}H_{36}O_3$	114	4.6
361.275	54.9	$C_{23}H_{38}O_3$	24	4.2
371.26	50.3	$C_{24}H_{36}O_3$	21	3.6
		P11/P12		
289.108	10.6	$C_{16}H_{18}O_5$	81	13.5
333.171	7.4	$C_{19}H_{26}O_5$	94	12.7
247.098	6	$C_{14}H_{16}O_4$	108	8.6
333.171	8.1	$C_{19}H_{26}O_5$	81	8.2
587.339	32.4	$C_{37}H_{48}O_6$	3	7.7
645.308	19.5	$C_{38}H_{46}O_9$	8	7.7
373.166	15.3	$C_{21}H_{26}O_6$	107	7.7
331.155	8.6	$C_{19}H_{24}O_5$	93	7.2

***(Table 3-14** Continued)*

313.145	15.2	$C_{19}H_{22}O_4$	117	6.4
349.166	6.6	$C_{19}H_{26}O_6$	102	6.1
		P3/P4/P9/P10		
619.438	47.9	$C_{40}H_{60}O_5$	1	1.5
347.187	19.5	$C_{20}H_{28}O_5$	531	1.2
763.551	57.9	$C_{48}H_{76}O_7$	1	1
707.474	9.1	$C_{40}H_{68}O_{10}$	5	0.9
763.551	53.6	$C_{48}H_{76}O_7$	1	0.8
369.301	47.9	$C_{22}H_{42}O_4$	8	0.7
397.223	12.4	$C_{21}H_{34}O_7$	26	0.7
333.207	14	$C_{20}H_{30}O_4$	776	0.6
379.213	20	$C_{21}H_{32}O_6$	52	0.6
187.098	6	$C_9H_{16}O_4$	31	0.5
		P8		
401.306	56.4	$C_{26}H_{42}O_3$	27	4.2
345.244	50	$C_{22}H_{34}O_3$	127	4.2
371.26	50.3	$C_{24}H_{36}O_3$	21	4.1
375.291	57.4	$C_{24}H_{40}O_3$	27	3.7
369.244	48.8	$C_{24}H_{34}O_3$	11	3.4
255.066	15.6	$C_{15}H_{12}O_4$	145	3.2
347.259	52.9	$C_{22}H_{36}O_3$	114	3.1
373.275	52.6	$C_{24}H_{38}O_3$	45	2.9
375.291	55.6	$C_{24}H_{40}O_3$	27	2.6
397.275	50.8	$C_{26}H_{38}O_3$	23	2.1

The twelve propolis sample (P1-P12) extracts were tested for their activity against *P. falciparum, T. brucei*, *L. donovani, C. fasciculata* and *M. marinum*. In addition, cellular toxicity assays were carried out using mammalian cells.

3.6 Biological profiling of crude Libyan Propolis

All the twelve crude propolis samples were subjected to biological screening using various biological assays and results exhibited some significant activity against different parasites such; *Trypanosoma brucei, Leishmania donovani, Plasmodium falciparum, Crithidia fasiculata* and *Mycobacterium marinum*. Also the activity against a range some pathogenic Gram-positive and Grame negative bacteria was assessed. In addition, the cytotoxicity activity was assessed for all extracts against a mammalian cell line (Section 3.5.7).

3.6.1 *In vitro* Antitrypanosomal assay of (P1-P12) samples against *T. brucei*

Tests of all EEP of P1-P12 were carried out in order to determine *in vitro* activity against the bloodstream form of *Trypanosoma brucei* using the well-established AlamarBlue® 96 well microplate assay as described in (section 2.6.1). For testing 1.0 mg of each sample was dissolved in 100 µl of DMSO to give a concentration of 10 mg/ml. The test samples were initially screened at a two concentrations of 10 and 20µg/ml (Table 3-15), compared with pentamidine as drug control. All the samples exhibited variable EC_{50} values of activity against trypanosome as compared to EC_{50} values ± SD (µg/ml) of pentamidine 0.00139± 0.000109 µg/ml samples P2 and P1 showed the strongest significant activity at 1.65 ± 0.042 and 8.24 ± 0.79 µg/ml respectively.

Table 3-15 EC_{50} values of Antitrypanosomal activity of P1-P12 samples against *T. brucei* (s427)

Propolis samples	**EC_{50} (μg/ml) ± SD**
P1	8.24 ± 0.797
P2	1.65 ± 0.0472
P3	13.49 ± 0.835
P4	32.29 ± 2.44
P5	18.25 ± 0.928
P6	13.7 ± 0.0416
P7	14.67 ± 0.837
P8	12.66 ± 0.603
P9	20.65 ± 2.88
P10	43.36 ± 3.45
P11	34.16 ± 4.75
P12	24.41 ± 0.541
Pentamidine (μg/ml)	0.00139± 0.000109

N.B.; Pentamidine; positive control, **(SD)**; standard deviation. (EC_{50}) values; (μg/mL), *(n=3)*

According the average EC_{50} results of all propolis samples are shown in Table 3-15, both P1 and P2 samples showed strong activity against *T. brucei* (s427). Therefore, both samples P1 and P2 further investigated against the wild resistant strain to pentamidine, (B48) which were adapted from Lister 427WT by *in vitro* exposure to pentamidine so that they have lost both main drug transporters, HAPT1 and TbAT1, and thus are highly resistant to pentamidine as displayed in Table 3-16.

All the samples exhibited variable EC_{50} values for anti-trypanosomal activity as compared to EC_{50} values ± SD (μg/ml) of the drug control pentamidine 5.433μM (1.84μg/ml). P2 sample showed stronger activity with an EC_{50} value of 0.964 μg/ml (Table 16).

Table 3-16 EC_{50} values of Antitrypanosomal activity of P1-P12 samples against *T. brucei* wild resistant strain to pentamidine (B48).

Propolis samples	**EC_{50} (μg/ml)**	**[a]SEM**	**[b]RF**
P1	5.615	0.1085	0.68
P2	0.9645	0.00685	0.58
Pentamidine	1.8497	0.389	2466

N.B.; Pentamidine; Drug control, [a](SEM); Averages and standard error of mean, [b](RF); Resistance factor,

3.6.2 In vitro Antileishmanial assay of (P1-P12) samples.against *L. donovani*

Tests of the EEPs of P1-P12 were carried out in order to determine *in vitro* activity against a *L. donovani* as described in (section 2.6.2) compared with Amphotericin B as drug control (Table 3-17). All the samples exhibited variable IC_{50} values for antileishmanial activity as compared to IC_{50} values ±SD (μg/ml) for Amphotericin B as drug control 0.024 ± 0.06 (n= 2) μM. samples P2 and P1 showed the highest activity again.

Table 3-17 IC_{50} values of Antileishmanial activity of (P1-P12) samples.against *L. donovani*.

Propolis Samples	**IC_{50} (µg/ml) ± SD**
P1	2.6 ± 0.18
P2	3.2 ± 0.27
P3	11.7± 1.1
P4	13.4± 1.3
P5	3.5 ± 0.73
P6	10.2 ± 0.26
P7	5.4 ± 0.06
P8	14.0 ± 1.6
P9	8.56 ± 0.34
P10	11.0 ± 0.60
P11	7.2 ± 0.27
P12	16.2 ± 1.5
Amphotericin B (µg/ml)	0.024 ± 0.06

N.B.; Amphotericin B; drug control, SD; standard deviation of IC_{50} (µg/ml) (n=3) ± SD.

3.6.3 *In vitro* Antimalarial assay of (P1-P12) samples.against *P. falciparum.*

Investigation of all the EEPs of P1-P12 was carried out in order to determine *in vitro* activity against a *P. falciparum* as described in (section 2.6.3), compared to chloroquine as drug control (Table 3-18). All the samples exhibited a variable range of antimalarial activities with EC_{50} values ±SD (μg/ml) compared with chloroquine 7.47 ± 0.07nM (0.00239 μg/ml). P2 and P1 had the highest activity again but were no where near as active as chloroquine.

Table 3-18 EC_{50} values of Antimalarial activity of P1-P12 samples against *P. falciparum*.

Propolis Samples	EC_{50} (μg/ml) ± SD
P1	6.05 ± 0.10
P2	3.42 ± 0.96
P3	8.57 ± 0.52
P4	14.53 ± 0.48
P5	28.73 ± 1.75
P6	42.81 ± 1.04
P7	10.64 ± 2.07
P8	53.61 ± 5.22
P9	8.66 ± 0.84
P10	22.68 ± 1.43
P11	14.66 ± 0.23
P12	14.19 ± 0.57
Chloroquine	2.389 ± 0.022

N.B.; Chloroquine; Drug control, SD; standard deviation of EC_{50} (μg/ml) (n=3) ± SD.

3.6.4 *In vitro* Anticrithidia assay of (P1-P12) samples against *C. fasciculata*

Assessments of all the EEPs of P1-P12 were carried out in order to determine activity against a *Crithidia fasciculata* as described in (section 2.6.4), compared with two drug controls pentamidine and menadione (Table 3-19). The P1-P12 samples exhibited a range of anti-crithidial activity with EC_{50} values (6.5-

75.5μg/ml) compared with pentamidine 18.3±3.8 μg/ml. P2, P1, P3 and P8 showed quite high activity although not as high as that on menadione.

Table 3-19 EC_{50} values of Anti- Crithidia activity of P1-P12 samples against *C. fasiculata*

Propolis Samples	**EC_{50} (μg/ml) ± SD**
P1	11.6 ± 2.1
P2	6.5 ± 1.3
P3	12.2 ± 2.6
P4	64.1 ± 5.3
P5	33.7 ± 2.3
P6	25.3 ± 3.2
P7	16.7 ± 1.1
P8	12.4 ± 1.6
P9	34.9 ± 3.8
P10	78.5 ± 8.6
P11	46.3 ± 5.5
P12	36.1 ± 3.8
Menadione	0.137 ± 0.034
Pentamidine	6.22 ± 1.293

N.B.; Pentamidine and Menadione; drug controls, SD; Standard deviation, (n=3).

3.6.5 *In vitro* Antimycobacterial assay of (P1-P12) samples.against *M. marinum*

Tests of all the EEPs of P1-P12 were carried out in order to determine activity against *M. marinum* as described in (section 2.6.5), compared with gentamycin as the drug control (Table 3-20). The samples exhibited a range of antimycobacterial activity as compared to MIC values of Gentamycin 6.25 μg/ml. The MIC value of samples P2 was much lower than those of the rest of the samples.

Table 3-20 MICs values of Anti-mycobacterium activity of P1-P12 samples against *M. marinum*.

Propolis Samples	MICs (μg/ml)
P1	62.5
P2	7.8
P3	18.75
P4	31.25
P5	50
P6	50
P7	50
P8	75
P9	>100
P11	>100
P12	>100
Gentamycin	6.25

N.B.; Gentamycin; Drug control, (n=2).

3.6.6 *In vitro* Antibacterial activity assay against Gram-positive and Gram-negative Bacteria

Investigations of all the EEPs of P1-P12 were carried out in order to determine activity against the Gram-positive bacteria *S. epidermidis* (ATCC 12228) and *S. aureus* (ATCC 29213) and Gram-negative bacteria *E. coli* (ATCC 8739) and *K. pneumoniae* (ATCC 13883) as described in (section 2.6.6), compared with gentamycin as drug control (Table 3-21). All the samples P1-P12 exhibited no activity against both strains of Gram-negative bacteria; *E. coli* (ATCC 8739) and *K. pneumoniae* (ATCC 13883), at the concentration used in this assay (500μg/ml) except for sample P2 that exhibited a MIC of 250μg/ml. While P2, P3 and P8-P10 had some activity against Gram-positive; *S. epidermidis* (ATCC 12228) and *S. aureus* (ATCC 29213) (Table 3-21).

Table 3-21 MICs of Antibacterial activity assay of P1-P12 samples against Gram-positive and Gram-negative Bacteria

Propolis samples	MIC (µg/ml)			
	Gram-positive		Gram-negative	
	S. epidermidis **ATCC 12228**	***S. aureus*** **ATCC 29213**	***E. coli*** **ATCC 8739**	***K. pneumoniae*** **ATCC 13883**
P1	>500	NA	NA	NA
P2	250	250	<500	250
P3	>500	NA	NA	NA
P4	500	NA	NA	NA
P5	500	NA	NA	NA
P6	500	NA	NA	NA
P7	NA	NA	NA	NA
P8	>500	250	NA	NA
P9	500	NA	NA	NA
P10	500	NA	NA	NA
P11	NA	NA	NA	NA
P12	NA	NA	NA	NA
Gentamycin	0.31	0.31	0.62	0.31

N.B.; **NA** = not active at top test concentration of 500µg/ml, **Gentamycin;** drug control.

3.6.7 *In vitro* Cytotoxicity assay of (P1-P12) samples.

Tests of the EEPs of P1-P12 were carried out in order to evaluate any toxic activity against mammalian cell line (U937 cells) and EC_{50} values (μg/ml), were calculate for all the tested samples as described in (section 2.6.7). The EEP P1-P8 exerts moderate cytotoxicity against the U937 cells. While the samples P9, P10, P11 and P12 are not cytotoxic to U937 cells exhibiting EC_{50} >100 μg/ml (Table 3-22).

Table 3-22 EC_{50} of Cytotoxicity of P1-12 samples against U937 cells.

Propolis Samples	**EC_{50} (μg/ml)**
P1	47.5
P2	53.2
P3	40.2
P4	50.08
P5	36.09
P6	47.92
P7	46.17
P8	34.1
P9	>100
P10	>100
P11	>100
P12	>100

N.B.; EC_{50} (μg/ml) (n=3)

3.6.8 *In vitro* Anti-inflammatory assay of (P1-P12) samples

Anti-inflammatory assays as described in section 2.6.8, were carried out for EEPs P1-P12 samples; samples P4, P6, P7 and P8 could not be assayed in this assay due to solubility issues with these samples in DMSO. Figure 3-29 shows the production of TNF-α by THP-1 cells stimulated with LPS compared with the medium alone. LPS displayed a significant $p \leq 0.001$, increase in production of TNF-α by THP-1 cells alone from 20.01 ± 2.35 pg/ml to 308.37 ± 35.79 pg/ml, cells with LPS, $p \leq 0.001$. While, Figure 3-29 shows the production of TNF-α by THP-1 cells stimulated with and without LPS and tested propolis samples (P1-P3, P5 and P9-P12), propolis samples exhibited a variable range of proinflammtory activity.

P9, P11 and P12, were showed proinflammatory properties by increasing TNF-α production compared with THP-1 cells alone from 20.01 ± 2.35 pg/ml to 764.55 ± 24.62 pg/ml, 483.48 ± 20.20 pg/ml and 431.10 ± 11.80pg/ml, $p \leq 0.001$, respectively.

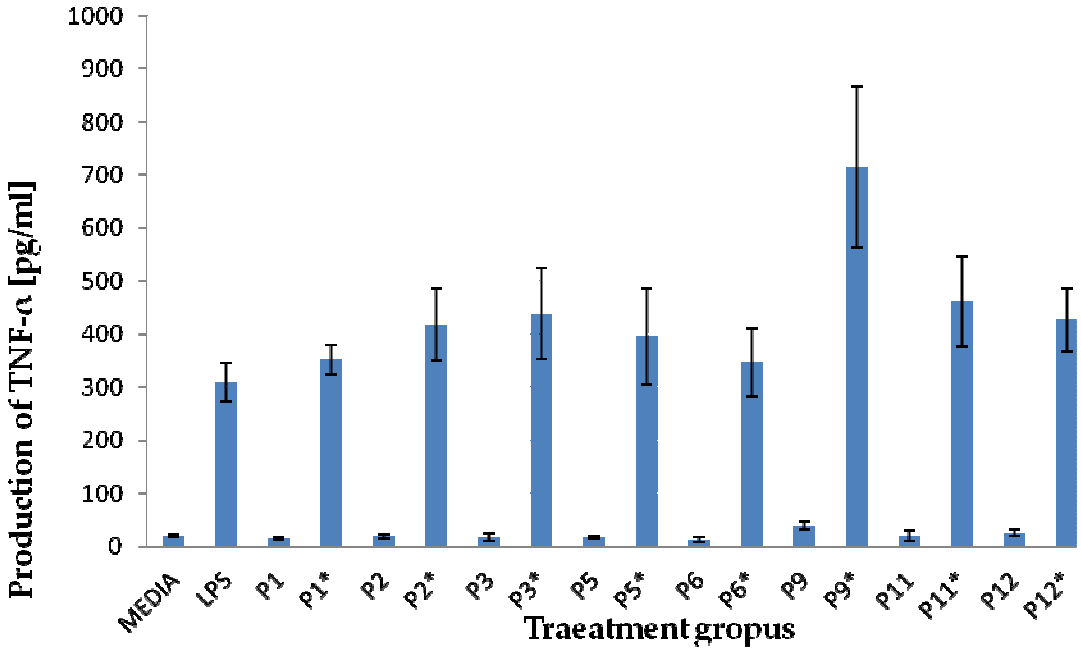


Figure 3-29 Effect of TNF-α production from THP-1 cells in the presences and absences of LPS and propolis samples (P1-P3, P5 and P9-P12).
Each bar represents the mean of P1, P2, P3, P5, P6, P9, P10, P11, P12 (n=3), LPS (n=3) and bar represent the media (n=3) measurements ± SD, LPS (positve control) and medium (negative control). ***P*<0.001 vs. LPS.

3.6.9 *In vitro* Anthelmintic activity assay of (P1-P12) samples

An anthelmintic assay was carried out for EEP P1-P12 against different pathogenic helminths such as *Trichinella spiralis* (*T. spiralis),* (Table 3-23) which cause a trichinosis disease and roundworm nematodes *Caenorhabditis elegans (C. elegans*) (Table 3-24)

3.6.9.1 *In vitro* Anthelmintic activity assay of (P1-P12) samples against *T. spiralis*

Anthelmintic assays were carried out for EEP of P1-P12 against a *T. spiralis* as described in (section 2.6.9.1). The EEPs P1-P12 exhibited a variable range of anthelmintic activity against *T. spiralis* as shown in Table 3-23.

Table 3-23 Anthelmintic activity of (P1-P12) samples against *T. spiralis*

Propolis samples	1µg/ml	10µg/ml	1µg/ml	10µg/ml	1µg/ml	10µg/ml
	% inhibition	% inhibition	% inhibition	% inhibition	% inhibition	% inhibition
P1	+	++++	++	++	+	+++
P2	++	+++	++++	+	++	+++
P3	+++	++++	+	+	+++	+++
P4	+++	++++	+	+	+++	+++
P5	+	+++	+	+	+	+++

Note; The % inhibition was categorized into 5 levels, which were **(+, ++, +++** and **++++);** represented less than 20% inhibition, 21-40% inhibition, 41-60% inhibition, 61-80% inhibition and 81-100% inhibition, respectively

The samples P1, P3 and P4 at concentration of 10µg/ml and sample P2 at concentration 1µg/ml exhibited mostly percentage of inhibition 81-100% of against *T. spiralis*. While samples P3 and P4 at concentration of 1µg/ml and sample P2 at concentration 1µg/ml exhibited percentage of inhibition 41-60% of against *T. spiralis*. EEPs P6-P12 did not show any activity against *T. spiralis*

According this finding, an EC_{50} value was determined for P1, P2, P3, P4 and P5 against *T. spiralis*. The EC_{50} results for samples P3 and P4 showed high activity against *T. spiralis*. The EC_{50} of P3 was 7.965µg/ml and EC_{50} of P4 was 29.22µg/ml, respectivly. While the rest of the samples P1, P2, and P5 no significant activity (Figures 3-30 and 3-31).

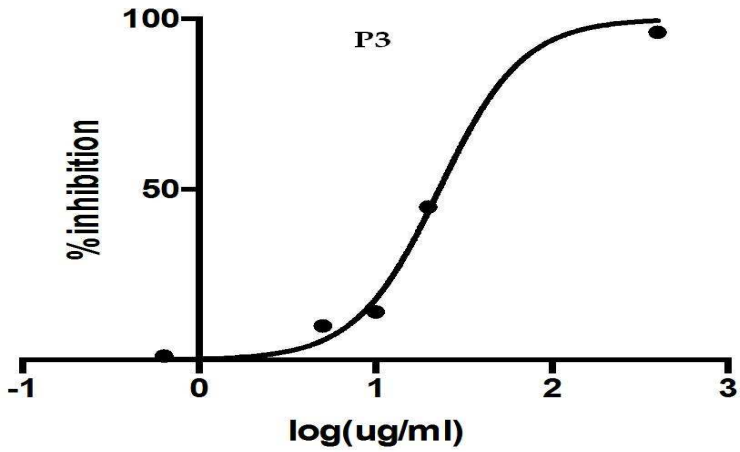


Figure 3-30 EC_{50} for anthelmintic assay of P3 sample against *T. spiralis* EC_{50} of P3 was 7.965µg/ml.

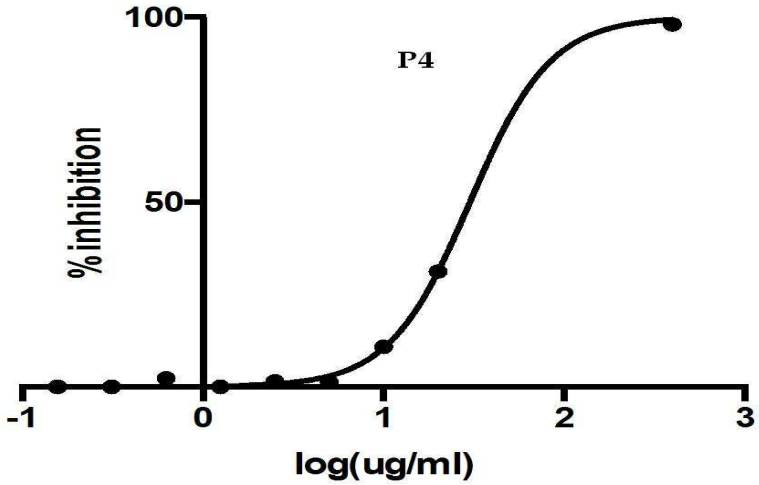


Figure 3-31 EC_{50} for anthelmintic assay of P4 sample against *T. spiralis* EC_{50} P4 was 29.22 µg/ml.

3.6.9.2 *In vitro* Anthelmintic activity assay against *C. elegans*

An anthelmintic assay was carried out for EEP of P1-P12 against *C. elegans* as described in (section 2.6.9.2). The samples demonstrated a range of activities against a *C. elegans* as shown in (Table 3-24) with all the samples being moderately active.

Table 3-24 Anthelmintic activity of (P1-P12) samples against *C. elegans*

Propolis	**1 µg/ml ± SD**	**10 µg/ml ± SD**
Samples	**% inhibition**	**% inhibition**
P1	20.4 ± 3.3	20.6 ± 3.7
P2	28.4 ± 4.2	20.5 ± 1.5
P3	26.2 ± 2.4	20.6 ± 3.6
P4	23.5 ± 1.3	20.7 ± 2.5
P5	24.6 ± 3.4	17.4 ± 3.2
P6	24.6 ± 0.4	18.3 ± 4.6
P7	26.1 ± 4.6	17.8 ± 3.8
P8	22.2 ± 3.6	19.1 ± 1.3
P9	26.5 ± 4.3	21.8 ± 0.2
P10	24.7 ± 3.5	20.3 ± 0.4
P11	24.1 ± 1.3	21.1 ± 0.5

Note; The % inhibition was categorized into 5 levels, which were (**+**, **++**, **+++** and **++++**), represented less than 20% inhibition, 21-40 % inhibition, 41-60% inhibition, 61-80% inhibition and 81-100% inhibition, respectively

From table 3-24 it can be seen that samples P1-P11 have no significant activity, all samples having inhibition in the range 21-40 %.

3.7 Modelling the anti-protozoal, anti-mycobacterial and cytotoxicity activity of (P1-P12) samples

3.7.1 OPLS model of activity of propolis extracts (P1-P12) against *P. falciparum*

Figure 3-32 shows an OPLS plot for the observed activity of the extracts against *P. falciparum* shown in (Table 3-18), constructed using 5 of the 300 variables used to produce Figure 3-32 by systematically discarding the variables with less impact on the model. The correlation between observed and predicted activity is very good with all the samples falling on the line.

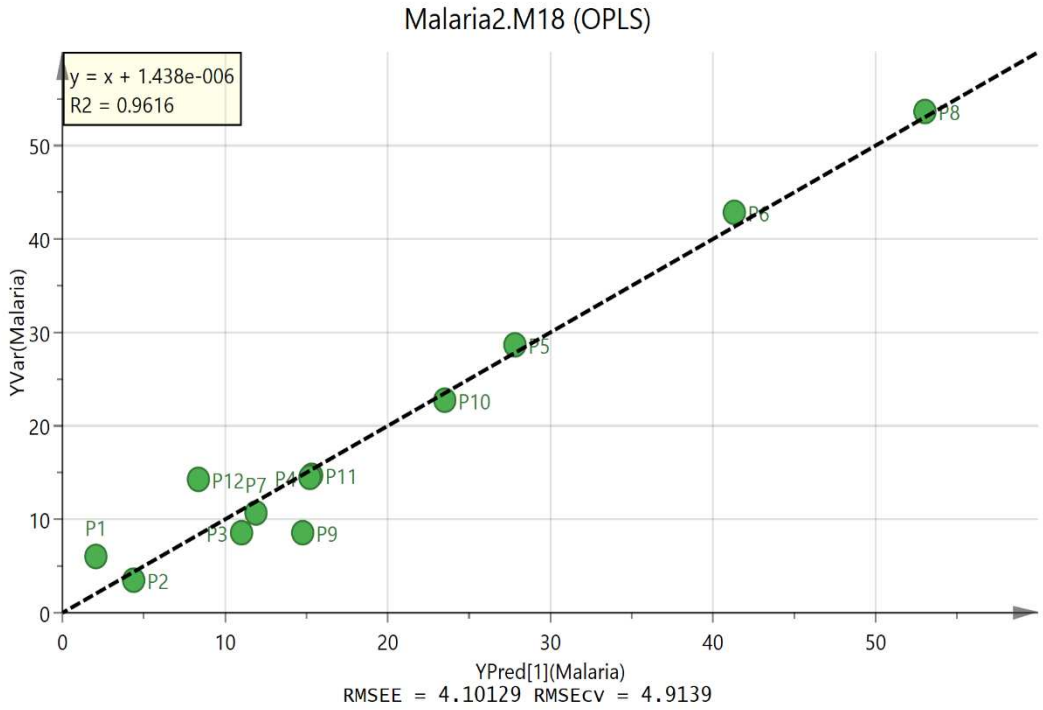


Figure 3-32 OPLS plot of observed against predicted activity against *P. falciparum* based on five compounds (A-E).

From the loadings plot the greatest activity was associated with compound D which is abundant in samples P1 and P2. As can be seen in the Figure 3-33 the more active samples have a greater abundance of compound D.

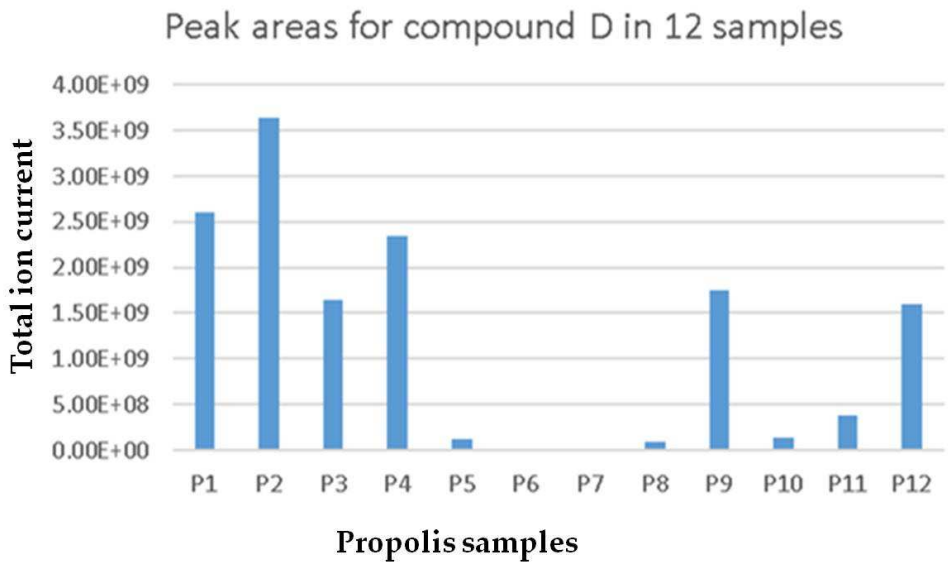


Figure 3-33 Abundance of compound D according to chromatographic peak area in the 12 Libyan propolis samples (P1-P12).

However, sample P11 is more active than would be predicted from levels of compound D and the activity appears to be based on a combination of the five marker compounds. Compound A seems to be associated with lower activity, but not always since it is high in P7 which has relatively high activity.

Table 3-25 shows the most important variables determining the activity of P2 in antiprotozoal and antimicrobial tests and important variables determining cellular toxicity based on sample P8 which was the most cytotoxic sample.

Table 3-25 The five most important variables contributing to the high activity of sample P2 in antiprotozoal and antimicrobial tests and important variables determining cellular toxicity based on sample P8 which was the most cytotoxic sample.

[m/z]	Rt (min)	Molecular Formula	Compounds
		P. falciparum	
373.275	52.6	$C_{24}H_{38}O_3$	**Compound A**
347.259	52.9	$C_{22}H_{36}O_3$	**Compound B**
345.244	50.0	$C_{22}H_{34}O_3$	**Compound C**
341.14	21.4	$C_{20}H_{22}O_5$	**Compound D**
301.217	43.6	$C_{20}H_{30}O_2$	**Compound E**
		T. brucei	
373.275	52.6	$C_{24}H_{38}O_3$	**Compound A**
329.067	13.1	$C_{17}H_{14}O_7$	**Compound F**
325.145	25.0	$C_{20}H_{22}O_4$	**Compound G**
301.217	43.6	$C_{20}H_{30}O_2$	**Compound E**
		L. donovani	
373.275	54.6	$C_{24}H_{38}O_3$	**Compound A**
325.145	25.0	$C_{20}H_{22}O_4$	**Compound D**
341.14	13.6	$C_{20}H_{22}O_5$	**Compound H**
341.103	10.5	$C_{19}H_{18}O_6$	**Compound I**
		C. fasciculata	
329.067	13.1	$C_{17}H_{14}O_7$	**Compound F**
325.145	25.0	$C_{20}H_{22}O_4$	**Compound G**
369.301	47.9	$C_{22}H_{42}O_4$	**Compound J**
		M. marinum	
341.14	21.4	$C_{20}H_{22}O_5$	**Compound D**
325.145	25.0	$C_{20}H_{22}O_4$	**Compound G**
289.108	10.6	$C_{16}H_{18}O_5$	**Compound K**
369.301	47.9	$C_{22}H_{42}O_4$	**Compound J**
		U937 Cells	
373.275	52.5936	$C_{24}H_{38}O_3$	**Compound A**
341.14	21.4344	$C_{20}H_{22}O_5$	**Compound D**
325.145	24.983	$C_{20}H_{22}O_4$	**Compound G**
397.275	50.7709	$C_{26}H_{38}O_3$	**Compound L**

MS^2 and MS^3 spectra were obtained for the marker compounds and are described below:

Compound A $C_{23}H_{37}O_3$, 45 isomers in DNP

MS^2 m/z 329.2850 (100) ($C_{23}H_{37}O$). MS^3 (329.2850) No fragmentation at the energy used. Not much information can be derived from the mass spectra since the base peak formed in MS^2 does not fragment, as showed in (Figure 3-34).

Compound B $C_{22}H_{36}O_3$, 114 isomers in DNP

MS^2 m/z 303.2689 (100) ($C_{21}H_{35}O$). MS^3 (303.2689) No fragmentation. Not much information can be derived from the mass spectra since the base peak formed in MS^2 does not fragment.as shown in (Figure 3-35)

Compound C $C_{22}H_{34}O_3$, 127 isomers in DNP

MS^2 m/z 301.2550 (100) ($C_{21}H_{33}O$). MS^3 (301.2550) No fragmentation. Not much information can be derived from the mass spectra since the base peak formed in MS^2 does not fragment as shown in (Figure 3-36).

Compound D $C_{20}H_{22}O_5$, 189 isomers in DNP

MS^2 323.1284 (100) ($C_{20}H_{19}O_4$) 313.1287 ($C_{19}H_{21}O_4$) 311.1287 ($C_{19}H_{19}O_4$) 242.0584 ($C_{14}H_{10}O_4$) MS^3 (311.1287) 216.0429 ($C_{12}H_8O_4$) 188.0479 ($C_{11}H_8O_3$) 144.0581 ($C_{10}H_8O$)

The ion at m/z 144.0581 is an important diagnostic fragment since it corresponds to naphthol and the ion at 188.0479 corresponds to a hydroxylated naphthoic acid. As shown in (Figure 3-37).

The ion at m/z 216.0429 has an additional CO suggesting a carbonyl is also substituted onto the hydroxynaphthoic acid and this fragment would arise from the molecular ion via the loss of a hydroxylated C_8H_{13} hydrocarbon chain. It was not possible to correlate this information to any structure in the literature.

Compound E $C_{20}H_{30}O_2$, 598 isomers in DNP

MS^2 220.1470 (100) ($C_{14}H_{20}O_2$), 205.1235 ($C_{13}H_{17}O_2$)

MS^3 (220.1470) 205.1235 (100) ($C_{13}H_{17}O_2$)

Not much structural information is revealed from the fragments produced as showed in (Figure 3-38).

Compound F $C_{17}H_{14}O_7$, 163 isomers in DNP

MS^2 m/z 314.0660(100) ($C_{16}H_{10}O_4$) m/z 299.0196 (14.3) ($C_{15}H_7O_7$)

MS^3 (299.0196) m/z 271.0246 (100) ($C_{14}H_7O_6$) m/z 255.0299 (6.3) ($C_{14}H_7O_5$),as showed in (Figure 3-39).

The structure could be related to dimethylquercetin which occurs in temperate propolis. However, there is a lack of the diagnostic fragments which usually arise from cleavage of the C ring in flavonoids (Hughes *et al*, 2001).

Compound G $C_{20}H_{22}O_4$, 109 isomers in the DNP

MS^2 m/z 242.0584 (6.1) ($C_{14}H_{10}O_4$) m/z 216.0427 (44.8) ($C_{12}H_8O_4$) m/z 188.0477 (65.4) ($C_{11}H_8O_3$) m/z 144.0581 (5) ($C_{10}H_8O$) MS^3 (188.0477) m/z 144.0581 (100) ($C_{10}H_8O$) as showed in (Figure 3-40).

This compound is related compound D and lacks the hydroxyl group in the side chain and it is a substituted hydroxy naphthoic acid.

Compound H $C_{20}H_{22}O_5$, 189 isomers in DNP

MS^2 m/z 271.0973 (100) ($C_{16}H_{15}O_4$) m/z 242.0584 (12.0) ($C_{14}H_{10}O_4$) m/z 216.0429 (10.8) ($C_{12}H_8O_4$) m/z 188.0479 (14.2) ($C_{11}H_8O_3$) m/z 144.0581 (0.8) ($C_{10}H_8O$)

MS^3 (271.0973) 242.0584 (100) ($C_{14}H_{10}O_4$) 216.0429 (30.0) ($C_{12}H_8O_4$) 188.0479 (46.0) ($C_{11}H_8O_3$) 144.0581 (1.8) ($C_{10}H_8O$), as showed in (Figure 3-41).

As Compound H is an isomer of compound D and has a very similar mass spectrum to compound D is clearly structurally closely related to it. As in Table 3-25

Compound I Isomers in DNP 128

MS^2 m/z 323.0923 (19.6) ($C_{19}H_{15}O_5$) m/z 311.0921 (52.8) ($C_{14}H_{10}O_4$) m/z 293.0818 (36.4) ($C_{18}H_{13}O_4$) m/z 265.0479 (10.7) ($C_{17}H_{13}O_3$) m/z 176.0478 (84.2) ($C_{10}H_8O_3$).

MS^3 (m/z 176.0478) m/z 147.0452 (100) ($C_9H_7O_2$), as showed in Figure 3-42 Compound I is most probably closely related to the lignan sesamin **(5)** which characterised in P1, P2 as **(6)** as shown in Table 3-33 and lacks one of the methylene groups, having a catechol structure in one of the aromatic rings rather than a methylene dioxy group.

Compound L $C_{26}H_{38}O_3$, 23 isomers in DNP.

MS^2 m/z 351.2715 (100) ($C_{25}H_{37}O$). MS^3 (m/z 351.2715) m/z 337.2557 (15.7) ($C_{24}H_{33}O_3$), m/z 323.2400 (2.9) ($C_{23}H_{31}O$), m/z 309.2243 (5.9) ($C_{22}H_{29}O$), m/z 295.2084 (7.3) ($C_{21}H_{27}O$), m/z 281.1929 (6.3) ($C_{20}H_{25}O$), m/z 267.1771 (5.9)

($C_{19}H_{23}O$), m/z 253.1613 (5.6) ($C_{18}H_{21}O$), m/z 239.1451 (5.5) ($C_{17}H_{19}O$), m/z 225.1299 (3.4) ($C_{16}H_{17}O$) m/z 133.0667 (0.8) (C_9H_9O), 119.0511 (2.3) (C_8H_7O), 107.0509 (2.2) (C_7H_7O).as in (Figure 3-43).

The MS^3 suggested a phenol substituted with a 17 carbon chain containing four units of unsaturation. The compound also contains a carboxylic acid shown by the loss of CO_2 in the MS^2 spectrum. The structure is consistent with an anacardic acid; these compounds are found in cashew oil (Kubo et al., 1993). On closer examination of the MS^3 spectrum of compound A it was also observed that very small ions corresponding at m/z 119.0511 and 107. 0509 could be observed.

Figures (3-34 to 3-43) represent the MS^2 and MS^3 Spectra obtained with for marker compounds **(A** to **L)**, respectively

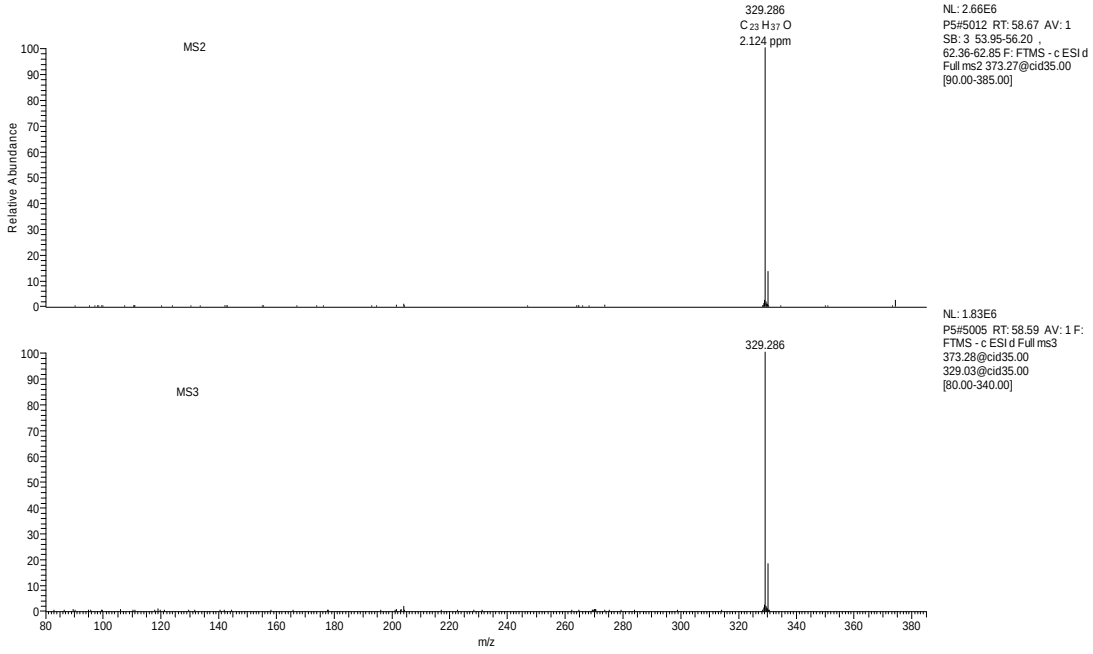


Figure 3-34 Compound A MS^2 and MS^3 spectra obtained with a collision energy of 35V.

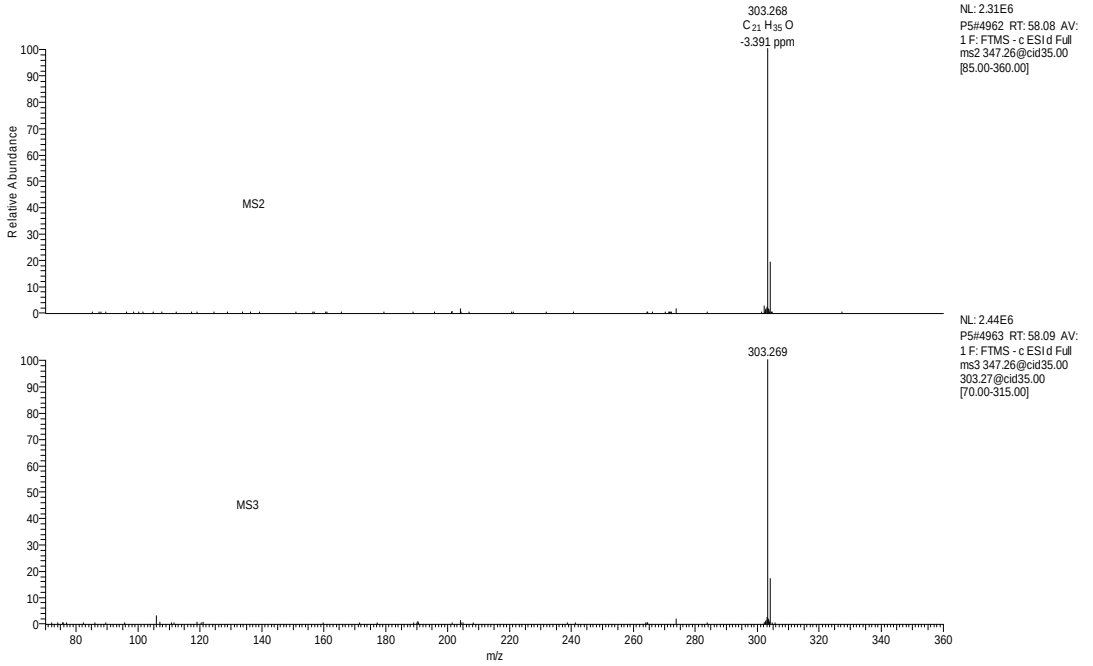


Figure 3-35 Compound B MS^2 and MS^3 spectra obtained with a collision energy of 35V.

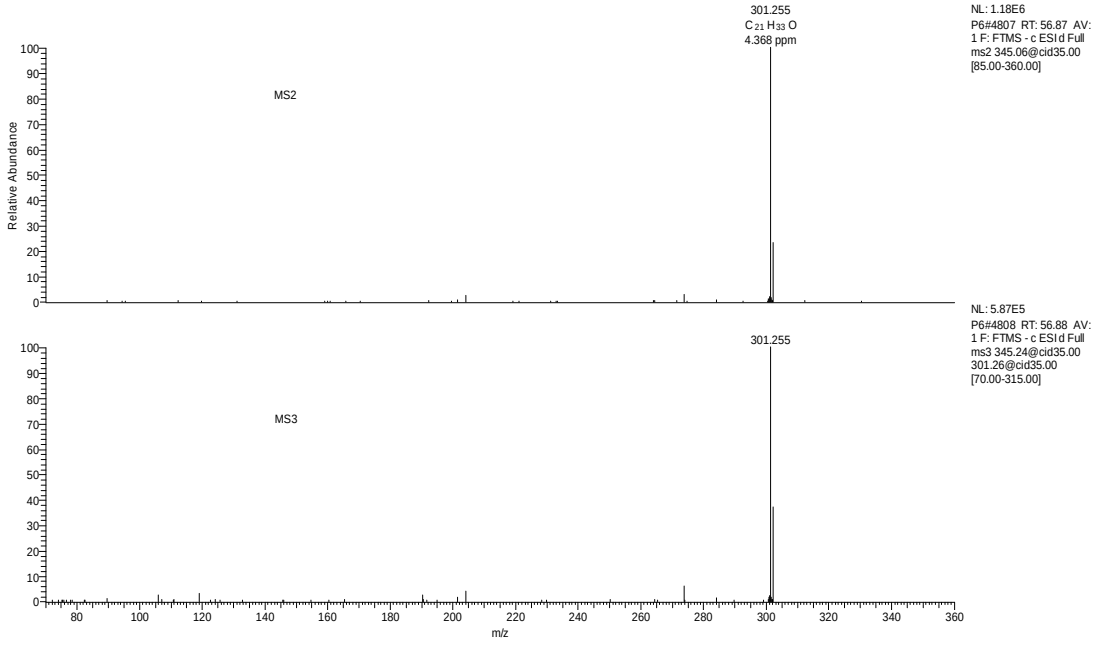


Figure 3-36 Compound C MS^2 and MS^3 spectra obtained with a collision energy of 35V.

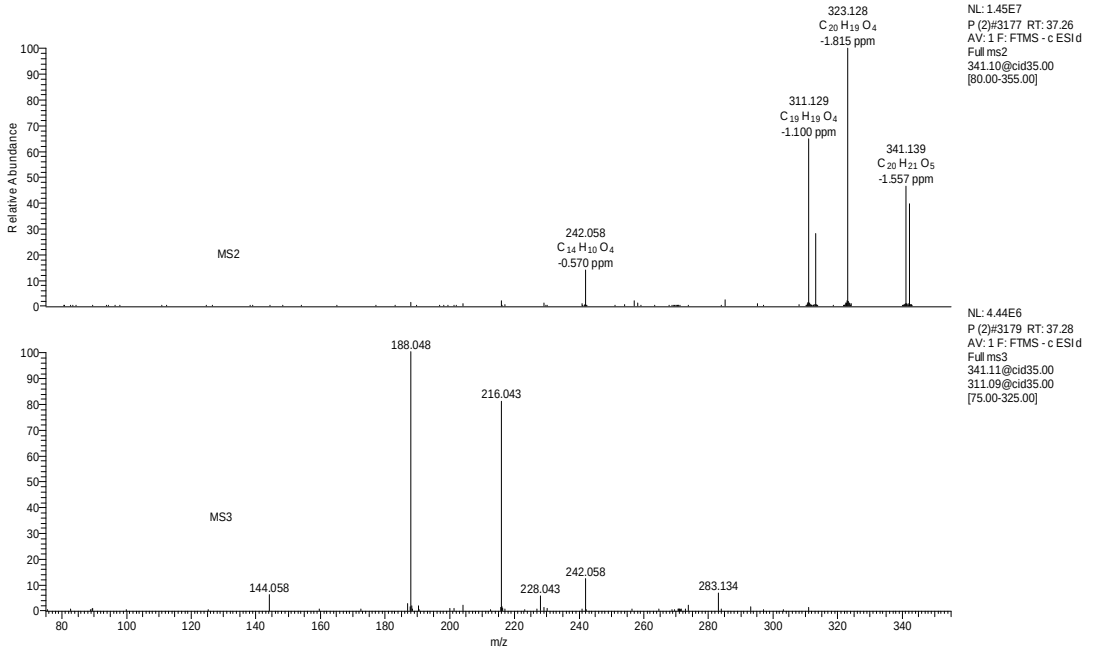


Figure 3-37 Compound D MS^2 and MS^3 spectra obtained with a collision energy of 35V.

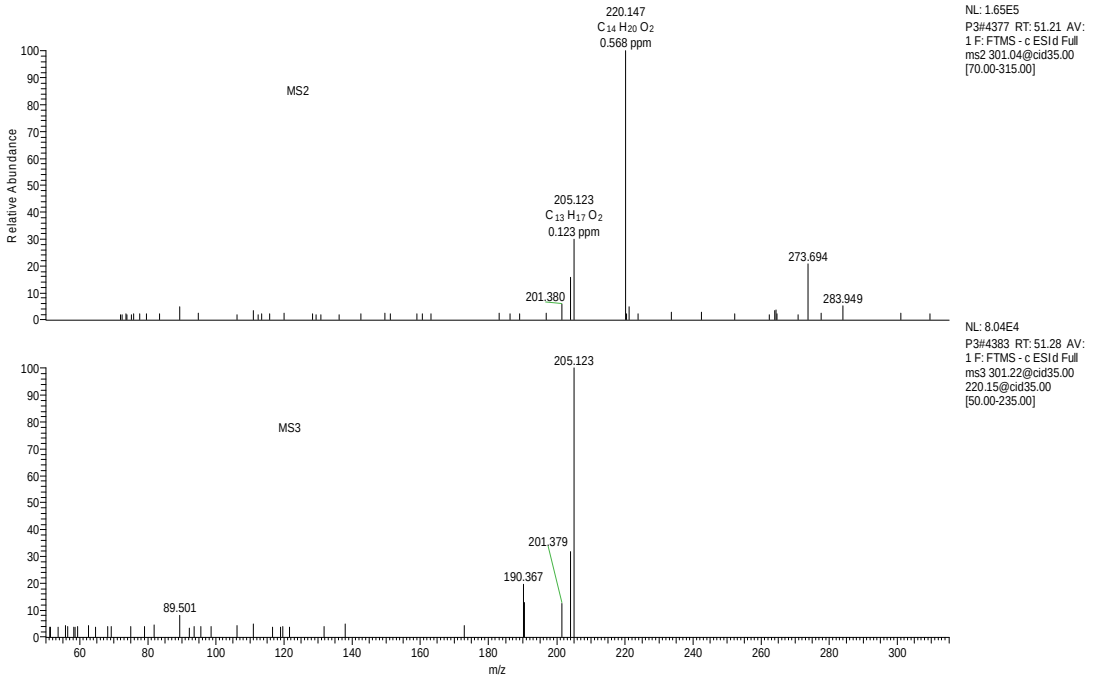


Figure 3-38 Compound E MS^2 and MS^3 spectra obtained with a collision energy of 35V.

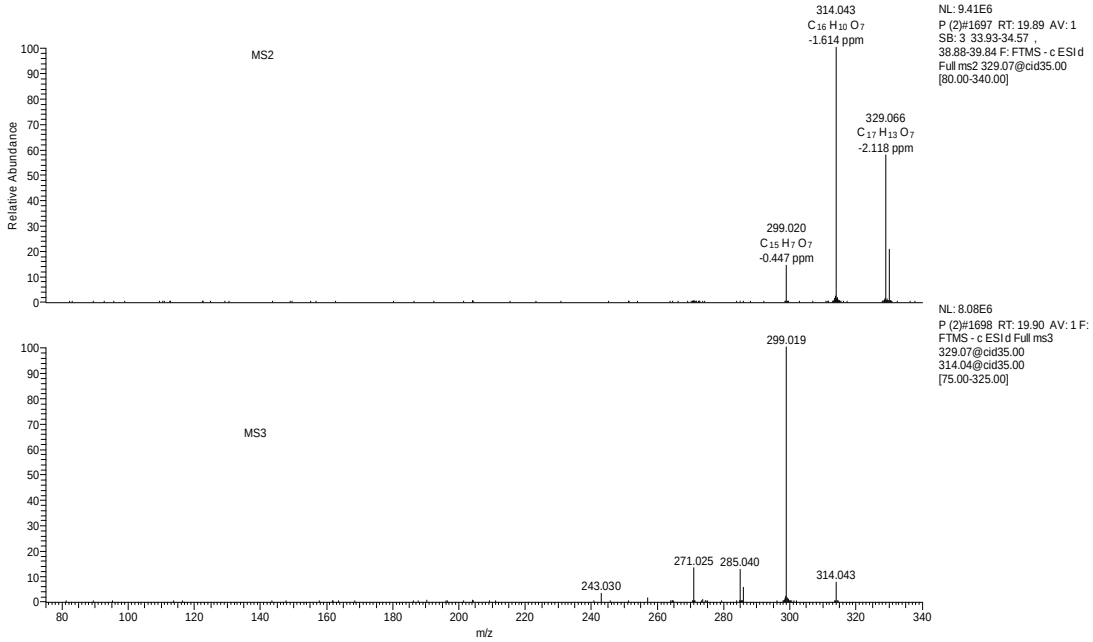


Figure 3-39 Compound F MS^2 and MS^3 spectra obtained with a collision energy of 35V

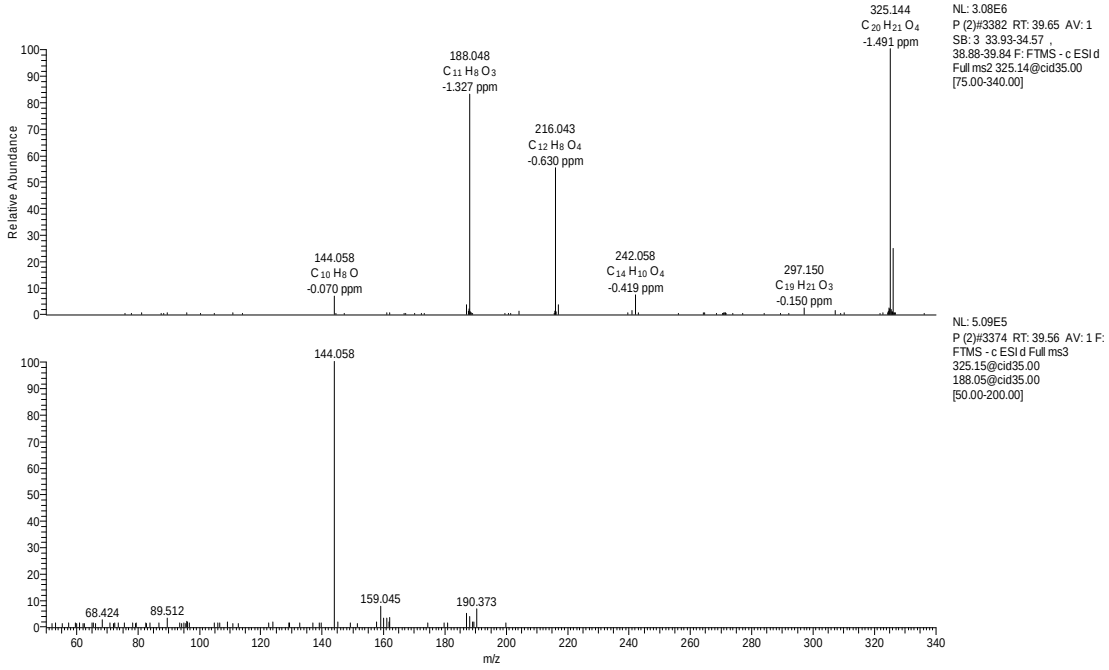


Figure 3-40 Compound G MS^2 and MS^3 spectra obtained with a collision energy of 35V.

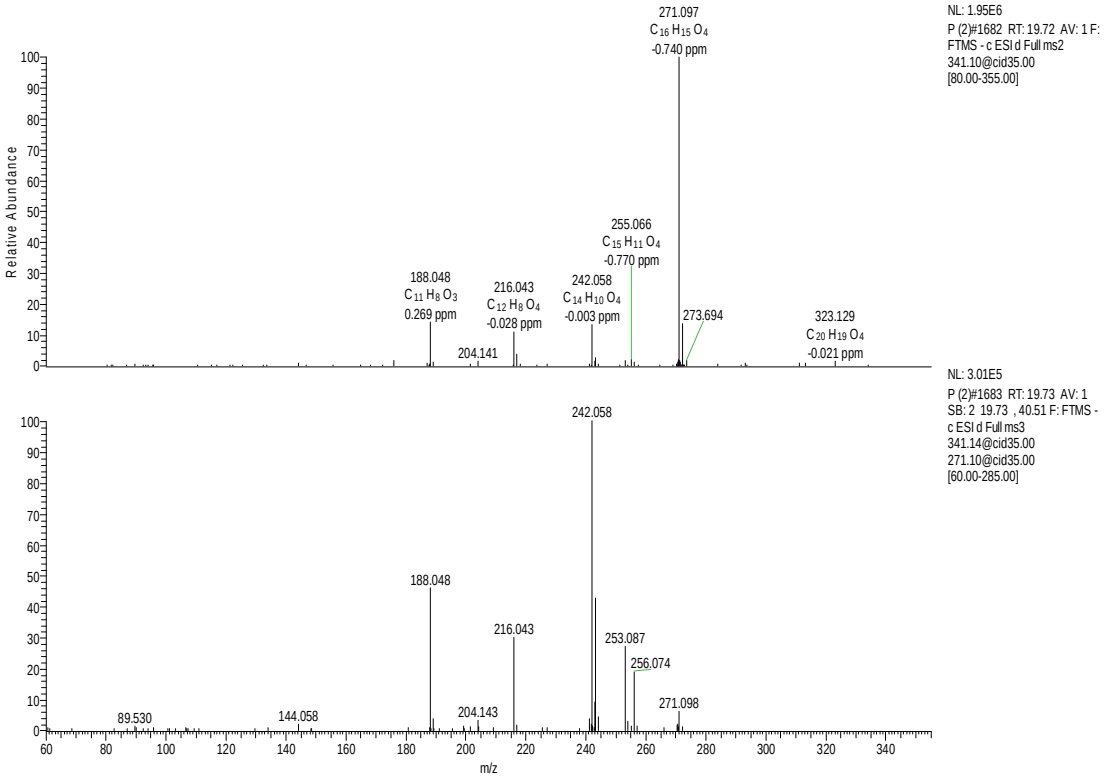


Figure 3-41 Compound H MS^2 and MS^3 spectra obtained with a collision energy of 35V.

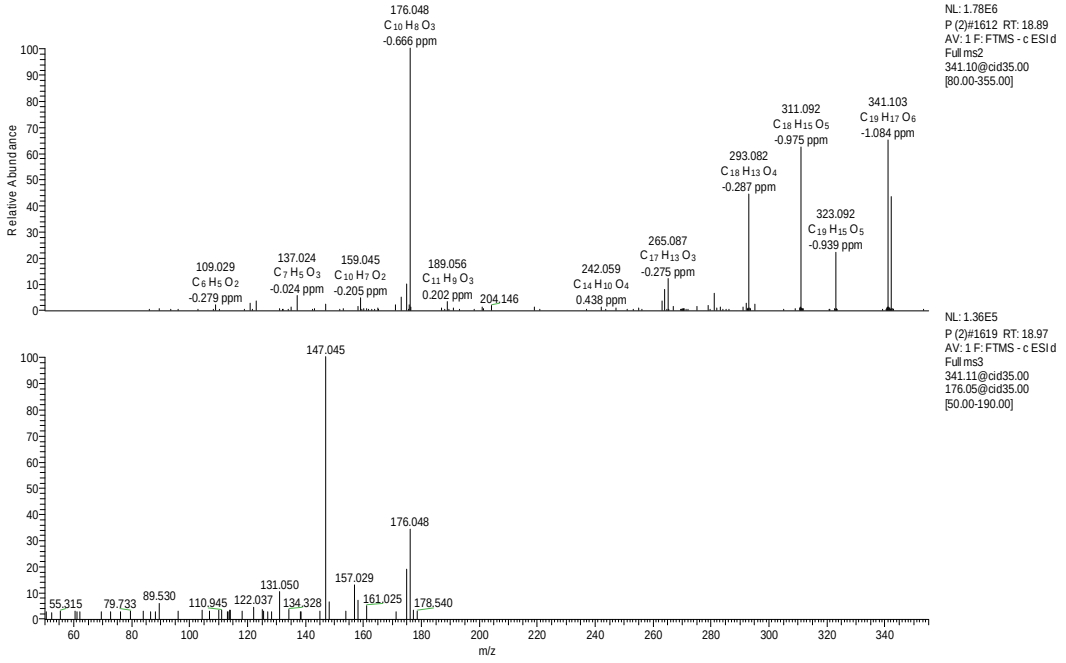


Figure 3-42 Compound I MS^2 and MS^3 spectra obtained with a collision energy of 35V.

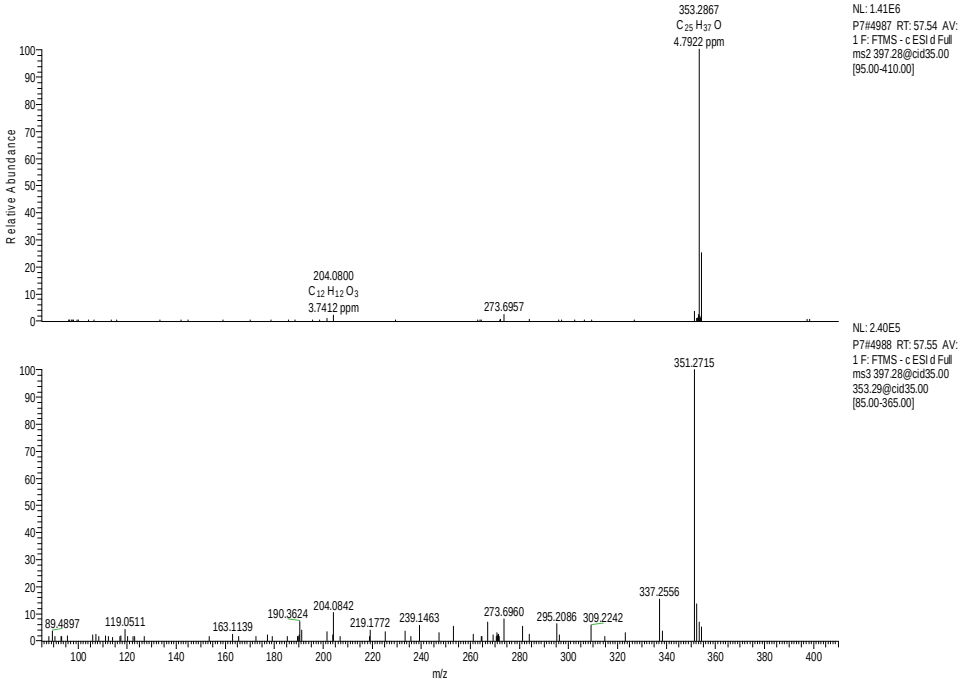


Figure 3-43 Compound L MS^2 and MS^3 spectra obtained with a collision energy of 35V.

3.7.2 OPLS model of activity of propolis extracts (P1-P12) against *T. brucei*

Figure 3-44 illustrates an Orthogonal Partial Least Squares (OPLS) model based on four compounds correlating strongly with activity against *T. brucei* as described in (Table 3-15). Two of these were compounds A and E, which were also important in the activity against *P. falciparum*. Compounds F and G were discussed above.

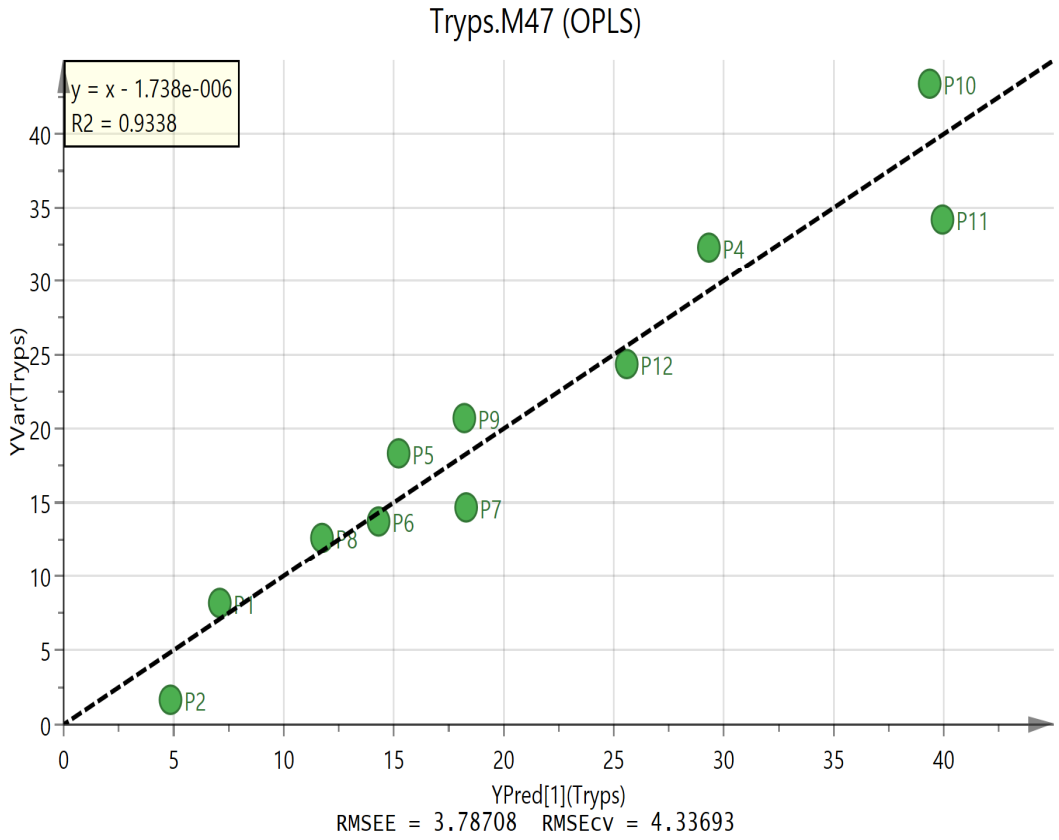


Figure 3-44 OPLS model of the activity of Libyan propolis samples against *T. bruce*i based on four compounds (A, E, G and F). Sample P3 was omitted in order to improve the fit of the model.

3.7.2.1 OPLS model of activity of propolis extracts (P1-P12) against *L. dovani.*

Only 9 out of 12 propolis samples could be fitted into and OPLS model with activity against *L. dovani* as described in (Table 3-17). As can be observed in;

Figure 3-45 in as Compounds A and D were important to the model and two additional compounds H and I were also important and are discussed above.

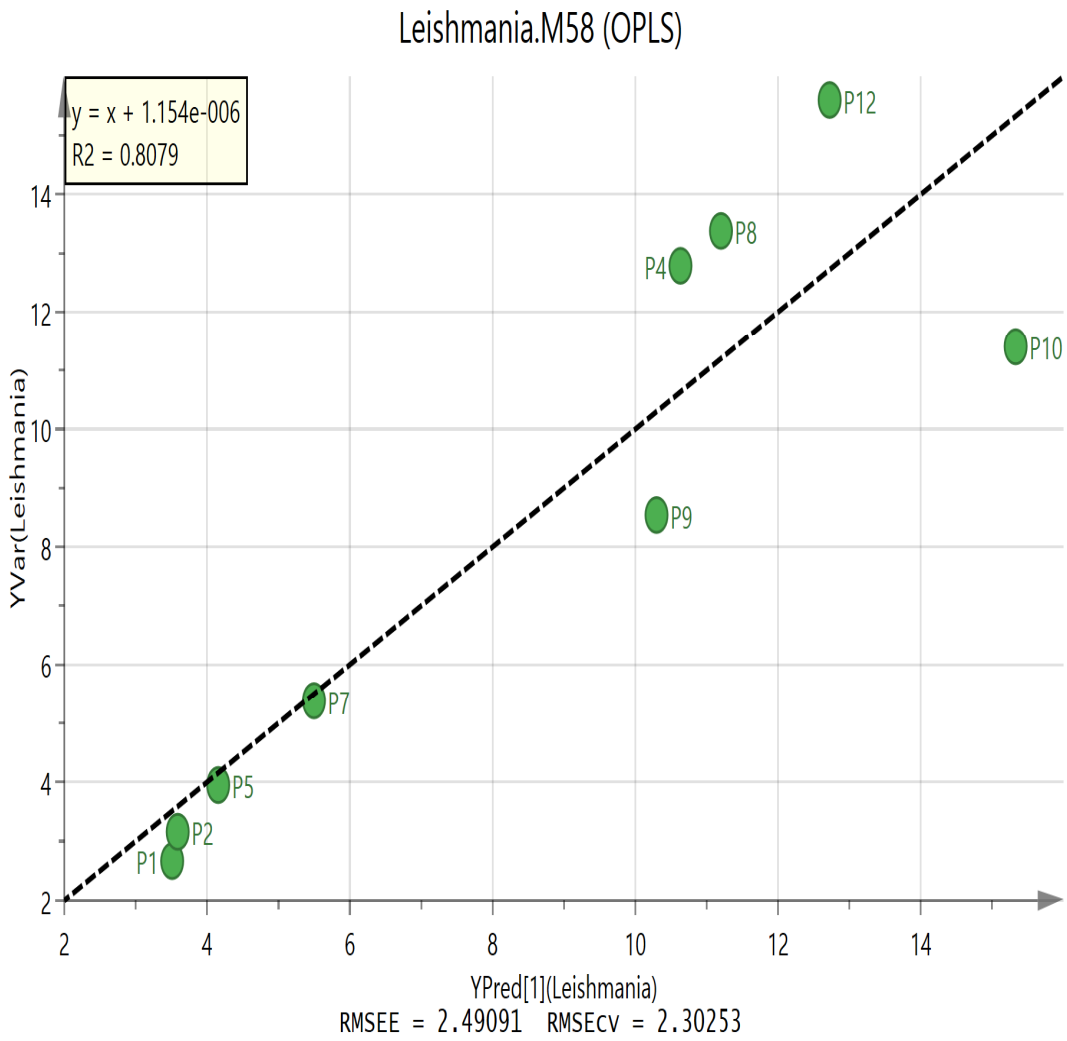


Figure 3-45 OPLS plot of observed against predicted activity of propolis samples against *L. donovani*. Sample P3, P6 and P11 were omitted in order to improve the fit of the model.

3.7.3 OPLS model of activity of propolis extracts (P1-P12) against *C. fasiculata*

The activity of P1-P12 against *C. fasiculata* as (Table 3-19), correlated strongly with three compounds in an OPLS model as in (Figure 3-46). Compounds F and G, which were important in other models of activity correlated with high activity and compound J which correlates with low activity. Compound J is a relatively minor peak and did not afford a clear MS^2 spectrum

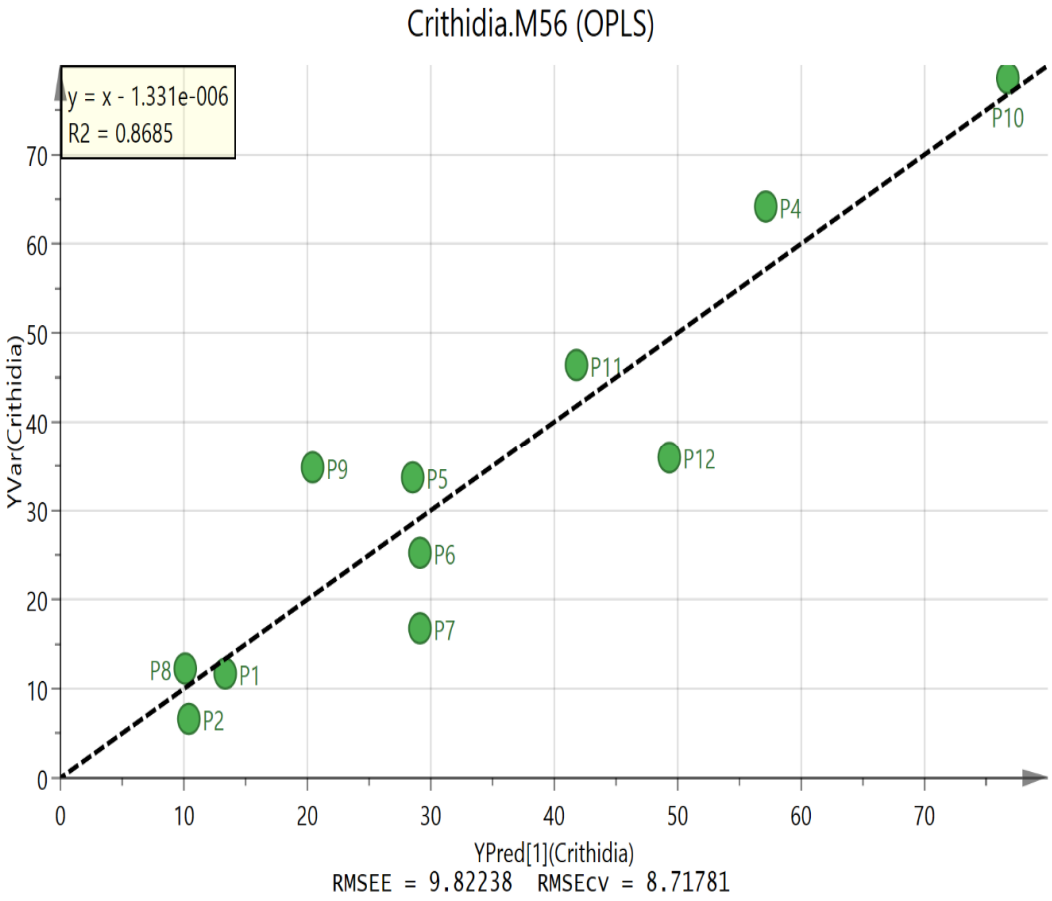


Figure 3-46 OPLS plot of observed against predicted activity of propolis samples against *C. fasciculata*. Sample P3 was omitted in order to improve the fit of the model.

3.7.4 OPLS model of activity of propolis extracts (P1-P12) against *M. marinum*

An OPLS model based on four components (Figure 3-47) gave a good fit to the activity against *M. marinum* (Table 3-20) Again compounds D and G were responsible for high activity while compounds J and K correlated with low activity.

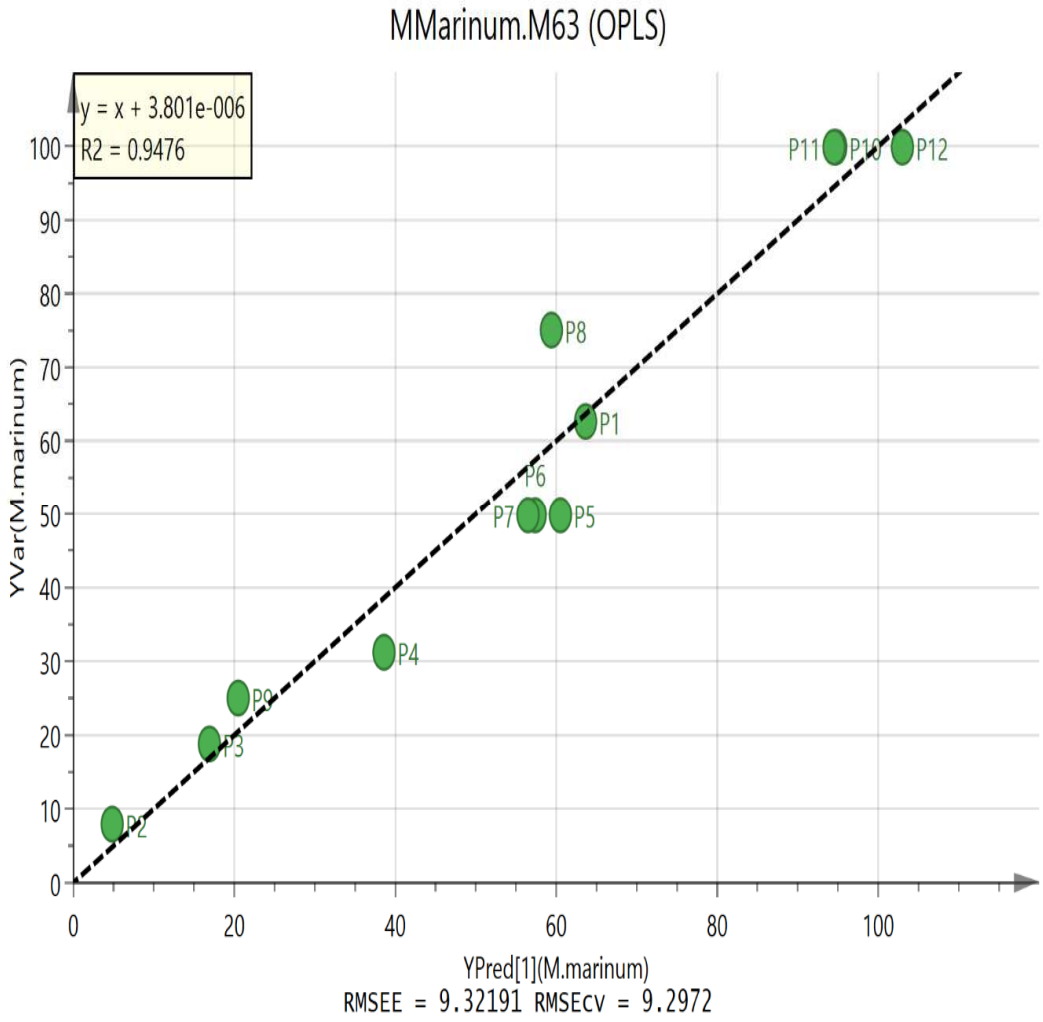


Figure 3-47 OPLS plot of observed against predicted activity of propolis samples against *M. marinum*

3.7.5 OPLS model of toxicity of propolis extracts (P1-P12) against mammalian cells (U937)

The toxicity of the propolis extracts was tested against a mammalian cell line (Table 3-22). For two of the samples, P9 and P11, there was no measurable toxicity up to 100µg/ml and thus they were excluded from the OPLS model as in (Figure 3-48) the most toxic sample was P8 which gave an IC_{50} value of 34.1 µg/ml of the samples showing toxicity below 100 µg/ml P2 was the least toxic. The main compounds responsible for the toxicity of the samples were compound A and compound L. From the similar elemental composition it seemed possible that compound A and compound L might be related. The mass spectrum of compound L was discussed above.

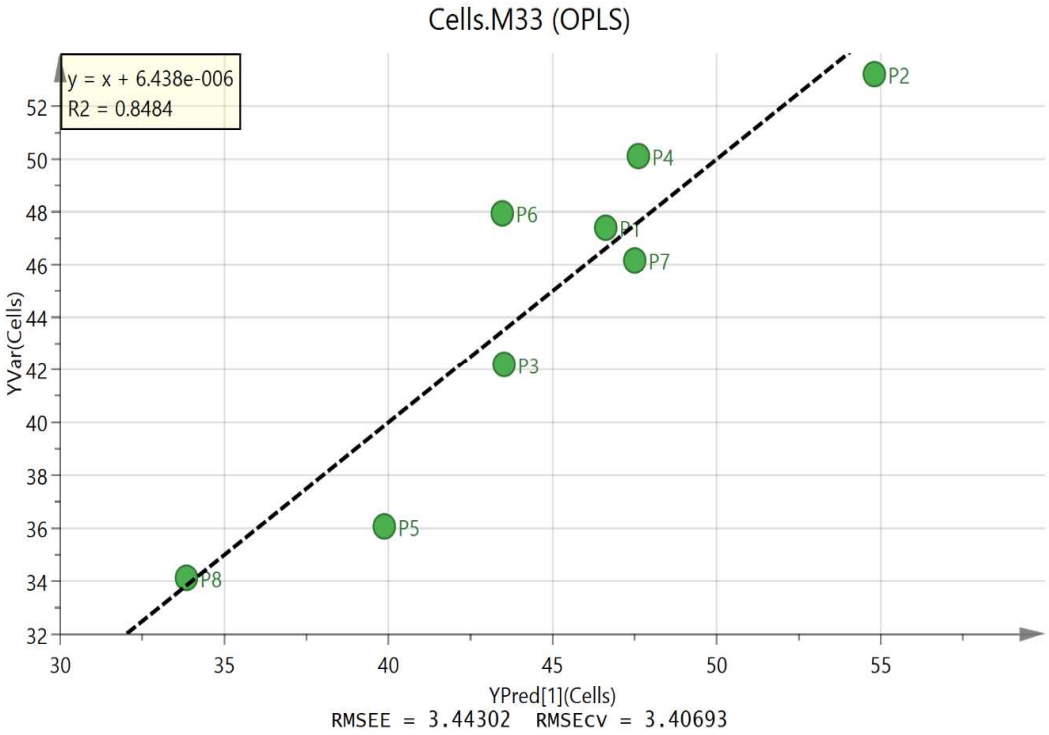


Figure 3-48 OPLS plot of observed against predicted activity of propolis samples against U937 cells. Samples P3 and P12 were omitted in order to improve the fit of the model.

Consequently, it seems likely that Compound **A** is also an anacardic acid substituted with a 17 carbon chain with two units of unsaturation. Looking at the marker compounds in Table 3-14, all but one of the top 10 VIPs for sample P8, the most toxic sample, have elemental compositions that would fit anacardic acids substituted with varying alkyl chains. Sample P8 is from the Southwest of the country from an oases area with a very dry climate; thus, there is nothing to suggest that cashew trees might grow in this area. However, pistachio trees (*Pistacia vera*) are cultivated in Libya and these contain anacardic acids (Khadem and Marles, 2010) A closely related series of alkylated phenols was recently observed in Cameroonian propolis (Kardar et al., 2014) and were thought to originate from the Anacardiaceae family of plants or possibly from mango. Anacardic acids have also been observed in propolis from Oman (Popova et al., 2013) and Brazil (Silva et al., 2008) Anacardic acids have been shown to exhibit cytotoxicity (Kubo et al., 1993) and their high levels in P8 would explain why it is the most cytotoxic sample. The samples from the other oasis area in the South East of the country P5, P6 and P7 also contain anacardic acids and are relatively cytotoxic.

A preliminary chemical screening was carried out following fractionation 2.56 g of the EEP of crude P1 and the collected fractions were evaporated and weighed as seen in Table 3-26 and based on the weight and then subsequent further chemical analysis and fractionation was carried out starting with highest weight fraction P1-3 and then the fractions P1-9, and P1-2.

3.8 Analysis of open column fractions isolated from P1 and P2 samples by LC-MS

Fractions were eluted from the open column collected and weighed and analysed LCMS and using a concentration of 1mg/ml for the analysis. Tables 3-26 and 3-27 show the weights of the fractions collected from open column chromatography of samples P1 and P2, respectively.

Table 3-26 Collected fractions from open column chromatography of P1 sample

P1 Fractions Codes	Weight (mg)
P1-1	33.3
P1-2	251.9
P1-3	1111.1
P1-4	185
P1-5	167.2
P1-6	64.1
P1-7	5
P1-8	2.3
P1-9	255.2
P1-10	1.7
P1-11	2.4
P1-12	2.4
P1-13	0.8
P1-14	4
P1-15	4.2
P1-16	4.3
P1-17	6.3
P1-18	0.3
P1-19	4.7
P1-20	154.2
P1-21	2.4
P1-22	32.4
P1-23	1
P1-24	3.2
P1-25	0.87

Table 3-27 Collected fractions from open column chromatography of P2 sample [a]

P2 Fractions Codes	Weight (mg)
P2-1	12.9
P2-2	8.6
P2-3	38.9
P2-4	97.4
P2-5	78.9
P2-6	26.2
P2-7	40.8
P2-8	87
P2-9	159.9
P2-10	154.8
P2-11	62.8
P2-12	354.2
P2-13	140.7
P2-14	84.9
P2-15	88.5
P2-16	60.4
P2-17	251.9
P2-18	50.6
P2-19	52.6
P2-20	45.5
P2-21	56.9
P2-22	64.7
P2-23	48.2
P2-24	207.56
P2-25	38.3
P2-26	30
P2-27	46.3
P2-28	17.4
P2-29	13.3
P2-30	9.7
P2-31	12.1
P2-32	7.4
P2-33	8.3
P2-34	17.8
P2-35	41.9

(Table 3-27 continued)

P2 Fractions Codes	Weight (mg)
P2-36	41.7
P2-37	12.9
P2-38	13.4
P2-39	14.2
P2-40	7.4
P2-41	17
P2-42	7.5
P2-43	7.7
P2-44	7.6
P2-45	27.3
P2-46	14.6
P2-47	17.1

N.B; [a]; column was eluted as described in section 2.4.1) and Table 2-3 the column was loaded with 2.56 g of EEP of P2.

3.9 Fractionation of P1 and P2 open column fractions by MPLC

All fractions were subjected to analysis by LC-HR- MS. P1-3 was found to contain an interesting range of compounds according to the analysis and had a high weight. Thus P1-3 as well as fractions P1-2 and P1-9, were selected for further fractionation by MPLC and 27 fractions were collected after using the programme as described above (section 2.5.3).

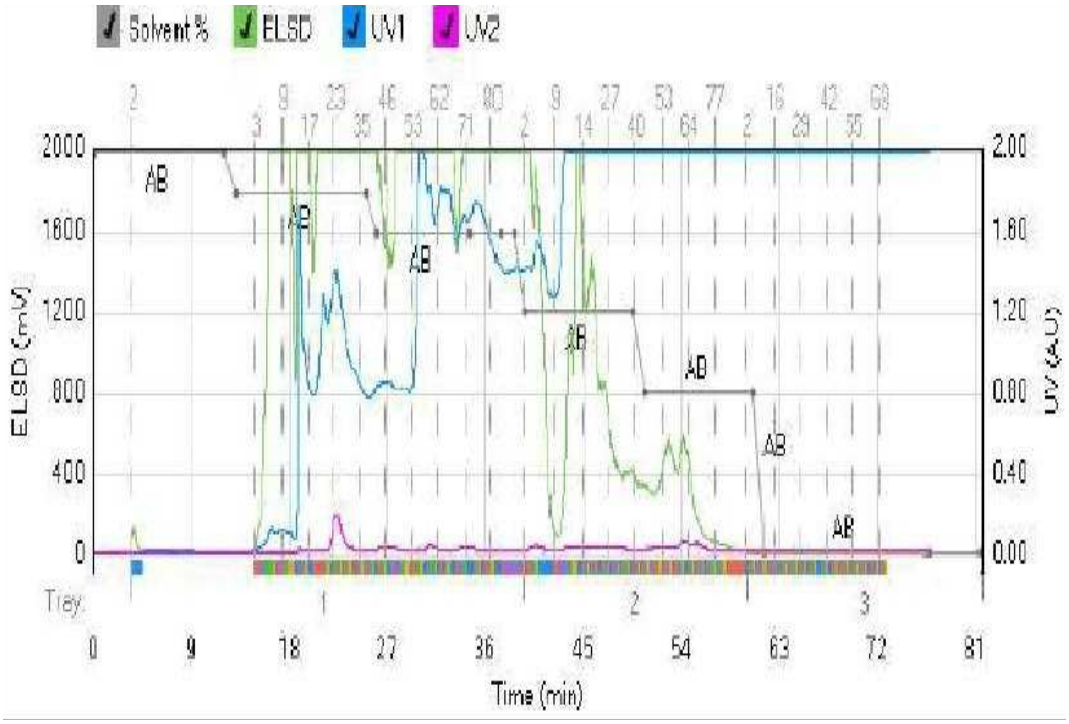


Figure 3-49 Chromatogram of Open Column fraction P1-3 on MPLC Grace Revelris®.

Figure 3-50 shows the chromatogram obtained in the fractionation of P1-3 by MPLC. The chromatogram looks to be quite a complicated mixture but it had strong UV absorption and strong ELSD absorption and 28 fractions were isolated in different vials, collected according to UV and ELSD peak absorption. Solvent was removed from the collected fractions by using a rotary evaporator and the weights are noted for each fraction in (Table 3-28).

Table 3-28 Collected fractions from MPLC of P1-3 fraction.

P1-3 Fractions codes	Pure Compounds	Weight (mg)
P1-3-1	-	2.1
P1-3-2	-	6.2
P1-3-3	-	6.7
P1-3-4	-	46.8
P1-3-5	-	21.2
P1-3-6	-	76
P1-3-7	-	13.7
P1-3-8	-	20.5
P1-3-9	**(1)**	13.8
P1-3-10	**(2)**	7
P1-3-11	-	17.4
P1-3-12	-	25.5
P1-3-13	-	7.2
P1-3-14	-	6.7
P1-3-15	**(3)**	101.71
P1-3-16	-	16.3
P1-3-17	**(4)**	17.7
P1-3-18	-	5
P1-3-19	-	16.7
P1-3-20	-	14.7
P1-3-21	-	9.4
P1-3-22	-	5.2
P1-3-23	-	4.6
P1-3-24	-	10.7
P1-3-25	-	12.1
P1-3-26	-	5.1
P1-3-27	-	6.6
P1-3-28	-	7

N.B; -; a mixture of compounds.

The fractions collected were dried and weighed and then subjected to analysis by LC-HRMS and GC-MS, and based on weight one of the attractive fractions was P1-3-15 **(3).** These fractions were analysed by ELSD, LC-HRMS and GC-

MS. Then subsequently chemical screening was carried out by different chromatographic techniques. Open column fractions P1-2 and P1-9 were also fractionated by MPLC and the results are shown in (Tables 3-29 and 3-30).

Table 3-29 Collected fractions from MPLC of P1-2 fraction

P1-2 Fractions codes	Pure Compounds	Weight (mg)
P1-2-1	-	26.4
P1-2-2	-	23.4
P1-2-3	-	2.2
P1-2-4	-	2.6
P1-2-5	-	15.3
P1-2-6	-	82.2
P1-2-7	-	13.8
P1-2-8	-	3.1
P1-2-9	**(2)**[a]	8.2
P1-2-10	**(3)**	107.3
P1-2-12	-	7.1
P1-2-13	-	3.8
P1-2-14	-	3.5
P1-2-15	-	6.8
P1-2-16	**(8)**	17.7
P1-2-17	-	0.4
P1-2-18	-	5.7
P1-2-19	-	7.3
P1-2-20	-	2.8
P1-2-21	-	2.3
P1-2-22	-	7.8
P1-2-23	-	4.4

N.B; -; a mixture of compounds, [a]; a compound (2) isolated from P2 sample as seen in (Table 3.28)

Table 3-30 Collected fractions from MPLC of P1-9 fraction

P1-9 Fractions codes	Pure Compounds	Weight (mg)
P1-9-1	-	12.5
P1-9-2	-	4
P1-9-3	-	3.8
P1-9-4	-	0.5
P1-9-5	-	3.5
P1-9-6	-	6.7
P1-9-7	**(5)**	19.3
P1-9-8	-	70.2
P1-9-9	-	57.9
P1-9-10	-	43.8
P1-9-11	-	22.7
P1-9-12	-	24.5
P1-9-13	-	11.8
P1-9-14	-	12

N.B; -; a mixture of compounds.

3.9.1 Fractionation of open column fractions obtained from P2 sample

Based on the yields shown in Table 3-27, fraction P2-12, (354.2 mg) was selected for further fractionation by MPLC and fractions were collected after using the programme was described above (section 2.4.3) pure compounds **(6)** and **(1)** were obtained as shown in Table 3-31.

Table 3-31 Collected fractions from MPLC of P2-12 fraction.

P2 Fractions codes	Pure Compounds	Weight (mg)
P2-12-37	**(6)**	22 .7
P2-12-29	**-**	17.5
P2-12-11	**-**	24.6.2
P2-12-9	**-**	15.3
P2-12-30	**-**	16.4
P2-12-6	**-**	14.8
P2-12-3	**-**	11.7
P2-12-4	**-**	16.7
P2-12-5	**-**	16.9
P2-12-55	**-**	14.8
P2-9	**-**	16.7
P2-10	**-**	20.1
P2-11	**(1)**[a]	12.8

N.B, [a]**;** Compound **(1)** was also isolated in P2 sample as seen in (Table), **-**; *a mixture of compounds.*

Fraction P2-24 was fractionated by MPLC as described in (section 2.5.2), yielding 20.3mg of compound P2-24-7 **(7)**

3.9.2 Direct Fractionation of EEP P7 by MPLC

A portion (1 g) of the EEP P7 from the South East of Libya was fractionated directly by MPLC. The fractions collected are shown in Table 3-32. This fractionation yielded 6 pure compounds (9-14).

Table 3-32 Collected fractions from MPLC of P7 sample.

P7 Fractions codes	Pure Compound	Weight (mg)
P7-15	**(9)**	25.7
P7-20	**(14)**	37.8
P7-21	**-**	31.5
P7-24	**-**	22.7
P7-25	**-**	26.6
P7-31	**(12)**	41.8
P7-32	**(10)**	29.22
P7-35	**(11)**	21.7
P7-51	**(13)**	33.8
P7-40	**-**	29.7
P7-5	**-**	32.3
P7-57	**-**	44.4

N.B; -; *[a] mixture of compounds.*

3.9.3 Direct Fractionation of EEP P9 by MPLC

A portion (1 g) of the EEP P9 from was fractionated directly by MPLC. The fractions collected are shown in (Table 3-33) yielding four pure compounds **(15)-(18).**

Table 3-33 Collected fractions from MPLC of P9 sample

P9 Fractions	Pure Compound	Weight (mg)
P9-5	-	27.43
P9-7	-	38.87
P9-8	-	23.8
P9-10	-	16.93
P9-11	-	19.45
P9-15	**(15)**	27.1
P9-16	-	43.1
P9-17	**(18)**	22.2
P9-19	-	55.5
P9-23	-	16.9
P9-27	**(17)**	22.3
P9-28	**(16)**	25.4
P9-55	-	80.34

N.B; -; *[a] mixture of compounds.*

3.10 High performance liquid chromatography with evaporative light scattering detectors (HPLC-ELSD).

HPLC was used for the analysis of crude samples and for checking the purity of fractions collected from the open column and also those fractions resulting from further purification by MPLC P1-3**.** Shows a HPLC ELSD analysis of the

open column fraction P1-3 there was no UV absorption in the chromatogram. The fraction was a complex mixture and it was fractionated further by MPLC.

ELSD traces for compounds **(1)**-**(5)** and **(8)** are shown in Figures 3-51 to 3-56. and those for compounds **(15)**–**(18)** as seen in (Appendix 1 to 4), and for compound **(6)**, as seen in (Appendix 6) Shows a HPLC ELSD analysis of the open column fraction P1-3. The fraction was a complex mixture and it was fractionated further by MPLC.

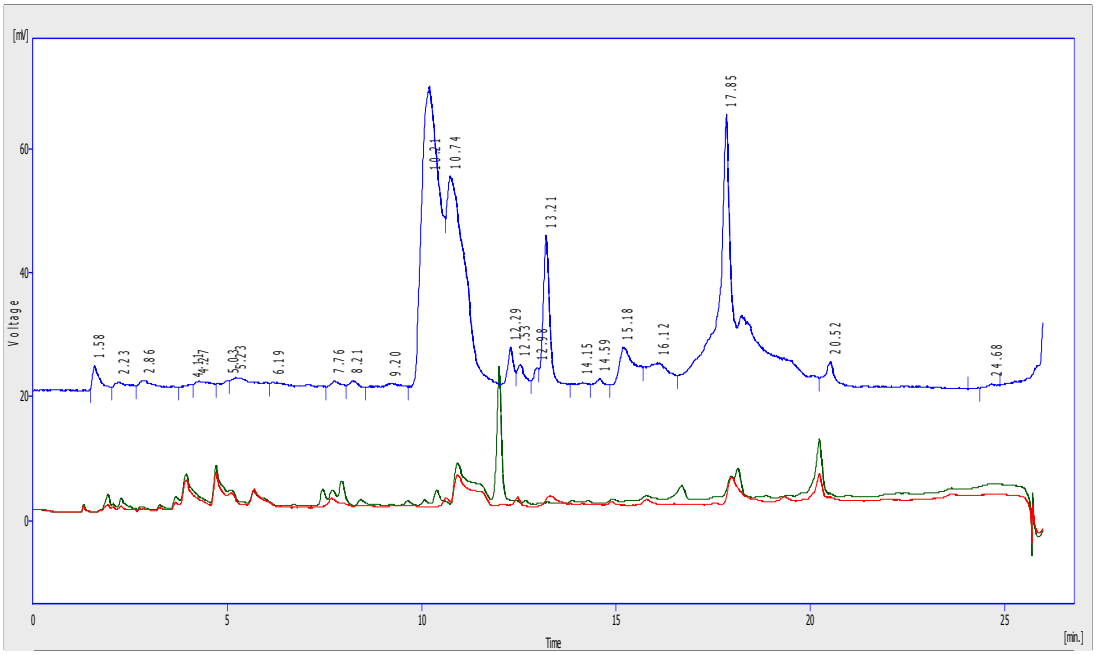


Figure 3-50 HPLC-UV-ELSD chromatogram of open column fraction P1-3 *(blue treces ELSD and pink trace UV at 290nm) (Conditions as in section 2.5.2)*

Some of the fractions obtained from MPLC were single peaks at a specific retention time, which confirmed the purity of these fractions, which were further analysed by using NMR. Figure 3-51 shows the HPLC-ELSD chromatogram for compound **(1)**, there was no UV response in the chromatogram.

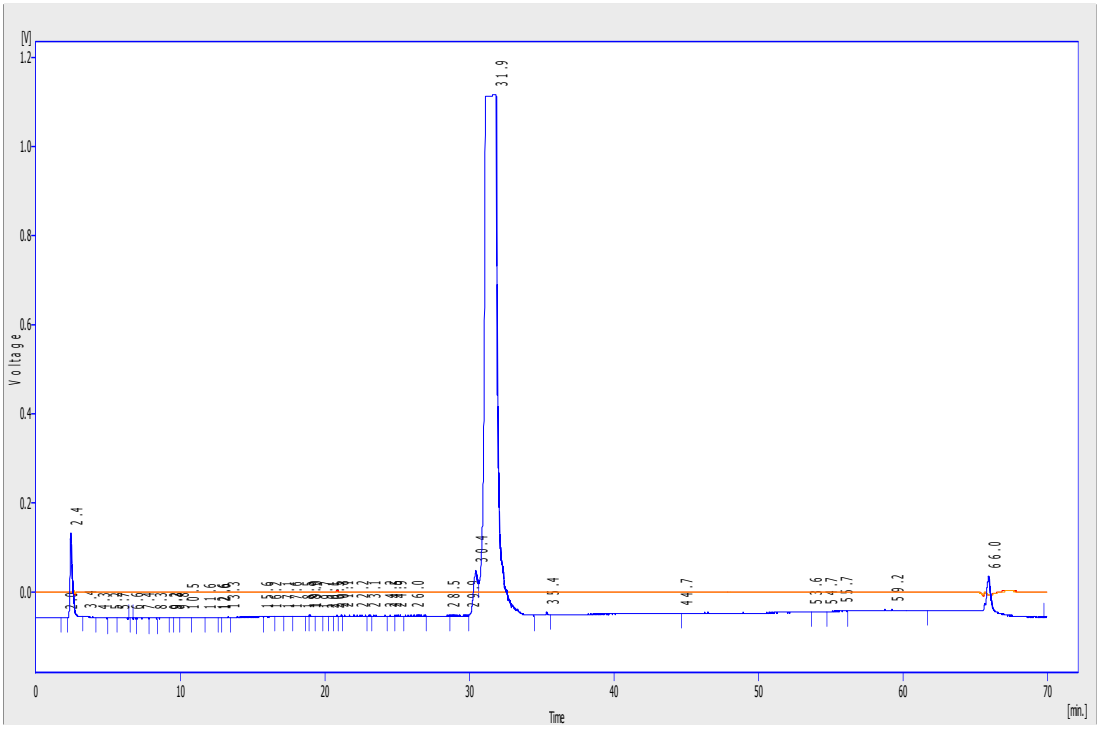


Figure 3-51 HPLC-UV-ELSD chromatogram of compound (1) *(blue treces ELSD and pink trace UV at 290nm)* gave a single peak at 31.9min *(Conditions as in section 2.5.2)*

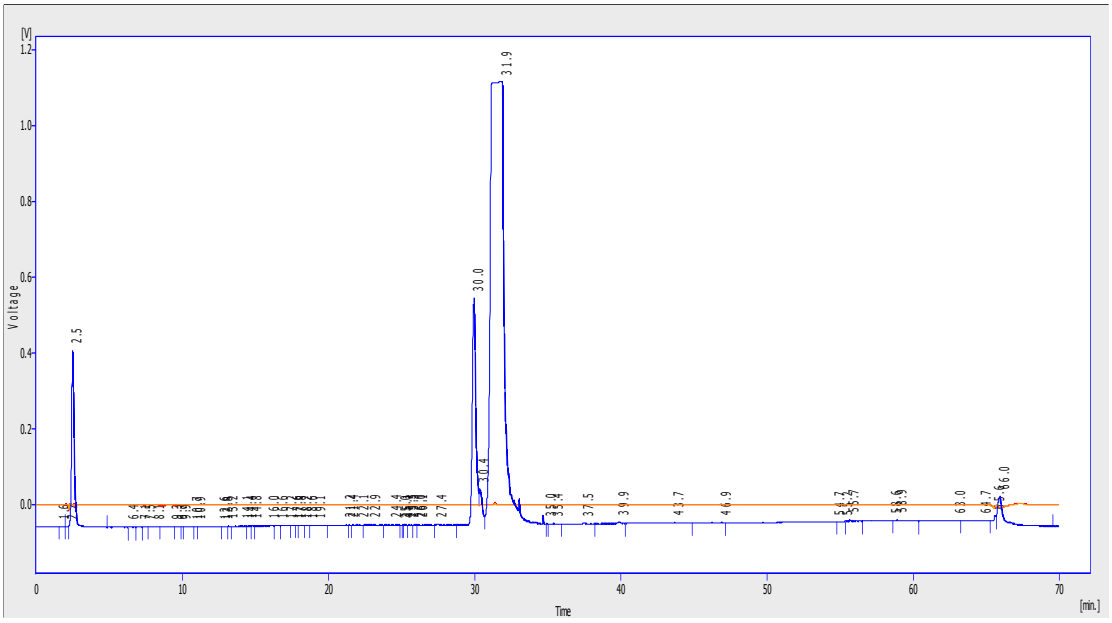


Figure 3-52 HPLC-UV-ELSD chromatogram of compound (2) *(blue treces ELSD and pink trace UV at 290nm)* gave a single peak at 31.9min *(Conditions as in section 2.5.2)*

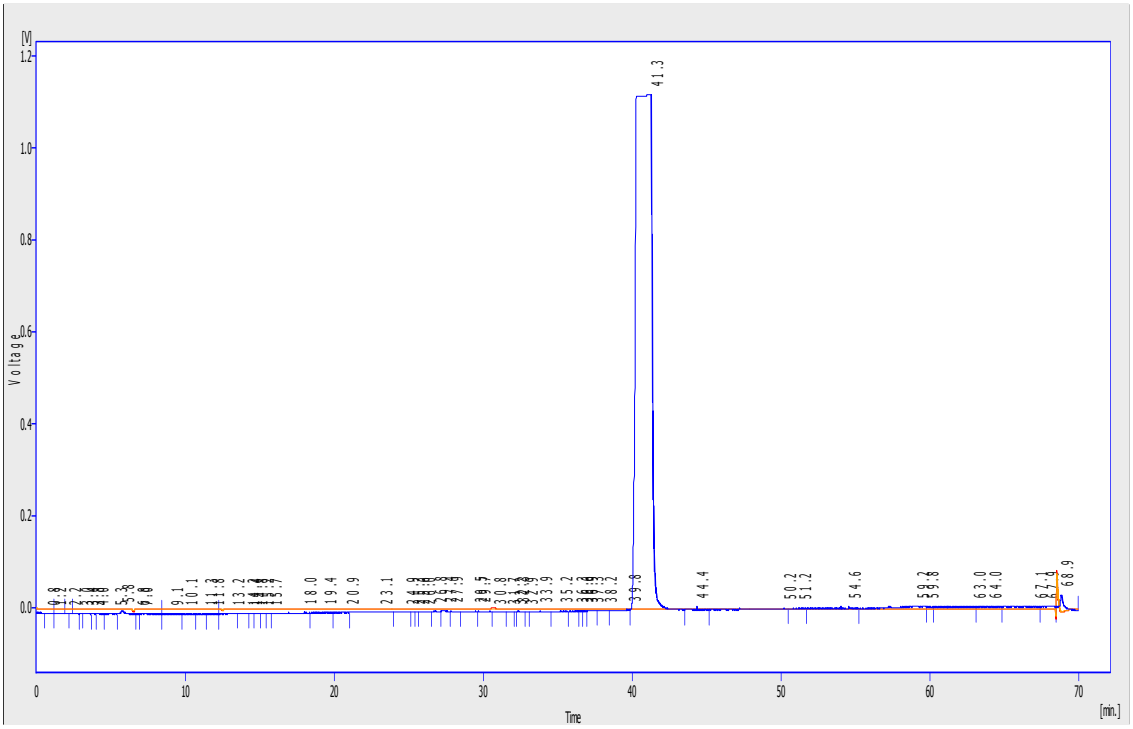


Figure 3-53 HPLC-UV-ELSD chromatogram of compound (3) *(blue treces ELSD and pink trace UV at 290nm)* gave a single peak at 41.3min *(Conditions as in section 2.5.2).*

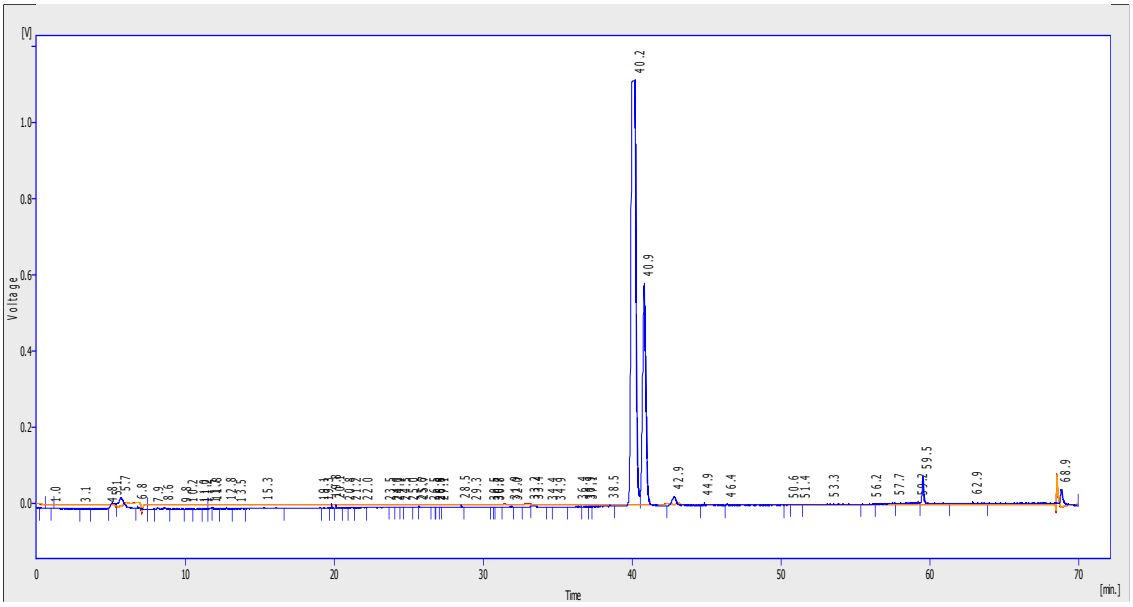


Figure 3-54 HPLC-UV-ELSD chromatogram of compound (4) *(blue treces ELSD and pink trace UV at 290nm*) gave a peak at 40.2min and 40.9min *(Conditions as in section 2.5.2).*

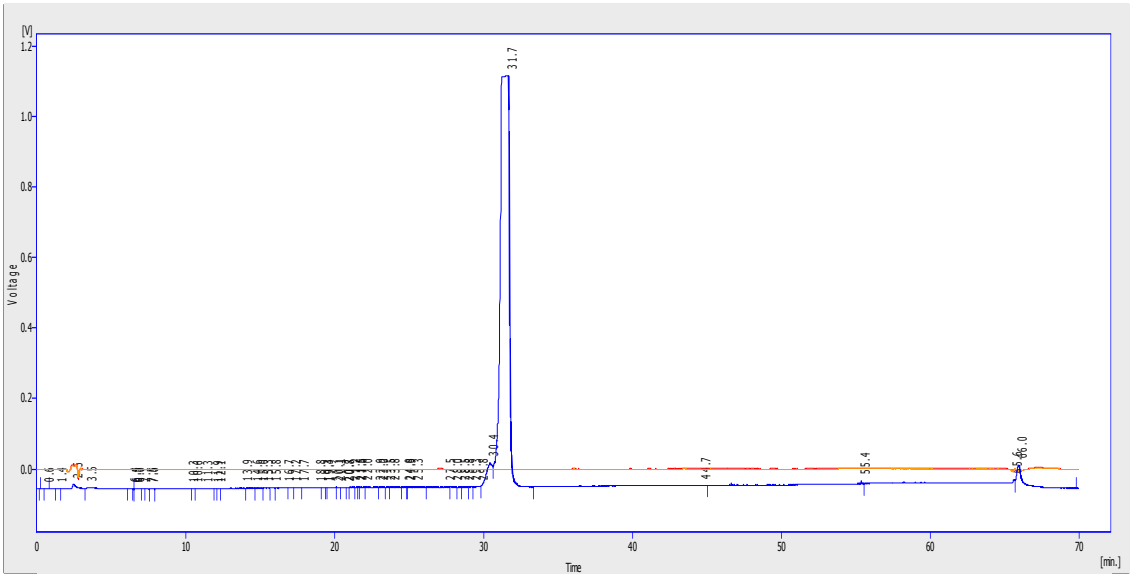


Figure 3-55 HPLC-UV-ELSD chromatogram of compound (5) *(blue traces ELSD and pink trace UV at 290nm)* gave a single peak at 31.7min *(Conditions as in section 2.5.2).*

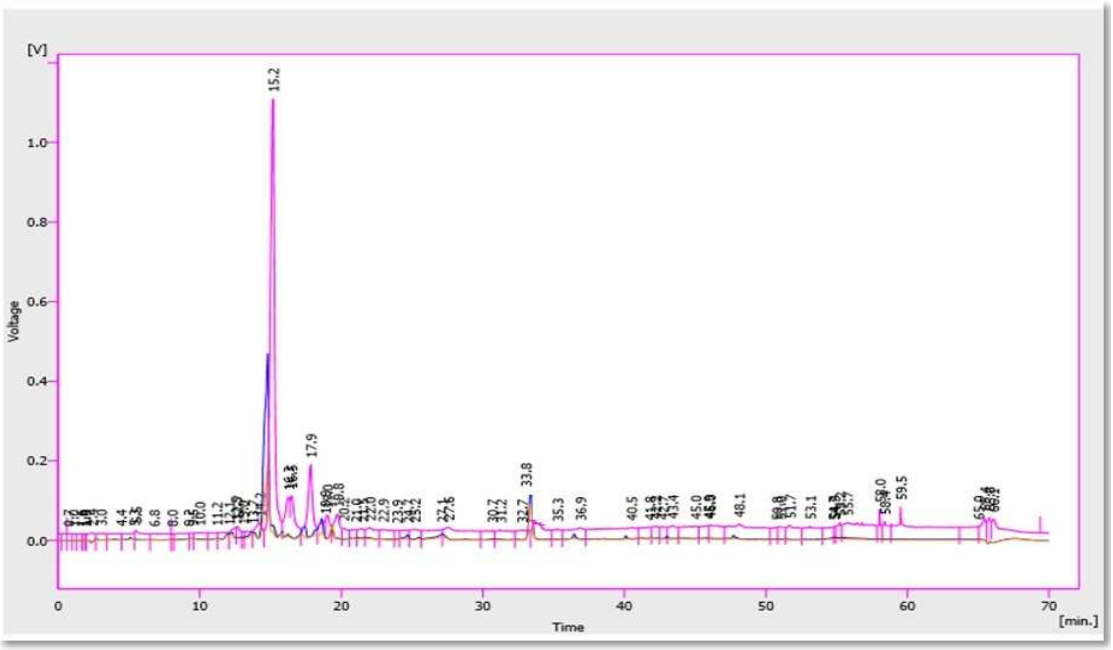


Figure 3-56 HPLC-UV-ELSD chromatogram of compound (8) from P1-2 by MPLC *(Pink trace UV at 290nm and Blue trace ELSD) (Conditions as in section 2.5.2)* gave a single peak at 15.2min.

3.11 LC-MS analysis of isolated compounds

The MPLC fractions containing largely pure compounds were investigated by high resolution LC-MS.

Figure 3-57 shows the mass spectrum of the compound **(3)** which has an elemental $C_{20}H_{32}O_3$ and an $[M-H]^-$ ion at m/z 319.2268

Compound **(3)** was considered as major compound in P1 sample as it gave a higher yield > 101mg in total weight and was collected from more than one fraction see (Table 3-28).

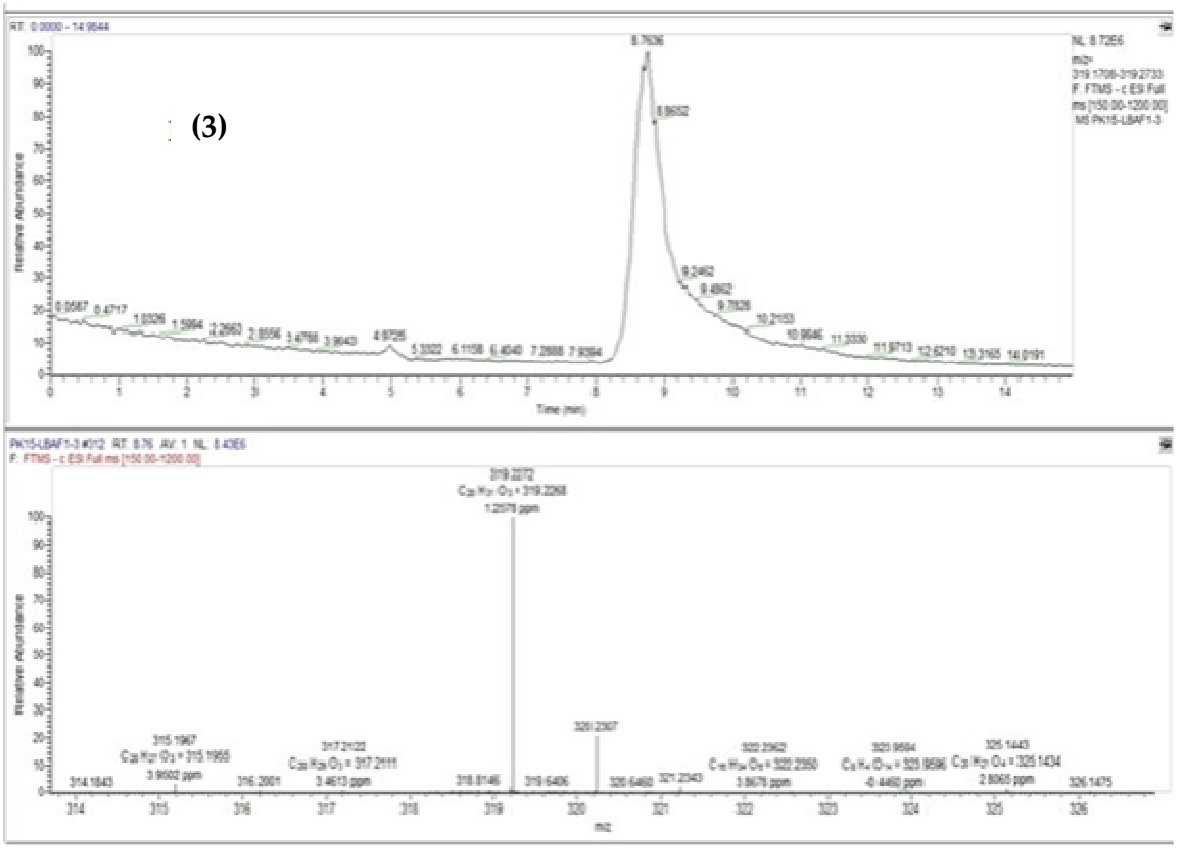


Figure 3-57 HR-ESIMS chromatogram of compound (3) with $[m-z]^{-1}$ -1 319.22 with RDB =7.5, Delta 1.23 ppm

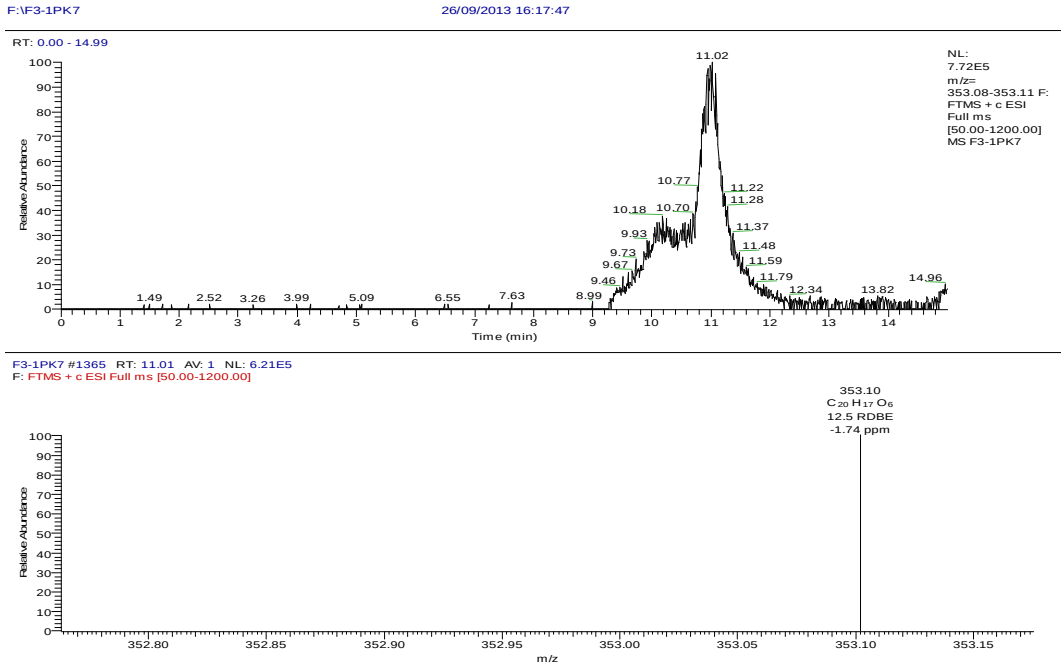


Figure 3-58 HR-ESIMS chromatogram of compound (5) Sesamin, with $[m-z]^{-1}$ 353.10.RDB=12.5 and delta ppm =-1.74

Figure 3-58 shows the LC-MS analysis of the compound in **(5)** which has and elemental composition of $C_{20}H_{17}O_6$ and a molecular ion with m/z 353.10 for its $[M-H]^-$ ion.

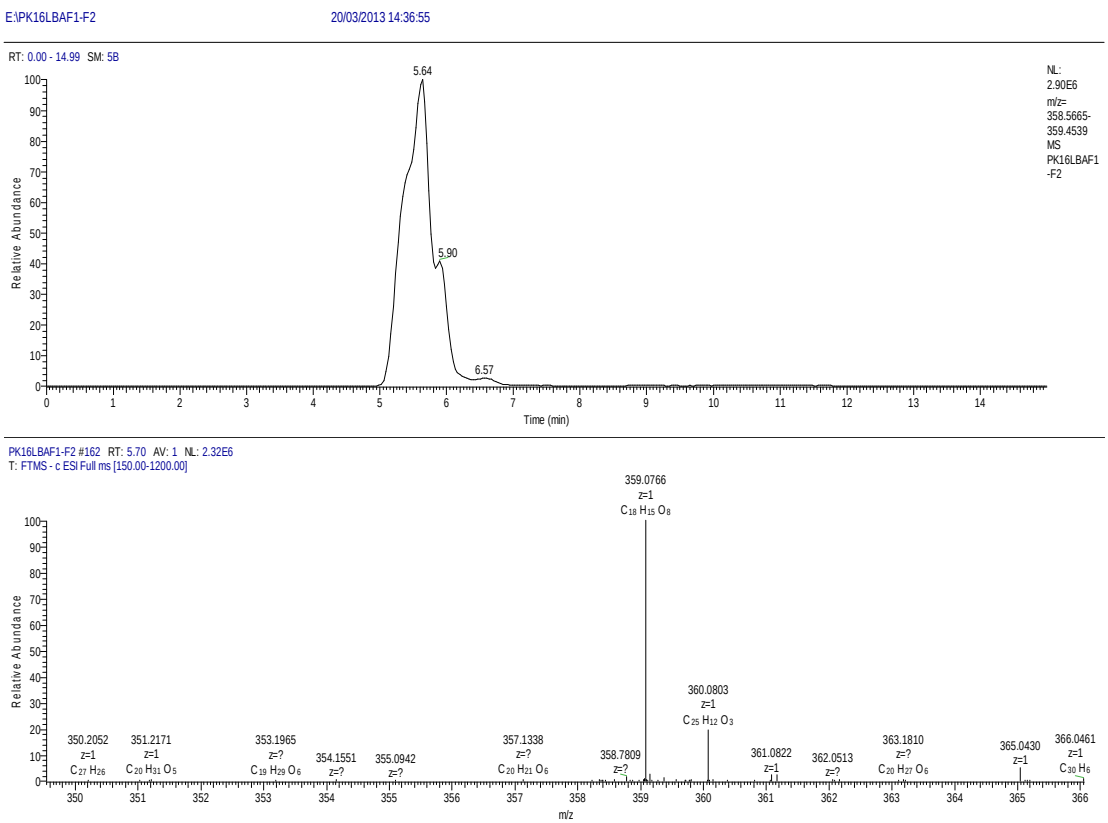


Figure 3-59 HR-ESIMS chromatogram of compound (8) with $[m-z]^{-1}$ 359.0766 and molecular formula $C_{18}H_{15}O_8$, RDB=10 and Delta ppm=1.784.

Figure 3-59 shows the HR-ESIMS analysis of compound **(8)** which has and elemental composition of $C_{18}H_{15}O_8$ and a molecular ion with m/z 359.0766 for its $[M-H]^-$ ion.

3.12 Gas Chromatography Mass Spectrometry (GC-MS) Analysis

Analysis by gas chromatography -mass spectroscopy (GC-MS) of samples P1, P2 was carried out and also for the MPLC fractions of P1 GC-MS analysis of P1 and P2 gave the chromatograms shown in (Figures 3-60 and 3-61) where most of components eluted at between 10 and 30 min.

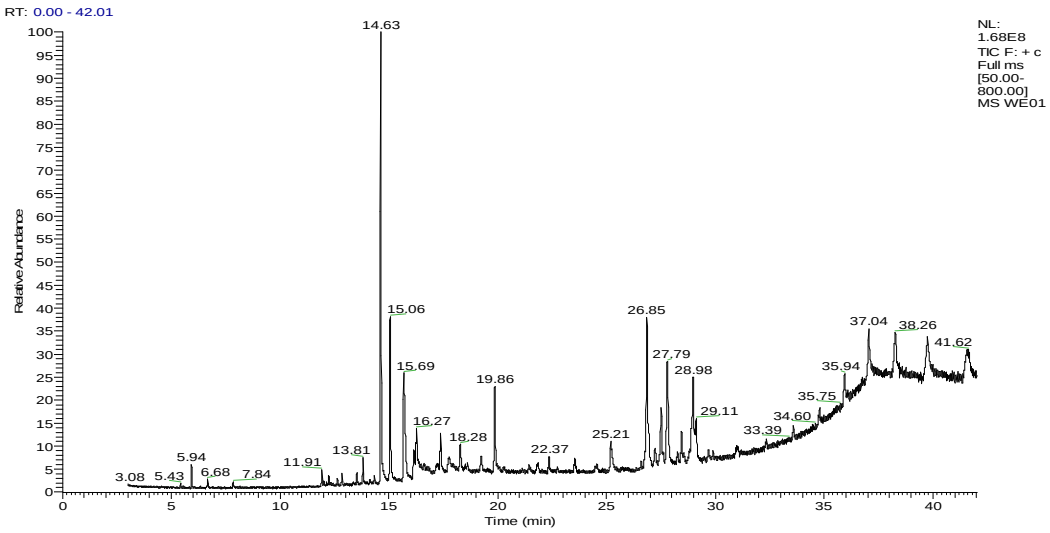


Figure 3-60 GC chromatogram of P1 Total ion current trace from GC-MS analysis of P1 on an Rtx-1 column.

Figure 3-61 shows the full GC-MS chromatogram of the crude EEP of P1 and the library matching indicated an abundance of diterpenes. In addition, there were also triterpenes in the extract.

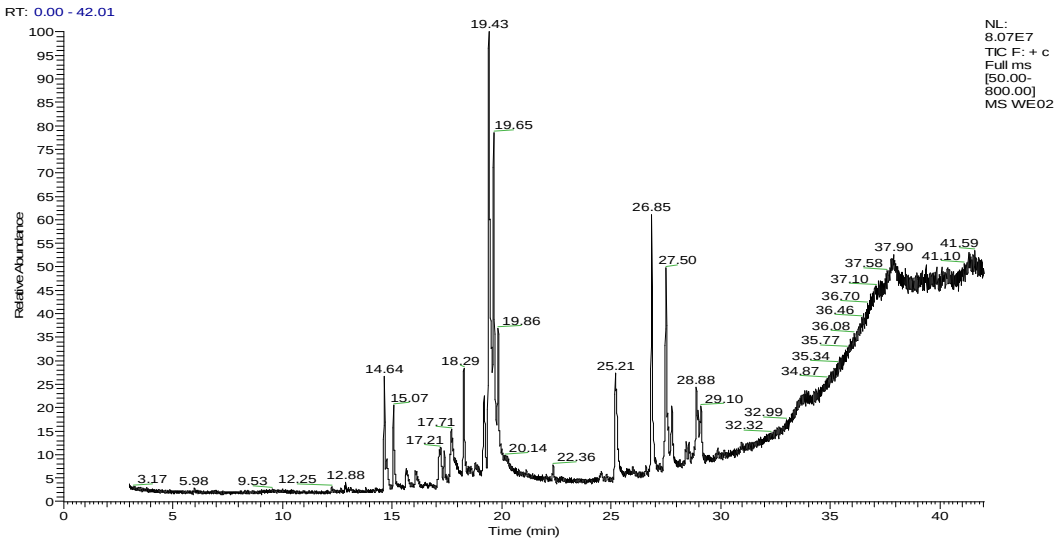


Figure 3-61 GC chromatogram of P2 Total ion current trace from GC-MS analysis of P2 on an Rtx-1 column.

Additionally, Figures 3-62 to 3-66 show the GC-MS chromatograms of compounds isolated from P1-3 fraction which are **(1)**, **(2), (3)** and **(4)** isolated by flash chromatography MPLC. The GC-MS analysis provided primary help in the structure elucidation of these isolated compounds. As can be seen from the chromatograms the GC-MS analysis confirmed a good degree of purity for the some compounds such as **(1)** (Figure 3-62) where it provides a clear peak at RT =14.8 min.

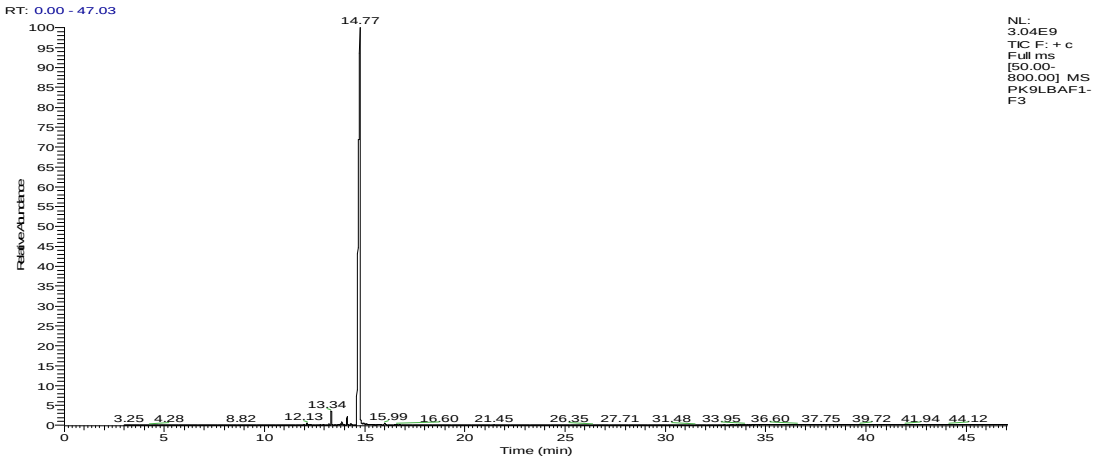


Figure 3-62 GC chromatogram of compound (1) RT=14.77 min Total ion current trace from GC-MS analysis of (1) on an Rtx-1 column.

As can be seen from the chromatogram (Figure 3-63) compound **(2)** also appears at retention time of 14.64 min.

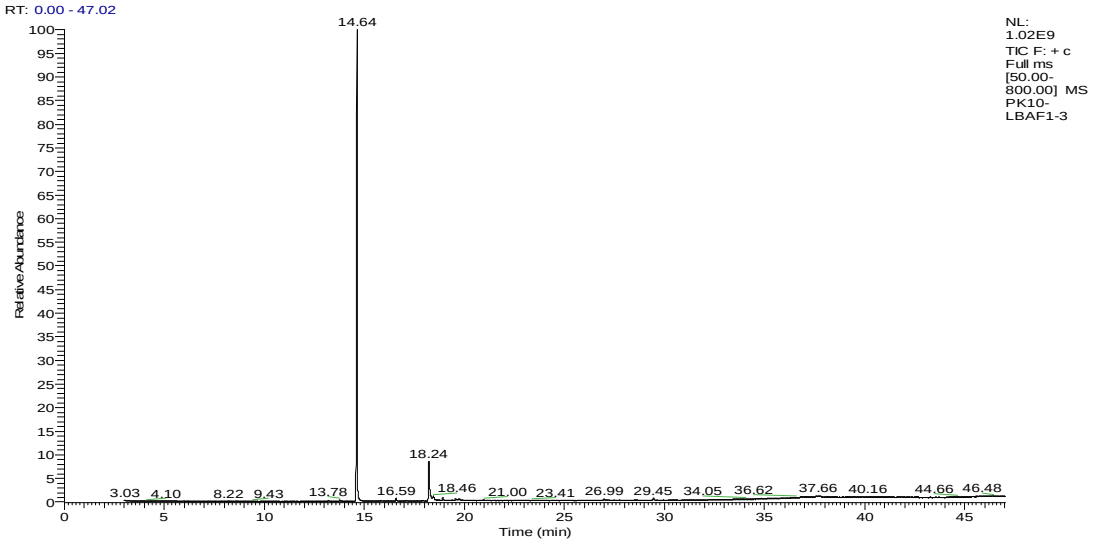


Figure 3-63 GC chromatogram of compound (2) RT=14.66 Total ion current trace from GC-MS analysis on a Rtx-1 column.

Also, the GC-MS trace shown in Figure 3-64 shows a peak at a retention time of 15.64min again indicating compound **(3).**

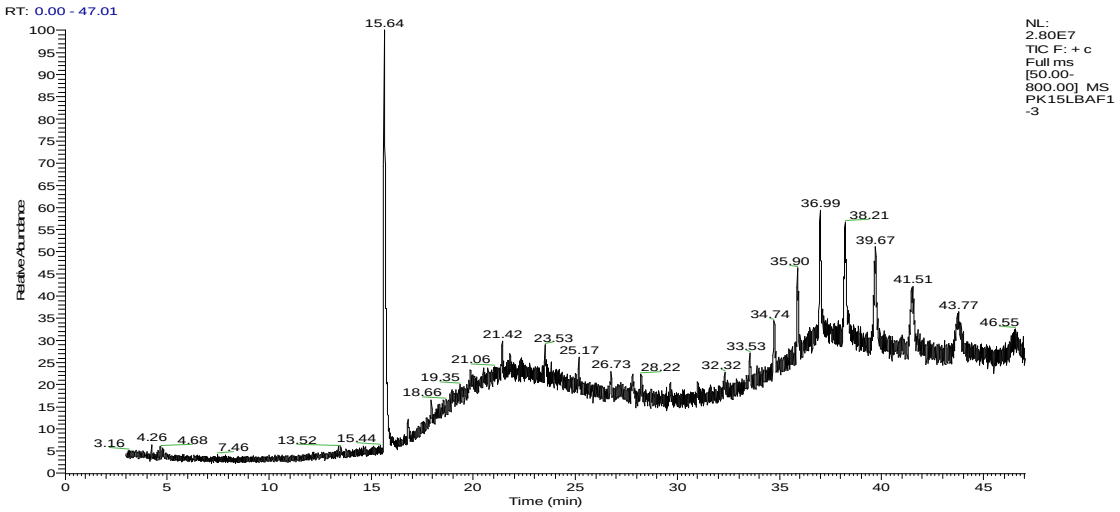


Figure 3-64 GC chromatogram of compound (3) at RT =15.58 Total ion current trace from GC-MS analysis of a Rtx-1 column.

The library matching for all four compounds indicated that these compounds were might be diterpenes.

Figure 3-65 of The GC-MS trace displayed a peak at a retention time of 15.72min again indicating compound **(4).**

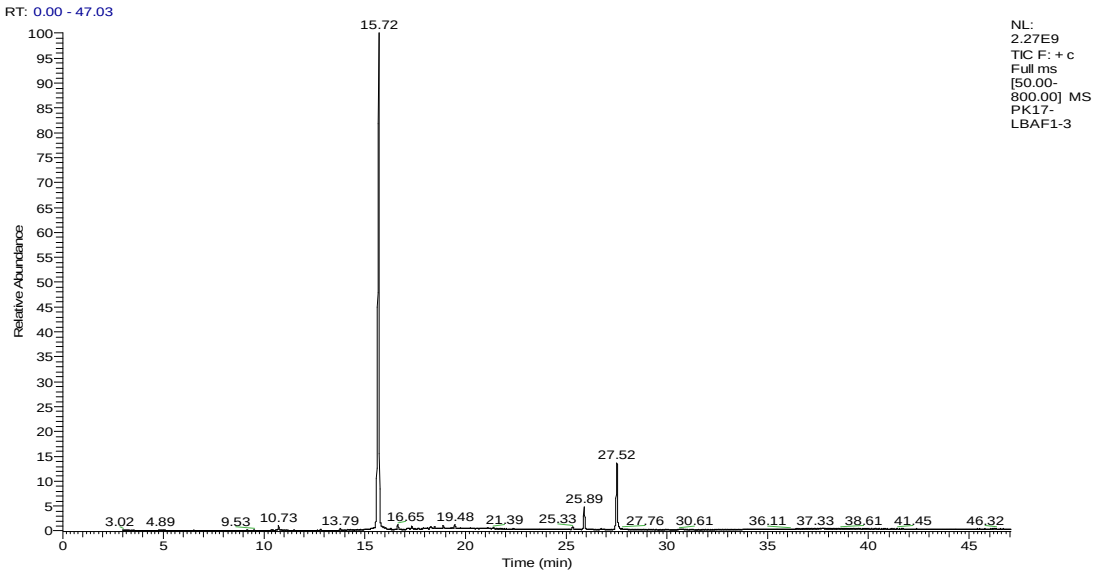


Figure 3-65 GC chromatogram of compound (4) Total ion current trace from GC-MS on an Rtx-1 column.

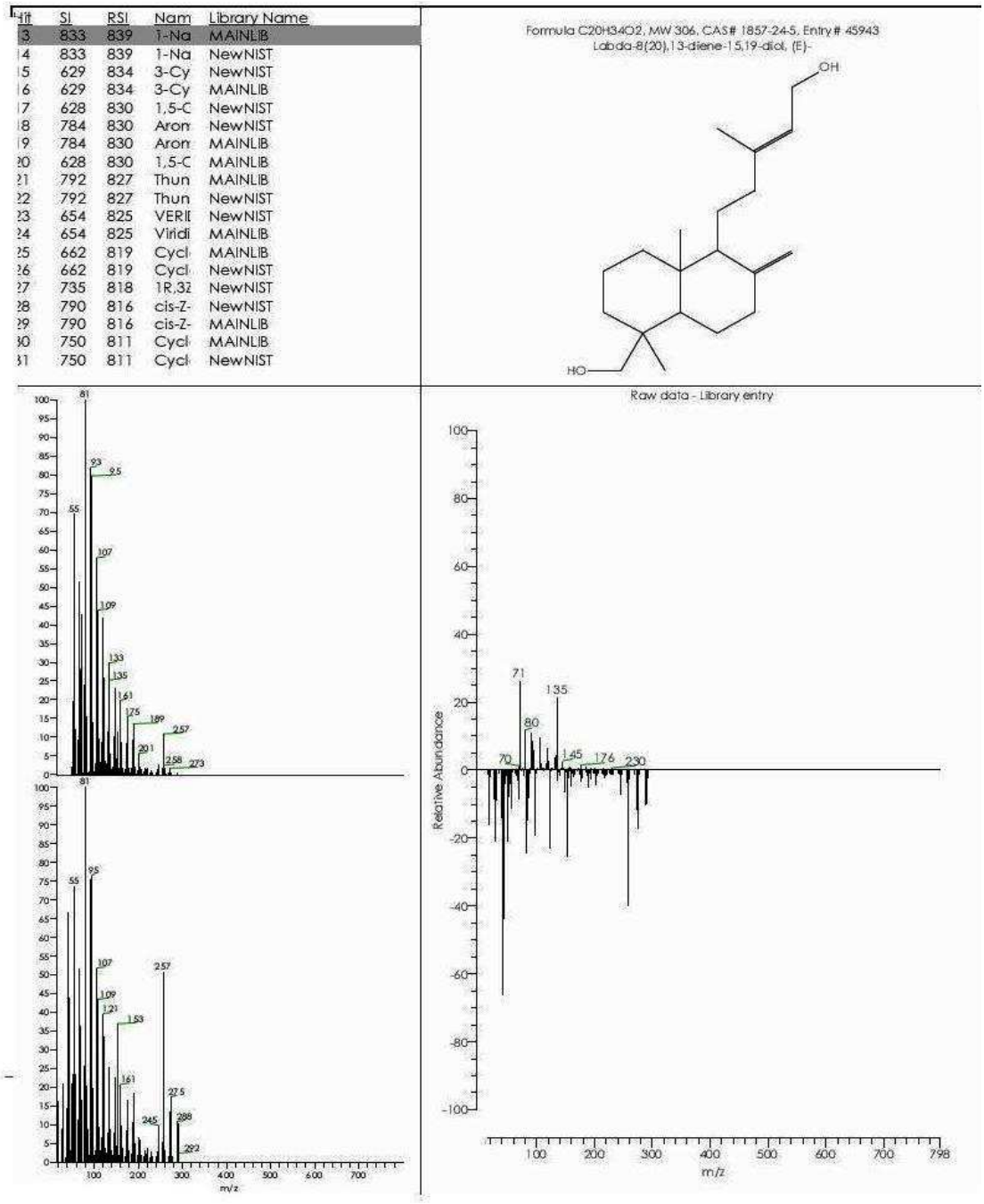


Figure 3-66 GC chromatogram of compound (4) Library match in NIST library

3.13 Characterisation of isolated compounds from P1, P2, P7 and P9 samples.

Table 3-34 summarises the 18 isolated compounds, which were isolated from P1, P2, P7 and P9 samples. Initially, P1 and P2 samples were chosen for a further purification using open column chromatorgraphy and MPLC based on their antitrypanosomal and antileishmanial activity (section 3.5.1, Table 3-15 to 3-16 and section 3.5.2, Table 3-17). Four pure diterpenes were isolated by MPLC from fraction P1-3 of the P1 sample (Table 3-33). These were **(1)** 13-epi-torulosal, **(2)** 13-O-Acetyl epi-cupressic acid, **(3)** 13-epicupressic acid and **(4)** 13-epitorulosol. Figure 3-67 shows ^{1}H NMR spectra in $CDCl_3$ (JEOL Delta GX 400 MHz) for the four pure diterpenes isolated by MPLC from P1 (P1-3) and chemical structures of compounds (**1), (2), (3)** and **(4)** are shown in Figure 3-68. What is immediately apparent from the spectra is that all the compounds contain two identical double bond systems can be seen by comparison of the proton spectra of **(1)-(4)** in the chemical shift region between 3-7ppm. The ^{1}H NMR spectra of the compounds **(1), (3)** and **(4)** showed three methyl singlets at $^{\delta}H$ at 1.22, 0.54 and 1.12 ppm which can be assigned to C-16, C-18 and C-19, also there are significant similarities between **(1), (2), (3)** and **(4)** in chemical shift between 0.5 and 2.42ppm.

In **(2)** there is a signal at chemical shift 2 ppm which is not present in **(1)**, **(3)** and **4**. Spectral data for **(2)** were very similar to those for **(3)** except for the presence of a downfield shifted methyl singlet at δ 1.96 ppm. The four spectra have an exomethylene group and vinyl group signals and in common with a methyl at $^{\delta}H$ approximately 0.6 ppm. The later is the signal of the methyl in position C10 (Figure 3-68)

However, there are significant differences in the chemical shift of the methyl groups in positions C4 and C13 due to their different environments.

The assignment of proton and carbon shifts was done by comparison of the data from this study with the previous literature as shown in Tables 3-34 to Table 3-37. Figure 3-68 shows ^{1}H NMR for compounds **(1)-(4)**. The spectra show a significant similarity between **(1), (2), (3**) and **(4)** in the region δ 4.4 ppm to 6 ppm there is similarity in peaks of **(1)**, **(2)** and **(3)** although the intensity of the peaks in **(3)** is lower. Thus the system of double bonds in these molecules is the same.

The NMR data of the pure compounds **(1)**, **(2)**, **(3)** and **(4)** are shown in Tables 3-35 – 3-38.

The experimental data of the current study of P1 pure fractions were compared to the data of a previous study carried out by Abdel-Sattar *et al*., (2009), and the study isolated these compounds from the resin of *Araucaria heterophylla* salisb (Abdel-Sattar et al., 2009).

GC-MS data (Figures 3-62 to 3-66) for compounds **(1)**, **(2)**, **(3)** and **(4)** indicated that they were diterpenes of the labdane group.

Table 3-34 Chemical characteristic of isolated pure compounds from P1, P2, P7 and P9 samples.

Pure Compound	Chemical Name	Propolis sample[a]	Molecular formula	Molecular weight	[M-1]	Chemical Group
(1)	13-epitorulosal	P1-3-9	$C_{20}H_{32}O_2$	304.4623	303.4623	Diterpene
(2)	13-O-epi-Cupressic acid	P1-3-10	$C_{22}H_{34}O_3$	346.1122	345.1122	Diterpene
(3)	13- Cupressic acid	P1-3-15	$C_{20}H_{32}O_3$	320.4731	319.4731	Diterpene
(4)	13-epitorulosal	P1-3-17	$C_{20}H_{34}O_2$	306.4812	305.4812	Diterpene
(5)	Sesamin	P1-9-7	$C_{20}H_{18}O_6$	354.1021	353.1021	Lignan
(6)	Demethylpiperitol	P2-12-37	$C_{19}H_{18}O_6$	342.1211	341.1211	Lignan
(7)	5′,methoxy pipertol	P2-24-7	$C_{21}H_{22}O_7$	386.1136	385.1136	Lignan
(8)	Taxifolin-3-acetate-4′methylether	P1-2-16	$C_{18}H_{16}O_8$	360.0766	359.0766	Flavone
(9)	Cycloartanol	P7-15	$C_{30}H_{50}O$	426.3821	425.3821	Cycloartan triterpene
(10)	Mangiferolic acid	P7-32	$C_{30}H_{48}O_3$	456.7123	455.7123	Cycloartan triterpene
(11)	Mangiferonic acid	P7-35	$C_{30}H_{46}O_3$	454.6934	453.6934	Cycloartan triterpene
(12)	Ambolic acid $C_{31}H_{51}O_3$	P7-31	$C_{31}H_{50}O_3$	470.738	469.729	Cycloartan triterpene
(13)	27-Hydroxymangiferonc acid	P7-51	$C_{30}H_{46}O_4$	470.6982	469.6982	Cycloartan triterpene
(14)	Cardol	P7-20	$C_{21}H_{34}O_2$	317.2421	316.2421	Resorcinols
(15)	Acetylisocuppressic acid	P9-15	$C_{20}H_{32}O_3$	320.4711	319.1711	Diterpene
(16)	Agathadiol	P9-28	$C_{20}H_{34}O_2$	306.4838	305.4838	Diterpene
(17)	Isocupressic acid	P9-26	$C_{20}H_{32}O_3$	320.4791	319.4791	Diterpene
(18)	Isoagatholal	P9-17	$C_{20}H_{32}O_2$	304.2412	303.2412	Diterpene

N.B; [a]; Propolis sample MPLC fraction codes

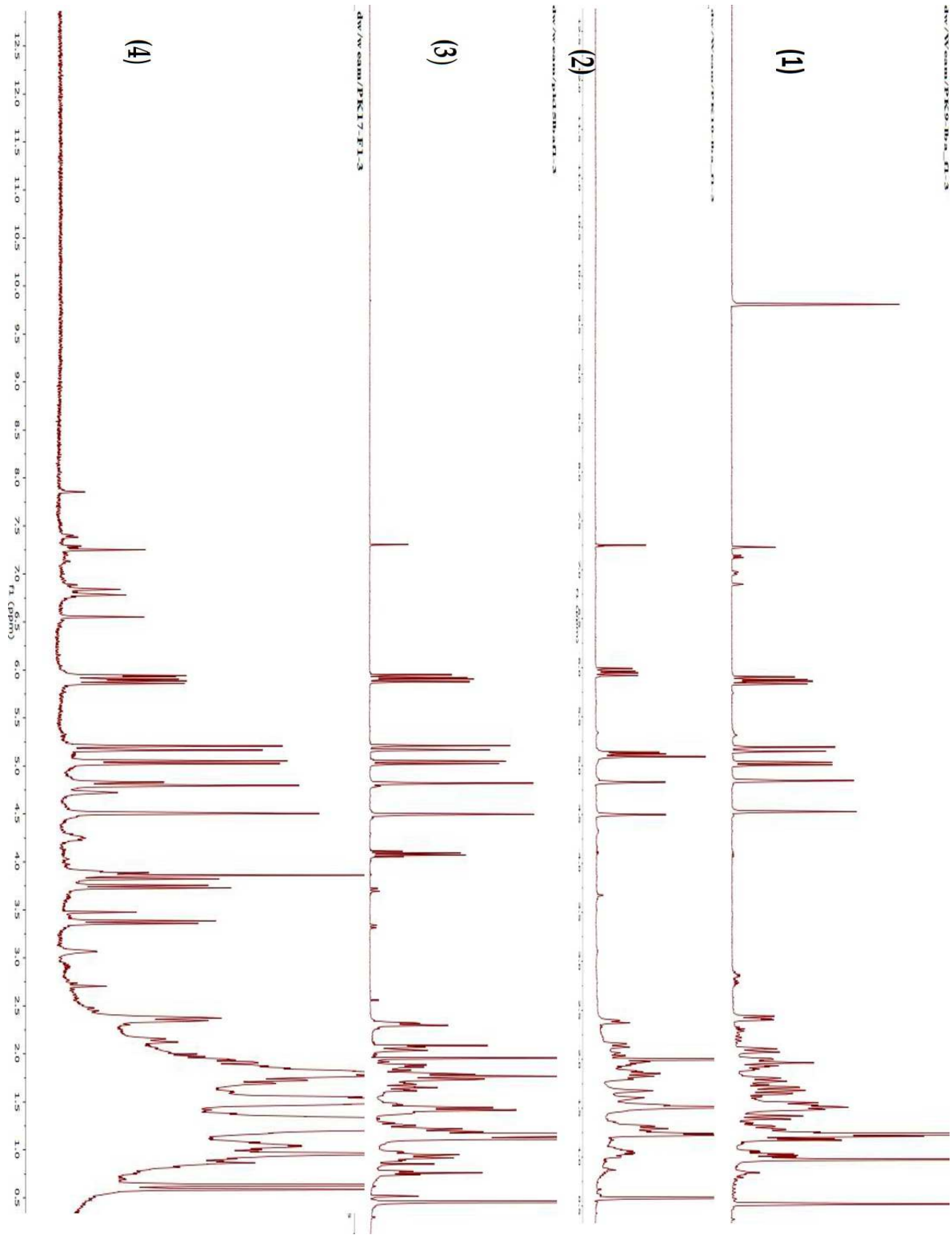
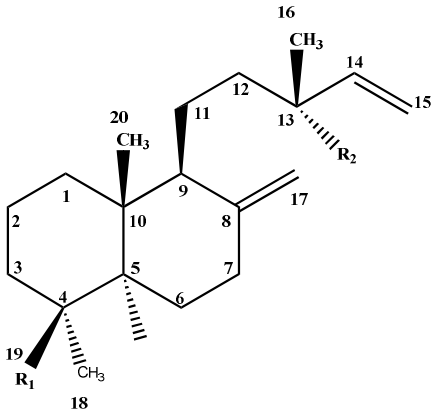
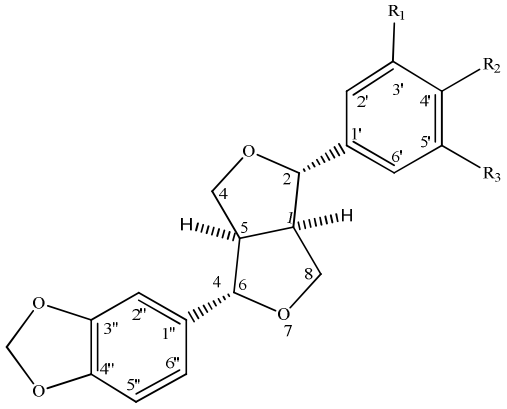


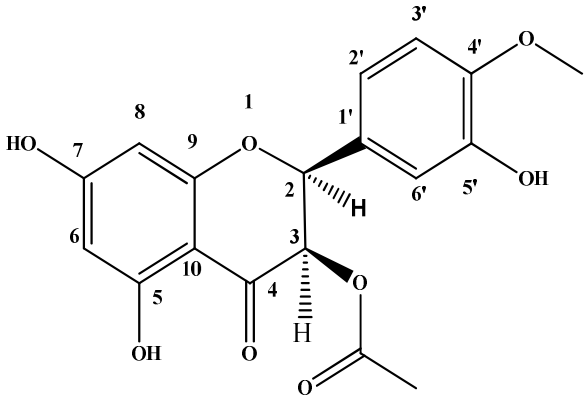
Figure 3-67 ^{1}H NMR spectra comparison of compounds (1), (2), (3) and (4) (0.5-12.5 ppm) (400 MHz, $CDCl_3$).



	Chemical names	R^1	R^2
(1)	13-epi-torulosal	CHO	OH
(2)	13 -O- Acetyl epi-cupressic acid	COOH	OAc
(3)	13-epicupressic acid	COOH	OH
(4)	13-epitorulosol	CH_2OH	OH



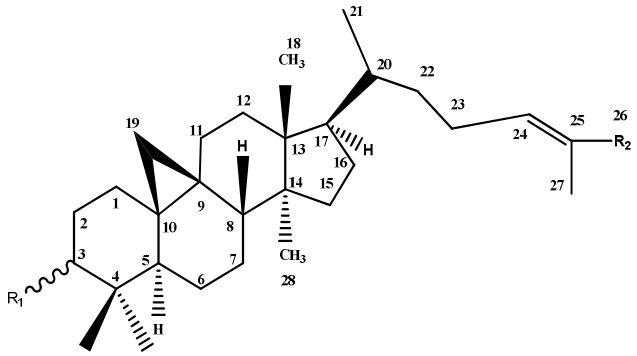
	Chemical names	R_1	R_2	R_3
(5)	Sesamin	OCH_2O		H
(6)	Demethylpiperitol	OH	OH	H
(7)	5′-methoxypiperitol	OCH_3	OH	OCH_3



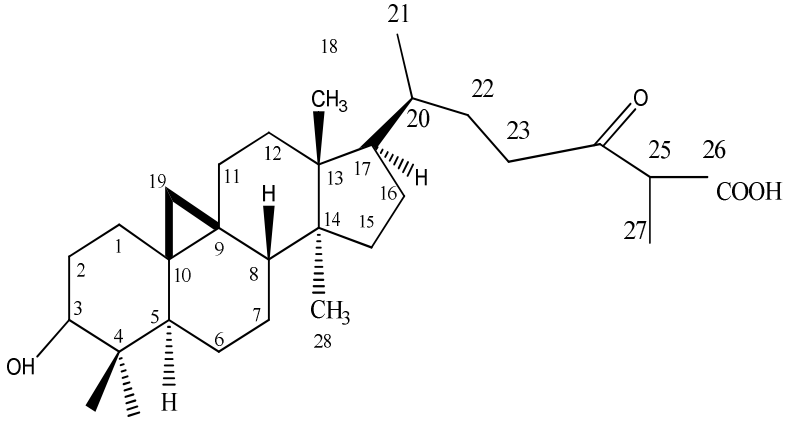
(8) Taxifolin-3-acetate-4′-methyl ether)

***(2S,3R)-5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-3- methyl-4-oxochroman-3-yl acetate**

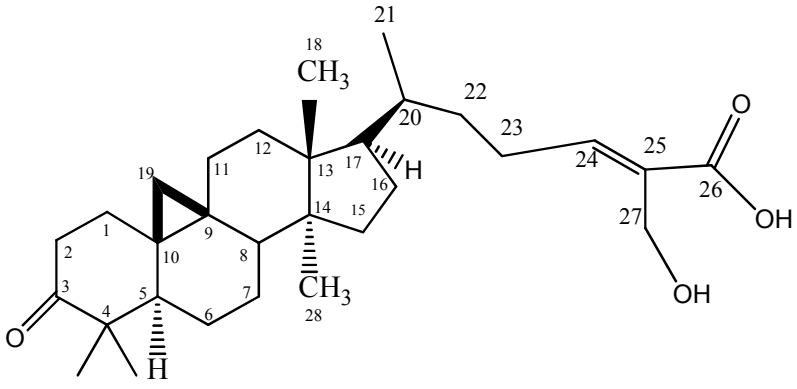
N.B, * (IUPAC) chemical name using ChemDraw Professional 15.1



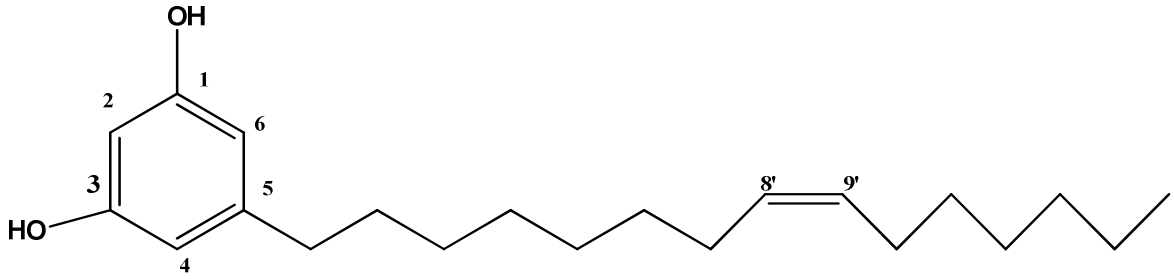
	Chemical names	R_1	R_2
(9)	**Cycloartanol**	**β-OH**	**CH_3**
(10)	**Mangiferolic acid**	**β-OH**	**COOH**
(11)	**Mangiferonic acid**	**=O**	**COOH**



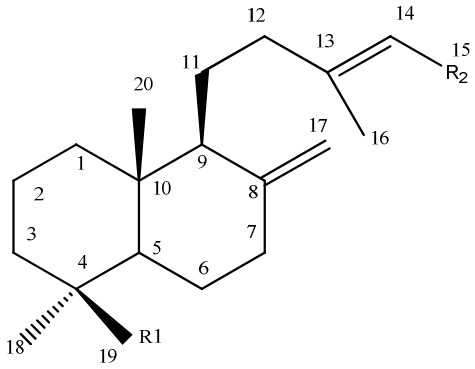
(12) Ambolic acid



(13) 27-hydroxymangiferonic acid



(14) Cardol



	Chemical names	R_1	R_2
(15)	**Acetylisocuppressic acid**	**COOH**	**CH_2OAc**
(16)	**Agathadiol**	**CH_2OH**	**CH_2OH**
(17)	**Isocupressic acid**	**COOH**	**CH_2OH**
(18)	**Isoagatholal**	**CHO**	**CH_2OH**

Figure 3-68 Structures of the isolated compounds from Libyan propolis P1, P2 and P7 and P9

Note, ChemDraw Professional 15.1 was used to draw the chemical structures followed by using the SciFinder® Data Base to find the previous occurrence of these compounds.

Table 3-35 ^{1}H and ^{13}C NMR of compound **(1)** (400 MHz, $CDCl_3$); 13-epi-torulosal compared to the literature[a]

No	^{1}H Experimental δ (ppm)	^{13}C Experimental δ (ppm)	^{1}H Literature[a] δ (ppm)	^{13}C Literature[a] δ (ppm)
1	2.15, 1.07	34.5 (CH_2)	-	34.6
2	1.58, 1.58	19.4 (CH_2)	-	19.4
3	1.86, 1.09	38.6 (CH_2)	-	38.6
4	—	48.8	—	48.9
5	1.60	56.0(CH)	-	56.0
6	2.03	24.1(CH)	-	24.2
7	1.85, 1.09	38.5(CH_2)	-	
8	—	147.5	—	147.7
9	1.47	56.2(CH)	-	56.3
10	—	40.4	—	40.4
11	1.59, 1.59	18.1(CH_2)	-	18.1
12	1.77,1.30	41.4(CH_2)	-	41.4
13	—	73.6	—	73.8
14	5.89(dd,*J*=17.3, 10.7Hz)	145.2	5.90(1H,dd,*J*=17.0, 10.5Hz	145.2
15	5.19(dd,*J*=17.3, 1.1Hz) 5.04(dd,*J*=10.7, 1.1Hz	111.8	5.20(1H,*dd*,*J*=17.5, 1.0 Hz 5.06(1H,*dd*,J=10.5, 1.0Hz)	111.9
16	1.26 (3H,s)	27.8	1.27(3H,s)	28.3
17	4.87(1H,s) 4.55 (1H,s)	107.6	4.86(1H,s) 4.52 (1H,s)	107.6
18	1.00(3H,s)	24.5(CH_3)	0.97(3H,s)	24.6
19	9.73(	205.9(CH)	9.74(1H,s)	206.0
20	0.55(3H,s)	13.6	0.56(3H,s)	13.7

N.B; [a]; (Woo et al., 2011), **-;** not mentioned before in literature, —; no proton.

Table 3-36 ^{1}H and ^{13}C NMR of compound **(2)** (400 MHz, $CDCl_3$); 13 -O-Acetyl epi-cupressic acid compared to the literature[a]

No	^{1}H Experimental δ (ppm)	^{13}C Experimental δ (ppm)	^{1}H Literature[a] δ (ppm)	^{13}C Literature[a] δ (ppm)
1	1.84, 1.08	39.1(CH_2)	-	38.9
2	1.87, 1.54	20.0(CH_2)	-	19.8
3	2.18, 1.17	38.1(CH_2)	-	37.9
4	—	44.2	—	44.1
5	1.34	56.4 (CH_2)	-	56.3
6	1.99,1.89	26.1 CH_2	-	26.0
7	2.42,1.89	38.8	-	38.6
8	—	148.0	—	147.8
9	1.55	56.6(CH)	-	56.3
10	—	39.3	—	39.2
11	1.53,1.35	17.7 (CH_2)	-	17.5
12	2.00,1.56	40.7(CH_2)	-	40.6
13	—	83.4	—	83.3
14	5.96 (1H,dd,*J*=17.6, 11.0 Hz	142.0	5.88(1H,*dd*,*J*=17.2, 10.8Hz)	141.8
15	5.12(1H,d,*J*=17.6Hz) 5.10(1H,d,*J*=11.0 Hz)	113.2	5.02(1H, d, J=17. Hz) 5.03(1H,d,*J*=10.8 Hz)	113.0
16	1.51(3H,s)	23.6	1.45(3H,s)	23.4
17	4.83(1H,s) 4.50(1H,s)	106.7	4.43(1H,brs) 4.76(1H,br s)	106.4
18	1.23 (3H,s)	29.1	0.52 (3H,s)	28.9
19	—	183.0	1.16	184.0
20	0.62(3H,s)	12.8	—	12.7
21($CO\underline{C}H_3$)	2.00(3H,s)	22.3	1.94 (3H,s)	21.0
22($\underline{C}OCH_3$)	—	170.7	—	171.1

N.B; [a]; (Abdel-Sattar et al., 2009), **-;** not mentioned before in literature, —; no proton.

Table 3-37 ^{1}H and ^{13}C NMR of compound **(3)** (400 MHz, $CDCl_3$); 13-epicupressic acid compared to the literature[a]

No	^{1}H Experimental δ (ppm)	^{13}C Experimental δ (ppm)	^{1}H Literature[a] δ (ppm)	^{13}C Literature[a] δ (ppm)
1	1.05, 1.81	39.2(CH_2)	-	39.0 (CH_2)
2	1.84,1.49	20.0(CH_2)	-	19.8 (CH_2)
3	1.03 (m)	38.1(CH_2)	-	37.9 (CH_2)
4	—	44.3 (C)	-	44.1 (C)
5	1.60	56.0 (CH)	-	56.3 (CH)
6	1.97,1.87	26.1 (CH_2)	-	25.9 (CH_2)
7	1.85 (m),2.38(m)	38.8 (CH_2)	-	38.7 (CH_2)
8	—	148.1 (C)	-	147.7 (C)
9	1.52	56.7 (CH)	-	56.5 (CH)
10	—	40.8 (C)	-	40.3 (C)
11	1.54 (m) ,1.32(m)	18.0 (CH_2)	-	17.8 (CH_2)
12	1.24 (m),1.74(m)	41.5 (CH_2)	-	41.3 (CH_2)
13	—	73.7 (C)	-	73.7 (C)
14	5.89 (1H,dd,*J*=17.2,10.8Hz)	145.39 (CH)	5.81 (1H,dd,*J*=17.2,10.8Hz)	144.9 (CH)
15	5.02 (1H,dd,*J*=17.2, 1.0Hz) 5.19 (1H,dd,*J*=10.8, 1.0 Hz)	111.7 (CH_2)	4.98 (1H,d,*J*= 17.2Hz) 5.13 (1H,d,*J*,= 10.8Hz)	111.6 (CH_2)
16	1.26 (s)	27.7 (CH_2)	1.22 (1H,s)	28.9 (CH_2)
17	4.51 (1H,s) 4.83 (1H,s)	106.8 (CH_2)	4.48 (1H,s) 4.79 (1H,s)	106.6 (CH_2)
18	1.22 (3H,s)	29.1 (CH_3)	1.18 (3H,s)	28.4
19	—	183.5 (C)	-	183.3 (C)
20	0.58 (3H,s)	12.8 (CH_3)	0.54(3H,s)	12.9 (CH_3)

N.B; [a]; (Abdel-Sattar et al., 2009), **-;** not mentioned before in literature.

Table 3-38 ^{1}H and ^{13}C NMR of compound **(4)** (400 MHz, $CDCl_3$); 13-epitorulosol compared to the literature[a]

No	^{1}H Experimental δ (ppm)	^{13}C Experimental δ (ppm)	^{1}H Literature[a] δ (ppm)	^{13}C Literature[a] δ (ppm)
1	1.80,1.06	39.0 (CH_2)	1.6,1.78 (m)	39.0 (CH_2)
2	1.51	19.0 (CH_2)	1.48 (m)	19.0 (CH_2)
3	1.05,1.51	35.7 (CH_2)	1.02,1.71 (m)	35.4 (CH_2)
4	—	39.1 (C)	—	38.8 (C)
5	1.27	56.4 (CH)	1.22 (m)	56.3 (CH)
6	1.80 (m) 1.27 (m)	24.5 (CH_2)	1.29 (m) 1.82 (m)	24.4 (CH_2)
7	1.91 (m) 2.38 (m)	38.5 (CH_2)	1.92 (td,*J*=4,12.5) 2.37 (td,*J*=4,12.5)	38.6 (CH_2)
8	—	147.5 (C)	—	148.2 (C)
9	1.56 (m)	57.4 (CH)	1.54 (m)	57.3 (CH)
10	—	39.1 (C)	—	39.7 (C)
11	1.54,1.35	17.9 (CH_2)	1.36,1.52 (2m)	17.8 (CH_2)
12	1.26,1.73	41.3 (CH_2)	1.24,1.76(2m)	41.3 (CH_2)
13	—	73.5 (C)	—	73.6 (C)
14	5.92 (dd,*J*=17.4,10.8Hz)	145.2 (CH)	5.90 (dd,*J*=10.8,17.4)	145.1(CH)
15	5.07 (*J*=10.8, 1.3Hz) 5.12 (*J*=17.4, 1.3Hz)	111.8 (CH_2)	5.04 (d,*J*=10.8) 5.19 (d,*J*=17.4)	111.6(CH_2)
16	1.26	27.7 (CH_3)	1.26 (s)	27.1 (CH_3)
17	4.83 4.55	107.7(CH_2)	4.51,4.81(s)	106.6 (CH_2)
18	1.00 (s)	27.2 (CH_3)	0.96 (s)	28.0 (CH_3)
19	3.74 3.37	65.8 (CH_2)	3.38,3.74 (2d,*J*=10.9 each)	65.0 (CH_2)
20	0.67 (s)	15.4 (CH_3)	0.64 (s)	15.2 (CH_3)

N.B; [a]; (Xue et al., 2004).

The open column fraction P1-9 was also fractionated by using MPLC as described in (section 2.4.3) and yielded a fraction that contained compound **(5)** which contained a single compound as judged by HPLC-ELSD. The structure was elucidated from the ^{1}H and ^{13}C NMR in comparison with the data in the literature (Table 3-39) and was found to correspond to the lignan sesamin.

Table 3-39 ^{1}H and ^{13}C NMR of compound **(5)** (400 MHz, $CDCl_3$); Sesamin compared to the literature[a]

No	^{1}H Experimental δ (ppm)	^{13}C Experimental δ (ppm)	^{1}H Literature[a] δ (ppm)	^{13}C Literature[a] δ (ppm)
1	3.04 m	54.40	3.11 (m)	55.1
2	4.71 d, *J*=4.4Hz	58.2	4.65 d, *J*=4.8	58.7
3 (O)	—	—	—	—
4	3.86 dd, *J*=9.2, 3.5Hz 4.23 m	71.8	3.82 (m) 4.20 (m)	71.7
5	3.04 m		3.09 (m)	55.1
6	4.71 d,*J*= 4.4Hz	85.9	4.64 d, *J* = 5.1	87.1
7(O)	—	—	—	—
8	3.80 4.23 m	71.8	3.83 (m) 4.23 (m)	72.4
1`	—	135.1	—	135.0
2`	6.84 d(J=1.2Hz)	106.4	6.877 d, *J* = 1.8	114.2
3`	—	147.9	—	147.1
4`		147.1	—	147.1
5`	6.77 d, *J*=8Hz	108.3	6.71 d, *J* = 8.1	116.2
6`	6.79 dd, *J*=8.12Hz	119.4	6.69 dd,*J* = 1.8,8.1	118.5
1``	—	135.11	—	136.2
2``	6.84 d(J=1.2 Hz)	106.6	6.87 d, 1.5	107.3
3``	—	147.9	—	147.9
4``	—	147.1	—	147.1
5``	6.77 d(J=8Hz)	108.3	6.77 d, 8.1	108.7
6``	6.79 dd(J=8, 1.2Hz)	119.4	6.84 dd, 8.1, 1.5	120.1
O-CH_2-O-	5.87 s(4H)		5.91s	

N.B; [a]; (Moazzami et al., 2007).

Compound **(6)**; 4-((1S,4S)-4-(benzo[d][1,3]dioxol-5-yl) tetrahydro-1H,3H-furo[3,4-c]furan-1-yl)benzene-1,2-diol was isolated from sample P2 and was found to have the chemical formula $C_{19}H_{18}O_6$ (m/z 341.11) this compound is a lignan similar to sesamin, but is asymmetrical due to absence of one of the methylenes of a methylenedioxy group. The structure was elucidated from the ^{1}H and ^{13}C NMR spectra in comparison with the data in the literature and was found to correspond to demethylpiperitol previously isolated by Nakai *et al* (2003) (Table 3-40).

Table 3-40 ^{1}H and ^{13}C NMR of compound **(6)** (400 MHz, $CDCl_3$); Demethylpiperitol (3, 4-Dihydroxy-3', 4'-methylenedioxy-7,9':7',9-diepoxylignan compared to the literature[a]

No	^{1}H Experimental δ (ppm)	^{13}C Experimental δ (ppm)	^{1}H Literature[a] δ (ppm)	^{13}C Literature[a] δ (ppm)
1	3.05	54.4	2.95 (m)	53.9
2	4.64	85.9	4.61 d (4.3)	85.0
3 (O)	—	—	—	—
4	3.80 (m)	71.6 (CH_2)	4.10 dd (6.8, 8.9) 3.69 dd (3.6, 9.1)	71.1
5	3.07(m)	54.3 (CH)	2.95 (m)	53.6
6	4.64 (d, 12.4, 8.16)	85.7 (CH)	4.52 d (4.3)	84.8
7 (O)	—	—	—	—
8	3.80	71.9 (CH_2)	4.06 dd (6.8, 8.9) 3.71 dd (3.6, 9.1)	70.8
1′	—	135.0		135.6
2′	6.84 (1H,d)	102 (CH)	6.90 d (1.4)	106.6
3′	—	147.0	—	147.5
4′	—	147.0	—	146.5
5′	6.77 d(8.0)	147.0	6.85 d (8.0)	147.4
6′	6.79 (1H,d)	102.3 (CH)	6.82 dd (1.4, 8.0)	119.4
1′′	—	135.0	—	132.3
2′′	6.85 d(*J*=1.5 Hz)	106.6 (CH)	6.71 d (2.0)	113.6
3′′	—	147.9	—	145.1
4′′	—	147.1		144.7
5′′	6.81 d,*J*=8Hz	108.4	6.66 d (8.0)	115.3
6′′	6.74 (dd,*J*=8.5Hz)	119.5	6.57 dd (*J*=2.0, 8.0 HZ)	117.1
OCH_2O	5.98 (2H,s)	101	5.97 (2H,s)	100.9
-OH×2	5.52 (1H,s) ×2	147.0	5.84 (s)	146.8

N.B; [a]; (Nakai et al., 2003)

The chemical structure of compound **(7)** was elucidated from the ^{1}H and ^{13}C NMR spectra in comparison with the data in the literature and was found to be similar to compound **(6)**. Compound **(7)** was isolated from P2-24 by MPLC fraction as shown previously in Table 3-27. It had the elemental composition $C_{21}H_{22}O_7$ (Li et al., 2007) and chemical name (1R, 2S, 5R, 6S)-2-(3, 4-methylenedioxyphenyl)-6-(4-hydroxy-3-5-dimethoxyphenyl)-3-7-dioxybicyclo [3.3.0] octane.

Table 3-41 ^{1}H and ^{13}C NMR of compound **(7)** (400 MHz, $CDCl_3$); C_{21} H_{22} O_7 (5'methoxy pipertol (1R,2S,5R,6S)-2-(3,4-methylenedioxyphenyl)-6-(4-hydroxy-3,5 dimethoxyphenyl)-3,7-dioxabicyclo[3.3.0] octane compared to the literature[a]

No	^{1}H Experimental δ (ppm)	^{13}C Experimental δ (ppm)	^{1}H Literature δ (ppm)	^{13}C Literature δ (ppm)
1	2.98 (m)	54.3	3.07 (1H,m)	54.4
2	4.73 (d, *J*=4.4Hz)	86.1	4.74 (1H,d,4.9)	85.8
3 (O)	—	—	—	—
4	4.55 3.89	71.6	4.24 (1H,dd,9.3,6.8) 3.89 (1H,m)	71.9
5	3.12 (1H,m)		3.07 (1H,m)	54.3
6	4.67 d, *J*=4.1Hz)	85.6	4.70 (1H,d,4.2)	86.1
7(O)	—	—	—	—
8	4.36 3,77		4.27 (1H,dd,9.0,7.1) 3.87 (1H,m)	71.6
1`	—	135.2	—	135.1
2`	6.88 d (*J*=1.5Hz)	102.7	6.85 (1H,brs)	106.5
3`		147.1	—	147.1
4`		147.3	—	148.0
5`	—	108.4	6.78 (1H,d,8.0)	108.2
6`	6.60 (1H,s)	102.7	6.81 (1H,dd,8.0,1.2)	119.3
1``	—	132.7	—	132.1
2``	6.60	106.6	6.58 (1H,s)	102.7
3``	—	147.0	—	147.1
4``	—	134.0	—	134.3
5``	—	147.0	—	147.1
6``	6.83(dd,*J*=8,1.5Hz)	119.5	6.58 (1H,s)	102.7
OCH_2-O	5.98 (2H,s)	101.5	5.95 (2H,s)	101.1
OCH_3-3	3.93 (3H,s)	56.4	3.90 (3H,s)	56.4
OCH_3-5	3.93 (3H,s)	56.4	3.90 (3H,s)	56.4

N.B; [a]; (Li et al., 2007).

Additionally, from P1, the open column fraction P1-2 was also fractionated by using MPLC and yielded compound **(8)** $C_{18}H_{16}O_8$ molecular weight 360.0766, which contained a single compound as judged by HPLC-ELSD. The structure was elucidated from the ^{1}H and ^{13}C NMR spectra in comparison with the data in the literature (Table 3-42) and was found to correspond to taxifolin-3-acetate-4′-methylether (Stevens et al., 1999).

Table 3-42 ^{1}H and ^{13}C NMR of compound **(8)** (600 MHz, $CDCl_3$); Taxifolin-3-acetate-4′-methylether

No	^{1}H Experimental δ (ppm)	^{13}C Experimental δ (ppm)
1	—	—
2	5.20 (1H,d,*J*=11.7Hz)	81.5
3	5.75 (1H,d,*J*=11.7Hz)	72.2
4	—	191.7
5-OH	11.34	164.4
6	6.10 (1H,s)	97.4
7	11.40	164.4
8	6.05 (1H,s)	95.8
9	—	163.1
10	—	101.8
1′	—	127.0
2′	6.68 (1H,d)	121.9
3′	6.68	146.8
4′	—	146.7
5′	6.75	114.3
6′	6.93	109.3
3-(OAC)CO-		169.2
3-(OAC)-CH_3	1.96 (3H,s)	20.2
4′-OCH_3	3.85 (3H,s)	56.13
5-OH	11.34	
7-OH	11.4	

N.B; [a]; n (Stevens et al., 1999).

In comparison to the literature the reference above Stevens *et al.*,(1999), there was no establishment of the position O-methyl (3′ or 4′) while in the current study by using 2D NMR it was confirmed that the compound **8** was Taxiolin-3-

acetate-4'methyl ether as shown in Table 3-42 and thus can be considerd to have been isolated for the first time (appendix 11-15).

3.14 MPLC Fractionation of propolis sample P7

Fractionation of P7 was carried out directly by MPLC. This led to the isolation of 6 pure compounds **(5)** which were triterpenes of the cycloartane type; **(9), (10), (11**), **(12)** and **(13)** and other was polyphenolic alkene **(14)**. The structures of the triterpenes were elucidated by comparison with the literature which included triterpenes isolated from propolis from Cameroon (Kardar et al., 2014, Li et al., 2009a, Li et al., 2009b).

Table 3-43 ^{1}H and ^{13}C NMR of compound **(9)** (400 MHz, $CDCl_3$); Cycloartanol compared to the literature[a]

No	^{1}H Experimental δ (ppm)	^{13}C Experimental δ (ppm)	^{1}H Literature δ (ppm)	^{13}C Literature δ (ppm)
1	1.50 (m), 1.18 (m)	32.0	-	31.9
2	1.72 (m) 1.62 (m)	30.5	-	30.4
3	3.22 dd	78.2	-	78.9
4	——	40.6	-	40.5
5	1.27	47.2	-	47.1
6	1.62 (m), 0.76 (m)	21.2	-	21.1
7	1.13 (m), 1.38 (m)	26.2	-	26.0
8	1.56	48.0	-	48.0
9	——	20.2	-	20.0
10	——	26.3	-	26.1
11	2.09, 1.12	26.6	-	26.6
12	1.75 (m)	33.1	-	32.9
13	——	45.5	-	45.2
14	——	48.91	-	48.7
15	1.43	35.7	-	35.9
16	1.48 (m), 2.08(m)	28.2	-	28.1
17	1.76	52.4	-	52.3
18	1.13 (3H,s	18.1	-	18.0
19	0.56 (d,4.1) 0.34 (d,4.3)	29.9	0.53 (d,4.1) 0.32 (d,4.3)	29.8
20	1.62	35.8	-	35.9
21	1.12	18.3	-	18.2
22	1.30, 1.73	36.4	-	36.3
23	2.18 (m), 2.30 (m)	25.0	-	24.9
24	5.12 (obsc)	124.8	5.10 (obsc)	125.2
25	——	130.6	-	130.9
26	1.52 (1H (s)	17.2	-	17.6
27	1.70 s	25.7	-	25.7
28	1.03	19.5	-	19.3
29	1.14	25.5	-	25.4
30	0.99	14.04	-	14.0

N.B; [a]; (Kardar et al., 2014), **-;** not mentioned before in literature

Table 3-44 ^{1}H and ^{13}C NMR of compound **(10)** (400 MHz, $CDCl_3$); Mangiferolic compared to the literature[a]

No	^{1}H Experimental δ (ppm)	^{13}C Experimental δ (ppm)	^{1}H Literature δ (ppm)	^{13}C Literature δ (ppm)
1	1.57 m,1.89 m	31.6	-	31.9
2	2.33 ,2 ,73	29.7	-	30.3
3	3.32 (dd)	79.0	3.29 m	78.9
4	-	40.5	-	40.5
5	1.33	47.0	-	47.1
6	-	21.4	-	21.1
7	1.09, 1.034	26.3	-	26.0
8	1.53 (dd)	47.9	-	47.9
9	-	20.1	-	19.9
10	-	26.1	-	26.0
11	1.91 (m), 1.16(m),	26.4	-	26.4
12	1.66	32.1	-	32.9
13	-	45.4	-	45.3
14	-	48.8	-	48.8
15	1.3 (m)	35.6	-	35.5
16	1.92 (m)	28.4	-	28.1
17	1.61 (m)	52.2	-	52.2
18	1.0 (3H,s (s)	18.1	0.95 (s)	18.1
19	0.53 (d,3.7)/0.35 (d6.0)	30.5	0.54 (d,3.7) 0.31 (d,4)	29.9
20	1.44	36.0	-	36.0
21	0.93 (d)	18.3	0.89 (d,6.6)	18.1
22	1.55 (m), 1.16 (m),	33.7	-	34.7
23	2.23 (m) ,2.20 (m)	26.2	-	25.9
24	6.93 (dd)	145.8	6.89 (brt)	145.8
25	-	126.6	-	126.5
26	-	173.2	-	172.8
27	1.86 (s)	11.9	1.8 (s)	11.9
28	0.99 (s)	25.0	0.95 (s)	25.4
29	0.83 (s)	14.0	0.80 (s)	14.0
30	0.93 (s)	19.3	0.89 (s)	19.3

N.B; [a]; (Kardar et al., 2014)**, -;** not mentioned before in literature.

Table 3-45 ^{1}H and ^{13}C NMR of compound **(11)** (400 MHz, $CDCl_3$); Mangiferonic acid compared to the literature[a]

No	^{1}H Experimental δ (ppm)	^{13}C Experimental δ (ppm)	^{1}H Literature δ (ppm)	^{13}C Literature δ (ppm)
1	188 (m),1.57(m),	32.0		34.4
2	2.73 (dt,*J*=6.4,13.9 Hz), 2.33 (m)	37.5	2.69 (m) 2.29 (m)	37.4
3	-	217.3	-	216.7
4	-	50.3	-	50.2
5	1.74 (m)	47.8	-	48.4
6	1.53 (m),1.21 (m)	21.5	-	21.4
7	1.42 (m),1.17 (m)	25.9	-	25.9
8	1.62 (m)	47.9	-	47.8
9	-	21.1	-	21.0
10	-	25.9	-	25.9
11	2.10 (m), 1.14 (m)	26.2	-	26.6
12	1.77 (m)	32.8	-	32.7
13	-	45.4	-	45.4
14	-	48.8	-	48.7
15	1.32 (m)	35.6	-	35.5
16	1.92 (m)	28.2	-	28.1
17	1.62 (m)	51.6	-	52.2
18	1.02 (m)	17.6	0.98 (s)	18.1
19	0.77 (d,4.6) 0.56 (d4.2)	29.8	0.77 (d,3.7)/ 0.56 (d,3.8)	29.5
20	1.47 (m)	35.5	-	35.9
21	0.95 (s)	17.8	0.90 (brt,3.7)	18.1
22	1.23 (m),1.62 (m)	34.8	-	34.7
23	2.24 (m) ,2.40 (m)	25.9	-	25.8
24	7.01 (br)	145.8	6.90 (brt,7.3)	145.7
25	-	126.6	-	126.6
26	-	171.9	-	172.8
27	1.85 (s)	11.9	1.83 (s)	12.0
28	1.07 (s)	22.2	1.05 (s)	22.1
29	1.12 (s)	20.78	1.10 (s)	20.8
30	0.93 (s)	18.3	0.90 (s)	19.2

N.B; [a]; (Kardar et al., 2014), -; not mentioned before in literature.

Table 3-46 ^{1}H and ^{13}C NMR of compound **(12)** (400 MHz, $CDCl_3$); Ambolic acid compared to the literature[a]

No	^{1}H Experimental δ (ppm)	^{13}C Experimental δ (ppm)	^{1}H Literature δ (ppm)	^{13}C Literature δ (ppm)
1	1.56 (m),124 (m)	31.9	-	31.9
2	1.76 (m),1.56 (m)	30.4	3.29 (m)	30.3
3	3.29 (m)	78.4	-	78.8
4	——	40.5	-	40.5
5	1.30 (m)	47.0	-	47.1
6	1.60 (m), 0.80 (m)	21.1	-	21.0
7	1.33 (m), 1.08 (m)	26.2	-	26.0
8	1.51 (m)	47.9	-	48.0
9	——	19.9	-	20.0
10	——	26.2	-	26.1
11	1.99 (m), 1.13 (m)	26.5	-	26.4
12	1.53 2H (m)	32.9	-	32.9
13	——	45.4	-	45.3
14	——	48.8	-	48.8
15	1.28 (m)	35.5	-	35.5
16	1.78 (m),1.28 (m)	28.1	-	28.1
17	1.61 (m)	52.3	-	52.2
18	0.98 (3H,s)	18.1	0.95 (s)	18.0
19	0.56 (d,4.1) 0.37 (d,4.1)	29.8	5.4 (d,4) 0.33 (d,4)	29.9
20	1.31 (m)	36.1	-	36.0
21	0.89 (d,6.5,3H)	18.4	0.89(d,6.6)	18.3
22	1.55 (m),1.21 (m),	34.6	-	34.5
23	2.12 (m),1.95 (m)	26.1	-	25.9
24	——	148.8	-	148.7
25	3.14 b (m)	45.5	3.16 (brq,6.6)	45.6
26	——	179.9	-	179.8
27	1.27 (d,6.5)	16.1	1.29 (d,7.1)	16.3
28	——	25.5	0.95 (s)	25.4
29	0.85 (s,3H)	14.2	0.80 (s)	14.0
30	0.89 (s,3H)	19.4	0.89 (s)	19.3
31	4.98 bs 4.94 bs	111.1	4.96 (brs) 4.92 (brs)	111.0

N.B; [a]; (Kardar et al., 2014), **-;** not mentioned before in literature.

Table 3-47 ^{1}H and ^{13}C NMR of compound **(13)** (400 MHz, $CDCl_3$); 27-Hydroxymangiferonic acid compared to the literature[a]

No	^{1}H Experimental δ (ppm)	^{13}C Experimental δ (ppm)	^{1}H Literature δ (ppm)	^{13}C Literature δ (ppm)
1	1.89 (m), 1.57 (m)	32.9	1.89 (m),1.54 (m)	33.4
2	2.73 (dt, *J*=6.4,13.9 Hz) 2.33 (m)	36.9	2.71 (m), 2.31 (m)	37.2
3	-	216.7	-	216.6
4	-	49.7	-	50.2
5	1.74 (m)	48.2	1.71 dd	48.4
6	1.55 (m),0.89 (m)	21.4	1.57 (m),0.95 (m)	21.5
7	1.33 (m),1.02 (m)	26.2	1.38 (m),1.14 (m)	25.9
8	1.62 (m)	47.6	1.59 (m)	47.8
9	-	21.0	-	21.2
10	-	25.9	-	26.00
11	189 (m),1.22 (m)	26.2	1.38 (m),1.14 (m)	26.8
12	1.65 (m)	32.7	1.61 (m)	32.8
13	-	45.4	-	45.4
14	-	48.7	-	48.8
15	1.30	35.5	1.32 (m)2H	35.6
16		28.4	1.92 (m)	28.2
17		51.6	1.61 (m)	52.2
18	1.02 (3H,s)	17.8	1.00 (3H,s)	18.12
19	0.77 (d,4.2) 0.56 (d,4.2)	29.5	0.79 (d,4.2) 0.58 (d,4.2)	29.6
20	1.47	35.9	1.44 (m)	36.00
21	0.94 (3H,s)	17.6 (CH_3)	0.92 (d,6.,3H)	18.2
22	1.23 (m) ,1.62 (m)	29.2	1.58 (m),1.17 (m)	34.8
23	2.24 (m), 2.40 (m)	25.8	2.26 (m),2.12 (m)	25.9
24	7.04	146.5	6.91 (brt)	145.80
25	-	129.3	-	126.6
26	-	171.1	-	173.1
27	4.38 (s)	56.6	4.22 (s)	57.2
28	0.93 (3H,s)	22.1	1.05 (3H,s)	22.2
29	1.07 (s)	20.8	1.02 (3H,s)	20.7
30	0.93 (s)	18.8	0.91 (3H,s)	19.3

N.B; [a]; (Anjaneyulu et al., 1992), **-;** not mentioned before in literature.

Table 3-48 ^{1}H and ^{13}C NMR of compound **(14)** (400 MHz, $CDCl_3$); Cardol compared to the literature[a]

No	^{1}H Experimental δ (ppm)	^{13}C Experimental δ (ppm)
1	-	156.8
2	6.22 t (*J* = 2.1Hz)	100.3
3	-	156.8
4	6.26 d (*J* = 2.1Hz)	107.9
5	-	146.0
6	6.26 d (*J* = 2.1Hz)	107.9
1′	2.48 brt (*J* = 7.8Hz)	35.9
2′	1.57 (m)	31.1
3′	1.34 (m)	29.4
4′-6′	1.30-1.39 env	
7′	2.04 (m)	27.2[a]
8′	5.37 (m) *	129.9*
9′	5.38 (m)*	129.88*
10′	2.04 (m)	26.9[b]
11′-13′	1.30-1.39 env	
14′	1.35 (m)	32.0
15′	0.92	14.0

N.B; [a]; (Pretsch et al., 2013), * [b] = interchangeable within columns.

3.15 MPLC fractionation of propolis sample P9

Fractionation of propolis sample P9 was carried by using MPLC directly. The following Table represents the isolated fractions obtained from P9 with their weights.

Table 3-49 Fractions isolated from propolis sample P9 using direct MPLC**.**

P9 Fractions Codes	Pure Compounds	Weight (mg)
P9-5	**-**	18.6
P9-7	**-**	11.5
P9-8	**-**	13.7
P9-10	**-**	17.65
P9-11	**-**	9.45
P9-15	**(15)**	17.10
P9-16	**-**	43.4
P9-17	**(18)**	120
P9-19	**-**	125
P9-23	**-**	16.7
P9-27	**(17)**	20
P9-28	**(16)**	48

N.B; **-**; [a] *mixture fraction compounds.*

The fractions contained four pure compounds. Compound **(15)**; had the molecular composition $C_{22}H_{22}O_4$ and the NMR indicated the presence of an acetate group at 2.07 ppm, and a two proton doublet at 4.60 was assigned to the CH_2OAc. By comparison of its ^1H, ^{13}C and 2D NMR spectra with the literature compound 15 was identified as acetyl isocuppressic acid (ent-labd-8(17), 13E-dine-15-acetoxy-18-oic acid) (Table 3-50).

Table 3-50 ^{1}H and ^{13}C NMR of compound **(15)** (400 MHz, $CDCl_3$); Acetylisocuppressic acid compared to the the literature[a]

No	^{1}H Experimental δ (ppm)	^{13}C Experimental δ (ppm)	^{1}H Literature δ (ppm)[a]	^{13}C Literature δ (ppm)[a]
1	0.99 (m), 1.66 (m)	39.4	0.98 (m), 1.76 (m)	39.1
2	1.35 (m).1.77 (m)	20.0	1.46 (m), 1.77 (m)	19.9
3	2.42 (m)	38.0	2.10 (m)	37.9
4	-	44.1	-	44.2
5	1.28	56.3	1.32	56.3
6	(m)	25.6	1.79 (m)	26.1
7	2.43 (m)	38.7	1.77 (m)	38.7
8	-	147.9	-	147.9
9	1.53 (m)	55.5	1.49 (m)	55.4
10	-	40.4	-	40.4
11	1.45 (m),1.62 (m)	21.8	1.38 (m), 1.56 (m)	21.8
12	1.84 (m), 2.16	38.4	0.98 (m), 1.81 (m)	38.4
13	-	142.9	-	143.0
14	5.28 tr	118.1	5.23 tr	118.0
15	4.56 (d)	61.4	4.51 d (7.1)	61.5
16	1.66 (s)	16.6	1.62 (s)	16.6
17	4.49 br (s), 4.83 br(s)	106.5	4.45 br(s),4.78 br(s)	106.5
18	1.23 (s)	28.9	1.17 (s)	29.0
19	-	182.4	-	183.5
20	0.57 (s)	12.8	0.53 (s)	12.8
21	-	171.4	-	171.2
22	2.03 (s)	21.4	2.23 (s)	21.1

N.B; [a]; (Popova et al., 2009)

By comparison, of ^{1}H and ^{13}C NMR spectra of compound 16 with the literature it was identified as agathadiol (Table 3-51).

Table 3-51 ^{1}H and ^{13}C NMR of compound **(16)** (400 MHz, $CDCl_3$); Agathadiol compared to the literature[a]

No	^{1}H Experimental δ (ppm)	^{13}C Experimental δ (ppm)	^{1}H Literature δ (ppm) [a]	^{13}C Literature δ (ppm) [a]
1	1.01 (m)	38.6(CH_2)	0.98 (m),176	38.5.
2	1.51 (m)	19.0 (CH_2)	1.46 (m),177	19.1
3	1.05,1.51	35.5 (CH_2)	2.10 (m)	35.6
4	-	38.8	-	39.6
5	0.96	56.3 (CH)	1.32	56.4
6	1.80, 1.27	24.1 (CH)	1.79 (m)	24.6
7	2.38, 1.91	38.7 (CH_2)	1.77 (m), 2.33 (m)	39.2
8	-	147.5 (CH)	-	148.2
9	1.56	56.3	1.49 (m)	56.6
10	-	40.00	-	38.9.6
11	1.54, 1.35	17.9 (CH_2)	1.38 (m),1.56 (m)	22.1
12	2.16, 2, 39	38.4.		38.7
13	—	140.6		140.3
14	5.39 t	123.2	*5.39 t(6.9)*	123.3
15	4.16, 4.15](ABq)(*J*=12)	59.5	4.15 d(6.9)	59.4
16	1.68 (brs)	17.00	1.67 (brs)	16.3
17	4.80 (brs) 4.51 (brs)	107.7	4.59 (brs) 4.92 (brs)	106.6
18	0.67 (s)	15.4	0.52 (3H,s)	27.2
19	3.75d (*J*=10.8) 3,40 dd (10.8,1Hz	65.1 (CH_2)	1.16 (3H,s)	65.1
20	0.65 (s)	19.0 (CH_2)	0.65 (s)	18.3

N.B; [a]; (San Feliciano et al., 1988)

Compound **(17)** had the elemental composition $C_{20}H_{32}O_3$. It had an exomethylenegroup, a carboxylic acid and propyl alcohol group. By comparison of ^{1}H and ^{13}C NMR spectra of compound **(17)** with the literature it was identified as isocupressic acid (ent-labd-18(17),13-*E*-dien-15-ol-18-oic acid) (Table 3-52).

Table 3-52 ^{1}H and ^{13}C NMR of compound **(17)** (400 MHz, $CDCl_3$); (isocupressic acid[a]) compared to the the literature[a]

No	^{1}H Experimental δ (ppm)	^{13}C Experimental δ (ppm)	^{1}H Literature δ (ppm)[a]	^{13}C Literature δ (ppm)[a]
1	1.01 (m)	38.6 (CH_2)	-	39.1
2	1.51 (m)	19.0 (CH_2)	-	19.3
3	1.05,1.51	36.9 (CH_2)	-	37.9
4	-	44.7	-	44.2
5	0.96 (s)	56.3 (CH)	-	56.3
6	1.80, 1.27	25.6 (CH)	-	26.1
7	2.38,1.91	38.7 (CH_2)	-	38.4
8	-	147.5 (CH)	-	147.9
9	1.56	56.3	-	55.5
10	-	40.00	-	40.4
11	1.54, 1.35	17.9 (CH_2)	-	24.1
12	2.16, 2, 39	38.4.	-	38.7
13	-	140.6	-	140.5
14	5.39t (br)	123.2	5.40 (t,*J*=7Hz)	122.9
15	4.16 d (7)	59.5	4.87 (s,1H)	59.3
16	1.67 (brs)	17.00	1.67 (3H s	16.3
17	4.86 (brs) 4.51 (brs)	107.7	4.17(d,*J*=7Hz,2H), 4,54(s,1H)	106.5
18	3.74, 3.337	29.0	1.25(3H s)	29.0
19	-	183.3	-	183.6
20	1.15 (s)	13.9	0.62 (3H s),	13.6

N.B; [a]; (Stegelmeiert, 1994), **-;** not mentioned before in literature.

The compound **(18)** is an isomer of the compound **(1)** with the OH group shifted from position 15 to 13 and the double bond from 13, 14 to 14, 15 (Table 3-53). By comparison, of ^{1}H and ^{13}C NMR spectra of compound **(18)** with the literature it was identified as isoagatholal (Table 3-53).

Table 3-53 ^{1}H and ^{13}C NMR of compound **(18)** in (400 MHz, $CDCl_3$); Isoagatholal compared to the literature[a]

No	^{1}H Experimental δ (ppm)	^{13}C Experimental δ (ppm)	^{1}H Literature δ (ppm)	^{13}C Literature δ (ppm)
1	1.05 (m)	38.4	-	38.4
2	1.41 (m)	19.4	-	19.3
3	1.01,1.51	34.4	-	38.
4	—	48.7	-	48.6
5	0.98 (s)	56.1	-	55.0
6	1.80 (m), 1.27 (m)	22.9	-	22.1
7	2.38,1.91	38.5	-	38.1
8	—		-	147.3
9	1.63	54.9	2.44 (m)	56.1
10	—	40.1	-	40.1
11	1.51 (m), 1.27 (m)		-	24.1
12	2.16 (m), 2,39 (m)	38.5	-	38.5
13	—	140.3	-	139.7
14	5.38	123.3	5.35 t (*J*=6.8)	123.5
15	4.16 (*J*=6.8)	59.5	4.11d (*J*=6.8)	59.2
16	1.67 (3H,s)	16.4	1.65 (3H,s)	16.3
17	4.89 (s),4.51 (s)	107.3	4.86 (s),4.53(s)	107.3
18	1.02 (3H,s)	24.4	1.02 (3H,s)	24.4
19	9.74 (s)	205.9 (CH)	9.70 (s)	205.5
20	0.57 (3.Hs)	13.8	0.57 (s)	13.6

N.B; [a]; (Hasegawa and Hirose, 1980), **-;** not mentioned before in literature.

3.16 Optical rotation of pure compounds

The optical rotations for the isolated compounds were determined using a Perkin Elmer 341 polarimeter. The compounds **(1)** to **(18),** were dissolved in 2ml of chloroform and the optical rotation was measured using the sodium D line. Table 3-54 shows the optical rotation for some of pure compounds isolated from P1, P2, P7 and P9 samples.

Table 3-54 Optical rotation of isolated pure compounds

Propolis Fractions Codes	**Pure Compounds**	**Optical Rotation[a]**
P1-3-9	**(1)**	[α] = +14°
P1-3-10	**(2)**	[α] = +92°
P1-3-15	**(3)**	[α] = +55.5°
P1-3-17	**(4)**	[α] = +15.8°
P1-9-7	**(5)**	[α] = +17°
P1-2-16	**(8)**	[α] = +1.6°
P2-24-7	**(7)**	[α] = +1.33°
P2-12-37	**(6)**	[α] = +0.11°
P7-15	**(9)**	[α] = +0.271°
P7-31	**(10)**	[α] = +78.2°
P7-32	**(11)**	[α] = +44.5°
P7-35	**(12)**	[α]= +68°
P7-51	**(13)**	[α] =+ 11°
P7-20	**(14)**	[α] = +123.5°
P9-15	**(15)**	[α] = +7.5°
P9-29	**(16)**	[α] = +28.5°
P9-27	**(17)**	[α] = +22.5°
P9-17	**(18)**	[α] = +10.5°

N.B, [a]; Optical rotation obtained by using a Perkin Elmer 341 polarimeter.

3.17 The biological activity testing of the fractions and pure compounds isolated from propolis samples P1, P2, P7 and P9

Biological screening was carried out for fractions of propolis samples P1, P2, P7 and P9 and the results showed some significant activity against different parasites such as *Trypanosoma brucei, Leishmania donovani, Plasmodium falciparum, Crithidia fasiculata* and *Mycobacterium marinum*. Also the fractions were tested against *Stapholococcus aureus and Klebsiella pneumoniae*. In addition, the cytotoxicity activity was asssessed for all extracts against a mammalian cell line.

3.17.1 In vitro Antitrypanosomal activity of P1, P2, P7 and P9 fractions against *T. brucei* (s427)

The fractions collected from sample P1 were tested against *T. brucei (*s427*)* in comparison with suramin as a drug control and MICs values were calculated the results showed that P1 open column fractions P1-2, P1-3, P1-4, P1-5, P1-7 and P1-11 exhibited variable significant activity against *T. brucei*. The pure compounds **(1), (2), (3)** and **(4),** had variable activity against *T. brucei* with MIC values of 2.5, 1.56, 10 and 2.5μg/ml, respectively (Table 3-55). Also compounds **(5)** and **(8)** of both showed MIC values of 6.25 μg/ml. Thus none of the fractions or isolated compounds had activity > than the crude P2 extract (Table 3-15) although compounds **(1), (2)** and **(4),** were more active than crude P1 (Table 3-15).

Table 3-55 MICs of the, P1 open column fractions and P1 MPLC fractions against *T. brucei* blood stream form (s427).

P1 Fractions Codes	**Pure Compounds**	**MICs (µg/ml)**
[a]P1-2	**-**	5
[a]P1-3	**-**	5
[a]P1-4	**-**	1.56
[a]P1-5	**-**	10
[a]P1-7	**-**	10
[a]P1-11	**-**	20.00
[b]P1-3-9	**(1)**	2.50
[b]P1-3-10	**(2)**	1.56
[b]P1-3-11	**-**	1.56
[b]P1-3-15	**(3)**	10
[b]P1-3-5	**-**	5.00
[b]P1-3-2	**-**	10.00
[b]P1-3-7	**-**	10.00
[b]P1-3-14	**-**	5
[b]P1-3-17	**(4)**	2.5
[b]P1-3-18	**-**	5
[b]P1-3-20	**-**	5
[b]P1-9-7	**(5)**	6.25
[b]P1-2-16	**(8)**	6.25
Suramin	**-**	0.178

N.B.; **-**; a mixture of compounds, [a]; Open Column Fractions and [b]; MPLC-Grace Reveleris® fractions. **(1), (2), (3), (4), (5)** and **(8)** as described in Table 3-33.

Screening fractions from P1, P2, P7 and P9 and the compounds isolated from these extracts compounds against *T. brucei* (s427) in comparison with a pentamidine resistant strain of *T. brucei* B48 was carried out. The results are summarised in Table 3-56. None of the compounds isolated from P2 or fractions were more active than the crude extract apart from compound **(1)** 13-epi-torulosal, which in this set of tests was slightly more active than the crude extract of P2 against both the pentamidine sensitive and resistant strains. Crude P7 had relatively weak antitrypanosomal activity with and EC_{50} value of 14.67μg/ml (Table 3-15). Three of its fractions and two of the compounds isolated from P7 had much higher activities than the crude extract with **(14)** cardol, having the strongest activity observed amongst any of the compounds isolated from Libyan propolis. The crude extract of P9 (Table 3-15) had relatively weak activity against *T. brucei (s427)* at 20.65 μg/ml. However, several of its fractions and compounds **(16)** (Agathadiol), compound **(17)** **(**Isocupressic acid) and compound **(18)** (isoagatholal)**,** which were isolated from it had much higher activities than the crude extract. In all cases the fractions from P1, P2, P7 and P9 and the compounds isolated from them had similar activities against both the sensitive and resistant strains of *T. brucei*.

Table 3-56 EC_{50} values for Antitrypanosomal activity of fractions from (P1, P2, P7 and P9) samples against *T. brucei* (s427) and *T. brucei* B48.

Tested propolis fractions	Pure Compounds	EC_{50} (µg/ml) of *T. brucei* (s427)	SEM[c]	EC_{50} (µg/ml) of *T. brucei* B48	SEM	RF[d]
P1-9-9[a]	-	13.5	0.23	11.9	0.28	
P1-9-10[a]	-	22.0	0.60	15.9	0.11	
P1-9-11[a]	-	25.6	0.53	21.5	0.91	
P1-9-13[a]	-	11.1	1.22	13.25	0.97	1.19
P1-2-10[a]	-	9.5	0.13	8.2	0.37	
P2-24-7	**(7)**	13.1	0.12	12.43	1.56	
P2-1-7	**(5)**	8.1	0.12	8.43	0.25	1.03
P2-12-37	**(6)**	2.7	0.23	2.68	0.04	0.98
P2-12-30[a]	-	2.1	0.31	2.03	0.16	0.98
P2-12-TA[a]	-	10.4	0.23	12.54	0.35	1.20
P2-12-T3[a]	-	8.6	0.07	8.79	0.22	1.02
P2-1-9[a]	-	3.6	0.65	2.9	0.38	
P2-1-10[a]	-	14.3	0.29	10.4	0.61	
P2-1-1	**(1)**	1.4	0.02	0.8	0.05	
P7-15-	**(9)**	3.7	0.09	3.42	0.08	0.92
P7-20	**(14)**	0.7	0.03	0.70	0.06	1.07
P7-21[a]	-	6.8	0.19	6.57	0.18	0.97
P7-24[a]	-	3.8	0.15	3.78	0.13	1.01
P7-25[a]	-	2.4	0.17	2.56	0.41	1.05
P7-40[a]	-	17.3	0.82	20.39	1.40	1.18
P7-35	**(11)**	14.6	0.24	14.66	0.41	1.01
P7-51	**(13)**	35.2	0.64	34.85	0.33	0.99
P9-8[a]	-	33.4	1.43	33.82	0.62	1.01
P9-10[a]	-	66.3	1.44	66.80	1.56	1.01
P9-11[a]	-	7.8	0.22	6.43	0.36	0.82
P9-15	**(15)**	25.0	0.24	25.62	0.91	1.02
P9-16[a]	-	6.5	0.25	5.30	0.66	0.82
P9-17	(**18)**	10.4	0.86	10.24	0.82	0.99
P9-18[a]	-	15.5	0.71	18.81	1.19	1.21
P9-23[a]	-	10.4	0.10	8.32	0.32	0.80
P9-27	**(17)**	3.0	0.07	2.73	0.11	0.90
P9-28	**(16)**	7.0	0.62	6.90	0.45	0.99
Pentamidine[b]	-	1.1574	0.034	0.00025	.000000034	74.26

N.B; [a]; a mixture of compounds, [b]; Pentamidine; positive control, [c]; SEM = Standard Error of Mean, [d]; RF; resistance. Average of EC_{50} (µg/ml).

3.17.2 *In vitro* Antimalarial activity of fractions from (P1, P2, P7 and P9) samples against *P. falciparum*

In vitro antimalarial assays were performed for the assessment of the activity of fractions and some of the compounds isolated from P1, P2, P7 and P9. The activity of the compounds and fractions isolated from P1, P2, P7 and P9 was in all cases lower than the activity of the crude extracts of these samples (Table 3-18). As seen in (Table 3-57), fraction P2-12-29 exhibited the highest activity against *P. falciparum* with EC_{50} 5.25 μg/mL. However, the fraction from P2-12-29 is a mixture of two or three lignan compounds in mixture such as P2-12-29 as shown in Appendix-9. The PLS model of activity against *P. falciparum* had indicated that the activity was associated with a compound which appeared to be a substituted naphthoic acid. Clearly none of the fractions where enriched with this compound.

Table 3-57 EC_{50} values of Antimalarial activity for propolis fractions from (P1, P2, P7 and P9) samples against *P. falciparum.*

Tested Propolis fractions	Pure Compounds	Average EC_{50} (µg/ml) ±[a]SEM
P1-9-11	**-**	41.47 ± 0.74
P2-12-29	**-**	5.25 ± 0.50
P2-12-55	**-**	22.11 ± 0.44
P2-12-6	**-**	11.21 ± 0.55
P2-12-9	**-**	10.79 ± 0.17
P2-12-11	**-**	18.07 ± 0.42
P2-12-37	**(6)**	17.54 ± 0.12
P2-12-5	**-**	34.43 ± 1.79
P7-32	**(10)**	-*
P7-24	**-**	9.79 ± 1.03
P7-51	**(13)**	-*
P7-20	**(14)**	73.01 ± 1.26
P7-21	**-**	12.41 ± 1.19
P7-35	**(11)**	49.21 ± 9.45
P7-15	**(9)**	-*
P7-31	**(12)**	-*
P9-23	**-**	32.83 ± 0.53
P9-27	**(17)**	-*
P9-11	**-**	29.09 ± 3.48
P9-19	**-**	34.09 ± 4.42
Chloroquine	**-**	3.405 ± 0.0255

N.B; Chloroquine; Positive control; *did not completely kill at 0.1mg/ml max conc., [a]SEM = Standard Error of Mean, Average of EC_{50} (µg/ml), *(n=3).*

3.17.3 *In vitro* Antileishmanial activity of P1 fractions against *L. donovani*

In vitro antileishmanial assays were performed for assessment the activity of some of the MPLC fractions from P1 against *L. donovani* from two different macrophage peritoneal infected macrophages and bone marrow-derived macrophages. EC_{50} (μg/mL) ±SEM were obtained and shows (Table 3-58).
The test was carried out against both LV82 and 200011 strains of *L. donovani. LV82* is susceptible to antimonial treatment and *200011* are resistant strains.

The EC_{50} value for the DMSO was determined because DMSO is known to kill Leishmania. The result from bone marrow macrophages is more accurate because they are easier to work with and it was possible to get better EC_{50} values. These EC_{50} values are for the parasite LV82 which is antimony susceptible. It should be mentioned that the intrapertonial macrophage results were only determined twice due to poor adhesion of the macrophages to wells.
As compounds **(1)** 13-epi-torulosal, **(2)** 13-O- acetyl epi-cupressic acid and **(3)** 13-epicupressic acid, were tested against Leishmania as well as the less pure MPLC fractions from P1, which could be mixure of two or more compounds P1-3-8, P1-3-7 and P1-3-11. Table 3-58 shows the results for these fractions against *L. donovani.* Table 3-58 shows the fractions were all active, and overall, their activity was greater in suppressing infection of peritoneal macrophages than in inhibiting infection of bone marrow macrophages. It can be seen that the EC_{50} values are somewhat variable, which can be attributed to the difficulty in getting the compounds to dissolve in the aqueous test medium.
Some of the fractions are purer than others so there might be even better activity if further purification were carried out. The fact that the compounds are all structurally related diterpenes compunds and small changes in structure as shown above in Figure 3-68 and Table 3-33 make a difference to activity is

fascinating which makes this an interesting series of lead compounds. Compounds **(1)** 13-epi-torulosal, **(2)** 13-O- acetyl epi-cupressic acid and **(3)** 13-epicupressic acid, are > 90% pure it may be the lipidome that is the target since they are lipophilic compounds. According to the results obtained by NMR, as compound **(1)** is an aldehyde diterpene so it is not surprising the aldehyde is most toxic given its reactive nature; however, it will probably be the least stable compound *in vivo*.

Table 3-58 EC_{50} values of Antileishmanial activity for P1 fractions against *L. donovani*

Tested P1 fractions	Pure Compounds	EC_{50} (μg/mL) ±[a]SEM	
		Peritoneal infected macrophages	Bone marrow-derived macrophages
P1-3-7	-	43 ± 38 *(n=2)*	33 ± 20 *(n=3)*
P1-3-8	-	10.4 ± 1.6 *(n=2)*	22.8 ± 7.8 *(n=3)*
P1-3-9	**(1)**	6.9 ± 3.7 *(n=3)*	7.4 ± 5.0 *(n=3)*
P1-3-10	**(2)**	7.0 ± 4.0 *(n=2)*	21.9 ± 12.3 *(n=3)*
P1-3-11	-	2.2± 1.8 *(n=2)*	32.2 ± 2.4 *(n=3)*
P1-3-15	**(3)**	5.1 ± 2.1 *(n=3)*	6.3 ± 3.7 *(n=3)*
Amphotericin B	-	0.01 ± 0.0 *(n=3)*	0.024 ± 0.06 *(n=3)*

N.B; Amphotericin B; drug control, [a]**SEM;** Standard Error of Mean.

The compounds show moderate activity compared with Amphotericin B (EC_{50} 0.01μg/mL). The LD_{50} value for DMSO was obtained at concentrations of 1-2%, which was between 50 and 200 μg/mL. None of the EC_{50} values for the compounds lie between these values so the DMSO did not interfere with the experimental effect.

3.17.4 In vitro Antibacterial activity of P2 fractions against *S. aureus* and *K. pneumoniae*

The antibacterial activity of fractions from sample P2 against both *S. aureus* and *Klebsiella pneumoniae* was tested as described in (section 3.6.6) and based on the results of screening of the results for P1-P12 against Gram-positive and Gram-negative bacteria are shown in Table 3-21. Some fractions from MPLC separation of the extract from P2 were randomly selected to investigate the antibacterial activity of P2. As shown in Table 3-59, Compound **(6)** Demethylpiperitol, showed promising activity against *S. aureus* with an MIC value at 25.2 μg/mL and against *K. pneumoniae* at 31.8 μg/mL.

The fraction P2-12-29 which a mixture of two or three lignan compounds was active against *S. aureus* with an MIC value of 6μg/mL and showed an MIC value of 2.3 μg/mL against *K. pneumonia*. (Table 3-31). Fraction P2-12-11, also another MPLC fraction of P2, showed high activity against *S. aureus* with and MIC value 8 μg/ml. Its MIC against the *K. pneumoniae* was much higher at 70.3 μg/ml. Also, P2-12-TA was also quite active against *S. aureus* and *K. pneumoniae.* Therefore, further purification for P2-12-29, P2-12-11 and P2-12-TA should be carried out in further study, which could lead to potential a strong antibacterial drug.

Table 3-59 MICs values of Antibacterial activity fractions from P2 against *S. aureus* and *Klebsiella pneumoniae*

P2 Fractions Compounds	Pure Compounds	MICs (μg/mL)	
		S. aureus	*K. pneumoniae*
P2-12-11	-	8	70.3
P2-12-29	-	6	2.3
P2-12-TA	-	9.5	33.9
P2-12-12	-	90.7	71.5
P2-12-34	-	101.6	52.2
P2-7	**(5)**	97	63.5
P2-12-37	**(6)**	25.2	31.8
P2-12-6	-	56	54.6
P2-12-44	-	103.7	80.6
P2-12-11	-	111.7	75.4
P2-12-3	-	51.6	71.2
Gentamycin		6.25	6.25

N.B; Gentamycin; drug control.

3.17.5 In vitro Antimycobacterial activity of P1 fractions against *M. marinum*

In vitro antimycobacterial activity of P1 fractions against *M. marinum* was tested as described in section 2.6.5. Table 3-60 shows the results obtained. The MICs of the open column fractions were either 100 or 50 μg/ml whereas the MPLC fractions demonstrate higher activity and further purification could potentially give more active compounds such as diterpenes, flavonoids or lignans against *M. marinum.*

Table 3-60 MICs values of Antimycobacterial activity of P1 fractions against *M. marinum*.

P1 Fractions	**MICs (μg/ml)**
[b]P1-1	50.0
[b]P1-2	100.0
[b]P1-3	50.0
[b]P1-4	50
[b]P1-5	100.0
[b]P1-8	100.0
[c]P1-3-5	25.0
[c]P1-3-6	25.0
[c]P1-3-8	25.0
[c]P1-3-13	50.0
[c]P1-3-25	100.0
[c]P1-3-26	100.0

N.B. MIC (μg/ml) *M. marinum* (ATCC.BAA535) [b]; Open Column Fractions and [c]; MPLC-Grace Reveleris® fractions.

4 Chapter 4

Discussion

4.1 Discussion

To date, the propolis from various geographical areas in the world e.g.; Europe, Asia, North and South America (especially from Brazil), (Salomão et al., 2009, Da Silva et al., 2013) has been studied extensively in the last decades. Meanwhile in Asia propolis from: China (Yang et al., 2011, Ahn et al., 2007, Banskota et al., 2000), Japan (Kumazawa et al., 2007, Hamasaka et al., 2004), Taiwan (Chen et al., 2008, Lu et al., 2003), Nepal (Shrestha et al., 2007b, Huang et al., 2014) and Myanmar (Li et al., 2009b), have recently become the subject of detailed studies. (Petrova et al., 2010).

Propolis in Libya is considered as the second bee product after honey in local traditional medicine markets and it has been used in folkloric medicine for treating some illnesses. Numerous beekeepers are dealing with bee products such as honey, royal jelly and propolis in many cities of Libya. The beekeepers are mostly found in the areas, which are rich in different plants where bees collect the exudates, from tree buds, sap flows, or other botanical sources in the fields and utilize them to make the propolis. All these factors are associated with the chemical composition variation in propolis. The fact that there was little publication of data on Libyan propolis led to the current study. The previous research work on Libyan propolis is summarised in section 1.9.1, Table 1-10 (Abd El-Rahman, 2010, Sarkez, 2014, Siheri et al., 2014, Azab et al., 2015a, Azab et al., 2015b, Siheri et al., 2016). The main goals of this research project were the comprehensive chemical profiling and the evaluation of the biological activity of propolis from different areas in Libya.

Due to the situation in Libya since starting this project it was not possible to collect as many samples as I would have liked but nonetheless twelve propolis samples (P1-P12) (section 2.3, Table 2-1 and Figure 2-2) were

collected from different localities which covered the North Eastern and South Eastern regions and the North Western and South Western areas of Libya (Figure 2-1).

Ethanolic extracts of the twelve propolis samples were prepared and these were profiled initially by NMR (see section 3.4), which gave some general indication of the type of compounds to be found in them providing signals typical of diterpene aldehydes and cycloartane triterpenoids as well as lignan depending on the origin of the sample. There were limited signals in the aromatic region between 6 and 8 ppm in contrast to Northern European samples where many signals from flavonoid compounds would be expected. The extracts were profiled by high resolution LC-MS and the LC-MS data (see section 3.5), was extracted and modelled by SIMCA-P software using PCA with HCA, which separated the samples into five main groups based on their chemical composition. The groups were according to Geographic origin which the samples from North East, North West, South East and Southwest Libya grouping together. The samples extracts were tested against a wide range of microorganisms including *T. brucei*, *L. donovani*, *P. falciparum*, *C. fasiculata*, *M. marinum*, *S. aureus*, *K. pneumoniae* and *T. spiralis* (see section 3.6). In addition, cell-based assays for cytotoxicity and anti-inflammatory activity were carried out. Almost all the extracts had anti-protozoal activity with the samples from North East Libya having the highest activities. Additionally, *in vitro* antibacterial assessment screening against Gram-positive and Gram-negative bacteria most of the samples exhibited no activity against both strains of Gram-negative bacteria; *E. coli* and *K. pneumoniae*, at the concentration used in this assay (500μg/ml) except for P2, which exhibited a MIC of 250μg/ml. While P2, P3 and P8-P10 had some activity against Gram-positive, *S. epidermidis* and *S. aureus* (Table 3-21). These results are similar to those previously observed (Fokt et al., 2010, Wagh, 2013). Greek propolis has shown remarkable activity

against Gram-positive bacteria and fungi which was mainly due to diterpenes (Melliou and Chinou, 2004). Samples P1-P8 exerted moderate cytotoxicity against the U937 cells while the samples (P9-P12) were not cytotoxic to U937 cells exhibiting EC_{50} >100 μg/ml (Table 3-22). There has been extensive research *in vitro* and *in vivo* into the cytotoxic activity of the propolis collected from different geographical areas with some samples exhibiting cytotoxicity (Bufalo et al., 2009, Banskota et al., 2001b, Banskota et al., 2000, Popova et al., 2005, Li et al., 2008, Kouidhi et al., 2010, Carvalho et al., 2011). Cytotoxic activity was evaluated against human laryngeal epidermoid carcinoma cell (Hep-2), human cervical adenocarcinoma (HeLa) and human normal epithelial embryonic kidney (Hek-293). Survival analysis for non-tumor cell lines showed greater IC_{50} compared to tumor cell lines, suggesting an increased sensitivity that may correlate with the higher proliferative index of the tumor against normal cells. The finding results of the research study indicated that the Brazilian red propolis is capable of inhibiting cancer cell growth and constitutes an excellent source of antioxidant and antitumor natural agent (da Silva Frozza et al., 2013). Another study Bufalo *et al*, (2009), has been carried out on green Brazilian propolis in order to evaluate *in vitro* cytotoxic action on human laryngeal epidermoid carcinoma (Hep-2) cells. Propolis samples exhibited *in vitro* a cytotoxic effect against Hep-2 cells, depending on both dose and time of exposure to propolis extracts (Bufalo et al., 2009).

Anti-inflammatory evaluation for all the EEPs P1-P12 samples using a TNF-α ELISA assay was carried out (section 3.6.8). Samples P4, P6, P7 and P8 could not be assayed it due to the insolubility of the samples in DMSO. The production of TNF-α by THP-1 cells stimulated with and without LPS, was assessed by a TNF-α ELISA assay. The samples exhibited a range of proinflammatory activity (increased production of TNFα by THP-1 cells). P9 exhibited a strongly significant proinflammatory (Figure 3-29). In contrast to

the proinflammtory findings that has been stated that propolis is commonly used for the treatment of various skin inflammatory diseases and has anti-inflammatory properties (Marcucci, 1995). It has been reported that propolis from different areas exhibits anti-inflammatory activity with a significant reduction of acute inflammation produced by zymosan in mice after the oral administration of water-soluble extracts of propolis at a dose of 150 mg/kg, (Khayyal et al., 1992, Castaldo and Capasso, 2002, Paulino et al., 2003, Ramos and Miranda, 2007, Paulino et al., 2008).

In vitro anthelmintic activity against *T. spiralis,* (Table 3-23) and *C. elegans*, (Table 3-24) was assessed in this thesis. Samples P1, P3 and P4 at concentration of 10μg/ml and sample P2 at concentration 1μg/ml exhibited mostly a percentage of inhibition 81-100% of against *T. spiralis*. Samples P3 and P4 at concentration of 1μg/ml and sample P2 at concentration 1μg/ml exhibited a percentage of inhibition 41-60% of against *T. spiralis*. P6-P12 did not show any activity against *T. spiralis*. According to this finding, an EC_{50} value was determined for P1, P2, P3, P4 and P5 against *T. spiralis.* The EC_{50} results for samples P3 and P4 showed high activity against *T. spiralis.* The EC_{50} of P3 was 7.96 and EC_{50} of P4 was 29.22, respectively. The rest of the samples P1, P2, and P5 had no significant activity (Figures 3-31 and 3-32).

The samples from North East Libya P1 and P2 were initially selected for futher fractionation by open column chromatography and MPLC. From these samples four labdane diterpenes, three lignans and and a flavonoid were isolated. All of these compounds were active to some extent against *T.brucei* and *L. donovani* but none of them had appreciably higher activity than the crude extracts. Compound **(5);** sesamin was isolated by Sartorelli *et al*. (2010), from the leaves of *Aristolochia cymbifera*, and was found to be the most active

compound against trypomastigotes of *Trypanosoma cruzi* (Sartorelli et al., 2010). Sesamin, has also been reported to exhibit moderate larvicidal activity, and to have antifeedant and growth-inhibition activities (Hassanali et al., 2013, ZHOU et al., 2004). Lignans have been isolated from Chilean propolis and Canary Islands propolis (Bankova et al., 1998, Christov et al., 1999, Bankova et al., 2000, Silva-Carvalho et al., 2015).

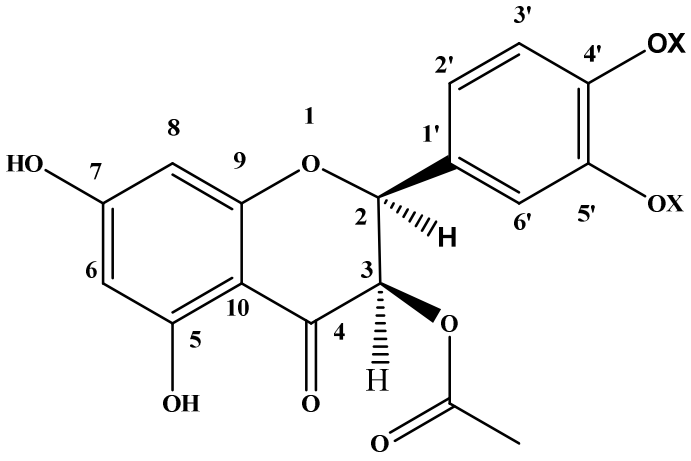
Interestinglly, compound **(8)** was indentified as (2S,3R)-taxifolin-3-acetate-4'-methyl ether [3-acetoxy-5,7,3'-trihydroxy-4'-methoxyflavanone. Another possible IUPAC name is, (2S,3R)-5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-3- methyl-4-oxochroman-3-yl acetate, (chemical name using ChemDraw Professional 15.1)

Additionally, from sample P1, the open column fraction P1-2 was further fractionated by MPLC (see section 3.8, Table 3-23) to yield a single compound **(8)** with molecular weight 360.0766 that had a compostion $C_{18}H_{16}O_8$. The ^1H NMR spectrum (Appendix 11), showed a very deshielded proton singlet at δ 11.44 together with two 3H singlets, a methoxy at δ 3.86 and acetoxy at δ 1.96, two coupled proton doublets (*J*=11.7Hz) at δ 5.20 and δ 5.73 and five aromatic protons. These latter were composed of two meta-split protons at δ 5.94 and 5.99, reminiscent of A-ring protons of a flavonoid, a multiplet for ^1H at δ 6.93 and a 2H multiplet at δ 6.86, each set of aromatics appearing to couple in the ^1H -^1H-COSY spectrum. The HSQC spectrum showed the carbon attachments of the carbon-bonded protons (see Table 3-42) and the fact that the latter two aromatic protons were attached to different carbons at 114.4ppm and 121.4ppm and therefore not part of a symmetrically substituted aromatic ring. The HMBC spectrum (Appendix 15), revealed that the deshielded phenolic proton (δ 11.44) coupled with three aromatic carbons, 97.4(CH), 101.8(C) and 164.4(C-O) corresponding to C-6, C-10and C-5, respectively. The proton H-6 at δ 5.99

and the proton at δ 5.94 (H-8) had strong ^{3}J correlations to C-10 and to each other's carbons and they both had a weak correlation to a carbon at 165.5ppm (C-7) and H-8 had a weak correlation to 162.3ppm (C-9).

The proton doublet at δ 5.75 had a cosy correlation to the proton doublet at δ 5.20 and was directly attached to C-3 (72.2ppm, as shown in HSQC) and they had HMBC correlation to a carbonyl at 169.2ppm which coupled to the methyl at δ 1.96ppm indicative of the 3-acetoxy moiety. The partner to this proton at δ 5.20 coupled to C-3 and C-4 (the carbonyl at 191.7ppm) and to 109.3, 121.4ppm, and 127.0ppm, C-6', C-2', and C-1', respectively. The protons attached to carbons, C-6'and C-2' at δ 6.93 and δ 6.86, coupled with the carbon 81.5 (C-2) and one another's carbons (109.3 and 121.4ppm) and 146.7ppm (C-4') and the latter carbon also coupled to the methoxy protons (δ 3.86). All assignments were made as shown in Table 3-42.

Thus, compound **(8)** was identified as the novel compound, 3-acetoxy-5,7,3'-trihydroxy-4'-methoxyflavanone. These data + the position of [α] = +1.6 optical rotation (Table 3-54), for this compound suggests the same configuration as published for (2R,3R)-taxifolin-3-acetate isolated from *Chrysothamnus viscidiflorus* ssp. *viscidiflorus* by Stevens, *et al* (1999). These authors also isolated a methyl ether which they called taxifolin-3-acetate-x'-methyl ether of their structure (**26)** as they could not establish the connection of the methoxy moiety to either position-3' or -4'.



X=H or CH_3

Compound **(26)** (Stevens, *et al* (1999)

However, we have established the attachment of the methoxy in compound **(8)** to the 4'-position. They did not publish any NMR data for their compound so it is still not possible to say which isomer they may have had.

It would be interesting to try find *Chrysothamnus viscidiflorus* or other closely related species of *Asteraceae*, that produce taxifolin-type flavonoids, growing in the vicinity of the hives.

Samples P1 and P2 had a composition typical of Mediterranean propolis (Popova et al., 2010, Miguel et al., 2010). Previous studies have found that Mediterranean Propolis exhibited biological properties such as antioxidant, ant proliferative, anti-inflammatory, neuroprotective effects (Miguel et al., 2010, Kujumgiev et al., 1999). The botanical origin of diterpenes in the propolis samples from Greece is yet unidentified, but on the basis of the diterpenic profile, the source plant should be the conifer species of the Cupressaceae

family, in which the flora of the region is very rich (Miguel et al., 2010). Wen-Chiung *et al* carried out the isolation of diterpenes compounds such as **(1)** 13-epi-torulosal and **(3)** 13-epicupressic acid, from the leaves of *Cryptomeria japonica juniperus rigida* from the family Cupressaceae which is distributed throughout Korea (Woo et al., 2011). From previous research studies the chemical name and chemical structure of this group of diterpenes was determined as follows. **(1)** 13-Epitorulosal, while **(3)** 13-Epicupressic which a related labdane diterpene. The previously published work could be used to identify **(1)** and **(3)** from previous NMR data for these compounds (Wen-Chiung et al., 1994, Caputo et al., 1974, Woo et al., 2011).

MPLC fractionation of P7 which was from the tropical region of Southeast Libya was carried out and this resulted in the isolation of a number of compounds of the cycloartane triterpene group: **(9)** cycloartanol, **(10)** mangiferolic acid, **(11)** mangiferonic acid, **(12)** ambolic acid and **(13)** 27-hydroxymangeferonic acid (Figure 3-68, Tables 3-34). These compounds were previously isolated from Cameroon propolis (Kardar et al., 2014). Although the crude extract of P7 did not have strong anti-protozoal activity most of the compounds isolated from it displayed significant anti-protozoal activity. Li *et al.,* (2009), isolated cycloartane-type tritepenes from Myanmar propolis, and evaluated their cytotoxic activity against a B16-BL6 melanoma cancer cell lines. The cycloartane-type triterpene, 3α,27-dihydroxycycloart-24*E*-en-26-oic acid showed the most potent cytotoxicity against B16-BL6 cells with an IC_{50} value of 5.91mM, comparable to those of positive controls, doxorubicin (IC_{50}, 5.66mM) and 5-fluorouracil (IC_{50}, 4.88mM) (Li et al., 2009a). Many studies, on propolis from different African regions such as Kenya, Cameroon, Congo and Ethiopia, showed that triterpenoids are major chemical components (Popova et al., 2013). In addition, (Trusheva *et al.,* 2011), three cycloartane-type triterpenes were isolated and identified from Indonesian propolis from East Java, and

concluded that Macaranga tanarius L. parasol tree a ''pioneer species''and *Mangifera indica* L. (mango), were plant sources of Indonesian propolis (Trusheva et al., 2011). In addition, **(14)** cardol, was isolated from P7. This compound was isolated previously by Maia *et al* from hydrogenated cashew nut shell (Maia et al., 2013). Of all the isolated compounds tested, this compound has the highest anti-trypanosomal activity of any that were isolated despite the fact that the crude propolis sample had relatively low activity.

Additionally, further purification of the P9 sample using using MPLC, resulted in four pure compounds **(15)** acetylisocuppressic acid, **(16)** Agathadiol, **(17)** Isocupressic acid and **(18)** isoagatholal**,** (Appendix 1 to 4). The structures of these compounds were determined using 2D NMR expirments HSQC and HMBC to identified the isolated compounds and then were comparison with previous literature and the data is summarised in (Tables 3-50 to 3-53), respectively. According to the results described in Table 3-49, other fractions were mixtures of two or more compounds and the ELSD traces for P9-16 and P9-18 are shown in Appendix 5 and 6, respectively. These fractions will be a target for further purification.

The chemistry of propolis can potentially reveal a great deal about the interaction between the bee and its environment, especially the flora with the problems of colony collapse affecting beehives in many parts of the world having a better understanding of the propolis chemical profile is of great importance. The biological finding of the current study has introduced additional data to the previous scientific knowledge of Libyan propolis. These results could lead to discovery of novel antiparisitic. The reason for propolis having anti-protozoal activity is supported by recent work in Scotland on the bee genome and microbiome. It has been observed that some of the more

abundant DNA in the bee microbiome corresponds to trypansomatid DNA (Regan, T. (2017) Roslin Institute, personal communication).

5 Chapter 5

Conclusion

5.1 Conclusion

Propolis shows a potential activity against a range of microorganisms. Its production is commercially viable and it is none destructive since bees collect it without damaging the plants they collect from. The main problem of exploiting the biological activity of a natural product like propolis is to guarantee a standardised supply of material. In addition, some of the active compounds isolated from propolis could be used as lead compounds for producing drugs that are more potent. During the preliminary stages of this research, we developed a method for rapid qualitative analysis of these propolis samples to determine their main components using various chromatographic and spectroscopic techniques. This was followed by conducting a series of chromatographic separations and assisted with spectroscopic detection methods in order to fractionate, purify and identify biologically active compounds from propolis extracts. Structure elucidation using NMR, identified the eighteen pure compounds, including four diterpens; **(1)** 13-epi-torulosal**, (2)** 13-O- Acetyl epi-cupressic acid**, (3)** 13-epicupressic acid, **(4)** 13-epitorulosol which were isolated from P1 sample and lignan compounds from P2 **(5)** Sesamin**, (6)** Demethylpiperitol, **(7)** 5',methoxy pipertol, a novel flavonoid flavan type **(8)** Taxifolin-3-acetate-4'-methyl ether, which was isolated from P1 and P2 samples also **(5)** Sesamin from P1 and P2. In addition, the compounds **(1)** and **(3)** were isolated from P2, the triterpenes cycloartan type such as **(9)** Cycloartanol, **(10)** Mangiferolic acid, **(11)** Mangiferonic acid, **(12)** Ambolic acid, **(13)** 27-hydroxymangeferonic acid **(14)** Cardol were isolated from P7 sample (South East-Libya). Another diterpenes were isolated from P9 which were **(15)** Acetylisocuppressic acid, **(16)** Agathadiol, **(17)** Isocupressic acid and **(18)** Isoagatholal.

The isolated pure compounds **(1)** to **(18)** are known compounds which habe been isolated before although this the first time that they were isolated from Libyan propolis a part from compound **(8)** taxifolin-3-acetate-4'-methyl ether**,** which is a novel compound. These isolated compounds all exhibited a range of *in vitro* activity against *T. brucei*. For the isolated pure compounds **(1)** to **(18),** further investigations are needed to study structure activity relationships in relation to parasite and anti-bacterial activity in order to determine the effect of changes in functional groups on their biological activity within and between classes of compounds. In addation, severeal other pure compounds have been isolated from the propolis extract during this work but their stractures have yet fully elucidated.

Generally, this research work has revealed Libyan propolis constituents that possess potential biological activity, and has confirmed the presence of an interesting range of compounds, which could be employed as lead compounds for new drug discovery. In addition, some of the isolated pure compounds were not isolated in sufficient quantity to be included in all the biological tests in this study; it is recommended that these should be scaled uptested against a range of pathogenic microorganaism and parasities. In addition, some of the fractions possessed strong biological activity and should be further purified.

A metabolomics study was carried out in order to try to understand the mechanism of action against *T. brucie*. The results were interesting and we intend to repeat this study at the first opportunity in order to gain firmer conclusions.

5.2 Future work

At the end of this project, I have gained a wide range of research experience. Therefore, a future plans will be continuous work on Libyan propolis to cover all the geographical areas, which were not covered in this project to discover the full mystery of the treasures behind Libyan propolis.

Moreover, further purification of the fractions mixtures isolated from P1, P2, P7 and P9 will subjected to use different chromatography techniqes.

Also fractionation and purification of the other Libyan propolis samples P3, P4, P5, P6, P8 and P8. And further biological testing using another pathogenic micoragnsims in order to investigation of other pharmacological activity of these samples.

Additionally, focusing on metabolomics study of isolated compounds posing significant anti trypanosomal activity against *T. brucei* would provide another interesting line of research.

6 Chapter 6

References

6.1 References

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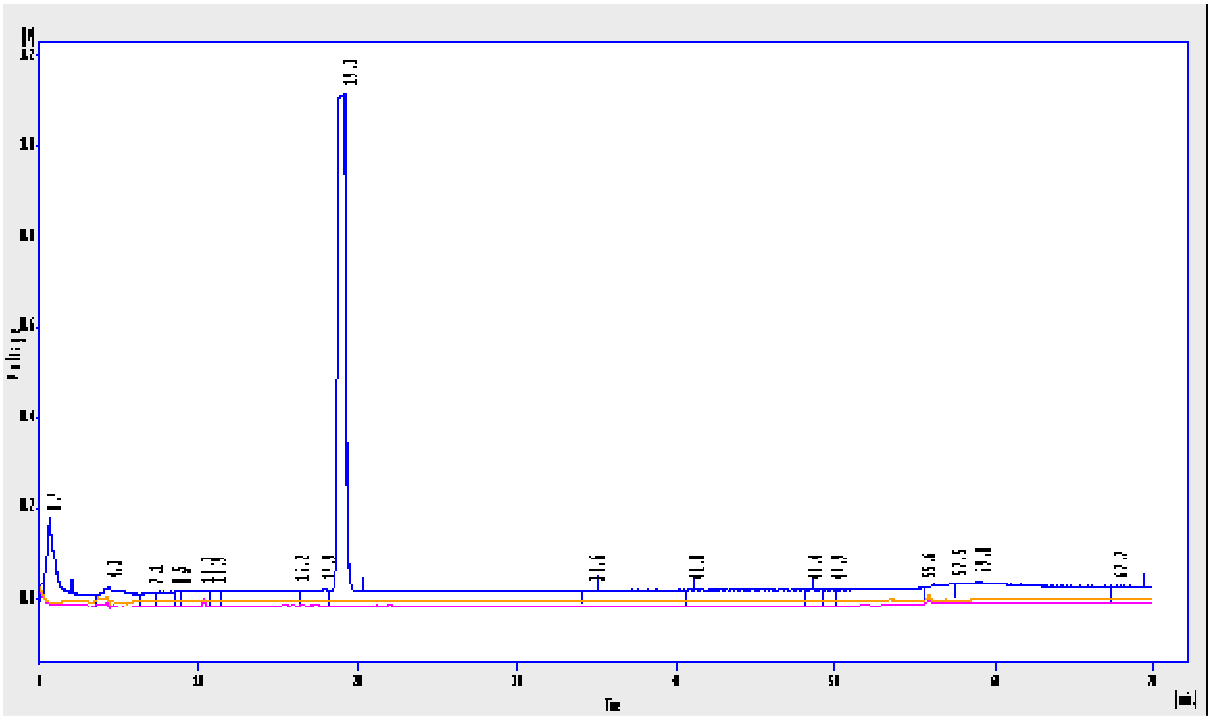
7 Chapter 7

Appendices

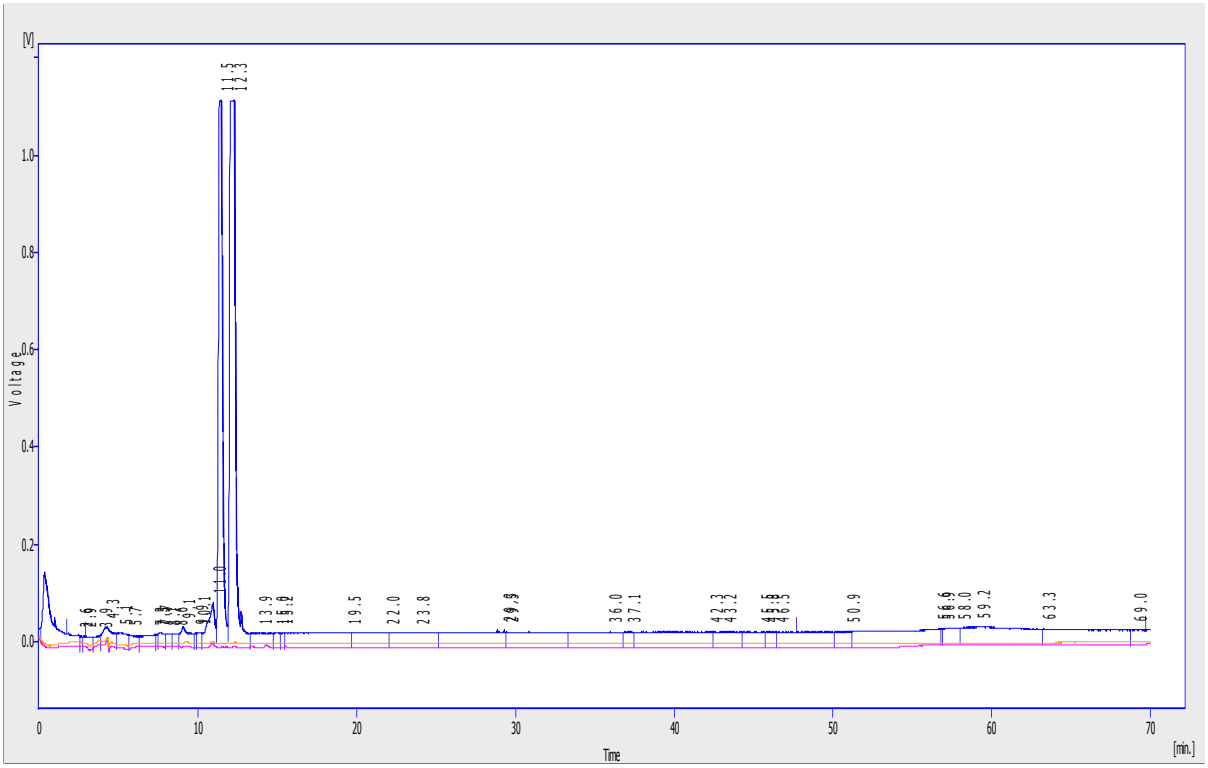
7.1 Appendices

7.2 HPLC –UV-ELSD Chromatograms of MPLC Fractions of P9

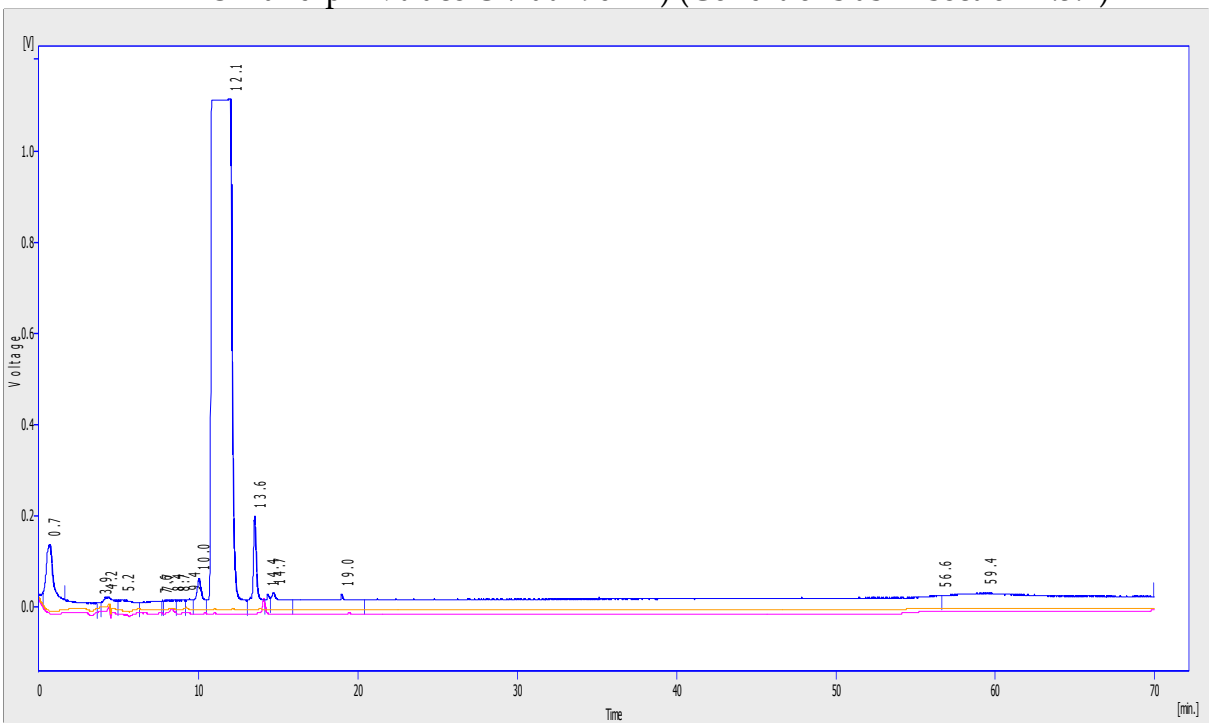
The following appendices of HPLC-UV-ELSD chromatograms in **(Conditions as in section 2.5.2)**, from the figures can show that the MPLC fractions purified and there were significant purity such as the follows;



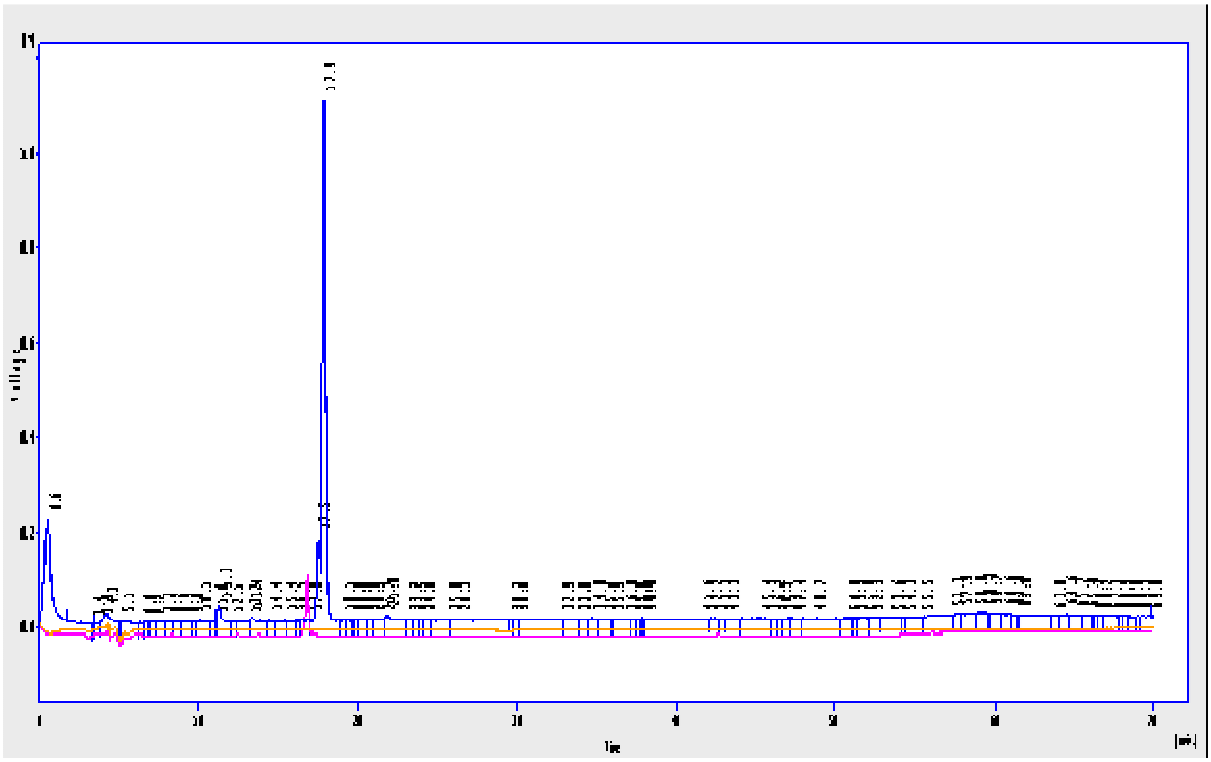
Appendix 1 HPLC –ELSD-UV chromatogram of compound (15) (blue treces ELSD and pink trace UV at 290nm) (Conditions as in section 2.5.2)



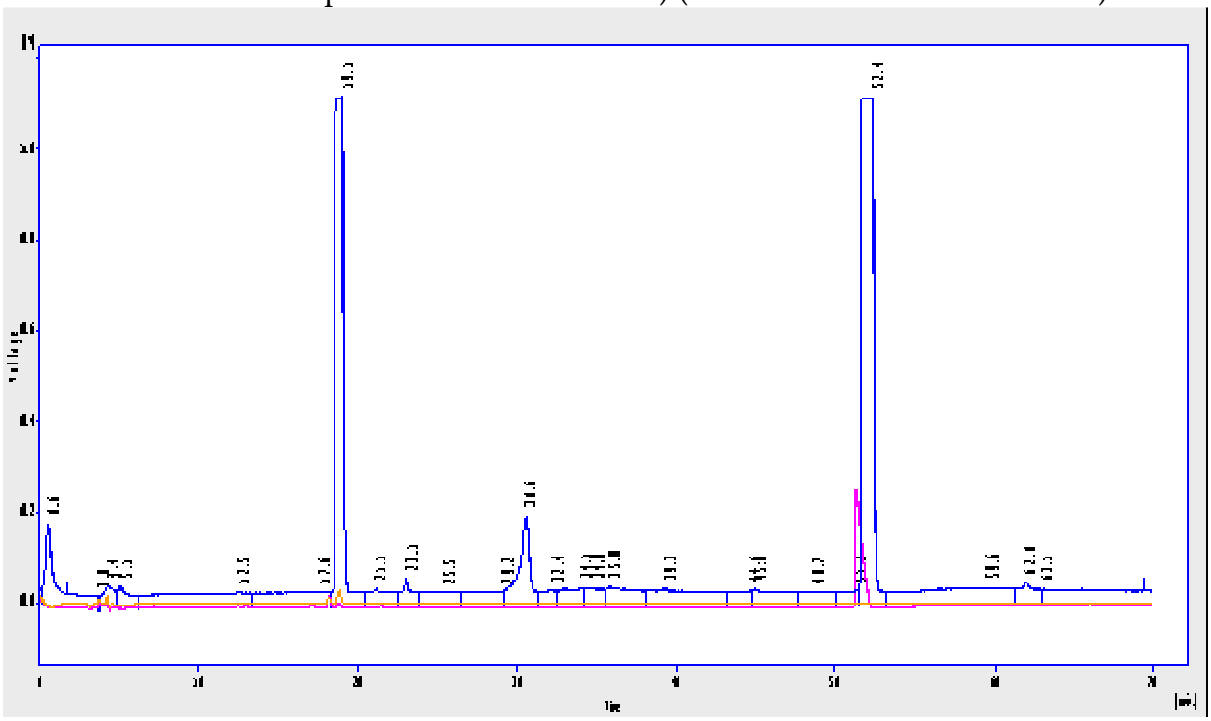
Appendix 2 HPLC –ELSD-UV chromatogram of compound (16) (blue treces ELSD and pink trace UV at 290nm) (Conditions as in section 2.5.2)



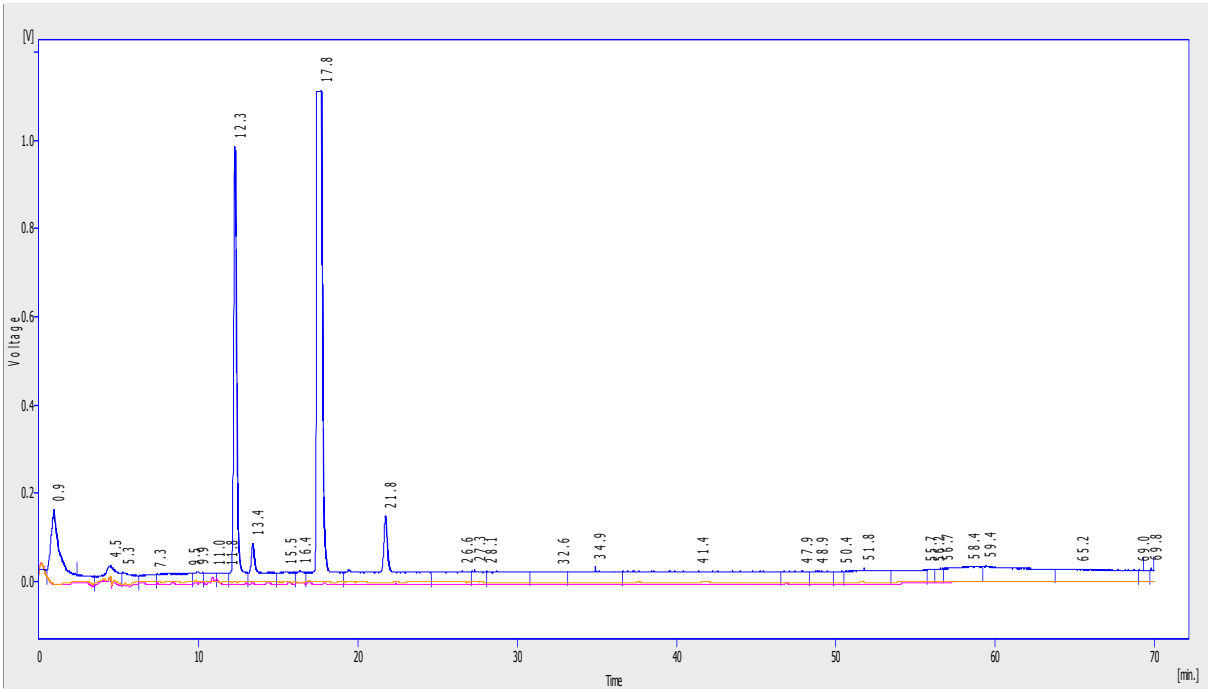
Appendix 3 HPLC –ELSD-UV chromatogram of compound (17) (blue treces ELSD and pink trace UV at 290nm) (Conditions as in section 2.5.2)



Appendix 4 HPLC –ELSD-UV chromatogram of compound (18) (blue treces ELSD and pink trace UV at 290nm) (Conditions as in section 2.5.2)

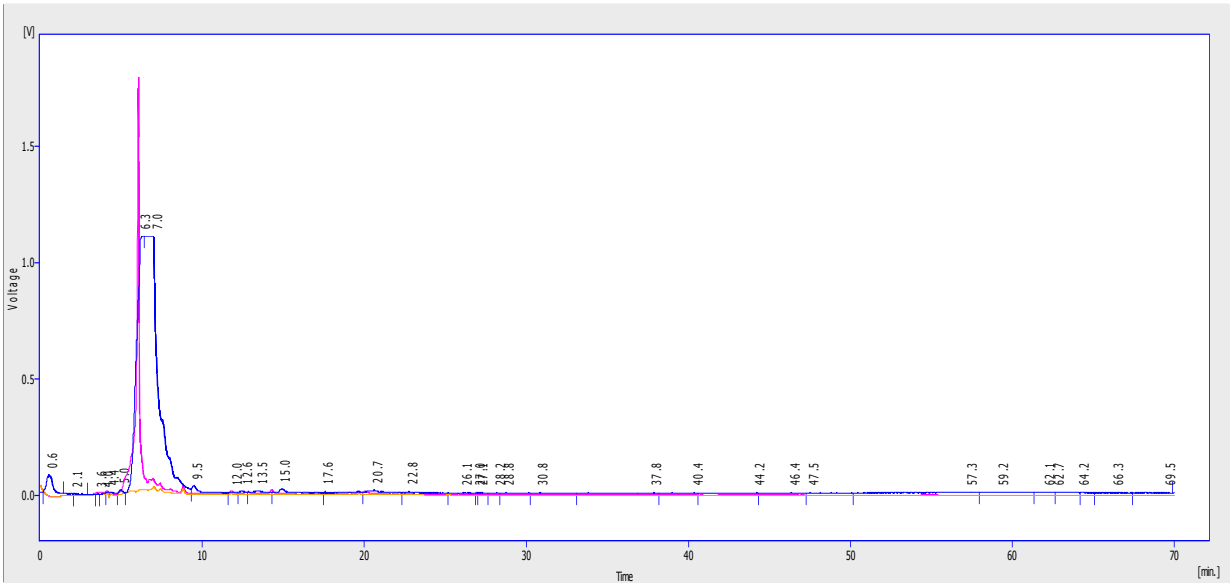


Appendix 5 HPLC –ELSD-UV chromatogram of fraction (P9-16) (blue treces ELSD and pink trace UV at 290nm) (Conditions as in section 2.4.2)

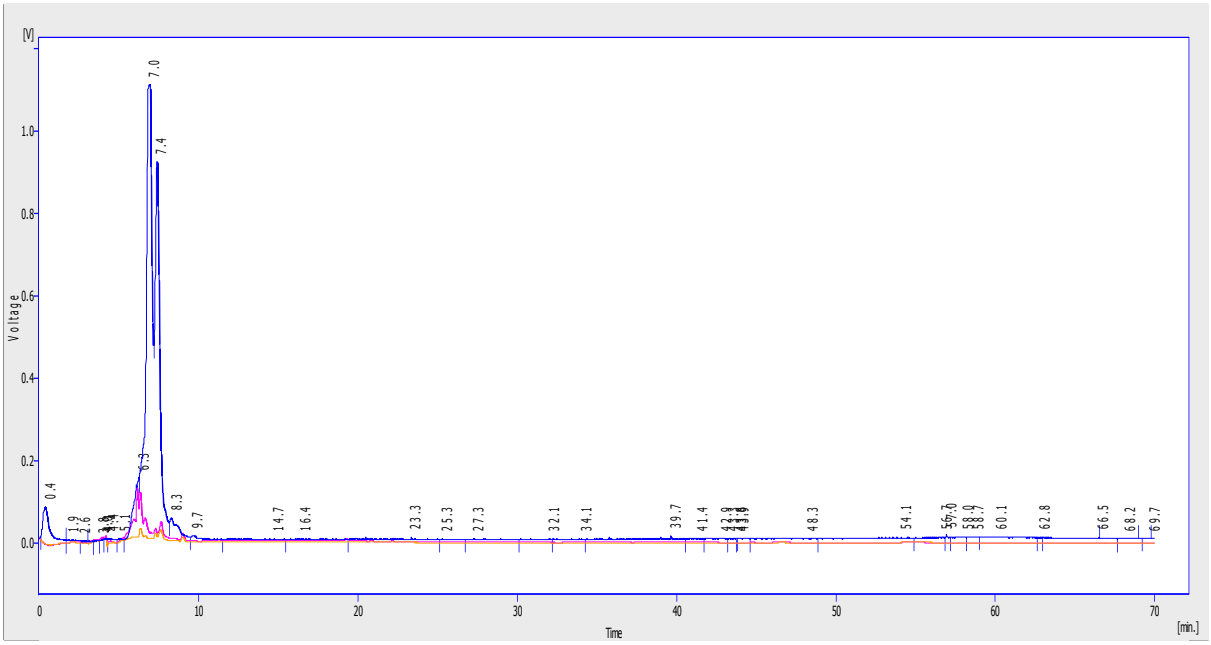


Appendix 6 HPLC –ELSD-UV chromatogram of P9 MPLC of (P9-18), (blue treces ELSD and pink trace UV at 290nm) (Conditions as in section 2.5.2)

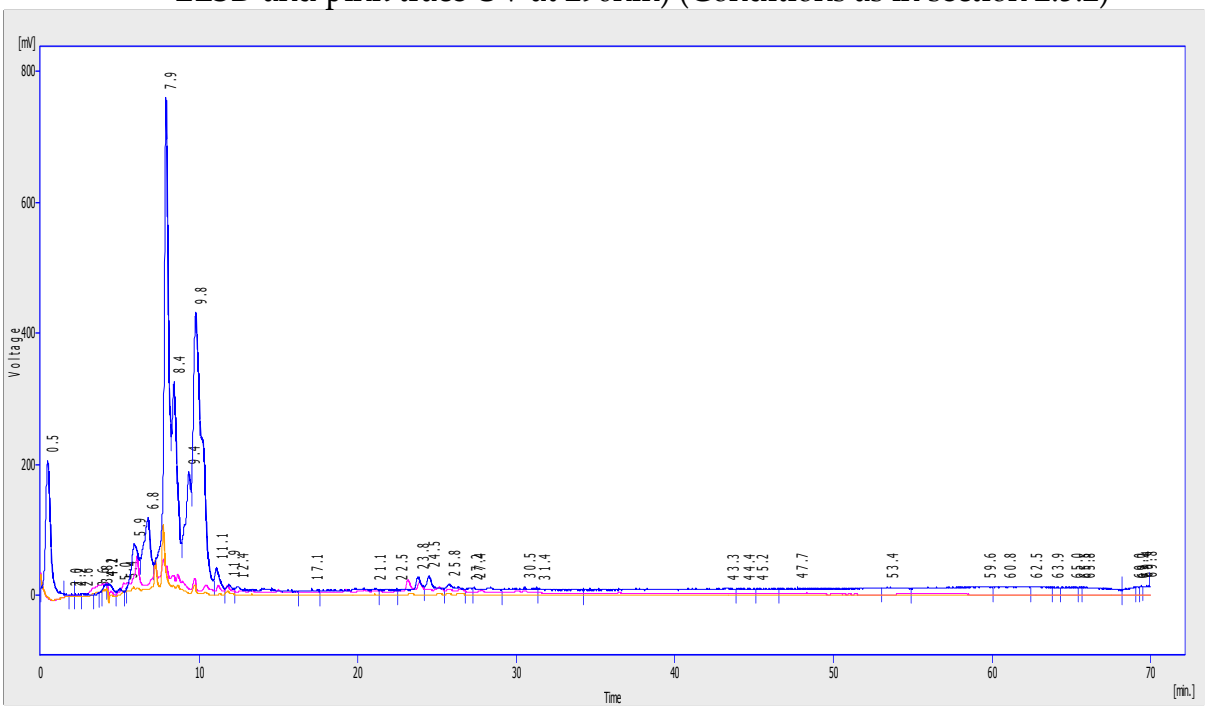
7.3 HPLC –UV-ELSD Chromatograms of MPLC Fractions of P2-12



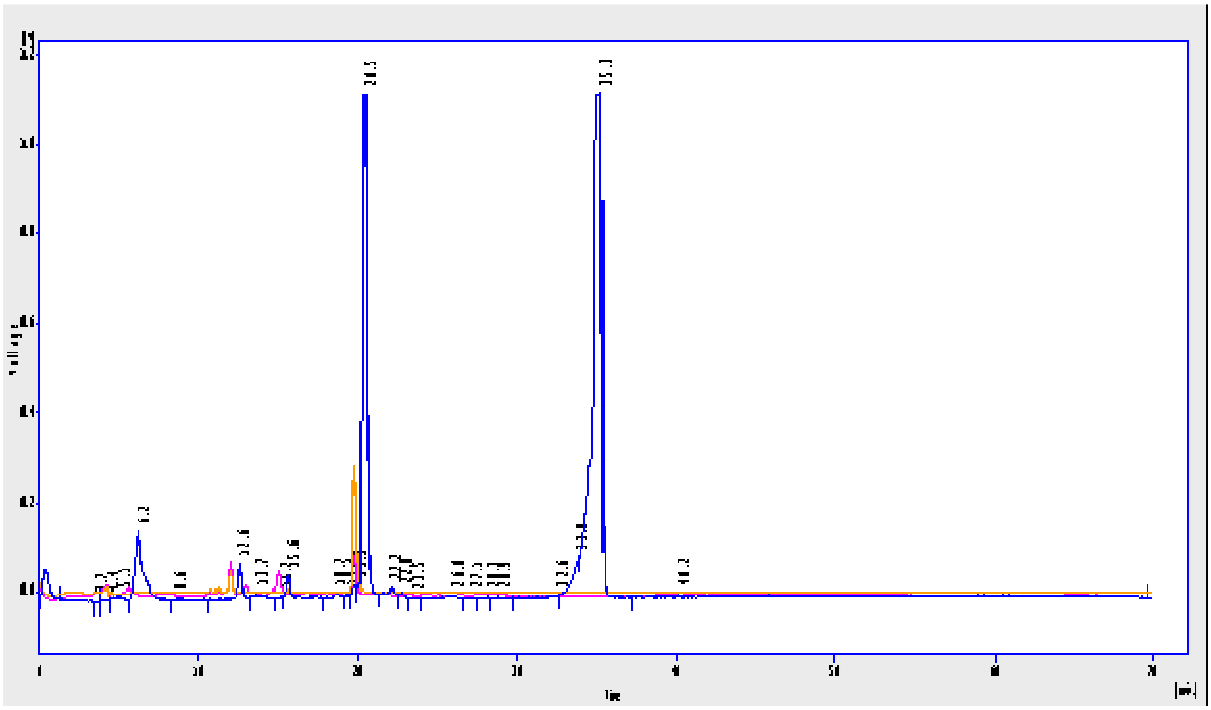
Appendix 7 HPLC –ELSD-UV chromatogram compound (6) (blue treces ELSD and pink trace UV at 290nm) (Conditions as in section 2.5.2)



Appendix 8 HPLC –ELSD-UV chromatogram of fraction F3-P2-TA (blue treces ELSD and pink trace UV at 290nm) (Conditions as in section 2.5.2)

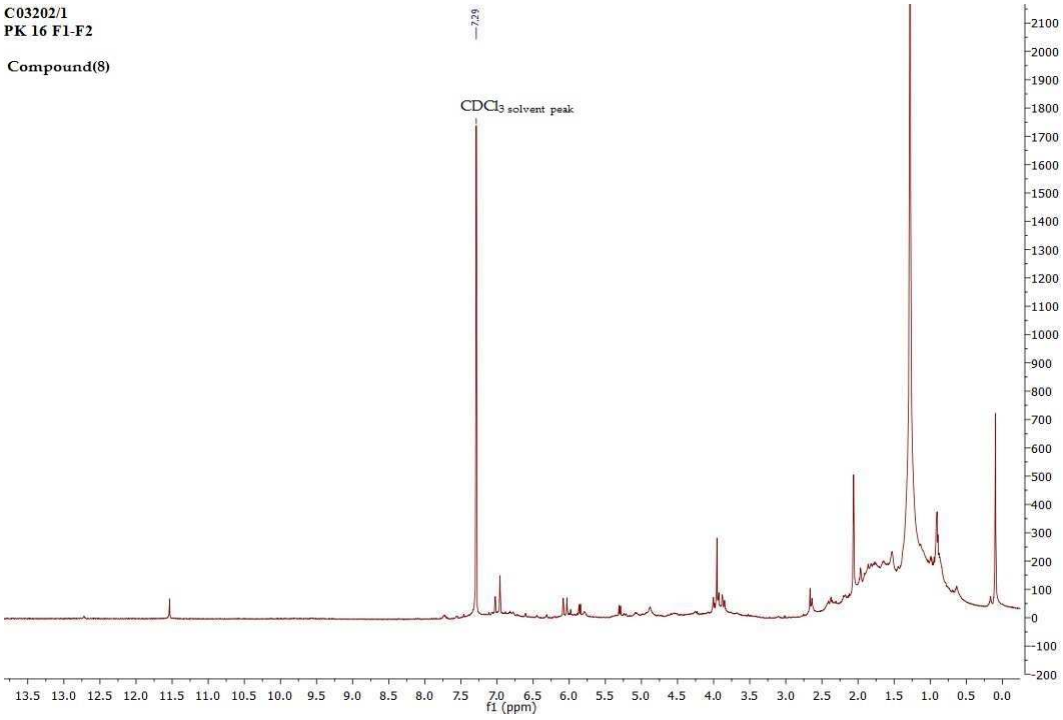


Appendix 9 HPLC –ELSD-UV chromatogram of fraction P2-12-29 (blue treces ELSD and pink trace UV at 290nm) (Conditions as in section 2.5.2).

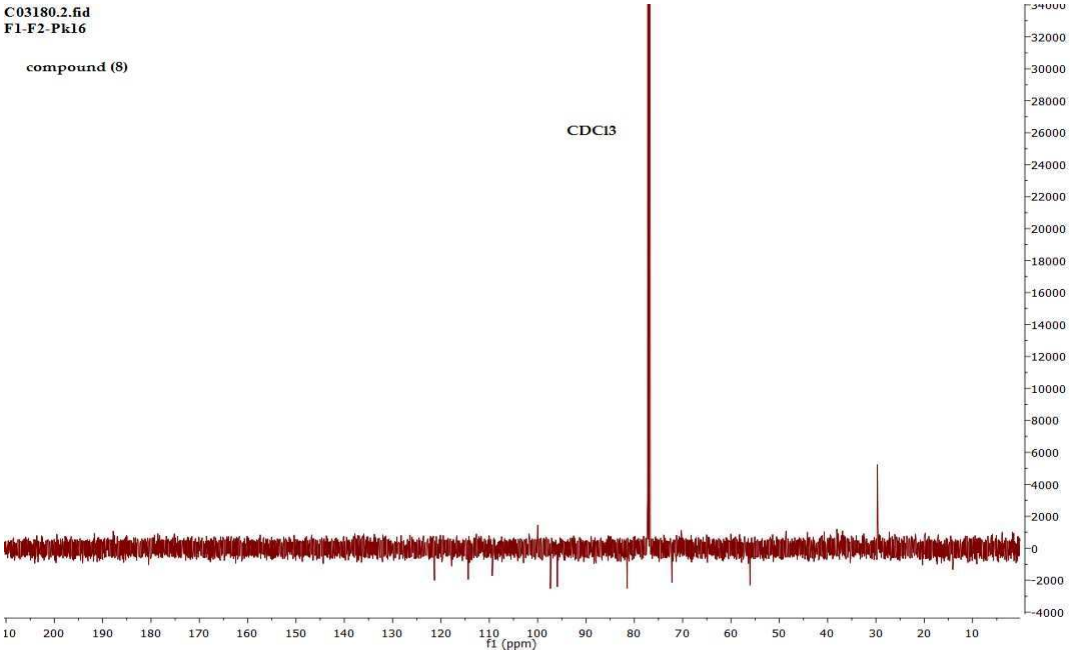


Appendix 10 HPLC –ELSD-UV chromatogram of fraction P2-12-6 (blue treces ELSD and pink trace UV at 290nm) (Conditions as in section 2.5.2)

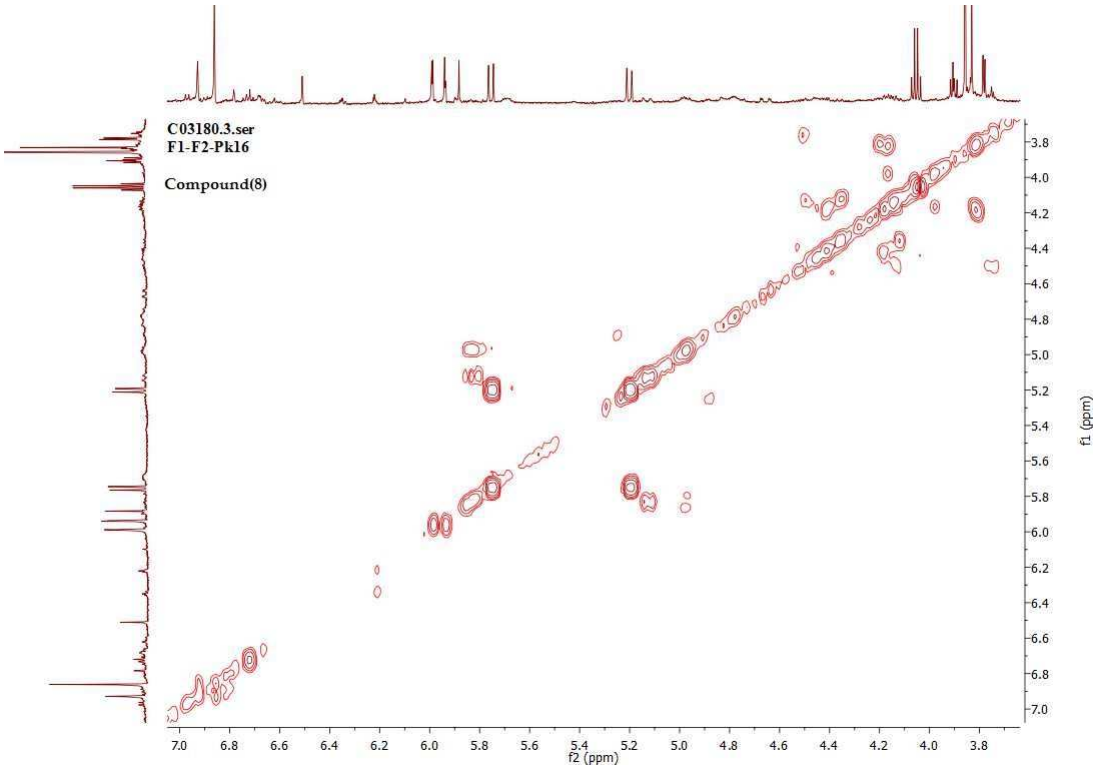
7.4 2D NMR data of compound (8)



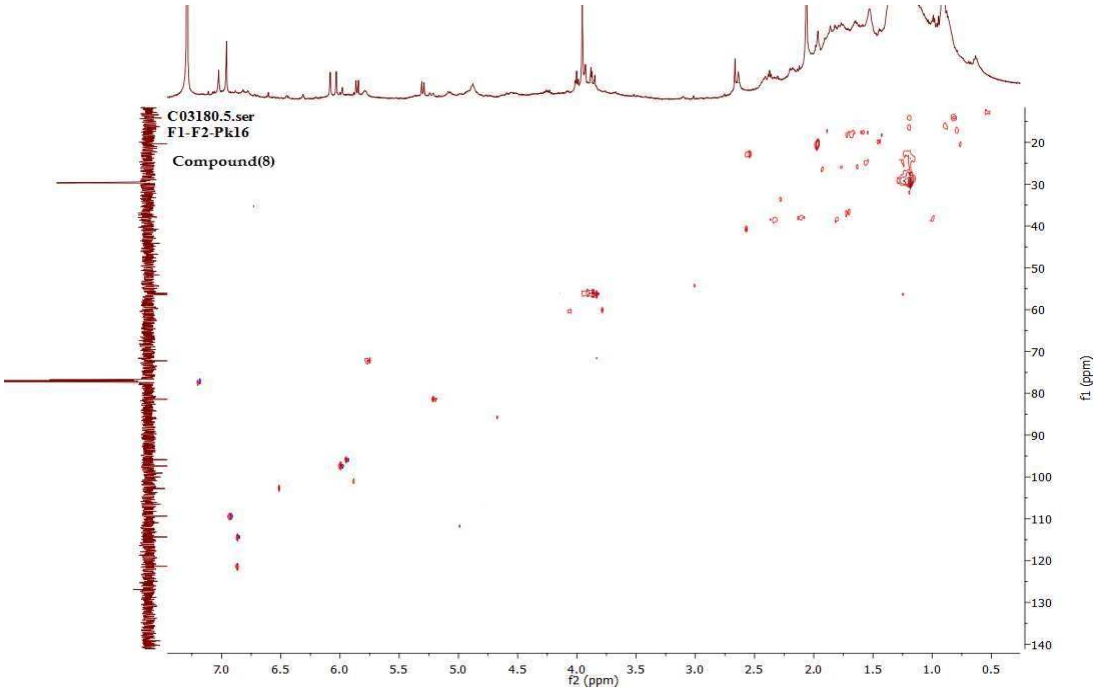
Appendix 11 ^{1}H NMR spectrum (600 MHz in CDCl3) of compound (8)



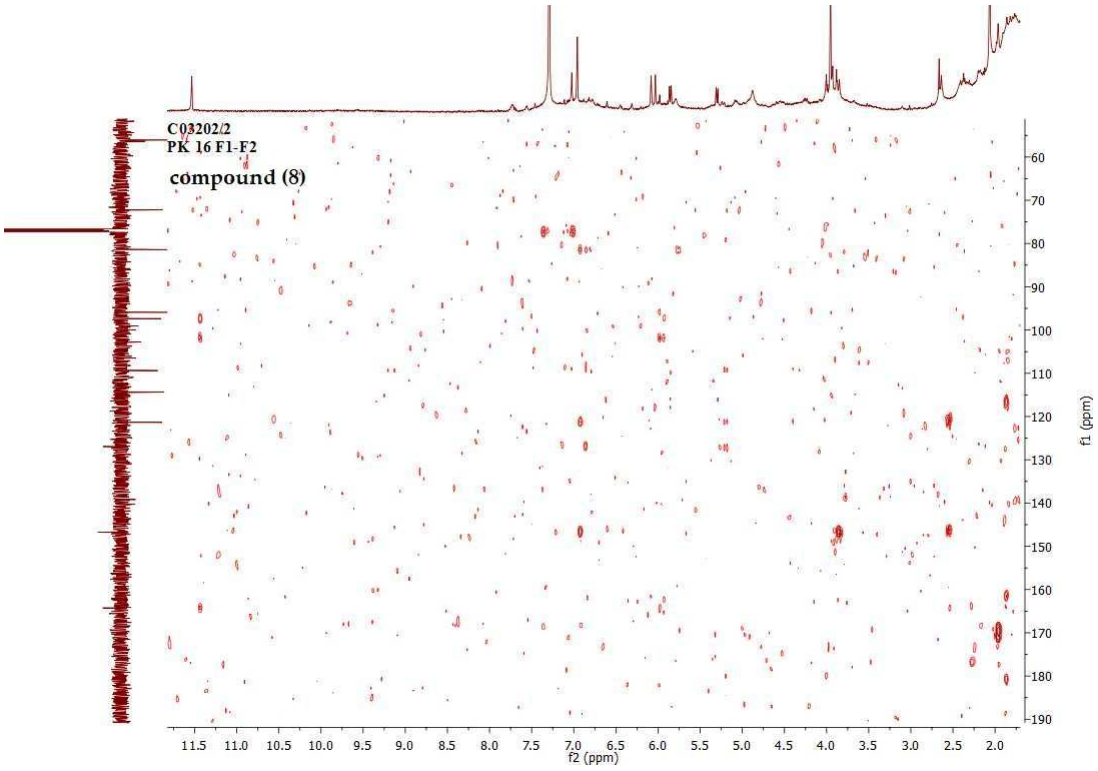
Appendix 12 The DEPT ^{13}C NMR spectrum **(600 MHz in CDCl3) of compound (8)**



Appendix 13 The correlation of the protons in a COSY spectrum of compound **(8)**.



Appendix 14 HSQC spectrum of compound (8)



Appendix 15 HMBC correlation spectrum of the compound (8).