University of Strathclyde

Strathclyde Institute of Pharmacy and Biomedical Science

A STUDY OF THE CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITY OF PROPOLIS OF VARYING GEOGRAPHIC ORIGIN

A thesis presented

By

WIAM FATHI MUSTAFA ALSIHERI

A thesis submitted in accordance with the regulations governing the award of degree of Master of Philosophy in pharmaceutical sciences

2010

ACKNOWLEDGMENT

First of all, I thank God for giving me the strength to complete this work.

I would like to express my great thanks and sincere appreciation acknowledgement to my supervisor Dr. Dave Watson for his precious guidance, supervision and motivation and invaluable encouragement in my research and his valuable advice and help in all my work.

I would like to express my special grateful thanks to my second supervisor Prof. Alexander Gray for his invaluable advice and expert comments in NMR analysis and interpretation of results. I would like to express my sincere appreciation to my husband Mr. Omar Alghmasy for his support, encouragement and help for always pushing me to perform at my best in my life. I could not have finished this degree without you. Thank you for always being there to listen, encourage and give continual support. Special thanks also go to my children for their unending patience.

I would like to show my appreciation to Dr.Ruan Edrada-Ebel for her help, advice and support in NMR interpretation. I am also obliged to thank Mrs. Carol Clements for her help and support in the biological analysis of my samples and all the colleagues and technicians in room 307 in SIPBS who have made my time there a delight. A special mention goes to my devoted parents who have made me the person I am today and have always supported me and prayed for my success from abroad. I wish you a lifetime of good health and prosperity.

Finally, I would like to thank the General People's Committee for High Education through the Faculty of Pharmacy Al-Fateh University Tripoli-Libya for funding this project.

ABSTRACT

Propolis is a resinous substance produced by honey bees from various plant sources. The present research focused on the chemical and biological study of ethanolic extracts of 22 different propolis samples obtained from different geographical regions. The samples were investigated initially by high performance liquid chromatography (HPLC) in order to see if they contained any of the common marker compounds used to assess the quality of propolis. The antimicrobial activity of the propolis samples was evaluated against some pathogenic microorganisms including *Staphylococcus aureus*, *Escherichia coli*, *Nocardia farcinica*, *Mycobacterium aurum* and *Trypanosoma brucei*. The cytotoxicity of the propolis samples was also determined. After preliminary screening, flash chromatography was used for chemical fractionation of two propolis samples S107 (Bulgaria) and S108 (China) which had been found to be active in the screens and the fractions were re-investigated for their anti-microbial and cytotoxic activity. S107(F6) and S107(F7) showed stronger antimicrobial activities than the crude samples. S107 and S108 were found to be typical temperate propolis samples containing the marker compounds caffeic acid, pinobanksin, cinnamic acid, chrysin, pinocemberin and galangin and esters of these compounds. Cytotoxicity assays were carried out on the fractions obtained from S107 and S108. Promising cytotoxic activities were found for S107(F9) reduced cell proliferation to 15% of the control against human foreskin cells. Towards the end of the study the propolis sample S263 originating from the Solomon Islands (tropical zone), was found to display promising activity against *S.areus, M. aurum* and *T.brucei.* This sample was found to be composed mainly of terpenoid components according to gas chromatography-mass spectrometry (GC-MS).

LIST OF CONTENTS

LIST OF FIGURES

Number *Pages*

LIST OF TABLES

Number *Pages*

ABBREVIATIONS

Abbreviations	*Definition*
1D ^{1}H NMR	One Dimensional Proton NMR Spectra
APCI-MS	Atmospheric pressure chemical ionization, ion trap–mass spectroscopy
MeCN	Acetonitrile
CAPE	Caffeic acid phenyl ester
CC	Column Chromatography
DME	Dulbecco' S Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
EEP	Ethanol extracted propolis
EI-MS	Electron Impact Mass Spectrometry
ESI-MS	Electro Spray Ionization Mass Spectrometry
EtOAc	Ethyl Acetate
FC	Flash Chromatography
GC	Gas Chromatography
GCMS	Gas Chromatography Mass Spectrometry
HPLC	High Performance Liquid Chromatography
LC	Liquid Chromatography
LCMS	Liquid Chromatography Mass Spectrometry
MeOH	Methanol
MHB	Muller Hinton Broth
MIC	Minimum Inhibition Concentration
MS	Mass Spectrometry
NaCl	Sodium Chloride
NMR	Nuclear Magnetic Resonance
NP	Normal phase chromatography
RP	Reverse Phase

TIC	Total Ion Current
TLC	Thin Layer Chromatography
UV	Ultra Violet
v/v	Volume Per Volume
w/v	Weight Per Volume
WHO	World Health Organization

1 Introduction

1.1 Biology of Propolis

Propolis, honey and royal jelly are bee products; that have been used for centuries in foods and in traditional medicine. These preparations contain biologically active constituents, such as flavonoids, as major components and have been used by physicians and healers in the treatment of human illnesses across the world. These products have recently been receiving increasing attention from the general population and researchers because of the health claims associated with them [1-3].

Propolis (bee glue) is a resinous natural substance that honeybees collect from different plant exudates and use it to fill the gaps and to seal parts of the hive [4]. The word propolis originates from the Greek *pro* meaning in front of and *polis* (the city) which relates to the defence of the hive. During the collection of propolis, 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 [5].

The composition of raw propolis varies with the plant source in general; it is composed of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% of various other substances [6]. Propolis contains a variety of chemical compounds such as polyphenols, (flavonoids aglycones, phenolic acids and their esters, fatty acids and terpenoids [7]. Propolis provides a chemical weapon for bees against pathogenic microorganisms. The first reports on the use of propolis in folk medicine were around 300 B.C [8]. Current medicines containing propolis include over the counter preparations such as for colds and flu, and dermatological preparations for the treatment of boils, herpes simplex infections, and neurodermatitis [9-12].

Propolis possesses strong antibacterial, antifungal, antiviral and antioxidant activities due to its content of bioactive materials [13]. Propolis has also been used as an astringent and a spasmolytic agent. It has been used in the treatment of Chagas disease, wounds, burns, neurodermatitis, leg ulcer and herpes simplex infections. Propolis has been used as in oral medicine in the treatment of dental problems and in toothpaste and mouthwash preparations as well as for the treatment of gingivitis, cheilitis and stomatitis [14]. Propolis is often defined by its colour and has different colours ranging from light and dark brown to creamy, Figure 1-1. There are some samples which have green colour such as Brazilian green propolis. The colour depends on the place where samples were collected and the age of the propolis. Additionally the texture of propolis samples differs from one sample to another. Some samples are sticky and some are hard and others are fragile. Propolis is also hard and brittle when cold and becomes soft and sticky when it is warm [5, 15, 16]. Propolis is considered a fascinating subject for study since the composition of propolis depends on the time of collection and the exact place of collection [5, 17, 18].

Some recent studies have focused on Brazilian propolis since it was reported that it has potent biological activity against different microorganisms that cause serious pathological conditions. The plant origin of Brazilian propolis has not been clarified, since there are no Poplar trees in Brazil, which are the main source of European propolis in Brazil. Recently it was reported that the plants of *Baccharis dracunculifolia*. DC (Compositae) is an important botanical source of proplis in southeastern Brazilian [17]. Researchers have specified more than 12 types of Brazilian propolis according to its geographical origin, chemical composition and plant sources [19].

(A)

www.naturaletz.com/_img/ft_propolis.gif

(B)

www.soorganic.com/blog/a-propolis-buzz-705.ht

(C)

http://www.mofaid.com/an/images/propolis.jpg

(D)

http://commons.wikimedia.org/wiki/File:Propolis

(E)

www.made-in-china.com/image/2f0j00PBsaQLdGJRgKM/Propolis.jpg

Figure 1-1 Different propolis morphology and colours.
(A). Light brown hard propolis, (**B).** Yellow hard propolis, **(C).** Fragile brown propolis, **(D).** Dark brown propolis, **(E).** Black hard propolis.

The most popular and widely studied Brazilian propolis is green propolis or Alecrim which originates from *Bacharis dracunculifolia* [20]. The red propolis collected from the other regions of Brazil such as in the Northern region has a red colour and its plant source was identified as *Clusia nerrorsa* (Clusiaceace) [11, 19, 21]. It was confirmed that triterpene alcohols are typical constituents of Brazilian propolis [19], Recent studies confirmed the fact that propolis, independently of its plant source and chemical composition, always possesses antimicrobial and antioxidant activities [22, 23].

1.2 Chemistry of Propolis

Propels has variable components and can contain up to 200 constituents [15]. 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, flavanones, dihydroflavonol and chalcones, Figure 1-3. Research on Brazilian propolis found that extracts of propolis contained 0.25% (w/w) of flavonoids and 0.5% (w/w) of wax and inactive materials in relation to the dry weight. It was observed that volatile oils in propolis have moderate antimicrobial activity and it was found that in tropical samples the volatile oil fraction is more important for antimicrobial activity than in samples from Europe.[24].

1.2.1 Flavonoids and Phenolics Occurring in Propolis and some of their biological effects

Flavonoids are a group of hetero cyclic 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 of the plant kingdom. Flavonoids consist of two benzene rings connected via pyran

oxygen containing heterocyclic ring (figure 1.2). The variation in the heterocyclic ring gives rise to flavonols (2-phenyl-3-hydroxy-chromones), flavones (2-phenyl-flavononones), flavan (2-phenyl-3-hydro-hydroxy-chromones)and flavanols, the structures of these are shown in Figure 1-3 [25]. They also occur in propolis and honey.

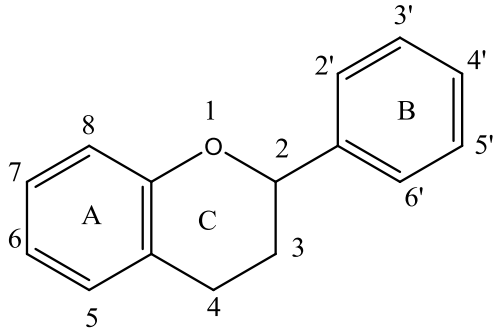


Figure 1-2 Flavan structure.

The types of flavonoids can be classified in accordance to the substitutions on rings A, B and C on the main nucleus of general flavonoids which is the flavan skeleton. Flavones have a substitution on rings A and B, but there are no oxygens at C3 e.g. chrysin, while flavonols are chemically called 3 – hydroxyflavans e.g. galangin. Flavanones can be described by the absence of the C2-C3 double bond with no hydroxyl substitutions at C3 of the flavan structure e.g. pinocembrin and naringenin. Pinobanksin is an example of a flavanone with hydroxyl substitution at C3 of flavan skeleton. There are other types of flavonoids e.g. prenylated flavonoids, which is class of flavonoids which have strong biological activities. C-prenylation is more common than o-prenylation of flavonoids. Prenylation involves the addition of a hydrophobic group to the organic molecule, which enhances the its biological activities by increasing its ability to pass through biological cell membranes [26]. Extensive studies were carried out on prenylated flavonoids in correlation to the biological activities [9, 27]. Figure 1-3 shows the marker compounds used to identify and control the quality of propolis.

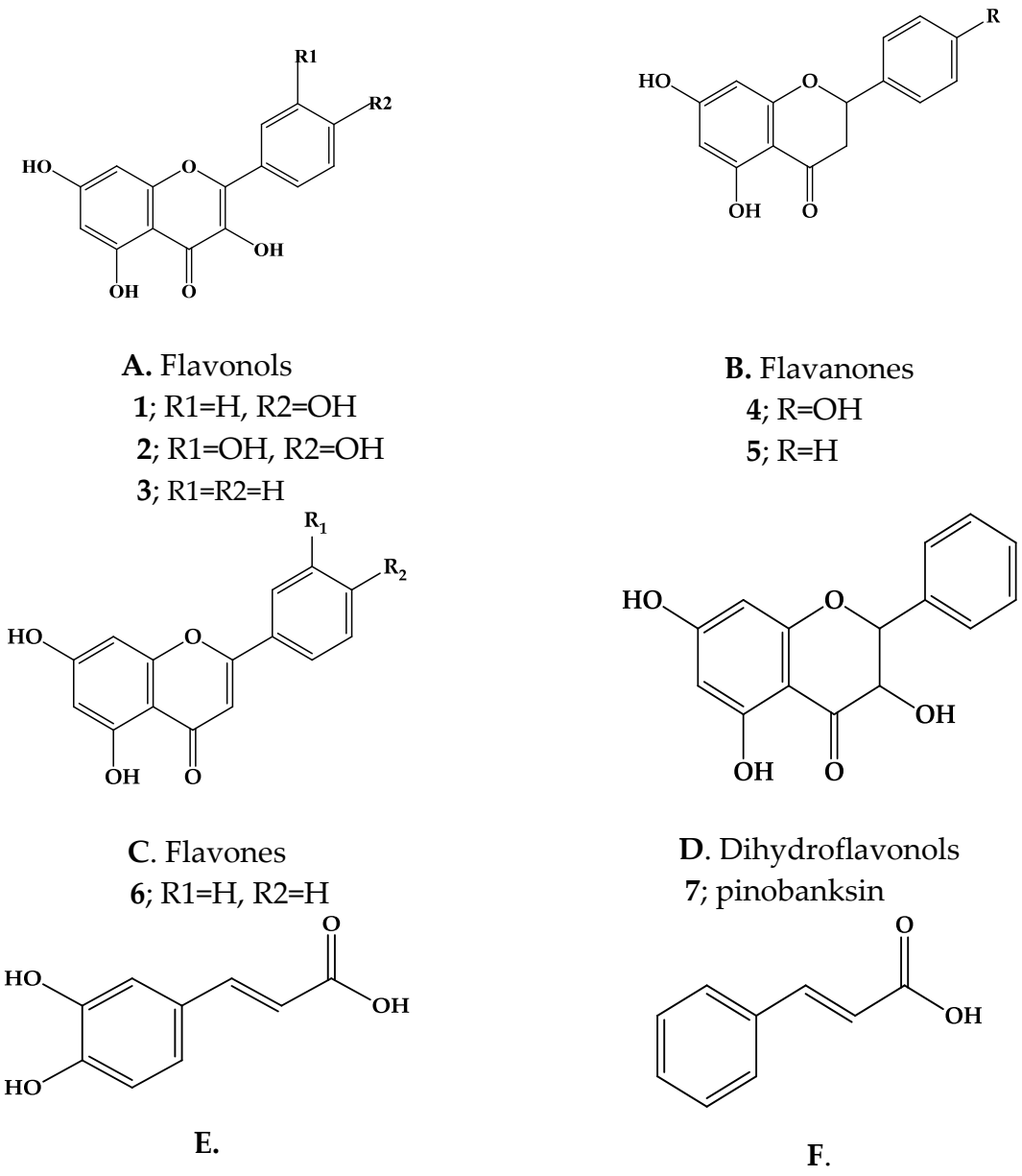
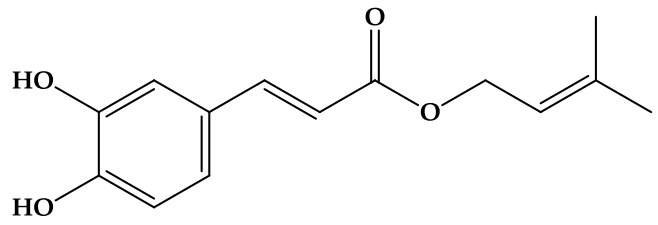


Figure 1-3 Chemical structures of flavonoids in present propolis.
A1. *Kaempferol,* **A2**. *Quercetin,* **A3**. *Galangin,* **B4**. *Naringenin,* **B5**. *Pinocembrin,* **C6**.*Chrysin,* **D7**. *Pinobanksin*, **E**. *Caffeic acid,* **F**. *Cinnamic acid.*

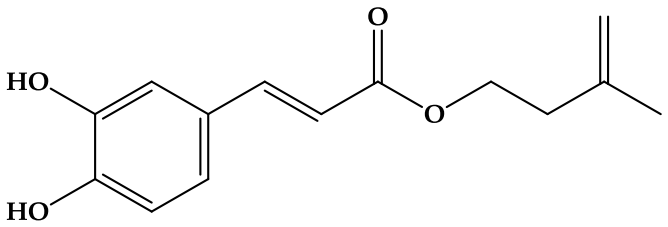
Among the marker compounds galangin (3, 5, 7 trihydroxyflavone), (Figure 1-3 (A3) has been found to have strong antibacterial and antifungal activity. Studies have concluded that galangin has antibacterial activity against Gram-positive *S.aureus* by increasing potassium loss through damaging the cytoplasmic membrane of the bacteria or by autolysis of the cell wall which leads to osmotic lysis [2]. Caffeic acid phenyl ester Figure 1-4 (C) is present in propolis as an active component and is one of the most important components which exert anti-oxidant and anti-inflammatory

activity in humans. Several studies were carried out on the effects of CAPE using a rat model by inducing CCl_4 kidney damage. These studies found that CAPE protected the kidney from the damage [28-30].

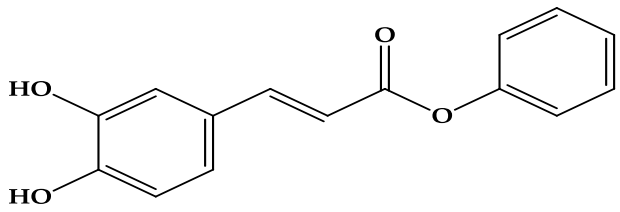
A study also found that CAPE and propolis components had the ability to penetrate, *in vitro*, the porcine buccal mucosa in and this indicated that propolis might be used in oral diseases because of its broad anti-microbial and anti-inflammatory effects in addition to its analgesic effects [31]. Tests were carried out on propolis samples from two different locations in Turkey. The activity of extracts of propolis was attributed to the presence of the caffeic acid esters, 3,3 dimethylallyl caffeate, Figure 1-4 (A) and isopent-3-enyl caffeate, Figure 1-4 (B) [29, 32].



(A)



(B)



(C)

Figure 1-4 Chemical structures of **A**. 3, 3'dimethylallyl caffeate, **B**. isopent-3-enyl caffeate and **C**. CAPE.

1.2.2 Terpenoids in propolis and some of their biological effects

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. Terpenes are a unique group of hydrocarbon-based natural products and are volatile constituents of plant essential oils and as they are volatile terpenes cause the aroma of the plant [33]. Monoterpenes and sesquiterpenes are composed of two and three isoprene units respectively. Monoterpene alcohols such as terpieneol have antiseptic properties [34].

A study was carried out on Brazilian propolis collected by stingless bees and found a range of monoterpenes including β-pinene [35]. Another study was carried out on Turkish propolis and found that thymol, α-terpieneol and myrtenol were among the monoterpenes, the sample was found limited activity against *Mycobacterium tuberculosis* [36]. Sesquiterpenes are composed of three isoprene units; eudesmol is an example of a sesquiterpene alcohol, which has been observed mainly in propolis samples from the southeast of Brazilian [36]. On the other hand studies on propolis from Egypt showed the presence of triterpenoids such as oleane and ursane [37]. Although volatile compounds are found only in low concentration in propolis samples their aroma and biological activities make them important for characterization of propolis. From the literature it was observed that the presence of the terpenoids in propolis can act with flavonoids in order to enhance biological activities [3]. Triterpenes such as lupeol and cycloartinol acid have antimicrobial activities, in addition, α-amyrin (Figure 1-5 (6)) is one of the most abundant triterpenes in propolis samples from Egypt [33]. Figure 1-5 shows some of the terpenoids which have been found to occur in propolis.

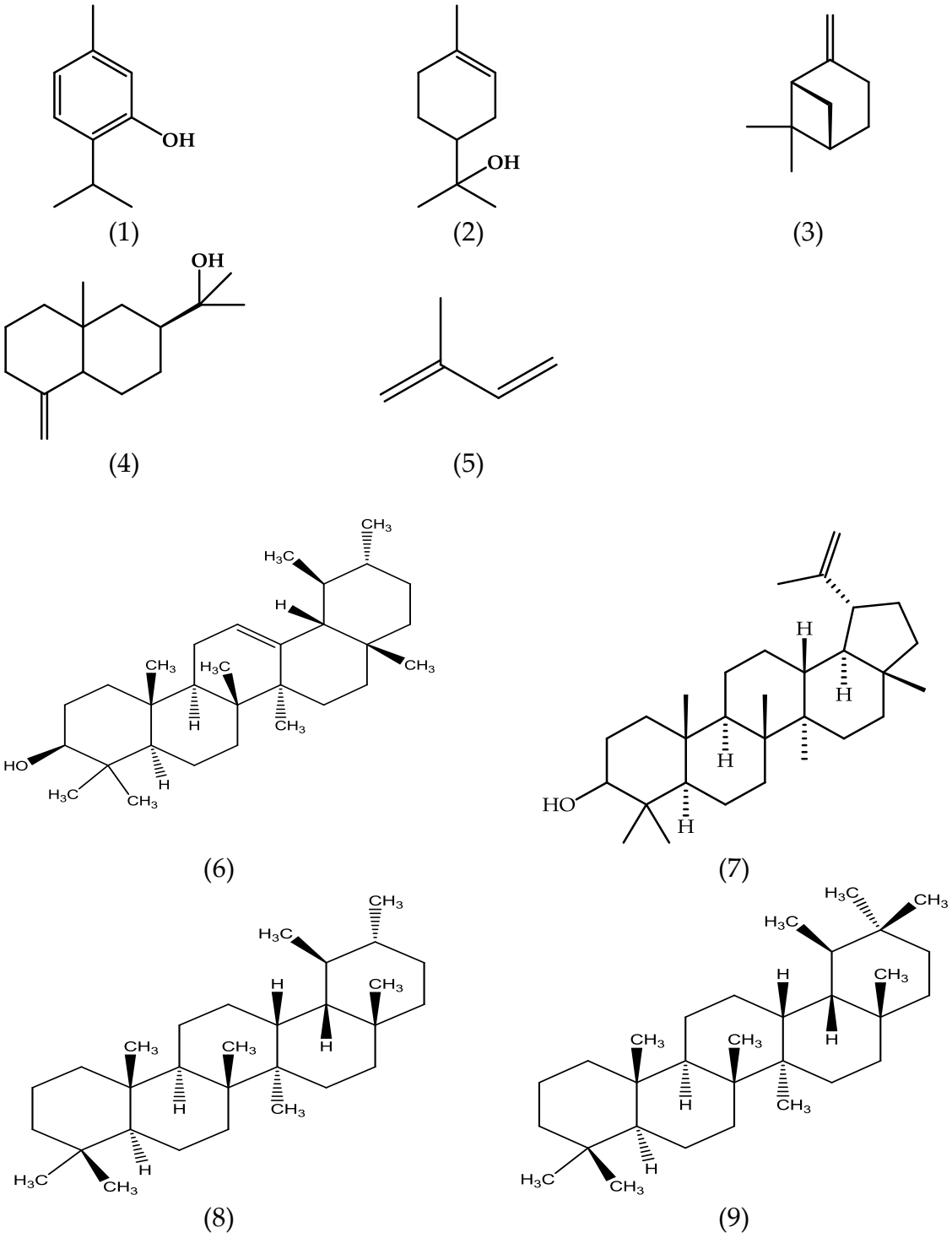


Figure 1-5 Chemical structures of terpen

1. Thymol, **2**. Terpineol, **3.** β-Pinene, **4** Eudesmol, **5.** Isoprenyl unit, **6**. β-amyrins, **7.** Lupeol, **8**. Ursane, **9.** Oleanane

1.3 Extraction of propolis

Raw propolis is considered to be difficult to use in cosmetics, foods or medicine unless it passes through various purification steps which extract materials such as wax, so the remaining extract is rich with the flavonoids that are often responsible for the biological activity of propolis [38, 39]

Before it can be analysed propolis should be extracted with solvents because the raw material cannot be analyzed due to presence of waxy materials. The extraction process aims to remove waxy materials and to preserve the polyphenolic fractions. Extraction with 80% ethanol is particularly suitable for obtaining dewaxed propolis extracts which is rich in polyphenolic materials [38]. Ethanol is the most widely used solvent in extraction of propolis among the previous and recent literature. The use of ethanol of 70% and 80% concentration in water for the extraction is suitable for obtaining dewaxed propolis and extracts rich in phenolic compounds [40]

Although different concentrations of ethanol have been used such as 95% and absolute ethanol, extraction with aqueous ethanol 70% and 80% has been found to give the best results. Extraction with aqueous ethanol results in wax free tinctures containing higher amounts of phenolic substances

Such extracts give highest absorption for flavonoids measured at 290 nm [4, 5, 12, 40-42]. Studies were carried out comparing water extracted propolis (WEP), propolis volatile fraction (PV) and ethanolic extract (EEP) [43]. It was found that the water ethanolic extract had the weakest biological activity, while the ethanolic extract of propolis had the highest activity [22, 25, 44-47].

1.4 Methods of analysis of propolis samples

Analysis of propolis has been carried out by the use of several instrumental and chemical techniques. These include high performance liquid chromatography (HPLC), mass spectrometry (MS) and liquid chromatography mass spectrometry (LCMS). Due to variations in the

chemical compositions of propolis, it is difficult to evaluate propolis from different geographical regions by a single instrumental technique.

Hence different chemical techniques have been used. For instance LCMS is useful in the determination of the different flavonoid components of propolis. It is considered to be versatile as the most reliable analytical technique in the quality control of different propolis samples [32].

GCMS 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 [9, 10, 38, 48, 49]. 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. Thin layer chromatography (TLC) and capillary electrophoresis (CE) have been used for determination of phenolic constituents in propolis. In addition atmospheric pressure chemical ionization, ion trap–mass spectrometry (APCI-MS), has been used, which allows typical fingerprints of complex natural materials [50].

1.5 Biological activity of propolis

Propolis has been used in folk medicine since ancient times and used in the treatment different diseases. Recently research has been carried out to investigate the activities of propolis samples and correlate the activities to the composition of different samples [11]. Despite the variations of the components of propolis, it has therapeutic value in treatment of different diseases caused by microorganisms, and also has cytotoxic activities [15, 51-53].

1.5.1 Antibacterial activity of propolis

It was confirmed that there was a strong linear relationship between total phenol content and the measured antibacterial activity of propolis. Although

considerable research has been devoted to the antibacterial activity of propolis against Gram-positive and Gram-negative bacteria, it has been found that activity is more against Gram-positive bacteria than Gram-negative bacteria. Flavonoids present in propolis such as pinocembrin, galangin and caffeic acid and its phenyl esters may work by the inhibition of bacterial RNA polymerase [15, 28, 54]. Other groups of researchers found that galangin had antibacterial action which involved the degradation of the cytoplasmic membrane of bacteria which led to a loss of potassium ions by cell autolysis [15]. Different methods have been used to investigate the anti-microbial activity of propolis samples including bioautography, agar dilution, agar diffusion and broth dilution. These methods give results which cannot be correlated for example results obtained from a disc diffusion assay cannot be correlated with the MIC data obtained from the broth dilution method [18].

The most common methods used to assess antibacterial activity are the diffusion and dilution methods, but these have limitations since they require the preparation of many agar plates. The diffusion method is affected directly by the solubility of constituents in the agar plates. Overall the broth micro dilution method which uses serial dilution in tubes is considered more suitable for comparing propolis extracts. Recently the broth micro dilution has been chosen to assess the microbial activity of propolis and it is considered as the method of choice for rapid and simultaneous screening of the multiple propolis samples in many studies [41, 54].

In one study samples of different geographical regions prepared as ethanolic extracts were tested using the broth micro dilution method. The results showed that some propolis samples possessed significant activity against Gram-positive bacteria but this activity is varied with the location of the propolis samples. The antibacterial activity of propolis samples was high for those samples originating from a collection place characterised by a wet-tropical rainforest type climate [1, 5, 18]. In another study Brazilian samples

were found to be active against oral bacteria, including: *Actinomycetem comitans* which is a Gram-negative bacterium associated with variety of diseases such as brain abscess and urinary tract infections. *Fusobacterium nucleatum,* which belongs to human micro biota which is found in the oral cavity, colon, genital tract and upper respiratory tract and *Porphyromonas gingivalis* and *Prevotella intermedia*, species of Gram-negative bacteria associated with destructive periodontitis. These strains of bacteria have resistance to different antibiotics. The antimicrobial activity of propolis constituents can be attributed to the synergistic effects of several phenolic compounds in the propolis extracts and this may lead in future to the use propolis as alternative treatment for these pathogens [7, 15, 25, 28, 52]. Several studies showed potential antibacterial and antifungal, activities of propolis on oral pathogens such as *C.albicans*, without any toxic effects on the gingival fibroblasts [2, 30, 54, 55].

1.5.2 Anti-Trypanosomal activity of Propolis

The activity of propolis against Chagas disease (caused by *Trypanosoma cruzi*) was assessed in comparison with crystal violet as a standard drug recommended to prevent the transmission of Chagas disease via blood transfusion [56]. Bulgarian propolis from Burgas Bur (Southeast Bulgaria) and from Lovetech Lov (West of Bulgaria) were studied. Four extracts , Hex-Bur, Ket-Bur Et.Bur and Et.lov were prepared by using different extraction solvents [57]. Stock solutions of the different extracts were prepared in DMSO and were tested against *T.cruzi*. The controls used were untreated and crystal violet treated parasites. Additionally, commercial North American propolis was tested. It was prepared in ethanol (Et.Sg) and Brazilian propolis (from Parana) which was prepared in methanol (Met-Pr) [58]. Et-Bur was richer in fatty acids and flavonoids, while Ket-Bur showed a high level of monosaccharides, glycerol and caffeic acid. In Met-Pr, the main constituents were monosaccharides and disaccharides. The non polar Hex-

Bur extract showed the presence of *n*-alkenes, *n*-alcohols and two series of wax acid esters. The results of the tests with the extracts against *T.cruzi* were significant. Ket-Bur was the most active extract against epimastigotes while Et-Bur and Et-Lov showed similar activities against each form of the parasite. The activity of each Bulgarian extract was differently affected by the presence of blood. For the Et-Bur extract no substantial interference was observed while for Et.Lov and Ket.Bur extracts a decrease of about two times was observed in the lytic effect against trypomastigotes when the assay was performed in the presence of blood [58, 59]. Additionally, the activity of Et.Bur was similar to that of the standard drug which is crystal violet. Inactivation of anti-trypanocidal activity by blood components has been previously described in studies with naphthoquinones and gossypol.

Met. Bur showed 7-10 times lower activity against *T.cruzi* in comparison with ethanol and acetone extracts. This was explained as being due to the chemical composition of the methanolic extract which has a huge abundance of monosaccharides and disaccharides, therefore implying a lower concentration of the active compounds.

Bulgarian propolis is rich in flavonoids which are considered as the main components of temperate zone propolis. In contrast the Brazilian samples have other classes of bioactive compounds, for instance, prenylated acetophenones and specific terpenoids with a wide range of antimicrobial activities. This result is enhanced by the fact that there were the differences in the trypanocidal activities according to the origin of the propolis samples [59]. Other studies confirmed that Brazilian green propolis has activity against *T.cruzi* [20, 56, 59].

1.6 Cytotoxic effect of propolis

Propolis has been found to have antitumor effects in both *in vivo* and *in vitro* studies [60]. CAPE isolated from propolis showed cytostatic effects on different types of cells but showed particularly strong effects on human cells

[29]. In addition other studies confirmed the cytostatic activity of the compounds [14, 61]. Several studies have confirmed that ethanol extracted propolis (EEP) has anticancer activity in animal models [22]. For instance in comparisons with other anticancer drugs such as bleomycin on mice infected with Erlich carcinoma, the rate of survival mice after around 55 days was 50% in the group treated with propolis and 44% in other group treated with bleomycin [10, 47]. A study was carried out using aqueous and alcoholic extracts of propolis against the cancer cells. All extracts were found to have limited *in vitro* cytotoxic and cytostatic effects against certain cancer cells and propolis extracts also slowed down the development of tumours in a mouse model [22]. Fractions of Brazilian propolis showed that it had limited cytotoxic effects on cancer cells, arresting tumour development at certain stages but having little effect on human diploid cells [23, 52].

Many reports showed the effectiveness of some propolis derivatives of CAPE in which the numbers of tumours in the lungs of mice were reduced by both preventative and curative therapy with CAPE and with less effect in the case of caffeic acid only [16, 62, 63].

1.7 Other biological activities

Studies have reported that propolis samples from Bulgaria and Brazil show activity as anti-leishmanial agents due to presence of flavonoids and amyrins [62]. Propolis also shows significant antioxidant activities because of its high content of polyphenolic compounds [21, 51]. Moreover, a study on Brazilian propolis demonstrated that a propolis displayed good anti-ulcer activity [64]. Thus the biological properties of propolis remain a suitable topic for study and the availability of a range of uninvestigated propolis samples of varying geographic origin provided by Bee Vital led to the current work.

1.8 Project Aims

Since propolis has been shown to have a variety of interesting and useful biological properties it was proposed to investigate a range of propolis samples of varying geographical origin for their biological activity. The propolis samples would be screened for anti-microbial and cytotoxic activities. In parallel with this the composition of these samples would be investigated by HPLC in order to characterise them with regard to their content of standard marker compounds. Having identified biologically active samples the samples would be fractionated by flash chromatography in order to enrich their biological activity and the sub-fractions screened for their activity. The chemical composition of the propolis fractions would be investigated using LC-MS, GC-MS and NMR. The ultimate goal would be the isolation of new anti-microbial or cytostatic compounds.

2 Material and Methods

2.1 Propolis samples

Nature's Laboratory (Whitby, N.Yorks) provided propolis samples from different geographical locations in the world. Sample S108 was from China, S107 from Bulgaria and S263 from Solomon Islands. The rest were collected from different geographical regions across the world but information on the origin of these was not available. These included: S240, S242, S243, S244, S245, S246, S248, S251, S252, S253, S254, S257, S259, S260, S261, S264 and S269. Propolis samples were stored at room temperature in the dark until required for analysis.

2.2 Materials and equipment

0.22 μm syringe filter, Millipore, UK

1.5 ml centrifuge tubes (Elkay, UK)

15 ml sterile centrifuge tubes (Greiner Bio-one, UK)

20 ml universal centrifuge tubes (Greiner Bio-one, UK)

5 ml glass vials (Kinesis Ltd,UK)

Absolute ethanol (Fisher Scientific, UK)

ACE C_{18} column (4.6x 150mm x5μm) (Hichrom, UK).

Alamarblue™ (Serotec, UK)

Anti-bumping granules (BDH, UK)

Automatic pipettes (Gilson, Anachem,UK)

Bench top Centrifuge (Heraeus instruments Labofuge 400) (Thermo scientific, UK)

Blue tip pipette (Star Lab, UK)

Caffeic acid (Sigma Aldrich, UK)

Chloroform (Fisher Chemicals, UK)

Chrysin (Sigma Aldrich, UK)

Cinnamic acid (Sigma Aldrich, UK)

Dimethylsulfoxide (DMSO, Sigma Aldrich, UK)

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

Ethanol (Sigma Aldrich,UK)

HPLC grade ethyl Acetate (Fisher Scientific, UK)

Ethyl acetate (Sigma Aldrich, UK)

Flash chromatography column (Isolute Flash SiII) (Biotage, Sweden)

Flash chromatography columns C_{18} reverse phase 200 gm (Biotage, Sweden)

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

Galangin (Sigma Aldrich, UK)

Hexane (HPLC grade) (Fisher Scientific, UK)

HPLC vials (Kinesis Ltd,UK)

M. aurum (ATCC SIP164482)(Fisher Scientific, UK)

Methanol (Sigma Aldrich, UK)

Methanol-d_4, 99.8+atom %D (Sigma-Aldrich, USA)

N.farcinica (ATCC 3318) (Fisher Scientific, UK)

Naringenin (Sigma Aldrich, UK)

Nylon filter membrane disc (Nylasorb™, USA)

Pasteur pipette (VWR International Lutterworth,UK)

Pinocembrin (Sigma Aldrich, UK)

Pre-coated plate, Silica Gel 60 F_{254} layer thickness 0.2mm (Sigma Aldrich, UK)

Rotary evaporator (Büchi, Switzerland)

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

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

Sonicator (Ultrasonic bath) (Banson 2510, USA)

Syringe filters (Acrodisc, Fisher Scientific,UK)

Syringes (Becton Dickinson, UK)

T.brucei (ATCC S427) blood stream form)(Fisher Scientific, UK)

Test tubes (Kinesis Cambridgeshire, UK)

TLC aluminium sheets 5x7.5 cm, RP-18 F_{254} (Sigma Aldrich, UK)

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

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

2.3 Biological activity assays

2.3.1 Anti-mycobacterial activity of propolis samples

A modification of the micro plate AlamarBlue™ (REDOX indicator) method for susceptibility testing of fast growing species of *Mycobacterium*. The tests were carried out with technical assistance according to standard protocols [65].

2.3.2 Antibacterial activity of propolis samples

The tests were carried out with technical assistance according to standard protocols using *S.aureus* and *E.coli* as the test organisms [66].

2.3.3 Anti-Trypanosome activity

The activity of propolis samples extracts and fractions against African Trypanosomes was determined *in vitro* using an AlamarBlue™ assay.

The tests were carried out with technical assistance according to standard protocols [67].

2.3.4 Cytotoxic activity tests

The cytotoxicity of crude propolis extracts and fractions was determined by using 96-well plates with the Alamarblue™. The tests were carried out with technical assistance according to standard protocols.[61] Table 2-1 summarises the types of cells that were used in the cytotoxicity assay. .

Table 2-1 Types of cells used in cytotoxicity assay.

HS27	Fibroblast	Human foreskin	Normal cells
L929	Fibroblast	Mouse, CH3/An connective tissue, areola and adipose	Normal cells
ZR75	Epithelial	Breast cancer cells	Cancer cells

2.4 Chromatographic methods

Flash chromatography was used to sub-fractionate biologically active propolis samples on a preparative scale. HPLC analysis was used in order to characterise propolis samples with respect to their content of standard marker compounds.

2.4.1 Normal phase Flash chromatography (NPFC)

NPFC was carried out using silica gel cartridges (20 g). A sample of propolis S263 (200 mg) was dissolved in 30 ml of hexane, ethyl acetate (1:1) by ground with silica gel (1 g) in a mortar and pestle until it was dispersed after it had dried the sample was loaded onto the top of the flash chromatography column. Isocratic elution with hexane: ethyl acetate, (95:5) was carried out to a volume of 1000 ml. The cartridge was filled and saturated with the mobile phase just prior to the sample loading.

2.4.2 Reverse phase Flash chromatography (RPFC)

Samples of propolis S108, S107 were fractionated by using RPFC with a C_{18} (20 g). Samples of propolis (200 mg) were dissolved in methanol (30 ml) and then dispersed in silica gel using a mortar and pestle to grind the sample until dissolved. The sample extract was then loaded onto a cartridge. A step gradient was carried out as shown in Tables 2-2 and 2-3. The cartridge was filled and saturated with the mobile phase just prior to the sample loading. The final propolis samples S107 and S108 fractions collected were labelled starting from F5, F6, F7, F8, F9 and F10. All solvents were HPLC grade the fractions were dried and used in chemical and biological assays as in Table 2-2 for sample S107 and Table 2-3 for sample S108.

Table 2-2 Fractions collected and mobile phases used in RPFC of propolis sample S107.

Fraction	Methanol (%)	Water (%)	Mobile phase volume (ml)
S107(F5)	50	50	200
S107(F6)	60	40	200
S107(F7)	70	30	200
S107(F8)	80	20	200
S107(F9)	90	10	200
S107(F10)	100	0	200

Table 2-3 Fractions collected and mobile phases used in RPFC of propolis sample S108.

Fraction	Methanol (%)	Water (%)	Mobile phase volume (ml)
S108(F5)	50	50	200
S108(F6)	60	40	200
S108(F7)	70	30	200
S108(F8)	80	20	200
S108(F9)	90	10	200
S108(F10)	100	0	200

The eluent was collected in pre-weighed round bottom flasks. Fractions were dried by using a rotary evaporator and then the dried residue in the flasks was weighed and then submitted for ^{1}HNMR in (section 2.4.7).

2.4.3 High Performance Liquid Chromatography (HPLC)

2.4.3.1 Preparation of solutions of marker compounds

Standards of marker compounds (50 mg) were placed in separate 50 ml volumetric flasks and 50 ml HPLC grade methanol added. The samples were shaken well and put into a sonicator for 5 min, giving a final concentration of 1mg/ml. Each stock solution of standards was diluted to 0.1 mg/ml with methanol: water (30:70), by diluting 1ml of stock solution in a 10 ml volumetric flask with methanol: water (30:70).

2.4.3.2 Selection of mobile phase conditions

The chromatographic mobile phase conditions were first selected by optimising them by using DryLab® software using the retention times of the marker compounds. The mobile phase consisted of filtered 0.1% (v/v) formic

acid in H_2O filtered through a nylon filter (Whatman, U.K.) and 0.1% (v/v) formic acid in methanol with gradient elution as shown in Table 2-4.

The auto sampler was set to inject 10 µl; peak detection was performed using a variable wavelength UV set at 290 and 350 nm an AS 3000 auto sampler and a P2000 pump. The system was fitted with an ACE5 C_{18} column and the oven temperature was 40˚C. Chromequest software was used to analyse the data.

Table 2-4 Chromatographic mobile phase conditions used for analysis of propolis samples.

Time (min)	A%(0.1% v/v formic acid in H_2O)	B% (0.1 % v/v formic acid in methanol)	Flow rate (ml/min)
0	46	54	1
20	46	54	1
35	0	100	1
40	0	100	1

A; aqueous phase and B; organic phase

2.4.3.3 Preparation of calibration curves for the standards

The standards used were: caffeic acid, cinnamic acid, naringenin, pinocembrin, chrysin and galangin. In order to create calibration curves 5 ml amounts of all standard solutions were transferred to a 200 ml volumetric flask and the mixture diluted with mobile phase to obtain the following concentrations, 0.02, 0.04, 0.05, .0.06, 0.08 and 0.1 mg/ml.

2.4.4 Preparation of samples for (HPLC) and liquid chromatography mass spectroscopy (LCMS)

Propolis samples (40 mg) were weighed into screw cap test tubes and then dispersed in absolute ethanol (3 ml). The sample was extracted by sonication at 70˚C for 60 min and then centrifuged for 30min. at 3500 rpm. The ethanolic solution (2 ml) was removed and 1ml of HPLC grade water mixed with it. The supernatant (1 ml) was diluted to 10 ml with the mobile phase (46% solvent A and 54% solvent B). All samples were analysed in duplicate. A 10 min. equilibration time was used between runs.

2.4.5 Gas Liquid Chromatography Mass Spectroscopy (GCMS)

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 to 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 MD 800 MS operated in electron impact mode at 70eV. The propolis sample was weighed (3 mg) and 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.

2.4.6 Liquid chromatography mass spectrometry (LCMS)

HPLC/ESI-MS was carried out using an LCQ-DECA (Thermo Finnegan) mass spectrometer in series with diode array UV detector and fitted with an Agilent 1100 pump. The propolis samples and fractions were dissolved in water/methanol mixtures and injected into the instrument. The instrument conditions are shown in Table 2-5. Xcalibur was used as the software to analyse the HPLC MS data. The marker compounds were analysed under the same conditions.

Table 2-5 LC/UV/MS system specifications.

HPLC system	**Agilent 1100 series pump, detector and auto sampler `**
Mass spectrometer	Finnegan MAT TSQ mass spectrometer equipped with TSP ASP pump and vacuum degasser
Column	ACE C_{18} column (4.6x150mm x5μm)
Oven temperature	40°C
Mobile phase	**A**. 0.1% v/v formic acid in H_2O **B.** 0.1% v/v formic acid in MeOH

2.4.7 Nuclear Magnetic Resonance Spectroscopy (NMR)

All one dimensional (1D) samples were obtained using Delta™ NMR software. Known amounts samples of propolis, fractions and marker

compounds (2-8 mg) were dissolved in 0.75 ml DMSO d6 in 4 ml sample tubes and transferred carefully to NMR tubes to be measured.

The observed chemical shift δ values were obtained in ppm and the coupling constant (J) in Hz. Spectra were referenced to residual solvent protons. ^{1}HNMR spectra were measured at a magnetic field strength of 400.13 MHz using a JEOL Delta GX 400 MHz FT NMR spectrometer. ^{1}HNMR spectra were initially measured for each sample while a ^{13}C NMR spectrum was obtained when further investigation of the structure of the compound was needed. ChemBioDraw Ultra, Version 11, was used to draw compound structures, and also to predict ^{1}H NMR data.

3 Results

3.1 Testing of Antibacterial activity

In order to narrow down the range of propolis samples to be investigated biological testing was carried out to identify those with the strongest biological activity. This testing was carried out in parallel with the analysis of the marker compounds by HPLC carried described in (section 3.2.)

3.1.1 General screening of propolis samples from different geographical regions against *S.aureus*.

Figure 3-1 shows the results of the screening of propolis samples from different geographic origins in comparison with the positive control gentamicin. Some of the samples have high activity against *S.aureus*. S263 shows very high activity (0-10% of the control). Propolis samples S108 and S107 gave high activity (10-30% of the control) 17.5 and 25.5% of the control. S251 had moderate activity (30-50% of the control) of 31.9% of the control. S260 and the other samples can be considered as having low activity (50-90% of the control) or being inactive. S108 (China) and S107 (Bulgaria) displayed high antibacterial activity against *S.aureus,* were available in good supply and thus were selected for further study and were fractionated using flash chromatography. They had similar activity although they were from different geographical origins.

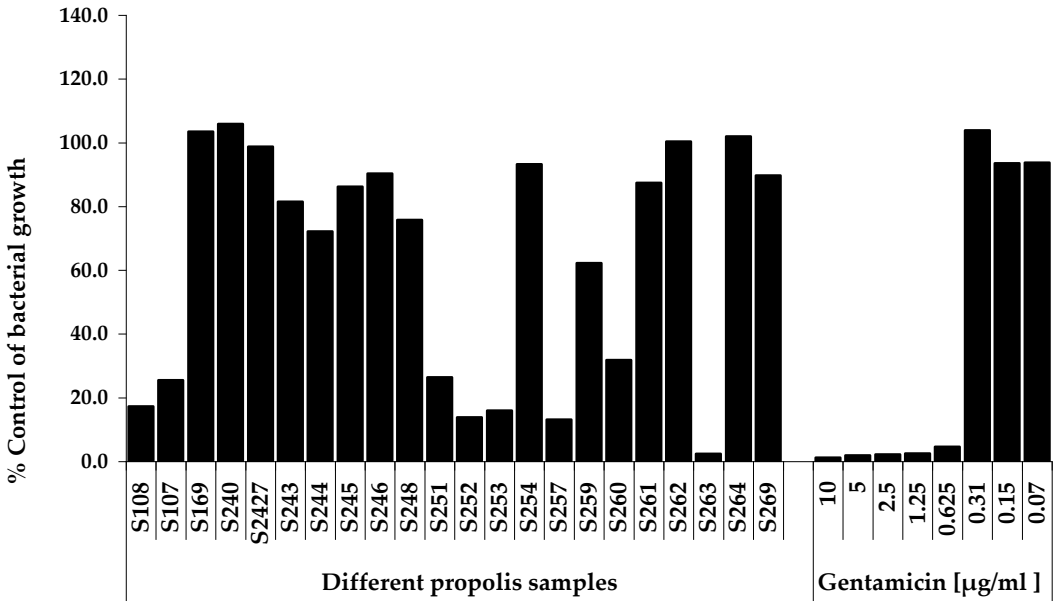


Figure 3-1 General screening of antibacterial assay of propolis samples from different geographical regions against *S.aureus.* An AlamarBlue™ susceptibility testing of ethanolic extract of propolis samples (200 µg/ml in DMSO) from different geographical origins against *S.aureus*. Results are represented as the percentage of the control of bacterial growth (n=1). Gentamicin (0.07-10 µg/ml) is the positive control. Plates were read at 530 nm and 590 nm.

3.1.2 Antibacterial activity of propolis sample S108 and its fractions against *S.aureus*.

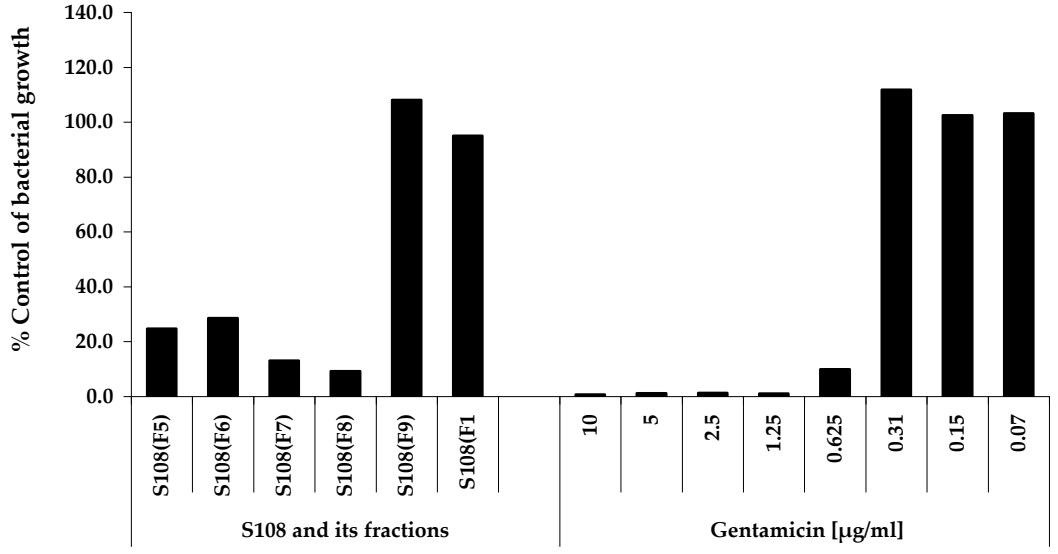


Figure 3-2 Antibacterial activity of propolis sample S108 and its fractions against *S.aureus.* The AlamarBlue™ susceptibility testing of ethanolic extract of propolis sample S108 and its fractions (F5,F6,F7,F8,and F9) (200 µg/ml in DMSO) against *S.aureus*. Results are represented as the percentage of the control of bacterial growth (n=1). Gentamicin (0.07-10 µg/ml) is the positive control. Plates were read at 530 nm and 590 nm.

Figure 3-2 shows the antibacterial activity of the S108 and its fractions at 200μg/ml. S108(F8) and S108(F7) gave 9.2 and 13%, respectively and are very highly active against *S.aureus,* while S108(F5) and S108(F6) gave 24.7 and 28% of control respectively and can be considered as highly active against *S.aureus* and fractions S108(F9) and S108(F10) were inactive. In view of the high activity of the fractions it was decided that the dose response of fractions would be investigated. Figure 3-3 shows the effect of S108 and its fractions against *S.aureus* in a dilution series S108 shows high activity down to a concentration of 125 μg/ml. Below this concentration it is not effective. All of the fractions display similar activity to the unfractionated materials, apart fraction S108(F8) which is more active than the crude S108 at 500 and 250 μg/ml but less active at 125 μg/ml/. Fraction S108(F7) is slightly more active than the crude S108 but it is inactive at 125 μg/ml.

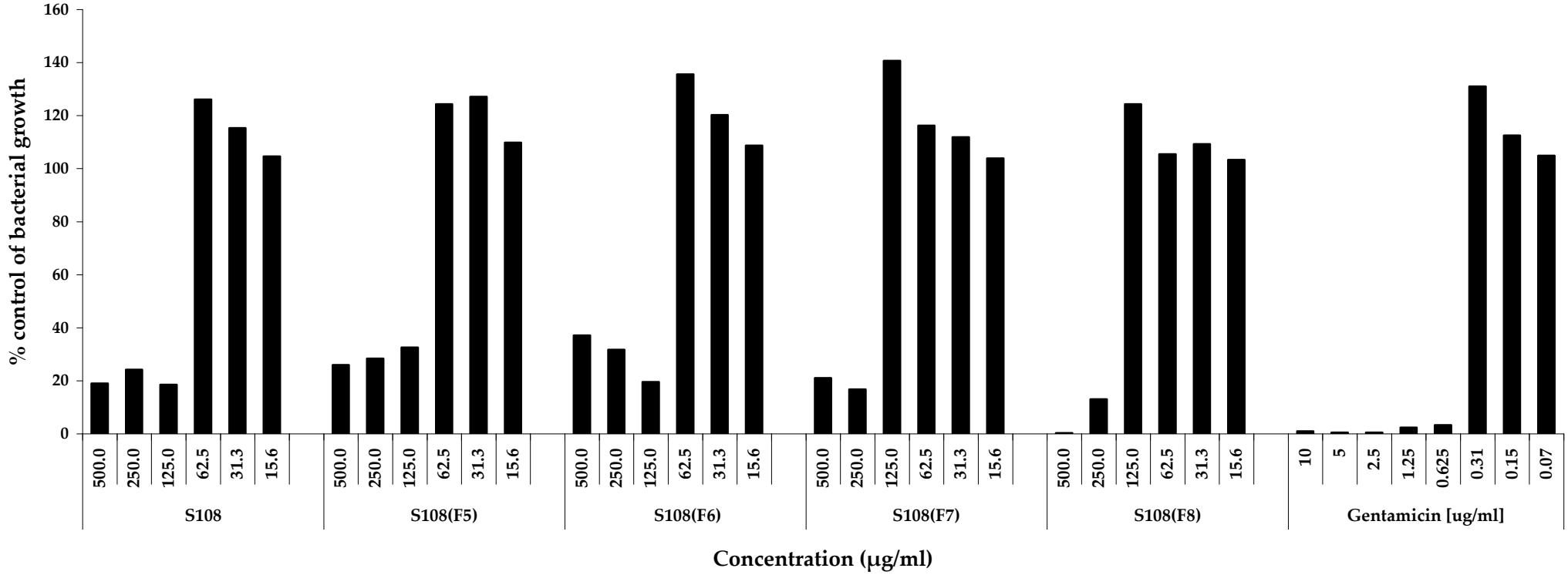


Figure 3-3 MIC values of fractions of S108 against *S.aureus*. The MIC Assay of ethanolic extract of propolis sample S108 and its fractions (F5, F6, F7, F8, and F9) in dilution series against *S.aurus*. Results are represented as percentage control of bacterial growth (n=1). Gentamicin (0.07-10 μg/ml) as the positive control and DMSO as solvent control. Plates were read at 530 nm and 590 nm**.**

3.1.3 Antibacterial activity of the fractions obtained from propolis sample S107 against *S.aureus*

Figure 3-4 shows the antibacterial activity of S107 fractions against *S.aureus.* Fraction S107 (F7) is the most active giving inhibition of 6% compared with the control value at a concentration of 200 μg/ml. Fraction S107 (F6) is also highly active giving inhibition equal to 23% of the control value. The other fractions were more or less inactive. In conclusion samples S107 and S108 show promising activity against *S.aureus.*

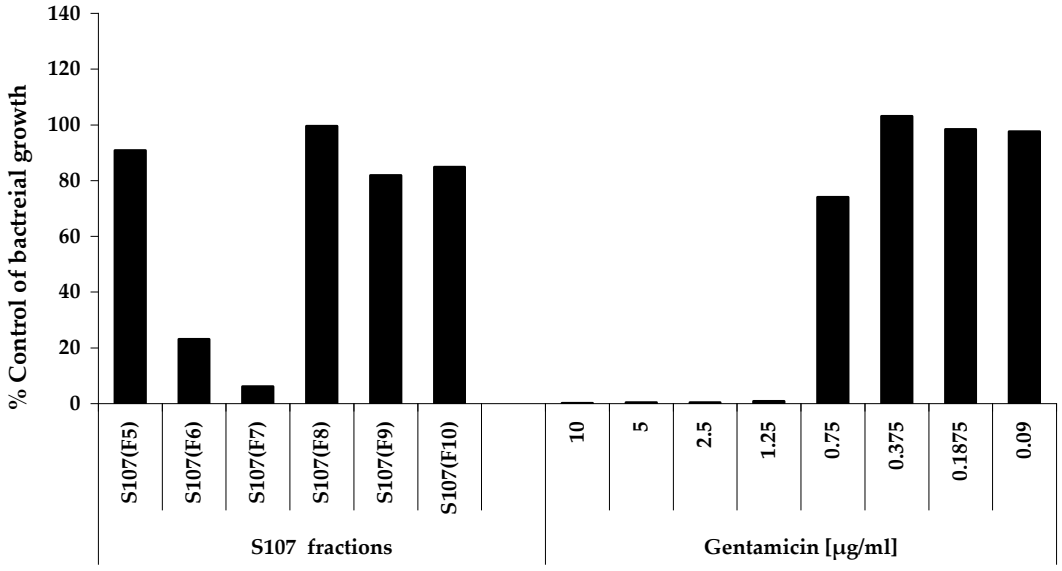


Figure 3-4 Antibacterial activity of the fractions propolis sample S107 against *S.aureus.* The AlamarBlue™ susceptibility testing of ethanolic extract of propolis sample S107 and its fractions (F5, F6, F7, F8, F9 and F10) (200μg/ml in DMSO) against *S.aureus*. Results are represented as percentage control of bacterial growth (n=1). Gentamicin (0.07-10 μg/ml) is the positive control. Plates were read at 530 nm and 590 nm.

3.1.4 General screening of propolis samples from different geographical regions against *E.coli*

Figure 3-5 shows the results of the screening of propolis samples against the Gram-negative organism *E.coli* using a concentration of 500 μg/ml. None of the samples were found to have appreciable activity against *E.coli.*

The fractions of S107 and S108 were also tested against *E.coli* and there was found to be no increase in anti-bacterial activity as a result of fractionation. Figure 3-6 and similarly Figure 3-7 show the results for the fractions from S108 where again there was no appreciable activity against *E.coli* at a concentration of 500 μg/ml apart from in S108(F5) and S107 (F9) where was some weak activity.

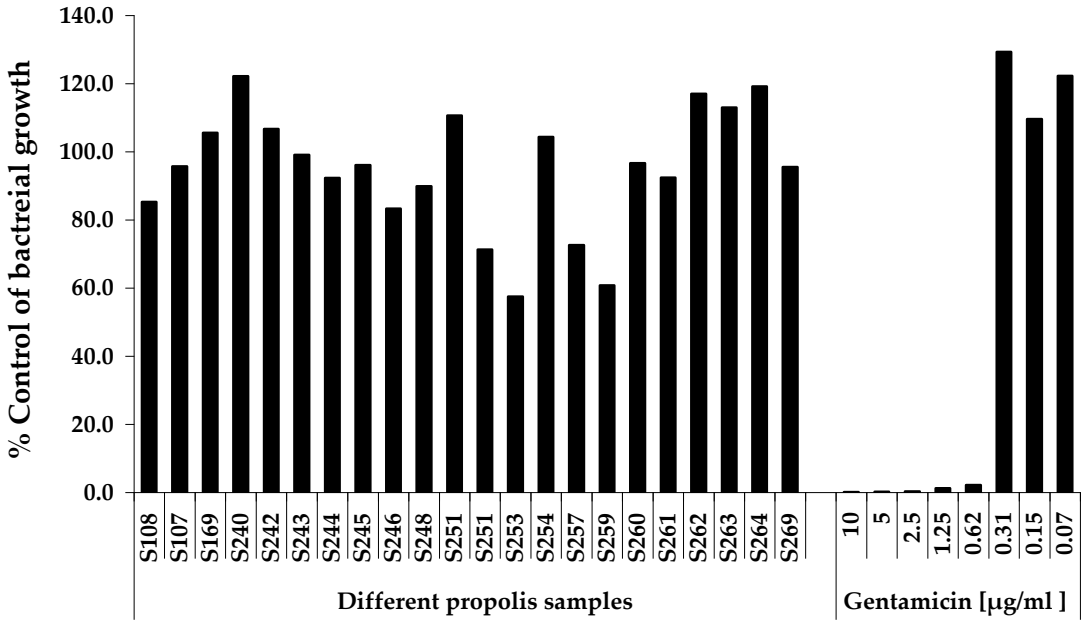


Figure 3-5 General screening for propolis samples from different geographical regions against *E.coli*. The AlamarBlueTM susceptibility testing of ethanolic extract of propolis samples (500 μg/ml in DMSO) from different geographical origins against E.coli. Results are represented as the percentage of the control of bacterial growth (n=1). Gentamicin (0.07-10 μg/ml) is the positive control. Plates were read at 530 nm and 590 nm.

3.1.5 Antibacterial activity of the fractions of propolis S108 against *E.coli*

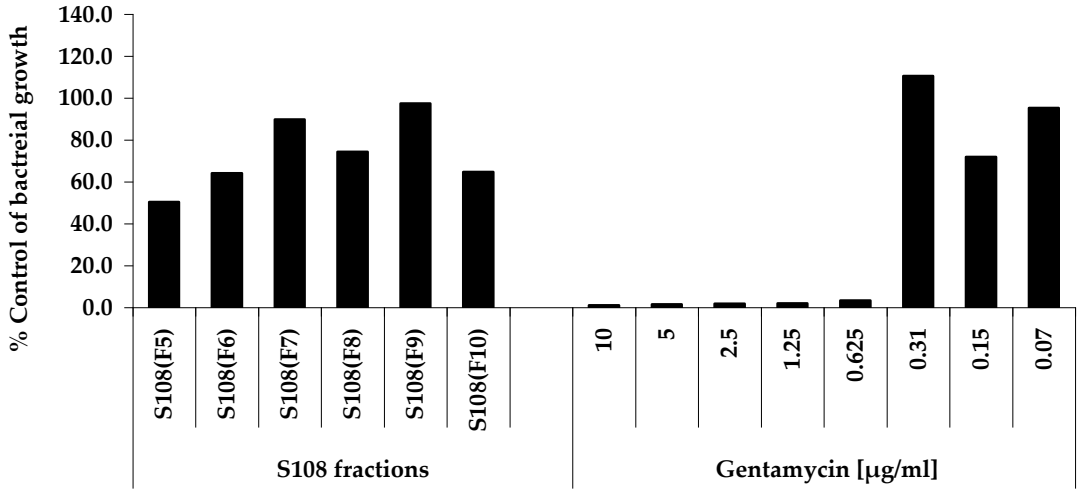


Figure 3-6 Antibacterial activity of the fractions of propolis sample S108 against *E. coli.* The AlamarBlue™ susceptibility testing of ethanolic extract of propolis sample S108 and its fractions (F5, F6, F7, F8, F9 and F10) (500 µg/ml in DMSO) against *E.coli*. Results are represented is the percentage of the control of bacterial growth (n=1). Gentamicin (0.07-10 µg/ml) is the positive control. Plates were read at 530 nm and 590 nm.

3.1.6 Antibacterial activity of the fractions of propolis sample S107 against *E.coli*.

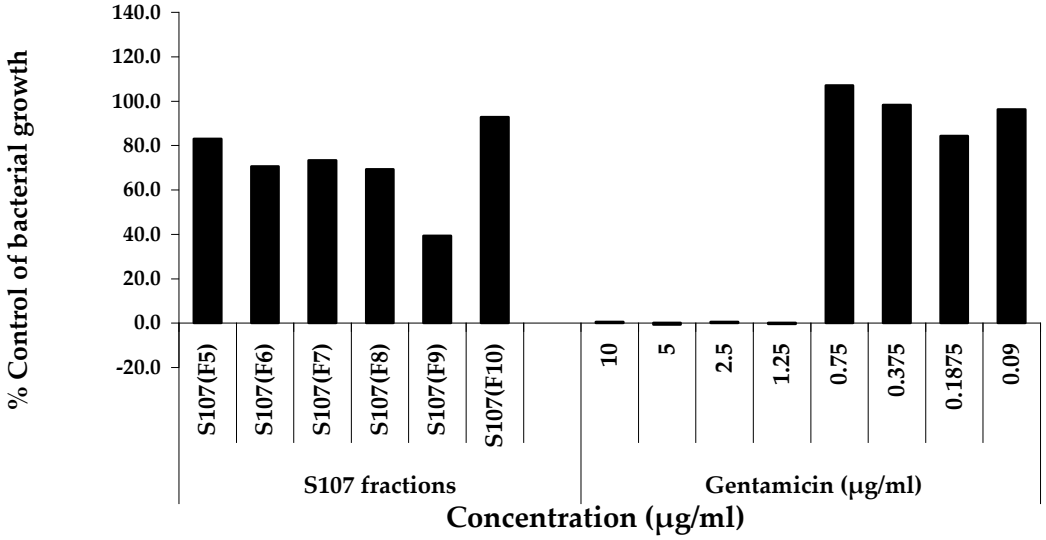


Figure 3-7 Antibacterial activity of the fractions of propolis sample S107 against *E.coli*. The AlamarBlue™ susceptibility testing of ethanolic extract of propolis sample S107 and its fractions (F5, F6, F7, F8, F9 and F10) (500 µg/ml in DMSO) against *E.coli*. Results are represented as percentage control of bacterial growth (n=1), Compared with gentamicin (100 µg/ml) as positive control. Plates were read at 530 nm and 590 nm

3.2 General screening for propolis samples from different geographical regions against *Mycobacterium aurum*

Some of the propolis samples of different geographical origin were screened against *M.aurum* Figure 3-8 shows the results which indicated that samples S108, S107, S251, S252, S257, S260 and S263 were active while S253 was not. The greatest activity was found in S263 which was completely inhibitory to a level of 31.3μg/ml and lost its effect completely below this level. S263 was much more active than the positive control ethambutol. S107 was also moderately active against *M.aurum* at concentration of 62.5 μg/ml. S108 displayed similar activity to S107. The concentration range of the tested samples was 1-500 μg/ml but only up to 15.6 μg/ml is shown and DMSO was not toxic as in Figure 3-8

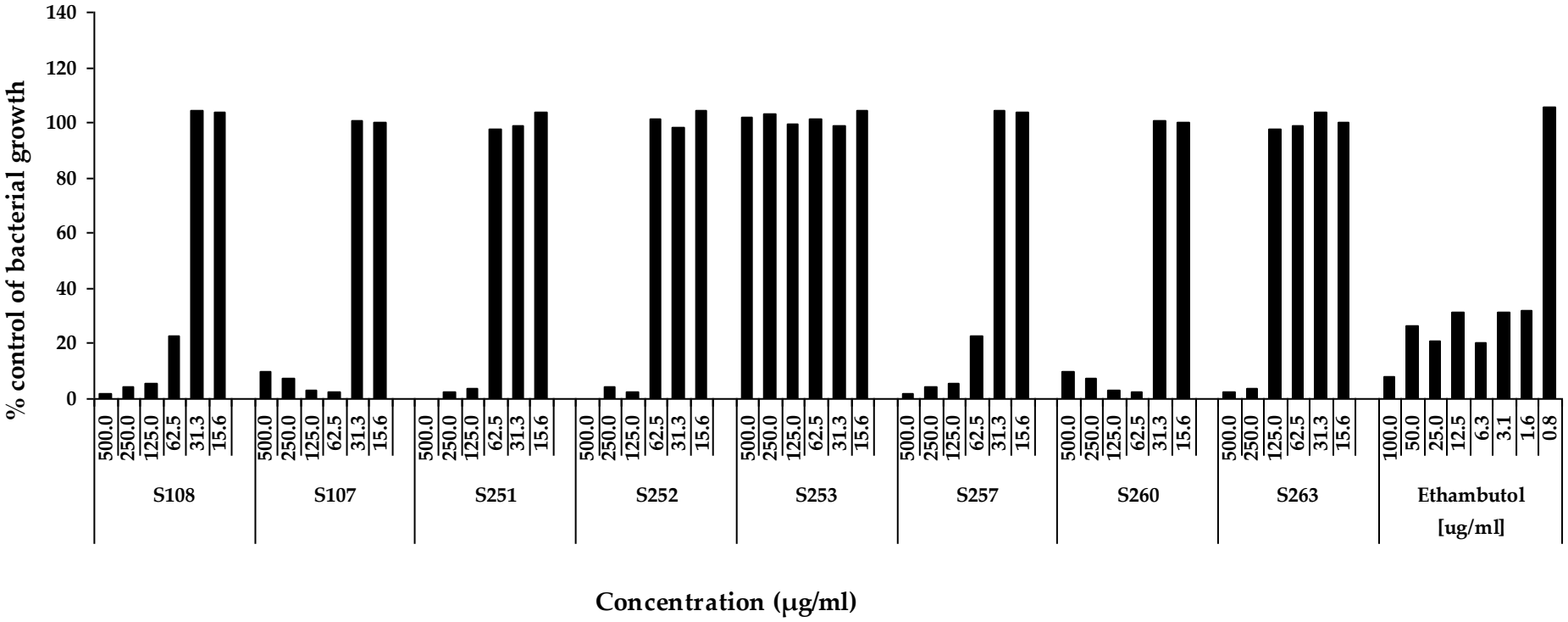


Figure 3-8 General screening for propolis samples from different geographical regions against *M. aurum.* The MIC assay of ethanolic extract of propolis samples from different geographical origins against *M.aurum*. Results are represented as the percentage of the control of bacterial growth (n=1). Compared with positive control ethambutol ug/ml and DMSO as solvent control. Plates were read at 530 nm and 590 nm**.**

3.3 Anti-trypanosmal activity of propolis samples and fractions against Trypanosome brucei

Figure 3-9 and Figure 3-10 show the results for propolis samples S107 and 108, respectively and their fractions which were tested against *T.brucei* with suramin as the positive control. The concentration range of the tested samples was 0.2-100 μg/ml but only up to 0.4 μg/ml is shown and DMSO was not toxic. The results showed activity for both the crude samples and the fractions of S107 and S108. Both S107 and S108 samples were moderately active and the activity increased with dilution down to a level of 3.1 μg/ml suggesting that the solubility of the active component in the test medium improved with dilution with less likelihood of the sample self associating. Moreover S108 displayed strong activity at a concentration of 6.2 1 μg/ml. There was no marked improvement in activity in the fractions compared with the unfractionated sample apart from in fraction S108(F8). Therefore further purification of fraction S108(F8) might yield further increases in activity. Figure 3-10 shows the activity of the fractions of S107 against *T.brucei*. S107 was considered to have moderate activity against *T.brucei* at and at 12.5μg/ml gave inhibition at 13.7% of the control. Again there was no marked improvement in activity from fractionation.

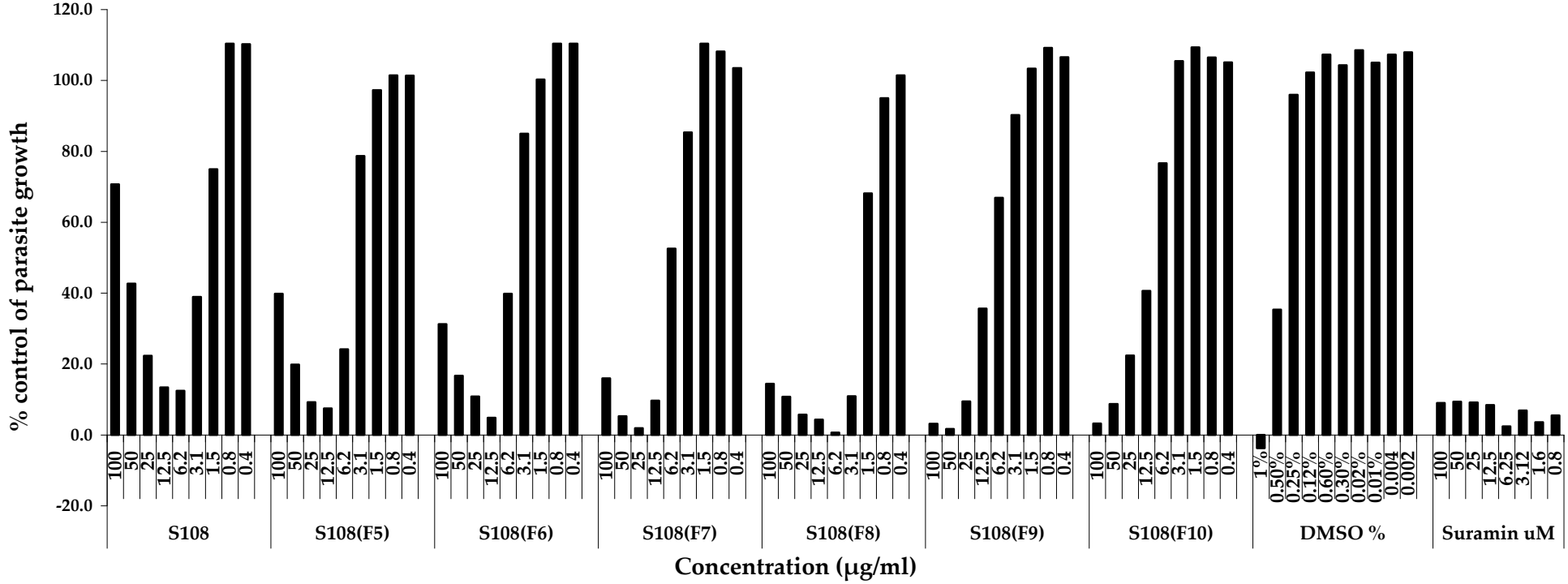


Figure 3-9 Anti-trypanosomal activity of propolis sample S108 and its fractions against *T.brucei*. The AlamarBlue™ susceptibility testing of ethanolic extract of propolis samples S108 and its fractions (F5, F6, F7, F8, F9 and F10) in dilution series *T.brucei*. Results are represented as percentage control of parasite growth (n=1). Suramin (100 µM) as a positive control and DMSO as solvent control. Plates were read at 530 nm and 590 nm.

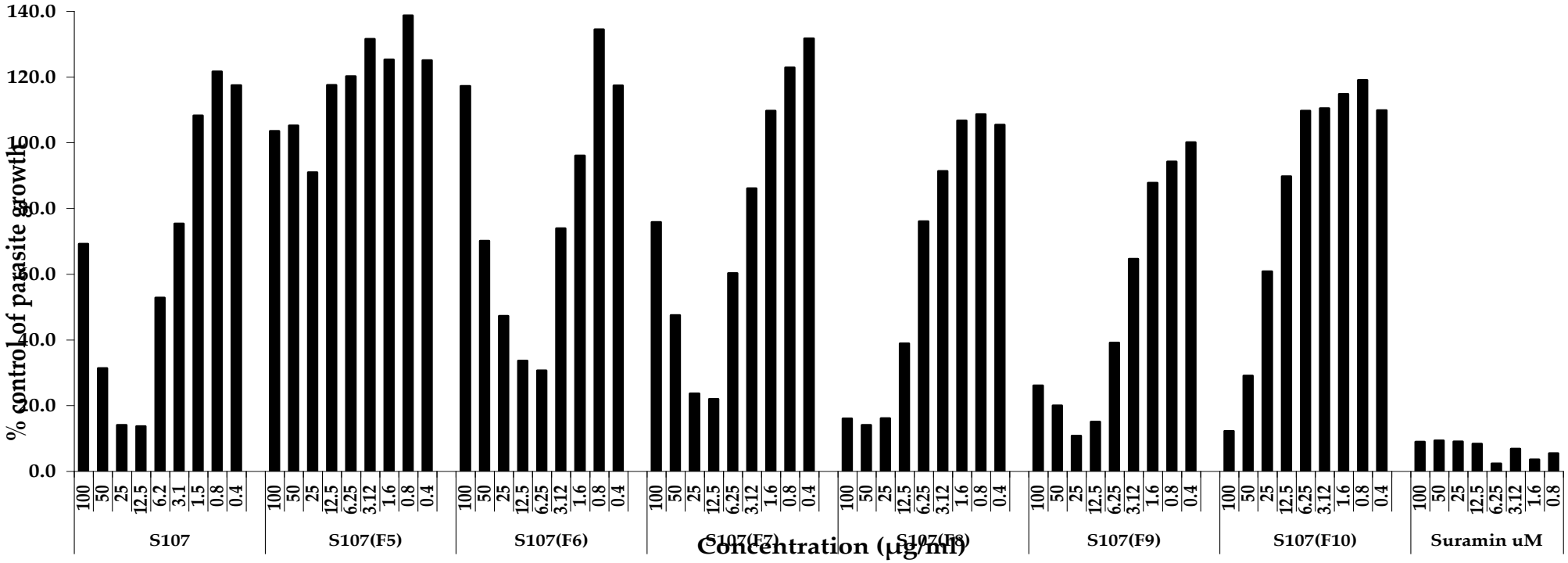


Figure 3-10 Anti-trypanosomal activity of propolis sample S107 and its fractions against *T.brucei*. The AlamarBlue™ susceptibility testing of ethanolic extracts of propolis sample S107 and its fractions (F5, F6, F7, F8, F9 and F10) in dilution series *T.brucei*. Results are represented as percentage control of parasite growth (n=1). Suramin (100 µM) as positive control and DMSO as solvent control. Plates were read at 530 nm and 590 nm.

3.4 Cytotoxicity activity

Three cell lines were studied HS27cells (fibroblast normal cells), L929 cells (fibroblast normal cells), and ZR75 cells (epithelial cancer cells). Figure 3-11 shows the results for propolis cytoxicity for the three cell lines. S108 did not have appreciable toxicity against the HS27 and the L929 cell lines at a concentration of 100 μg/ml, but it did display toxicity against ZR75 cells. S107 displayed some toxicity against all three cell lines (Figure 3-10).

Figure 3-12 shows the toxicity of the fractions of S108 against the three cell lines there was no increase in toxicity resulting from the fractionation of the sample. Figure 3-11 shows the results obtained for the toxicity of fractions from S107 against ZR75 cells. Fraction S107(F9) was quite strongly cytotoxic at a concentration of 100 μg/ml.

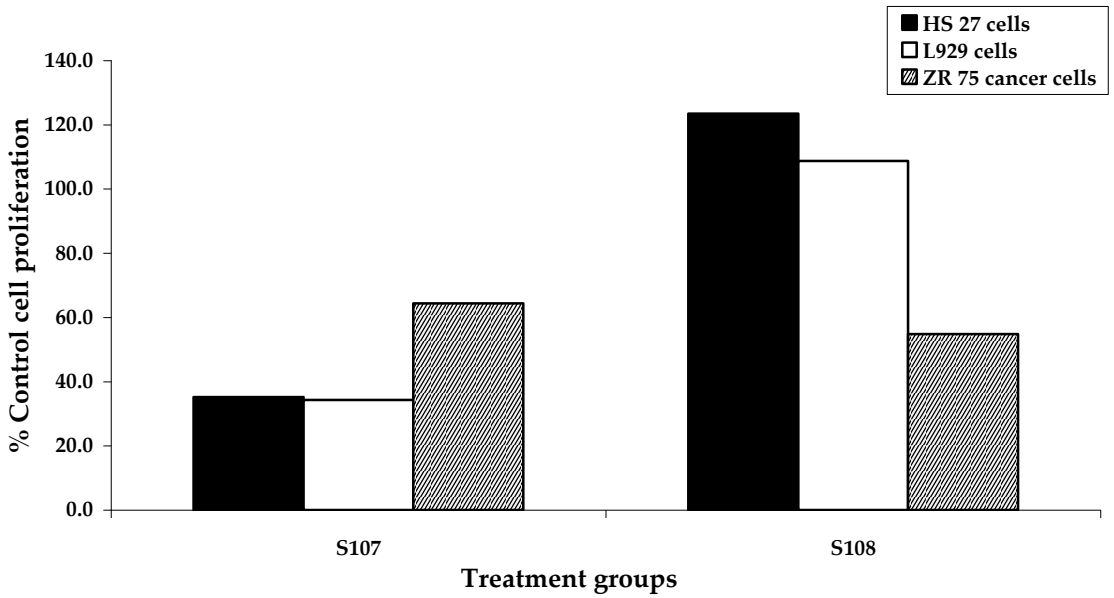


Figure 3-11 Comparison of cytotoxicity activity between propolis samples S107 and S108. (100 μg/ml). Three different cell types were tested (HS27, L929 and ZR75). Results are represented as percentage control of cell proliferation. Plates were read at 530 nm and 590 nm.

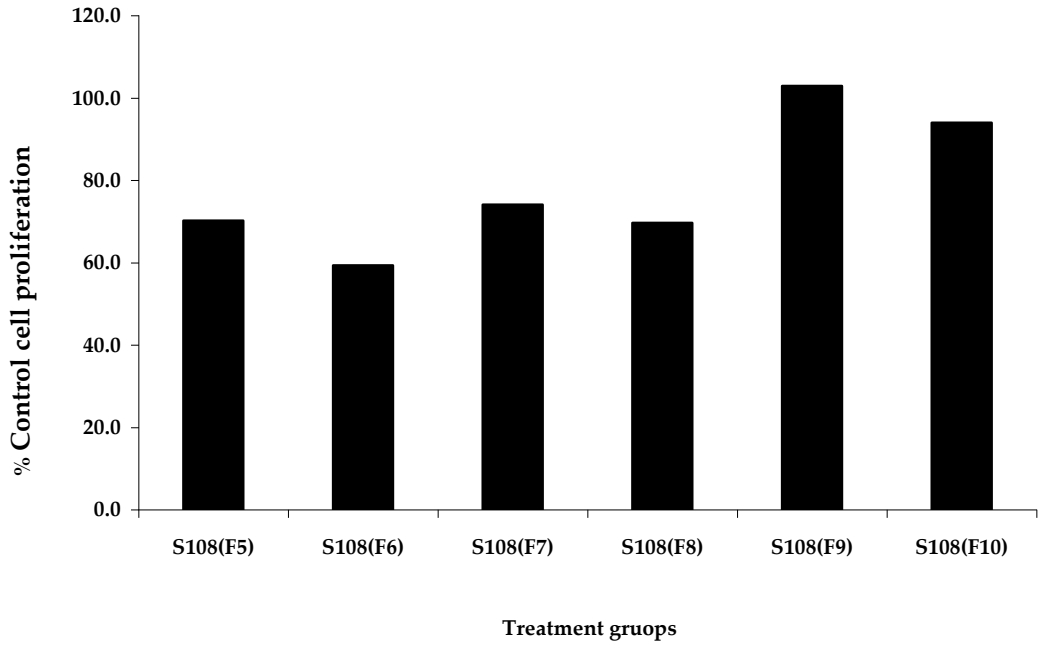


Figure 3-12 Cytotoxic activity of propolis samples S108 fractions. Cytotoxicity results of ethanolic extract propolis sample S108 and its fractions (100 μg/ml), cancer cell (ZR75) was tested Results are represented as percentage control of cell proliferation (n=1). Plates were read at 530 nm and 590 nm.

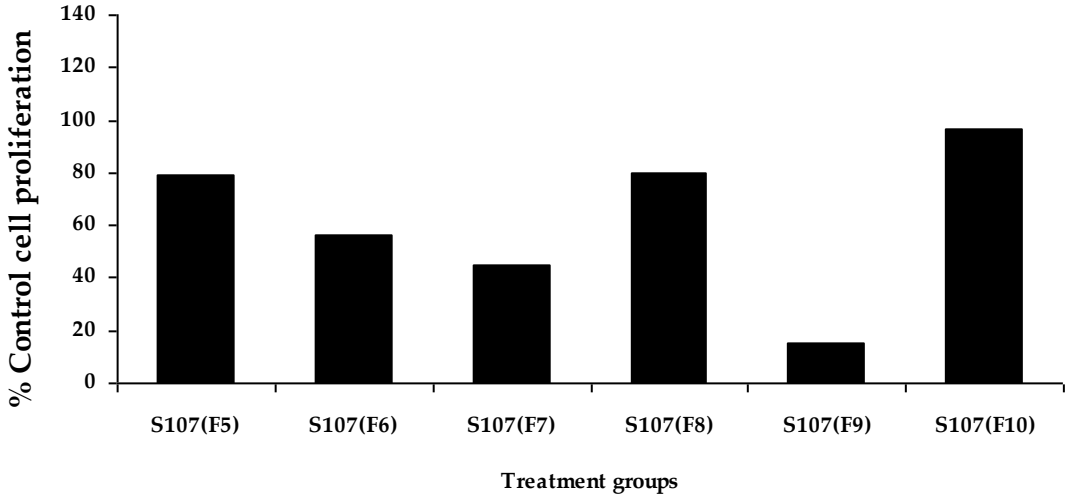


Figure 3-13 Cytotoxic activity of propolis sample S107 fractions. Cytotoxicity results of ethanolic extract propolis sample S107 and its fractions (100 μg/ml) cancer cell (ZR75) was tested Results are represented as percentage control of cell proliferation (n=1). Plates were read at 530 nm and 590 nm.

3.5 Chemical results

3.5.1 Optimisation of the HPLC analysis of the marker compounds in propolis

The general composition of propolis samples was investigated in parallel with the biological screening experiments. The previous method used for the analysis of the marker compounds in propolis [5] involved a run time up to 70min with many of the marker compounds eluting between 30 and 40 min. The method was shortened so that the marker compounds eluted within 20 min, by using Drylab® to model the chromatography. The prediction was based on two isocratic runs for the standards at 50% methanol / 50% 0.1% formic acid and 60% methanol /40% 0.1% formic acid. Drylab® predicted an optimum performance, while still preserving separation between caffeic and cinnamic acid, at 54% methanol/46% containing 0.1% v/v formic acid. The conditions predicted were practically applied in the HPLC method. Figure 3-1 shows the chromatogram obtained using an ACE C_{18} column (150×4.6mmx 5μm particle size) and isocratic elution with methanol / 0.1% formic acid (54: 46).

3.5.2 HPLC analysis of propolis

Propolis quality was assessed against a series of marker compounds: caffeic acid, cinnamic acid, pinocembrin, chrysin and galangin. Linearity for the calibration curves was obtained in the range 0.02-0.12 mg/ml for all the marker compounds. Figure 3-15 shows the retention times for marker compounds on a C_{18} column and Figure 3.16 shows the calibration curves for the marker compounds.

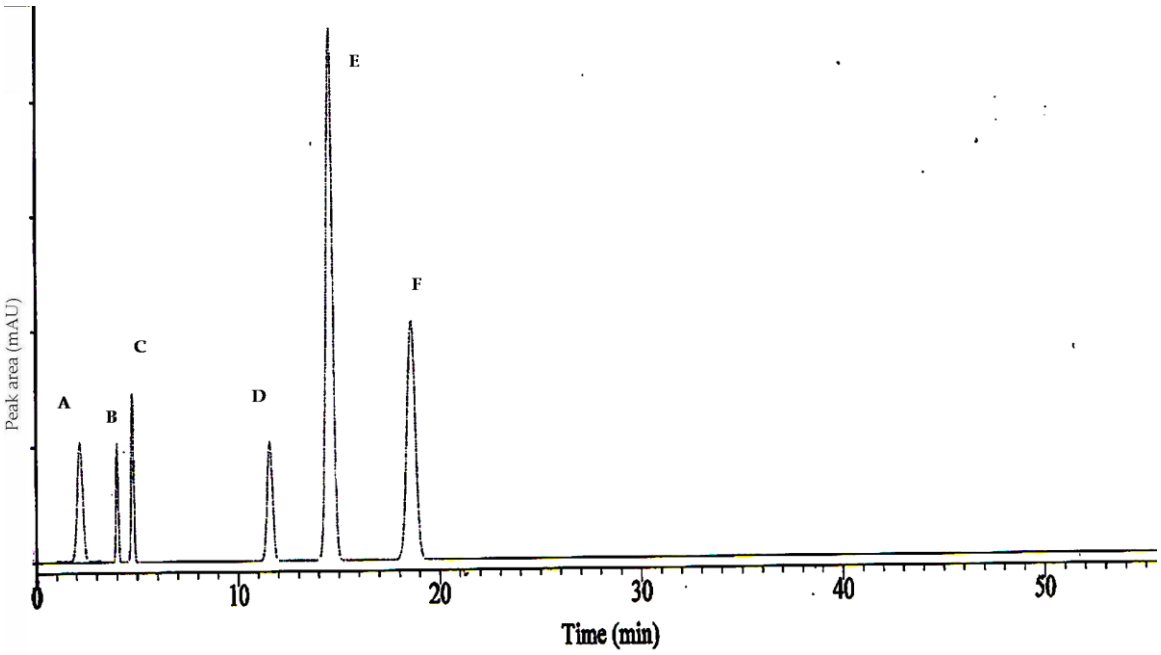


Figure 3-14 Drylab® chromatogram of mixture of marker compounds. Predicted for 54% methanol, 46% 0.1 formic acid. **A**. Caffeic acid, **B**. Naringenin, **C**. Cinnamic acid, **D**. Pinocembrin, **E**. Chrysin, **F.** Galangin

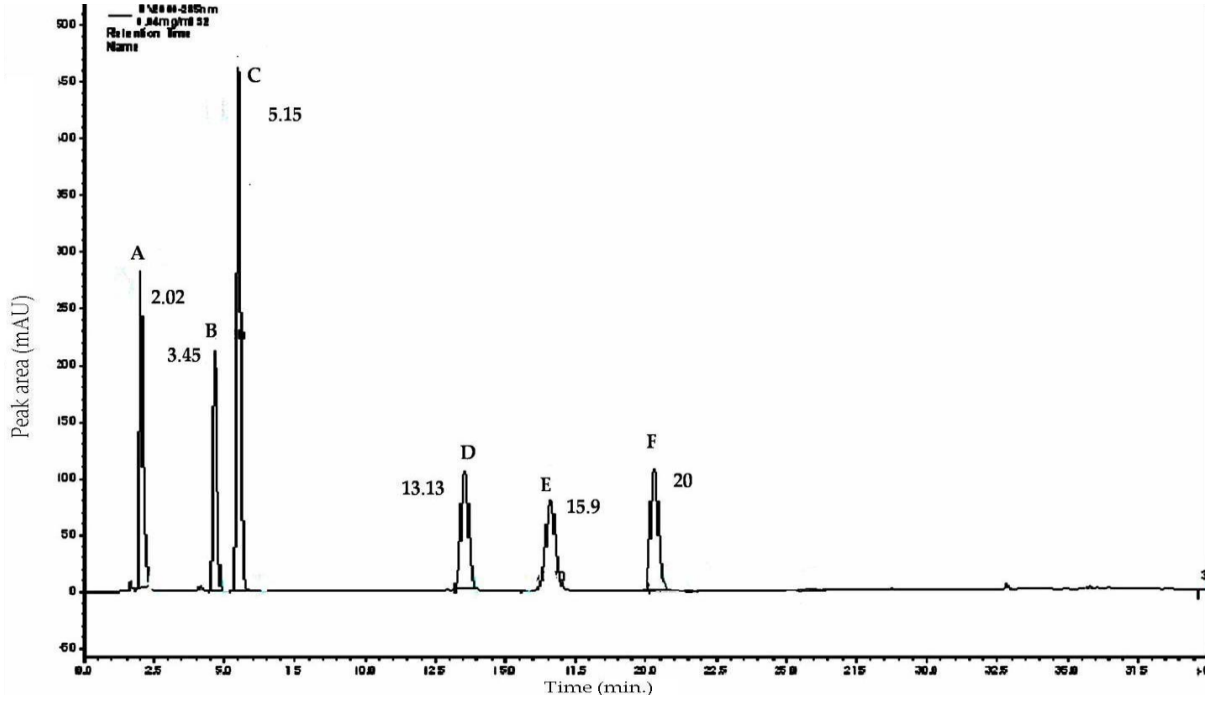
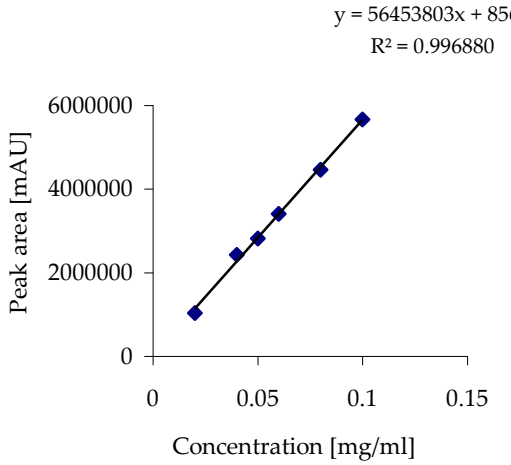


Figure 3-15 HPLC Chromatogram of a mixture of marker compounds**.** Obtained using an ACE C_{18} column (150×4.6mm) and isocratic elution with methanol / 0.1% formic acid (54: 46). **A**. Caffeic acid, **B**. Naringenin, **C**. Cinnamic acid, **D**. Pinocembrin, **E**. Chrysin, **F.** Galangin

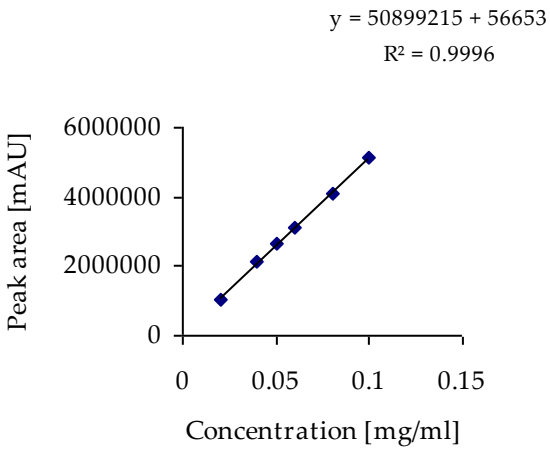
There was quite good agreement with the DryLab® prediction although naringenin eluted much earlier than predicted (Table 3-1).

Table 3-1 Retention times of marker compounds analysed by HPLC. (ACE C_{18} column (150×4.6mmx 5μm particle size) and isocratic elution with methanol / 0.1% formic acid (54: 46)).

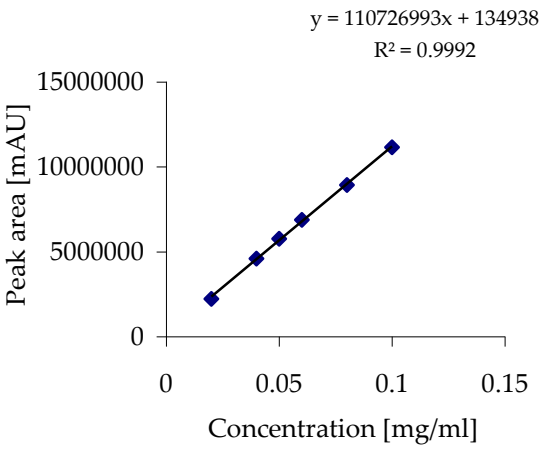
Marker Compounds	Retention Times (min)
Caffeic acid	2.02
Naringenin	3.25
Cinnamic acid	5.15
Pinocembrin	13.13
Chrysin	15.9
Galangin	19.9



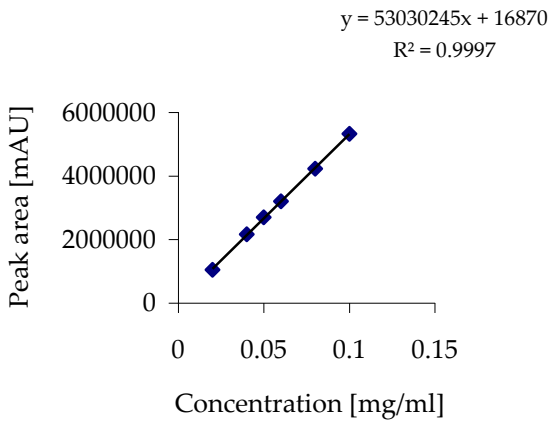
(A)



(B)



(C)



(D)

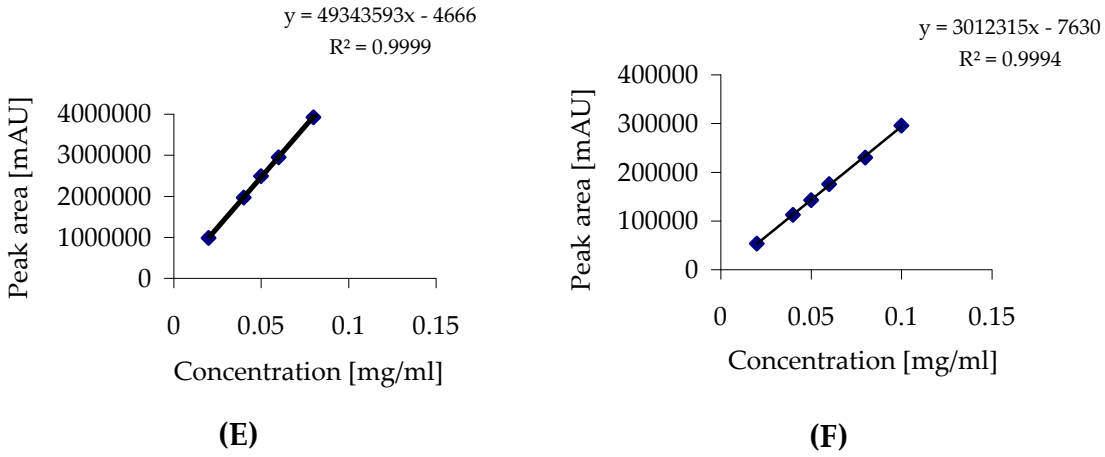


Figure 3-16 Calibration curves for the marker componds.
A. Caffeic acid, **B.** Naringenin, **C.** Cinnamic acid, **D.** Pinocembrin, **F.** Chrysin, **G.** Galangin

3.5.3 HPLC chromatograms of propolis

HPLC analysis was carried out in order to get some idea of the variation in chemical profile of the various propolis samples under test. The amounts of marker compounds in the samples as % w/w, calculated according to equation1. These are shown in Table 3-2.

Table 3-2 The quantification of some marker compounds in various propolis samples.

Propolis samples	Caffeic acid *%w/w	Naringenin *%w/w	Cinnamic acid *%w/w	Pinocembrin *%w/w	Chrysin *%w/w	Galangin *%w/w
S107	1.79	0.12	1.23	11.64	9.68	4.29
S108	2.36	0.17	0.64	7.51	9.03	5.75
S260	3.92	10.74	3.50	13.67	7.09	22.23
S210	5.64	0.13	2.54	15.50	0.86	0.40
S208	9.80	ND	3.854	5.41	1.14	4.55
S263	ND	ND	ND	ND	ND	ND

Figure 3-17 shows the HPLC chromatogram obtained for propolis sample S107 (Bulgaria). Although the marker compounds are present in this sample, there are also many unidentified compounds present. Sample S107 is a typical temperate region propolis with many of the standard marker compounds present in large amounts.

Equation 1 Equation used for the quantification of marker compounds (% w/w).

$$*\%W/W = \frac{(mg\,in\,1ml)\times 10\times 3}{40mg}\times 100$$

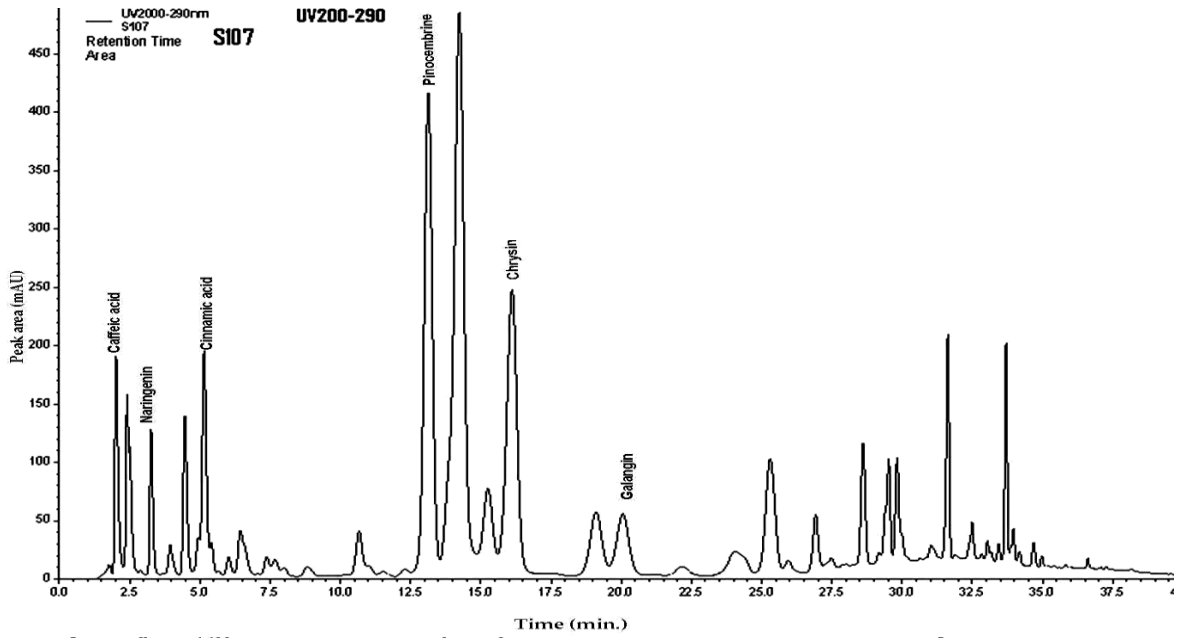


Figure 3-17 The HPLC chromatogram of propolis sample S107

Figure 3-18 shows a HPLC trace for an extract from S108 (Table 3-2) shows the amounts of marker compounds (% w/w) in addition to the marker compounds there are also many unidentified compounds also present. S108 is also a typical temperate region propolis having substantial amounts of marker compounds. Even through S107 came from Bulgaria and S108 came from China there are many similarities in their profiles with the most abundant compounds eluting between 10 and 20 min.

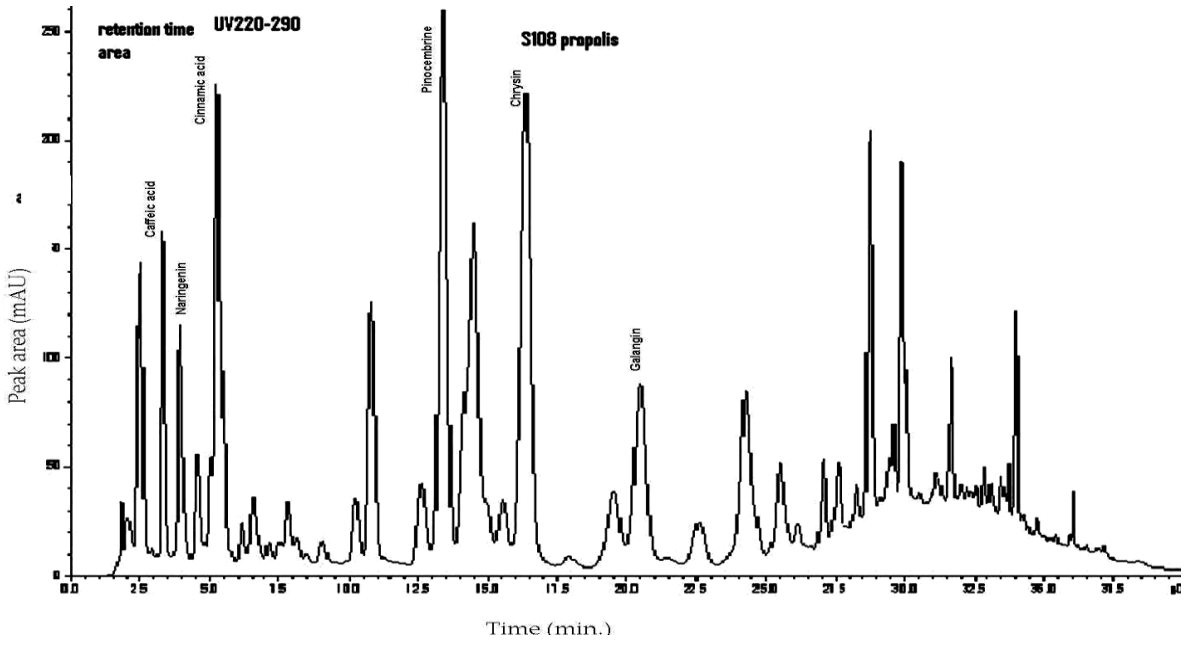


Figure 3-18 The HPLC chromatogram of propolis sample S108.

Figure 3-19 shows the HPLC chromatogram obtained S263 which is from the Solomon Islands and thus of tropical origin. The chromatogram is almost devoid of any UV absorbing peaks, apart from one dominating peak running late in chromatogram. Thus although S263 is a propolis sample it has none of the marker compounds which were proposed for the quality control of propolis from temperate regions.

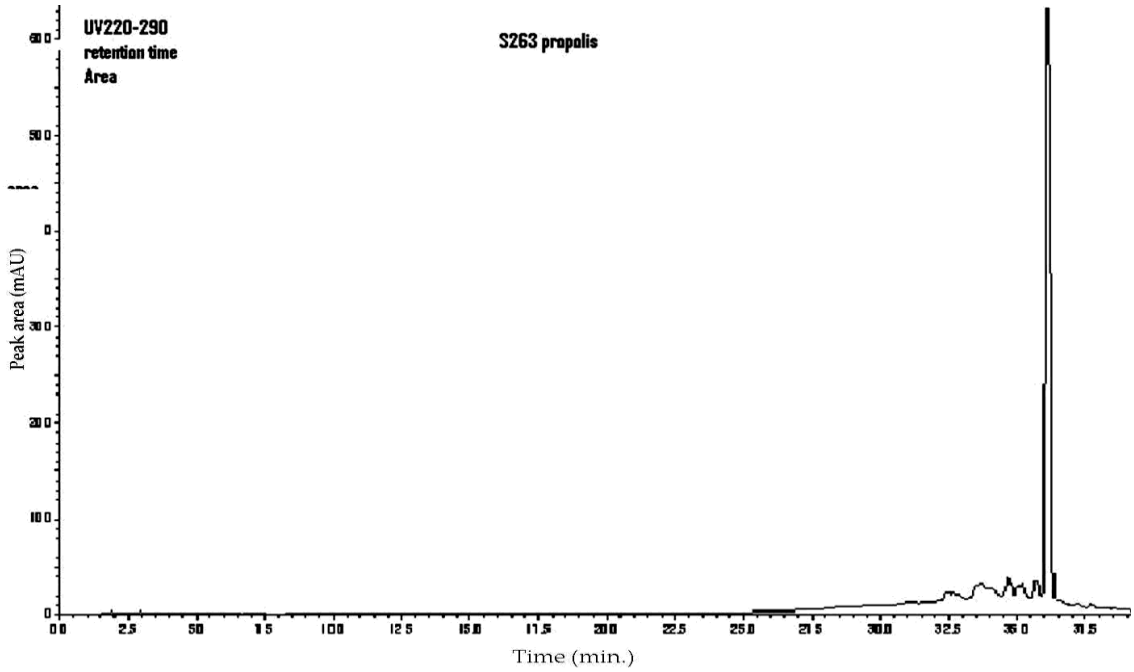


Figure 3-19 The HPLC chromatogram of propolis sample S263.

Figure 3-20 shows the chromatogram obtained for S260 which is of unknown origin and which contains all six marker compounds. It is more typical of temperate propolis with two large peaks for marker compounds in the range 10-20 min. Samples of S210 Appendix 3 and S208 Appendix 3 shows HPLC traces which do not fit into the categories of tropical or temperate propolis and are characterised as containing large amounts of cinnamic acid. Thus there remains a great deal of work to be carried out in characterising these samples. Samples S107, S108 and S263 were investigated further since they displayed interesting biological activity.

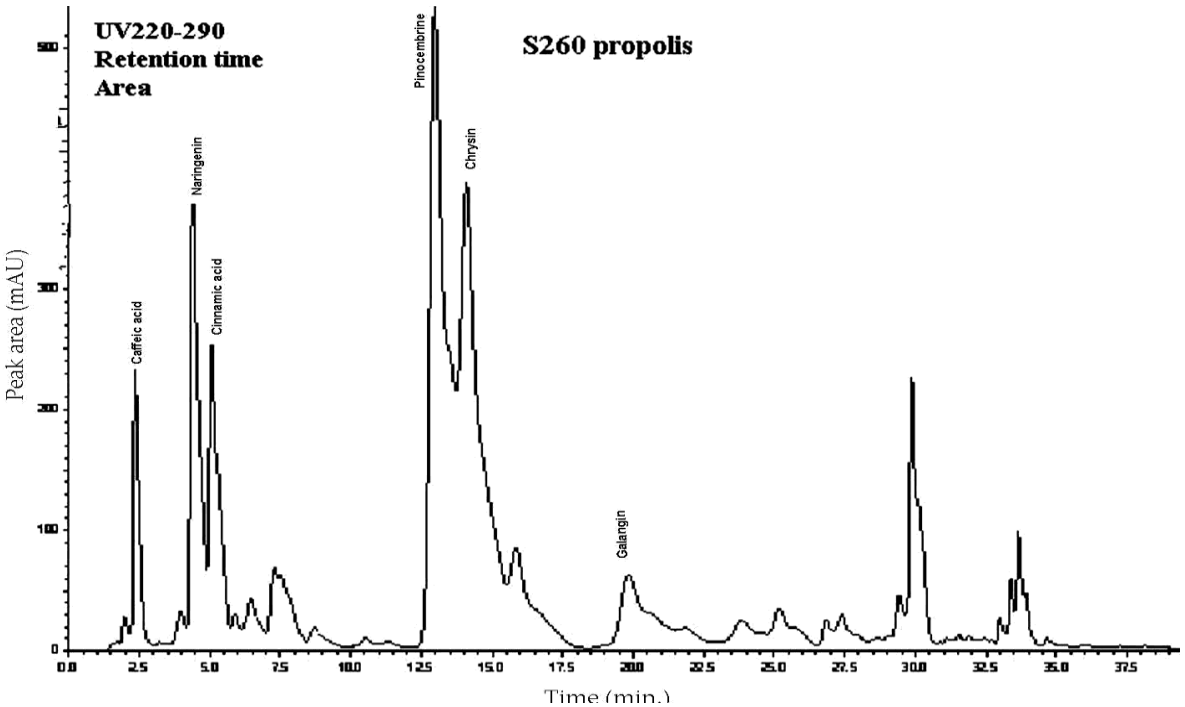


Figure 3-20 The HPLC chromatogram of propolis sample S260**.**

3.5.4 Analysis of Sample S263 by GC-MS

S263 did not give any UV absorbing peaks upon HPLC analysis or any peaks from LC-MS analysis. Thus GC-MS analysis of the sample was carried out. The GC-MS trace for the sample is shown in (Figure 3-21) unlike the typical temperate propolis samples which contain eudesmane sesquiterpenes as typical volatile components this sample was found to contain compounds which were probably sesquiterpenes but did not closely match the mass spectra of the compounds in the National Institute of Standards (NIST) library. The major components in the extract elute between 12 and 14 min and are all related to the triterpene lupeol. Components include lupeol acetate and possibly cycloursane (Figure 3-22). Figure 3-23 shows the EI MS of the lupeol acetate.

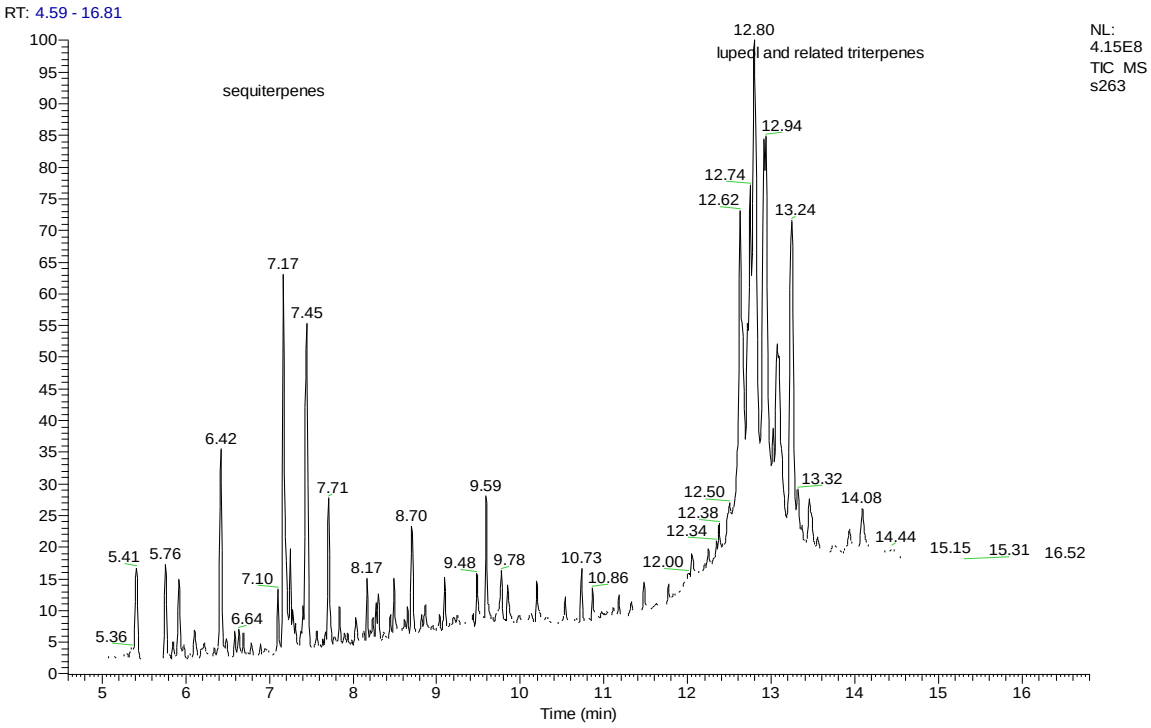


Figure 3-21 Total ion current trace from GC-MS analysis of S263 on an Rtx-1 column.

The sequiterpenes in the sample would require isolation and NMR analysis in order to confirm their structures.

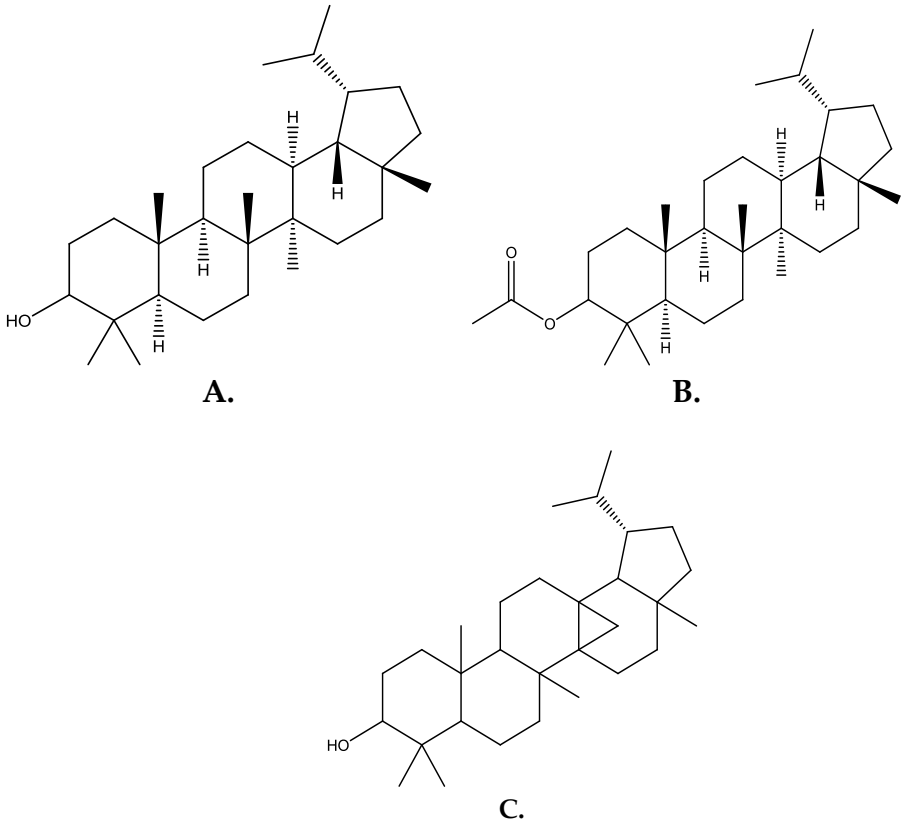


Figure 3-22 Structures of lupeol triterpenes found in propolis sample S263.
A. Lupeol, **B.** Lupeol acetate and **C**. Cycloursane.

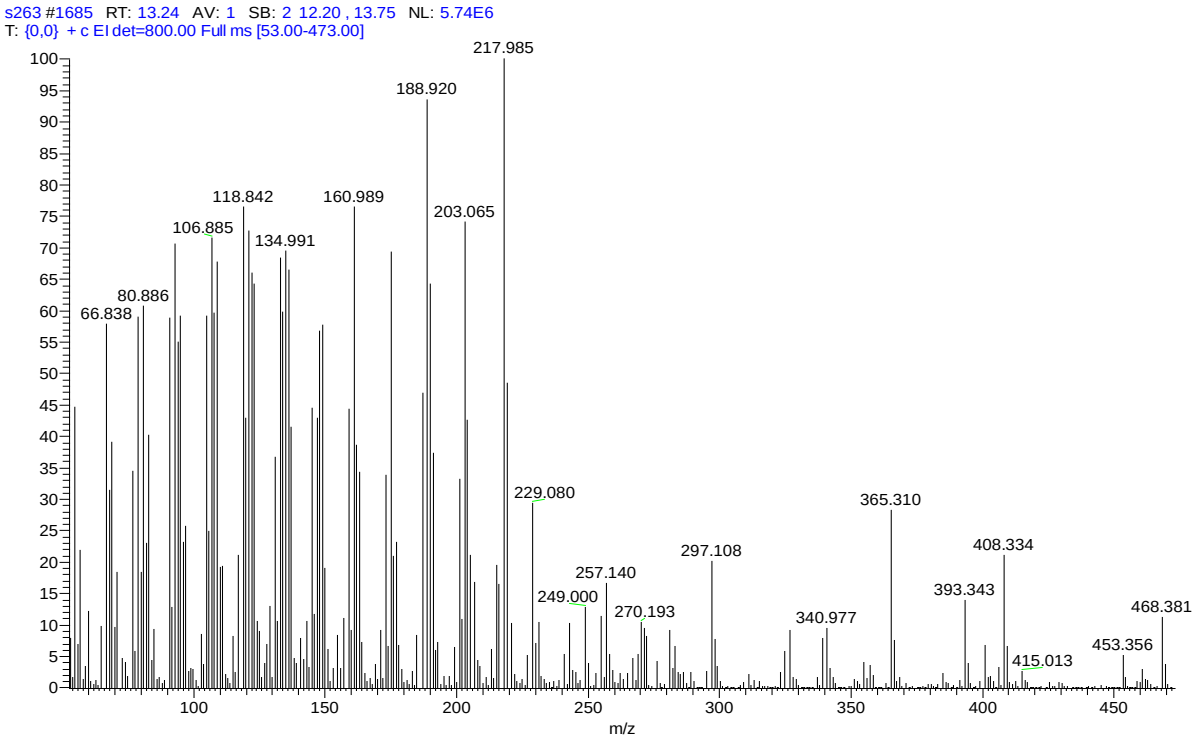


Figure 3-23 EI Mass Spectrum of lupeol acetate in S263 obtained using a MD800 MS at 70eV.

3.5.5 Flash chromatography

Samples which were found to display interesting biological activity were subjected to preparative scale fractionation. Sample S108 was fractionated and (Table 3-3) shows the weights of the fractions obtained.

Table 3-3 Percentage yield for each fractions of S108 sample separated by RP Flash column chromatography.

Fractions	Weight (g)	% yield of each fraction
S108(F5)	0.48	39.1
S108(F6)	0.072	5.9
S108(F7)	0.060	5.0
S108(F8)	0.18	14.5
S108(F9)	0.073	6.0
S108(F10)	0.36	29.6

Total weight 1.22 g

S107 was fractionated by flash chromatography and (Table 3-4) shows the yield of the fractions obtained.

Table 3-4 The weights of fractions collected after fractionation of S107 sample By RP Flash column chromatography.

Fractions	weight (g)	% yield of each fraction
S107(F5)	0.045	11.9
S107(F6)	0.094	24.9
S107(F7)	0.060	16.0
S107(F8)	0.046	12.2
S107(F9)	0.0429	11.3
S107(F10)	0.0430	11.3

Total weight of the sample was 0.38 g

3.5.6 LC-MS analysis of propolis fractions from S107 and S108.

Table 3-5 shows the retention times and the molecular ions obtained for the propolis marker compounds under the conditions used for LC-MS in positive and negative ion modes.

Table 3-5 The retention times and the molecular ions obtained for the propolis marker compounds.

Compound	**Molecular Weight (amu) ***	**Time (min.)**	**M+H+ + additional ions**	**M-H- + additional ions**
Caffeic acid	180	2.4	ND	179, 214 ($^{+}$Cl), 225 ($^{+}$ HCOOH)
Cinnamic acid	148	6.4	ND	147, 182($^{+}$Cl),193 ($^{+}$HCOOH)
Pinocembrin	256	17.2	257	255, 290 ($^{+}$Cl), 301 ($^{+}$HCOOH)
Chrysin	254	21.8	255	253, 288 ($^{+}$Cl), 299 ($^{+}$HCOOH)
Galangin	270	25.8	271	269
Naringenin	272	5.6	273	271, 306($^{+}$Cl),317($^{+}$HCOOH)

ND= not detected, * (amu) = atomic mass units

LC-MS analysis of the fractions obtained from flash chromatography separation of S107 was carried out. The HPLC UV chromatograms obtained during the LC-MS analysis of fractions S107(F6)-S107(F9) are shown in (Figure 3-24), the associated mass spectra and UV spectra are in Appendix 2. S107(F6) contained the marker compounds caffeic acid, kaempferol, pinobanksin, pinocembrin and galangin used to assess the quality of propolis

as can be seen in the UV chromatograms shown in (Figures 3-24 and 3-25) and also some the acetate ester of pinobanksin. Tentative identifications are given for the major peaks observed in the active fractions of S107sample in (Table 3-6). The most abundant peaks in the UV trace included some of the standard marker compounds but there was also a large amount of the acetate and propionate esters of pinobanksin as well as some unidentified components in the sample (Figure 3-26). The esters of pinobanksin in most cases showed neutral loss of the ester moiety from the negative molecular ion e.g. (Figure 3-27) suggesting that the ester is on the aliphatic hydroxyl group on the 3-position. Fractions S107(F8) and S107(F9) can be observed to contain an extended series of esters (Figures 3-28 and 3-29) which are also probably esters of pinobanksin. Table 3-6 summarises the retention and mass spectral data for some of the compounds observed in fractions F6-F9. Appendix 2 shows the corresponding mass spectra and UV spectra data obtained for the major compounds observed in fractions 6-9 S107.

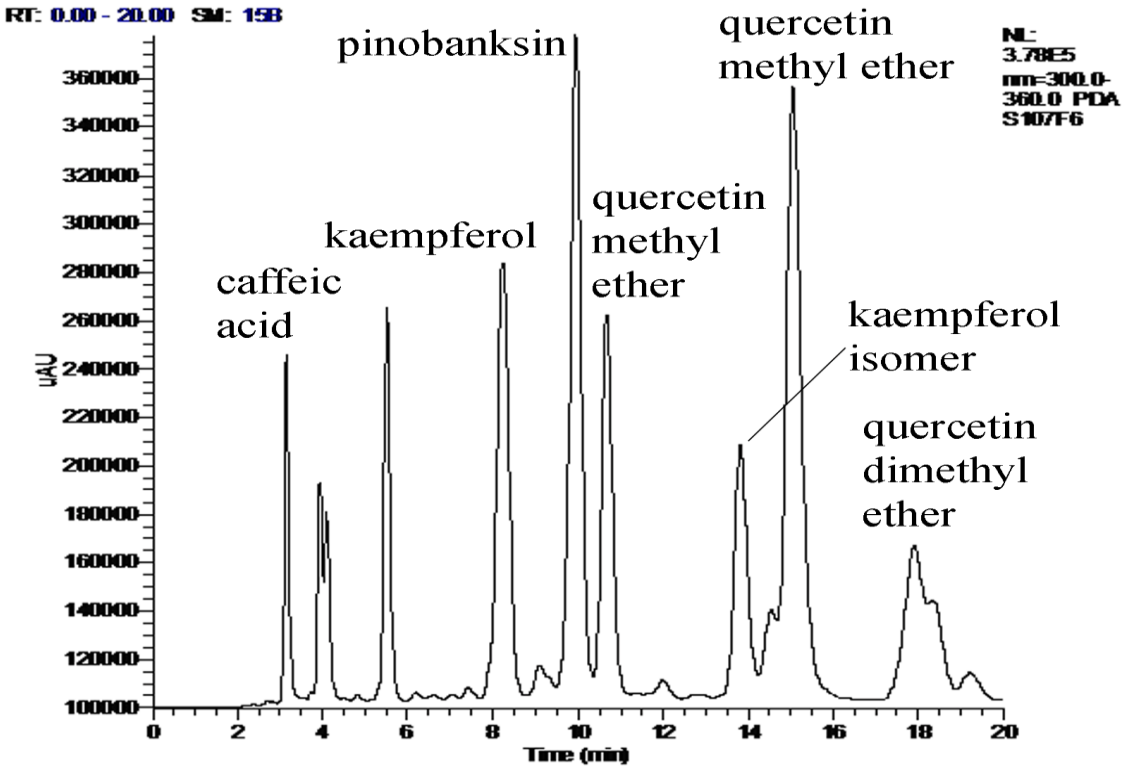


Figure 3-24 UV trace of fraction S107(F6) 0-20 min (300-360 nm) .

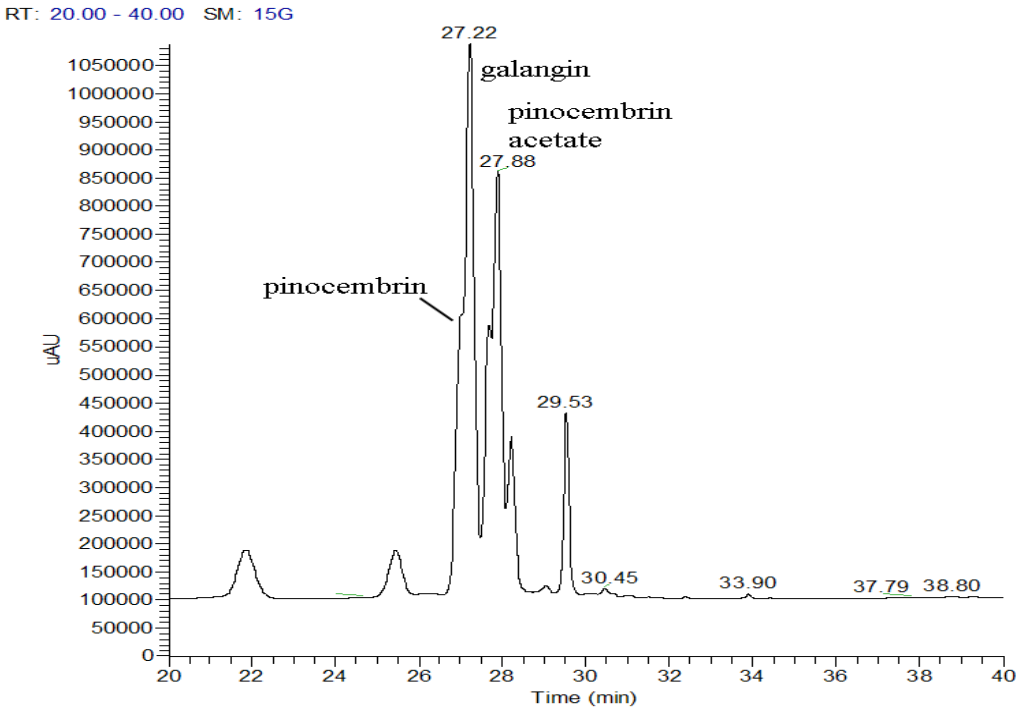


Figure 3-25 UV trace of fraction S107(F6) 20-40 min (300-360 nm).

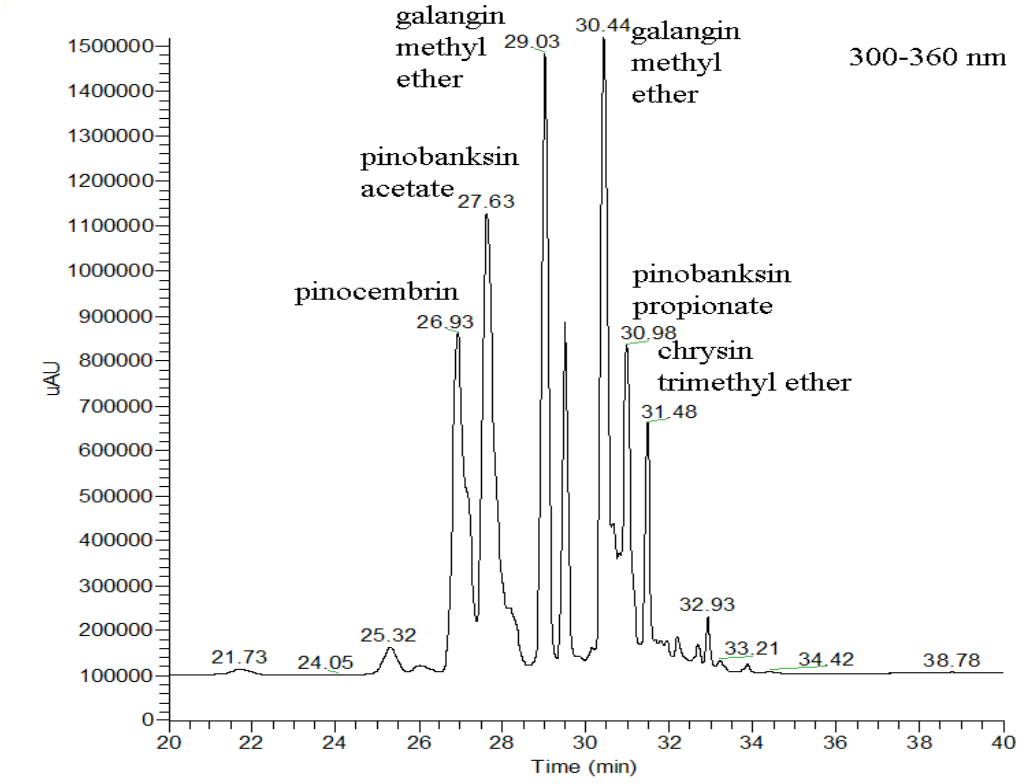


Figure 3-26 UV trace of fraction S107(F7) 20-40 min (300-360 nm)

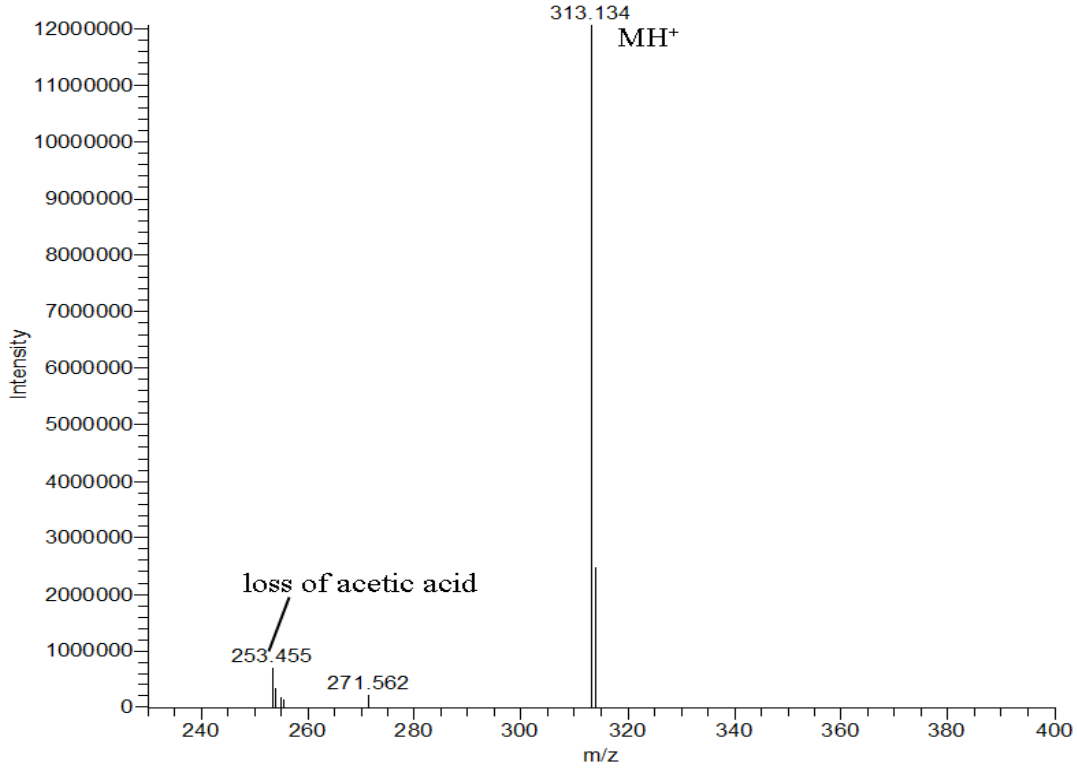


Figure 3-27 MS² spectrum of pinobanksin acetate at 35V showing neutral loss of acetic acid

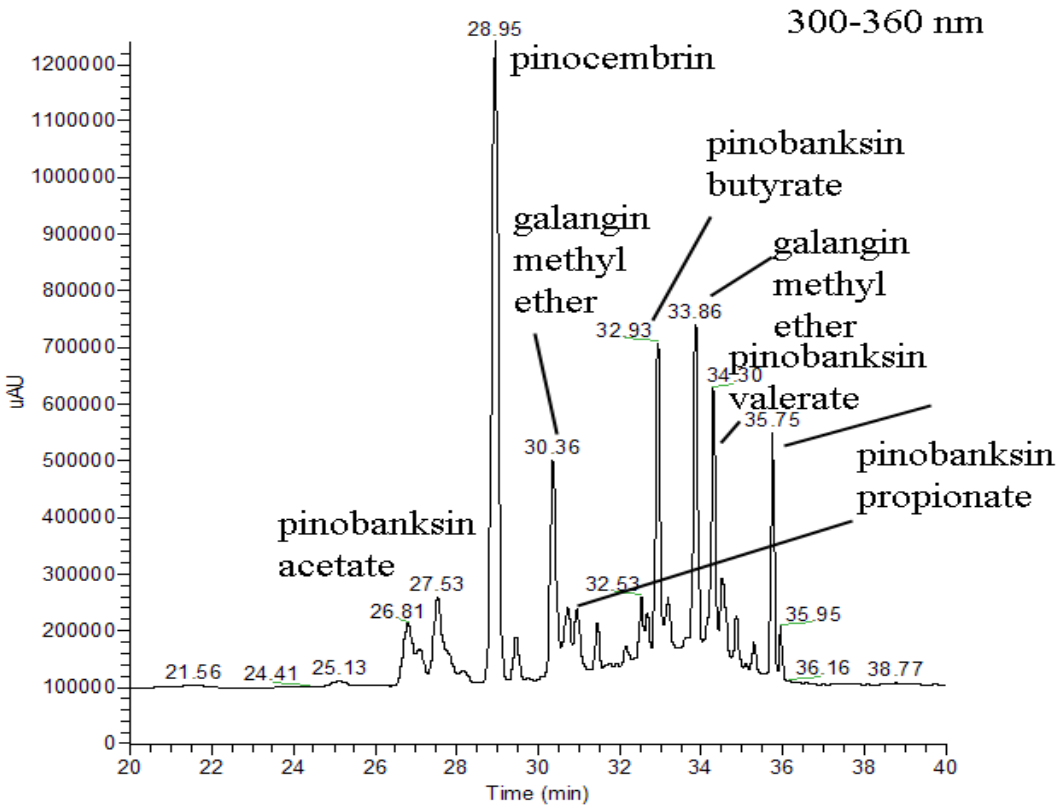


Figure 3-28 UV trace of fraction S107(F8) 20-40 min (300-360 nm).

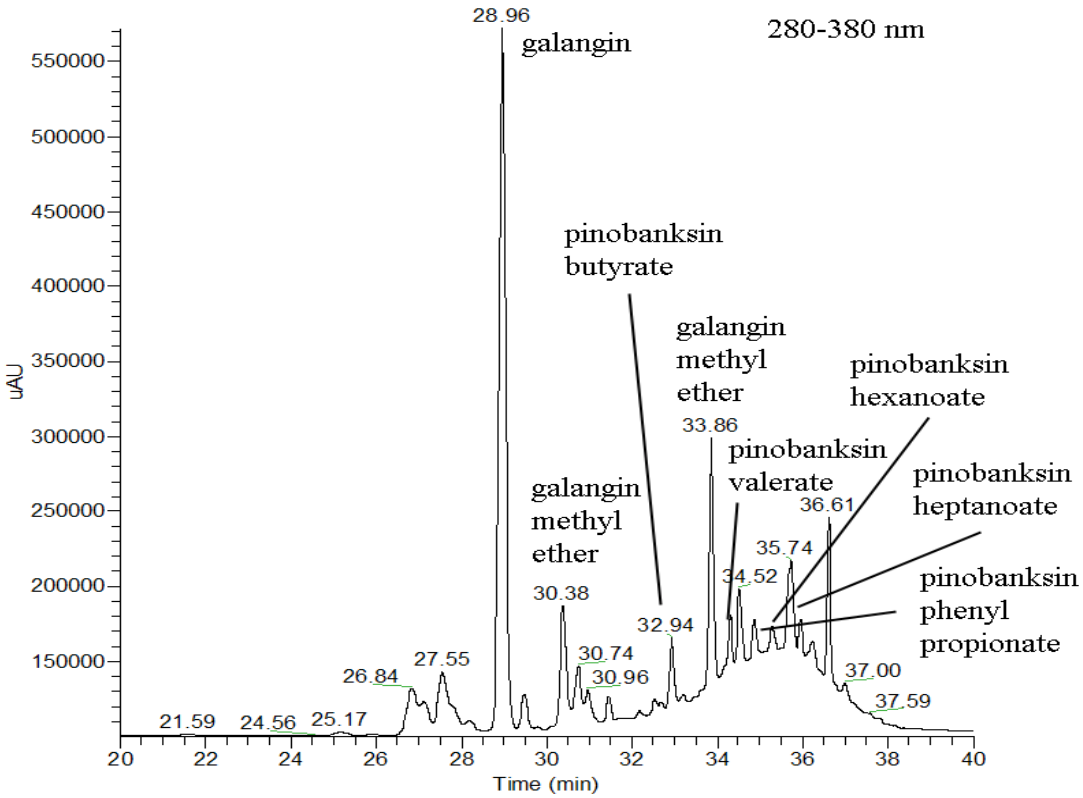


Figure 3-29 UV trace of fraction S107(F9) 20-40 min (280-380 nm).

Table 3-6 Marker compounds and esters of pinobanksin present in sample S107 fractions S107(F6), S107(F7) and S107(F9).

Tentative Identification	Time (min)	M+H+	+MS2	M-H-	-MS2
Pinocembrin	26.9	257		255	
Pinobanksin acetate	27.6	315	ND	313	253
Pinocembrin isomer	29.0	257		255	
Methyl galangin	29.5	ND	ND	283	ND
Galangin	30.5	271		269	
Pinobanksin propionate	31.0	329		327	
Trimethyl chrysin	31.5	295			
Pinobanksin valerate	32.7			353	253
Pinobanksin butenoate	32.9			341	253

ND=not detected.

3.5.7 NMR data of marker compounds

In order to gain additional information about the components in the fractionated propolis NMR spectra were obtained using. Firstly the NMR

spectra for the standard marker compounds were obtained in order to assist in the interpretation of the NMR spectra of the mixtures of compounds in the propolis fractions.

The data from NMR spectra of the propolis marker compounds are shown in Table 3-7 – Table3-12

Naringenin

The signals obtained from the NMR spectrum can be assigned as shown in table 3-7.[68]

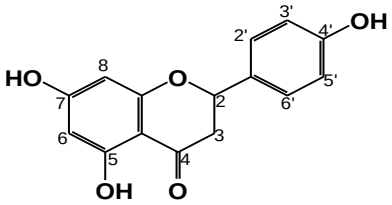


Table 3-7 ^{1}H NMR data for Naringenin

position	δ (ppm)	multiplicity	J-coupling(Hz)
2	5.45	dd	12.8, 2.9
3A	3.25	dd	17.1, 2.9
3B	2.68	dd	17.1, 12.8
5OH	12.15	s	
6, 8	5.86 (2H)	s	
7OH	10.53	s	
2′/6′	7.33 (2H)	d	8.1
3′/5′	6.83 (2H)	d	8.1
4′	9.54	s	

s singlet, d doublet, dd doublet of doublet

Cinnamic acid

The signals obtained from the NMR spectrum can be assigned as shown in table 3-8.

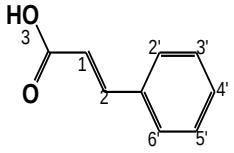


Table 3-8 ^{1}H NMR spectra for Cinnamic acid.

position	δ (ppm)	Multiplicity	J-coupling(Hz)
1	6.51	d	16.3
2	7.61	d	16.3
2′/6′	7.70 (2H)	m	
3′-4′-5′	7.44(3H)	m	
3	12.20	s	

Ss singlet, d doublet, m multiplet

Caffeic acid

The signals obtained from the NMR spectrum can be assigned as shown in table 3-9.

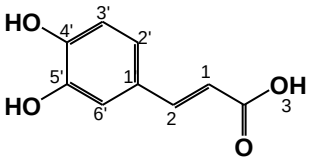


Table 3-9 ^{1}H NMR spectra for Caffeic acid.

position	δ (ppm)	Multiplicity	J-coupling(Hz)
1	6.22	d	15.8
2	7.43	d	15.8
3 -OH	12.10	bs	
2′	6.97	dd	7. 2, 3.2
3′	6.76	d	7.2
4′-OH	9.51	bs	
5′-OH	9.51	bs	
6′-H	7.06	d	1.8

d doublet, dd doublet of doublets, bs= broad singlet.

Chrysin

The signals obtained from the NMR spectrum can be assigned as shown in table 3-10.

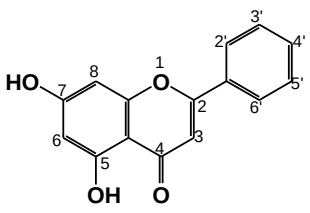


Table 3-10 ^{1}H NMR spectra of Chrysin.

position	δ (ppm)	Multiplicity	J-coupling(Hz)
3	6.95	s	
7-OH	11.14	s	
6	6.24	d	2.4
8	6.56	d	2.4
2′/6′	8.14	d	8.3
3′-4′-5′	7.63	m	
5-OH	13.25	s	

s singlet , d doublet, m multiplet

Galangin

The signals obtained from the NMR spectrum can be assigned as shown in table 3-11.

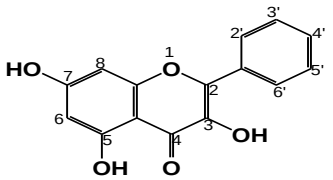


Table 3-11 ^{1}H NMR spectra for Galangin.

position	δ (ppm)	Multiplicity	J-coupling.(Hz)
3	8.34	s	
5,7	12.10	s	
6	6.19	s	
8	6.45	s	
2′/6′	8.10 (2H)	m	
3′-4′-5′	7.54 (3H)	m	

s singlet, m multiplet

Pinocembrin

The signals obtained from the NMR spectrum can be assigned as shown in table 3-12.

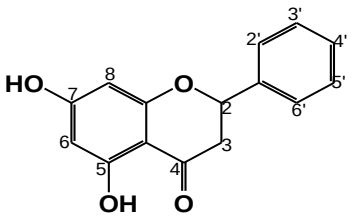


Table 3-12 [1]H NMR spectra for Pinocembrin.

position	δ (ppm)	Multiplicity	J-coupling(Hz)
2	5.60	dd	13.2, 3.1
3B	2.80	dd	17.2 ,3.1
3A	3.25	dd	17.6,13.2
5-OH	12.13	s	
6,8	5.92	d	2.3
7	12.13	s	
2′/6′	7.52 (2H)	m	
3′-4′-5′	7.45 (3H)	m	

s singlet, d doublet, dd doublet of doublets, m multiplet.

3.5.8 [1]H NMR spectra of propolis sample S107 fractions F6, F7 and F9

Figure 3-30 shows the [1]H NMR spectra of S107 fractions F6, F7 and F9 and indicates the presence of a mixture of flavonoid compounds. The [1]HNMR spectra show the presence of the most prominent proton signals observed at 3.25 ppm in ring C, 6.8 ppm, 7.9 ppm in ring B, in addition to the major 5-OH proton signal at around 12-13 ppm. The sub fractionations of S107 contain a mixture of flavonoids in each fraction, containing more than one compound with the same flavonoid nucleus.

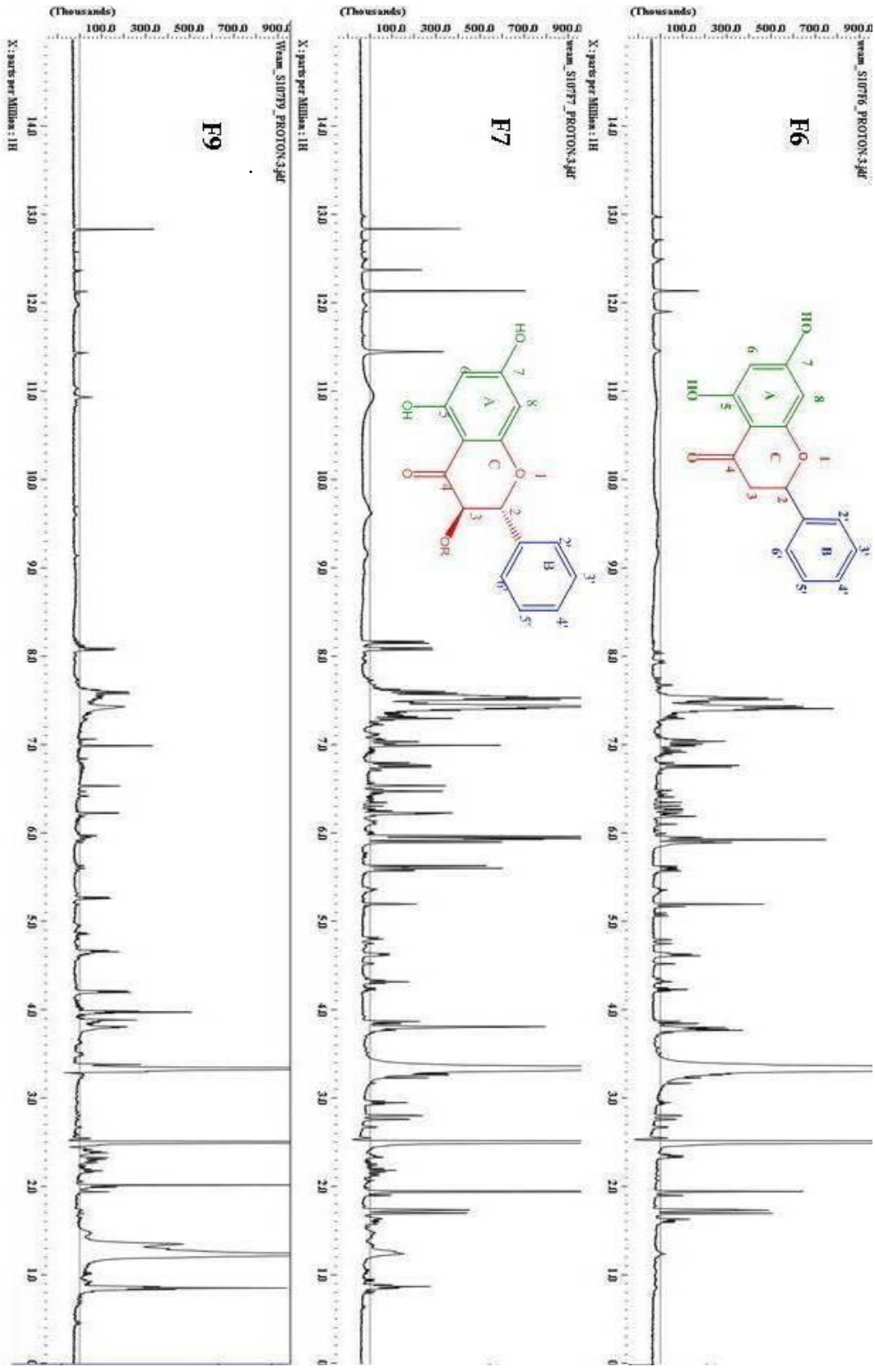


Figure 3-30 ^{1}H NMR spectra of S107 fractions F6, F7 and F9 in DMSO (1-15ppm)**.** (JEOL Delta GX 400 MHz).

Two sharp aliphatic proton doublets for compound (1) at 3.7 ppm are most prominent in fraction S107(F7) indicating the presence of (H-3B) in Ring C. An aliphatic proton doublet at 4.6 ppm due to (H-3A) (1) in ring C of a flavonol can be observed. This doublet is more predominant in S107(F6) than in F7 and F9. The presence of this doublet is consistent with the presence of esters of pinobanksin. This signal is more prominent in F6>F7>F9 (Figure 3-31).

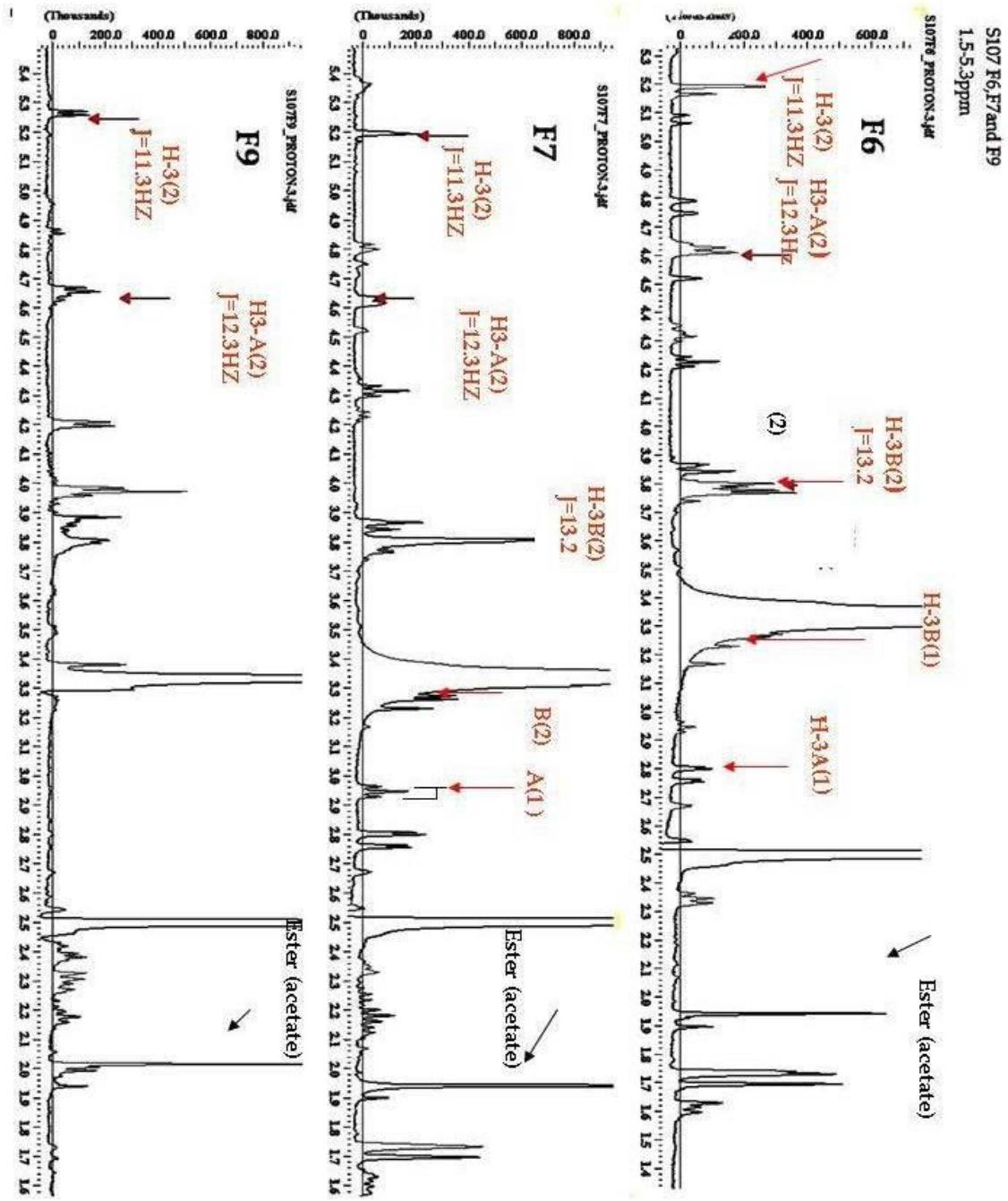


Figure 3-31 An expansion of the ^{1}H NMR spectrum of S107 fractions F6, F7 and F9 (1-5.4 ppm). (JEOL Delta GX 400 MHz)

Figure 3-32 shows an expansion of the ^{1}H NMR spectrum of S107 active fractions F6, F7 and F9 in (5-8.2 ppm). The NMR spectra of S107 fractions F6, F7 indicated the presence of mixture of compounds at 5.6 ppm with two sharp olefinic peaks.

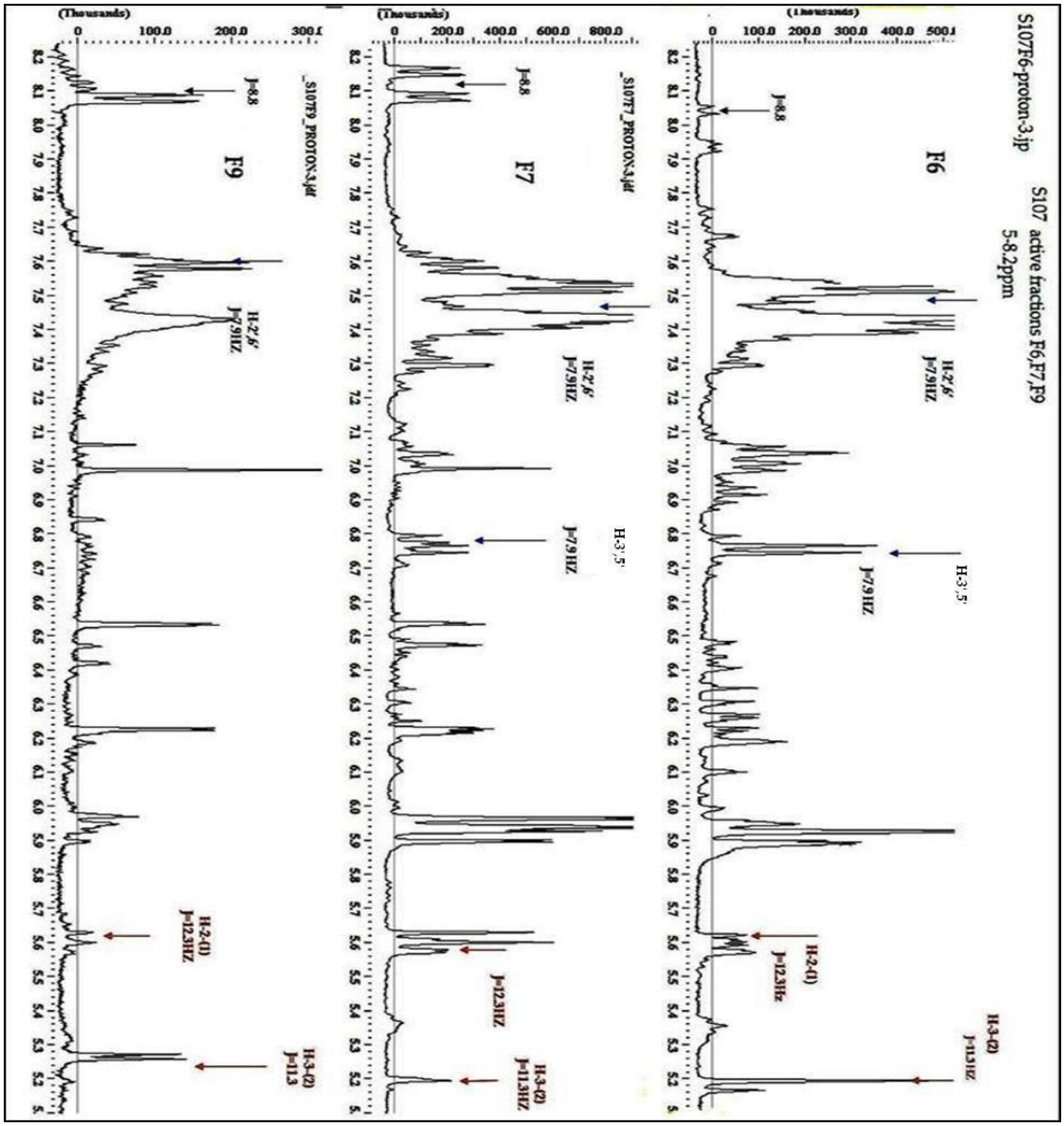


Figure 3-32 An expansion of the ^{1}H NMR spectra of S107 fractions F6, F7 and F9 in DMSO (5.2-8.2ppm). (JEOL Delta GX 400 MHz)

Figure 3-32 shows an expansion of the ^{1}H NMR spectrum of S107 active fractions F6, F7 and F9 in (5-8.2 ppm). The NMR spectra of S107 fractions F6, F7 indicated the presence of mixture of compounds at 5.6 ppm two sharp olefinic peaks.

Figure 3-33 shows the ^{1}H NMR spectra of the fractions, S107(F6), S107(F7) and S107(F9) followed almost similar pattern with peaks mainly appearing in the region of 6.0 to 8.0 ppm with different substitution patterns, singlets at 11.50-13.0 ppm. A few peaks were also observed between 3.5 to 5.8 ppm and 1.5 to 2.5 ppm.

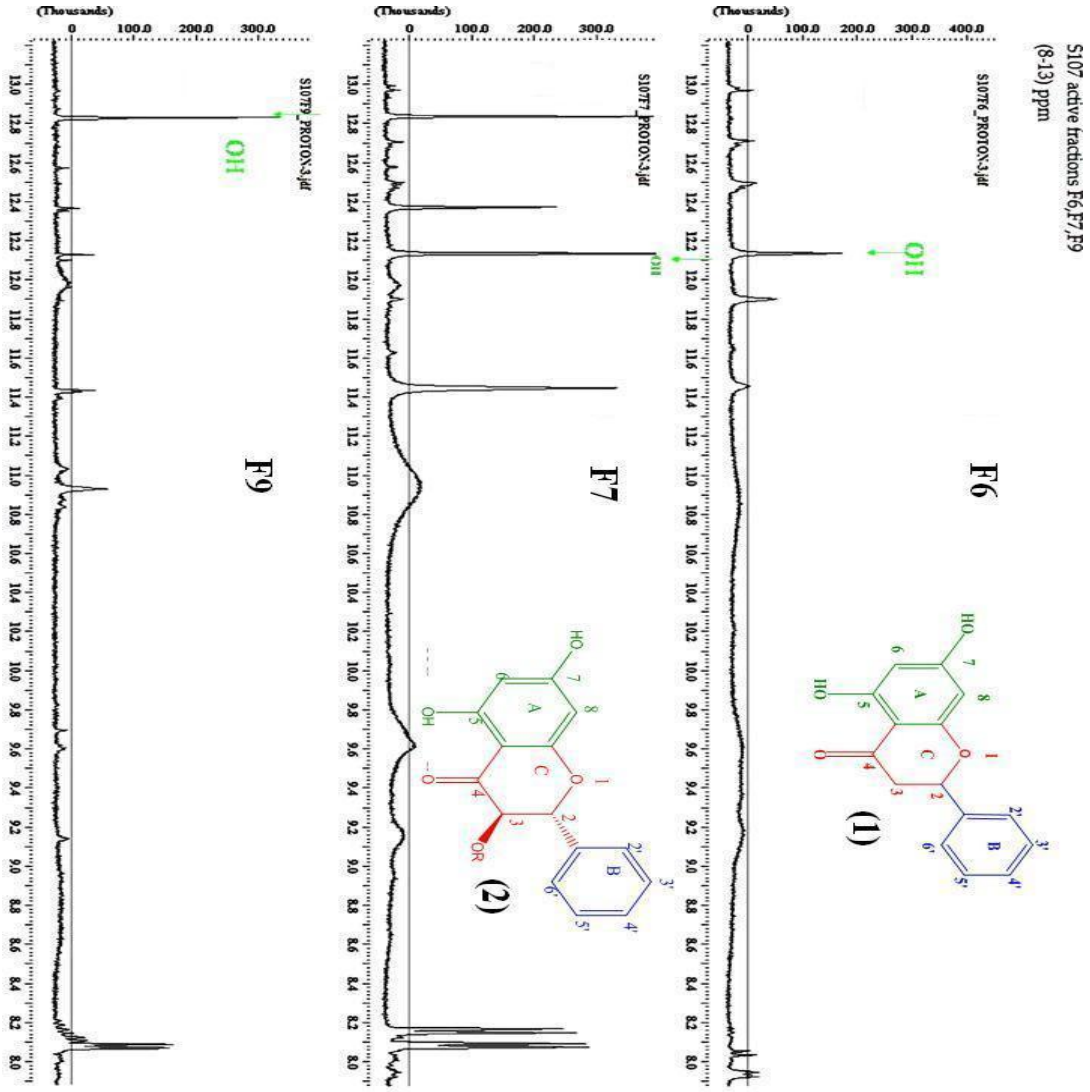


Figure 3-33 An expansion of the^{1}H NMR spectra of S107 active fractions F6, F7 and F9 in DMSO (8-13ppm). (JEOL Delta GX 400 MHz)

When studied in detail, the fraction S107(F6) showed relatively smaller peaks in the region of 11.50-13.0 ppm suggesting that few of the flavonoids present had 5-hydroxy substitution. The LCMS data proves the presence of chrysin (MW-254, RT- 21.8) galangin (MW-270, RT-26.1) pinocembrin (MW-256, RT-17). However they may represent a minor proportion as the peaks belonging to 5-OH of ring A, doublets belonging to 2' and 6' 7.9-8.1 ppm were relatively

small in size. Fraction S107 (F7) indicated a greater proportion of 5-OH flavonoids with large peaks in the region of 11.50-13.0 ppm.

3.5.9 ^{1}H NMR spectra of propolis sample S108 fractions, F5, F6, F7, F8 and F9

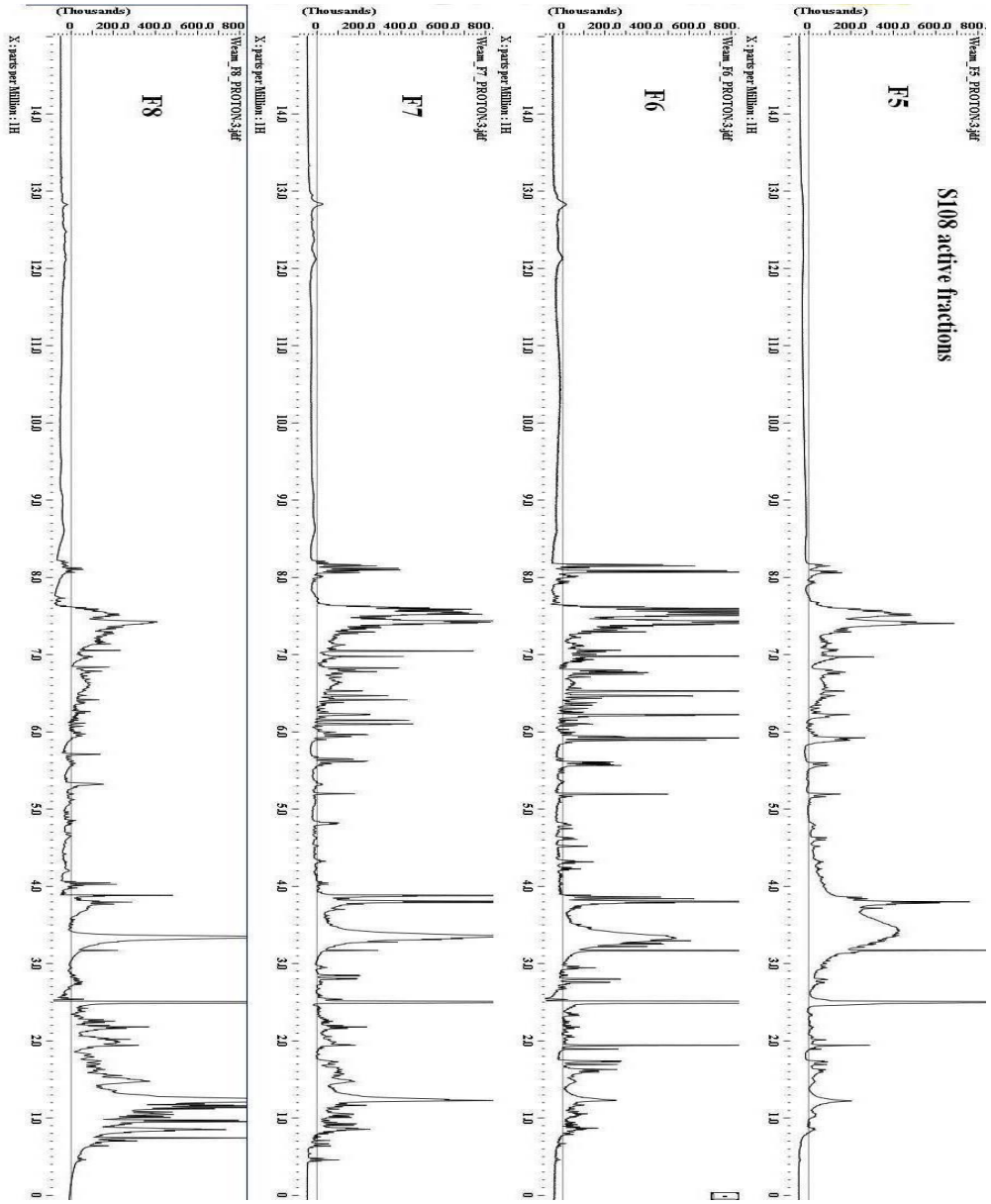


Figure 3-34 ^{1}H NMR spectra of S108 active fractions F5, F6, F7 and F8 in DMSO (1-15ppm). (JEOL Delta GX 400 MHz)

Figure 3-34 S108 propolis sample looks to be more crude than S107 large signals due to aliphatic protons suggest that it is rich in the fatty acid and terpenoids in addition to mixtures of variety of flavonoids which is observed in the region of chemical shift of abundant peaks between 1-3 ppm. In

addition significant peaks between 5-8 ppm indicate the presence of different flavonoids at this chemical shift.

3.5.10 ^{1}HNMR spectrum of propolis sample S263

Figure 3-35 shows the ^{1}H NMR spectrum of propolis S263 sample and reveals that the majority of the compounds of this sample are aliphatic in nature representing triterpenoid or steroid compounds, very few peaks in the aromatic region were observed. Some signals in the region of δH 3-5.5 ppm suggest the presence of oxymethines and olefinic protons.

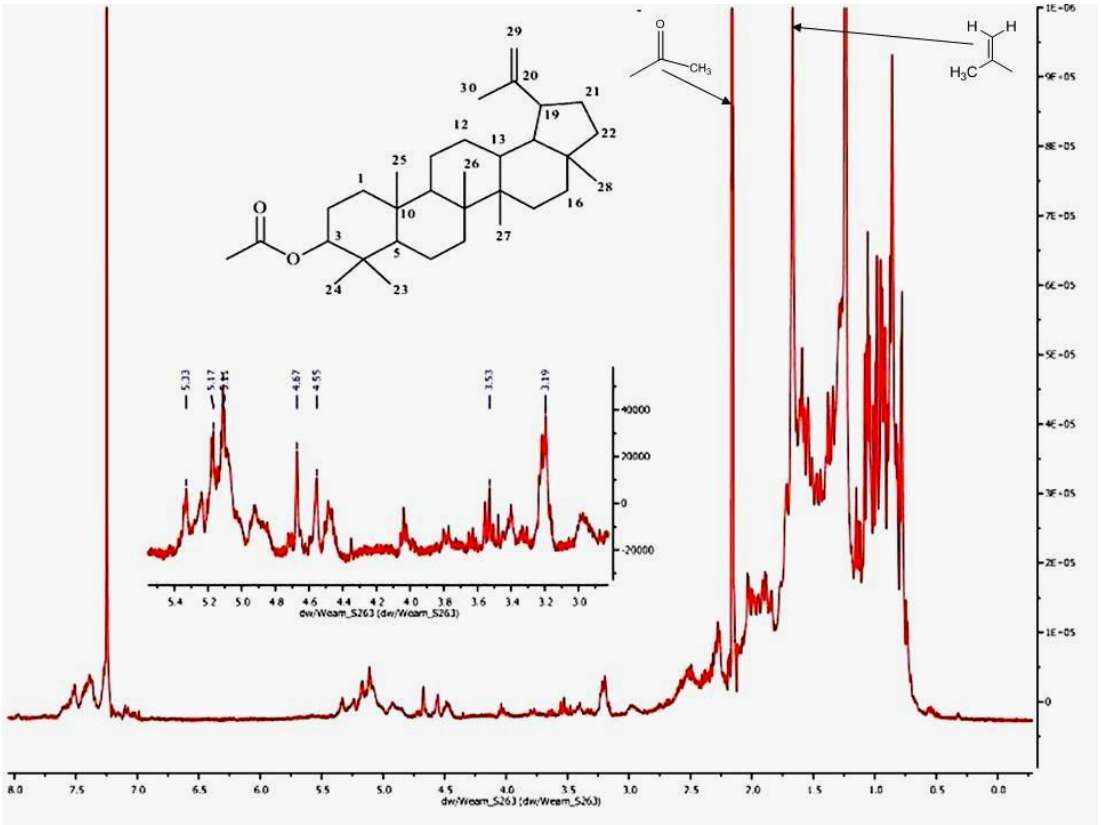


Figure 3-35 ^{1}H NMR spectrum of propolis sample S263 in chloroform (0-8ppm).

The EI-MS spectrum showed some molecular ion peaks between *m/z* 400-500, further indicating the presence of triterpenes in S263 the oxymethines present in the spectrum may belong to the H-3 proton of the triterpenes.

As suggested by a GC-MS spectral library search, lupane and ursanolic type triterpenes and their acetates are present. The presence of acetates is

suggested by a strong singlet at around 2.1 ppm. A strong signal at δH 1.7 ppm probably corresponds to the methyl groups at position 2 in the A ring which are present in lupeol type triterpenes. While some of the peaks present in the olefinic region are due to the methylene protons of the exomethylene group.

4 Discussion

Ethanolic extracts from twenty two samples of propolis were chemically and biologically evaluated. The samples displayed varying degrees of biological activity against Gram-positive bacteria and in particular two samples S107 and S108, from Bulgaria and China respectively, were found to be very active. The antimicrobial activity of propolis is well established and is attributed to phenolic compounds in the propolis extracts [7, 15, 25, 28, 52], however, S107 and S108 were particularly active. The mechanism of antibacterial activity of propolis is complex and could be attributed to synergism between phenolic and other compounds mainly pinocembrin, galangin and pinobanksin [69]. Galangin was found to exert its antibacterial effect by damaging the cell membrane [2]. The samples were also screened against *E.coli* where there was found to be limited activity in all the samples which reflects previous observations [18].

Screening against *M.aurum* indicated that S107 and S108 were very active against this organism. In addition sample S263 from the Solomon Islands was very active against this organism. S107 and S108 were also found to display cytotoxic activity and anti-trypanosomal activity. The anti-trypanosomal activity of propolis is well established [56-58]. *In vivo* and *in vitro* study on effect of propolis against Trypanosoma cruzi was shown to cause a loss of plasma membrane integrity of the trypomastigotes of the trypanosoma parasite [20].

In parallel with the biological testing a rapid HPLC-UV screening method was developed to profile propolis samples with regard to their content of the standard marker compounds caffeic acid, cinnamic acid, chrysin, pinocembrin and galangin. The method allowed the marker compounds to elute within 20 min instead of within 50 min as previously reported [5]. However, it was still necessary to include a final gradient step to remove

traces of wax from the column. Overall the run time was reduced to 40 min from the original run time of 70 min [5]. The compounds were quantified in a number of propolis samples however some propolis samples such as S263 were found not to contain any of the marker compounds. Volpi and Bergonzini (2007) investigated the components of propolis using HPLC and LCMS. Phenolic acids and the most abundant flavonoids; chrysin, pioncembrin, pinobanksin and galangin were detected and they concluded that these compounds should be used as quality markers [70]. However, from our results they are not quality markers for all types of propolis. Similar conclusions were drawn in a previous study where Watson *et al*. (2006) investigate the propolis samples obtained from different geographical origin using HPLC to analyse propolis samples and also by using ^{1}HNMR spectra for profiling samples [5]. Wide variations in sample composition were found according to geographical origin. From the preliminary screening propolis samples S107 and S108 were selected for fractionation. HPLC analysis showed the presence of all six marker compounds in both S107 and S108 samples in different proportions, Fractionation of S107 was carried out using reverse phase flash chromatography. In sample S107, the active fractions S107(F6) and S107(F7) were shown to have significant activity against *S.aureus* and at a concentration of 200 μg/ml they gave inhibition at 23.0 and 6.1% of the control respectively) as shown in (Figure 3-4). Fraction S107(F7) had stronger antibacterial activity against Gram positive bacteria than the crude sample S107. Fractions S107(F6), S107(F7) showed no activity against *E.coli* but had strong activity against *T.brucei* as shown in (Figure 3-7 and Figure 3-10). The collected fractions from S107 were further investigated by LCMS which confirmed the presence of the marker compounds. LCMS data also indicated the presence of esters most probably of pinobanksin which as expected were more abundant in the later fractions from the reverse phase flash column. The esters ranged from acetate to octanoyl and were probably substituted at the 3-position in the C-ring of the flavanol since they

exhibited neutral loss of the ester moiety under MS^2 conditions. Previous workers have observed the presence of esters in propolis [28]. There were also a number of methyl ethers in the samples. NMR spectra confirmed the presence of pinocembrin and pinobanksin esters in the samples. The presence of lipophilic esters might have facilitated the penetration of active components through bacteria cell membrane. The most active fraction was S107(F7) which contained abundant acetate and propionate esters the longer chain esters in fraction 8 and 9 were less active which fits with the idea that there is an optimal hydrophilic-lipophilic balance for antibacterial activity. Results from previous studies showed that antibacterial activity in Brazilian propolis was due to the presence of pinocembrin, galangin, pinobanksin, pinobanksin-3-acetate, and caffeic acid esters [39]. There is agreement between our results finding and the literature, were the propolis sample has significant activities against Gram positive bacteria while there are no remarkable activities against Gram negative bacteria [18], except for fraction S107(F9), were some activity against *E.coli* was detected, which may be due to the presence of components of the esters of pinobanksin as shown in LCMS. The pinobanksin esters in S107(F9) had long chains with high lipophilicity and the penetration of the cell membrane gram negative cell membrane might become possible. To confirm this, further purification of the fractions would be required.

Fractionations of crude S108 was carried out using reverse phase flash chromatography. In case of the S108 fractions, it seems that crude S108 had moderate activity against *S.aureus*. Fraction S108(F8) had stronger activity than the crude S108 while in fraction S108(F7) was slightly more active than crude material. Fractions S108(F5) and S108(F6) were slightly less active than crude material as shown in (Figure 3-3). There were no remarkable activities of S108 fractions against Gram negative bacteria except for fraction S108(F5), which had weak activity against *E.coli* as shown in (Figure 3-6) . Further chemical separation and fractionations for this fraction would be required to

identify the components that can penetrate cell wall of Gram negative bacteria. Regarding anticancer activities, propolis has been widely investigated for its anticancer activity. Antitumor action *in vivo* and *in vitro* has also been reported using propolis extracts or its isolated compounds[60]. Another study confirmed the cytotoxic activity of Brazilian red propolis against cancer cells as a synergetic effect of propolis components [60]. Other studies proved that methanolic propolis extract exhibited stronger cytotoxicity than a water extract [22]. The flavonoids and cinnamic acid derivatives, including CAPE have been found to exhibit anticancer activity. In addition, some terepenes were reported to possess some anticancer activity [52].

S108 has moderate cytotoxic activity and selectivity towards cancer cells as compared to normal epithelium cells, while the S107 displayed cytotoxic activity with less selectivity between normal cells and cancer cells Investigation of crude S107 and its fractions against different cell lines was carried out. Crude S108 had more cytotoxic activity than its fractions. Fractions of S107 sample showed more cytotoxic activity than the crude S107. Fractions S107(F6), S107(F7) have moderate activity while S107(F9) had significant activity. Fraction S107(F9) showed some activity against *E.coli* and cytotoxic activity.

Sample S263 (Solomon Islands) was investigated towards the end of the research work. It showed very interesting activity against *M.aurum* and *S.aureus.* The activity of S263 against *M.aurum* (Figure 3-8) was even stronger than that of ethambutol (positive control. Chemical screening of S263 by HPLC indicated there were no flavonoid compounds present since no peaks were observed in the HPLC trace. Thus the strong antimicrobial activity was not related to the flavonoid class of compounds. The analysis of sample by GCMS data and from ^{1}HNMR spectrum indicated that the sample contained triterpenes such as lupeol and lupeol acetate which are the main components of this sample along with sesquiterpenes. The EI Mass Spectrum of

confirmed presence of the lupeol acetate in S263 (Figure 3-23) [33]. Further fractionation and separation of components is recommended to obtain pure compound responsible for this valuable biological activity. Synergistic effects of terpenes within a sample can produce a more powerful effect than a single terpene [33]. Thus from this study it is evident that terpenoids alone, without flavonoids, can have potent antibacterial activity.

5 Conclusion

The propolis samples investigated had strong biological activity. It is potentially a rich source of biologically active lead compounds which could be developed into drugs. There is an advantage in propolis samples in that the bees have done the work of extracting biologically active materials from the plants. The finding that flavonoid esters have strong anti-microbial activity is interesting since flavonoid esters are accessible by chemical synthesis. Thus it would be of interest to take different commercially available flavonoids and make esters from them and test their anti-microbial action. The hydrophilic-lipophilic balance of the esters is important with regard to antimicrobial action. One sample from Solomon Islands S263 was found to have no UV absorbing peaks by HPLC but was highly active against *M.aurum, S.aureus,* and trypanosomes. This sample was examined by GC-MS and NMR was found to be rich in triterpenoids related to lupeol. S263 gave higher activity than ethambutol the positive control. This sample certainly merits further investigation and indeed this is currently continuing under another project with very promising results. The main problem of exploiting the biological activity of a natural product like propolis is to guarantee a secure supply of the material. However, a commercially promising drug might simplify this.

6 References

1. Viuda-Martos, M., Y. Ruiz-Navajas, J. Fernandez-Lopez, and J.A. Perez-Alvarez, *Functional properties of honey, propolis, and royal jelly.* Journal of Food Science, 2008. **73**(9): p. 117-124.
2. Cushnie, T.P.T. and A.J. Lamb, *Detection of galangin-induced cytoplasmic membrane damage in Staphylococcus aureus by measuring potassium loss.* Journal of Ethnopharmacology, 2005. **101**(1-3): p. 243-248.
3. Bankova, V., *Chemical diversity of propolis and the problem of standardization.* Journal of Ethnopharmacology, 2005. **100**(1-2): p. 114-117.
4. Marcucci, M.C., F. Ferreres, C. Garc a-Viguera, V.S. Bankova, S.L. De Castro, A.P. Dantas, P.H.M. Valente, and N. Paulino, *Phenolic compounds from Brazilian propolis with pharmacological activities.* Journal of Ethnopharmacology, 2001. **74**(2): p. 105-112.
5. Watson, D.G., E. Peyfoon, L. Zheng, D. Lu, V. Seidel, B. Johnston, J.A. Parkinson, and J. Fearnley, *Application of principal components analysis to 1H-NMR data obtained from propolis samples of different geographical origin.* Phytochemical Analysis, 2006. **17**(5).
6. Isla, M.I., J.F. Paredes-Guzman, M.I. Nieva-Moreno, H. Koo, and Y.K. Park, *Some chemical composition and biological activity of northern Argentine propolis.* Journal of Agricultural and Food Chemistry, 2005. **53**(4): p. 1166.
7. Kartal, M., S. YIldIz, S. Kaya, S. Kurucu, and G. Topçu, *Antimicrobial activity of propolis samples from two different regions of Anatolia.* Journal of Ethnopharmacology, 2003. **86**(1): p. 69-73.
8. Bankova, V.S., S.S. Popov, and N.L. Marekov, *A study on flavonoids of propolis.* Journal of Natural Products, 1983. **46**(4): p. 471-474.
9. Gardana, C., M. Scaglianti, P. Pietta, and P. Simonetti, *Analysis of the polyphenolic fraction of propolis from different sources by liquid*

chromatography–tandem mass spectrometry. Journal of Pharmaceutical and Biomedical Analysis, 2007. **45**(3): p. 390-399.

10. Repollés, C., J.M. Herrero-Martínez, and C. Ràfols, *Analysis of prominent flavonoid aglycones by high-performance liquid chromatography using a monolithic type column.* Journal of Chromatography A, 2006. **1131**(1-2): p. 51-57.
11. Trusheva, B., M. Popova, V. Bankova, S. Simova, M.C. Marcucci, P.L. Miorin, F.R. Pasin, and I. Tsvetkova, *Bioactive constituents of Brazilian red propolis.* Evidence-based Complementary and Alternative Medicine, 2006. **3**(2): p. 249-254.
12. Volpi, N. and G. Bergonzini, *Analysis of flavonoids from propolis by on-line HPLC-electrospray mass spectrometry.* Journal of Pharmaceutical and Biomedical Analysis, 2007. **42**(3): p. 354-361.
13. Banskota, A.H., Y. Tezuka, J.K. Prasain, K. Matsushige, I. Saiki, and S. Kadota, *Chemical constituents of Brazilian propolis and their cytotoxic activities.* Journal of Natural Products, 1998. **61**(7): p. 896-900.
14. Burdock, G.A., *Review of the biological properties and toxicity of bee propolis (propolis).* Food and Chemical Toxicology, 1998. **36**(4): p. 347-363.
15. Kujumgiev, A., I. Tsvetkova, Y. Serkedjieva, V. Bankova, R. Christov, and S. Popov, *Antibacterial, antifungal and antiviral activity of propolis of different geographic origin.* Journal of Ethnopharmacology, 1999. **64**(3): p. 235-240.
16. Oršoli, N. and I. Baši, *Water-soluble derivative of propolis and its polyphenolic compounds enhance tumoricidal activity of macrophages.* Journal of Ethnopharmacology, 2005. **102**(1): p. 37-45.
17. Kumazawa, S., M. Yoneda, I. Shibata, J. Kanaeda, T. Hamasaka, and T. Nakayama, *Direct evidence for the plant origin of Brazilian propolis by the observation of honeybee behavior and phytochemical analysis.* Chemical & Pharmaceutical Bulletin, 2003. **51**(6): p. 740-742.

18. Seidel, V., E. Peyfoon, D.G. Watson, and J. Fearnley, *Comparative study of the antibacterial activity of propolis from different geographical and climatic zones.* Phytotherapy Research, 2008. **22**(9).

19. Alencar, S.M., T.L.C. Oldoni, M.L. Castro, I.S.R. Cabral, C.M. Costa-Neto, J.A. Cury, P.L. Rosalen, and M. Ikegaki, *Chemical composition and biological activity of a new type of Brazilian propolis: Red propolis.* Journal of Ethnopharmacology, 2007. **113**(2): p. 278-283.

20. Salomao, K., E.M. de Souza, A. Henriques-Pons, H.S. Barbosa, and S.L. de Castro, *Brazilian Green Propolis: Effects In Vitro and In Vivo on Trypanosoma cruzi.* Evidence-based Complementary and Alternative Medicine, 2009. **102**(1).

21. Isla, M.I., M.I. Nieva Moreno, A.R. Sampietro, and M.A. Vattuone, *Antioxidant activity of Argentine propolis extracts.* Journal of Ethnopharmacology, 2001. **76**(2): p. 165-170.

22. Banskota, A.H., Y. Tezuka, I.K. Adnyana, K. Midorikawa, K. Matsushige, D. Message, A.A.G. Huertas, and S. Kadota, *Cytotoxic, hepatoprotective and free radical scavenging effects of propolis from Brazil, Peru, the Netherlands and China.* Journal of Ethnopharmacology, 2000. **72**(1-2): p. 239-246.

23. Park, Y.K., S.M. Alencar, and C.L. Aguiar, *Botanical origin and chemical composition of Brazilian propolis.* J. Agric. Food Chem, 2002. **50**(9): p. 2502-2506.

24. Salomao, K., P.R.S. Pereira, L.C. Campos, C.M. Borba, P.H. Cabello, M.C. Marcucci, and S.L. De Castro, *Brazilian propolis: correlation between chemical composition and antimicrobial activity.* Evidence-based Complementary and Alternative Medicine, 2008. **5**(3): p. 317.

25. Bohm, B.A., *Introduction to flavonoids*. Vol. 2. 1998: Harwood Academic Publishers

26. Kumazawa, S., H. Goto, T. Hamasaka, S. Fukumoto, T. Fujimoto, and T. Nakayama, *A new prenylated flavonoid from propolis collected in*

Okinawa, Japan. Bioscience, Biotechnology, and Biochemistry, 2004. **68**(1): p. 260-262.

27. Simons, R., J.P. Vincken, E.J. Bakx, M.A. Verbruggen, and H. Gruppen, *A rapid screening method for prenylated flavonoids with ultra-high-performance liquid chromatography/electrospray ionisation mass spectrometry in licorice root extracts.* Rapid Communications in Mass Spectrometry, 2009. **23**(19): p. 3083-3093.
28. Santos, F.A., E.M.A. Bastos, M. Uzeda, M.A.R. Carvalho, L.M. Farias, E.S.A. Moreira, and F.C. Braga, *Antibacterial activity of Brazilian propolis and fractions against oral anaerobic bacteria.* Journal of Ethnopharmacology, 2002. **80**(1): p. 1-7.
29. Ogeturk, M., I. Kus, N. Colakoglu, I. Zararsiz, N. Ilhan, and M. Sarsilmaz, *Caffeic acid phenethyl ester protects kidneys against carbon tetrachloride toxicity in rats.* Journal of Ethnopharmacology, 2005. **97**(2): p. 273-280.
30. Sonmez, S., L. Kirilmaz, M. Yucesoy, B. Yücel, and B. Yilmaz, *The effect of bee propolis on oral pathogens and human gingival fibroblasts.* Journal of Ethnopharmacology, 2005. **102**(3): p. 371-376.
31. Ceschel, G.C., P. Maffei, A. Sforzini, S. Lombardi Borgia, A. Yasin, and C. Ronchi, *In vitro permeation through porcine buccal mucosa of caffeic acid phenetyl ester (CAPE) from a topical mucoadhesive gel containing propolis.* Fitoterapia, 2002. **73**: p. S44-S52.
32. Ivanauskas, L., V. Jakstas, J. Radusiene, A. Lukosius, and A. Baranauskas, *Evaluation of phenolic acids and phenylpropanoids in the crude drugs.* Medicina (Kaunas, Lithuania), 2008. **44**(1): p. 48.
33. El Hady, F.K.A. and A.G. Hegazi, *Egyptian propolis: 2. Chemical composition, antiviral and antimicrobial activities of East Nile Delta propolis.* Extraction, 2000.

34. Kobayashi, A., H. Okamoto, and F. Okada, *Antiseptic bactericides and cosmetics, drugs and foods containing the antiseptic bactericides*, in *EUROPEAN PATENT APPLICATION*. 2005

35. Salatino, A., E.W. Teixeira, and G. Negri, *Origin and chemical variation of Brazilian propolis.* Evidence-based Complementary and Alternative Medicine, 2005. **2**(1): p. 33.

36. Yildirim, Z., S. Hacievliyagil, N.O. Kutlu, N.E. Aydin, M. Kurkcuoglu, M. Iraz, and R. Durmaz, *Effect of water extract of Turkish propolis on tuberculosis infection in guinea-pigs.* Pharmacological Research, 2004. **49**(3): p. 287-292.

37. Hegazi, A.G. and F.K.A. El Hady, *Egyptian propolis: 3. Antioxidant, antimicrobial activities and chemical composition of propolis from reclaimed lands.* Zeitschrift für Naturforschung - C, 2002. **57**(3/4): p. 395-402.

38. Vaher, M. and M. Koel, *Separation of polyphenolic compounds extracted from plant matrices using capillary electrophoresis.* Journal of Chromatography A, 2003. **990**(1-2): p. 225-230.

39. Park, Y.K., M.H. Koo, J.A.S. Abreu, M. Ikegaki, J.A. Cury, and P.L. Rosalen, *Antimicrobial activity of propolis on oral microorganisms.* Current Microbiology, 1998. **36**(1): p. 24-28.

40. Qi, Y., A. Sun, R. Liu, Z. Meng, and H. Xie, *Isolation and purification of flavonoid and isoflavonoid compounds from the pericarp of Sophora japonica L. by adsorption chromatography on 12% cross-linked agarose gel media.* Journal of Chromatography A, 2007. **1140**(1-2): p. 219-224.

41. Uzel, A., K. Sorkun, Ö. Önça, D. Ço ulu, Ö. Gençay, and B. Sali h, *Chemical compositions and antimicrobial activities of four different Anatolian propolis samples.* Microbiological Research, 2005. **160**(2): p. 189-195.

42. Castaldoa, S. and F. Capasso, *Propolis, an old remedy used in modern medicine* Fitoterapia, 2002. **73**: p. S1-S6

43. Orsi, R.O., J.M. Sforcin, V.L.M. Rall, S.R.C. Funari, L. Barbosa, and J.R.A. Fernandes, *Susceptibility profile of Salmonella against the antibacterial activity of propolis produced in two regions of Brazil.* Journal of Venomous Animals and Toxins including Tropical Diseases, 2005. **11**: p. 109-116.

44. Murad, J.M., S.A. Calvi, A. Soares, V. Bankova, and J.M. Sforcin, *Effects of propolis from Brazil and Bulgaria on fungicidal activity of macrophages against Paracoccidioides brasiliensis.* Journal of Ethnopharmacology, 2002. **79**(3): p. 331-334.

45. Hayacibara, M.F., H. Koo, P.L. Rosalen, S. Duarte, E.M. Franco, W.H. Bowen, M. Ikegaki, and J.A. Cury, *In vitro and in vivo effects of isolated fractions of Brazilian propolis on caries development.* Journal of Ethnopharmacology, 2005. **101**(1-3): p. 110-115.

46. Blonska, M., J. Bronikowska, G. Pietsz, Z.P. Czuba, S. Scheller, and W. Krol, *Effects of ethanol extract of propolis (EEP) and its flavones on inducible gene expression in J774A. 1 macrophages.* Journal of Ethnopharmacology, 2004. **91**(1): p. 25-30.

47. Hu, F., H.R. Hepburn, Y. Li, M. Chen, S.E. Radloff, and S. Daya, *Effects of ethanol and water extracts of propolis (bee glue) on acute inflammatory animal models.* Journal of Ethnopharmacology, 2005. **100**(3): p. 276-283.

48. Maciejewicz, W., M. Daniewski, K. Bal, and W. Markowski, *GC-MS identification of the flavonoid aglycones isolated from propolis.* Chromatographia, 2001. **53**(5): p. 343-346.

49. Kartal, M., S. Kaya, and S. Kurucu, *GC-MS analysis of propolis samples from two different regions of Turkey.* Zeitschrift Fur Naturforschung C-Journal of Biosciences, 2002. **57**(9-10): p. 905-909.

50. Volpi, N., *Separation of flavonoids and phenolic acids from propolis by capillary zone electrophoresis.* Electrophoresis, 2004. **25**(12).

51. Nilesh kumar, Mueen Ahmad K. K., Raman Dang, and A. Husain, *Antioxidant and antimicrobial activity of propolis from Tamil Nadu zone.* Journal of Medicinal Plants Research, 2008. **2**(12): p. 361-364.

52. Banskota, A.H., T. Nagaoka, L.Y. Sumioka, Y. Tezuka, S. Awale, K. Midorikawa, K. Matsushige, and S. Kadota, *Antiproliferative activity of the Netherlands propolis and its active principles in cancer cell lines.* Journal of Ethnopharmacology, 2002. **80**(1): p. 67-73.

53. Boyanova, L., S. Derejian, R. Koumanova, N. Katsarov, G. Gergova, I. Mitov, R. Nikolov, and Z. Krastev, *Inhibition of Helicobacter pylori growth in vitro by Bulgarian propolis: preliminary report.* Journal of Medical Microbiology, 2003. **52**(5): p. 417.

54. Miorin, P.L., N.C. Levy Junior, A.R. Custodio, W.A. Bretz, and M.C. Marcucci, *Antibacterial activity of honey and propolis from Apis mellifera and Tetragonisca angustula against Staphylococcus aureus.* Journal of Applied Microbiology, 2003. **95**(5): p. 913-920.

55. Orsi, R.O., J.M. Sforcin, S.R.C. Funari, A. Fernandes Junior, and V. Bankova, *Synergistic effect of propolis and Antibiotics on the Salmonella typhi.* Brazilian Journal of Microbiology, 2006. **37**: p. 108-112.

56. De Castro, S.L. and K.O. Higashi, *Effect of different formulations of propolis on mice infected with Trypanosoma cruzi.* Journal of Ethnopharmacology, 1995. **46**(1): p. 55-58.

57. Dantas, A.P., K. Salomão, H.S. Barbosa, and S.L. De Castro, *The effect of Bulgarian propolis against Trypanosoma cruzi and during its interaction with host cells.* Memórias do Instituto Oswaldo Cruz, 2006. **101**: p. 207-211.

58. Prytzyk, E., A.P. Dantas, K. Salomão, A.S. Pereira, V.S. Bankova, S.L.D. Castro, and F.R.A. Neto, *Flavonoids and trypanocidal activity of Bulgarian propolis.* Journal of Ethnopharmacology, 2003. **88**(2-3): p. 189-193.

59. Dantas, A.P., B.P. Olivieri, F.H.M. Gomes, and S.L. De Castro, *Treatment of Trypanosoma cruzi-infected mice with propolis promotes changes in the immune response.* Journal of Ethnopharmacology, 2006. **103**(2): p. 187-193.

60. Bufalo, M.C., J.M.G. Candeias, and J.M. Sforcin, *In vitro Cytotoxic Effect of Brazilian Green Propolis on Human Laryngeal Epidermoid Carcinoma(HEp-2) Cells.* Evidence-based Complementary and Alternative Medicine, 2009. **6**(4): p. 483.

61. O'Brien, J., I. Wilson, T. Orton, and F. Pognan, *Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity.* FEBS Journal, 2000. **267**(17): p. 5421.

62. De Carvalho Machado, G.M., L.L. Leon, and S.L. De Castro, *Activity of Brazilian and Bulgarian propolis against different species of Leishmania.* Memorias-instituto oswalodo cruz, 2007. **102**(1): p. 73.

63. Oršoli, N. and I. Baši, *Immunomodulation by water-soluble derivative of propolis: a factor of antitumor reactivity.* Journal of Ethnopharmacology, 2003. **84**(2-3): p. 265-273.

64. de Barros, M.P., J.P.B. Sousa, J.K. Bastos, and S.F. de Andrade, *Effect of Brazilian green propolis on experimental gastric ulcers in rats.* Journal of ethnopharmacology, 2007. **110**(3): p. 567-571.

65. Collins, L. and S.G. Franzblau, *Microplate alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against Mycobacterium tuberculosis and Mycobacterium avium.* Antimicrobial Agents and Chemotherapy, 1997. **41**(5): p. 1004-1009.

66. Baker, C.N. and F.C. Tenover, *Evaluation of Alamar colorimetric broth microdilution susceptibility testing method for staphylococci and enterococci.* Journal of Clinical Microbiology, 1996. **34**(11): p. 2654-2659.

67. Räz, B., M. Iten, Y. Grether-Bühler, R. Kaminsky, and R. Brun, *The Alamar Blue® assay to determine drug sensitivity of African trypanosomes*

(Tb rhodesiense and Tb gambiense) in vitro. Acta Tropica, 1997. **68**(2): p. 139-147.

68. Eberhard, B., *Structure elucidation by NMR in organic chemistry: a practical guide*. 1993, England: Wiley Press.

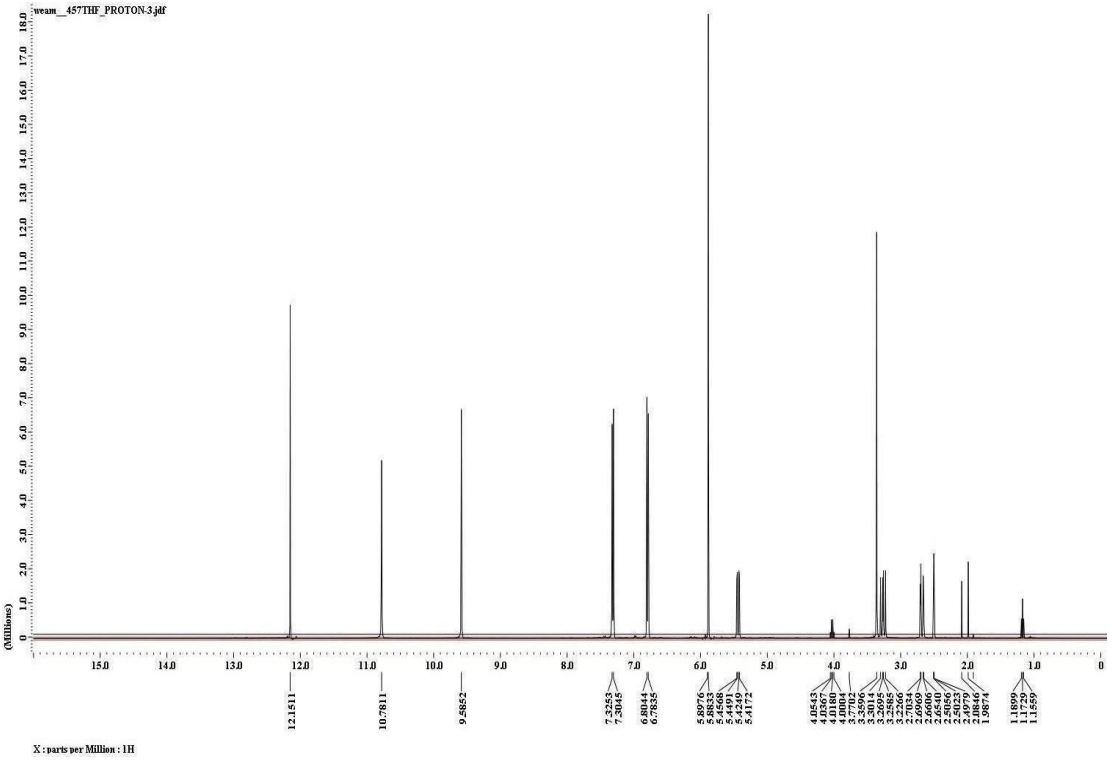
69. Fernandes Júnior, A., E.C. Balestrin, J.E.C. Betoni, R.O. Orsi, M. Cunha, and A.C. Montelli, *Propolis: anti-Staphylococcus aureus activity and synergism with antimicrobial drugs.* Memórias do Instituto Oswaldo Cruz, 2005. **100**: p. 563-566.

70. Pietta, P.G., C. Gardana, and A.M. Pietta, *Analytical methods for quality control of propolis.* Fitoterapia, 2002. **73**: p. 7-20.

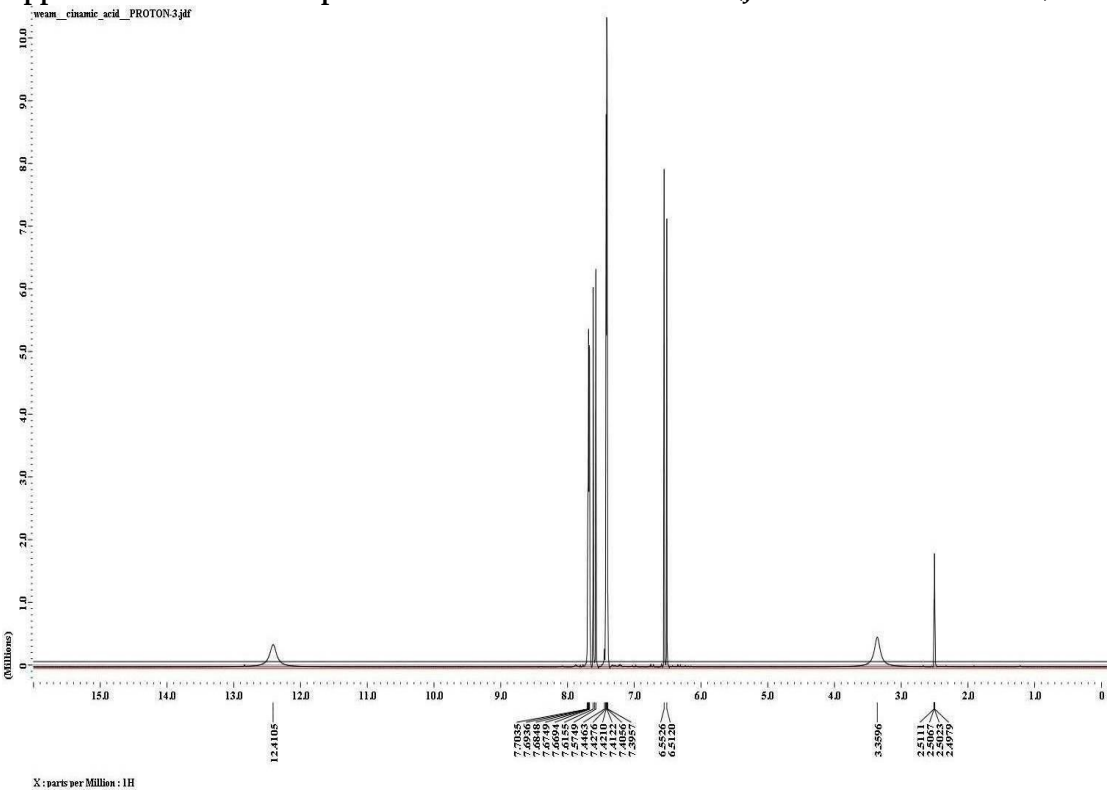
7 Appendix

7.1 Appendix 1 ^{1}HNMR Spectra

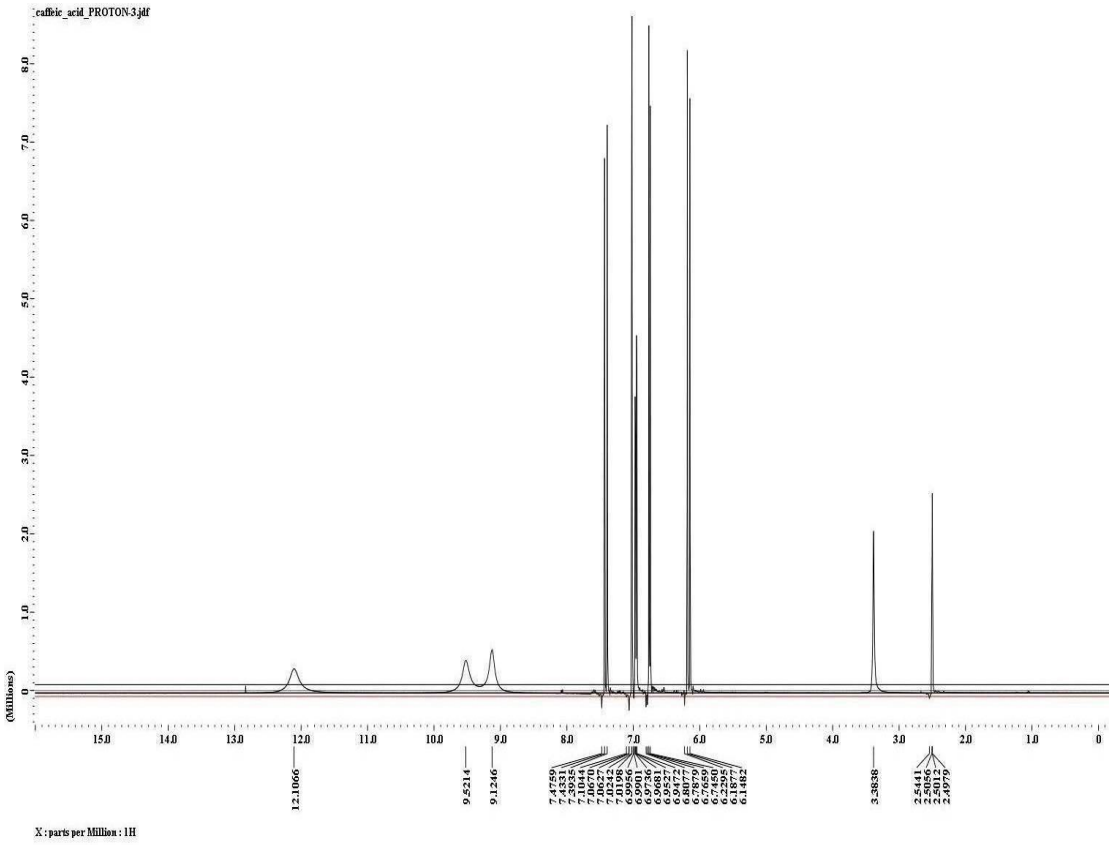
Appendix 7.1.1 ^{1}H NMR spectra of naringenin in DMSO (JEOL Delta GX 400 MHz).



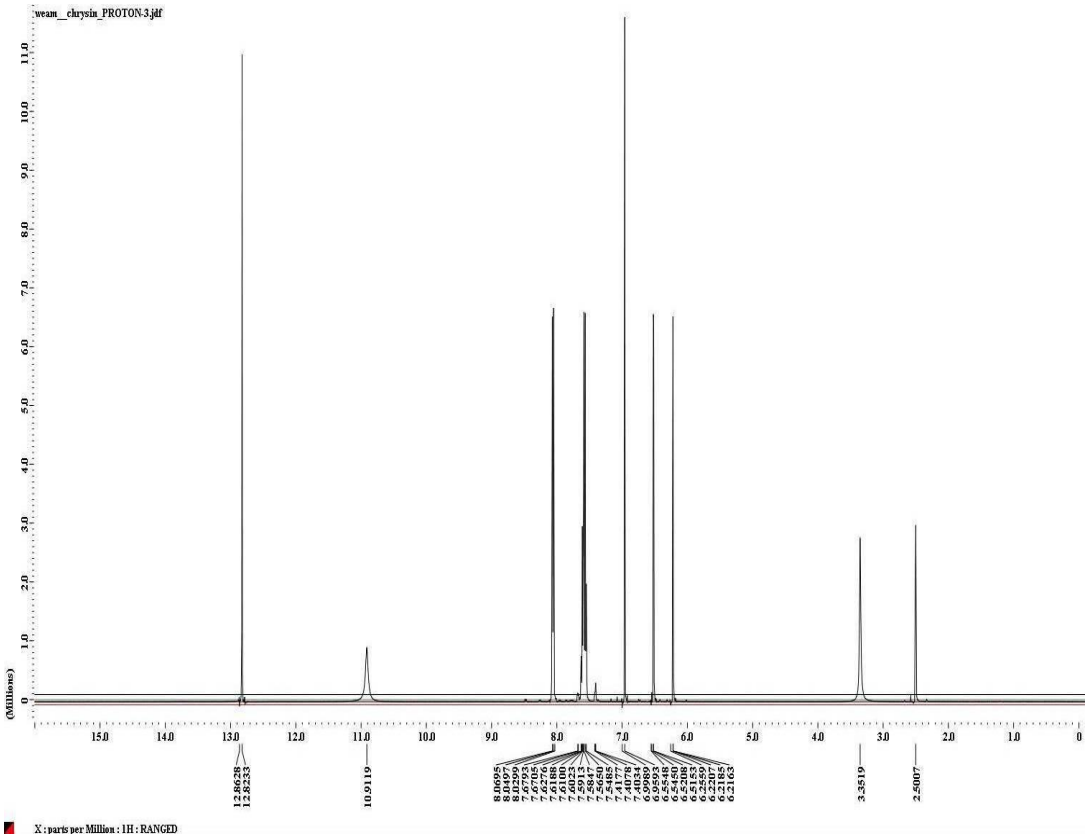
Appendix 7.1.2 ^{1}H NMR spectra of cinnamic acid in DMSO (JEOL Delta GX 400 MHz).



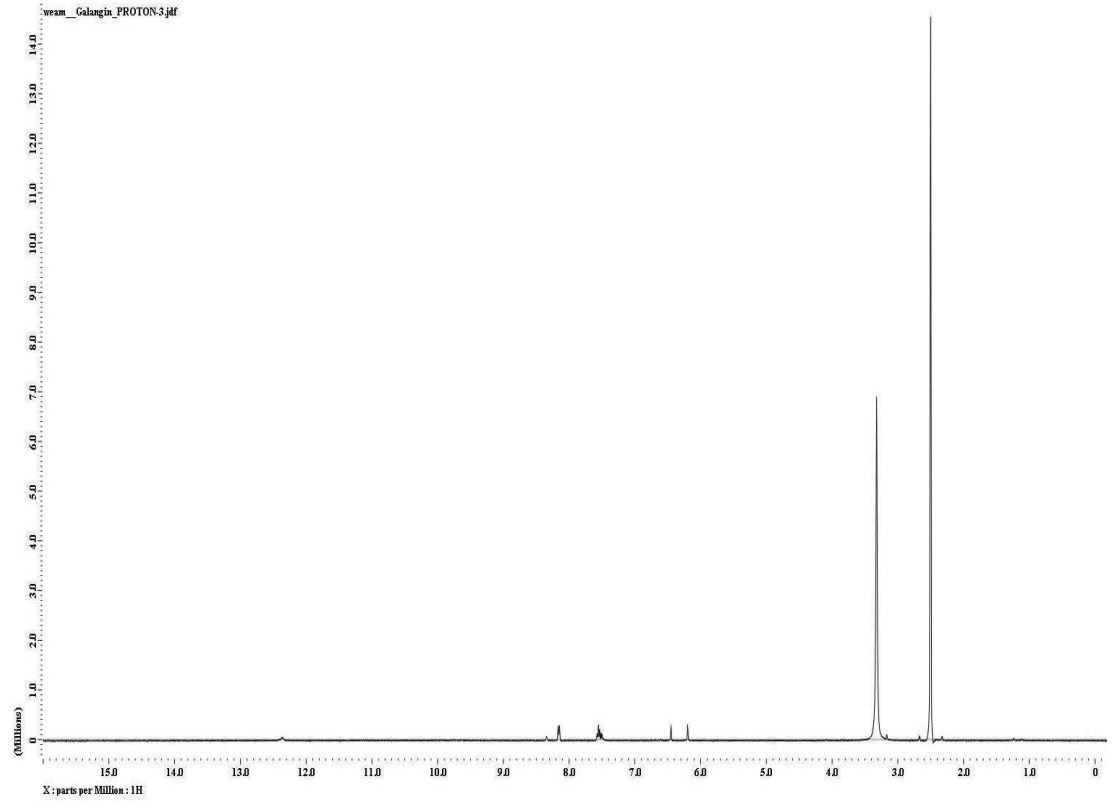
Appendix 7.1.3 ^{1}H NMR spectra of caffeic acid in DMSO (JEOL Delta GX 400 MHz).



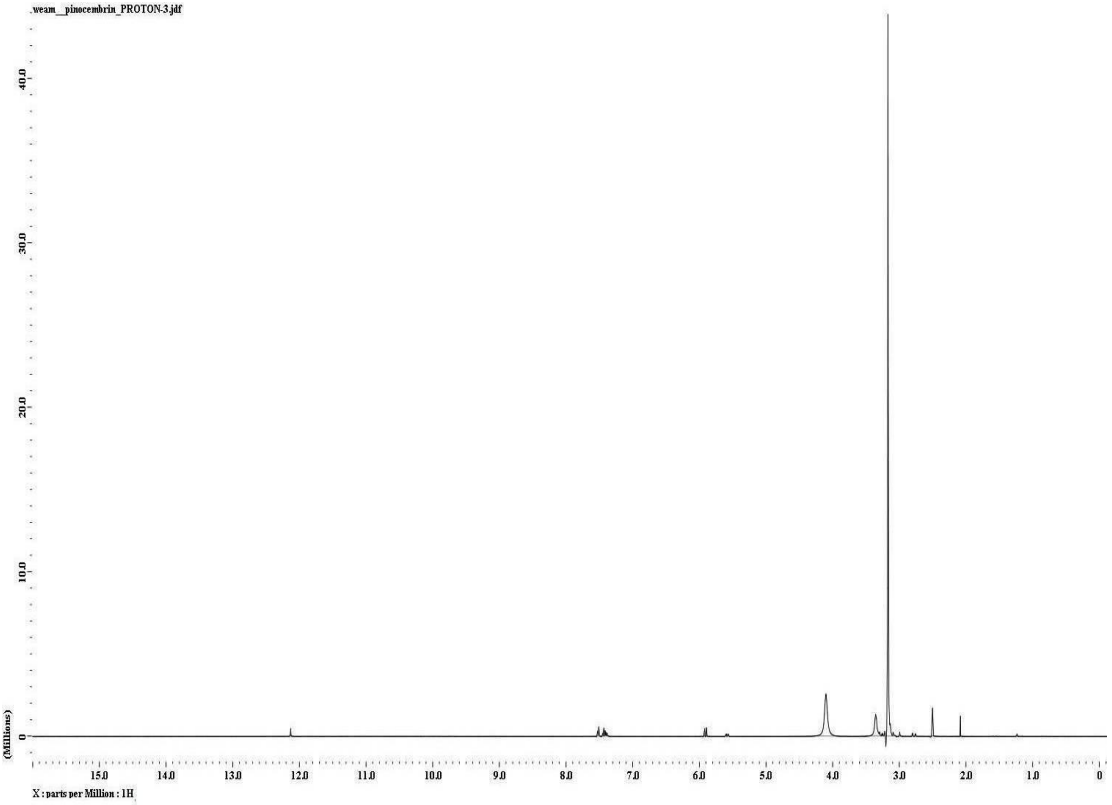
Appendix 7.1.4 ^{1}H NMR spectra of chrysin in DMSO (JEOL Delta GX 400 MHz).



Appendix 7.1.5 ^{1}H NMR spectra of galangin in DMSO) (JEOL Delta GX 400 MHz).

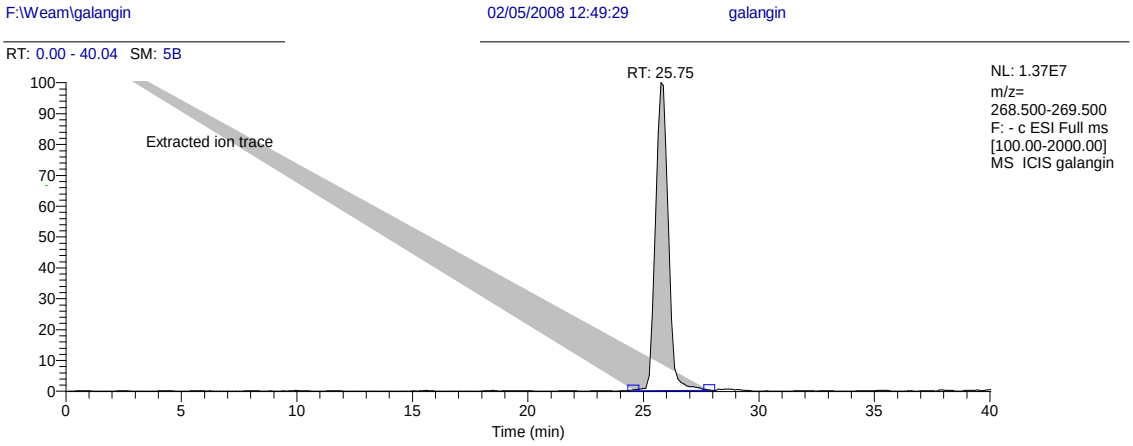


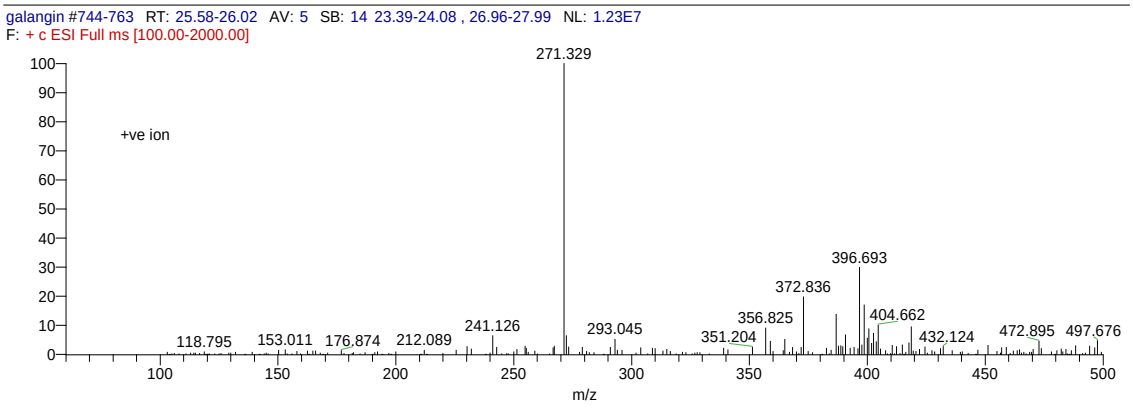
Appendix 7.1.6 ^{1}H NMR spectra of pinocembrin in DMSO (JEOL Delta GX 400 MHz).

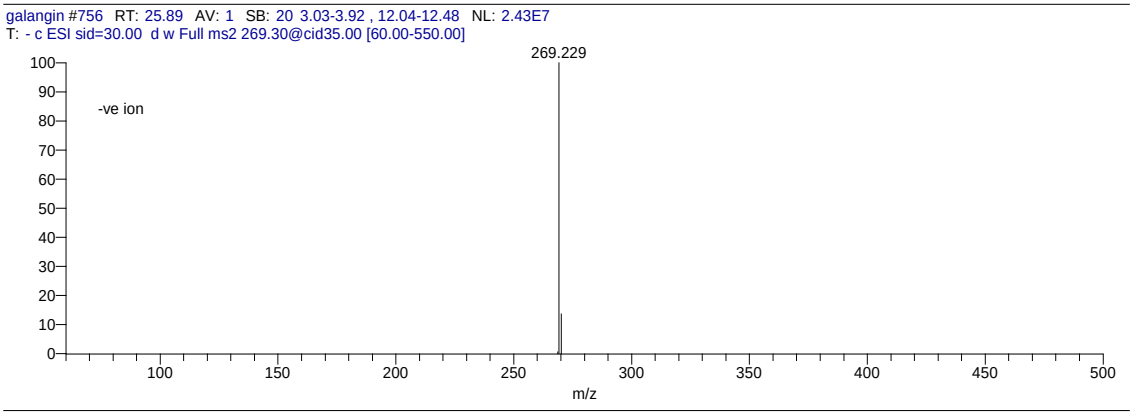


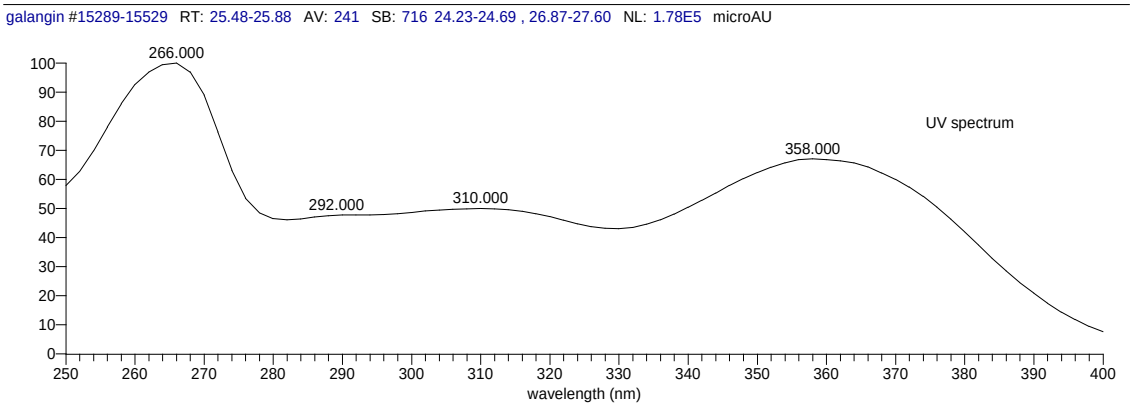
7.2 Appendix 2 LC-MS data for standard marker compounds and the fractions from S107

Appendix 7.2.1 Mass spectral and UV spectrum data for galangin

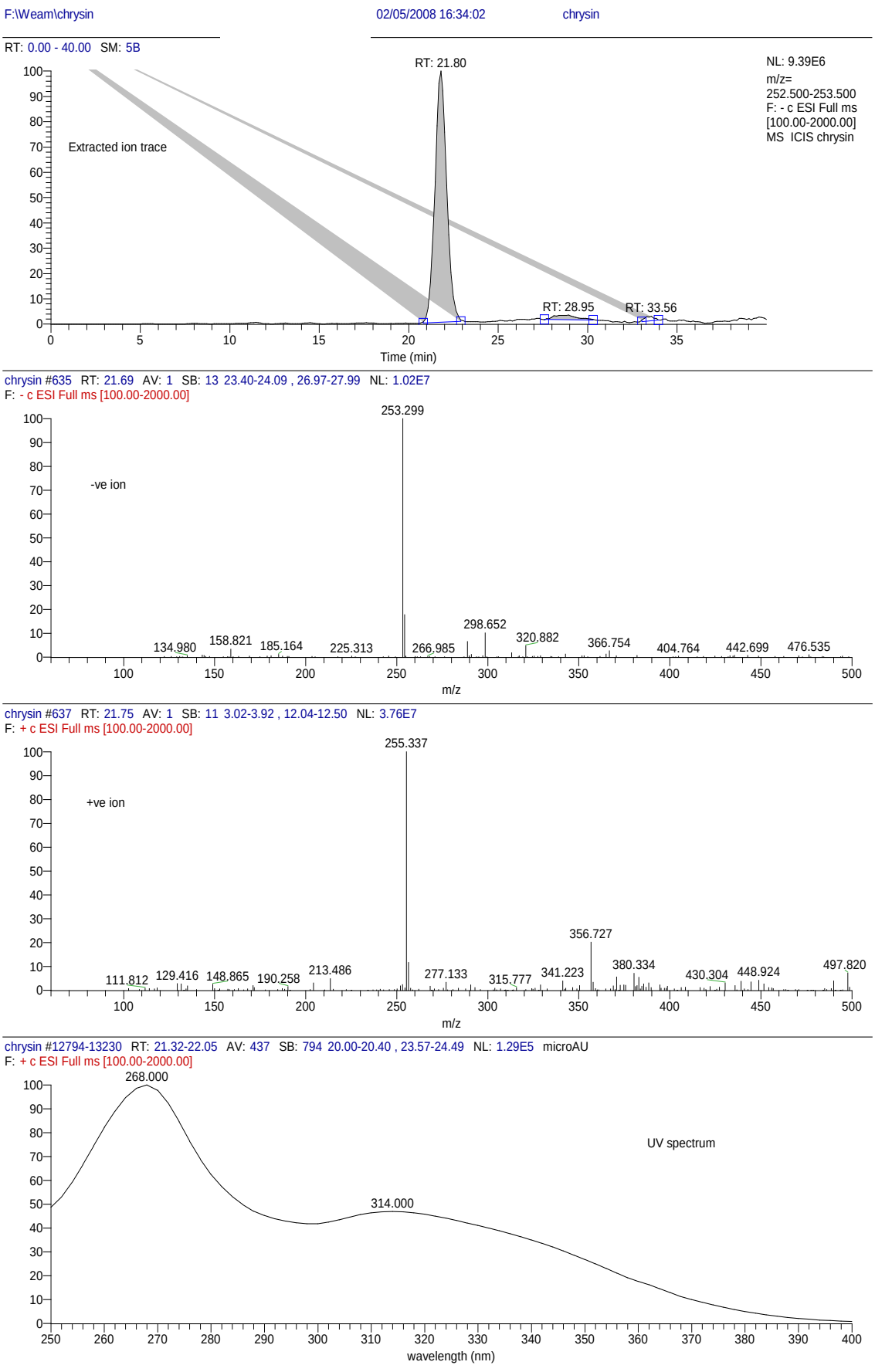




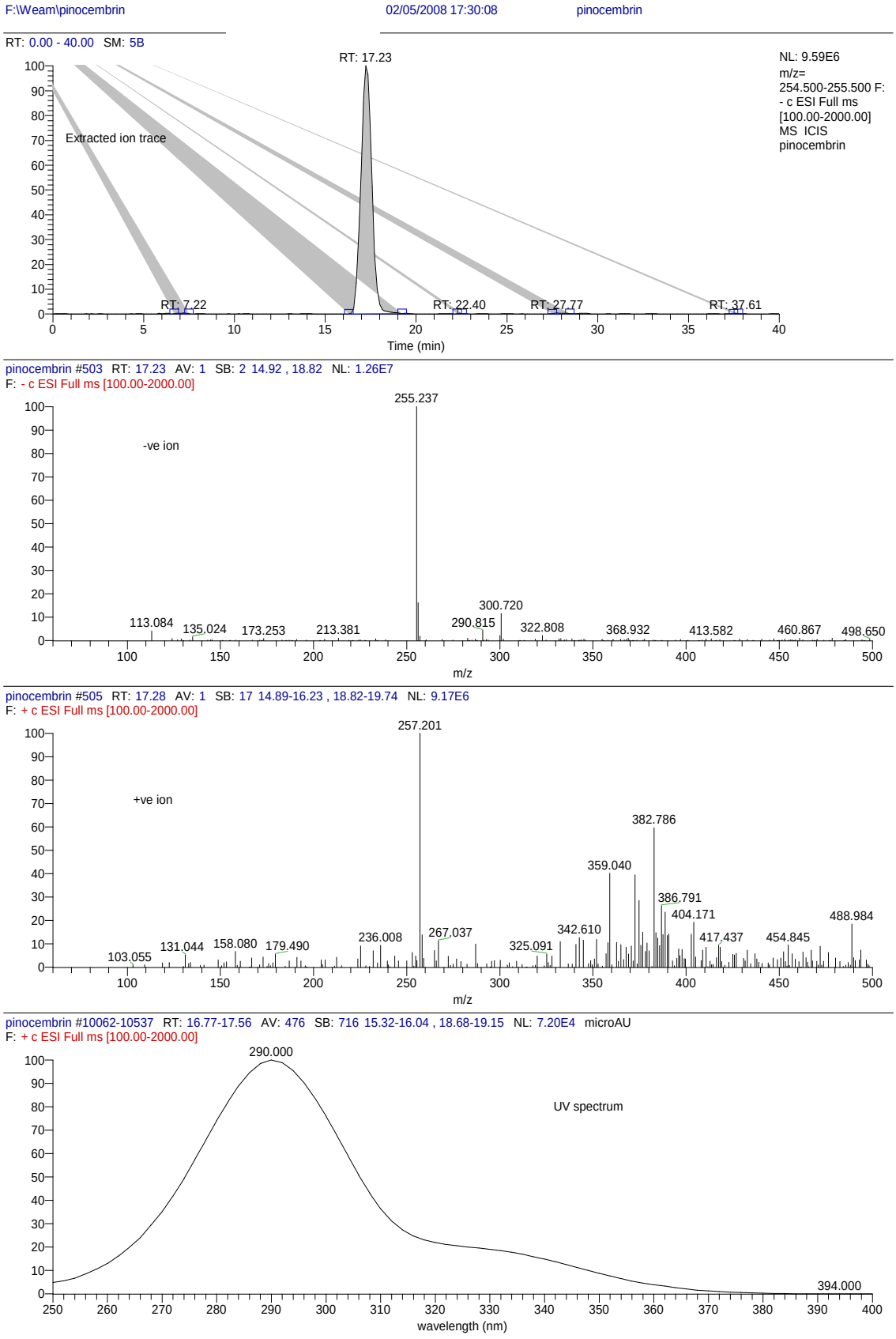




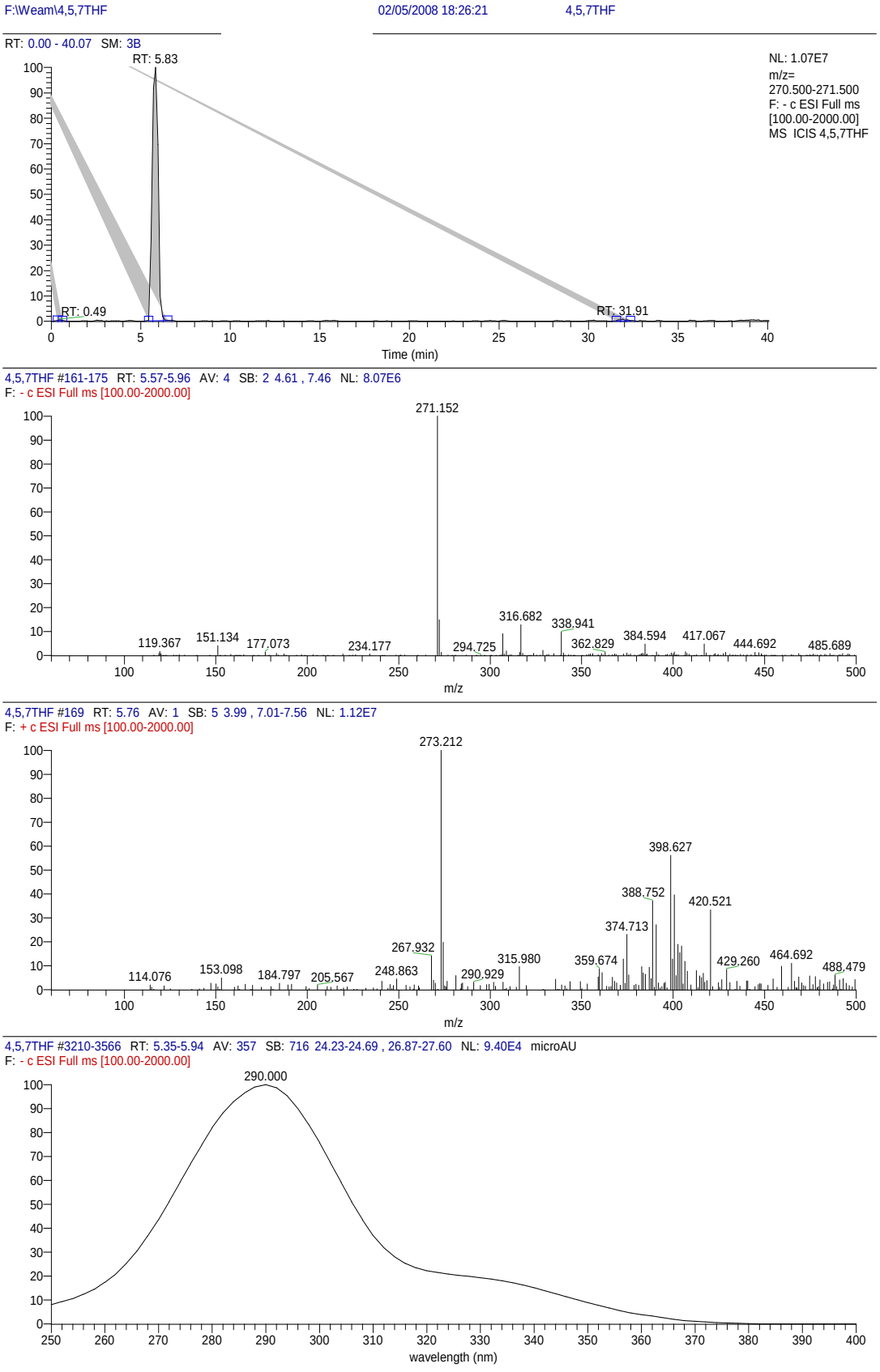
Appendix 7.2.2 Mass spectral and UV data for chrysin



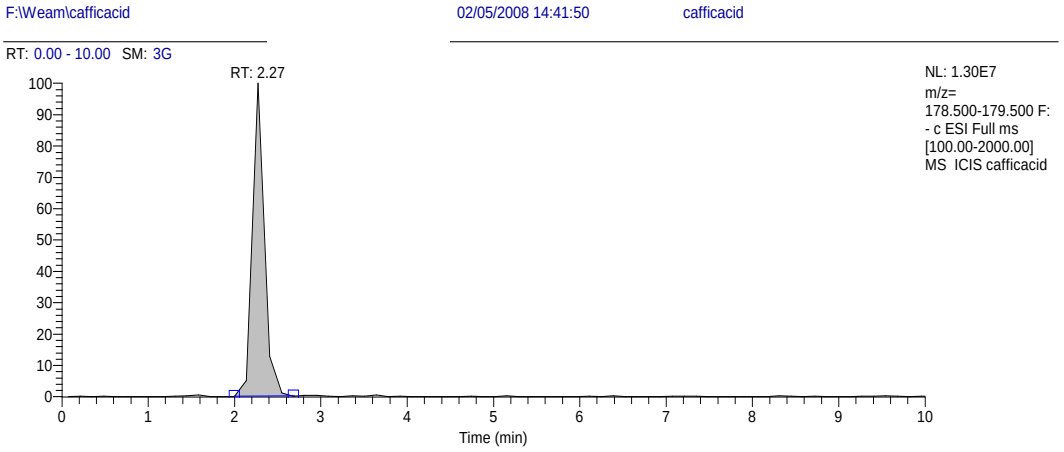
Appendix 7.2.3 Mass spectral and UV data for pinocembrin

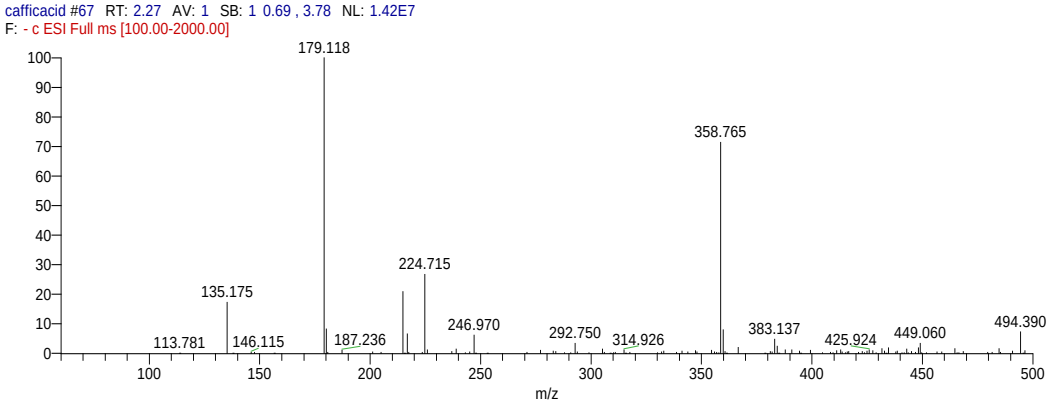


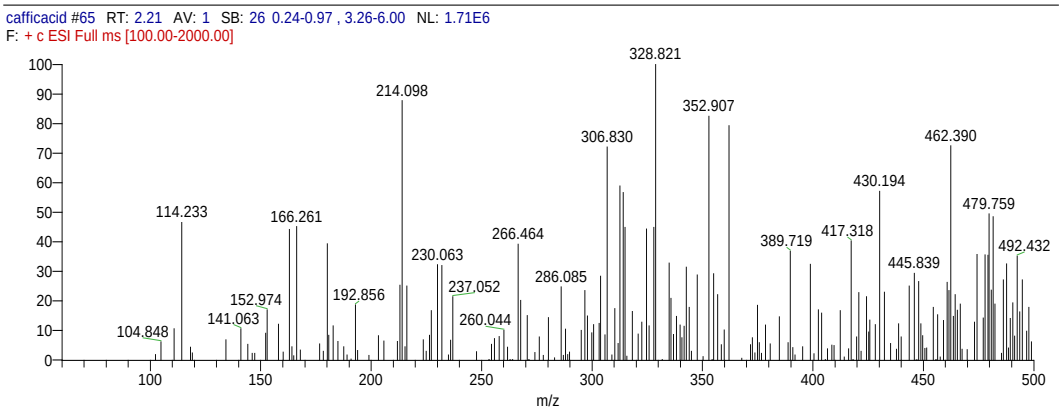
Appendix 7.2.4 Mass spectral and UV data for 4,5,7-trihydroxy flavanone

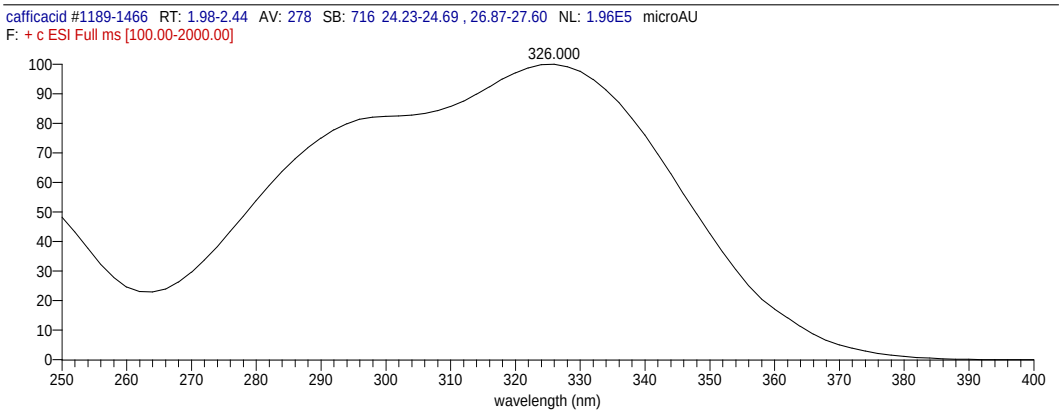


Appendix 7.2.5 Mass spectral and UV data for Caffeic acid





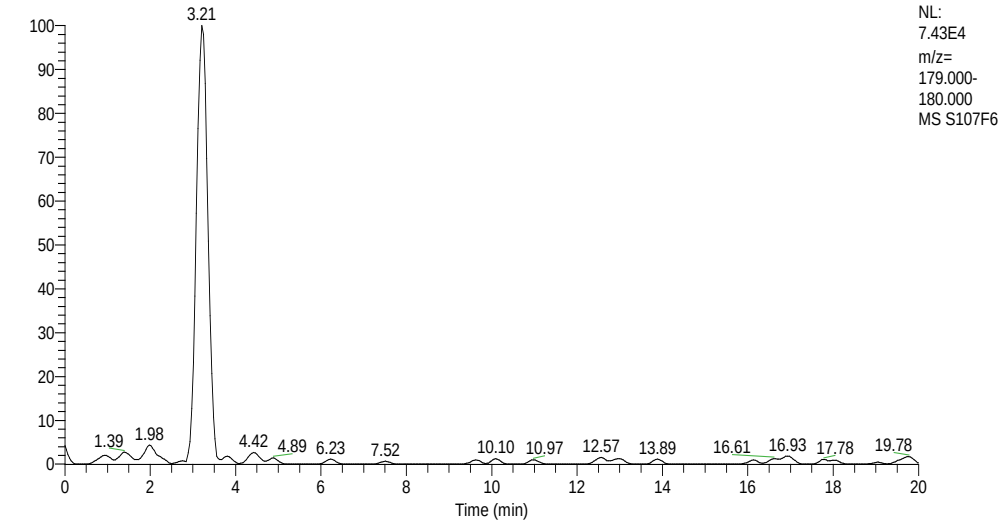




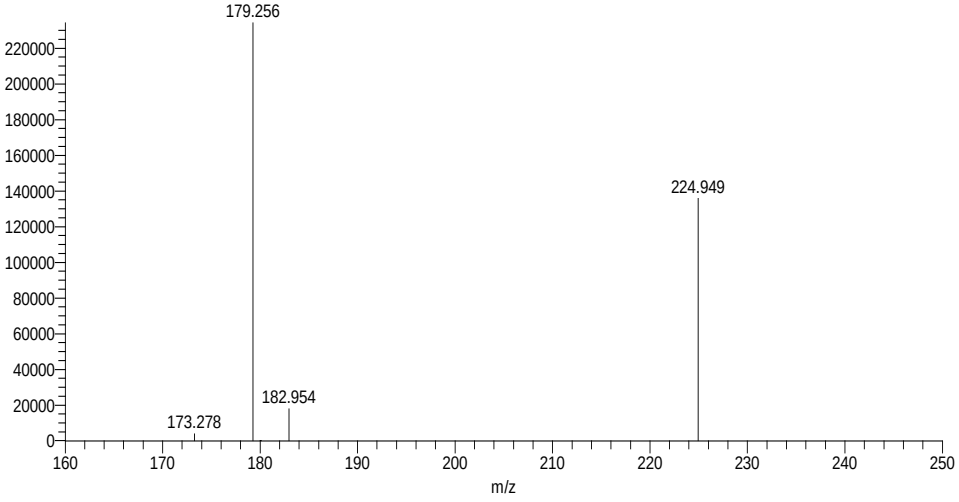
Appendix 7.2.6 UV and -ve ion MS spectra of caffeic acid in S107 fraction 6

F:\Weam\S107F6 17/10/2008 12:26:27 SIO7F6

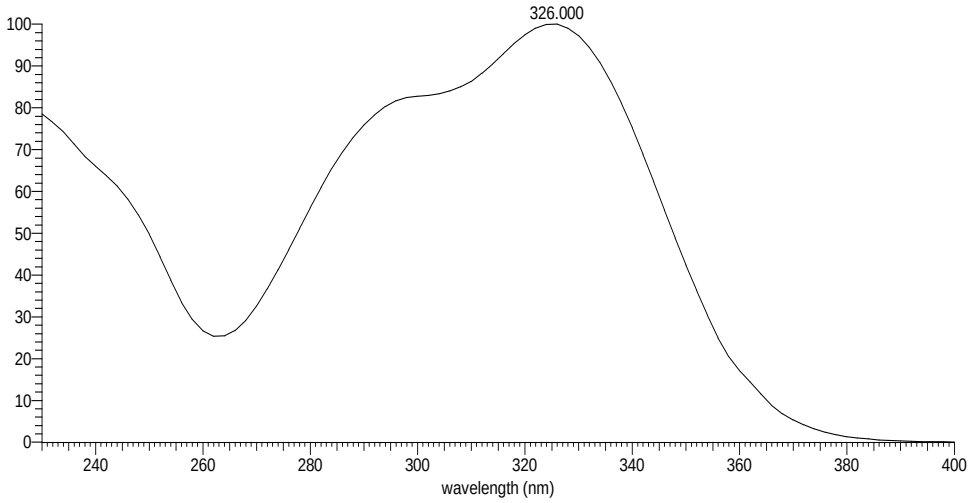
RT: 0.00 - 20.00 SM: 15G



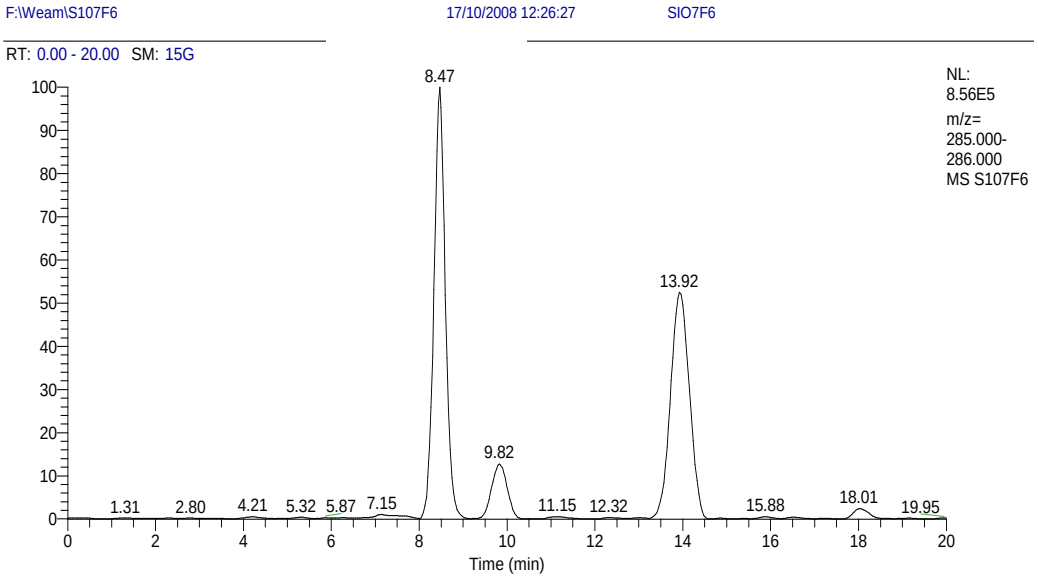
S107F6 #80 RT: 3.16 AV: 1 SB: 2 2.63 , 3.68 NL: 2.34E5
T: - c ESI Full ms [100.00-2000.00]

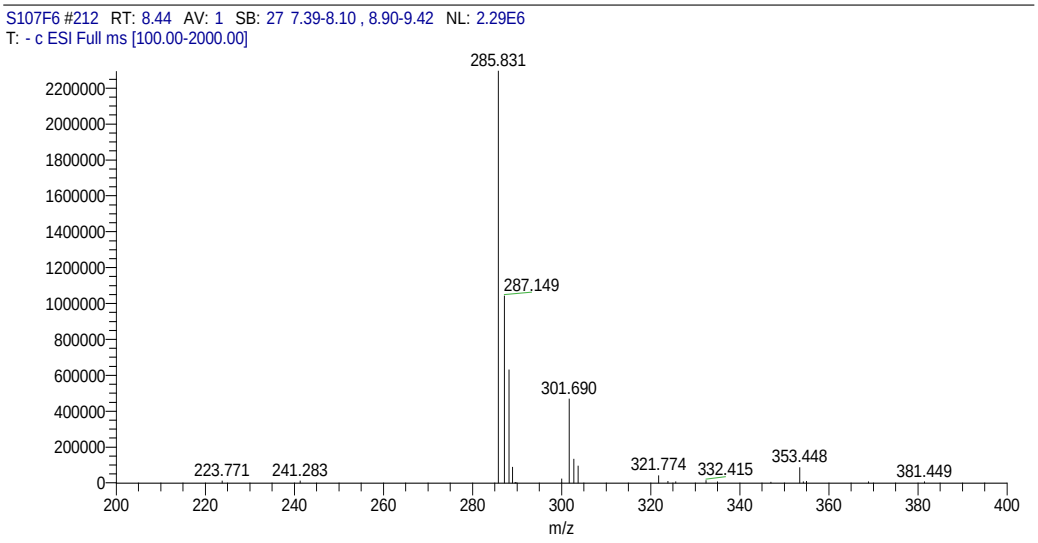


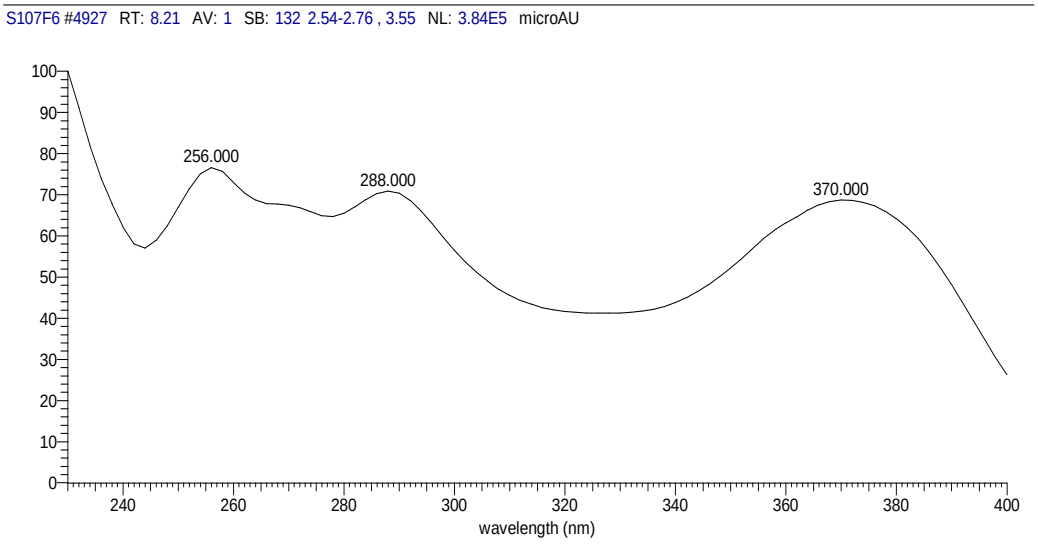
S107F6 #1844-1931 RT: 3.07-3.22 AV: 88 SB: 132 2.54-2.76 , 3.55 NL: 1.33E5 microAU



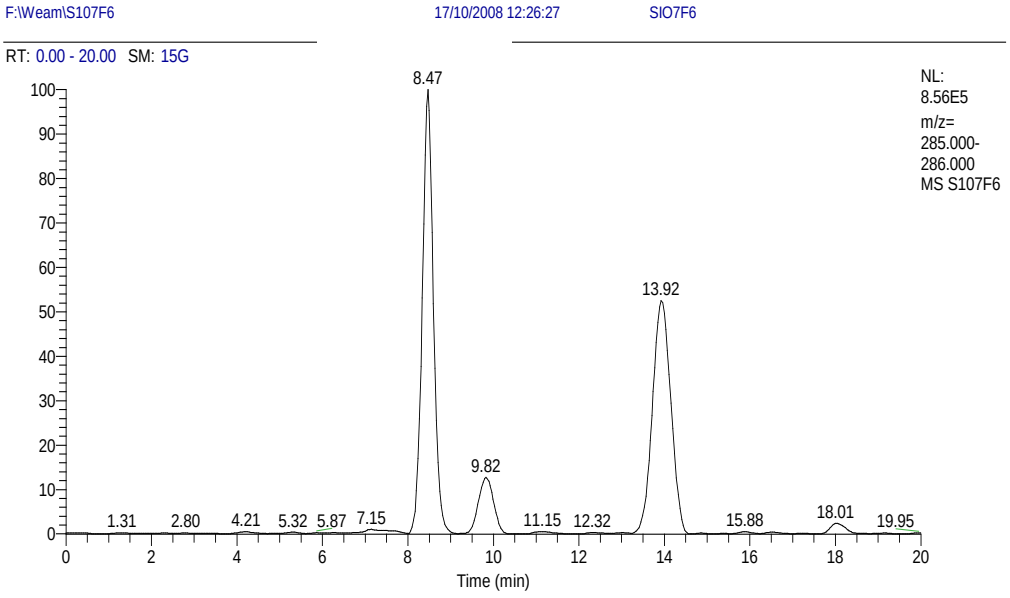
Appendix 7.2.7 UV and -ve ion MS spectra of kaempferol isomer 1 in S107 fraction 6

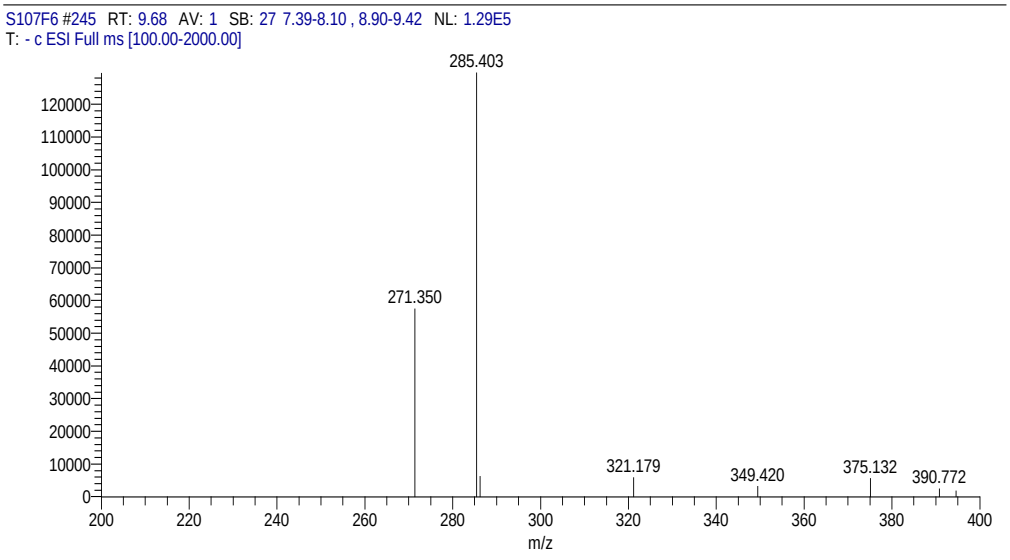


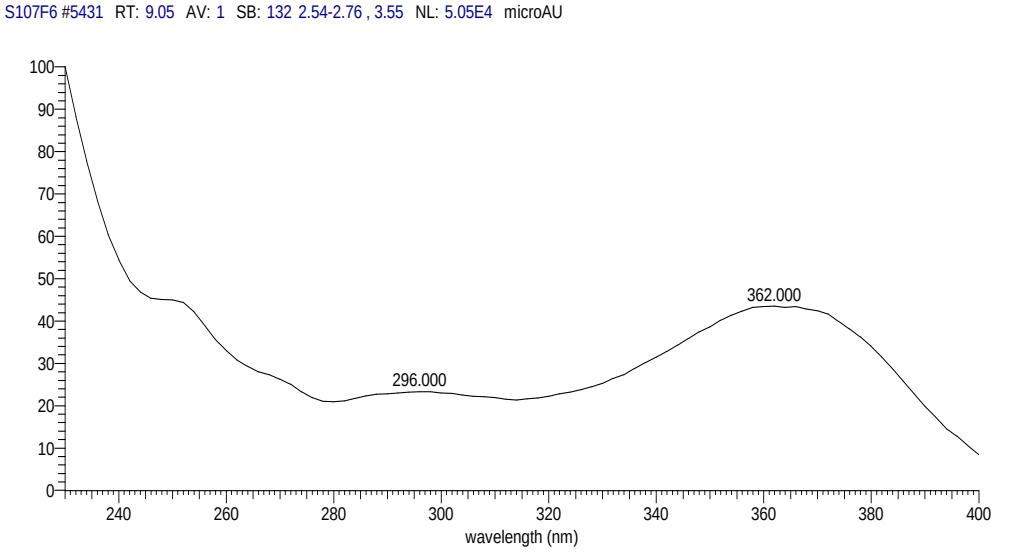




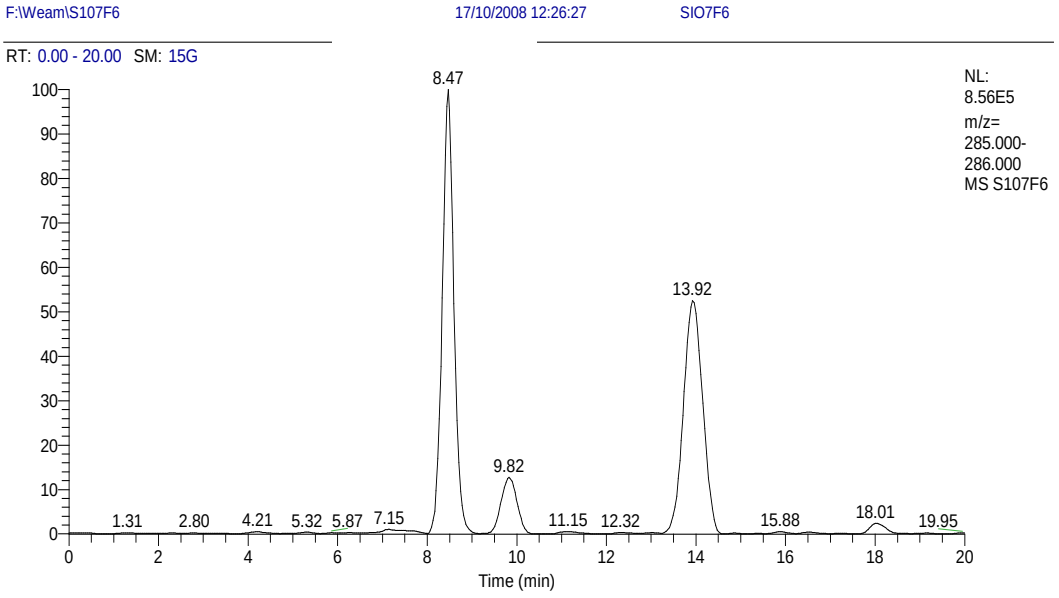
Appendix 7.2.8 UV and -ve ion MS spectra of kaempferol isomer 2 in S107 fraction 6

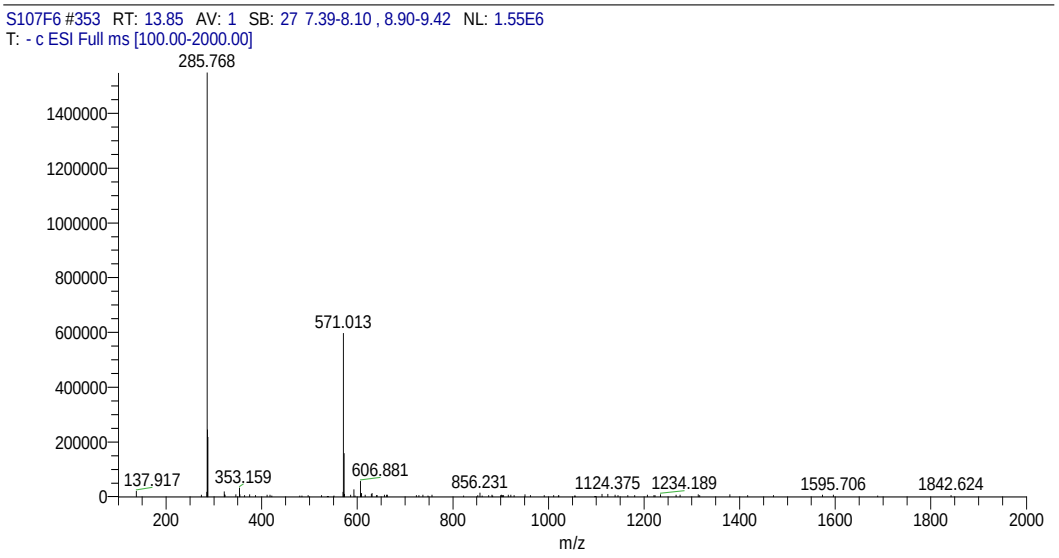


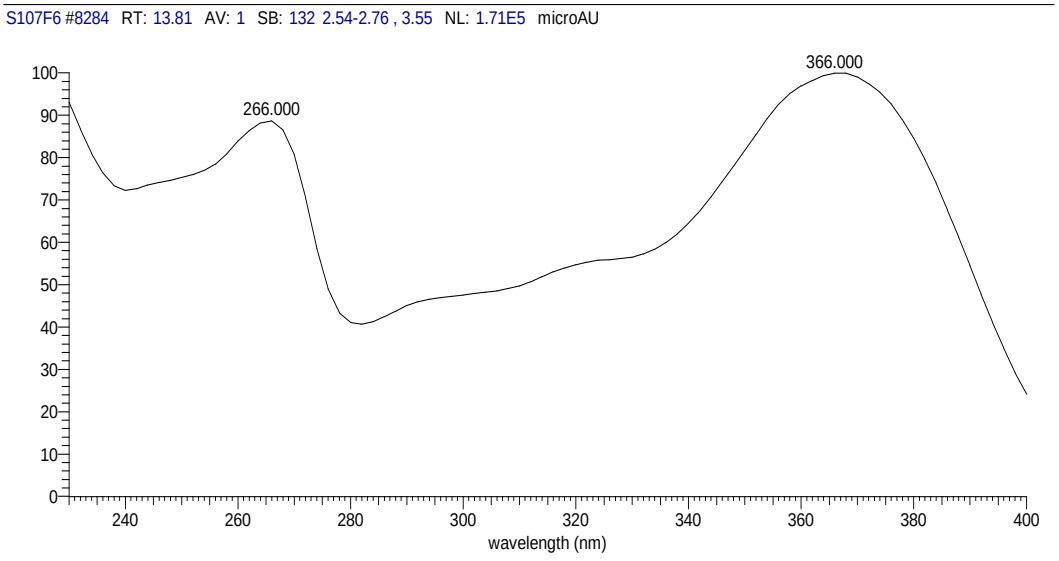




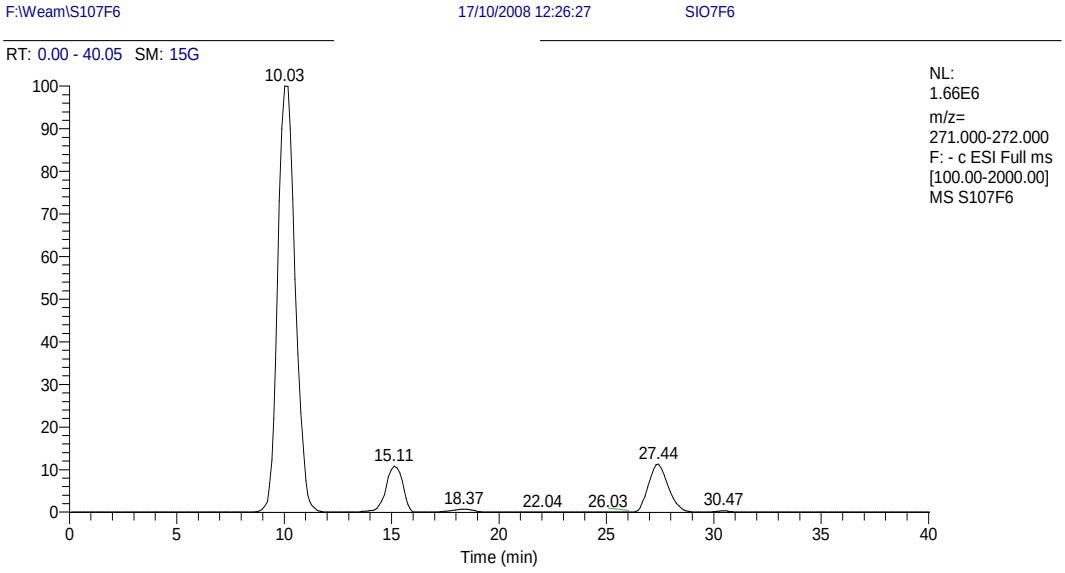
Appendix 7.2.9 UV and -ve ion MS spectra of kaempferol isomer 3 in S107 fraction 6

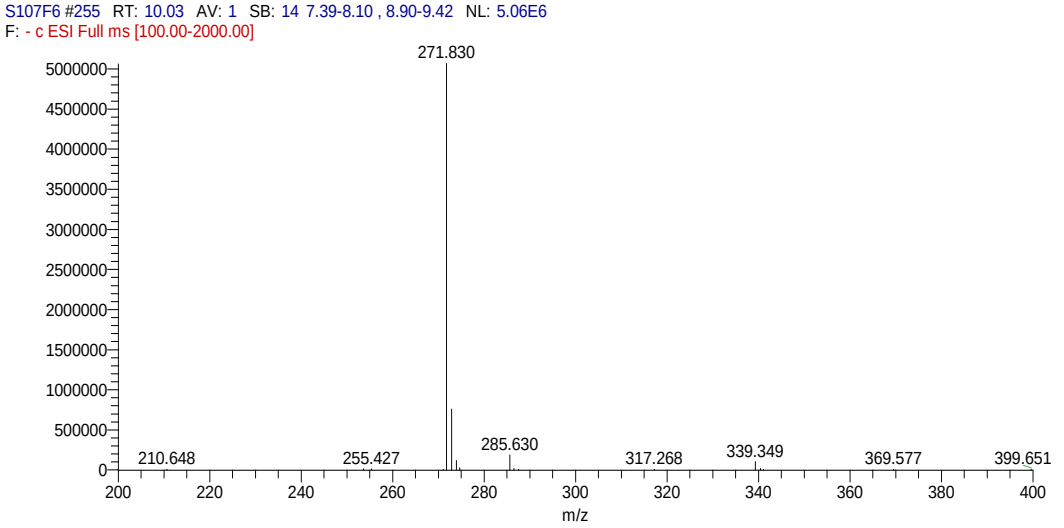


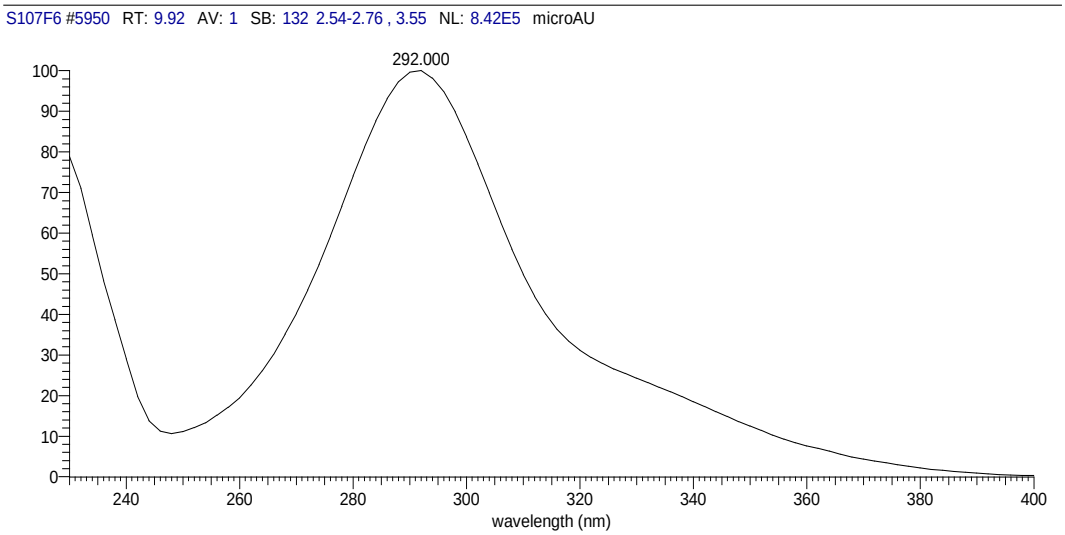




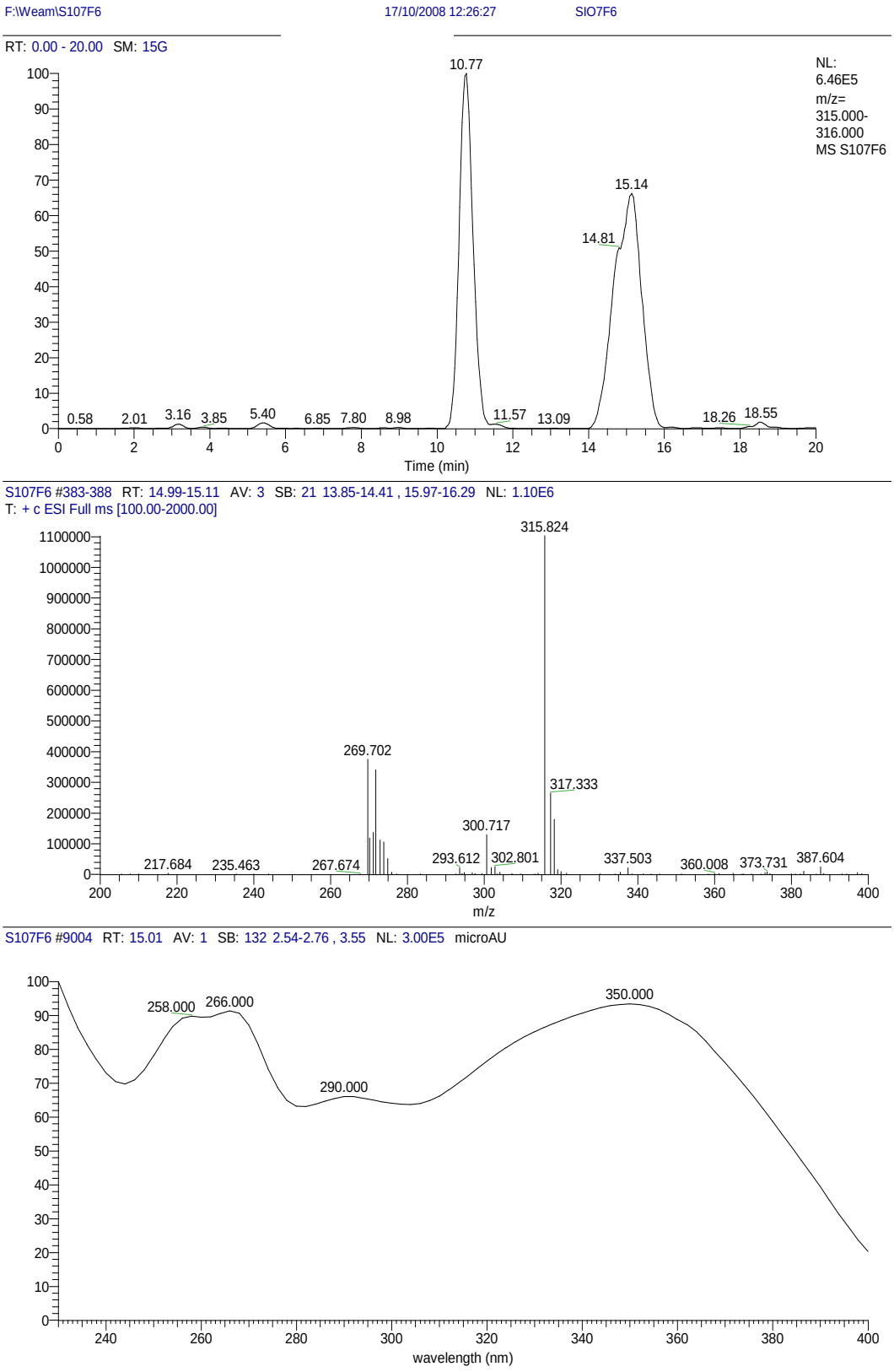
Appendix 7.2.10 UV and -ve ion MS spectra of pinobanksin in S107 fraction 6



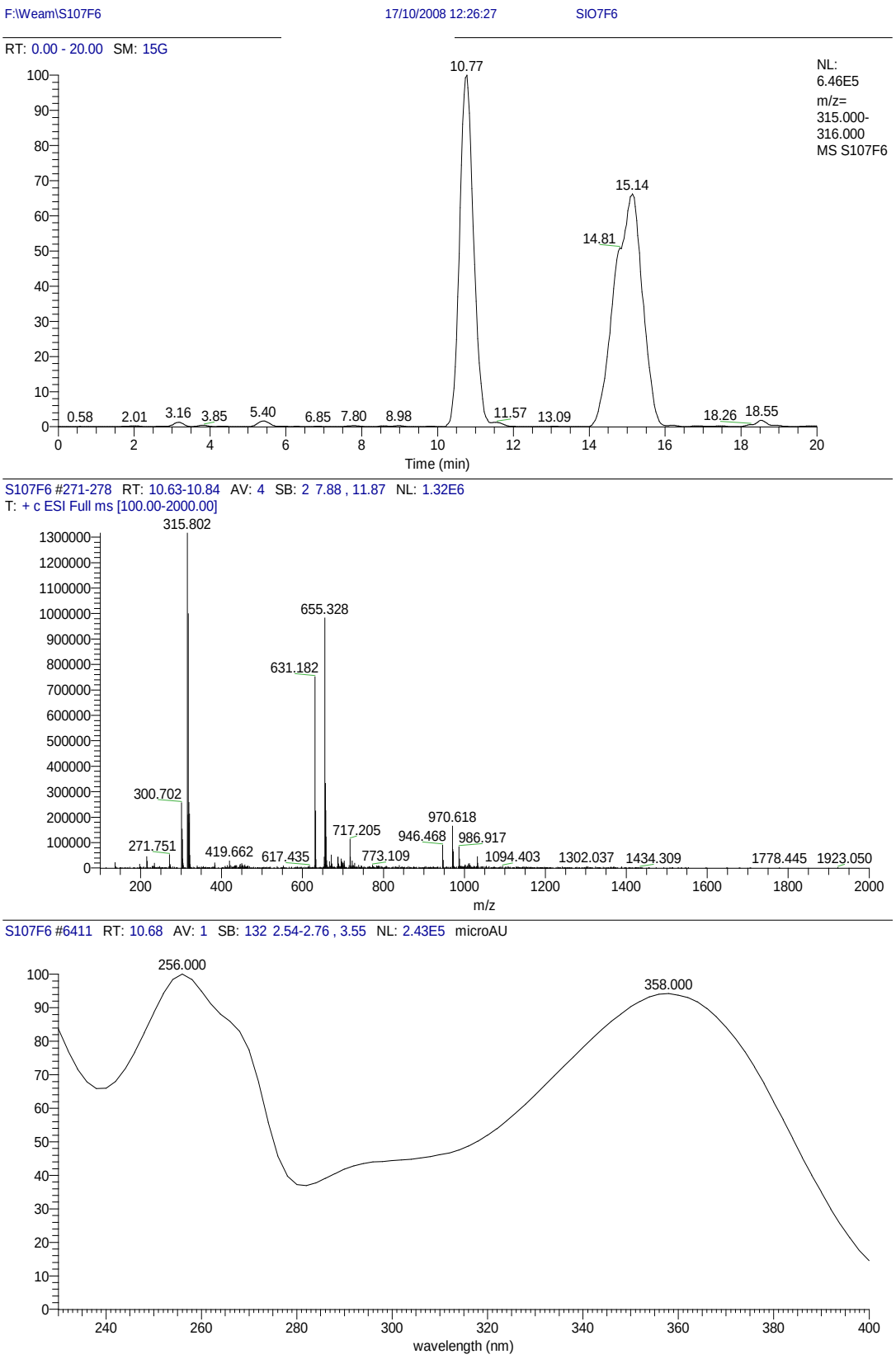




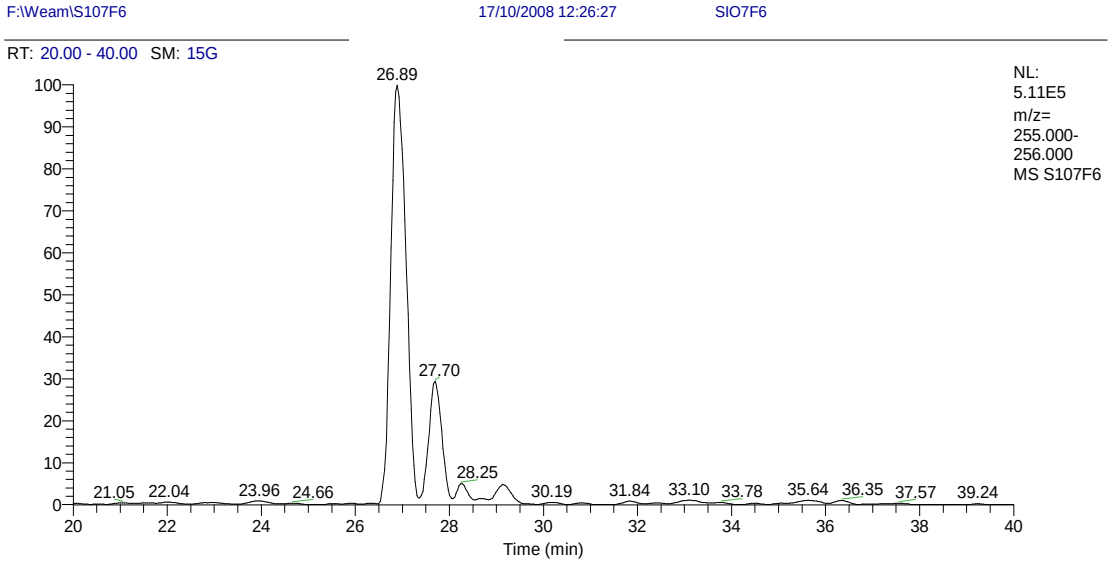
Appendix 7.2.11 UV and -ve ion MS spectra of galangin in S107 fraction 6 (base peak in mass spectrum is due to the formic acid adduct)

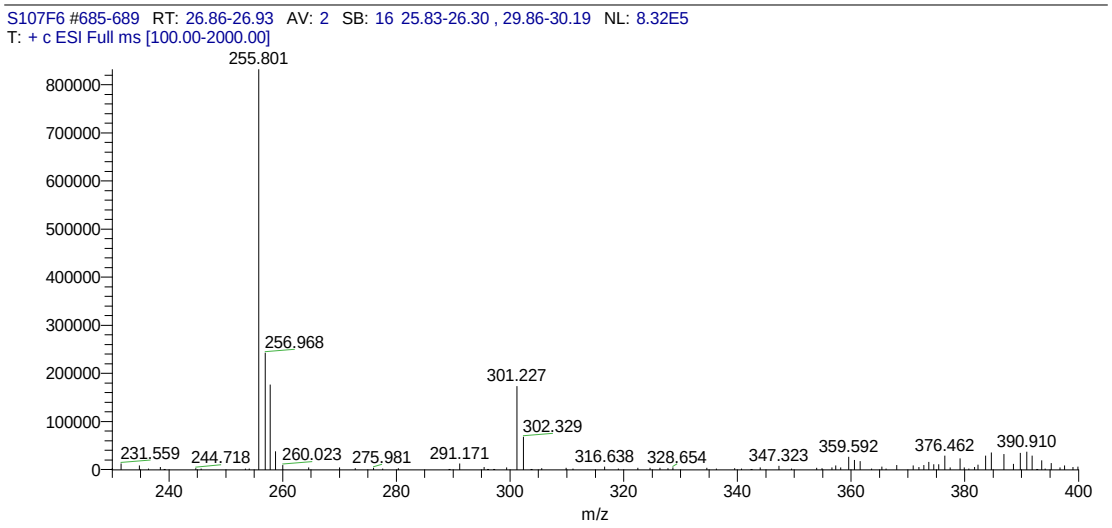


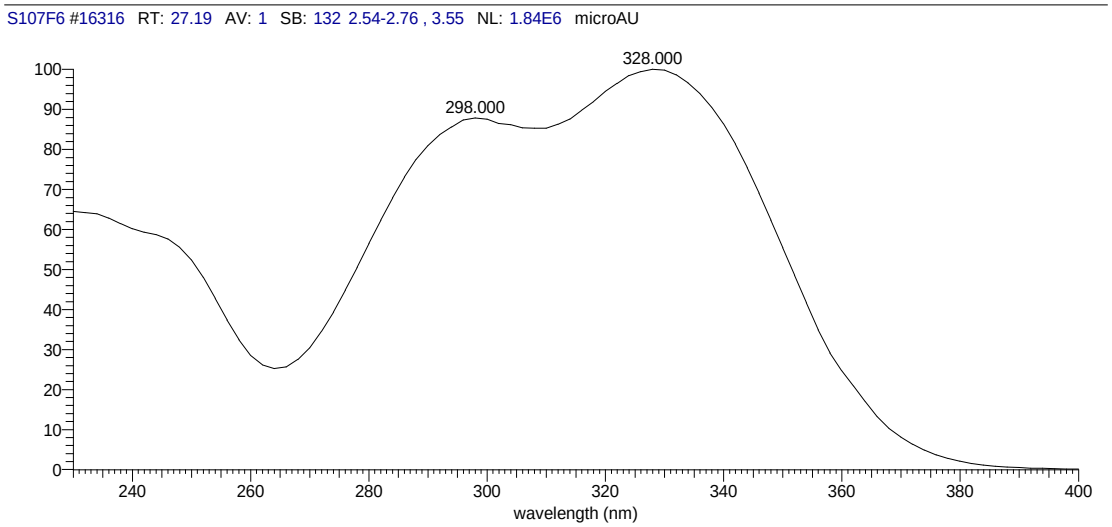
Appendix 7.2.12 UV and -ve ion MS spectra of quercetin methyl ether in S107 fraction 6 (base peak in mass spectrum is due to the formic acid adduct)



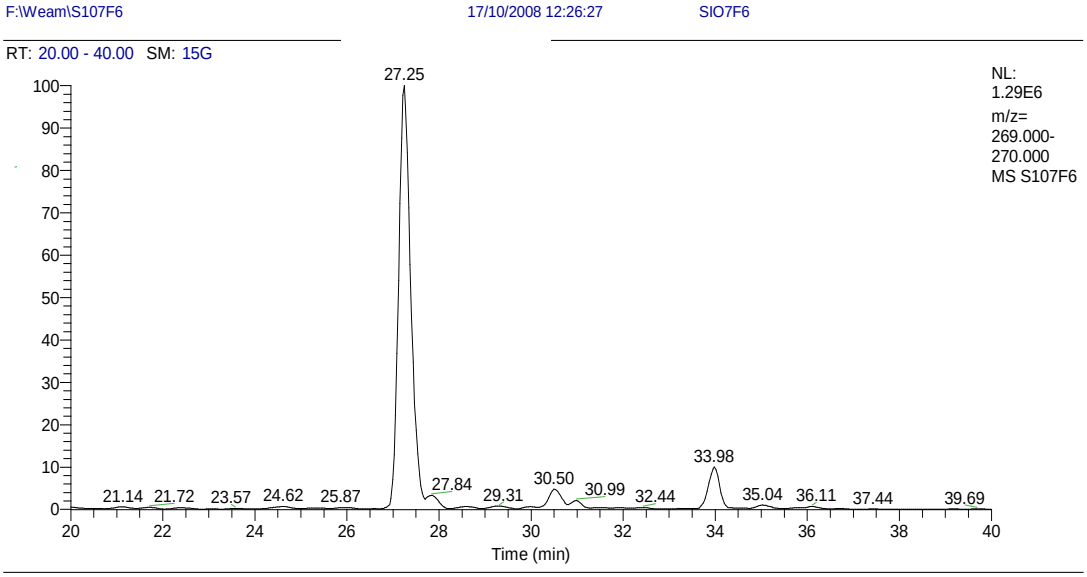
Appendix 7.2.13 UV and -ve ion MS spectra of pinocembrin in S107 fraction 6

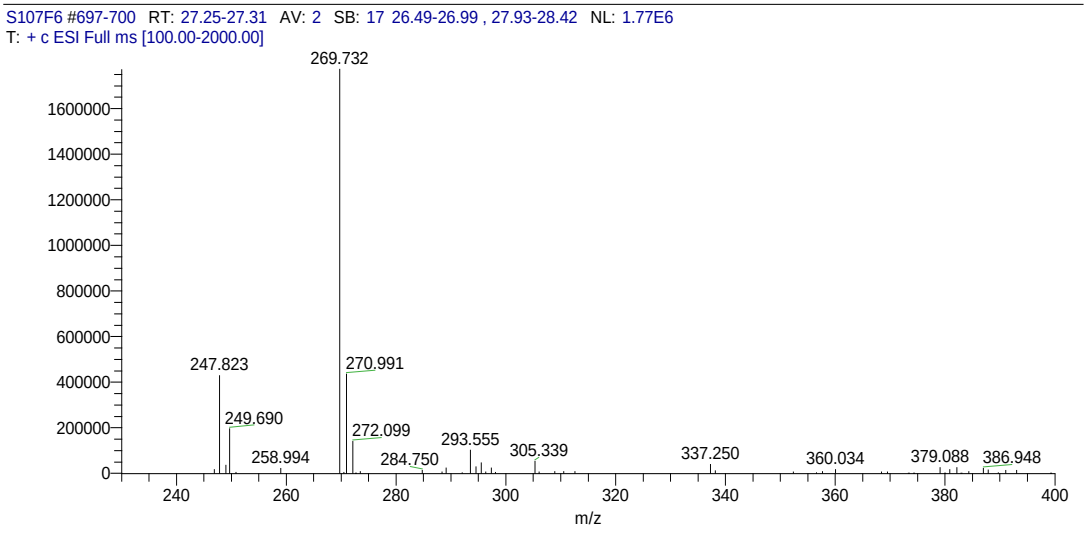


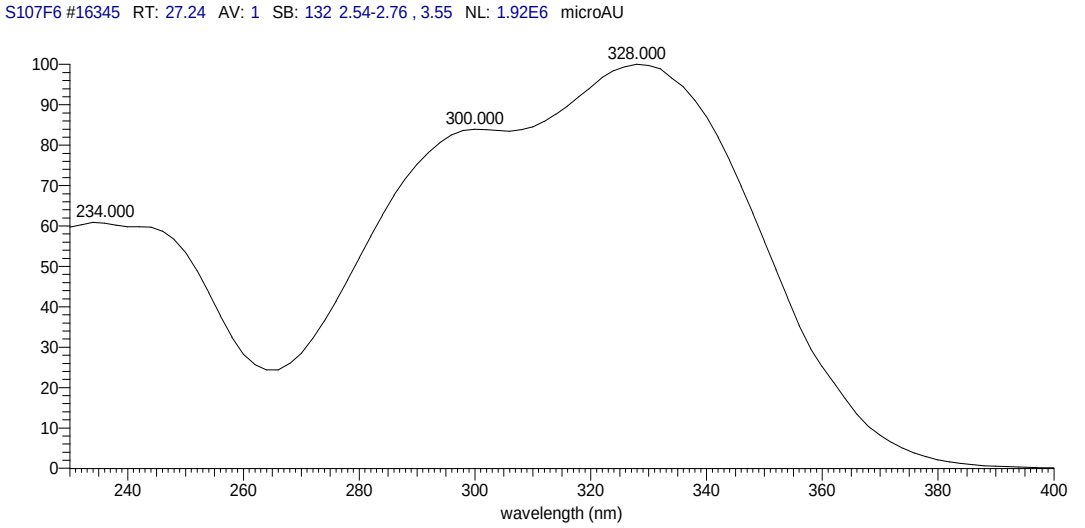




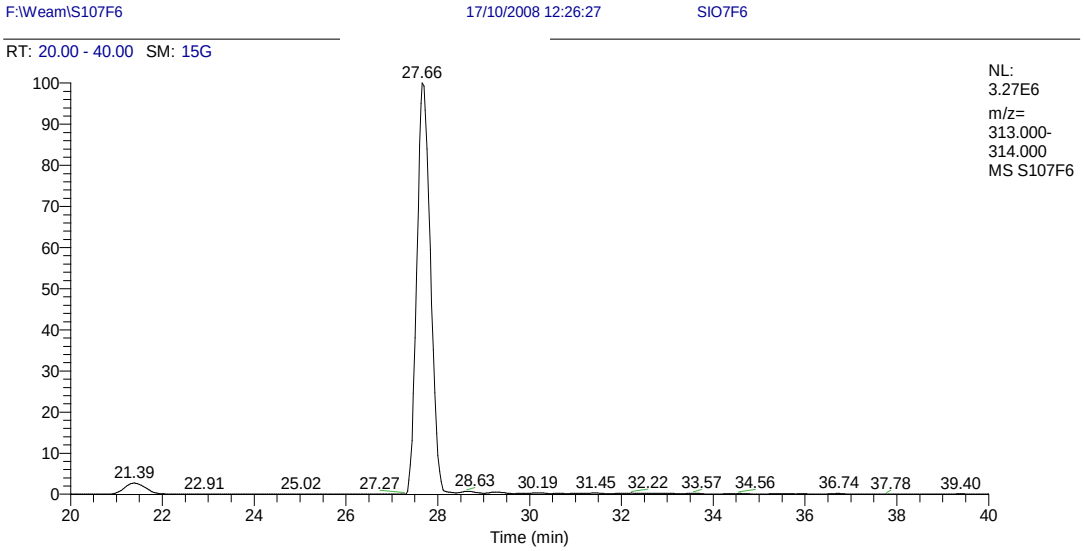
Appendix 7.2.14 UV and -ve ion MS spectra of galangin in S107 fraction 6

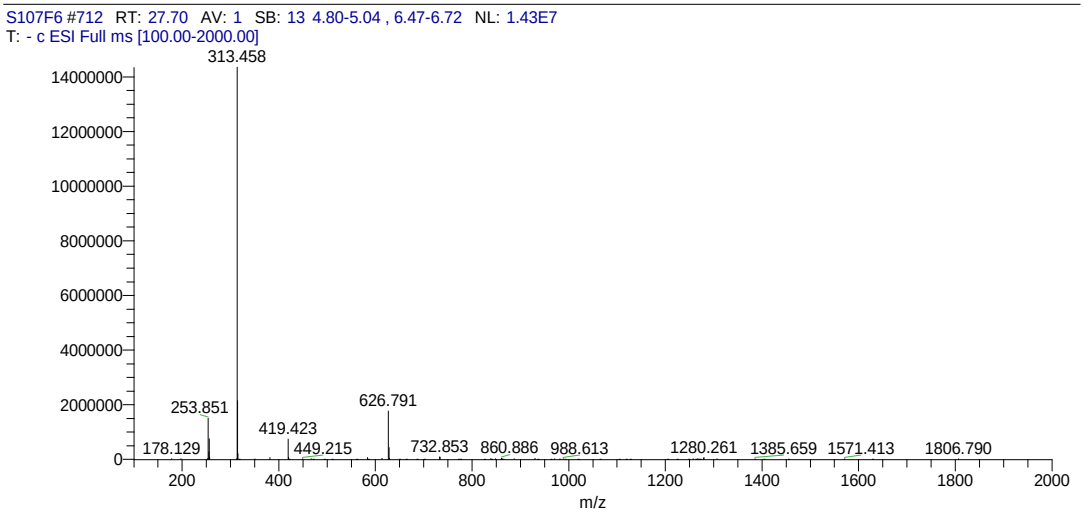


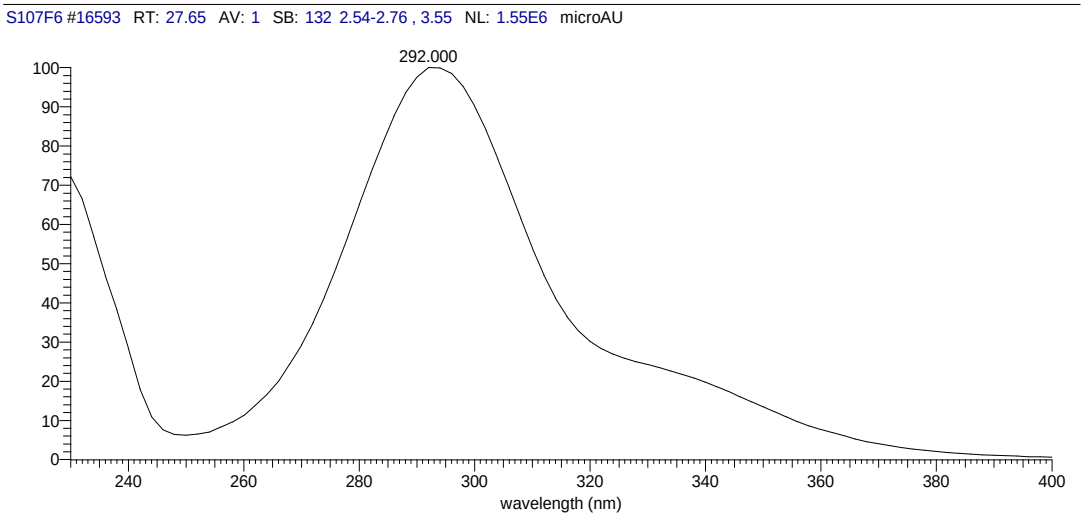




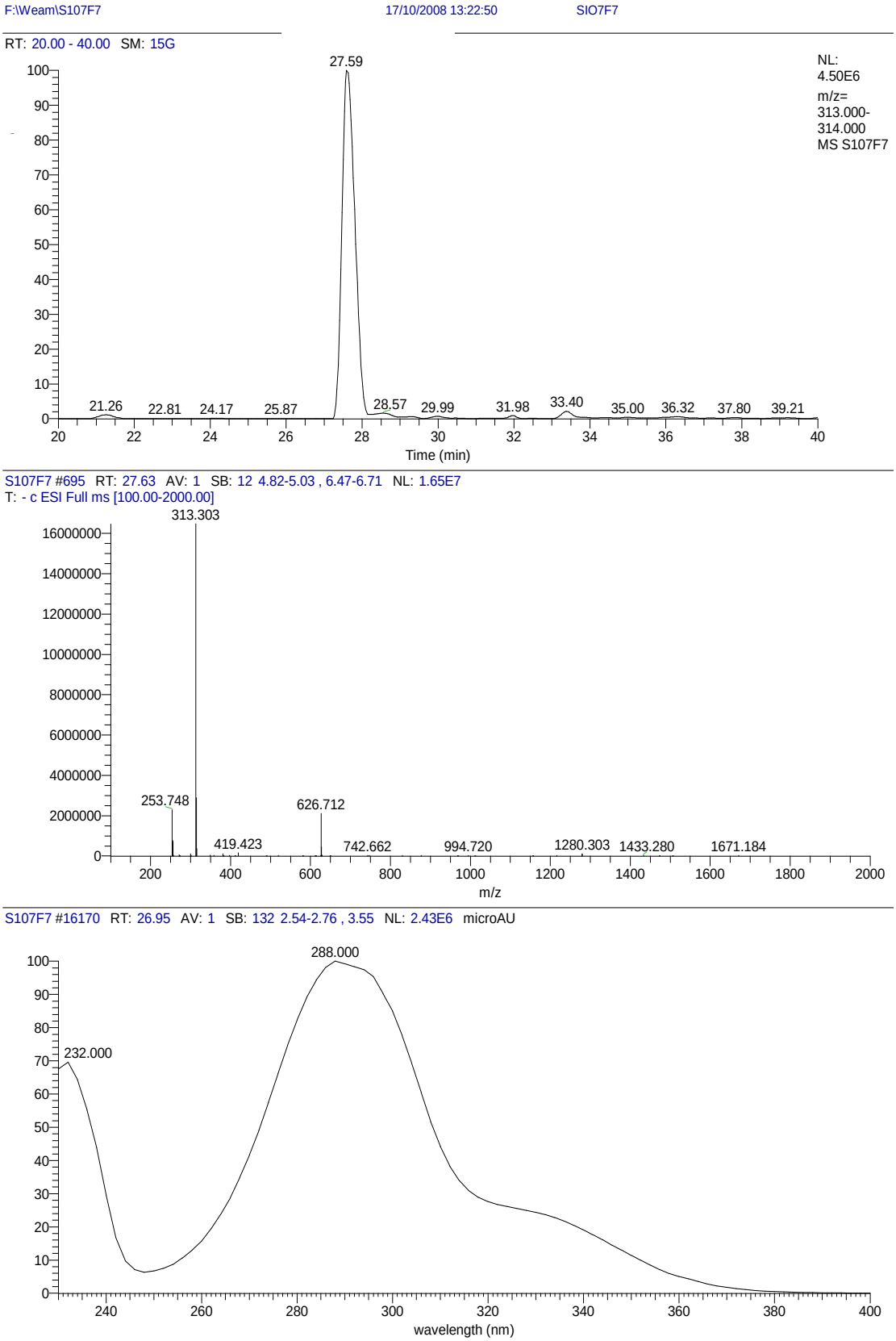
Appendix 7.2.15 UV and -ve ion MS spectra of pinobanksin acetate in S107 fraction 6



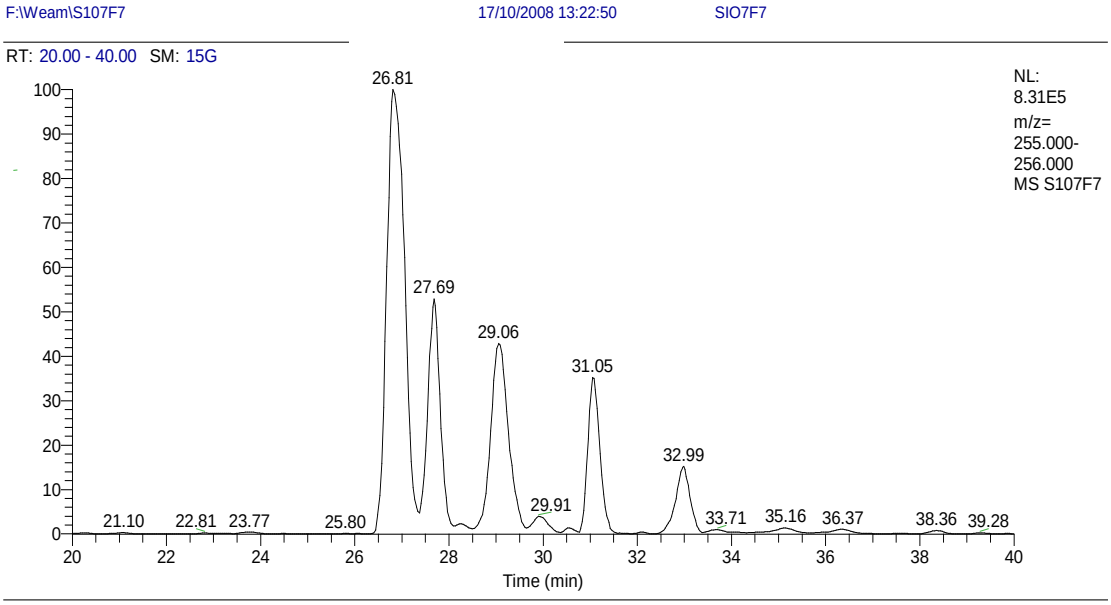


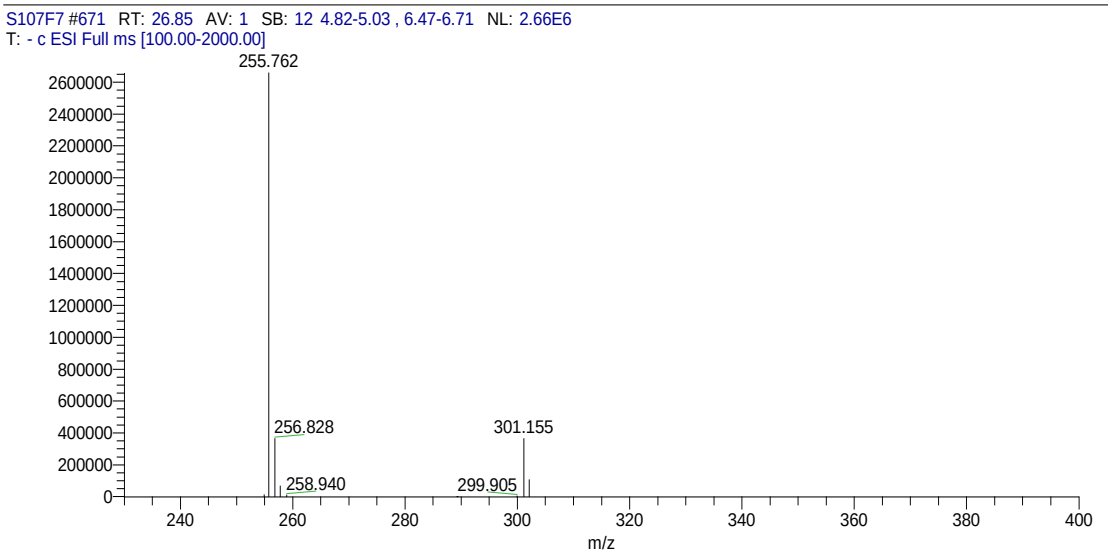


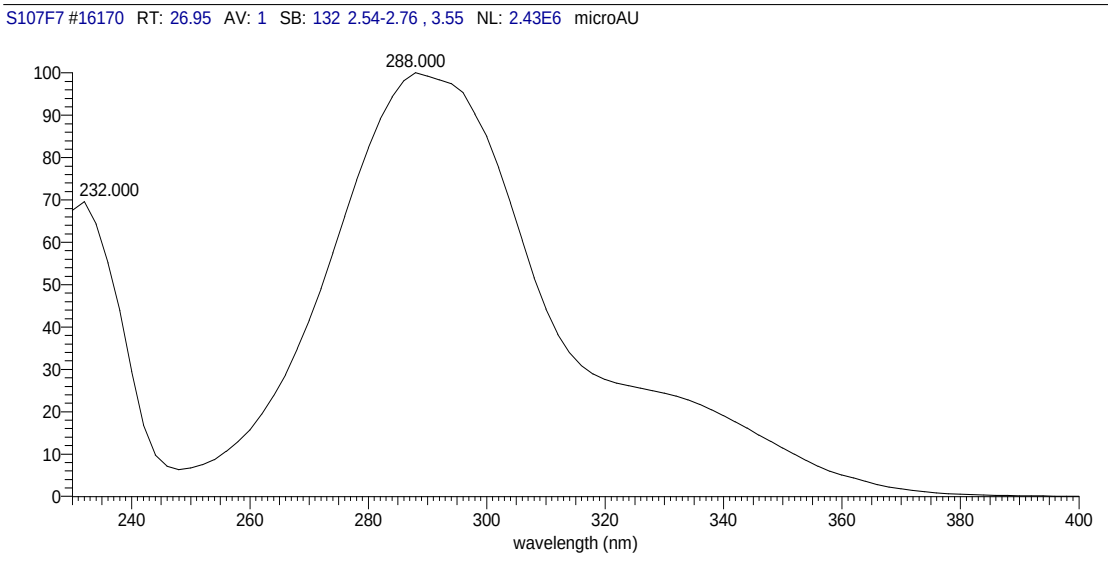
Appendix 7.2.16 UV and -ve ion MS spectra of pinobanksin acetate in S107 fraction 7



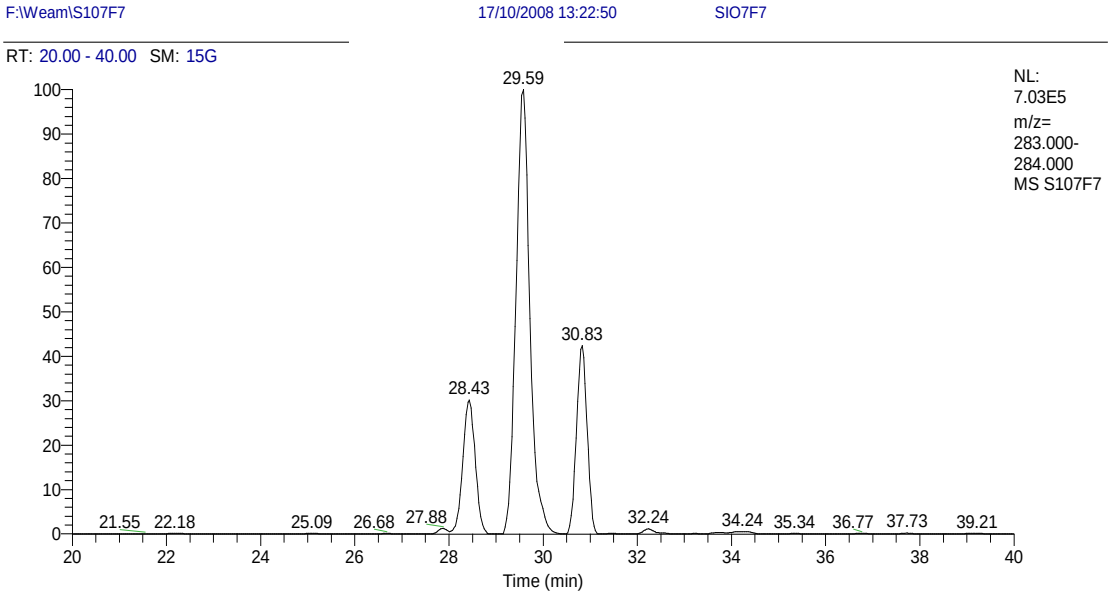
Appendix 7.2.17 UV and -ve ion MS spectra of pinocembrin in S107 fraction 7

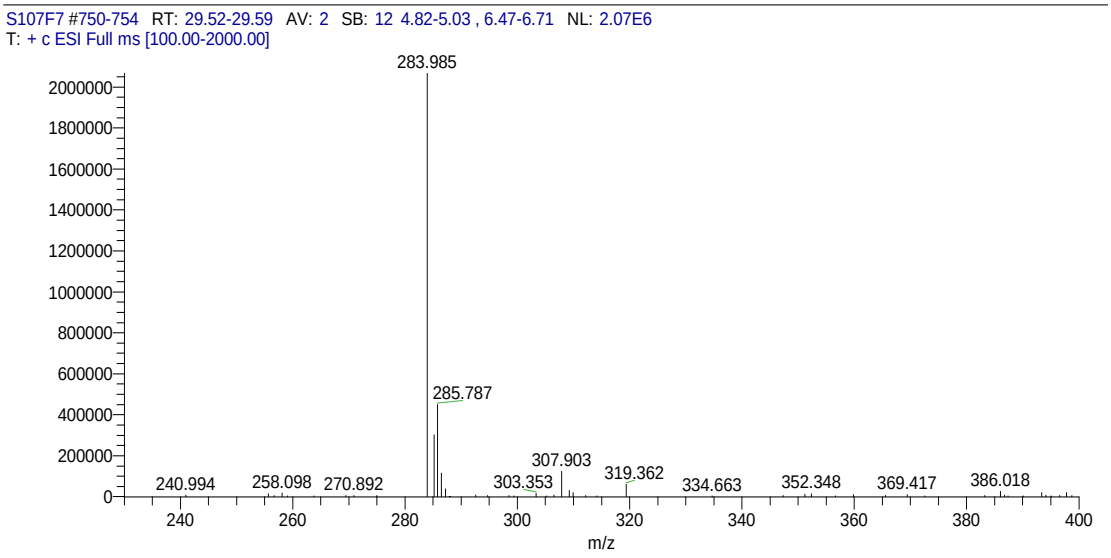


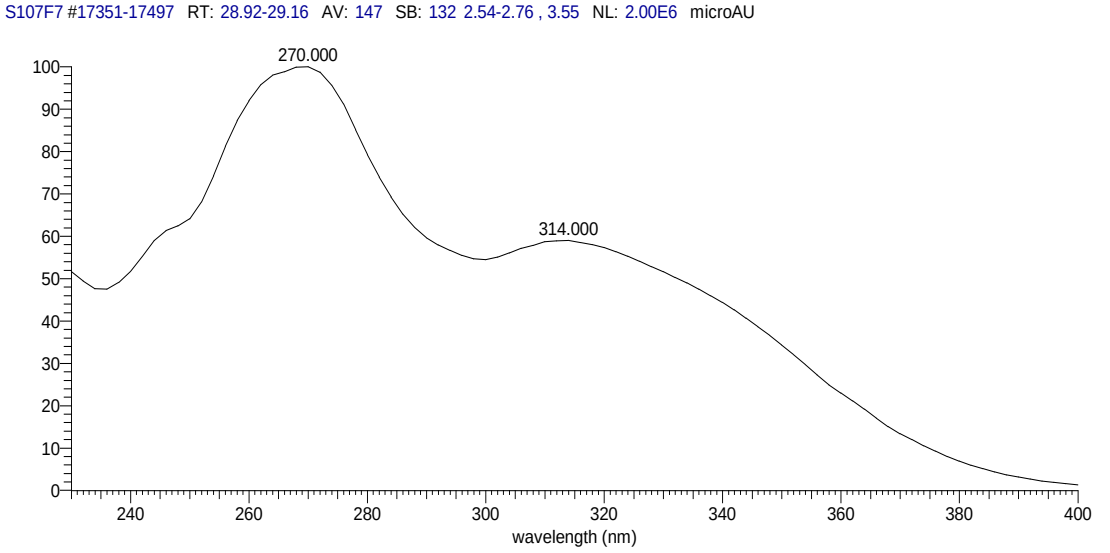




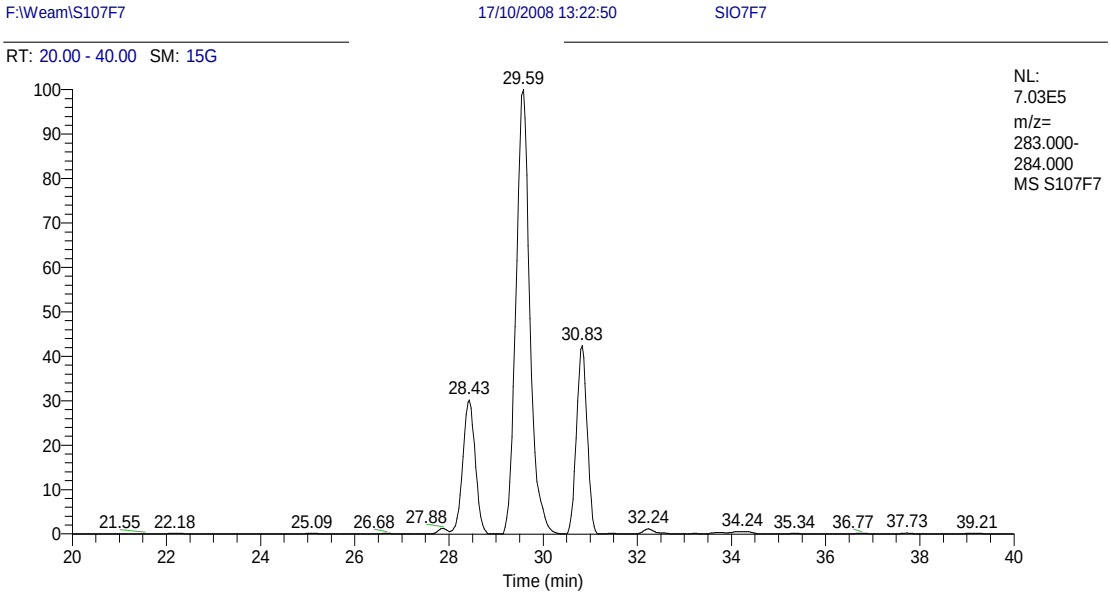
Appendix 7.2.18 UV and -ve ion MS spectra of galangin methyl ether in S107 fraction 7

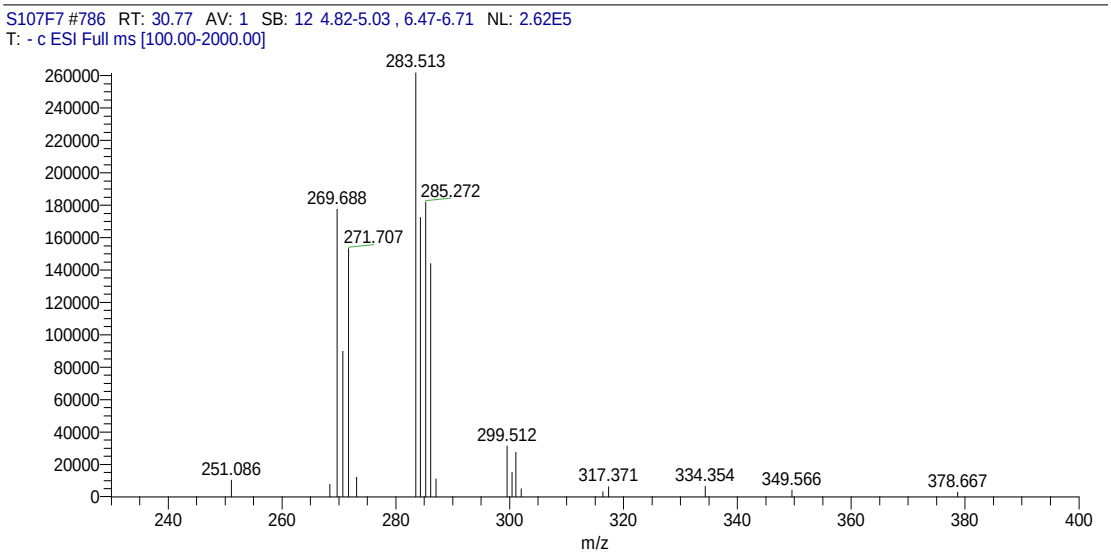


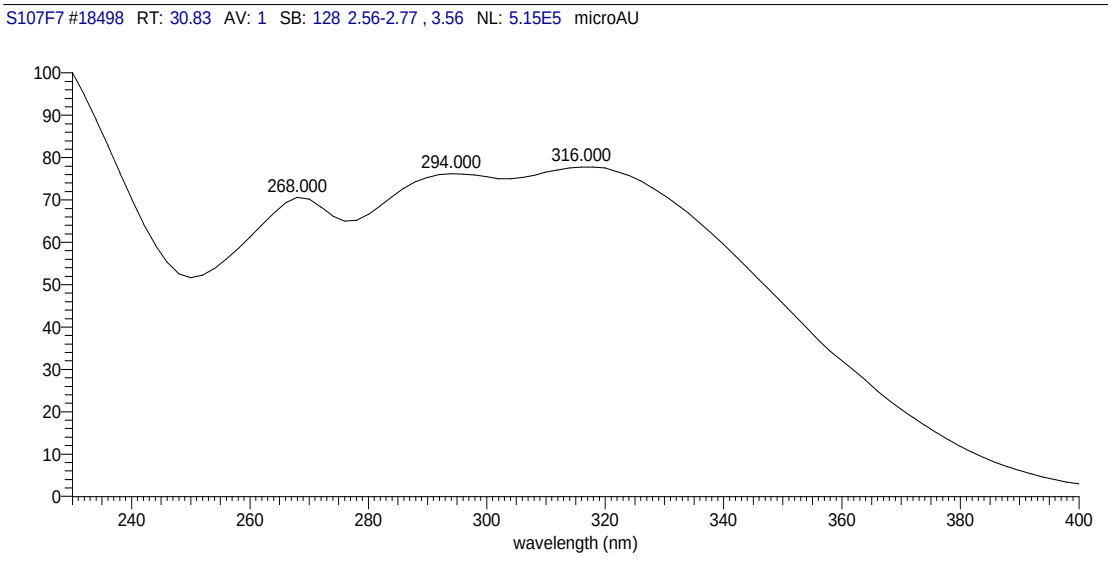




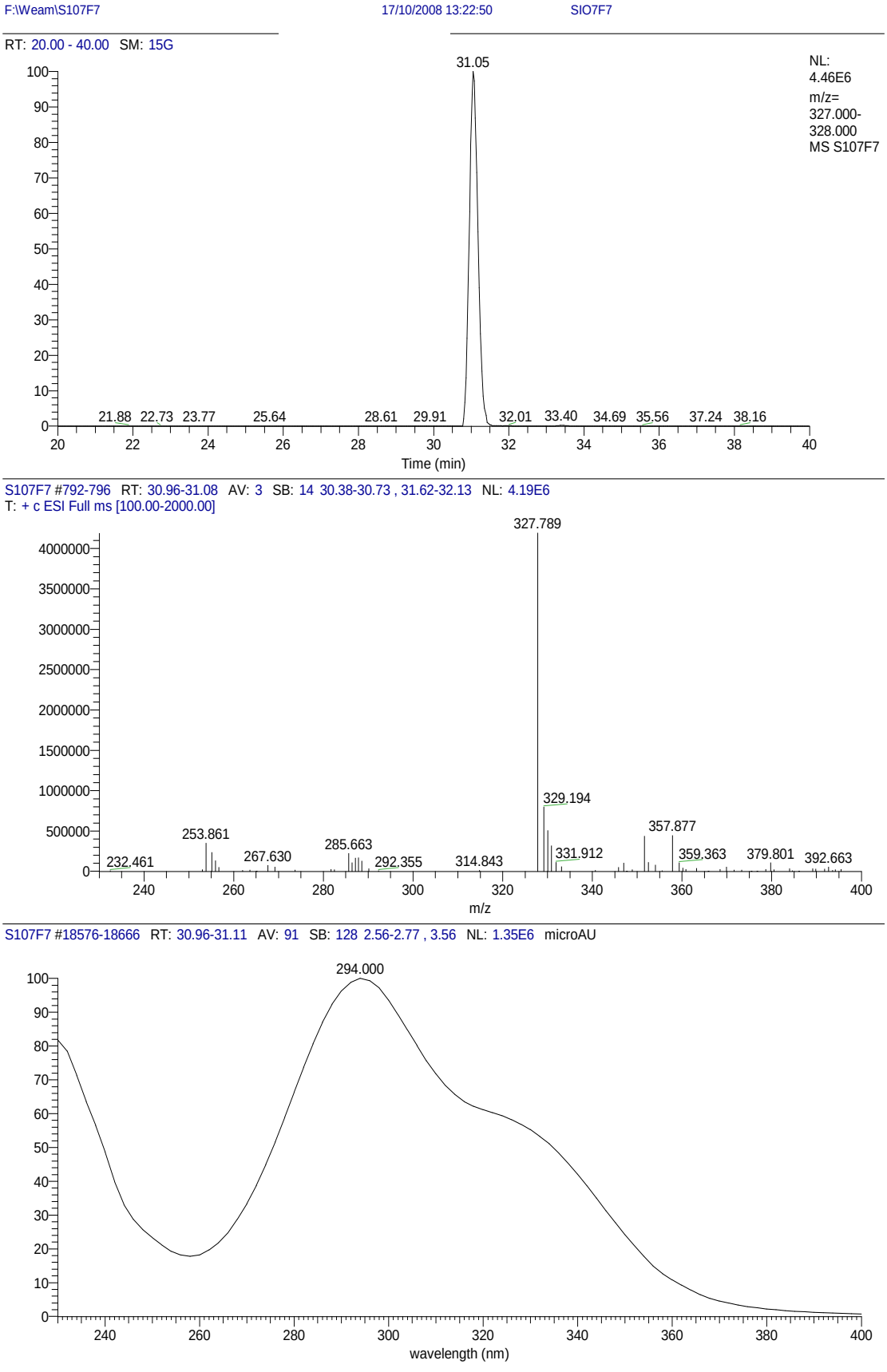
Appendix 7.2.19 UV and -ve ion MS spectra of galangin methyl ether in S107 fraction 7



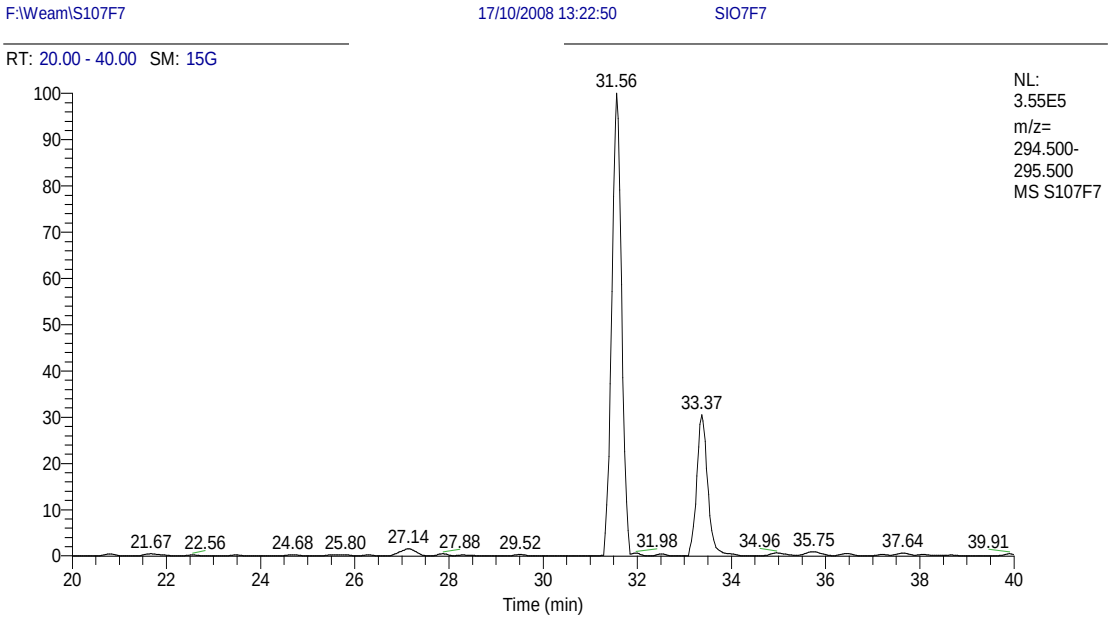


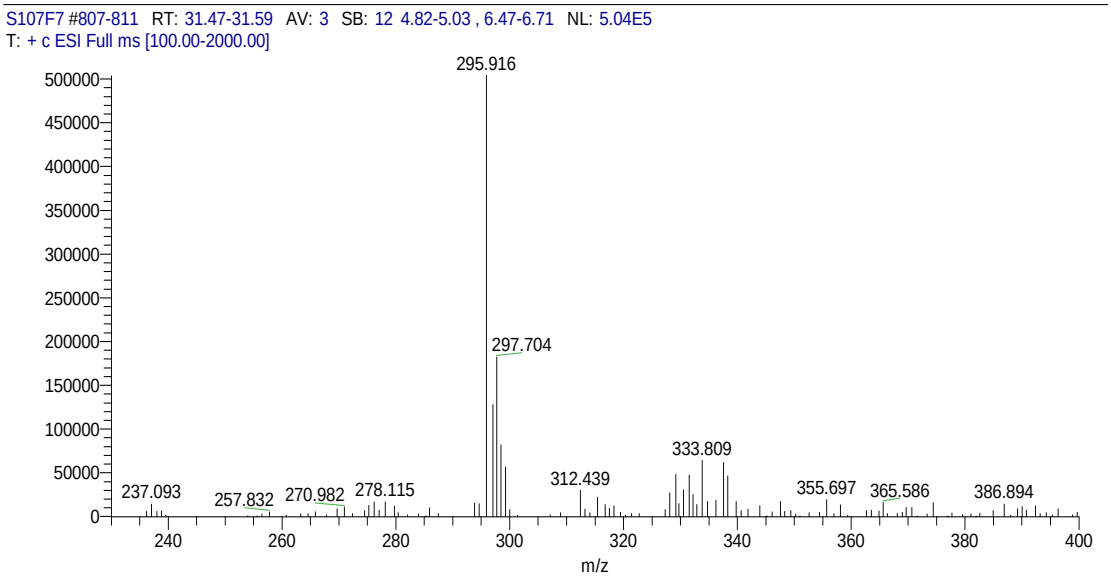


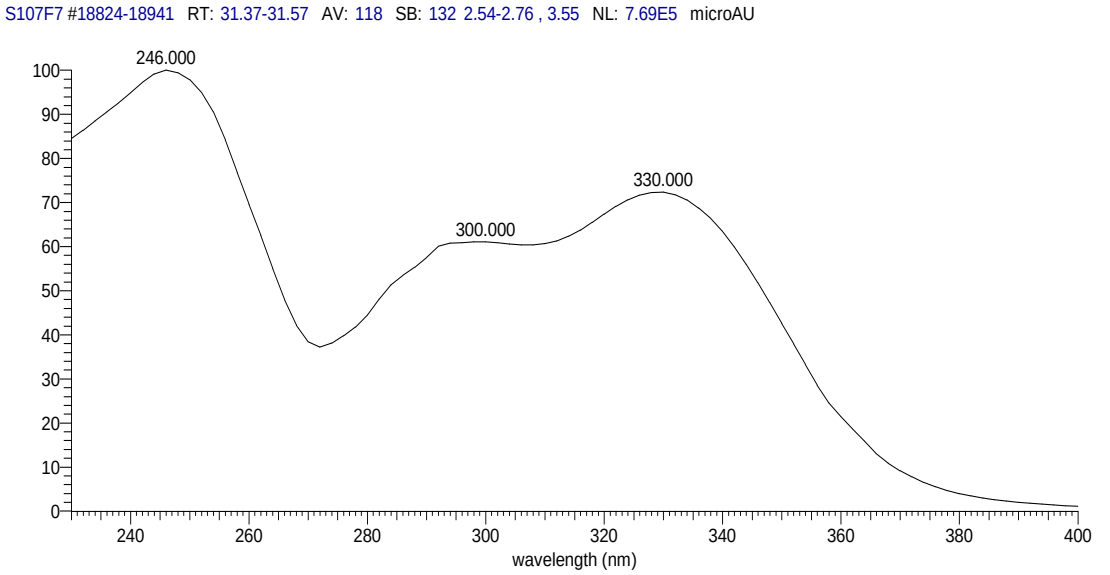
Appendix 7.2.20 UV and -ve ion MS spectra of pinobanksin propionate in S107 fraction 7



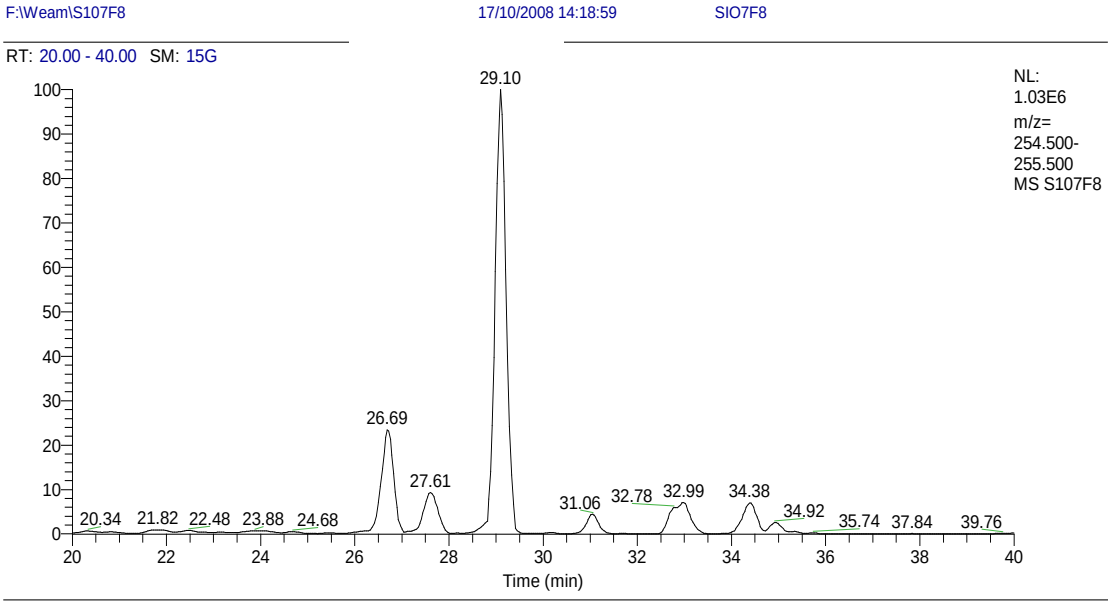
Appendix 7.2.21 UV and -ve ion MS spectra of chrysin trimethyl ether in S107 fraction 7

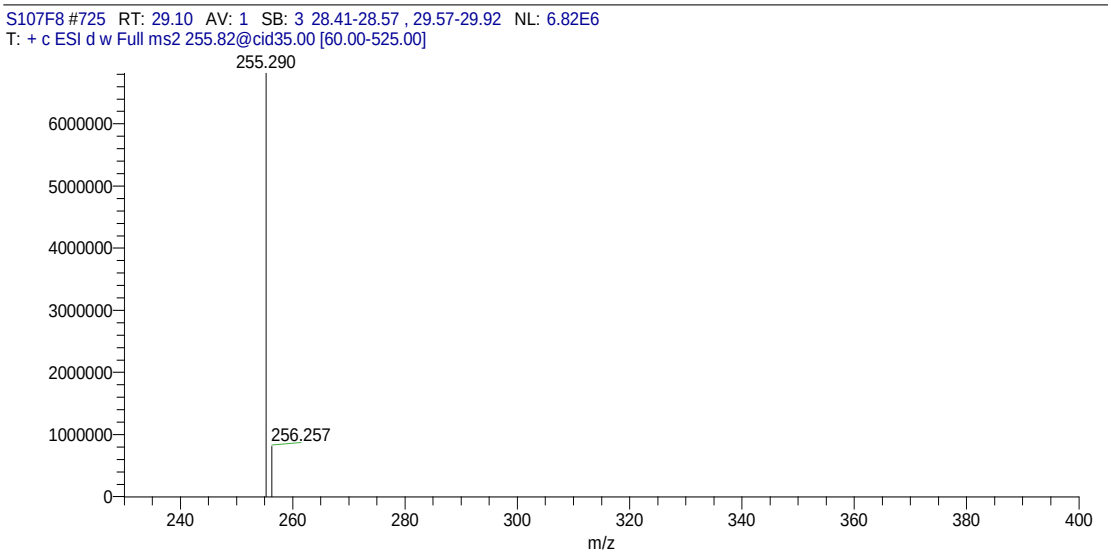


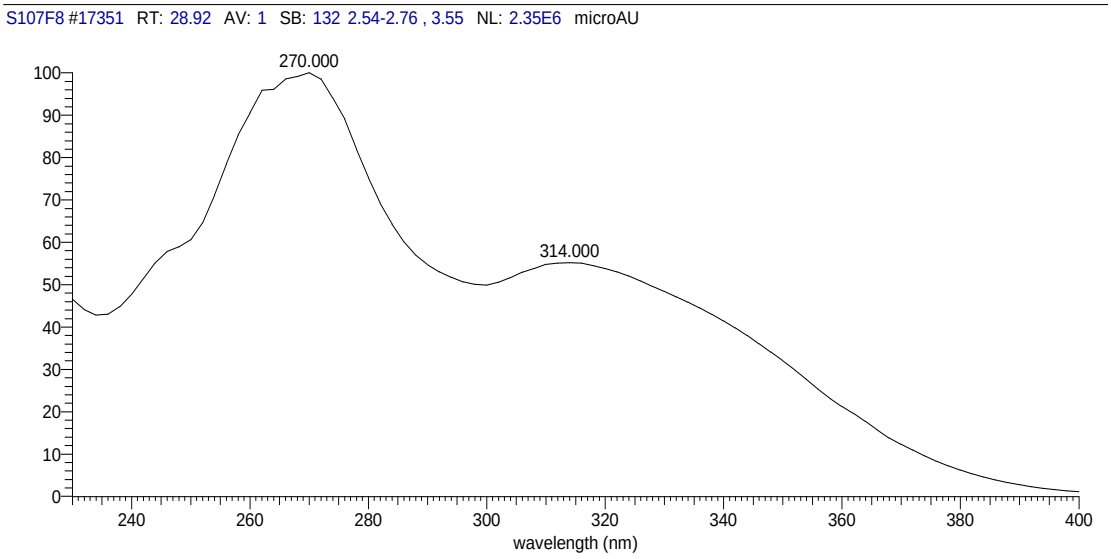




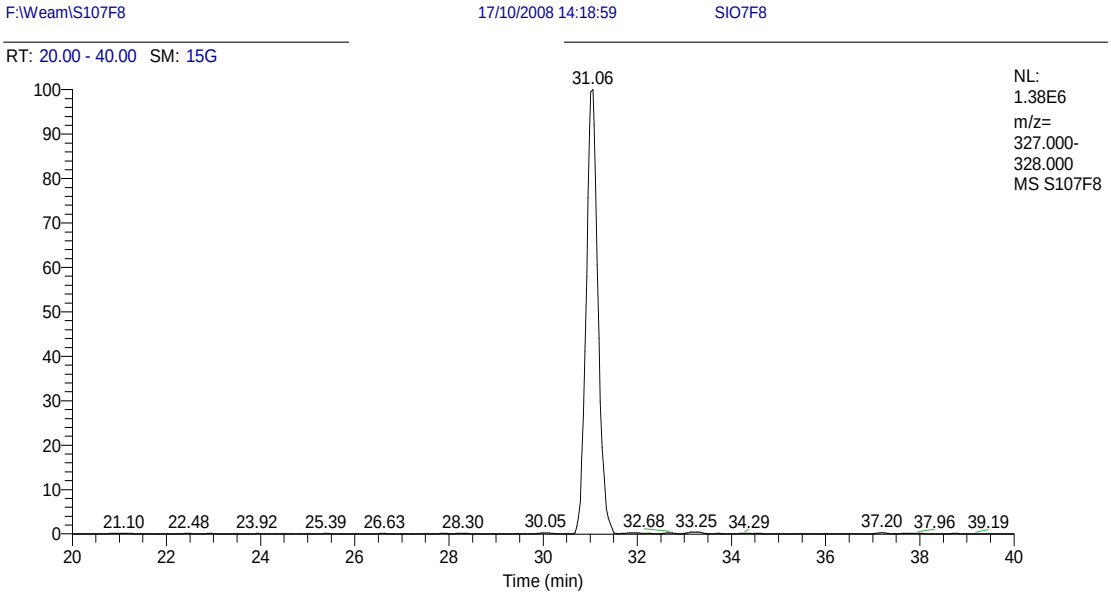
Appendix 7.2.22 UV and -ve ion MS spectra of pinocembrin in S107 fraction 8

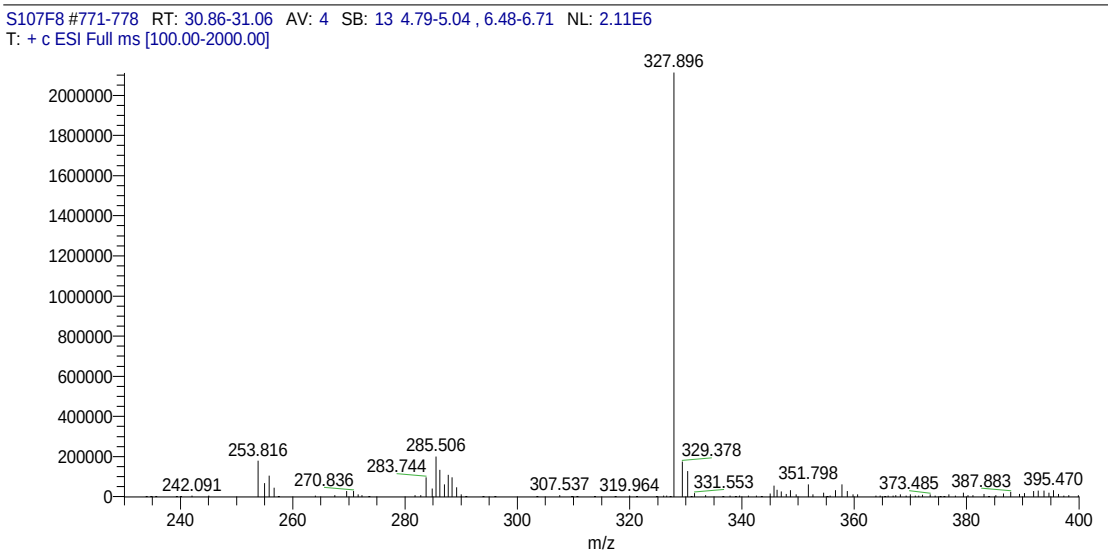


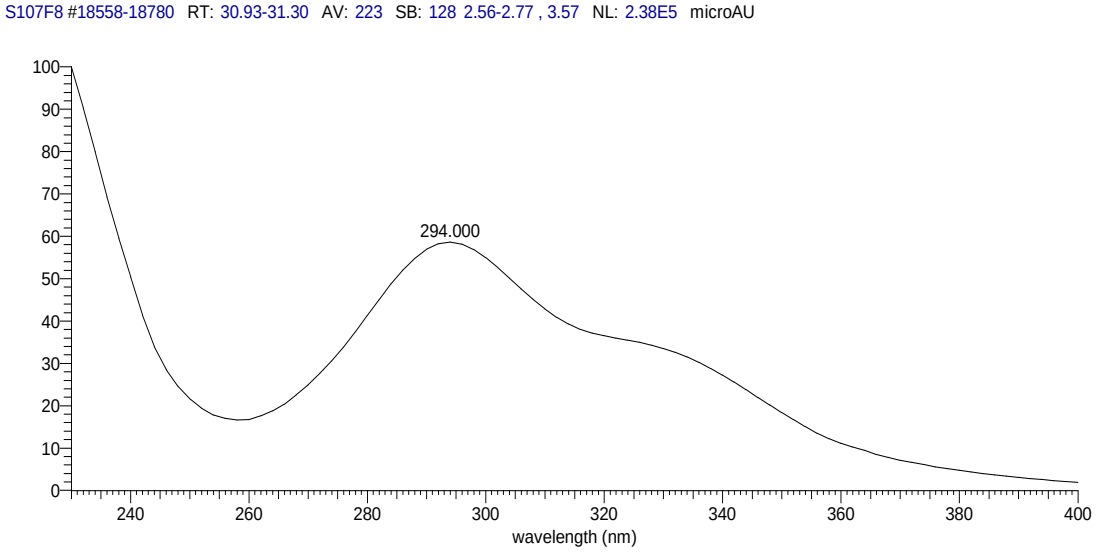




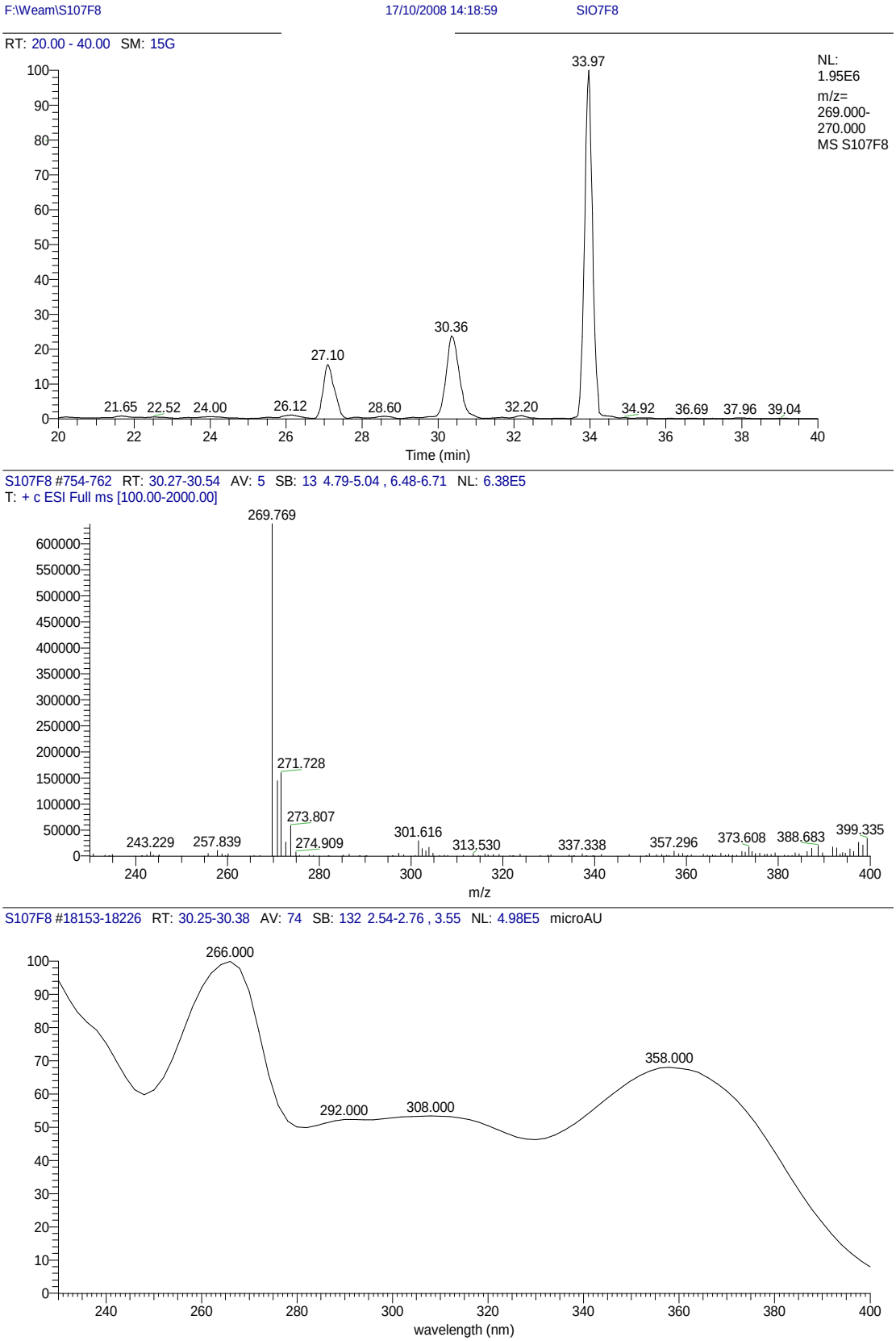
Appendix 7.2.23 UV and -ve ion MS spectra of pinobanksin propionate in S107 fraction 8



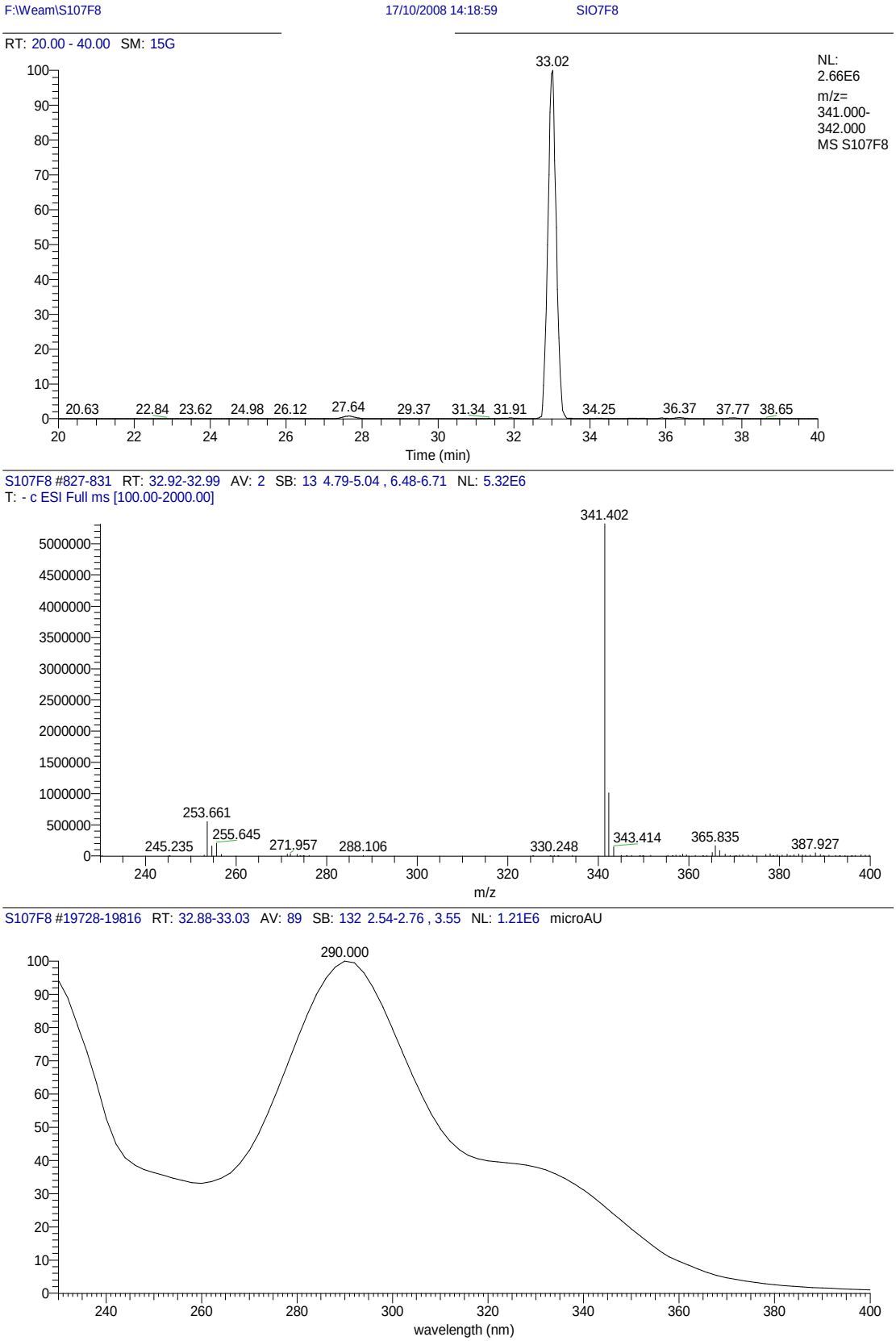




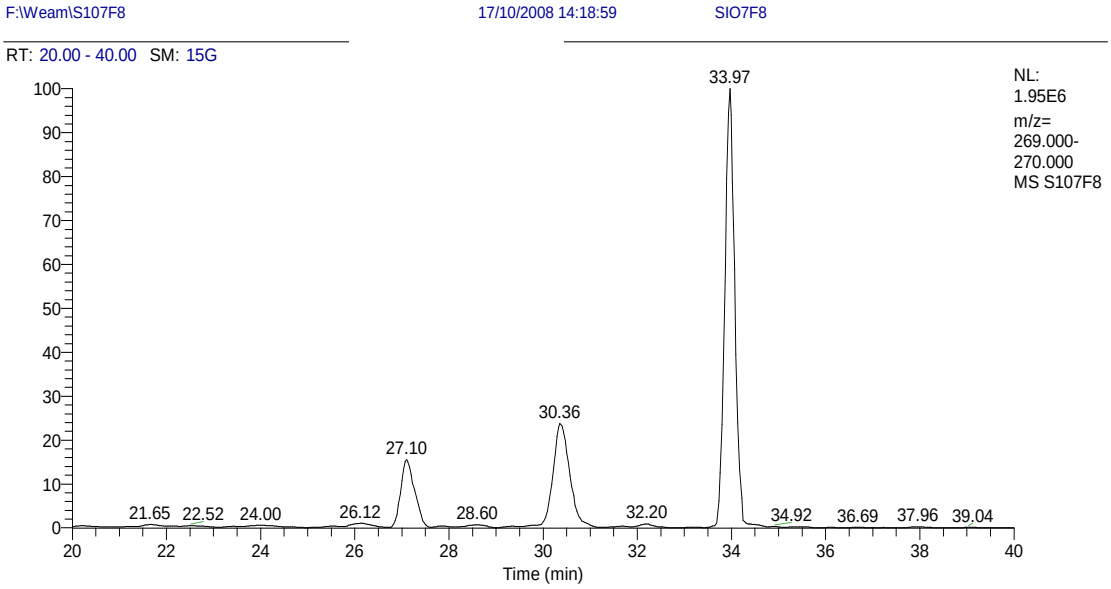
Appendix 7.2.24 UV and -ve ion MS spectra of galangin methyl ether in S107 fraction 8

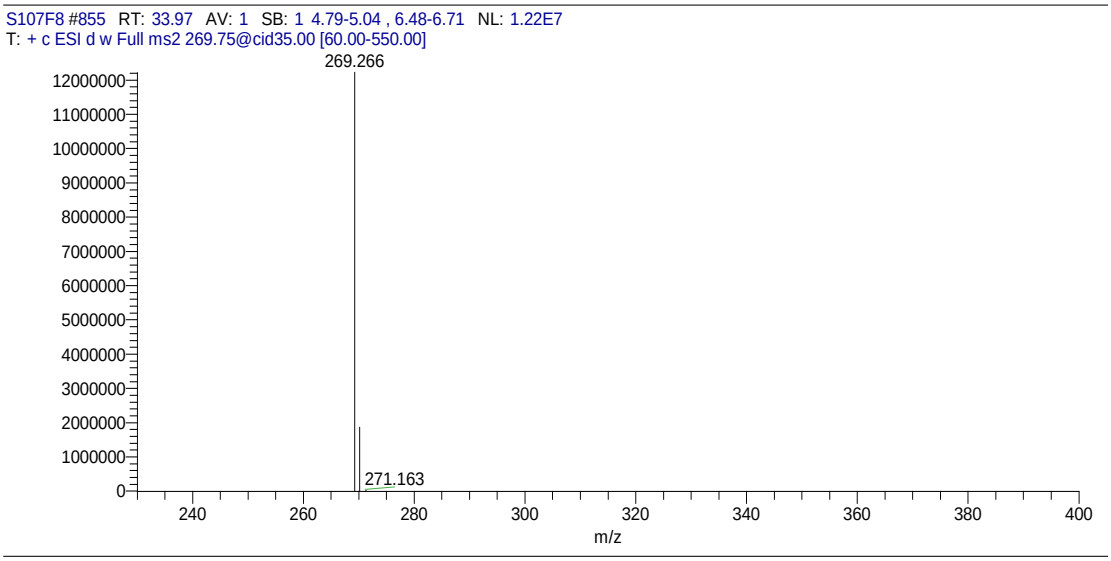


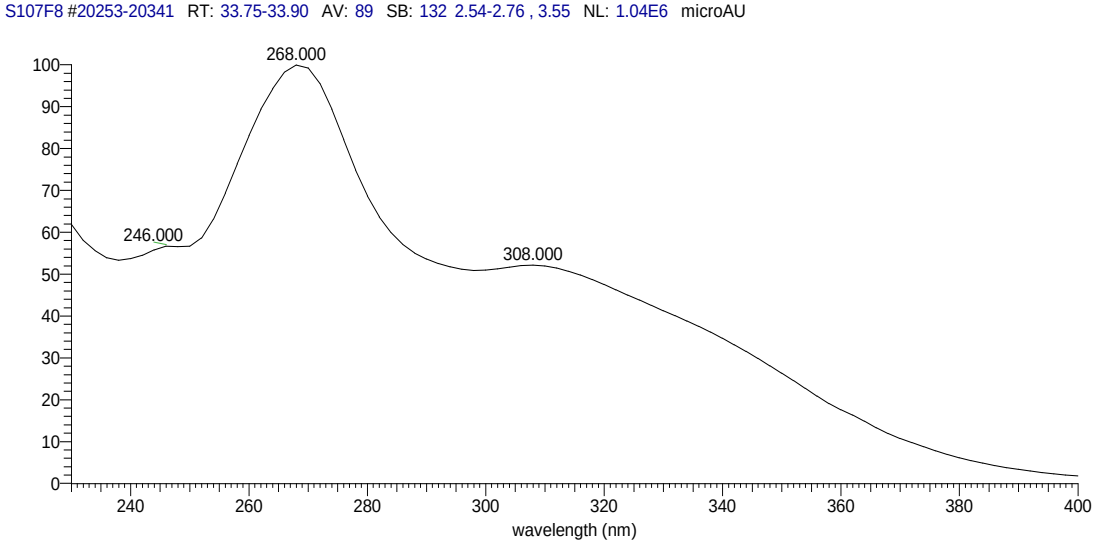
Appendix 7.2.25 UV and -ve ion MS spectra of pinobanksin butyrate in S107 fraction 8



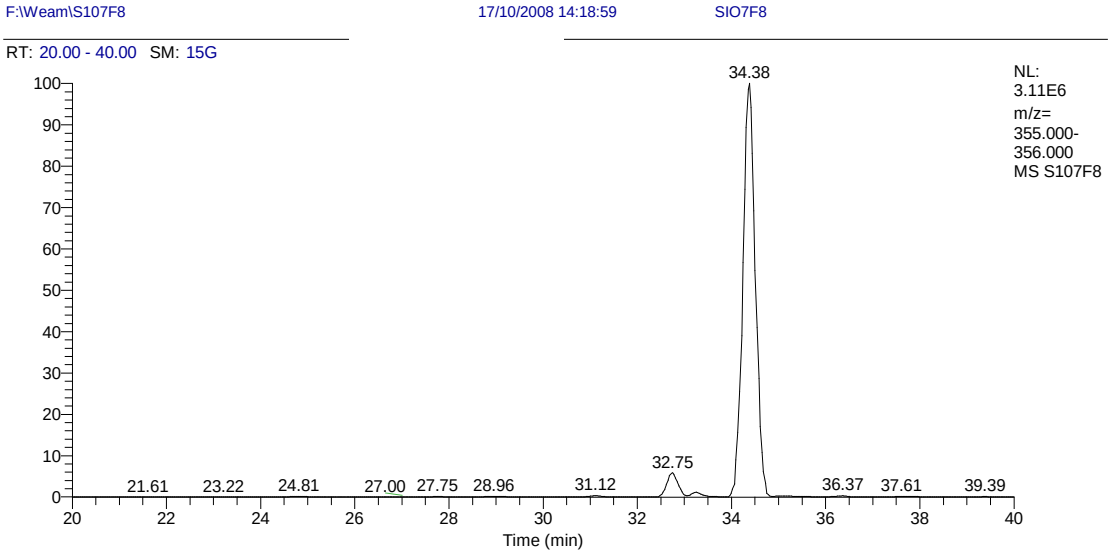
Appendix 7.2.26 UV and -ve ion MS spectra of galangin methyl ether in S107 fraction 8

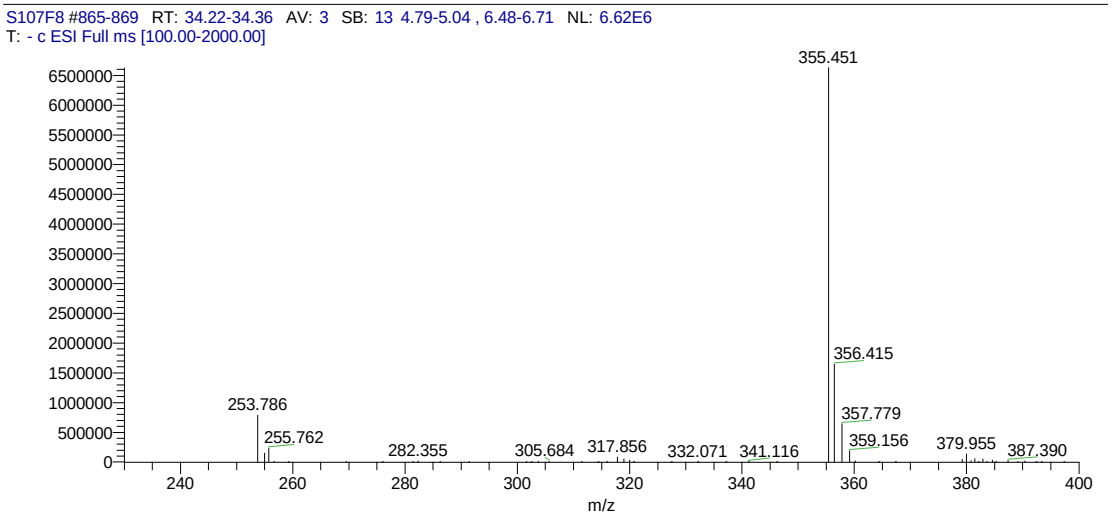


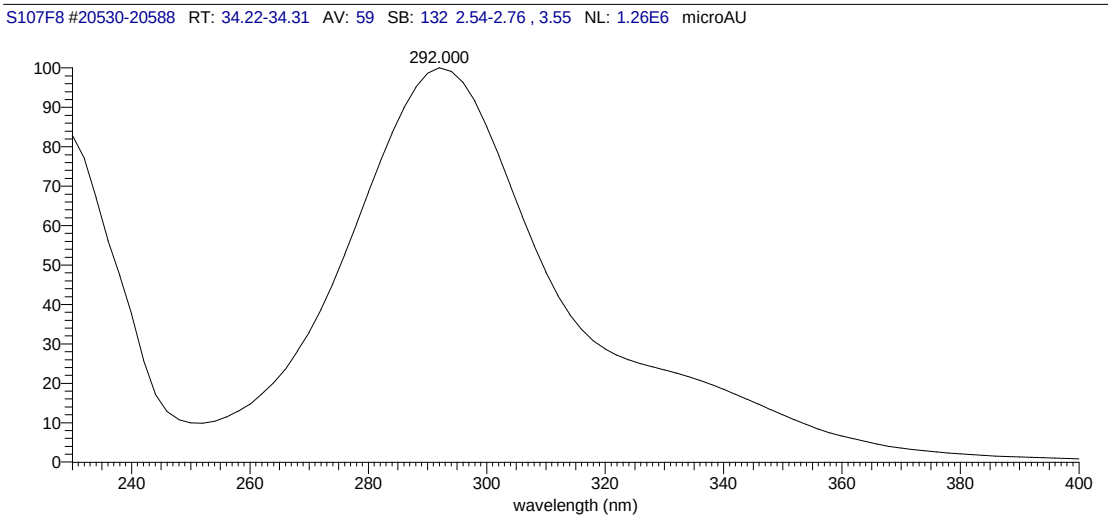




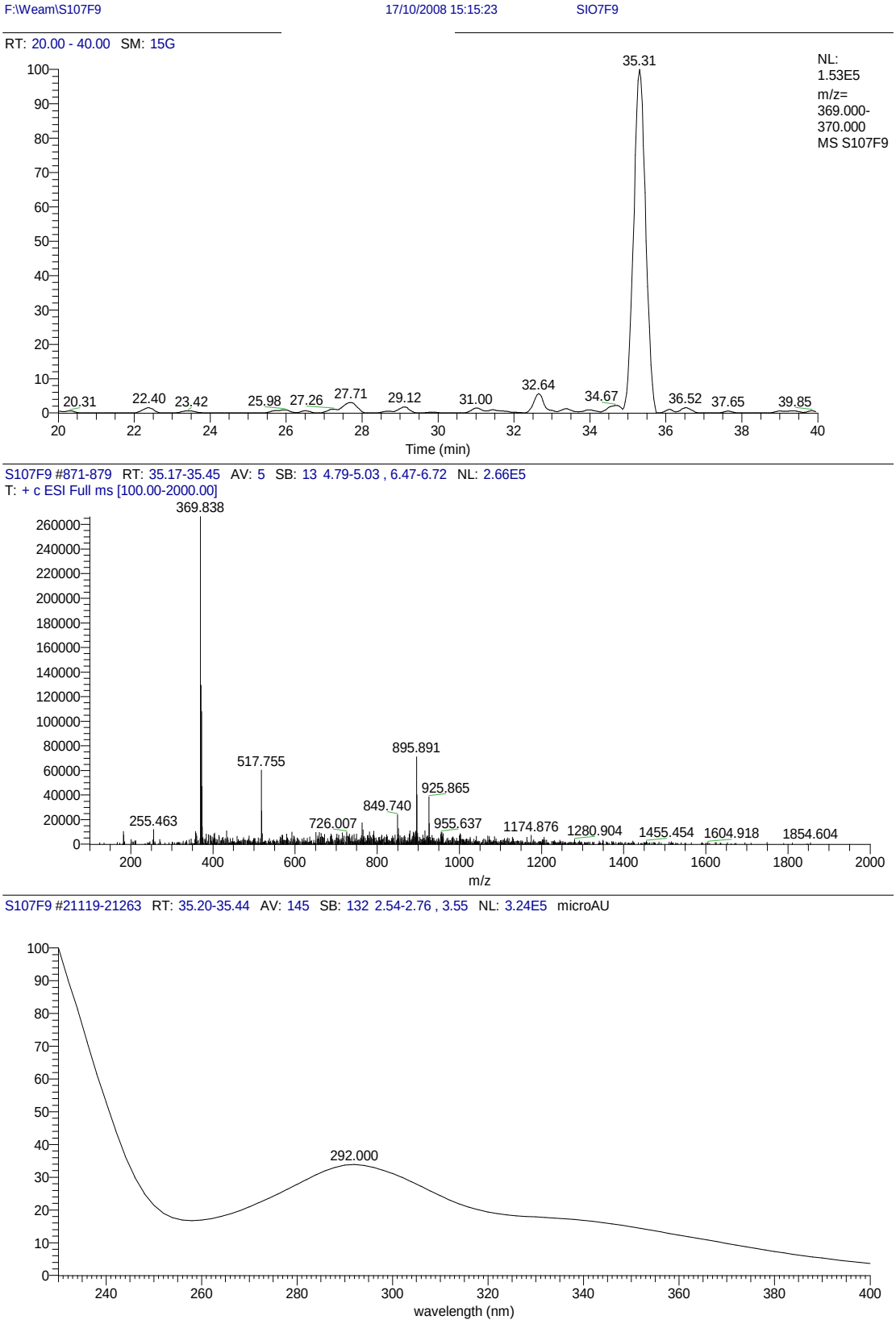
Appendix 7.2.27 UV and -ve ion MS spectra of pinobanksin valerate in S107 fraction 8



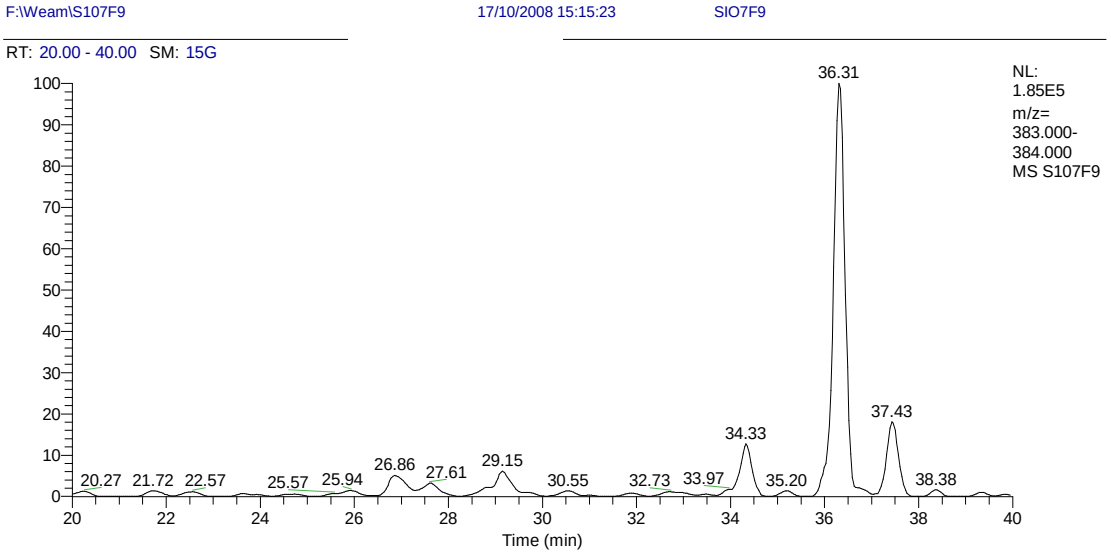


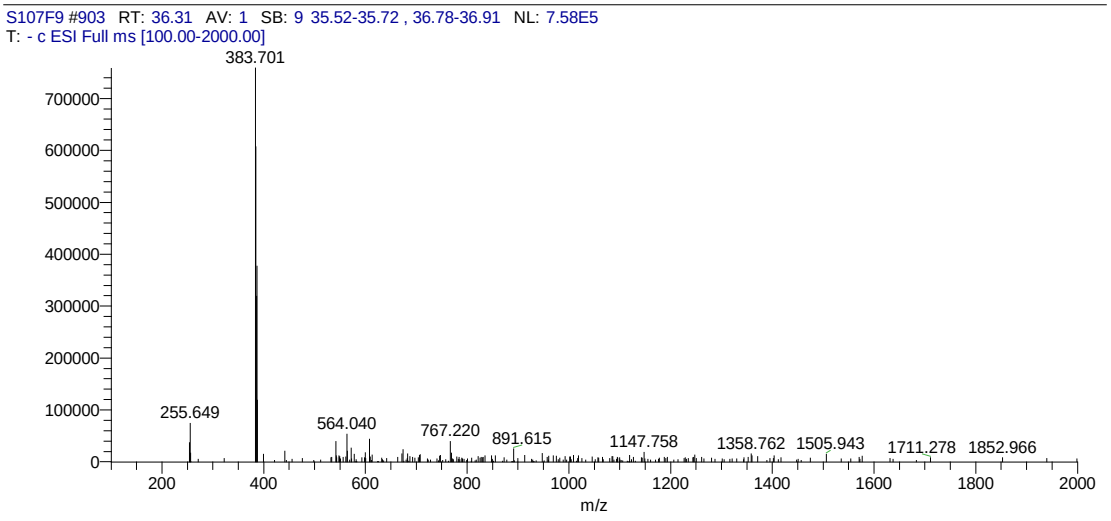


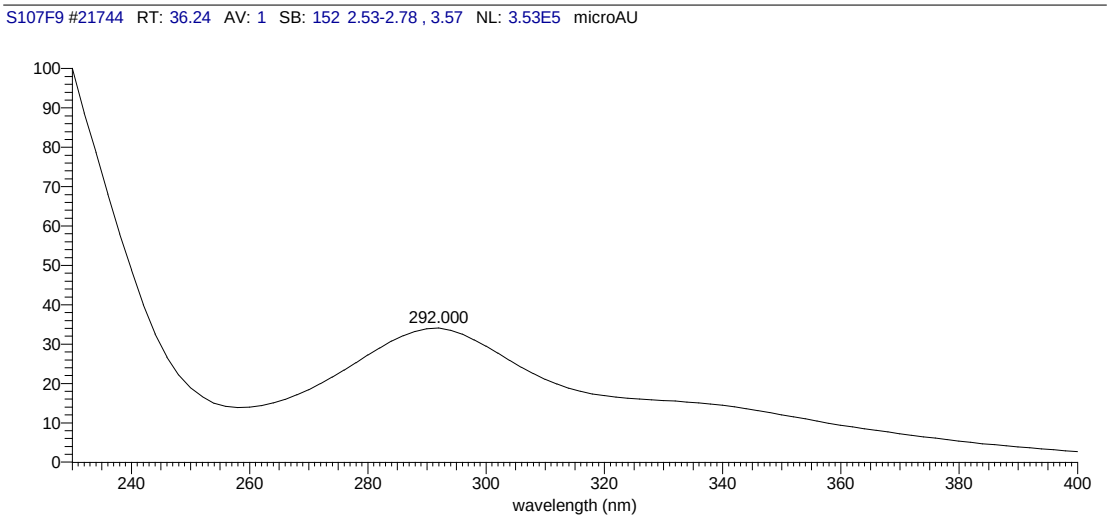
Appendix 7.2.28 UV and -ve ion MS spectra of pinobanksin hexanoate in S107 fraction 9



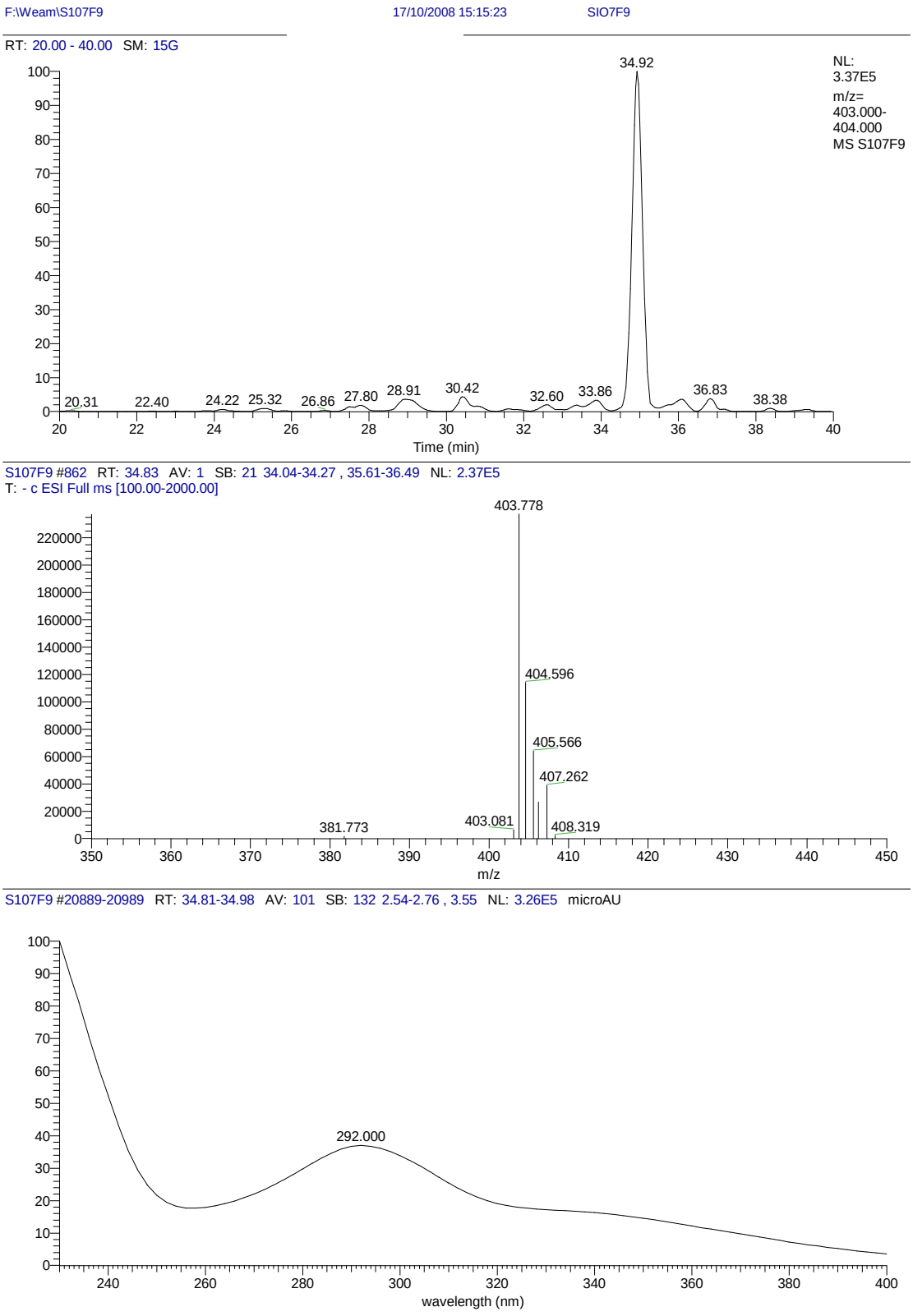
Appendix 7.2.29 UV and -ve ion MS spectra of pinobanksin heptanoate in S107 fraction 9





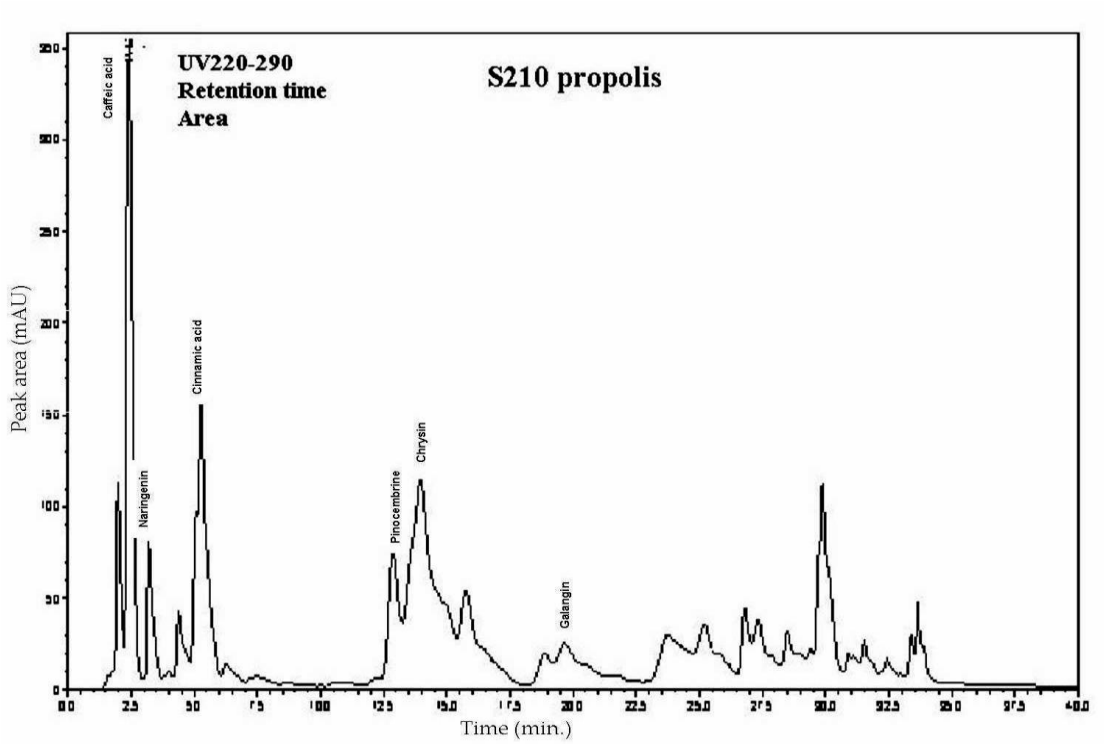


Appendix 7.2.30 UV and -ve ion MS spectra of pinobanksin phenypropionate in S107 fraction 9



7.3 Additional HPLC UV Chromatograms of Propolis Samples

Appendix 7.3.1 The HPLC chromatogram of propolis sample S210



Appendix 7.3.2 The HPLC chromatogram of propolis sample S208